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par

Laurine Blanchard

Impact of lightning on evolution, structure and function of bacterial communities

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JURY

Rapporteur	Odile Berge	Chargée de Recherche CNRS - INRA d'Avignon
Rapporteur	Paolo Nannipieri	Professeur - Université de Florence (Italie)
Examinateur	Cindy Morris	Directrice de Recherche - INRA d'Avignon
Examinateur	Pierre Amato	Chargé de Recherche CNRS – ICCF
Encadrant	Sandrine Demanèche	Ingénieure de Recherche - Laboratoire Ampère
Directeur de thèse	Pascal Simonet	Directeur de Recherche CNRS – Laboratoire Ampère
Président du jury	François Buret	Professeur - Ecole Centrale de Lyon

IT
ALWAYS
SEEMS
Impossible
UNTIL IT'S
Done.

-Nelson Mandela

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LIST OF ACRONYMS

A: Ampere

AIDA: Aerosol Interaction and Dynamics in the Atmosphere

aph3ia: Aminoglycoside O-phosphotransferase gene

BGA: Between Group Analysis

BLAST: Basic Local Alignment Search Tool

BOX: repetitive sequences highly conserved among diverse bacteria

bp: base pair

CCC: Covalently Closed Circular

CCN: Cloud Condensation Nuclei

CFU: Colony Forming Unit

CI: confidence intervals

CSA: Côte-Saint-André soil

CsCl: Cesium Chloride

d/D: Day(s)

DDOL: dichlorocyclohexadiene-diol

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

DNOL: trichlorocyclohexadiene-ol

E: Electrotransformation rate

EDTA: Ethylenediaminetetraacetic acid

EtBr: Ethidium Bromide

F: Farad

FAO: Food and Agriculture Organization

g: gram

g: gravitational

GC-MS: Gas Chromatography –Mass spectrometer

h: hour

H: Humidity

HCH: Hexachlorocyclohexane

HGT: Horizontal Gene Transfer

HMP: sodium hexametaphosphate

IGS: Intergenic Spacer

IN: Ice Nuclei

INA: Ice Nucleation Activity

IS: Insertion Sequence

Kan: Kanamycin

Kb: Kilobase

KB: King's B

KBC: King's B medium modified by Mohan and Schaad (1987)

KBCTK: KBC supplemented with Tetracycline and Kanamycin

KBTK: King's B supplemented with Tetracycline and Kanamycin

L: liter

LB: Lysogeny Broth

LBa: LBagar

LBC: LB supplemented with Cycloheximide

LBCTK: LB supplemented with Cycloheximide, Tetracycline and Kanamycin

LBTK: LB supplemented with Tetracycline and Kanamycin

lin: lindane degradation genes

LSU: Large Subunit

m: meter

M: molar concentration

MGEs: Mobile Genetic Elements

min: minute

MLST/MLSA: Multilocus Sequence Typing/Analyses

mob: mobilization gene

mol: mole

MON: Montrond soil

mRNA: messenger Ribonucleic Acid

NB: Nutrient Broth

NBG: Nutrient Broth supplemented with Glycerol

NBGTK: Nutrient Broth supplemented with Glycerol, Tetracycline and Kanamycin

NCBI: National Center for Biotechnology Information

NOx: mono-nitrogen oxides NO and NO_2

OC: Open Circular

OD: Optical Density

p: p-value

PAMDB: Plant Associated and Environmental Microbes Database

PCA: Principal Component Analysis

PCCH: Pentachlorocyclohexene

PCR: Polymerase Chain Reaction

PEF: Pulsed Electric Field

pH: potential hydrogen

PhyML: Phylogenetic estimation using Maximum Likelihood

qPCR : quantitative Polymerase Chain Reaction

rDNA: ribosomal DNA

RDP: Ribosomal Database Project

REP: Repetitive Extragenic Palindromic sequences

rep: replication gene

RISA: Ribosomal Intergenic Spacer Analysis

RNA: Ribonucleic Acid

Roth: Rothamsted soil

rpm: revolutions per minute

rrs: SSU ribosomal RNA

RT: Reverse Transcription

RTG: Ready-To-Go

s: seconds

S: Siemens

SbyE: South by East

sd: standard deviation

SSU: Small Subunit

SW: SouthWest

SWbyS: SouthWest by South

SWbyW: SouthWest by West.

TBE: Tris-borate-EDTA buffer

TBS: Tris-buffered saline

TCB: Trichlorobenzene

TCDN: Tetrachlorocyclohexadiene

TE: Tris EDTA

Tet: Tetracycline

tet: tetracycline resistance gene

TK: Tetracycline and Kanamycin

Tn: Transposon

TS: Tris NaCl buffer

TSA-C: Trypticase soy agar supplemented with Cycloheximide

TSA-CTK: Trypticase soy agar supplemented with Cycloheximide, Tetracycline and Kanamycin

TSA-L: Trypticase soy agar supplemented with Lindane

TSA: Trypticase soy agar

UV: Ultraviolet

V: Volt

v/v: volume/volume

w/v: weight/volume

α: Alpha risk in statistics (Type I error)

Ω: Ohm

PREAMBULE - FRANÇAIS

Cette thèse, située dans le domaine de la microbiologie environnementale et effectuée dans un laboratoire pluridisciplinaire, a pour but d'étudier les interactions entre phénomènes physiques et organismes biologiques. Cette introduction générale a pour vocation de contextualiser le sujet en apportant les éléments essentiels de compréhension des différentes disciplines abordées. Le rôle et l'importance des bactéries dans l'environnement y seront notamment abordés et nous décrirons les transferts horizontaux de gènes dont l'électrotransformation et ses potentielles implications dans l'évolution des bactéries sur Terre et plus particulièrement dans les nuages. Le manuscrit étant principalement rédigé en langue anglaise, un résumé de la thèse, écrit en français, a été ajouté. Le choix de la langue anglaise pour l'écriture a été motivé par la présence d'un membre non francophone dans la composition du jury qui va évaluer ce travail, et pour permettre une large divulgation de ces travaux de recherche à l'étranger.

PREAMBLE - ENGLISH

This thesis, set in the field of environmental microbiology and performed in a multidisciplinary laboratory, aims at studying the interactions between physical phenomena and biological organisms. This general introduction aims to contextualize the subject by bringing essential elements of understanding about the different disciplines. The role and importance of bacteria in the environment will be approached and we will describe the horizontal gene transfers, of which the electrotransformation and its potential implication in the evolution of bacteria on Earth and especially in clouds will be discussed. The manuscript being mainly written in English, a French summary of the thesis has been added. The choice of the English language was motivated by the presence of a non-French-native member in the jury who is going to evaluate this work and to also allow a wide divulgation of these research works abroad.

SYNTHESE

INTRODUCTION :

Pendant plus de 3,5 milliards d'années d'évolution sur Terre, les bactéries se sont continuellement adaptées à un large spectre de conditions environnementales ce qui leur a permis de coloniser les écosystèmes les plus divers (Whitman *et al.*, 1998; Wiedenbeck and Cohan, 2011). Elles sont détectables presque partout dans la biosphère : dans le sol (Torsvik *et al.*, 1990), l'eau (LeChevallier *et al.*, 1980), l'air (Imshenetsky *et al.*, 1978), la pluie (Ahern *et al.*, 2007), la neige (Yan *et al.*, 2012) et les nuages (Amato *et al.*, 2005) ; leur abondance globale étant actuellement estimée à 5×10^{30} cellules (Whitman *et al.*, 1998). Les bactéries sont également rencontrées dans des environnements extrêmes tels que les mines acides (Baker and Banfield, 2003), les déserts (Chanal *et al.*, 2006), les sources hydrothermales aux températures extrêmes (Stetter, 1999), les milieux hyper-salins (Sorokin *et al.*, 2006), les grottes (Urzì *et al.*, 2010), les glaces polaires (Staley and Gosink, 1999), les sédiments des fonds sous-marins (Quigley and Colwell, 1968), les sites pollués (Hassen *et al.*, 1998), la croûte terrestre (Edwards *et al.*, 2003) et même les volcans (Lösekann *et al.*, 2007) et les déchets radioactifs (Fredrickson *et al.*, 2004). Outre leur large distribution, les bactéries ont une importance considérable sur les écosystèmes ; en effet, elles y occupent des fonctions primordiales telles que la dégradation et la minéralisation de la matière organique, évitant ainsi l'accumulation de molécules récalcitrantes difficiles à décomposer (Amon and Benner, 1996; Štursová *et al.*, 2012). Elles sont également les principaux acteurs des grands cycles biogéochimiques, tels ceux du carbone, de l'azote, du phosphore, du soufre, du magnésium… (Evans, 1976; Falkowski *et al.*, 2008; Godfrey and Glass, 2011). Nous leur devons aussi « l'invention » de la photosynthèse qui, dans les prémices de la vie sur terre, a permis l'enrichissement de l'atmosphère en oxygène (Edwards, 2004). Outre leur importance indéniable au sein des différents écosystèmes, les microorganismes sont utilisés dans plusieurs secteurs économiques. En effet, ils sont impliqués dans l'industrie agro-alimentaire et notamment dans la fabrication de produits issus de la fermentation : fromage, yaourt, bière, vin et cacao (Leroy and De Vuyst, 2004) ainsi qu'en biotechnologie et dans la production industrielle de nombreux médicaments et d'antibiotiques (Ishige *et al.*, 2005). Du point de vue environnemental, les bactéries jouent un rôle dans la bioremédiation (Furukawa, 2003; Mikesková *et al.*, 2012) avec pour exemple, le traitement des eaux usées et la dissolution des marées noires. On les emploie aussi dans le secteur minier pour la récupération de nombreux métaux (Rawlings, 2002). Ils occupent naturellement une place importante dans le secteur de

l’agriculture où leurs actions influencent la mise en place et le maintien de la fertilité des sols (Babalola, 2010), améliorent la croissance des plantes cultivées (Lucy *et al.*, 2004) et permettent de contrôler certains ravageurs (Raaijmakers *et al.*, 2002). Ils peuvent aussi interagir avec les insectes et d’autres organismes (Slater, 1984; Lerouge and Vanderleyden, 2002; O'Hara and Shanahan, 2006; Douglas, 2009; Grice *et al.*, 2009; Mansfield *et al.*, 2012; Vorholt, 2012) et sont également retrouvés chez l'Homme et les animaux, sur la peau et dans le tube digestif où ils vont jouer un rôle important de défense naturelle contre les pathogènes (Thompson, 1978) mais aussi d’aide à la digestion de certains aliments (Tappenden and Deutsch, 2007). Malheureusement, ils sont également responsables de bon nombre de maladies infectieuses, touchant aussi bien l’Homme (World Health Organization, 2013) que les animaux et sont responsables d’importantes pertes économiques en agriculture par l’infection de nombreuses espèces de plantes (Mansfield *et al.*, 2012).

Contrairement à la transmission verticale « traditionnelle » de caractères génétiques des deux parents aux descendants menant à un certain degré de diversité chez les êtres supérieurs, la division bactérienne par scissiparité aboutit à un faible degré de variations génétiques entre les cellules. Pour diversifier leur matériel génétique, s’adapter aux perturbations environnementales et coloniser de nouvelles niches (Wiedenbeck and Cohan, 2011), les processus évolutifs bactériens comprennent notamment l’évolution par mutations, se traduisant par modification de la séquence nucléique du génome. Plusieurs facteurs sont connus comme étant mutagènes: l’exposition aux rayons UV, les conditions de stress oxydatif (Demple, 1991) mais aussi les enzymes cellulaires comme l’ADN polymérase impliquée dans le maintien, la réplication et la réparation des molécules d’ADN et connues pour commettre des erreurs dans la séquence d’ADN (1 erreur par 10 000 000 de bases) (Voliotis *et al.*, 2012). Dans la plupart des cas, ces mutations sont délétères car elles modifient les gènes clés entraînant leur inactivation ou la production de protéines altérées ou non fonctionnelles ce qui peut ainsi empêcher les bactéries de passer la barrière de la sélection naturelle (Loewe *et al.*, 2003). Cependant, certaines mutations peuvent être neutres ou bénéfiques et augmenter rapidement la valeur adaptative ou « fitness » des bactéries leur permettant ainsi de s’adapter aux conditions environnementales fluctuantes (Matic *et al.*, 1997; Gordo *et al.*, 2011). Les processus endogènes comme le déplacement d’éléments génétiques mobiles (y compris les transposons et séquences d’insertions) d’un endroit à un autre du génome sont également impliqués dans la variabilité génétique (Mahillon and Chandler, 1998; Fehér *et al.*, 2012). Toutefois, l’adaptation par mutations est un phénomène lent et ces deux mécanismes

(mutations et processus endogènes) ne peuvent expliquer à eux seuls l'incroyable potentiel d'évolution et d'adaptation des bactéries à leur environnement.

Les transferts horizontaux de gènes (THG) ont ensuite été proposés comme moteur important de l'évolution des génomes procaryotes par acquisition de nouvelles informations génétiques (Ochiai *et al.*, 1959; Syvanen, 1985; Jain *et al.*, 2002; Aminov, 2011; Zhaxybayeva and Doolittle, 2011). Ces transferts se produisent rapidement et de manière constante comparés aux mutations, à des taux relativement élevés. Au sein d'espèces proches, ces transferts peuvent contrecarrer les effets délétères des instabilités génomiques dans le core-génome (Fall *et al.*, 2007; Treangen *et al.*, 2008). Les THG provoquent souvent des changements drastiques dans le caractère écologique des espèces bactériennes par l'introduction de nouveaux traits physiologiques provenant d'organismes lointainement apparentés, promouvant ainsi la diversification et la spéciation microbienne (Dutta and Pan, 2002; Gogarten *et al.*, 2002). Ces transferts permettent la dissémination de gènes appropriés parmi les populations bactériennes menant à leur adaptation rapide aux fluctuations environnementales ainsi qu'aux conditions les plus sévères (Koonin *et al.*, 2001). Le THG a été reconnu comme étant une force majeure qui permet aux bactéries de coloniser avec succès la plupart des niches écologiques disponibles sur Terre (Wiedenbeck and Cohan, 2011). L'évolution des études sur les gènes et les génomes indique que de nombreux THG ont eu lieu entre procaryotes et que ces transferts ont joué un rôle majeur dans l'évolution des génomes bactériens (Lan and Reeves, 1996; Ochman *et al.*, 2000; Koonin *et al.*, 2001; Zaneveld *et al.*, 2008). Différents types de matériel génétique, les éléments génétiques mobiles (EGMs ou mobilome) peuvent être transférés entre les cellules bactériennes: les transposons, les intégrons, les séquences d'insertion, les îlots génomiques, les éléments liés aux bactériophages, les plasmides… Ces divers éléments ne sont pas impliqués de la même façon dans les différents types de transferts de gènes (Top *et al.*, 2002; Nojiri *et al.*, 2004; Popa and Dagan, 2011). Les plasmides sont des éléments prépondérants au sein du mobilome car ils présentent l'avantage de se répliquer de manière autonome et peuvent être intégrés dans les chromosomes bactériens. Ils codent des protéines clés impliquées dans de nombreux aspects de l'écologie microbienne notamment la détoxification, la virulence, la fertilité, les interactions écologiques, la production de bactériocines et la résistance aux antibiotiques (Smillie *et al.*, 2010; Andersson and Hughes, 2011; Liang *et al.*, 2012).

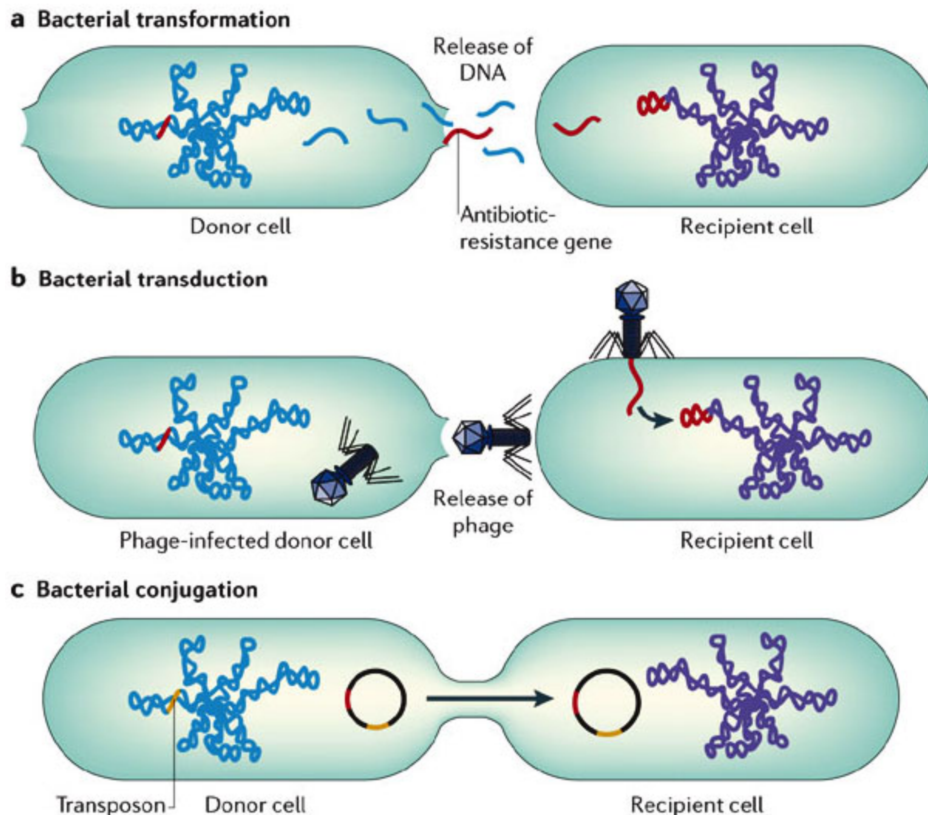


Figure 1: Transferts horizontaux de gènes entre bactéries

a) La transformation se produit quand de l'ADN exogène est libéré d'un organisme et est pris en charge par un autre. La séquence d'ADN peut être intégrée au chromosome ou au plasmide de la cellule réceptrice.

b) Lors de la transduction, les gènes sont transférés d'une bactérie à une autre grâce aux bactériophages et peuvent être intégrés dans le chromosome de la cellule réceptrice.

c) La conjugaison est le transfert de plasmides entre deux bactéries ayant établi un contact physique temporaire, pouvant entraîner l'acquisition de gènes de résistance aux antibiotiques par la cellule réceptrice. Les transposons sont des séquences d'ADN qui portent leurs propres enzymes de recombinaison permettant la transposition d'un site à un autre; les transposons peuvent également porter des gènes de résistance aux antibiotiques (Furuya and Lowy, 2006).

Il existe trois types de mécanismes pour l'acquisition d'information génétique par transfert horizontal (Figure 1) (Popa and Dagan, 2011):

- La **conjugaison** qui implique l'échange direct d'ADN entre deux bactéries *via* la constitution d'un *pilus* sexuel (Lederberg and Tatum, 1953).

- La **transduction :** le transport de matériel génétique entre deux bactéries par des vecteurs appelés bactériophages.

- La **transformation naturelle,** qui est l'intégration d'ADN nu présent dans l'environnement proche de la cellule. Ce procédé implique quatre étapes principales : (i) la liaison entre l'ADN exogène et la surface de la cellule, (ii) la prise en charge par la cellule, (iii) l'intégration dans le génome bactérien (ou la réplication autonome dans le cas de l'acquisition d'un plasmide) et (iv) l'expression des gènes acquis (Claverys *et al.*, 2009). L'intégration de l'ADN entrant dans des structures génétiques existantes, comme le chromosome ou les plasmides, requiert la mise en place de recombinaison homologue ou illégitime (Lawrence, 2002; Thomas and Nielsen, 2005). Des représentants naturellement transformables ont été détectés dans environ 1% des espèces bactériennes décrites (Thomas and Nielsen, 2005). La transformation naturelle requiert l'expression de gènes spécifiques pour le développement du stade de compétence nécessaire à la prise en charge de l'ADN. Cet état est induit par des signaux spécifiques et nécessite une machinerie de régulation qui n'est pas présente chez toutes les bactéries (Claverys *et al.*, 2009). Outre la nécessité de cellules spécifiques, la disponibilité de l'ADN exogène dans l'environnement est un autre obstacle à la transformation naturelle (Paget *et al.*, 1992; Paget and Simonet, 1994). Il est difficile de prédire le développement de la compétence dans un système hétérogène et complexe comme le sol (Johnsborg *et al.*, 2007; Levy-Booth *et al.*, 2007). Dans le sol, l'ADN est préférentiellement libéré par la matière en décomposition ou la désintégration cellulaire (de plantes, champignons, insectes, bactéries, particules virales…), ou par l'excrétion active de certaines cellules (Thomas and Nielsen, 2005). Une partie importante de l'ADN libéré est rapidement dégradée mais une autre partie est adsorbée sur les particules de sol et peut persister dans l'environnement pendant des semaines voire des mois après sa libération hors des cellules (Romanowski *et al.*, 1993). Il a été montré que l'ADN adsorbé était partiellement disponible pour la transformation bactérienne mais aussi accessible aux nucléases (Demanèche *et al.*, 2001a).

Ces trois mécanismes naturels peuvent être utilisés en laboratoire mais leur efficacité dépend de plusieurs facteurs comme l'expression d'un lot de fonctions génétiquement

régulées, la proximité entre cellules pour la conjugaison ou encore la spécificité d'hôte (Thomas and Nielsen, 2005; Johnsborg *et al.*, 2007; Levy-Booth *et al.*, 2007; Claverys *et al.*, 2009; Popa and Dagan, 2011). Du fait de ces limites, des méthodes ont été développées afin d'introduire du matériel génétique dans les cellules (procaryotes et eucaryotes). En effet, l'entrée d'ADN dans les cellules peut être stimulée par différents facteurs: thermiques, chimiques (e.g., les sels) ou électriques par exemple, provoquant la perméabilisation des membranes cellulaires (Aune and Aachmann, 2010). Parmi ces techniques, l'électroporation, développée dans les années 60, est l'application d'un choc électrique entraînant la perméabilisation des membranes (Maniatis *et al.*, 1982). Les effets d'un champ électrique sur une cellule peuvent être décrits en considérant la cellule comme le corps conducteur (le cytoplasme) entouré par une couche diélectrique (la membrane) (Deng *et al.*, 2003). L'application d'un champ électrique entraîne l'accumulation de charges électriques de polarités opposées de chaque côté de la membrane créant ainsi une chute du voltage dans celle-ci. Si ce voltage membranaire n'est pas excessif et la durée d'impulsion limitée, les importants changements de structure de membrane qui en résultent sont réversibles. Ainsi, les champs électriques au-dessus d'une valeur seuil perméabilisent les membranes, et le courant permet la migration passive de l'ADN exogène à travers la membrane : c'est l'électrotransformation. Bien que beaucoup d'études montrent l'efficacité de l'électroporation sur de nombreux taxons bactériens, ainsi que sur les cellules de champignons, de plantes ou d'animaux (Maniatis *et al.*, 1982; Dower *et al.*, 1988; Chakraborty and Kapoor, 1990; Gilchrist and Smit, 1991; Drury, 1996; Weaver and Chizmadzhev, 1996; Lurquin, 1997; Newell, 2000; Villemejane and Mir, 2009), l'efficacité d'électrotransformation diffère souvent entre espèces, nécessitant généralement des préparations spécifiques des cellules pour une efficacité maximale de transformation (Drury, 1996).

Ces méthodes de laboratoire introduisant de l'ADN dans les bactéries grâce à des décharges électriques imitent ce qui peut se passer naturellement dans l'environnement pendant les décharges de foudre, durant les orages. Un orage est une turbulence dont le processus comprend un développement progressif de nuages orageux (de cumulus à cumulonimbus) suivi de décharges de foudre et d'un effet acoustique dans l'atmosphère terrestre appelé tonnerre. Ces phénomènes sont généralement accompagnés par des vents forts et des précipitations (Gary, 1999). Les paramètres électriques relatifs à ce processus ont été largement étudiés étape par étape et sont maintenant bien caractérisés (Rhouma and Auriol, 1997).

Dans les nuages orageux, les rapides courants d'air provoquent des collisions et clivages entre les cristaux, entraînant la séparation des charges électriques (Gary, 1999; Aguet and Ianoz, 2001). Cette séparation des charges accroit la quantité de charges électriques de signes opposés en bas et au sommet du nuage, entraînant une accumulation de charges au sol. Par exemple, dans les climats tempérés, les nuages sont chargés positivement dans la zone supérieure du nuage et négativement en bas du nuage, ce qui entraîne des modifications de charges (positives) et une distribution du champ électrique au sol. Les champs électriques élevés résultant de l'accumulation de charges électriques à l'intérieur des nuages ou entre les nuages et la Terre, induisent la formation de canaux ioniques. En effet, la différence de voltage entre le nuage et le sol peut atteindre jusqu'à 100 MV, ce qui correspond à environ 16 $kV.m^{-1}$ pour les surfaces terrestres plates, et jusqu'à 700 $kV.m^{-1}$ localement, dû à un effet de pointe. La foudre (éclair et bruit) est due aux flux de courant électrique élevés à l'intérieur du canal ionique qui relie le sol au nuage. Plusieurs impulsions de courant d'intensités élevées peuvent être délivrées et correspondent aux éclairs (Gary, 1999; Aguet and Ianoz, 2001).

Au niveau terrestre, ces courants pénètrent le sol sur une petite surface (quelques centimètres carrés) avec un pic d'intensité de courant des éclairs variant de 10 à 200 kA (Rhouma and Auriol, 1997). Tout près du point d'impact de la foudre, il se peut que les cellules bactériennes soient détruites mais la foudre crée un champ électrique dont la valeur est du même ordre que ceux associés à l'électroporation (6 $kV.cm^{-1}$*versus* 12.5 $kV.cm^{-1}$) avec une injection de courant supposée se répandre sur environ 2 m^2 de surface de sol, conduisant également à des valeurs similaires (12 $kA.m^{-2}$ *versus* 5 $kA.m^{-2}$)(Demanèche *et al.*, 2001c). Ces données suggèrent que la foudre pourrait agir naturellement en tant qu'« électroporateur » *in situ* pour servir de médiateur au transfert de gènes chez les bactéries environnementales. L'environnement étant régulièrement soumis à des orages et décharges de foudre qui induisent d'énormes perturbations électriques, la possibilité d'un mécanisme d'« électrotransformation naturelle » des bactéries pourrait expliquer la nette divergence entre les fréquences de THG déduites d'analyses *in silico* et celles relevées d'expériences *in situ* (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994; Davison, 1999). Basés sur cette hypothèse, Demanèche *et al.* (2001c) ont testé le potentiel d'électrotransformation de cellules d'*Escherichia coli* inoculées dans des microcosmes de sol *via* des injections de courant simulant la foudre et des champs électriques de nuages orageux. Le générateur haute-tension utilisé pour simuler la foudre délivrait un champ électrique avec des paramètres électriques (voltage 7 $kV.cm^{-1}$, courant 2.5 $kA.m^{-2}$) similaires à ceux de la foudre grandeur nature (6 $kV.cm^{-1}$et12 $kA.m^{-2}$). Ces expériences ont réussi à démontrer la présence d'événements de

transferts de plasmides dans le sol par électrotransformation des bactéries, obtenue par simulation des paramètres électriques de la foudre. Le courant injecté dans le sol par la foudre se répand de façon plus ou moins uniforme en fonction de l'homogénéité du sol (Cérémonie *et al.*, 2006), son intensité diminuant avec la distance du point d'impact de la foudre. Ceci a conduit les auteurs à estimer le volume de sol affecté par des paramètres électriques compatibles avec l'électrotransformation variant de 2 à 950 m^3 par décharge de foudre. L'efficacité d'électrotransformation *in situ* dépend également de la densité et de la localisation des cellules réceptrices potentielles dans les compartiments internes et externes du sol mais aussi de la concentration et de la disponibilité de l'ADN extracellulaire libre dans le sol ou adsorbé sur les particules de sol. Les décharges de foudre pourraient également augmenter le pool d'ADN libre en tuant les bactéries présentes dans la zone d'impact, libérant ainsi leur contenu génomique, et peuvent aussi contribuer à la désorption de l'ADN des particules de sol (Cérémonie *et al.*, 2008).

En considérant ces différentes données, ainsi que la fréquence des décharges de foudre (vingt impacts de foudre atteignent le sol chaque seconde à travers le monde), l'électrotransformation a été proposée comme quatrième mécanisme potentiel de transfert de gènes chez les bactéries du sol (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006). Cérémonie *et al.* (2004) ont isolé deux bactéries électrocompétentes du sol et identifié comme étant des *Pseudomonas* sp. Elles présentaient une fréquence d'électrotransformation dans le sol de quelques ordres de grandeur de plus qu'*E. coli* (10^{-4} to 10^{-5} *versus* 10^{-5} to 10^{-8} electrotransformants par cellule réceptrice) ce qui a permis de confirmer le rôle potentiel de l'électrotransformation naturelle comme mécanisme adaptatif des bactéries du sol (Figure 2A). Ce potentiel d'électrotransformation naturelle lié à la foudre pourrait dont être utilisé pour diverses applications notamment la bioremédiation des sols pollués par ajout d'ADN contenant des gènes cataboliques.

De plus, tandis que le sol est soumis à seulement un cinquième des impacts de foudre mondiaux, les nuages sont bien plus exposés avec cent canaux de foudre formés à l'intérieur des nuages toutes les secondes à travers le monde (Gary, 1999). Etant données les conditions de température (environ 2000°C), d'intensité (>200 A) et de voltage (>100 kV) rencontrées dans un canal de foudre, la survie et donc l'électroporation ne serait pas possible (Gary, 1999; Aguet and Ianoz, 2001). Cependant, hormis au centre du canal de foudre, de nombreuses bactéries sont capables de survivre, de persister et de se développer dans les nuages (Whitman *et al.*, 1998; Sattler *et al.*, 2001; Amato *et al.*, 2005; Amato *et al.*, 2007b; Amato *et al.*, 2007a; Vaïtilingom *et al.*, 2013).

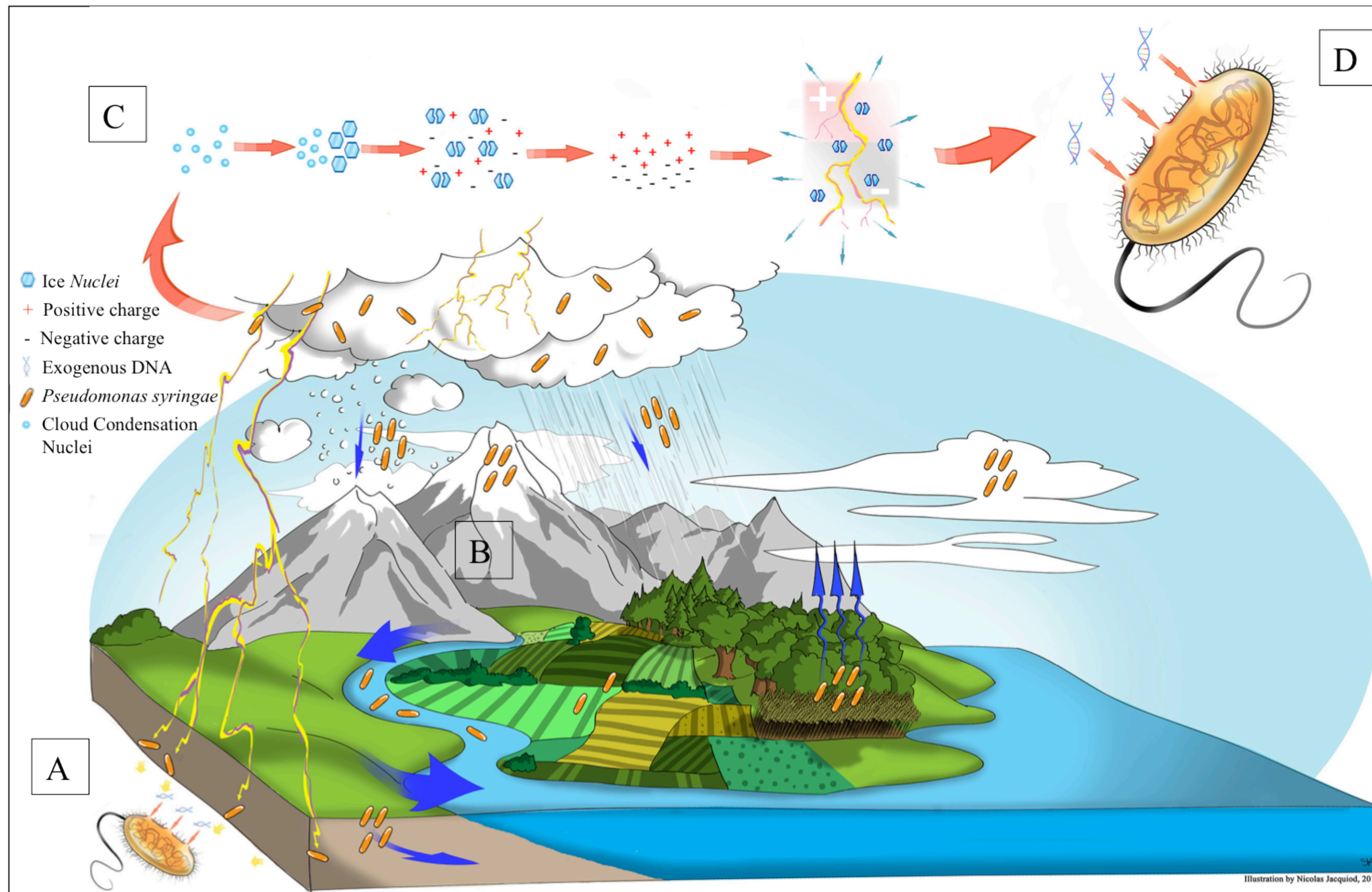


Figure 2: Cycle terrestre d'électrotransformation des bactéries par la foudre

(A) Les bactéries du sol peuvent être soumises à des décharges électriques associées aux décharges de foudre et compatibles avec l'électrotransformation *in situ*. (B) *Pseudomonas syringae* est retrouvée dans les eaux douces, sur les plantes, dans les champs et les forêts, et dans les nuages révélant ainsi son association avec le cycle de l'eau. (C) L'activité glaçogène de *P. syringae* dans les nuages peut causer des collisions et clivages de la glace, entraînant la séparation et l'accumulation des charges électriques pouvant générer des décharges de foudre. (D) Les impulsions électriques formées avant et pendant le foudroiement peuvent potentiellement électrotransformer les bactéries des nuages avec de l'ADN exogène.

En effet, certaines bactéries sont aérosolisées à des altitudes de 40 km et plus (Imshenetsky *et al.*, 1978) grâce à des courants d'air convectifs ascendants provenant du sol, de la végétation et aussi grâce aux éclatements de bulles au niveau des surfaces aqueuses (Lighthart, 1997; Delort *et al.*, 2010; Morris *et al.*, 2011). Des microorganismes ou des particules de glaces chargées pouvant contenir des microorganismes peuvent aussi être aérosolisées dans la stratosphère par les champs électriques liés à la formation des orages (Dehel *et al.*, 2008). Le nombre total de procaryotes dans l'air atteint 10^{19} à l'échelle mondiale, ce qui pourrait suffire à affecter les processus biogéochimiques (Whitman *et al.*, 1998; Bauer *et al.*, 2002). Les nuages peuvent ainsi jouer un rôle majeur dans la dissémination transitoire des microorganismes aéroportés sur de longues distances (Bovallius *et al.*, 1978; Griffin *et al.*, 2001; Griffin *et al.*, 2002; Smith *et al.*, 2011; Hara and Zhang, 2012; Smith *et al.*, 2012; Yamaguchi *et al.*, 2012; Creamean *et al.*, 2013) en les ramenant ensuite sur Terre par dépôt humide (Vali *et al.*, 1976; Delort *et al.*, 2010). Ces microorganismes trouvent leurs nutriments durant leur transport sur les poussières et d'autres ont été trouvés fonctionnellement stables dans des grêlons (Šantl-Temkiv *et al.*, 2012; Šantl-Temkiv *et al.*, 2013), dans des gouttelettes de brouillard servant de milieu de culture (Fuzzi *et al.*, 1997), ainsi qu'en croissance active dans l'eau des nuages et les précipitations de neige et de pluie (Casareto *et al.*, 1996; Carpenter *et al.*, 2000; Sattler *et al.*, 2001; Amato *et al.*, 2007b; Amato *et al.*, 2007a; Vaïtilingom *et al.*, 2013). Pour se disséminer à travers la haute atmosphère, les bactéries doivent survivre à des conditions stressantes comme le manque de nutriments, le vent, les conditions d'oxydation élevée, la dessiccation, les températures basses, le pH acide, les radiations UV, les phases répétées de gel-dégel et les chocs osmotiques (Deguillaume *et al.*, 2008; Delort *et al.*, 2010; Wilson *et al.*, 2012). Parmi les bactéries de l'atmosphère, certaines exploitent les propriétés des processus de condensation afin d'être protégées contre la dessiccation, les radiations UV et les phases répétées de gel-dégel. Elles peuvent agir comme noyaux de condensation en induisant la formation de gouttelettes par dépôt de vapeur d'eau sur leur surface (Bauer *et al.*, 2003; Sun and Ariya, 2006; Möhler *et al.*, 2007) et comme noyaux glaçogènes catalysant la formation de cristaux de glace à partir d'eau surfondue à des températures supérieures à l'activité des noyaux glaçogènes minéraux (jusqu'à -2°C *versus* -10°C) (Maki and Willoughby, 1978; Vali, 1996; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Möhler *et al.*, 2008). Ces deux mécanismes de production de noyaux permettent la formation de nuages et de précipitations (pluie et neige) par croissance et multiplication des cristaux de glace dans les nuages (Mossop and Hallett, 1974; Vali *et al.*, 1976; Morris *et al.*, 2004; Möhler *et al.*, 2007; Christner *et al.*, 2008).

Certaines des bactéries fréquemment isolées d'environnements où les conditions sont rigoureuses comme la surface des plantes ou le permafrost sont d'ailleurs détectées dans la haute atmosphère (Lighthart, 1997; Amato *et al.*, 2005; Amato *et al.*, 2007c; Amato *et al.*, 2010; Delort *et al.*, 2010). Parmi elles, *Pseudomonas syringae*, est une des bactéries glaçogènes les plus connues et appartient également à un des complexes d'espèces les plus phytopathogènes causant d'importantes pertes économiques sur une large gamme de cultures de fruits et légumes, notamment par sa contribution à la congélation des bourgeons à des températures supérieures à l'activité des glaçogènes minéraux (Maki *et al.*, 1974; Arny *et al.*, 1976; Maki and Willoughby, 1978; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Kennelly *et al.*, 2007; Möhler *et al.*, 2008). *P. syringae* pourrait se disperser sur les cultures par la pluie du fait de sa présence à presque toutes les étapes du cycle de l'eau *i.e.,* au niveau des épilithons des rivières, des ruisseaux, des lacs, des fossés d'irrigation, du manteau neigeux, des aérosols ascendants et des nuages (Maki and Willoughby, 1978; Constantinidou *et al.*, 1990; Morris *et al.*, 2004; Morris *et al.*, 2008; Morris *et al.*, 2010; Morris *et al.*, 2011; Monteil *et al.*, 2012; Morris and Sands, 2012) (Figure 2B). Il se peut que les bactéries glaçogènes y compris *P. syringae* soient impliquées dans le déclenchement de la foudre dans les nuages (Figure 2C) comme cela a été suggéré par modélisation (Gonçalves *et al.*, 2012), les impulsions électriques (de quelques $kV.cm^{-1}$) associées aux courants loin du cœur du canal de foudre pouvant potentiellement induire une électrotransformation génétiques des bactéries (Nucci *et al.*, 1988; Gonçalves *et al.*, 2012) (Figure 2D) de la même manière que ce qui a été décrit chez les bactéries du sol (Demanèche *et al.*, 2001c).

Cela nous a conduits à émettre l'hypothèse qu'un pathogène de plantes tel que *P. syringae* pourrait utiliser son potentiel glaçogène afin d'induire le déclenchement de phénomènes pouvant contribuer à l'augmentation de son potentiel adaptatif par l'acquisition de nouveaux gènes par électrotransformation. Combiné à un fort potentiel de dissémination par le biais de précipitations provenant des nuages, *P. syringae* pourrait améliorer ses capacités phytopathogènes ainsi que d'autres propriétés liées à son potentiel adaptatif et coloniser de nouvelles niches (Jackson, 2009), notamment les sols, les lacs, la neige et les océans. Cependant, de telles capacités adaptatives nécessiteraient la survie des bactéries aux décharges de foudre et certaines capacités d'électrotransformation afin qu'elles puissent tirer avantage de ces impulsions électriques associées aux courants de foudre.

C'est dans ce contexte qu'est située ma thèse de doctorat, financée par le Ministère Français de l'Education. Ce projet a été développé dans l'objectif de contribuer à une meilleure compréhension des mécanismes gouvernant l'évolution des bactéries notamment

l'impact de la foudre pour favoriser le transfert de gènes chez les bactéries environnementales.

Mon travail de thèse a été organisé autour de trois axes de recherche majeurs:

I : L'étude *in vitro* de paramètres électriques sur les bactéries potentiellement soumises à la foudre dans les nuages et/ou au sol.

II : L'étude de l'impact de la foudre sur la survie et l'électrotransformation des bactéries de la pluie.

Nous avons donc conduit des expériences *in vitro* afin de déterminer si *Escherichia coli*, *Pseudomonas* sp. N3, *P. syringae* (Chapitre I) et les bactéries de la pluie (Chapitre II) seraient capables de survivre aux conditions particulièrement rigoureuses rencontrées durant leur transport et leur séjour dans les nuages, et notamment aux impulsions électriques relatives aux décharges de foudre, et si elles pourraient profiter de ces impulsions associées aux courants électriques pour acquérir de nouveaux gènes.

III : Le développement d'un outil basé sur l'électrotransformation pour la bioremédiation de sols contaminés par du lindane, un pesticide qui a été largement utilisé avant son interdiction en 2006, incluant l'étude de l'impact de décharges électriques simulant la foudre sur les structures des communautés bactériennes.

Une conclusion générale clôt cette étude, où des perspectives sont évoquées.

Tableau 1: Correspondance entre les conditions testées en laboratoire et la littérature

	Expérience de laboratoire (*in vitro*)	**Etat de l'art**
a-	9,5 *versus* 12,5 $kV.cm^{-1}$	Quelques $kV.cm^{-1}$ (Nucci *et al.*, 1988; Wendt-Potthoff *et al.*, 1992; Bassett and Janisiewicz, 2003)
b-	Conditions glacées	Noyau glaçogène (Morris *et al.*, 2004)
c-	Un à cinq chocs	Plusieurs impacts de foudre (Gonçalves *et al.*, 2012)
d-	Phase exponentielle *versus* stationnaire	Bactéries en croissance active (Sattler *et al.*, 2001)
e-	De 10^4 à 10^8 bactéries	10^4 cellules par m^3 de nuage (Amato *et al.*, 2005)
f-	Ajout de plasmide 30 s à 30 min après le choc	Pores métastables (Smith *et al.*, 2004)
g-	Plasmide provenant de *P. syringae versus E. coli*	Provenance du plasmide (Bassett and Janisiewicz, 2003)

I. ETUDE DES PARAMETRES D'ELECTROPORATION SUR LES BACTERIES SOUMISES A LA FOUDRE DANS LES NUAGES ET SUR TERRE.

Introduction

Dans ce chapitre, l'objectif a été de réaliser des expériences *in vitro* afin de déterminer le comportement de la bactérie glaçogène *P. syringae* CC0094, utilisée comme modèle, en terme de survie et de capacité à être génétiquement transformée dans différentes conditions d'électroporation (simulant une gamme de paramètres électriques rencontrés durant les décharges de foudre affectant les nuages), comparée à la bactérie hautement électrocompétente *Pseudomonas* sp.N3 isolée du sol (Cérémonie *et al.*, 2004, 2006) et à la bactérie type de laboratoire *E. coli* TOP10.

Etant données les conditions physiques et chimiques auxquelles sont soumises les bactéries dans les nuages, des tests d'électroporation ont été développés *in vitro* avec l'objectif de simuler certaines des conditions pouvant être rencontrées dans les nuages (Tableau 1) notamment la température et l'état du milieu (tests dans la glace), la possibilité d'être choquées électriquement plusieurs fois (application de 2 à 5 chocs électriques successifs), différents voltages (9.5 *versus* 12.5 $kV.cm^{-1}$), un contact différé entre bactéries et ADN (ajout du plasmide de 30 s à 30 min après le[s] choc[s] électrique[s]), une densité variable de bactéries (entre 10^4 et 10^8 UFC par réaction), le stade physiologique des bactéries (phase stationnaire *versus* phase active de croissance) et une origine exogène d'ADN transformant (plasmides extraits d'*E. coli versus P. syringae*). Les paramètres tels que la survie et la fréquence de transformation ont été étudiés après soumission des bactéries à ces différents tests d'électroporation. De plus, une étude de l'évolution de l'intégrité du génome de *P. syringae* a été réalisée.

1. Potentiel de survie

Le potentiel de survie des bactéries après une décharge de foudre est un des plus importants paramètres nécessaires à l'adaptation des bactéries dans les nuages. Selon Sambrook et Russell (2001), les facteurs électriques létaux pour les bactéries sont la force du champ électrique et la durée d'électroporation. Au-dessus d'une certaine limite, chaque facteur seul ou en combinaison, détruit la cellule par rupture de la membrane et libère des composants cellulaires. Certaines bactéries peuvent mieux survivre aux impulsions électriques

Tableau 2: Pourcentage de survie des trois bactéries étudiées après différents traitements.

Taux de survie	*P. syringae* CC0094	*Pseudomonas* sp.N3	*E. coli* TOP10
Liquide (condition standard)	15,00 ± 8,62%	11,55 ± 9,36%	19,25 ± 9,12%
9.5 $kV.cm^{-1}$	17,77 ± 4,34%	/	/
Milieu glacé	60,21 ± 7,72%*	33,44 ± 1,64%	18,52 ± 10,65%
3 chocs	1,60 ± 0,73%	5,25 ± 3,87%	6,45 ± 3,54%
5 chocs	1,53 ± 0,80%	3,16 ± 2,08%	3,34 ± 1,37%
Ajout de plasmide 30 sondes après choc	16,55 ± 9,98%	4,84 ± 2,09%	11,19 ± 2,91%
10^6 bactéries	/	0,88 ± 0,18%	/
10^5 bactéries	/	2,16 ± 1,18%	/
10^4 bactéries	4,09 ± 0,38%	1,16 ± 0,49%	33,86 ± 13,44%
10^3 bactéries	3,41 ± 1,19%	20,46 ± 24,02%	22,84 ± 2,24%
10^2 bactéries	5,13 ± 0,45%	1,39 ± 1,96%	9,80 ± 6,06%
Phase stationnaire	37,29 ± 5,05%	/	/
pBLN de *P. syringae* CC0094	33,56 ± 9,14%	/	/

Les valeurs correspondent aux moyennes et écarts types des taux de survie d'au moins trois expériences. Les astérisques montrent quelles valeurs sont significativement différentes ($p<0.05$) de la condition standard.

Tableau 3: Pourcentage de survie des trois bactéries contenant pBLN étudiées après zéro à cinq chocs.

	P. syringae CC0094 + pBLN	*Pseudomonas* sp. N3 + pBLN	*E. coli* TOP10 + pBLN
Sans choc	100,00 ± 0,00%*	100,00 ± 0,00%*	100,00 ± 0,00%*
1 choc (condition standard)	26,48 ± 15,20%	19,98 ± 18,86%	87,94 ± 97,06%
2 chocs	14,41 ± 9,00%	16,96 ± 8,43%	63,13 ± 70,50%
3 chocs	14,40 ± 10,64%	15,31 ± 11,82%	59,02 ± 71,33%
4 chocs	5,92 ± 5,11%	20,56 ± 18,04%	49,99 ± 63,62%
5 chocs	5,47 ± 6,90%	2,59 ± 1,63%	16,24 ± 11,39%

Les valeurs correspondent aux moyennes et écarts types des taux de survie d'au moins trois expériences. Les astérisques montrent quelles valeurs sont significativement différentes ($p<0.05$) de la condition standard.

et d'autres y sont extrêmement sensibles, probablement à cause de dommages irréversibles subis par leur membrane. Le pourcentage de survie après choc électrique a été calculé comme le ratio du nombre de bactéries survivantes sur le nombre de bactéries totales non choquées. Nos résultats ont montré que les trois bactéries étudiées *i.e., P. syringae* CC0094, *P.* sp. N3 et *E. coli* TOP10 présentaient les mêmes taux de survie (entre 11 et 20% - Tableau 2) quand un même nombre de cellules en suspension dans un volume identique était soumis à une électroporation en condition standard (Drury, 1996) comprenant l'utilisation de cuvettes d'électroporation de 2mm pour une décharge de 9,5 ou 12,5 $kV.cm^{-1}$ (Wendt-Potthoff *et al.*, 1992; Bassett and Janisiewicz, 2003), 25 µF, 200 Ω, 5 ms et une incubation immédiate des cellules électroporées dans les milieux de croissance pour favoriser leur récupération. Les bactéries glaçogènes ne semblaient donc pas avoir un avantage sur les bactéries non glaçogènes après soumission à une décharge électrique.

Puisque Gonçalves *et al.* (2012) ont démontré l'augmentation significative du nombre d'impulsions électriques en présence de bactéries glaçogènes dans les nuages, nous avons ensuite voulu évaluer si les bactéries de notre étude répondraient de la même manière aux chocs électriques multiples (2 à 5 chocs répétés à 12,5 $kV.cm^{-1}$, 25 µF, 200 Ω durant 5 ms). Le taux de survie d'*E. coli* TOP10, *P.*sp.N3, *P. syringae* CC0094 (contenant ou ne contenant pas le plasmide pBLN) n'a pas diminué significativement après des chocs répétés, comparé à la condition standard avec un seul choc (Tableaux 2 & 3). Par conséquent, les trois bactéries pourraient survivre à au moins 5 chocs, sans avantage spécifique de la part des *P. syringae* glaçogènes.

Après un choc dans la glace (électroporation standard mais après congélation de la suspension pendant 10 minutes à -20°C), le taux de survie de *P. syringae* CC0094 a augmenté de manière significative pour atteindre 60.21% tandis que la survie d'*E. coli* TOP10 était identique en milieu liquide ou glacé et *Pseudomonas* sp. N3 a montré un comportement intermédiaire (augmentation modérée du taux de survie en condition glacée mais non significativement différente de la condition standard liquide) (Tableau 2). Etant donné que *P. syringae* est capable de former des noyaux glaçogènes à des températures proches de -6°C (Vali, 1996; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Möhler *et al.*, 2008), tandis que les deux autres en sont incapables, nous avons émis l'hypothèse que les cristaux de glace formés pourraient protéger les cellules de *P. syringae* de la décharge de foudre. Ce phénomène pourrait être expliqué par la faible conductivité de la glace puisque la résistance

de la pluie glacée que nous avons mesurée (45 MΩ) correspondait à une conductivité (1.26 $\mu S.m^{-1}$) plus faible que celle de l'eau liquide. Les conductivités des eaux souterraines et de l'eau distillée atteignent environ 5 $mS.m^{-1}$et 10 $\mu S.m^{-1}$, respectivement, tandis que l'eau de mer est très conductrice (4.80 $S.m^{-1}$) dû à la présence de sels (Cox *et al.*, 1967; Maxwell *et al.*, 1971; Saxena and Ahmed, 2001; Pashley *et al.*, 2005). La protection de la glace aux décharges électriques pourrait également être expliquée par la faible disponibilité des molécules d'eau liquide puisque la formation réversible de pores est supposée être due au gradient électrique à l'interface entre les couches lipidiques et aqueuses et au déplacement conséquent des molécules d'eau dans le gradient et probablement à travers la membrane qui accélère le processus de formation des pores (Tieleman, 2004). Ainsi, les bactéries ayant un pouvoir glaçogène pourraient posséder un avantage pour survivre dans les environnements froids comme les nuages. Afin de confirmer cette hypothèse de survie relative à la protection de la glace, des essais plus approfondis devraient se focaliser sur l'évaluation du taux de survie de mutants *ina-* de *P. syringae* CC0094 (ayant perdu leur propriété glaçogène) et de souches environnementales.

Un autre facteur à prendre en considération est le stade de croissance des bactéries. Bien qu'elles aient été isolées en croissance active dans les nuages (Sattler *et al.*, 2001; Amato *et al.*, 2007b; Amato *et al.*, 2007a; Vaïtilingom *et al.*, 2013), toutes les bactéries ne sont sûrement pas en phase exponentielle de croissance. Calvin et Hanawalt (1988) ont montré que les cellules étaient plus affectées par une impulsion électrique en début de croissance mais qu'ensuite les taux de survie se stabilisaient lorsque les cellules atteignent la fin de la phase exponentielle. Ainsi, nous nous sommes demandés si *P. syringae* CC0094 en phase stationnaire (après 60h de croissance) serait toujours capable de survivre à un choc électrique. Le taux de survie des *P. syringae* CC0094 en phase stationnaire n'était pas significativement différent de celui en condition standard (Tableau 2), indiquant que, quelle que soit la phase de croissance de cette bactérie dans les nuages, elle serait capable de survivre aux décharges de foudre.

Finalement, nous avons vérifié l'impact de la densité bactérienne sur la survie. La densité de bactéries totales dans les nuages étant plus faible que les quantités utilisées pour les électroporations en laboratoire(Amato *et al.*, 2005), nous avons vérifié si un plus faible nombre de bactéries était plus sensible à un choc électrique dû au manque de protection par les cellules environnantes. Nous avons montré que les taux de survie n'étaient pas

Tableau 4: Taux d'électrotransformation des trois bactéries étudiées après différents traitements.

Taux d'électrotransformation	*P. syringae* CC0094	*Pseudomonas* sp.N3	*E. coli* TOP10
Liquide (condition standard)	$2{,}55\times10^{-3} \pm 3{,}34\times10^{-3}$	$5{,}83\times10^{-3} \pm 6{,}87\times10^{-3}$	$1{,}68\times10^{-3} \pm 2{,}38\times10^{-3}$
9.5 $kV.cm^{-1}$	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	/	/
Milieu glacé	$7{,}93\times10^{-6} \pm 1{,}01\times10^{-6}$*	$3{,}94\times10^{-6} \pm 2{,}57\times10^{-6}$*	$2{,}85\times10^{-3} \pm 2{,}88\times10^{-3}$
3 chocs	$3{,}20\times10^{-3} \pm 2{,}07\times10^{-3}$	$9{,}32\times10^{-5} \pm 8{,}94\times10^{-5}$*	$5{,}10\times10^{-3} \pm 2{,}89\times10^{-3}$
5 chocs	$9{,}82\times10^{-4} \pm 7{,}59\times10^{-4}$	$1{,}36\times10^{-4} \pm 1{,}40\times10^{-4}$*	$2{,}74\times10^{-3} \pm 1{,}41\times10^{-3}$
Ajout du plasmide 30 min, 3 min, 75 ou 45 s après choc	/	/	$0{,}00\times10^{0}$*
Ajout du plasmide 3 min or 54 s après choc	$0{,}00\times10^{0}$*	/	/
Ajout du plasmide 30 s après 5 chocs	$0{,}00\times10^{0}$*	$0{,}00\times10^{0}$*	$0{,}00\times10^{0}$*
Ajout du plasmide 30 s après choc en milieu glacé	$0{,}00\times10^{0}$*	$0{,}00\times10^{0}$*	$0{,}00\times10^{0}$*
Ajout du plasmide 30 s après choc	$2{,}01\times10^{-7} \pm 1{,}57\times10^{-7}$*	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	$1{,}32\times10^{-7} \pm 1{,}14\times10^{-7}$*
10^{6} bactéries	/	$4{,}39\times10^{-2} \pm 1{,}80\times10^{-2}$	/
10^{5} bactéries	/	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	/
10^{4} bactéries	$8{,}95\times10^{-4} \pm 4{,}78\times10^{-4}$	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	$8{,}07\times10^{-7} \pm 1{,}14\times10^{-6}$*
10^{3} bactéries	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*
10^{2} bactéries	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*	$0{,}00\times10^{0} \pm 0{,}00\times10^{0}$*
Phase stationnaire	$5{,}42\times10^{-6} \pm 8{,}95\times10^{-7}$*	/	/
pBLN de *P. syringae* CC0094	$1.18\times10^{-2} \pm 9.91\times10^{-3}$	/	/

Les valeurs correspondent aux moyennes et écarts types des taux d'électrotransformation d'au moins trois expériences. Les astérisques montrent quelles valeurs sont significativement différentes ($p<0.05$) de la condition standard.

significativement différents entre les différentes concentrations de cellules (10^2 à 10^8 bactéries par électroporation – Tableau 2) pour aucune des trois bactéries étudiées montrant que les faibles densités de cellules dans les nuages n'impactent pas leur capacité à survivre aux chocs électriques.

2. Potentiel d'électrotransformation

Nous avons étudié la capacité des bactéries à acquérir de l'ADN plasmidique par électroporation en utilisant le plasmide à large spectre d'hôte pBLN de 8,5kb construit par Lyon *et al.,* (2010) et contenant les gènes de résistance à la tétracycline (*tetA*), à la kanamycine (*aph3ia*) et un gène de dégradation du lindane (*linA*). Notre but était de comparer les fréquences de transfert entre les trois espèces bactériennes en simulant les conditions susceptibles d'être rencontrées par les bactéries *in situ* (dans les nuages) (Tableau 1). Les taux d'électrotransformation ont été déterminés par le nombre de colonies résistantes à la tétracycline et à la kanamycine divisés par le nombre de bactéries survivantes. Avant et pendant la foudre, des impulsions électriques de quelques $kV.cm^{-1}$sont générées dans les nuages (Nucci *et al.*, 1988), correspondant aux valeurs compatibles avec l'électrotransformation. Etant donné qu'aucun transformant de *P. syringae* CC0094 n'a été obtenu lorsque les électroporations ont été conduites à 9.5 $kV.cm^{-1}$ (Tableau 4), le voltage conseillé par Bassett et Janiesiewicz (2003), il a été augmenté à 12.5 $kV.cm^{-1}$ (Calvin and Hanawalt, 1988; Wendt-Potthoff *et al.*, 1992) pour tenter d'améliorer l'efficacité de transformation. Ceci a produit des transformants avec succès. Les paramètres électriques (12.5 $kV.cm^{-1}$, 25 µF, 200 Ω, 5 ms et cuvettes de 2 mm) appliqués aux trois souches bactériennes ont produit des taux d'électrotransformation similaires (de 1×10^{-3} à 6×10^{-3}) dans la condition standard d'un seul choc en milieu liquide (Tableau 4).

Puisque les souches bactériennes étaient capables de survivre à au moins 5 chocs, nous avons testé si plusieurs chocs pouvaient influencer la quantité d'ADN que les bactéries étaient capables d'acquérir. Les fréquences d'électrotransformation n'étaient pas significativement augmentées quand les cellules d'*E. coli* et de *P. syringae* étaient soumises à un ou plusieurs chocs (Tableau 4), comme pour Calvin and Hanawalt (1988) qui ont essayé d'appliquer des chocs répétés pour améliorer les taux de transformation d'*E. coli* mais ont conclu qu'une seule impulsion était optimale pour l'efficacité de transformation. Par contre, les taux d'électrotransformation de *Pseudomonas* sp. N3 ont été significativement réduits après trois et cinq chocs (Tableau 4) ce qui est étonnant. Chez les cellules mammifères, les chocs multiples

augmentent significativement les efficacités de transformation mais entraînent également un déclin drastique de la survie cellulaire (Baron *et al.*, 2000; Song *et al.*, 2010), contrairement à nos résultats montrant un taux de survie conservé après plusieurs impulsions électriques pour les trois souches. Des phénomènes plus complexes peuvent être mis en jeu, comme chez les levures où des résultats variables sont rapportés quand de multiples impulsions sont appliquées, dépendant des macromolécules impliquées et des champs électriques (Brown *et al.*, 1992).

L'ADN exogène pouvant entrer dans les cellules après des décharges électriques grâce à la perméabilisation de la membrane, nous avons recherché si les décharges de foudre successives dans les nuages pouvaient entraîner une perte de plasmide. Les pourcentages de perte de plasmide ont été déterminés comme les proportions de bactéries survivantes sensibles à la tétracycline et à la kanamycine par rapport au nombre total de cellules survivantes après incubation.

Tableau 5: Taux de perte de plasmide des trois bactéries étudiées contenant pBLN après zéro à cinq chocs.

	P. syringae **CC0094-pBLN**	***Pseudomonas*** **sp. N3-pBLN**	***E. coli*** **TOP10-pBLN**
Sans choc	-20.32 ± 24.50%	-35.74 ± 40.22%	-21.38 ±29.53%
1 choc	18.89 ± 22.23%	-14.39 ± 36.99%	-10.32 ± 19.20%
2 chocs	5.65 ± 38.78%	-52.47 ± 66.10%	20.49 ± 23.14%
3 chocs	17.07 ± 59.06%	-55.74 ± 74.54%	-9.52 ± 46.05%
4 chocs	18.39 ± 30.76%	-6.46 ± 83.03%	-3.10 ± 29.94%
5 chocs	5.10 ± 36.76%	-32.72 ± 101.83%	12.76 ± 27.53%

Les valeurs correspondent aux moyennes et écart types des taux de perte de plasmide d'au moins quatre expériences.

Les trois souches étudiées contenant pBLN et soumises à des décharges électriques répétées n'ont pas montré de perte de plasmide significativement différentes du contrôle (sans choc) (Tableau 5). Il est donc peu probable que les décharges de foudre successives comme celles qui peuvent se produire dans les nuages (Gonçalves *et al.*, 2012) entraînent des pertes de plasmides, contrairement aux résultats de Heery *et al.* (1989), qui ont appliqué des électroporations haute tension provoquant 80-90% de perte de plasmide chez *E. coli.* Cette

contradiction peut être expliquée par le fait que certains des plasmides à large spectre d'hôte comme pBLN peuvent être très stables dans les bactéries (Eynard and Teissie, 2000). L'utilisation d'un plasmide à simple copie pourrait améliorer la détection des pertes de plasmides des bactéries cultivées car pBLN est un plasmide à nombre de copies faible impliquant que, comme pour les plasmides à nombre de copies élevé, toutes les copies doivent être perdues pour détecter une diminution du nombre d'UFC sur milieu avec antibiotiques. On peut donc estimer que l'ADN exogène internalisé dans les nuages lors d'électrotransformation par des bactéries ne serait pas suivi de perte significative lors de décharges de foudre successives.

En conditions glacées, les deux souches de *Pseudomonas* présentaient des taux d'acquisition de plasmide réduits (Tableau 4), comme attendus à cause de la conductivité basse de la glace. Par contre, le taux d'électrotransformation *d'E. coli* dans le milieu glacé était le même qu'en condition liquide (standard) (Tableau 4) ce qui est surprenant. Ce résultat est parallèle à la conservation du taux de survie d'*E. coli* indépendamment de la condition du milieu liquide ou glacé d'électroporation, tandis que les souches de *Pseudomonas* montrent des tendances opposées de survie et d'électrotransformation. Il se peut que moins de pores aient été créés en conditions glacées, ce qui a pu réduire la capacité de ces bactéries à acquérir le plasmide. De plus, la différence de composition de membrane et de présence de mucus entre les genres *Escherichia* et *Pseudomonas* pourraient expliquer les différences de comportement face au choc en conditions glacées. Des explorations plus poussées comme des analyses microscopiques sont requises pour comprendre si ces phénomènes inversement liés (survie augmentée et succès d'électrotransformation plus faible) dépendent de mécanismes communs. Toutefois, nos résultats, à notre connaissance les premiers sur l'électroporation en conditions glacées, soutiennent l'hypothèse de survie et d'électrotransformation des bactéries dans les noyaux glaçogènes des nuages. De plus, les températures basses des gouttelettes d'eau en surfusion observées dans les nuages (Delort *et al.*, 2010) pourraient augmenter les taux d'électrotransformation (Shi *et al.*, 2003).

Bien qu'avec une efficacité d'électrotransformation réduite comme précédemment observée pour *E. coli* (Calvin and Hanawalt, 1988)*, P. syringae* CC0094 était encore capable d'acquérir des plasmides en phase stationnaire, après 60 heures sans remplacement du milieu (Tableau 4). Par contre, le taux moyen d'électrotransformation des *Pseudomonas* sp. N3 en phase stationnaire était maintenu en comparaison des cellules en phase exponentielle

(Cérémonie *et al.*, 2004). De la même façon, *Pseudomonas* sp. N3 s'est démarqué en ce qui concerne le nombre minimum de cellules requises pour la transformation (10^6) (Tableau 4). En effet, *E. coli* TOP10 et *P. syringae* CC0094 étaient encore transformés à une concentration cellulaire initial de 10^4 bactéries, à un taux plus faible (*E. coli* TOP10) ou similaire (*P. syringae* CC0094) à la condition standard (10^8 bactéries) (Tableau 4). Il se pourrait que *Pseudomonas* sp. N3 ait besoin d'atteindre une densité suffisante de bactéries avant d'être électrotransformé avec succès, probablement à cause d'une quantité minimale de mucus nécessaire. Chez *E. coli* TOP10, le taux d'électrotransformation significativement réduit observé à une densité cellulaire de 10^4 peut être facilement expliqué. En effet cette souche est une des plus efficaces pour l'électroporation et elle est vendue à des concentrations très hautes (10^8-10^9 UFC.mL^{-1}), peu rencontrées dans l'environnement, qui optimisent son efficacité de transformation (Drury, 1996) et qui correspondent aux pratiques standards de laboratoire. Dans les nuages, le dénombrement des bactéries totales atteint 10^4 cellules par m^3 de nuage (Amato *et al.*, 2005). *P. syringae* CC0094 serait donc la seule des trois souches étudiées à être efficacement transformée dans les nuages à des densités cellulaires faibles. Etant capable d'acquérir de l'ADN exogène quels que soient son stade de croissance et sa densité, *P. syringae* CC0094 semble être la souche la mieux adaptée à l'électrotransformation parmi les trois souches étudiées dans les conditions simulées des nuages.

Aucun transformant n'a été récupéré lorsque le plasmide a été ajouté 30 min, 3 min, 75 ou 45 s après l'impulsion électrique pour *E. coli* TOP10, et 3 min ou 54 s après l'impulsion électrique pour *P. syringae* CC0094 (Tableau 4). L'échange d'ADN ne se produit donc pas après la décharge de foudre, bien que les pores créés par le choc électrique pourraient rester ouverts pour un période suffisante afin de permettre à la transformation de se produire plus tard dans d'autres conditions (Smith *et al.*, 2004). En effet, plusieurs études impliquant des cellules mammifères ou bactériennes ont montré que certains pores hydrophobes pourraient être convertis en pores hydrophiles métastables, nécessitant moins d'énergie pour se former et se maintenir et pouvant rester ouverts pendant quelques minutes ou quelques heures, voire ne jamais se refermer. Nous nous attendions à ce que ces pores longue durée permettent à l'ADN d'entrer dans la cellule bien après que le voltage transmembranaire soit revenu à des valeurs faibles tout en étant suffisamment petits pour éviter une libération du cytoplasme et la mort de la cellule (Benz and Zimmermann, 1980; Kwee *et al.*, 1992; Saulis, 1997; Ryttsén *et al.*, 2000; Sambrook and Russell, 2001; Rae and Levis, 2002; Langham, 2004; Smith *et al.*, 2004; Schmeer, 2009). Les deux laps de temps testés pour *P. syringae* CC0094 (3 min et 54 s)

représentent le temps que la pluie mettrait à tomber sur les zones plates, calculés en utilisant la distance des nuages de la Terre (entre 3 et 5 km au-dessus du niveau de la mer) et la vitesse de tombée de la pluie (entre 100 et 200 $km.h^{-1}$). Dans de telles conditions, *P. syringae* ne serait pas capable d'incorporer de l'ADN après les décharges de foudre. La possibilité que ce temps avant disparition des pores puisse influencer l'acquisition d'ADN a été testée plus amplement par ajout du plasmide 30 s après des chocs électriques répétés ou après une simple impulsion électrique en condition glacée. La répétition de chocs électriques augmente la durée d'ouverture des pores (Ryttsén *et al.*, 2000; Smith *et al.*, 2004) et ne réduit pas la survie des trois souches étudiées, de même que le refroidissement des cellules à 0°C peut retarder la fermeture stochastique des pores (Saulis, 1997). Dans les deux cas, aucun transformant n'a été récupéré pour l'une ou l'autre des trois bactéries étudiées (Tableau 4). Par contre, l'ajout du plasmide 30 s après une simple décharge a entraîné des résultats différents selon les souches bactériennes : *Pseudomonas* sp.N3 n'était pas capable d'incorporer des plasmides tandis que *E. coli* TOP10 et *P. syringae* CC0094 l'étaient, mais avec une réduction d'efficacité de 4 logs (Tableau 4). Ceci suggère que *P. syringae* pourrait encore électrotransformer de l'ADN après dépôt par la pluie de nuages orageux bas sur des montagnes, qui constituent un environnement propice pour l'incorporation de gènes. En effet, les densités de population de cette bactérie dans la litière de surface des prairies alpines sont de 10^9 $bacteries.m^{-2}$ et celles des bactéries totales sont cent fois plus riches (Monteil *et al.*, 2012).

Les transformants de *P. syringae* CC0094 contenant le plasmide pBLN ont été utilisés pour réisoler pBLN afin d'évaluer l'efficacité d'électrotransformation en fonction de la provenance et de la topologie du plasmide. Afin d'obtenir des plasmides non dégradés par la présence potentielle de nucléases (Casse *et al.*, 1979), nous avons utilisé le protocole de Schwinghamer (1980) pour extraire les pBLN de *P. syringae* CC0094 contrairement à ceux d'*E. coli* TOP10 qui avaient été extraits avec le kit de purification 'Nucleospin plasmid'. Le protocole de Schwinghamer impliquant une séparation des différentes formes de plasmides par gradient de Chlorure de Césium en ultracentrifugation, seule la forme CCC (covalente circulaire fermée) a été récupérée de *P. syringae* CC0094 alors que les trois formes (linéaire, ouvert circulaire OC et une majorité de CCC) du même plasmide ont été récupérées d'*E. coli* TOP10 avec le kit de purification 'Nucleospin plasmid'. En utilisant le plasmide réisolé de *P. syringae,* le taux d'électrotransformation de *P. syringae* CC0094 n'était pas significativement augmenté (Tableau 4) contrairement aux rapports précédents impliquant *P. syringae* L-59-66 où une augmentation de 2 à 3 ordres de grandeur était notée avec un plasmide isolé de la même espèce et d'un *E. coli* déficient pour la méthylation.

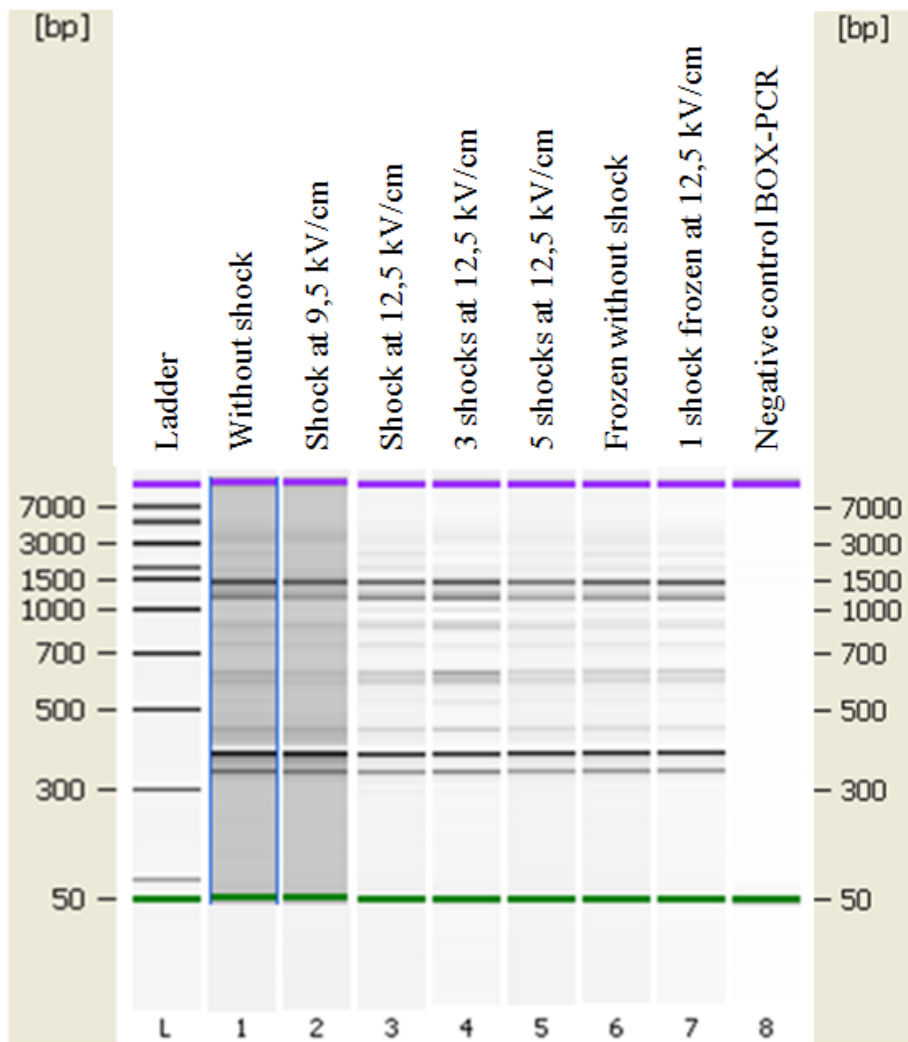


Figure 3: BOX PCR (rep-PCR) sur les ADN extraits de *P. syringae* CC0094.
Control sans choc (ligne 1), avec un choc à 9.5 kV.cm^{-1} (ligne 2), un, trois et cinq chocs à 12.5 kV.cm^{-1} (lignes 3, 4 et 5), glacé sans choc (ligne 6) et un choc glacé (ligne 7); marker (ligne L); contrôle négatif de la BOX-PCR (ligne 8)

(Bassett and Janisiewicz, 2003). Les différences de topologie de plasmide et de systèmes de méthylation (*P. syringae versus E. coli*) ne semblent donc pas influencer les efficacités de transformation, bien que les topologies de molécules d'ADN soient connues pour affecter les transformations bactériennes (Drury, 1996). La forme linéaire du plasmide est moins efficacement transformée que les formes OC et CCC quand les plasmides se répliquant de façon autonome sont utilisés (Demanèche *et al.*, 2002) probablement parce qu'elle est plus sensible aux nucléases (Sambrook *et al.*, 1989). Ce résultat confirme et supporte le fait que *P. syringae* CC0094 est mieux adapté à l'acquisition d'ADN exogène.

Les paramètres relatifs à l'intégrité de la membrane (tels que les stress nutritionnels, la présence de cations et de surfactants, ainsi que le refroidissement du milieu) connus pour augmenter les taux d'électrotransformation (Shi *et al.*, 2003), n'ont pas été testés dans cette étude mais devraient être considérés par la suite afin d'obtenir un aperçu plus approfondi des mécanismes impliqués dans l'efficacité d'électrotransformation dans les nuages. En effet, dans cet environnement, les températures sont basses (Delort *et al.*, 2010), des cations peuvent être présents (Amato *et al.*, 2007a; Vaïtilingom *et al.*, 2013) et certaines bactéries produisent des surfactants qui facilitent la formation des noyaux de condensation (Ahern *et al.*, 2007).

3. Stabilité génomique de *P. syringae* CC0094

Finalement, nous avons vérifié si l'électroporation dans les différentes conditions testées (une impulsion en liquide ou en glace ou même après 5 décharges électriques), affectait la structure génomique de *P. syringae* CC0094 (Figure 3). Basé sur les profils de BOX-PCR (Louws *et al.*, 1994), aucune modification visible n'a pu être détectée, attestant du maintien des distances des séquences palindromiques extragéniques répétées, et donc de la stabilité de la structure génomique globale de cette bactérie.

Conclusion

Dans l'ensemble, ces résultats fournissent une preuve de concept que l'acquisition d'ADN basée sur l'électrotransformation des bactéries pourrait se produire dans les nuages grâce aux décharges électriques relatives à la foudre.

Les expériences conduites avaient pour but de déterminer si *P. syringae* CC0094 était capable d'acquérir du nouveau matériel génétique dans les nuages en utilisant des décharges de foudre simulés. Premièrement, *P. syringae* CC0094 se comportait de la même manière que

Pseudomonas sp.N3 et *E. coli* TOP10 en termes de taux de survie et d'électrotransformation après un choc à 12.5 kV.cm^{-1} (condition standard) simulant la foudre. Par contre, seule *P. syringae* CC0094 présentait une résistance élevée aux décharges de foudre simulées en conditions glacées, sans doute grâce à sa capacité à former des noyaux glaçogènes, tout en restant capable d'acquérir de l'ADN plasmidique. *P. syringae* CC0094 était également capable de résister à plusieurs chocs comme ceux pouvant avoir lieu dans les nuages (particulièrement en présence de bactéries glaçogènes) sans modification de sa structure génomique, ni changement de taux d'acquisition ou de perte de plasmide. Sa capacité d'électrotransformation était maintenue en phase stationnaire et à faible densité. A cause des conditions rigoureuses rencontrées dans les nuages, de l'ADN bactérien peut être libéré par lyse cellulaire. Bien que la densité bactérienne soit probablement trop faible dans un noyau glaçogène pour qu'un échange de gènes puisse avoir lieu, la perméabilisation de la membrane due à la foudre semble se maintenir plus de 30 secondes après le choc, permettant aux transformations d'avoir lieu ultérieurement. Au contraire, *Pseudomonas* sp. N3, une bactérie naturellement transformable isolée du sol, est moins adaptée aux conditions du nuage, particulièrement à cause de sa faible survie en conditions glacées, la fermeture rapide de ses pores et la densité minimale de bactéries nécessaires à son électrotransformation. *Escherichia coli*, quant à elle, également connue comme étant une des souches les plus efficacement électrotransformables, n'est pas protégée par l'activité de nucléation de la glace et est moins efficacement transformée à de faibles densités, contrairement à *P. syringae.*

Pour conclure, *P. syringae* CC0094 montre des potentiels de survie et d'électrotransformation plus importants que ceux d'*E. coli* TOP10 et *Pseudomonas* sp. N3 pour les différents paramètres étudiés. Toutes nos observations soutiennent l'hypothèse qu'un pathogène de plantes tel que *P. syringae* est un bon candidat à la survie et à l'électrotransformation dans les nuages. Sa capacité à utiliser son pouvoir glaçogène pour augmenter sa fitness en pathogénie et coloniser de nouveaux écosystèmes a encore besoin d'être confirmée par une approche mutationnelle. Cependant, la capacité de *P. syringae* à survivre et évoluer tout en étant transporté dans les nuages pourrait expliquer comment cette bactérie ubiquitaire et glaçogène est aussi un des phytopathogènes des plus importants, causant de très importantes pertes économiques à travers le monde.

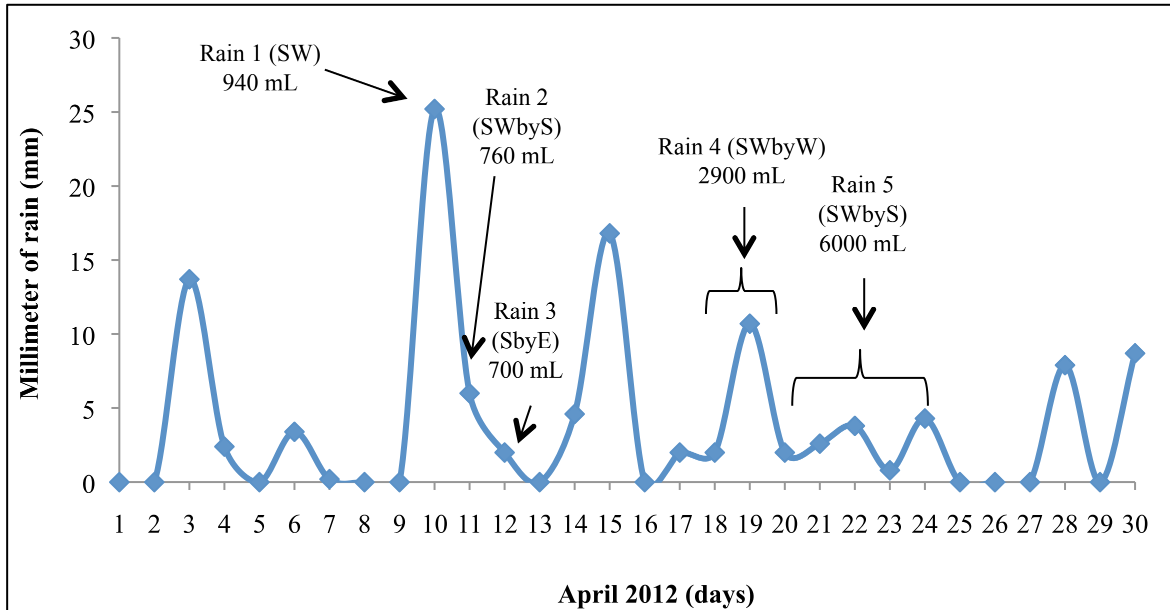


Figure 4: Quantité de précipitations journalières collectées à Ecully (45.784921 N 4.767873 E) au cours du mois d'Avril 2012 (données de METEO FRANCE de la plus proche station, Bron).

Les cinq épisodes de pluies échantillonnés sont indiqués par des flèches, avec d'autres paramètres comme la direction du vent (entre parenthèses) et le volume de pluie collecté (mL). SW: Sud-Ouest; SWbyS: Sud-Ouest par le Sud; SbyE: Sud par l'Est; SWbyW: Sud-Ouest par l'Ouest.

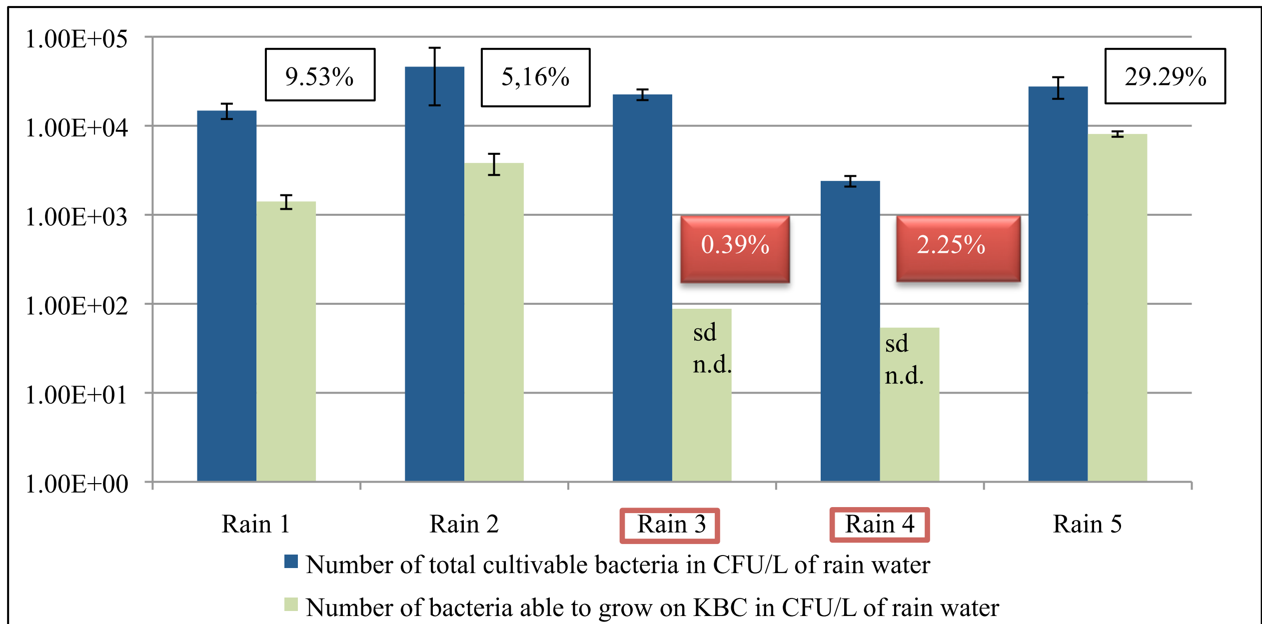


Figure 5: Nombre de bactéries totales cultivables sur LBC (LB avec cycloheximide) et de *Pseudomonas* spp. supposés, poussant sur KBC (milieu semi sélectif crée par Mohan and Schaad (1987) pour les cinq épisodes de pluie.

Les écart-types ont été déterminés en utilisant le dénombrement de trois boîtes sauf pour les dénombrements des *Pseudomonas* spp. putatifs sur milieux KBC des pluies 3 et 4 où les écart-types n'ont pas été déterminés (sd n.d.). Les pourcentages de *Pseudomonas* spp. putatifs dans chaque épisode de pluie sont indiqués en encadrés.

II. IMPACT DE LA FOUDRE SUR LA DIVERSITE, LA SURVIE ET LE POTENTIEL D'ELECTROTRANSFORMATION DES BACTERIES DE LA PLUIE.

Introduction

Afin de déterminer si les bactéries de la pluie seraient capables de survivre et de profiter des impulsions électriques relatives aux décharges de foudre pour acquérir de nouveaux gènes, nous avons isolé *in vitro* les communautés bactériennes de cinq épisodes de pluie comme substitut des bactéries des nuages déclenchant les précipitations. En effet, les bactéries de la pluie sont probablement les plus représentatives des communautés des nuages étant donné que la récupération d'aérosols par la pluie est très inefficace pour des petites particules (inférieures à quelques microns en diamètre) (McDonald, 1962; Respondek *et al.*, 1995). Nous avons caractérisé certaines de leurs propriétés adaptatives y compris leur capacité à survivre à des décharges simulées de foudre, à résister à deux antibiotiques et à être naturellement et électriquement transformées et également déterminé la proportion de *Pseudomonas* sp. et plus particulièrement de *P. syringae* dans ces épisodes de pluie. Tous les tests ont été réalisés avant culture, directement sur les fractions des communautés bactériennes collectées lors des épisodes de pluie et concentrées, pour éviter une sélection de souches habituées à la croissance en laboratoire sur milieux de culture.

1. Concentration des bactéries cultivables de la pluie

Les bactéries cultivables récupérées des cinq épisodes de pluie, collectés en Avril 2012 (Figure 4), présentaient une concentration moyenne de $2,27\times10^{4}$ $UFC.L^{-1}$ (Figure 5). En considérant le taux accepté de 1% de bactéries cultivables dans les environnements atmosphériques (Lighthart, 1997; Bauer *et al.*, 2002; Amato *et al.*, 2005; Amato *et al.*, 2007a), la concentration de bactéries totales serait 100 fois supérieure, environ 2×10^{6} $cellules.L^{-1}$, ce qui correspond aux 2.10^{5}-10^{7}cellules bactériennes totales par litre de précipitations (neige ou pluie), habituellement observés (Casareto *et al.*, 1996; Bauer *et al.*, 2002) et aux 10^{6}-10^{8} bactéries par litre d'eau des nuages (Sattler *et al.*, 2001; Bauer *et al.*, 2002; Amato *et al.*, 2005; Amato *et al.*, 2007a; Hill *et al.*, 2007; Vaïtilingom *et al.*, 2013). La différence entre les concentrations de bactéries des nuages et celles des précipitations, bien que négligeable, est probablement due à la capacité de seulement une partie des gouttelettes des nuages contenant les bactéries qui déclenchent les précipitations (c'est à dire celles ayant une activité

glaçogène) de tomber (Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Morris *et al.*, 2011). Cette uniformité de concentration bactérienne entre toutes les précipitations suggère des contraintes générales dans ces environnements. Les variations saisonnières et diurnes comme celles observées chez les microorganismes atmosphériques totaux (Amato *et al.*, 2007a), dans les échantillons d'aérosol(Bowers *et al.*, 2012), ou autour de midi (Lighthart and Shaffer, 1995; Lighthart, 1999), ne sont pas pertinentes dans le cas des concentrations de bactéries récupérées de la pluie puisque les cinq épisodes ont été échantillonnés le même mois et sur 24 heures.

La concentration des microorganismes atmosphériques est fortement influencée par l'orientation du vent, l'emplacement du site d'échantillonnage, la salinité et la pollution, ce qui a permis d'identifier 3 types de masses d'air au centre de la France, maritime, continental et urbain en fonction de l'orientation des vents de l'Ouest, du Sud-Ouest et du Nord-Est (Marinoni *et al.*, 2004; Amato *et al.*, 2005; Vaïtilingom *et al.*, 2013). Quatre des cinq échantillons de pluie collectés provenaient de masses d'air du Sud ou de l'Ouest (Figure 4), censés transporter les plus hautes concentrations de microorganismes grâce aux fortes émissions de l'océan Atlantique. L'autre échantillon (troisième épisode de pluie) a été recueilli de nuages provenant de vents du Sud-Est (Figure 4) dépourvus d'une contribution océanique et potentiellement soumis à des pollutions notamment de SO_2 et NOx qui affectent la multiplication et la survie des microorganismes (Marquardt *et al.*, 2001; Amato *et al.*, 2007a; Delort *et al.*, 2010). Cependant, les concentrations de bactéries totales cultivables étaient du même ordre de grandeur pour les 5 épisodes de pluie.

Parmi les bactéries cultivables de la pluie, une attention spécifique a été portée sur *Pseudomonas syringae*, un important pathogène des plantes et une des bactéries les plus glaçogènes. Sa croissance est favorisée par l'utilisation d'un milieu de culture semi sélectif créé pour l'isolation de pathovars de *P. syringae* par Mohan and Schaad (1987). Les résultats de dénombrements confirment l'importance de cette bactérie dans la pluie (10^2-10^4 *P. syringae*.L^{-1} de pluie) (Morris *et al.*, 2008) puisqu'elle représente presque un dixième (10^3 UFC.L^{-1} de pluie) de l'ensemble des bactéries cultivables (2.27×10^4 UFC.L^{-1}) des trois échantillons de pluie collectés avec des vents provenant du Sud-Ouest et étudiés en détail. Non sans surprise, le nombre de *P. syringae* putatifs a diminué d'un facteur 100 quand le vent soufflait du Sud-Est. Il est possible que ce soit dû soit à une émission urbaine plus importante que les surfaces marines et végétales soit à une sensibilité particulièrement marquée des bactéries aux produits chimiques présents dans les nuages pollués.

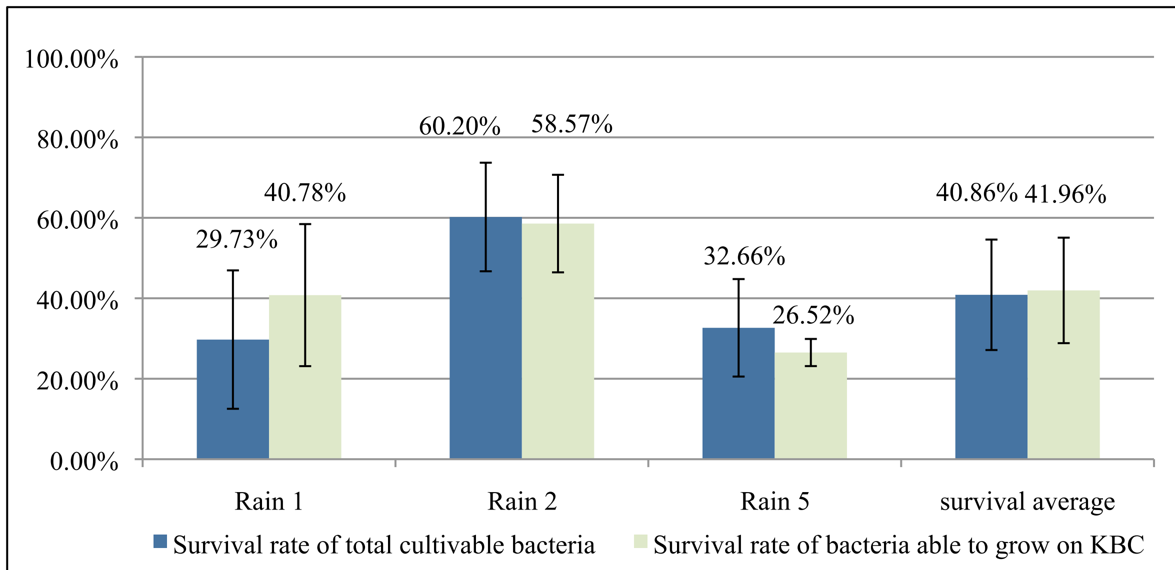


Figure 6: Pourcentage de survie des bactéries totales cultivées sur LBC et de *Pseudomonas* putatifs cultivés sur KBC des différents épisodes de pluie après une impulsion électrique à 12.5 kV.cm^{-1}.
Les écarts types pour chaque épisode de pluie ont été déterminés en utilisant le dénombrement après choc électrique de trois boîtes de LBC ou KBC pour les bactéries totales cultivables et les *Pseudomonas* spp. supposés, respectivement. Les moyennes des taux de survie des triplicats biologiques sont aussi montrées avec les écarts types comme barres d'erreur.

Le quatrième épisode de pluie a été échantillonné, comme les trois autres, lorsque les vents soufflaient du Sud-Ouest, mais n'a pas pu être préparé de la même manière que les autres à cause d'une rupture de stock temporaire de sucrose 0.5M au laboratoire au moment de sa récupération. Les bactéries ont donc été lavées et concentrées dans du glycérol 10% (w/v) au lieu du sucrose 0.5 M. Le glycérol est fréquemment utilisé comme solution osmotique pour laver les cellules bactériennes et notamment *E. coli*, mais le sucrose 0.5 M est plus adapté à la préparation des *Pseudomonas* (Bassett and Janisiewicz, 2003). Cette étude étant principalement focalisée sur *Pseudomonas* spp., les épisodes de pluie 1, 2 et 5 contenant la plus forte proportion de *Pseudomonas* supposés, ont été étudiés plus en détail tandis que les dénombrements de bactéries les plus bas des épisodes de pluie 3 et 4 nous a amenés à les exclure des analyses plus poussées.

2. Survie des bactéries de la pluie à la foudre

L'adaptation à l'environnement du nuage nécessite que les bactéries aérosolisées soient capables de survivre aux décharges de foudre. Nous avons évalué le potentiel de survie des communautés bactériennes de la pluie (des épisodes de pluie 1, 2 et 5) en comparant les estimations de bactéries cultivables directement avec les bactéries concentrées (5000 fois dans le sucrose 0.5 M) ou après électroporation à 12.5 $kV.cm^{-1}$, 25 µF, 200 Ω dans des cuvettes de 2 mm. Les paramètres électriques de l'électroporation *in vitro* (12.5 $kV.cm^{-1}$ et 5 $kA.m^{-2}$) sont du même ordre de grandeur que ceux mesurés durant les décharges de foudre (6 $kV.cm^{-1}$ et 12 $kA.m^{-2}$) (Demanèche *et al.*, 2001c). Nous avons donc simulé les phénomènes électriques se produisant dans les nuages en utilisant l'électroporation.

Les taux de survie que nous avons observés pour les bactéries de la pluie dépassaient largement les 5% des bactéries de laboratoire telles *E. coli* K12 (Calvin and Hanawalt, 1988). Tous nos résultats montrent une tolérance remarquable des bactéries (dont *P. syringae*) récupérées de pluies fraiches face aux décharges électriques étant donné qu'en moyenne 40% des bactéries isolées survivent au choc. Il se peut que cette opposition reflète la différence entre bactéries « domestiquées » en laboratoire et bactéries des nuages avec la possibilité que le potentiel de résistance aux chocs électriques ait été modifié par les conditions rencontrées dans les nuages dont les limitations en nutriments, l'environnement très oxydatif, la nucléation de la glace, la dessiccation, la température, le pH, les radiations UV, les cycles répétés de congélation-décongélation et les chocs osmotiques, qui pourraient agir séparément, ou en synergie. De plus, des tendances identiques ont été remarquées en moyenne entre bactéries totales cultivables et *Pseudomonas* supposés. Ceci suggère que les *Pseudomonas*

isolés sur KBC et censés comporter des bactéries glaçogènes ne soient pas plus avantagées que les autres bactéries des nuages en ce qui concerne le stress électrique.

Ainsi, il se peut que les bactéries des échantillons de pluie soient mieux capables de survivre à des chocs électriques simulant la foudre grâce à une sélection s'étant opérée durant leur transport atmosphérique (Šantl-Temkiv *et al.*, 2012).

3. Impact de la foudre sur la diversité bactérienne

Nous avons également étudié la structure taxonomique des communautés bactériennes des *Pseudomonas* putatifs de la pluie avant et après impulsion électrique simulant la foudre afin de rechercher un effet possible des stress électriques comme ceux rencontrés pendant un véritable épisode de foudre sur la diversité bactérienne.

Distribution contrôle des Pseudomonas spp. putatifs de la pluie

Lorsque c'était possible, trois colonies par phénotype poussant sur KBC ont été analysées par amplification, séquençage et analyse phylogénique des séquences d'ADNr SSU pour chaque épisode de pluie.

Une diversité de *Pseudomonas* spp. (Mulet *et al.*, 2010) a été identifiée parmi les isolats des cinq épisodes de pluie cultivés sur KBC (Figure 7). Ce milieu de culture est supposé être spécifique des pathovars de *Pseudomonas syringae* (Mohan and Schaad, 1987) et nous avons effectivement récupéré certaines bactéries de cette espèce. Cependant, nos isolats représentaient un échantillonnage taxonomique bien plus large au sein du genre *Pseudomonas.* Plus surprenant encore, une actinobactérie liée à l'espèce *Plantibacter* (*Microbacteriaceae*), a également été récupérée, soulignant les limites du milieu King's en ce qui concerne la spécificité pour les *Pseudomonas*.

Parmi les isolats de *Pseudomonas* identifiés des cinq épisodes de pluie, la lignée *P. syringae* semblait être la plus abondante (Figure 7). Cependant, *P. syringae* n'est pas le microorganisme le plus abondant isolé de l'atmosphère (Lighthart, 1997) et ne constitue pas plus de 5-9% des bactéries de type *P. syringae* (Constantinidou *et al.*, 1990). De même, aucune population cultivable de *P. syringae* n'a été récupérée d'échantillons de neige fraîche alors que des niveaux significatifs de bactéries glaçogènes étaient détectés (Christner *et al.*, 2008). Par contre, lorsque les 2 échantillons les plus petits (pluies 3 et 4 – seulement 1,25% des isolats) sont omis, cette lignée est seulement le troisième groupe de *Pseudomonas* le plus

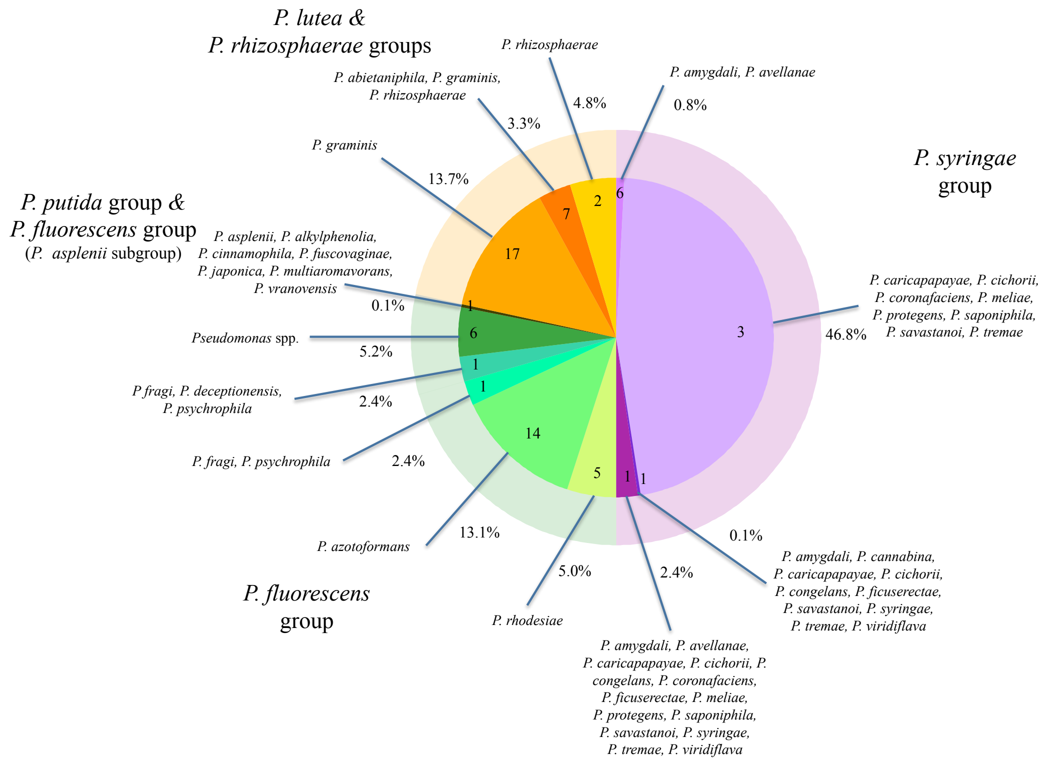


Figure 7: Représentation des espèces de *Pseudomonas* parmi les isolats des cinq épisodes de pluie combinés.

Isolats cultivés sur le milieu KBC (échantillons non choqués). Les nombres d'isolats pour chaque lignée phylogénique sont donnés, ainsi que les pourcentages calculés après correction de la taille de l'échantillon. Les lignées sont colorées en fonction des données de regroupement phylogénique.

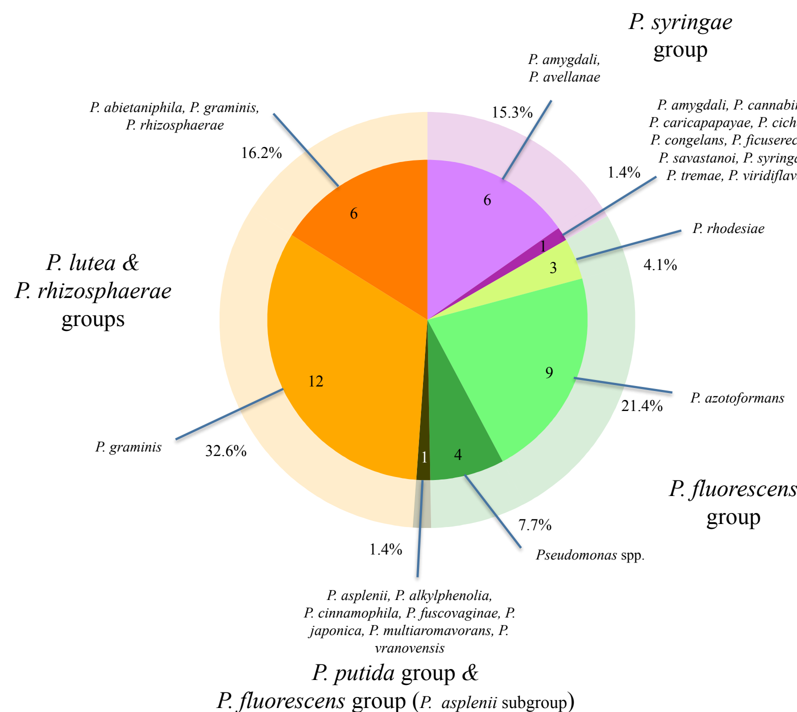


Figure 8: Représentation des espèces de *Pseudomonas* parmi les isolats combinés des épisodes de pluie 1, 2 et 5 combinés.

Isolats cultivés sur le milieu KBC (échantillons non choqués). Les nombres d'isolats pour chaque lignée phylogénique sont donnés, ainsi que les pourcentages calculés après correction de la taille de l'échantillon. Les lignées sont colorées en fonction des données de regroupement phylogénique.

représenté, derrière les lignées combinées de *P. lutea* et *P. rhizosphaerae* (presque la moitié des isolats) et le groupe *P. fluorescens* (un peu plus de 30%) (Figure 8). Il est intéressant de noter que des bactéries des espèces *P. graminis*, *P. rhizosphaerae,* ainsi que *P. syringae* ont été isolées des nuages par Amato *et al.* (2007b), bien que ces auteurs n'aient pas utilisé de milieux de culture spécifiques des *Pseudomonas.*

Au sein du groupe *P. syringae*, les espèces les plus proches des isolats de la pluie étaient des phytopathogènes (Figures 7 & 8) qui ont une large gamme d'hôtes et peuvent avoir un impact économique important sur différentes récoltes (O'Brien *et al.*, 2011; Mansfield *et al.*, 2012). La seule espèce de *P. syringae* a été sous divisée en plus de 50 pathovars et est responsable de nombreuses et différentes maladies de plantes.

La plupart des isolats de *Pseudomonas* obtenus dans cette étude sont plus proches des espèces *P. rhizosphaerae, P. abietaniphila* et/ou *P. graminis* (Figure 8). Ils sont probablement issus d'aérosolisation à partir des végétaux (Lindemann *et al.*, 1982; Lighthart and Shaffer, 1995; Morris *et al.*, 2008) étant donné que *P. graminis* et *P. rhizosphaerae* ont été respectivement isolés de la phyllosphère et de la rhizosphère de l'herbe (Behrendt *et al.*, 1999; Peix *et al.*, 2003). *P. graminis* a aussi été identifié comme agent de contrôle du feu bactérien (Mikiciński *et al.*, 2011). L'identification de *P. abietaniphila*, un dégradeur de résine acide isolé d'eaux usées d'une fabrique de pâte à papier (Muttray *et al.*, 2001), est également compatible avec une provenance liée aux plantes.

Les autres espèces de plantes isolées identifiées étaient affiliées à la lignée *P. fluorescens* (Figures 7 & 8)*,* qui contient une large diversité de sous-groupes et d'espèces aux styles de vie différents. Le plus représenté dans notre étude était le pathogène et promoteur de la croissance des plantes *P. azotoformans* (Levenfors *et al.*, 2011) et le saprophyte *P. rhodesiae* (Coroler *et al.*, 1996). Nos autres isolats appartiennent aux sous-groupes *P. fragi* et *P. asplenii* et sont proches de biodégradateurs (*P. vranovensis, P. asplenii, P. japonica* et *P. alkylphenolia)* (Tvrzová *et al.*, 2006; Pungrasmi *et al.*, 2008; Cho *et al.*, 2011), psychrophiles (*P. fragi, P. deceptionensis* et *P. psychrophila*) (Eichholz, 1902; Yumoto *et al.*, 2001; Carrión *et al.*, 2011) ou de phytopathogènes (*P. asplenii* et *P. fuscovaginae*) (Miyajima *et al.*, 1983; Beattie, 2006).

Dans l'ensemble, en plus de *Pseudomonas* potentiellement glaçogènes, il se peut que d'autres bactéries adaptées au froid, saprophytes ou promotrices de la croissance des plantes et certaines avec des capacités de biodégradation ainsi que divers phytopathogènes pouvant constituer une menace pour les écosystèmes, soient présentes parmi les bactéries de la pluie (Kellogg and Griffin, 2006; Brodie *et al.*, 2007).

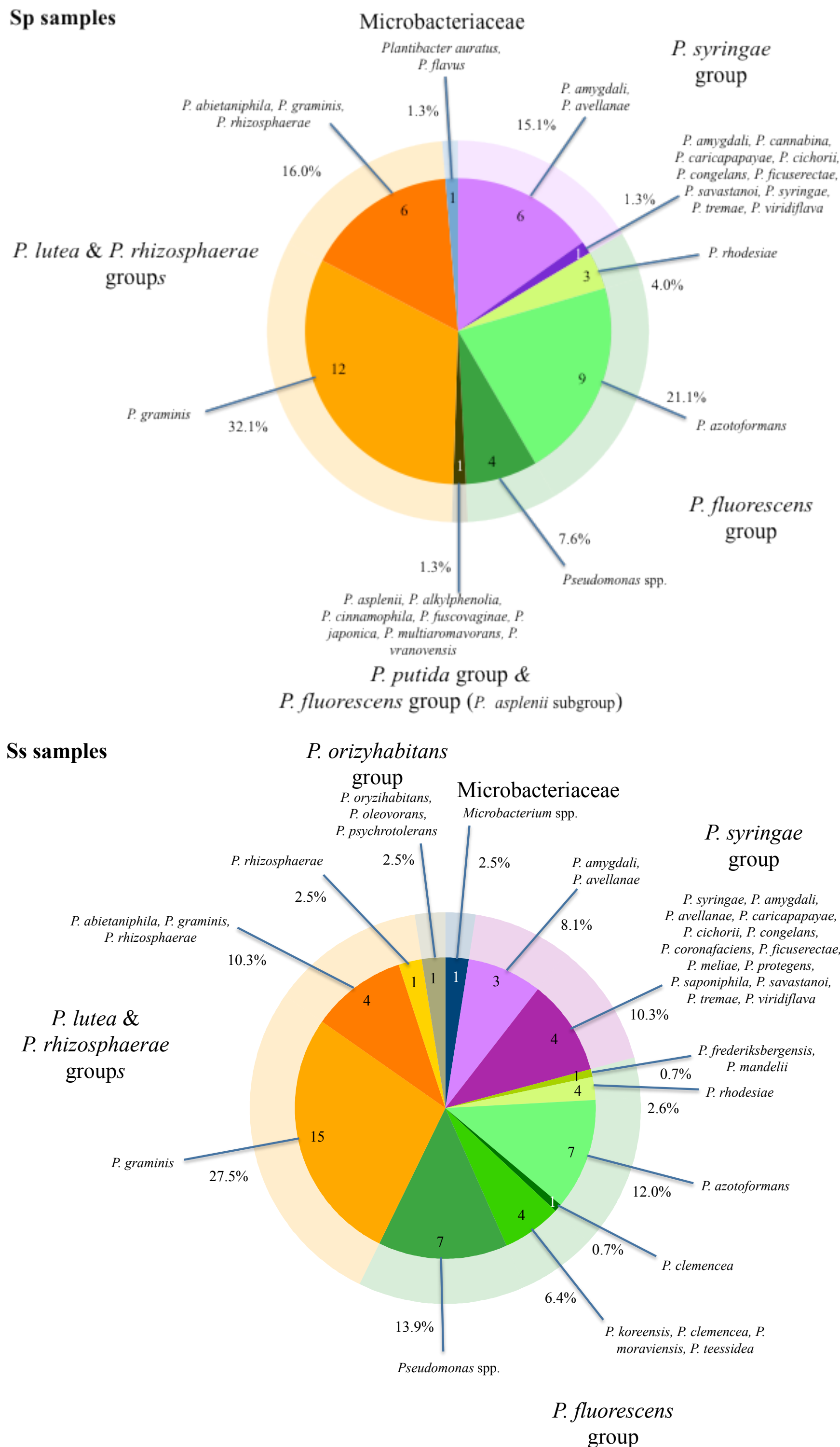


Figure 9: Représentation générale des différentes lignées identifiées parmi les isolats des épisodes de pluie 1, 2 et 5 non soumis à un choc électrique ("Sp" samples) et soumis à un choc électrique ("Ss" samples).
Isolats cultivés sur le milieu KBC. Les nombres d'isolats pour chaque lignée phylogénique sont donnés, ainsi que les pourcentages calculés après correction de la taille de l'échantillon. Les lignées sont colorées en fonction des données de regroupement phylogénique.

Distribution des Pseudomonas spp. putatifs de la pluie après impulsion simulant la foudre

La distribution des isolats soumis à une décharge électrique chez les principales lignées phylogéniques de *Pseudomonas* a peu varié par rapport à ce qui était observé en l'absence de choc électrique (Figure 9). Cependant, le traitement a révélé une plus grande diversité taxonomique visible notamment au sein des lignées de *P. fluorescens* et *P. lutea* & *P. rhizosphaerae*. Ceci peut être lié à la diminution du nombre total de bactéries, qui peut ainsi révéler des groupes mineurs moins facilement détectables auparavant. Sinon, il se peut que certaines lignées soient plus résistantes au stress électrique et puissent avoir été sélectionnées durant l'électroporation. Le sous-groupe *P. putida/P. fluorescens asplenii* (un seul isolat dans la distribution précédente) n'était plus représenté quand les bactéries de la pluie étaient soumises à une décharge électrique, ce qui suggère un manque de résistance de ces espèces face à la foudre. Au contraire, la lignée de *P. oryzihabitans* (un seul isolat), qui contient des pathogènes opportunistes humains et des espèces psychrotolérantes (Kodama *et al.*, 1985; Freney *et al.*, 1988; Hauser *et al.*, 2004) ainsi que les sous-groupes *P. koreensis* et *P. mandelii* au sein de la distribution du groupe *P. fluorescens*, ont été nouvellement récupérées après choc. Les espèces les plus proches phylogéniquement au sein de ces derniers groupes sont des espèces ayant des capacités de biocontrôle (*P. koreensis*) (Kwon *et al.*, 2003; Hultberg *et al.*, 2010b; Hultberg *et al.*, 2010a) et de biodégradation *(P. frederiksbergensis* et *P. moraviensis*) (Andersen *et al.*, 2000; Tvrzová *et al.*, 2006) ainsi que des psychrophiles (*P. mandelii*) (Verhille *et al.*, 1999; Jang *et al.*, 2012).

4. Potentiel d'électrotransformation des isolats de pluie

Jusqu'à présent, l'efficacité de l'électrotransformation par la foudre a été prouvée seulement chez les bactéries du sol (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006). Etant donné que les nuages sont susceptibles d'être cinq fois plus soumis aux décharges de foudre que le sol (Gary, 1999), nous avons émis l'hypothèse que les bactéries pouvaient également être électrotransformées dans les nuages. L'électrotransformation des isolats de pluie était donc le deuxième paramètre que nous avons étudié afin de déterminer si les conditions électriques rencontrées par les bactéries dans les nuages pourraient contribuer à leur potentiel adaptatif et leur sélection spécifique.

Afin de réaliser les transformations, nous avons choisi d'utiliser un plasmide donneur à large spectre d'hôte (pBLN) (Lyon *et al.*, 2010) contenant deux gènes de résistance à des

Tableau 6: Taux de bactéries totales cultivables et de *Pseudomonas* putatifs naturellement résistants à la tétracycline et à la kanamycine, naturellement transformés et electrotransformés par pBLN pour chaque épisode de pluie.

Episode de pluie	Milieu de croissance	Taux de résistance naturelle (R)	Taux de transformation naturelle (N)	Taux d'électrotransformation (E)
R1	LBCTK[1]	$2,70\times10^{-4}$	$1,14\times10^{-3}$	$2,00\times10^{-3}$
	KBCTK[2]	$0,00\times10^{0}$	$0,00\times10^{0}$	$0,00\times10^{0}$
R2	LBCTK[1]	$0,00\times10^{0}$	$8,43\times10^{-4}$	$9,81\times10^{-4}$
	KBCTK[2]	$0,00\times10^{0}$	$0,00\times10^{0}$	$3,58\times10^{-4}$
R5	LBCTK[1]	$0,00\times10^{0}$	$0,00\times10^{0}$	$0,00\times10^{0}$
	KBCTK[2]	$0,00\times10^{0}$	$0,00\times10^{0}$	$0,00\times10^{0}$

[1]LBCTK : LBC avec Tétracycline et Kanamycine
[2]KBCTK : KBC avec Tétracycline et Kanamycine

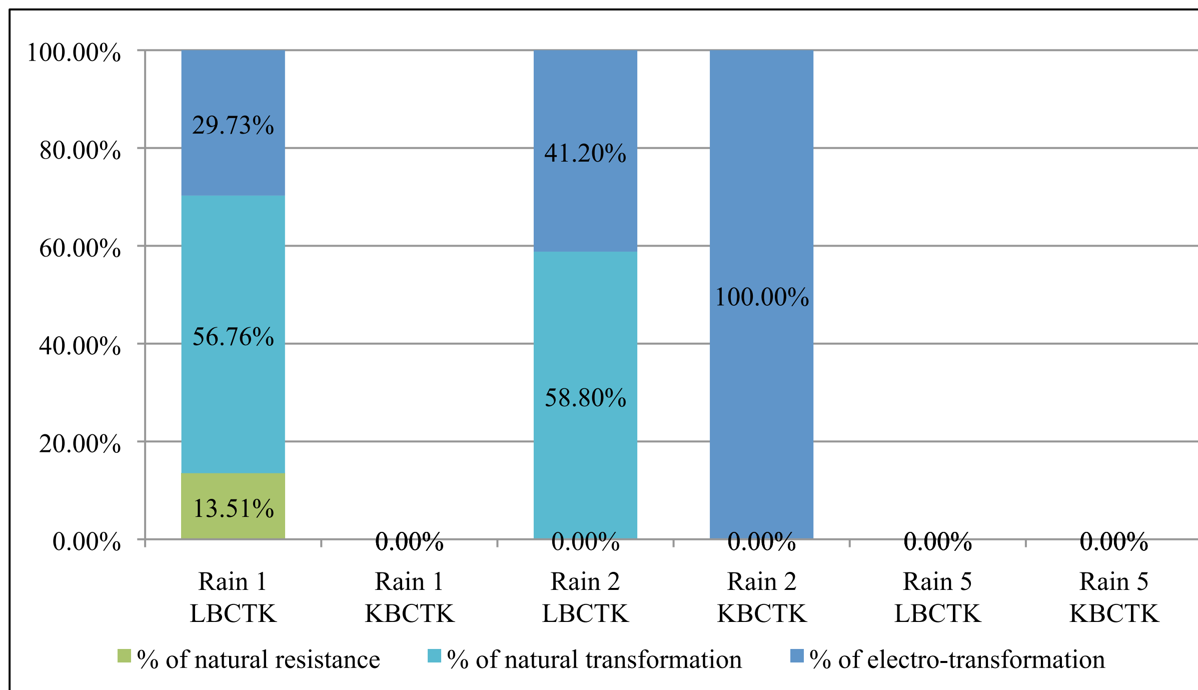


Figure 10: Distribution en pourcentages des trois types de résistance envers la tétracycline et la kanamycine parmi les bactéries cultivables et les *Pseudomonas* putatifs isolés sur LBCTK et KBCTK à partir des épisodes de pluie 1, 2 et 5.

antibiotiques (*tetA* et *aph3ia*) et un gène mosaïque et spécifique dont la présence est rare chez les bactéries environnementales, le gène *linA* (Boubakri *et al.*, 2006), utilisé pour les confirmations par PCR sur les isolats présentant une résistance aux deux antibiotiques. Ceci a été utilisé pour distinguer ces évènements d'électrotransformation des mutations spontanées ou des résistances naturelles à plusieurs antibiotiques (jusqu'à 18), fréquemment rencontrées dans l'environnement (Dantas *et al.*, 2008). L'électrotransformation a donc été vérifiée par comparaison des niveaux de résistance des isolats électrotransformés à ceux obtenus après transformation naturelle du plasmide ainsi qu'à ceux observés en absence de transformation.

La présence d'ADN nu et suffisamment préservé dans l'environnement des cellules est nécessaire pour la transformation naturelle et l'électroporation. De l'ADN dissous de différentes origines a été détecté dans l'eau de mer, l'eau douce, les sédiments et le sol à des concentrations significatives (Ogram *et al.*, 1987; Selenska and Klingmüller, 1992; Nielsen *et al.*, 2007), ces données manquent pour les nuages, la pluie ou l'air extérieur. Les concentrations d'ADN nu sont probablement faibles dans l'atmosphère mais des molécules d'ADN protégées par leur liaison avec la poussière pourraient être une source d'ADN transformant, comme dans le sol (Paget *et al.*, 1992; Demanèche *et al.*, 2001a). De plus, l'émission forte de bactéries de différentes surfaces terrestres comme des plantes, du sol et de l'eau peut également impliquer celle d'ADN exogène et l'ADN libéré par les bactéries mourantes incapables de s'adapter aux conditions rigoureuses rencontrées dans les nuages peut cependant enrichir le réservoir atmosphérique potentiel d'ADN. Des travaux plus approfondis devraient donc évaluer la présence d'ADN nu dans les échantillons atmosphériques, de nuages et de pluie.

Conditions contrôles

Nous avons d'abord défini le niveau de résistance naturelle aux antibiotiques utilisés comme marqueurs dans notre procédure expérimentale et trouvé seulement un isolat résistant à la tétracycline et à la kanamycine (représentant 13.51% des résistants de la pluie 1) (Figure 10). Tous les autres, des échantillons de pluies 1 et 2 (aucun résistant isolé de la pluie 5), ont été obtenus soit par transformation naturelle (la majorité, soit 56.76% et 58.80% respectivement pour les pluies 1 et 2 – Figure 10) soit par électrotransformation.

Les taux de transformation naturelle des bactéries totales (sur milieu LBC avec antibiotiques - LBCTK) obtenus pour les échantillons de pluie 1 et 2 étaient du même ordre de grandeur et étonnamment élevés (1.10×10^{-3} et 8.43×10^{-4} respectivement) (Tableau 6) en

ce qui concerne les taux de transformation connus obtenus avec des bactéries transformables données (de 1.90 x 10^{-9} pour *Vibrio parahaemolyticus* à 9.50 x 10^{-2} pour *Azotobacter vinelandii*) (Lorenz and Wackernagel, 1994). Les taux que nous avons détectés correspondent à la proportion de bactéries transformables de la communauté bactérienne entière de la pluie et peuvent varier individuellement d'une espèce isolée à une autre. Les conditions de laboratoire que nous avons utilisées correspondent aux conditions standards appliquées pour le développement de la compétence d'*Acinetobacter* (Demanèche *et al.*, 2002). Nous n'avons pas pris en considération les conditions nécessaires et spécifiques au développement de la compétence des autres bactéries telles que la phase de croissance spécifique, l'exigence nutritionnelle, les conditions défavorables ou le besoin de facteurs de compétence présents dans l'environnement cellulaire (Lorenz and Wackernagel, 1994; Solomon and Grossman, 1996). Il se peut que notre estimation représente donc une sous-estimation du potentiel actuel de transformabilité naturelle des isolats de la pluie.

Concernant le potentiel de transformation des *Pseudomonas* spp. putatifs, aucun transformant naturel n'a été détecté au sein des trois épisodes de pluie. Ce n'est pas très surprenant étant donné que la plupart des bactéries n'acceptent pas d'ADN exogène sans être soumises à un traitement physique ou chimique entraînant le développement de leur stade de compétence. Cependant, nombre d'espèces de *Pseudomonas* ont montré une capacité à être transformées génétiquement naturellement, par exemple *P. fluorescens* dans les microcosmes de sol, (Demanèche *et al.*, 2001b), *P. stutzeri, P. mendocina, P. alcaligenes,* et *P. pseudoalcaligenes in vitro* (ainsi que dans le sol pour *P. stutzeri*) (Carlson *et al.*, 1983; Stewart and Sinigalliano, 1989; Lorenz and Wackernagel, 1990; Lorenz and Wackernagel, 1991; Lorenz and Wackernagel, 1994).

Taux d'électrotransformation des isolats de la pluie

L'efficacité d'électrotransformation a atteint 2 x 10^{-3}pour les bactéries totales cultivables (Tableau 6). Cependant, l'électrotransformation peut être affectée par des facteurs tels que la qualité de l'ADN, les conditions d'électroporation, le nombre de bactéries et leur stade de croissance (comme montré dans la première partie de cette synthèse), les meilleurs taux étant atteints lorsque les bactéries sont concentrées et cultivées en phase exponentielle (Cérémonie *et al.*, 2004). De plus, les conditions rencontrées dans les nuages (les températures basses, la présence de cations et de surfactants, le stress nutritionnel) pourraient augmenter les taux de transformation comme vu précédemment (Shi *et al.*, 2003). Ainsi, 30 à 40% (épisodes de

pluie 1 et 2 respectivement) des bactéries de la pluie cultivables résistantes à des antibiotiques sont capables d'incorporer et d'exprimer le plasmide après un stress électrique (Figure 10), ce qui soutient notre hypothèse que les bactéries de la pluie possèdent un potentiel augmenté d'électrotransformation. Une telle efficacité d'acquisition d'ADN exogène grâce aux impulsions électriques associées aux courants de foudre dans les nuages pourrait avoir des conséquences considérables sur l'adaptation et l'évolution des bactéries disséminées.

Un seul isolat de *Pseudomonas* spp. putatif (épisode de pluie 2) a été récupéré après électroporation sur le milieu semi-sélectif KBC complété des deux antibiotiques, bien que la méthode utilisée pour préparer les cellules électrocompétentes soit censée être particulièrement efficace pour les espèces du genre *Pseudomonas* (Bassett and Janisiewicz, 2003). Ceci se traduit par une efficacité d'électrotransformation de 10^{-4}, un assez faible niveau considérant le potentiel d'électrotransformation des cellules électrocompétentes commerciales qui sont utilisées à des concentrations d'environ 10^{9} et qui peuvent atteindre jusqu'à 80% de transformation des bactéries survivantes (Drury, 1996) et les taux d'électrotransformation très élevés (10^{-2}) de certaines souches de *Pseudomonas* isolées du sol (Cérémonie *et al.*, 2006). En revanche, cette valeur est plus importante que les meilleures efficacités d'électrotransfert de 10^{-5} connues pour *P. syringae* (Wendt-Potthoff *et al.*, 1992) (Bassett and Janisiewicz, 2003).

Diversité des bactéries électrotransformées de la pluie

Ici, seules les souches contenant le gène *linA* ont été analysées. Cependant, nous ne pouvons clairement différencier les bactéries capables d'être transformées par le plasmide pBLN de façon naturelle ou grâce à l'électroporation. Cependant, nous pouvons comparer la distribution des espèces bactériennes isolées sur milieu avec antibiotiques avec ou sans choc électrique. Les taxons bactériens identifiés parmi les bactéries totales cultivables (milieu LBC avec les deux antibiotiques - LBCTK) différaient significativement de ceux caractérisés parmi les isolats cultivés sur milieu KBC. Les communautés bactériennes transformées naturellement ou électriquement étaient constituées de membres de deux phyla bactériens *Actinobacteria* et *Proteobacteria*, conformément avec les rapports précédents sur la diversité des bactéries de l'atmosphère, des nuages, de la pluie, des grêlons ou de la neige (Amato *et al.*, 2005; Maron *et al.*, 2005; Amato *et al.*, 2007b; Brodie *et al.*, 2007; Jones *et al.*, 2008; Bowers *et al.*, 2011; Vaïtilingom *et al.*, 2012). Contrairement à ces études, nous n'avons récupéré aucun représentant des groupes des *Firmicutes* ou des *Bacteroidetes*

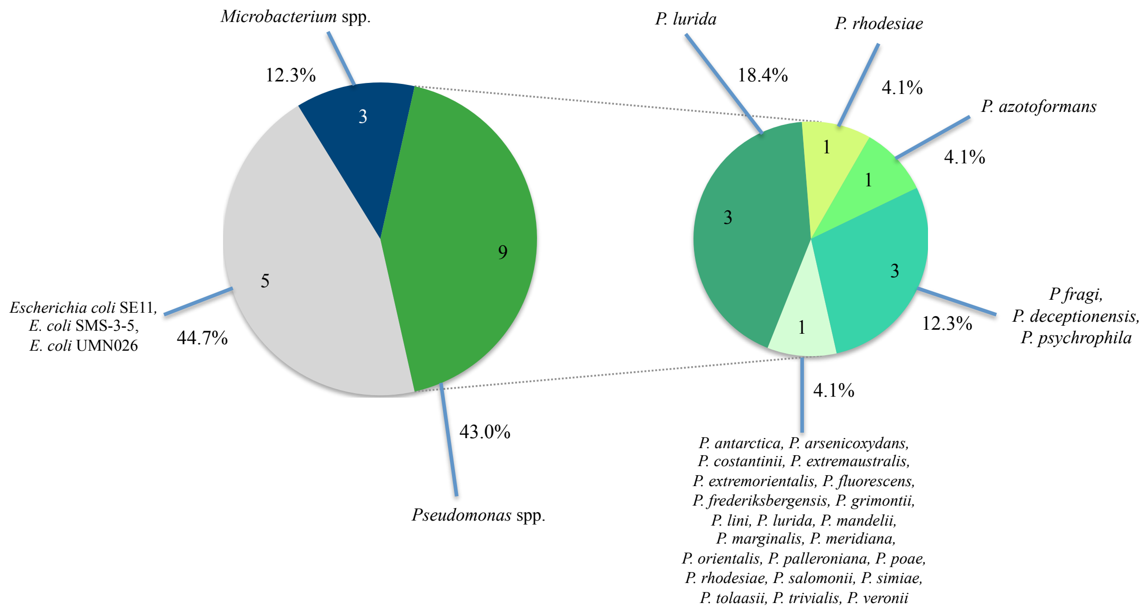


Figure 11: Distribution des transformants naturels des pluies 1 et 2 combinées isolés sur milieu LBCTK.
Isolats cultivés sur le milieu LBCTK. Le nombre d'isolats pour chaque lignée phylogénique est donné, ainsi que les pourcentages calculés après correction de la taille de l'échantillon. Les lignées sont colorées en fonction des données de regroupement phylogénique.

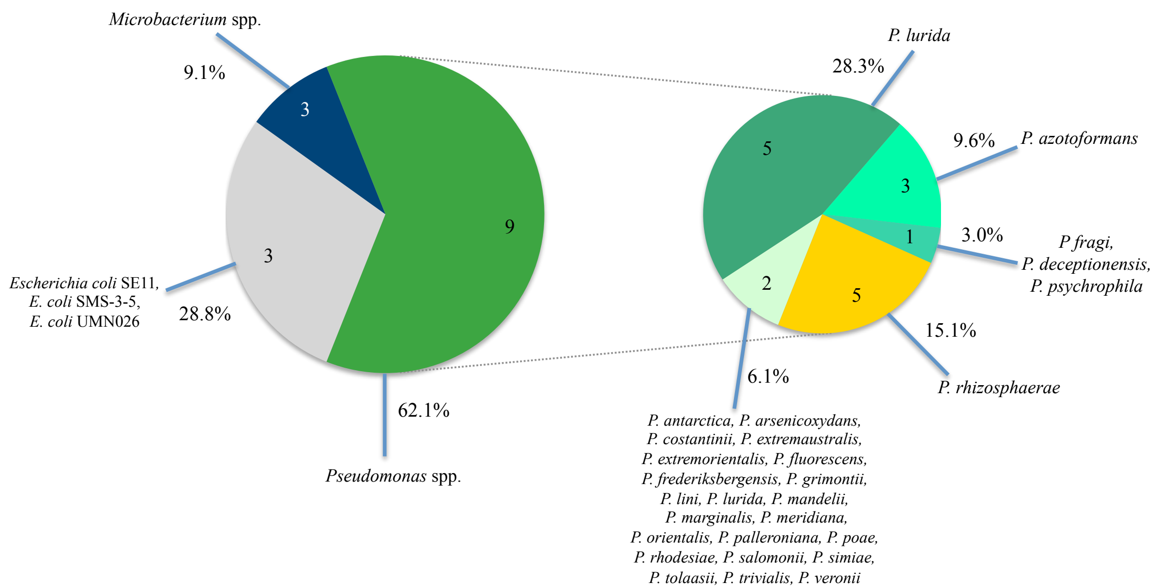


Figure 12: Distribution des transformants électroporés des pluies 1 et 2 combinées isolés sur milieux LBCTK et KBCTK.
Isolats cultivés sur les milieux LBCTK et KBCTK. Le nombre d'isolats pour chaque lignée phylogénique est donné, ainsi que les pourcentages calculés après correction de la taille de l'échantillon. Les lignées sont colorées en fonction des données de regroupement phylogénique.

(Fuzzi *et al.*, 1997; Amato *et al.*, 2007b; Šantl-Temkiv *et al.*, 2012), ni de *Burkholderiales* ou de *Moraxellaceae* et plus généralement de *Beta-proteobacteria* qui sont des composants majeurs des communautés bactériennes aéroportées (Bowers *et al.*, 2011), ou encore d'*Alpha-proteobacteria* (Amato *et al.*, 2007c; Šantl-Temkiv *et al.*, 2012). Il se peut que l'absence de *Firmicutes, Bacteroidetes* et d'autres *Proteobacteria* de nos échantillons résulte de leur incapacité à accepter de l'ADN exogène dans les conditions employées. Bien que la fraction de germes à Gram positif parmi les bactéries cultivables de l'air, de l'eau de nuages, de précipitations ou de neige de glacier soit généralement très importante, probablement parce qu'ils comprennent des bactéries sporulantes qui sont plus résistantes, nous n'avons récupéré qu'environ 10% de bactéries à Gram positives, toutes appartenant au genre *Microbacterium* de la famille *Microbacteriaceae* au sein des *Actinobacteria*. *Microbacterium* spp. représentait 21% (échantillons électroporés) à 25% (transformants naturels) des bactéries transformées de l'épisode de pluie 2, ce qui est inhabituel pour ce taxon. Les espèces les plus proches phylogéniquement de nos isolats représentent divers modes de vie et habitats comme les plantes (riz, rhizosphère du ginseng et du palétuvier), les champignons, le hamster, les nuages et l'air, le sol en tant que saprophytes, les eaux de mer, les boues marines et les sédiments profonds, certaines ayant été identifiées comme pathogènes humains ou animaux ou contaminants de laque à cheveux ; d'autres ont montré des propriétés telles que la dégradation d'insecticide et la résistance à la vancomycine (Takeuchi and Hatano, 1998; Behrendt *et al.*, 2001; Laffineur *et al.*, 2003; Ushakova *et al.*, 2004; Amato *et al.*, 2005; Bakir *et al.*, 2008; Buczolits *et al.*, 2008; Wu *et al.*, 2008; Cáceres *et al.*, 2009; Cabrera *et al.*, 2010; Srinivasan *et al.*, 2010; Woo *et al.*, 2010; Young *et al.*, 2010; Wu *et al.*, 2011; Yasuma *et al.*, 2011). Parmi ces espèces proches, *M. oxydans* a été signalé ces dernières années pour un potentiel de pathogénie en hausse, ce qui souligne le risque que comporte sa dissémination par la pluie et les bactéries aéroportées (Woo *et al.*, 2010; Yasuma *et al.*, 2011).

Les bactéries à Gram négatif dominaient la distribution d'espèces de bactéries capables d'acquérir de l'ADN, comme cela a déjà été trouvé dans les aérosols par Jones *et al.* (2008) ou dans l'eau des nuages (Lighthart, 1997), et constituaient environ 90% des communautés de transformants naturels ou induits électriquement, ce qui est comparable avec la proportion de 80% des bactéries aéroportées (Bauer *et al.*, 2003). Cette conclusion doit cependant être prise avec prudence: étant donné que nous avons focalisé sur les bactéries cultivables capables d'incorporer de l'ADN exogène, nous pouvons seulement conclure que les bactéries cultivables à Gram négatif de la pluie semblent être plus transformables que celles à Gram positif. Tous les isolats à Gram négatif sont des *Gamma-proteobacteria*, avec au moins la

moitié d'entre eux appartenant aux *Pseudomonadaceae*, contrairement au faible pourcentage de récupération observé dans les échantillons de neige (1%), d'air (3%) par Bowers *et al.* (2009), mais en accord avec l'abondance des bactéries du genre *Pseudomonas* décrites dans les gouttelettes d'eau (Amato *et al.*, 2007b), les échantillons d'air (Shaffer and Lighthart, 1997) et d'eau de brouillard (Fuzzi *et al.*, 1997). En plus des *Pseudomonadaceae*, une seule autre gamma-protéobactérie, *E. coli*, a été récupérée et constitue le second taxon le plus abondant dans les échantillons de pluie. Cette entérobactérie n'est habituellement pas détectée dans les communautés aériennes et sa présence dans la pluie n'a jamais été notée. A cause de son écologie, nous pouvons imaginer qu'elle provient de contaminations fécales d'oiseaux (Kaper *et al.*, 2004). De telles particules seraient suffisamment grandes pour être récupérées par la tombée des gouttes de pluies (McDonald, 1962; Respondek *et al.*, 1995); il se peut donc que ces bactéries ne soient pas soumises à la foudre dans les nuages. Cependant, bien qu'en proportion plus faible, certaines ont été détectées dans l'échantillon traité par la simulation de foudre. Il est intéressant de noter que les *E. coli* que nous avons récupérées après transformation naturelle et électrique sont plus proches phylogéniquement de souches virulentes et multirésistantes (SMS 3-5 and UMN026) (Fricke *et al.*, 2008; Touchon *et al.*, 2009). La dissémination par aérosolisation et pluie de telles bactéries pourrait constituer une menace pour la santé humaine.

Contrairement à *Microbacterium* spp. et *E. coli*, la proportion de *Pseudomonadaceae* parmi les transformants a augmenté de 43% à 62% quand la décharge électrique a été appliquée aux échantillons de pluie, suggérant des capacités d'électrotransformation supérieures. La plupart des isolats appartenaient au groupe *P. fluorescens*, ce qui n'est pas très étonnant étant donné que *P. fluorescens* a été identifié parmi les bactéries glaçogènes (Beattie, 2006; Bowers *et al.*, 2009). *P. lurida* était la seule espèce à être identifiée dans tous les échantillons de transformants. Cette bactérie, non récupérée quand le milieu de croissance spécifique des *Pseudomonas* putatifs a été utilisé, est un promoteur de la croissance des plantes, psychrotolérant qui a été isolé de la phyllosphère des graminées ainsi que dans le sol rhizosphérique à haute altitude (Behrendt *et al.*, 2007; Selvakumar *et al.*, 2011). Sa proportion a augmenté dans les échantillons électroporés. Une tendance similaire a été observée dans le cas des isolats de la pluie 2 affiliés au sous-groupe *P. fluorescens.* Les membres de cette lignée sont, pour la plupart, des saprophytes non pathogènes produisant un pigment fluorescent. Le plus connu d'entre eux, *P. fluorescens,* est ubiquitaire et peut être isolé du sol, de l'eau, de la nourriture comme agent d'altération et de l'Homme en tant que pathogène opportuniste (Baggi *et al.*, 1983; Gershman *et al.*, 2008). Comme *P. trivialis* (Behrendt *et al.*,

2003; Mejri *et al.*, 2012), il peut présenter des propriétés de biodégradation (Beattie, 2006) et de biocontrôle, prévenir les maladies des plantes et promouvoir leur croissance (Baggi *et al.*, 1983; Haas and Défago, 2005). Au sein de ce groupe, les espèces les plus proches phylogéniquement de nos isolats correspondaient à des bactéries psychrophiles ou psychrotolérantes isolées de l'Antarctique, de chambre froide pour la conservation de la nourriture ainsi qu'à des isolats altérant la nourriture (Ismail, 1998; Yumoto *et al.*, 2001; Reddy *et al.*, 2004; López *et al.*, 2009; Broekaert *et al.*, 2011; Carrión *et al.*, 2011; Jang *et al.*, 2012; Tribelli *et al.*, 2012), espèces qui sont probablement adaptées à des conditions environnementales rigoureuses. En revanche, la fraction de ce dernier groupe était plus importante dans l'échantillon de bactéries capables de transformation naturelle. Les isolats correspondant à l'espèce *P. azotoformans,* un phytopathogène des grains de céréales également capable de fixer l'azote comme un promoteur de croissance des plantes, ont été trouvés parmi les électrotransformants de la pluie 2 et les transformants naturels de la pluie 1. De même, le saprophyte des eaux minérales naturelles, *P. rhodesiae*, a été isolé d'un seul échantillon.

De façon plus significative, les seuls autres *Pseudomonadaceae* que nous avons identifiés appartenaient à la lignée d'espèces de *P. rhizosphaerae*. Cette espèce a été isolée du sol rhizosphérique des herbacées en Espagne et solubilise activement le phosphate *in vitro*, agissant sans doute en tant que promoteur de la croissance des plantes. En plus de leur isolement sur le milieu spécifique des *Pseudomonas,* les isolats de *P. rhizosphaerae* de la pluie 2 ont été récupérés seulement après électrotransformation sur les deux milieux additionnés des antibiotiques.

Contrairement à ce qui était attendu pour cette bactérie hautement adaptative, aucun *P. syringae* n'a été transformé naturellement ou électriquement probablement parce qu'ils n'étaient pas dominants dans la distribution globale des bactéries de la pluie.

Conclusion

À notre connaissance, ce travail est le premier à rapporter le comportement de communautés bactériennes face à la foudre. Nous avons montré que les bactéries de la pluie étaient plus résistantes à la foudre que les bactéries du laboratoire et qu'elles étaient capables d'acquérir de l'ADN par transformation naturelle et électrotransformation. Les concentrations de bactéries cultivables de la pluie étaient similaires à celles d'autres études sur les aérosols ou les précipitations mais les structures de communautés bactériennes étaient différentes. Toutefois, les structures de communautés bactériennes ne peuvent pas valablement être

comparées puisque nous avons seulement cherché la diversité des bactéries capables d'acquérir de l'ADN exogène en focalisant spécialement sur *Pseudomonas* et plus particulièrement *P. syringae*. Les bactéries isolées différaient en origines et modes de vie : en plus de capacités glaçogènes, certaines pourraient être adaptées au froid, d'autres auraient des capacités de biodégradation et d'autres encore seraient saprophytes, promoteurs de la croissance des plantes ou encore pathogènes de plantes ou de mammifères notamment *P. syringae.* En plus des substances nutritives libérées des plantes à cause des blessures causées durant les gelées et de la création de gouttelettes de pluies qui servent de vecteurs de dissémination pour la descente sur la surface terrestre, la catalyse de la formation de la glace dans les nuages pourrait augmenter la diversité génétique bactérienne, même si aucun transformant de *P. syringae* n'a été retrouvé. Ces différentes utilisations de l'activité glaçogène pourraient expliquer le caractère ubiquitaire de *P. syringae,* son potentiel d'adaptation à différents habitats et son important pouvoir pathogène pour les plantes, cause de considérables pertes économiques.

Le séquençage et les analyses phylogéniques basées sur les séquences d'ADNr SSU ne sont pas suffisants pour identifier les souches de *P. syringae*, surtout au niveau du pathovar. La prochaine étape serait donc de confirmer l'identité des souches et d'évaluer leur pathogénie. Par ailleurs, il serait intéressant de confirmer l'activité glaçogène des isolats par des expériences *in vitro* et de rechercher la présence du gène *ina*, responsable de la nucléation de la glace.

Cette étude confirme que les bactéries sont aérosolisées de différents écosystèmes terrestres et qu'elles se répandent vers de nouveaux habitats par la pluie. Nous souhaitons attirer l'attention sur le fait que la dispersion de bactéries pathogènes pourrait présenter un risque pour les écosystèmes et la santé humaine et accroître l'impact économique lié aux maladies des plantes. En plus des conditions rigoureuses que les bactéries rencontrent dans l'atmosphère, la protection contre la foudre ainsi que la transformation naturelle, chimique et électrique conditionnent probablement l'évolution microbienne.

III. EVALUATION DU POTENTIEL DE BIOAUGMENTATION GENETIQUE BASEE SUR L'ELECTROTRANSFORMATION POUR LA BIOREMEDIATION DU LINDANE

Introduction

La bioremédiation dépend de la capacité des microorganismes à dégrader ou transformer les contaminants en composés chimiques moins nocifs et, ainsi, à nettoyer les sites pollués. Les bactéries impliquées dans la bioremédiation doivent porter dans leur génome des gènes codant les enzymes nécessaires à la dégradation et doivent être capables de les exprimer sur le site pollué. Une façon de s'assurer que les fonctionnalités désirées soient disponibles sur le site est d'ajouter ou d'augmenter la présence des gènes de dégradation par « bioaugmentation », c'est à dire par l'ajout de bactéries connues pour exprimer les enzymes d'intérêt (Roane *et al.*, 2001). Le principal obstacle à la bioaugmentation est la survie de l'inoculum *in situ* (Cases and de Lorenzo, 2005; Park *et al.*, 2008). En effet, les inocula sont généralement évincés, consommés, privés de nutriments dans leur « nouvel » environnement par les bactéries indigènes mieux adaptées, ce qui entraîne fréquemment l'inefficacité de la méthode (Vogel, 1996; El Fantroussi and Agathos, 2005).

Pour contourner cette difficulté, il a été proposé l'introduction de gènes de dégradation dans les bactéries indigènes, afin de leur procurer les fonctions enzymatiques et un avantage compétitif supplémentaire. Dans la nature, des transferts de gènes ont lieu entre cellules et espèces bactériennes *via* les THG à travers différents mécanismes et jouent un rôle majeur dans l'évolution des génomes bactériens (Lan and Reeves, 1996; Ochman *et al.*, 2000; Koonin *et al.*, 2001). Il se peut que l'utilisation de ce processus naturel améliore le succès de la bioremédiation. De nombreuses méthodes impliquant les THG et les éléments génétiques mobiles (EGM) tels que les plasmides, les transposons, les éléments liés aux bactériophages et les îlots génomiques (Aminov, 2011; Popa and Dagan, 2011) sont utilisées pour la « bioaugmentation génétique » (Top *et al.*, 2002; Nojiri *et al.*, 2004). La conjugaison a été testée avec succès au laboratoire pour le transfert de gènes de dégradation (Top *et al.*, 1998; Pepper *et al.*, 2002; Desaint *et al.*, 2003; Bathe *et al.*, 2005; Nancharaiah *et al.*, 2008). Cependant, les méthodes de bioremédiation basées sur la conjugaison dépendent toujours d'un inoculum bactérien pour l'introduction des gènes de dégradation dans le sol et, parce que seulement certaines espèces bactériennes sont capables de conjuguer entre elles, un nombre limité de bactéries serait impliqué. Concernant la transduction, seule la transduction

généralisée (Ebel-Tsipis *et al.*, 1972) impliquant un bactériophage tempéré permet le transfert d'ADN entre deux bactéries mais nécessite leur proximité et leur appartenance à la même gamme d'hôte spécifique du bactériophage, ce qui limite l'utilisation de ce mécanisme de THG pour son utilisation en bioremédiation (Davison, 1999). La transformation naturelle, quant à elle, peut avoir lieu sans influence extérieure lorsque les bactéries sont naturellement compétentes. Ce mécanisme de THG a été utilisé avec succès pour introduire un plasmide contenant les gènes de dégradation de l'atrazine dans les bactéries d'un biofilm (Perumbakkam *et al.*, 2006). Cependant, la compétence naturelle est induite par des signaux spécifiques et nécessite des machineries de prise en charge et de régulation non présentes chez toutes les bactéries (Claverys *et al.*, 2009). Etant donné qu'environ 1% des espèces bactériennes décrites est naturellement transformable (Thomas and Nielsen, 2005), l'expression des gènes de compétence est difficile à prédire dans un système complexe et hétérogène comme le sol (Johnsborg *et al.*, 2007; Levy-Booth *et al.*, 2007).

Pour affecter une plus grande diversité bactérienne, l'utilisation de l'électrotransformation a été proposée pour le transfert d'ADN plasmidique libre directement dans les bactéries *in situ* par l'application de décharges électriques à décroissance exponentielle (Lyon *et al.*, 2010). En effet, c'est une des méthodes de transformation les plus puissantes puisqu'elle est indépendante de l'état physiologique des cellules et peut être appliquée à tous types de cellules (Prasanna and Panda, 1997; Yuan, 2007). Les instruments d'électroporation commercialement disponibles sont capables de délivrer des impulsions électriques haute tension sur de faibles volumes de liquide. Par ailleurs, il a été montré que l'application d'une telle impulsion par un générateur haute tension (Figure 13) afin de simuler la foudre sur un volume de sol, facilitait le transfert de gènes entre les bactéries du sol (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006).

Ainsi, l'application d'une impulsion électrique facilite l'entrée des EGM dans les bactéries qui ne participeraient pas habituellement aux THG. De l'ADN « bénéfique » pourrait donc être introduit dans le sol et intégré dans les bactéries par électrotransformation dans le but de leur apporter des fonctions essentielles pour la réalisation d'une nouvelle activité. Cette méthode pourrait être particulièrement utile pour les essais de remédiation des composés xénobiotiques (structures chimiques entièrement nouvelles synthétisés en laboratoire), pour lesquels les gènes de dégradation ne sont pas répandus ou encore indisponibles (Leisinger, 1983; Timmis *et al.*, 1994).

Dans leur pilote expérimental, Lyon et al. (2010) (Appendix III-1) ont testé la faisabilité de l'électrotransformation *in vitro* (milieu liquide) et *in situ* (sol) par un plasmide codant un

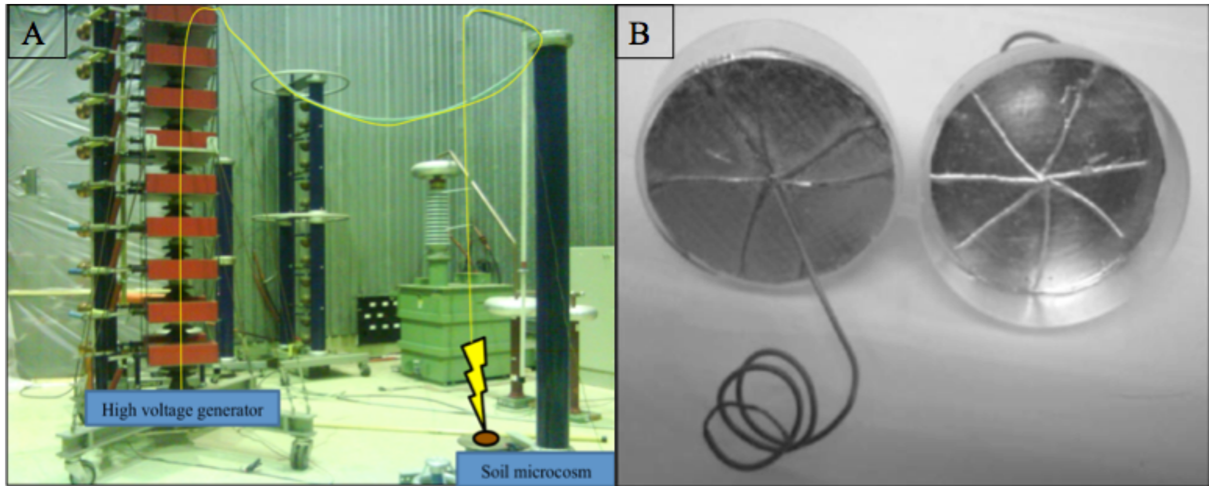


Figure 13: (A) Générateur haute tension et localisation de l'échantillon de sol; (B) vue de face et de dos des boîtes de Petri modifiées recouverte d'aluminium pour l'électrotransformation du sol.

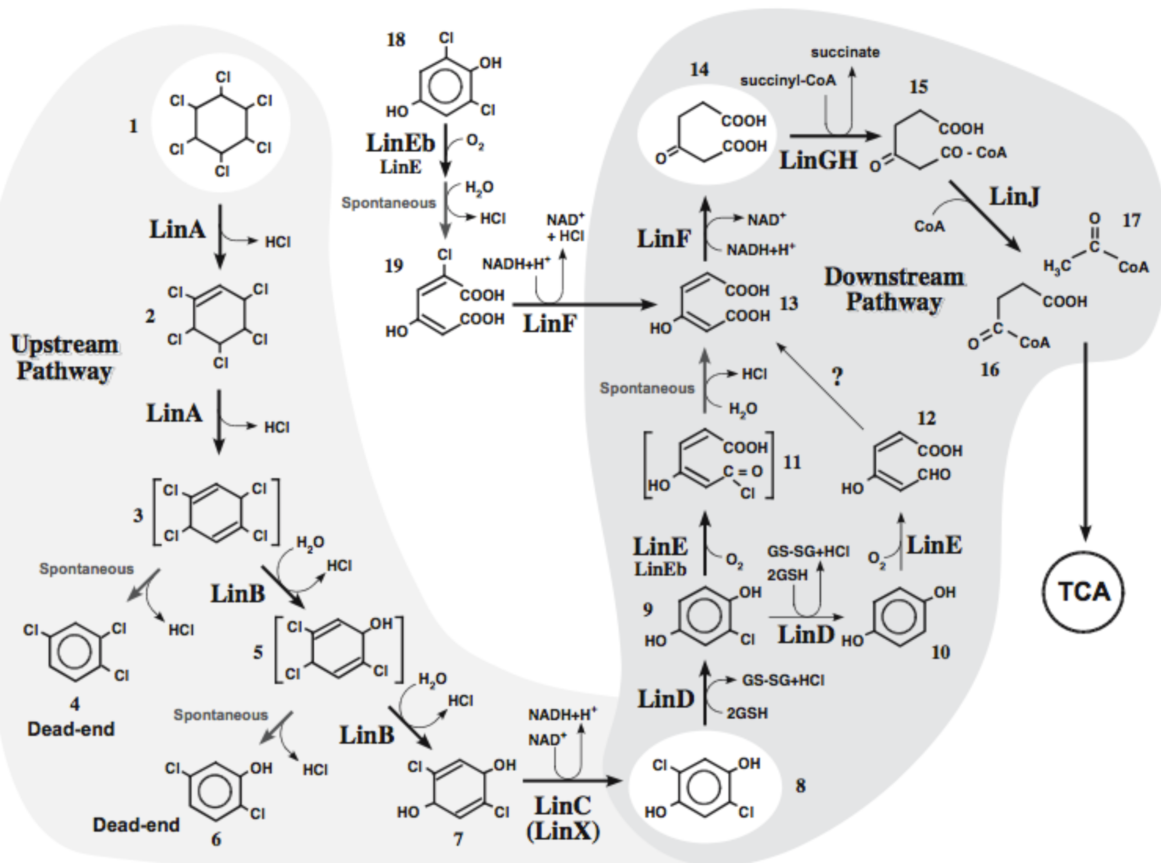


Figure 14: Voies de dégradation de γ-HCH proposées chez *Sphingobium japonicum* UT26 (tirée de (Nagata *et al.*, 2007).

Composés: **1** γ-Hexachlorocyclohexane (γ-HCH), **2** pentachlorocyclohexene (γ-PCCH), **3** 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN), **4** 1,2,4-trichlorobenzene (1,2,4-TCB), **5** 2,4,5-trichloro- 2,5-cyclohexadiene-1-ol (2,4,5-DNOL), **6** 2,5-dichlorophenol (2,5- DCP), **7** 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL), **8** 2,5-dichlorohydroquinone (2,5-DCHQ), **9** chlorohydroquinone (CHQ), **10** hydroquinone (HQ), **11** acylchloride, **12** γ-hydroxymuconic semialdehyde, **13** maleylacetate (MA; 2-maleylacetate, 4-oxohex-2-enedioate), **14** β-ketoadipate (3-oxoadipate), **15** 3-oxoadipyl-CoA, **16** succinyl-CoA, **17** acetyl-CoA, **18** 2,6-dichlorohydroquinone (2,6- DCHQ), et **19** 2-chloromaleylacetate (2-CMA). TCA: Cycle du citrate/ acide tricarboxylique; GSH: glutathione (réduit de); GS-SG: glutathione (oxydé de). Les crochets montrent les composés instables dont la présence reste à démontrer. LinE et LinEb sont principalement impliquées dans la dégradation de CHQ et de 2,6-CHQ, respectivement, chez UT26 (Endo *et al.*, 2005). Les zones claires et foncées indiquent les voies hautes et basses de dégradation, respectivement.

gène de dégradation d'un polluant vers les bactéries indigènes colonisant deux sols différents (La Côte St-André (CSA) et Montrond (MON)). Dans les deux expériences, les bactéries électrotransformées affichaient une augmentation de la dégradation du polluant (le lindane) et du nombre de copies du plasmide, l'activité de dégradation du lindane étant apparemment plus élevée dans le sol de la CSA. Bien que les expériences d'électrotransformation en milieu liquide étaient plus efficaces que celles réalisées *in situ,* Lyon *et al.* (2010) ont démontré que l'utilisation de l'électrotransformation *in situ* pouvait améliorer les taux de dégradation du polluant et potentiellement fournir un autre outil à la bioremédiation.

Forts de ces résultats préliminaires, nous avons exploré cette nouvelle voie de bioremédiation en évaluant la bioaugmentation génétique basée sur l'électrotransformation des bactéries, la contamination des sols en lindane servant de modèle d'étude. Le lindane, ou γ-hexachlorocyclohexane est un pesticide qui a été largement utilisé en agriculture avant son interdiction en 2006. La pollution persistante des sols en lindane est un problème relativement commun dû à l'étendue des applications agricoles et à sa stabilité chimique. Malgré sa libération relativement récente dans l'environnement (moins de 60 ans), plusieurs bactéries capables de dégrader et de minéraliser le lindane ont été isolées (Lal *et al.*, 2006). Une de ces espèces, *Sphingobium japonicum*, contient un gène, *linA*, qui code une protéine réalisant les deux premières étapes de déchlorination du lindane, probablement les étapes limitantes de la voie de dégradation du composé (Nagata *et al.*, 1999; Nagata *et al.*, 2007) (Figure 14). Dans cette étude, un plasmide à large spectre d'hôtes (pBLN) contenant *linA* et deux gènes de résistance à des antibiotiques (*tetA* and *aph3ia*) a été ajouté aux communautés microbiennes du sol qui n'avaient pas été préalablement exposées au lindane, afin d'évaluer la capacité de bioremédiation par bioaugmentation génétique basée sur l'électrotransformation. L'impulsion électrique a été appliquée directement sur les sols (*in situ*) grâce aux matériels adéquats disponibles au laboratoire mais aussi sur les bactéries indigènes extraites par la méthode du gradient de nycodenz pour une électrotransformation par électroporation *in vitro.* Les microcosmes avec ou sans bactéries indigènes réintroduites ont ensuite été incubés jusuqu'à plusieurs mois, la dégradation du lindane étant évaluée par extraction du composé et analyses de chromatographie en phase gazeuse et spectrométrie de masse (GC-MS). La présence et l'expression du gène *linA* ont été vérifiées et quantifiées en utilisant la qPCR et la RT-qPCR respectivement sur des ADN et ARN extraits des sols et la structure des communautés bactériennes établies par la technique des empreintes RISA. Le gène *linA* code une enzyme qui effectue les deux premières étapes de déchlorination du lindane dont la production successive de γ-pentachlorocyclohexene (γ-PCCH) et 1,3,4,6-tetrachloro-1,4-cyclohexadiene

Tableau 7: Humidité et paramètres électriques pour chaque sol utilisé.

Sol	CSA	Roth 1	Roth 2
Date d'échantillonnage	Novembre 2009	Juin 2010	Mars 2012
% d'humidité	18,67 ± 0,29	17,92 ± 0,20	28,76 ± 0,11
% d'humidité requit pour une impulsion optimale	9	21,5	16,5
Voltage d'entrée (kV)	25	40-65	40-50
Voltage de sortie (kV)	9,4-9,6	4-9	7-10
Intensité de sortie (A)	1,34-1,36	3-8	3-4

(1,4-TCDN) spontanément transformé en 1,2,4-trichlorobenzene (TCB) (Figure 14). La présence et l'abondance de ces différents composés ont également été analysées par GC-MS dans les différents échantillons de sol.

1. Détermination des conditions expérimentales optimales:

Baker *et al.* (2010) ont montré que la dégradation de pesticide dépend de la composition des communautés microbiennes mais aussi des propriétés physicochimiques du sol. Du fait de sa composition différente en argile et en matière organique, le sol limoneux argileux de Rothamsted pourrait se comporter différemment de la marne sableuse du sol de la Côte Saint-André (CSA). En effet, l'activité de dégradation du lindane s'est montrée plus efficace dans le sol CSA que dans le sol de prairie de Montrond (MON) (Lyon *et al.*, 2010).

Un autre paramètre important est la teneur en eau du sol, qui peut en affecter l'activité microbienne (Butenschoen *et al.*, 2011) et la diffusion des paramètres électriques (Cérémonie *et al.*, 2004). Dans un sol sec, la décharge électrique se concentre en un point d'impact et suit un canal de moindre résistance, alors que dans un sol humide, l'impulsion électrique se répand de façon beaucoup plus homogène; cependant, dans un sol trop humide, l'impulsion électrique présente une tension électrique diminuée. Les essais d'electrotransformation ont donc été réalisés sur des échantillons présentant pour chaque sol testé des pourcentages d'humidité variables afin d'atteindre des voltages et intensités de courant optimaux (respectivement 4-8 kV et ≈5 A). Le tableau 7 présente les pourcentages d'humidité optimaux qui ont permis d'atteindre les meilleurs voltages et intensités de sortie pour les trois sols utilisés dans notre étude : CSA, Roth 1 et Roth 2.

2. Suivi de la déchlorination du lindane par LinA:

Dans une étude pilote (Lyon *et al.*, 2010), nous avons vérifié que certaines bactéries du sol étaient capables d'acquérir le plasmide pBLN par électrotransformation dans un milieu liquide et qu'elles pouvaient exprimer *linA* (mesure de la déchlorination du lindane évaluée *via* une augmentation de chlore dans le surnageant des cultures). Tandis qu'aucune augmentation de chlore n'était détectée dans les contrôles négatifs et dans l'essai de transformation naturelle, les essais d'électrotransformation *in vitro* des deux sols montraient une augmentation significative de concentration de chlore, indiquant que le lindane avait été au moins en partie dégradé.

La seconde étape de notre étude pilote consistait en l'addition du plasmide pBLN dans le sol, l'application d'une impulsion électrique en utilisant la génératrice haute tension et le suivi

de la dégradation du lindane en parallèle de l'estimation de la présence du gène de dégradation parmi les bactéries du sol et son expression. La dégradation du lindane a été évaluée *via* une augmentation des concentrations de chlore dans le surnageant des solutions sol-eau. Après 3 semaines dans le sol CSA, l'échantillon sans choc (contrôle de transformation naturelle), l'échantillon soumis à électrotransformation et les contrôles positifs pour l'électrotransformation et la dégradation du lindane (*Pseudomonas* sp. N3 electroporés avec pBLN et *Sphingobium francense* sp+, respectivement, ajoutés dans les sols avec lindane) ont tous montré des augmentations significatives de la dégradation de lindane en comparaison du contrôle négatif avec lindane. Dans l'ensemble, la décharge électrique ne semble pas améliorer l'efficacité de transformation. En effet, la capacité à dégrader le lindane du sol CSA observée lors de l'électrotransformation *in situ* n'était pas significativement augmentée par rapport à la transformation naturelle.

Pour le sol MON, la seule augmentation significative dans la dégradation du lindane a été détectée avec l'échantillon de sol utilisé comme contrôle positif dans lequel la souche *S. francense* sp+ avait été inoculée. L'activité de dégradation du lindane semble donc avoir été plus efficace dans le sol CSA que celui de MON. D'après ce résultat, le sol CSA a été sélectionné pour une expérience à plus long terme et a montré une augmentation de dégradation de lindane au cours du temps mais pas autant que ce qui était attendu pour un résultat de deux mois. Nous en avons donc conclu que la majorité de l'activité de dégradation due à LinA s'était déjà produite durant les trois semaines d'incubation en présence de lindane après électrotransformation de pBLN. La première partie de notre étude avait pour but de mieux évaluer la période de temps nécessaire pour la déchlorination du sol par LinA et d'améliorer la détection de la dégradation du lindane et l'expression du gène *linA.*

Suivi de dégradation du lindane sur deux semaines

Dans une nouvelle série d'expériences, nous avons évalué la dégradation du lindane après électrotransformation du sol CSA et incubation sur une période de deux semaines, dégradation estimée par chromatographie en phase gazeuse couplée à de la spectrométrie de masse (GC-MS), une technique qui permet la quantification précise du lindane et une estimation de ses produits de dégradation (γ-PCCH, 1,4-TCDN et 1,2,4-TCB).

Même si ce n'était pas significativement différent, les concentrations de lindane ont diminué dans tous les échantillons au cours du temps. Ceci suggère une adsorption du lindane

sur les particules de sol au cours du temps, comme décrit par Kay and Elrick (1967) et limite donc les conclusions qui peuvent être tirées concernant la biodégradation.

Le γ-PCCH, provenant de la première étape de déchlorination du lindane par LinA, a augmenté de manière significative dans le contrôle positif de bioaugmentation (Sol avec lindane et avec *Pseudomonas* sp. N3 contenant le plasmide pBLN) après 14 jours. Ceci suggère que la dégradation du lindane pourrait être promue par l'inoculation de bactéries adaptées. Le 1,4-TCDN, produit durant la deuxième étape de déchlorination n'a pas été détecté. Ceci est sûrement dû à la troisième déchlorination spontanée du lindane produisant du TCB (Figure 14) (Nagata *et al.*, 1999; Nagata *et al.*, 2007).

Seuls les contrôles positifs d'électrotransformation (Sol avec lindane, *P.* sp. N3 et pBLN soumis à une impulsion électrique) et de dégradation du lindane (Sol avec lindane et *P.* sp. N3 contenant pBLN) respectivement, ont montré une augmentation dans l'abondance de TCB après 14 jours – bien que, encore une fois, ceci n'était pas statistiquement significatif – indiquant une tendance à la dégradation du lindane dans ces échantillons. Il se peut que cette faible production de TCB reflète un succès faible de l'électrotransformation et de la bioaugmentation. Sinon, ceci pourrait provenir de la dégradation ultérieure du TCB par les bactéries indigènes du sol (Brunsbach and Reineke, 1994; Wang *et al.*, 2007) suite à sa production par *P.* sp. N3 *via* l'expression de *linA*.

Cette nouvelle série d'expériences a montré que qu'une incubation de deux semaines était trop courte, bien que l'électrotransformation et la majorité de l'activité de dégradation due à LinA s'était déjà produite dans les trois semaines de notre étude pilote (Lyon *et al.*, 2010). La légère augmentation des produits de dégradation (en dépit de la diminution de lindane dans tous les échantillons) indique cependant une tendance prometteuse de l'efficacité potentielle des traitements de bioaugmentation ou d'électrotransformation *in situ* impliquant des bactéries inoculées.

Suivi de dégradation du lindane via électrotransformation sur une période de deux mois

Après vérification de l'absence d'adsorption du lindane par les composés du sol de Rothamsted (Roth) et l'absence de capacité de bioremédiation naturelle par les bactéries indigènes de ce sol sur une période de 23 jours, une nouvelle étude optimisée a été entreprise sur le sol Roth. Cette étude était composée (i) d'une électrotransformation directe *in situ* des bactéries du sol et (ii) d'une électrotransformation *in vitro* des bactéries extraites du sol avant

ré-inoculation dans les échantillons de sol. La dégradation du lindane était suivie sur des microcosmes de sol incubés sur une période de deux mois dans différentes conditions.

Le lindane, le TCB et le γ-PCCH ont été détectés mais pas les autres produits de dégradation (1,4-TCDN; 2,4,5 DNOL; 2,5 DDOL).

Après un mois, le lindane était présent en quantité équivalente dans tous les échantillons y compris le témoin négatif contenant uniquement du sol avec lindane. Un mois plus tard, presque tous les échantillons présentaient une diminution de la concentration en lindane, suggérant qu'une adsorption du lindane sur les particules de sol a lieu lorsque les microcosmes sont incubés pour une période plus longue (1 à 2 mois *versus* l'expérience préliminaire de 23 jours). De même, aucun des échantillons n'a montré d'augmentation significative de TCB et γ-PCCH comparé au contrôle contenant seulement le sol avec lindane après 1 ou 2 mois.

Dans l'ensemble, cette expérience de 2 mois n'a pas réussi à confirmer la faible dégradation du lindane observée pendant l'étude de 3 semaines sur le sol CSA. Comme pour l'expérience avec le sol CSA, aucun autre produit de dégradation n'a été détecté, rejetant ainsi l'hypothèse d'une dégradation plus poussée de TCB. Il se peut que cet échec dans cette tentative de bioremédiation du sol contaminé par le lindane soit dû aux propriétés physicochimiques du sol et à la composition de sa communauté microbienne, comme montré par (Baker *et al.*) en 2010.

3. Suivi du nombre de copies et de l'expression du gène *linA*:

Les extraits microbiens de CSA et MON ont tous deux montré une présence basale du gène *linA* dans le test *in vitro* de notre étude pilote (Lyon *et al.*, 2010). En accord avec les résultats de l'étude chimique ne détectant pas un accroissement de la concentration en chlore, aucune augmentation significative du nombre de copies du gène *linA* n'a été notée dans les contrôles négatifs des deux sols (extraits microbiens des sols électroporés avec lindane, extraits microbiens des sols avec pBLN pour contrôler la transformation naturelle). Dans les essais de bactéries électroporées par le plasmide pBLN et ajoutées aux sols avec lindane (essais d'électrotransformation), une augmentation significative du nombre de copies du gène *linA* a été observée seulement pour l'échantillon du sol CSA, indiquant qu'un nombre significatif de copies du gène *linA* a du être introduit dans les bactéries indigènes du sol. L'électrotransformation pourrait ainsi augmenter le potentiel de bioremédiation seulement si les gènes incorporés sont fonctionnels. La comparaison du nombre de copies du gène *linA* et des concentrations de chlore correspondantes montre clairement que les bactéries du sol CSA

sont capables d'incorporer le vecteur pBLN portant *linA* et d'exprimer le gène. Par contre, pour l'échantillon du sol MON, une libération de chlore était détectée suggérant une augmentation de l'expression de *linA* par les bactéries en dépit de l'absence d'augmentation significative du nombre de copies d'ADN.

Dans le test *in situ* de notre étude pilote (Lyon *et al.*, 2010), le fragment *linA* ciblé par qPCR était détecté dans tous les échantillons des sols MON et CSA. Pour le sol MON, le niveau de ce gène *linA* était significativement augmenté dans les échantillons de sol du fait de la transformation naturelle et de l'électrotransformation, ce qui signifie que les deux mécanismes étaient impliqués. Le niveau de *linA*, bien que plus faible dans tous les échantillons après 3 semaines, était encore significativement supérieur dans l'échantillon électrotransformé. Cependant, seul le témoin positif avec *S. francense* sp+ a montré une augmentation significative de libération de chlore. Ainsi, la bioaugmentation génétique basée sur l'électrotransformation n'était pas efficace contrairement à la bioaugmentation utilisant *S. francense* sp+ qui contient tous les gènes cataboliques pour une dégradation complète du lindane. Dans les échantillons de sol de CSA, les niveaux initiaux du gène *linA* étaient constants dans tous les échantillons, même si l'électrotransformation était censée introduire le gène *linA* dans certaines bactéries. Cette présence dans tous les échantillons provient probablement d'une contamination du sol dans notre laboratoire car le gène *linA* est un gène mosaïque et spécifique dont la présence dans les bactéries environnementales est extrêmement faible, étant restreinte aux bactéries des sols contaminés (Boubakri *et al.*, 2006). Après trois semaines comme après deux mois d'incubation avec du lindane, le fragment *linA* était toujours détecté dans tous les échantillons en accord avec les mesures de chlore. Cependant, le nombre de copies du gène *linA* continuait à diminuer au cours du temps, en contradiction avec les mesures chimiques sur le chlore. Ceci suggère une augmentation de l'expression du gène *linA* par les bactéries indigènes ou ajoutées malgré l'absence d'augmentation du nombre de copies d'ADN, donc du nombre de bactéries ayant acquis ce gène.

Forts de cette étude préliminaire, nous avons voulu améliorer la bioremédiation basée sur l'électrotransformation des bactéries indigènes du sol CSA en optimisant les conditions expérimentales. Nous avons d'abord émis l'hypothèse que le nombre de copies d'ADN *linA* ne varierait pas entre les échantillons, comme montré précédemment, tandis que l'expression du gène pourrait augmenter dans les échantillons électrotransformés et les témoins positifs. Après traitement par la DNase du sol CSA pour retirer toutes molécules de plasmides non intégrées, l'ADN et l'ARN ont été extraits et les copies d'ADN et d'ARNm de *linA* ont été

quantifiées au cours du temps par qPCR et RT-qPCR, respectivement. Les ADNr SSU ont aussi été quantifiés et utilisés pour normaliser les mesures de *linA* et afin d'évaluer de possibles biais expérimentaux.

Au cours des deux semaines de l'étude, aucun échantillon n'a présenté de différences significatives par rapport au témoin négatif contenant seulement le sol avec lindane pour les trois paramètres étudiés (nombre de copies d'ADN *linA*, nombres de copies d'ARN *linA* et ratio ADN *linA*/ADNr SSU) excepté le témoin positif de dégradation du lindane (Sol avec lindane et *P.* sp. N3 contenant pBLN) qui, après un jour, montrait des taux significativement supérieurs dans les trois cas. En effet, concernant les autres échantillons et les autres temps d'analyses, les nombre de copies d'ADN et d'ARN *linA* étaient similaires et décroissants au cours du temps et les ratios ADN *linA*/ADNr SSU étaient similaires et constants suggérant un manque d'expression de *linA* et donc de dégradation de lindane par les bactéries du sol dans tous les échantillons et à chaque temps de prélèvement. Ainsi, seul le témoin positif a montré une présence et une expression significative de *linA*, suggérant sa capacité à dégrader le lindane.

D'un point de vue général, il y avait une diminution systématique du nombre de copies au cours du temps dans chaque échantillon, comme précédemment montré par Lyon *et al.* (2010), après une période de trois semaines et qui s'intensifie après deux mois. Nous suggérons une perte de plasmides soit à cause de la mort des bactéries électrotransformées soit par un processus actif d'excrétion/exclusion (De Gelder *et al.*, 2007). Cette perte de plasmides pourrait être liée au manque d'avantage sélectif *in situ* des bactéries portant et exprimant les gènes codés et/ou au fardeau métabolique causé par pBLN dans sa bactérie hôte, entraînant l'acidification du microenvironnement local des bactéries (Cirpka *et al.*, 1999).

Quelques biais peuvent être notés afin d'expliquer ces résultats : (i) il se peut que le traitement DNase effectué avant extraction d'ADN et d'ARN manque d'efficacité à cause de l'adsorption des DNases sur les particules de sol (Demanèche *et al.*, 2001a). (ii) Vu le nombre élevé de copies des molécules d'ARN présents dans les échantillons, il est possible qu'une contamination de l'ARN par de l'ADN co-extrait se soit produite. (iii) Bien qu'aucun pBLN n'ait été rajouté dans le témoin négatif contenant seulement du sol avec lindane, comme pour Lyon *et al.* (2010), le nombre de copies d'ADN et d'ARNm de *linA* dans cet échantillon était également élevé. Dans notre étude pilote (Lyon *et al.*, 2010), il était suggéré que l'ajout de lindane sélectionne et augmente immédiatement la proportion de bactéries indigènes contenant *linA*. Etant donné l'importance du nombre de copies et le manque de dégradation

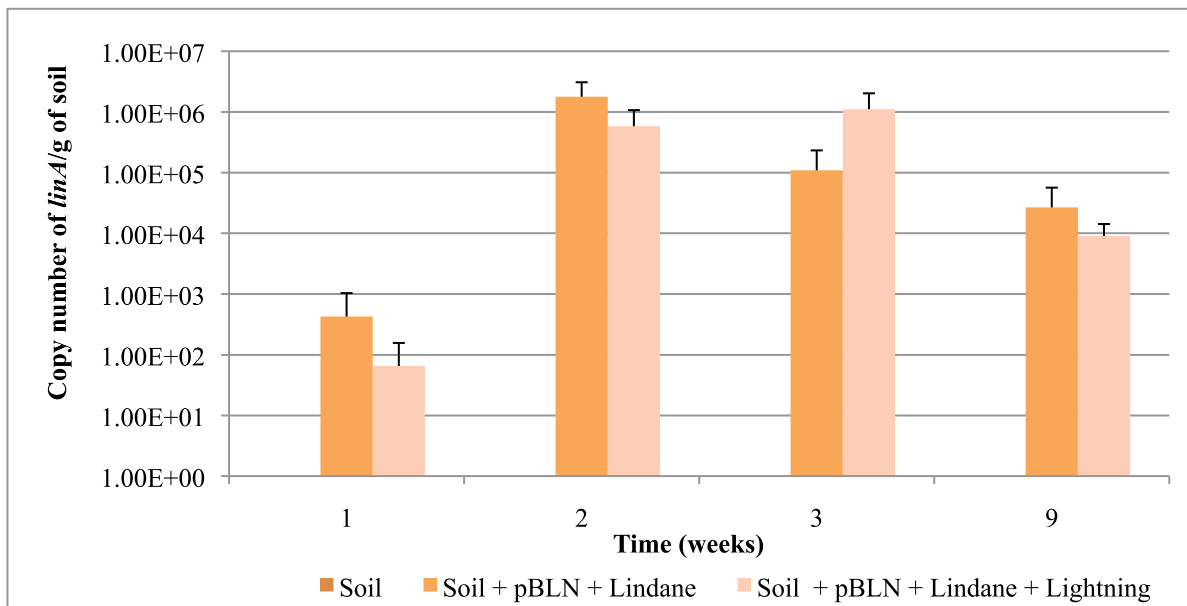


Figure 15: Copies d'ADN *linA*.g^{-1}de sol évaluées par quantification par qPCR au cours du temps pour le sol Roth dans trois différents échantillons.

montré par les analyses de GC-MS, l'explication la plus plausible implique une contamination du sol par le plasmide pBLN.

Tandis que Lyon *et al.* (2010) ont conclu à une contribution mutuelle de transformation naturelle et d'électrotransformation *in situ* dans l'augmentation de la capacité de dégradation du lindane dans le sol CSA, nous avons observé une présence non significative d'ADN et d'ARNm de *linA* dans tous les échantillons et à toutes les dates d'échantillonnage, ce qui nous empêche de tirer des conclusions. Cependant, les analyses statistiques utilisées ici sont plus rigoureuses que celles de Lyon *et al.* (2010).

4. Evaluation de l'application répétée de décharges électriques:

Nous avons évalué l'impact de chocs répétés (trois chocs, deux fois par semaine, sur trois semaines) sur la quantité de plasmides incorporés dans les bactéries du sol et sur la diversité bactérienne du sol de Rothamsted sur une période de trois semaines. En effet, des expériences *in vitro* avec des cellules mammifères ont montré que les électroporations multiples entraînaient une plus haute efficacité de transformation.

Nous avons de nouveau augmenté la quantité de plasmides ajoutés afin d'optimiser la proximité entre cellules bactériennes et plasmides et donc l'électrotransformation. Du fait des résultats précédents suggérant un manque d'efficacité du traitement DNase avant extraction des acides nucléiques, nous avons optimisé cette étape. Les qPCR ont été réalisées une fois par semaine pendant les 3 semaines d'impulsions électriques répétées puis six semaines plus tard. Nous avons vérifié au préalable et pendant toute la durée de l'étude que le sol avec ou sans lindane ne contenait aucune copie du plasmide pBLN.

Suivi de l'incorporation du gène linA:

Après une ou deux semaines, les échantillons de transformation naturelle et d'électrotransformation (respectivement sol avec plasmide et lindane et, sol avec plasmide et lindane soumis à des impulsions électriques répétées simulant la foudre), contenaient une quantité équivalente de nombre de copies de *linA* (Figure 15). Le nombre de copies de *linA* de l'échantillon d'électrotransformation augmentait au cours des trois semaines, mais n'était pas significativement différent du nombre de copies dans l'échantillon où seule la transformation naturelle pouvait être impliquée, suggérant, comme dans les travaux de Lyon *et al.* (2010), une contribution mutuelle des deux mécanismes à l'incorporation du plasmide pBLN dans les bactéries indigènes. Une incubation additionnelle de six semaines sans choc supplémentaire a

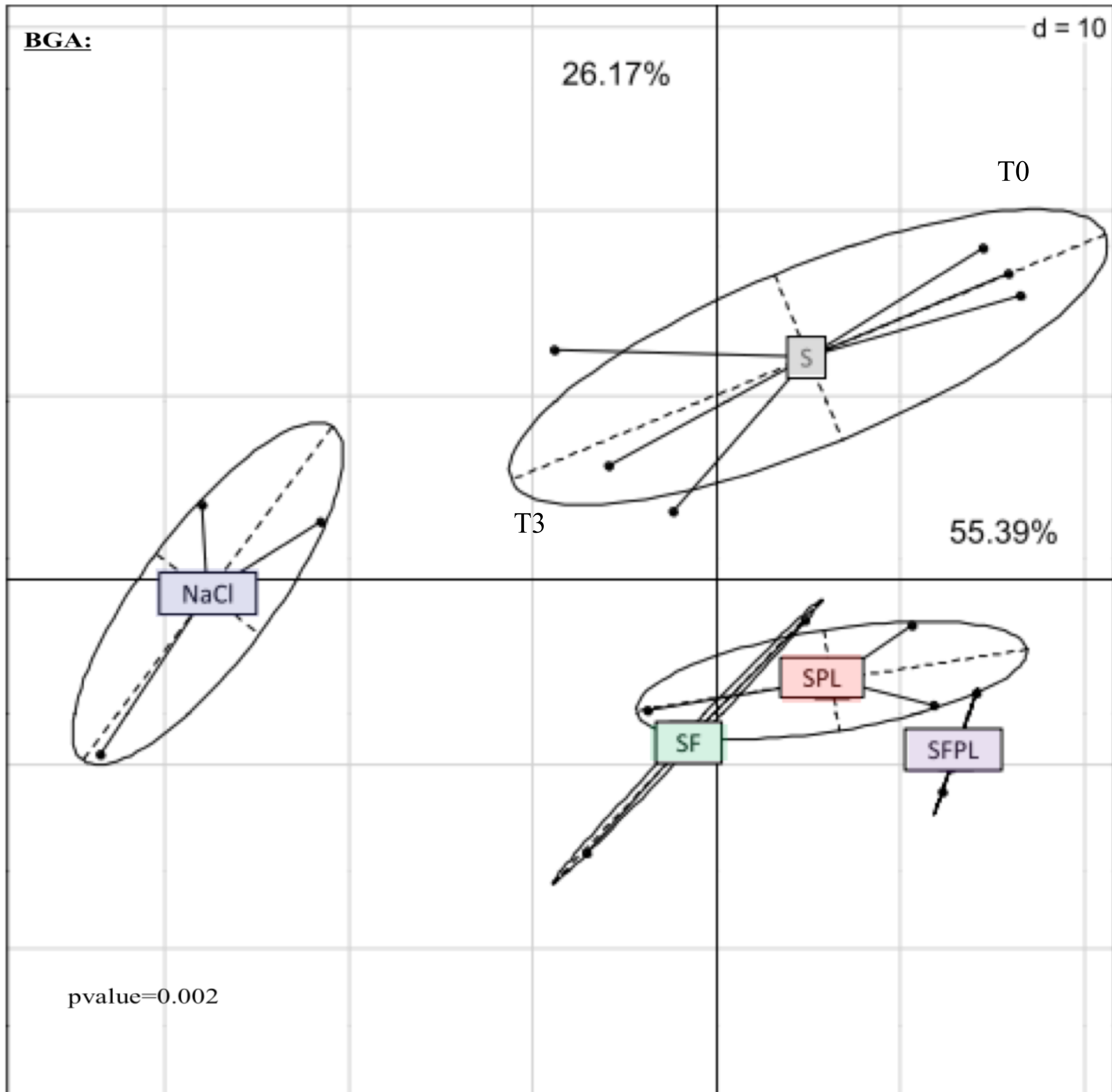


Figure 16: 'Between Group Analyses' des RISA des différents échantillons (SF, SPL et SFPL) après trois semaines de chocs répétés comparés au contrôle négatif (S à 0 et 3 semaines) et aux témoins positifs (SNaCl).

S: sol; SF: Sol soumis aux impulsions électriques; SPL: Sol avec pBLN et lindane; SFPL: Sol soumis aux impulsions électriques avec pBLN et lindane; SNaCl: Sol avec NaCl.

montré une diminution significative du nombre de copies de *linA* par gramme de sol dans l'échantillon électrotransformé. Ceci peut être dû à la perte du plasmide à cause du manque d'avantage sélectif et l'absence de chocs électriques.

Impact sur la diversité bactérienne du sol:

En parallèle à cette étude, nous avons suivi la structure des communautés bactériennes du sol Roth par amplification et comparaison des profils de RISA (analyses des espaces intergéniques ribosomaux) afin d'apprécier un possible effet des chocs répétés. Après trois semaines de chocs répétés, des modifications dans la diversité bactérienne pouvaient être observés (Figure 16) en comparaison aux témoins négatif (Sol à 0 et 3 semaines) et positif (Sol avec NaCl). En effet, la salinité, connue pour changer la conductivité électrique dans le sol, peut significativement réduire la diversité bactérienne (Arshad and Farooq, 2003).

La différence majeure observée concernait le contrôle positif (SNaCl séparé des autres sur le 1er axe expliquant 55,39% de la variabilité d'échantillons RISA), la deuxième différence la plus marquante (axe 2 expliquant 26,17% de la variabilité) étant observée entre le témoin négatif (Sol S) et les échantillons traités (SF : Sol soumis aux impulsions électriques; SPL : Sol avec pBLN et lindane; SFPL : Sol soumis aux impulsions électriques avec pBLN et lindane). De plus, la diversité bactérienne a changé au cours des trois semaines d'incubation (échantillons de sol T0 *versus* T3). Parmi les échantillons incubés trois semaines, SFPL était le seul séparé du témoin négatif (S3T) le long du 1er axe, tandis que les échantillons SF et SPL chevauchaient avec ce dernier. Les deux traitements ont altéré la structure des communautés microbiennes, le changement le plus important étant dû aux électrotransformants. Il se peut que les bactéries capables de résister aux impulsions électriques répétées, principalement après électrotransformation, se soient multipliées au sein de la communauté bactérienne, comme rapporté par Cérémonie *et al.* (2008), l'impulsion électrique induisant une redistribution des composés du sol favorisant une augmentation de certaines populations bactériennes.

Après six semaines d'incubation additionnelle, sans décharge électrique supplémentaire (neuf semaines au total), tous les échantillons présentaient globalement une structure de communauté bactérienne similaire (données non montrées). Le seul effet visible était lié à la période d'incubation additionnelle de six semaines. La modification des communautés bactériennes n'est donc maintenue que par des applications répétées de chocs électriques.

Tableau 8: Nombre de cellules bactériennes du sol viables, cultivables, résistantes aux antibiotiques et dégradant le lindane après différents traitements.

	Cellules viables.mL^{-1} avec le kit live/dead	UFC.mL^{-1} sur TSA-C 1/10ème	UFC.mL^{-1} sur TSA-CTK 1/10ème	UFC.mL^{-1} sur TSA-L 1/10ème	Présence de zones de dégradation du lindane	Survie des bactéries (%)
Bactéries du sol (Contrôle)	$2,06\times10^{8} \pm 5,57\times10^{1}$	$5,52\times10^{6} \pm 4,82\times10^{6}$	0	$2,88\times10^{6}$	+	/
Bactéries du sol + pBLN (Contrôle de transformation naturelle)	/	$7,00\times10^{5}$	0	$4,60\times10^{5}$	+	/
Bactéries du sol électroporés avec pBLN (Echantillon d'électro-transformation)	/	$3,01\times10^{5} \pm 2,55\times10^{4}$	$2,00\times10^{1} \pm 1,63\times10^{1}$	$2,36\times10^{5} \pm 2,83\times10^{4}$	++	43,00 ± 3,64%

Les valeurs avec écart types sont la moyenne d'au moins trois répétitions d'expériences. TSA: milieu Trypto-caséine soja ; TSA-C : milieu TSA avec de la cycloheximide ; TSA-CTK : milieu TSA avec de la cycloheximide, de la tétracycline et de la kanamycine ; TSA-L : TSA avec du lindane.

5. Potentiel de bioremediation des bactéries indigènes du sol bioaugmentées génétiquement:

Potentiel d'électrotransformation in vitro des bactéries indigènes du sol:

Le potentiel d'électrotansformation des bactéries indigènes du sol a été vérifié *in vitro* en comparant la capacité de dégradation du lindane et le niveau de résistance à des antibiotiques des isolats électrotransformés à ceux obtenus après transformation naturelle du plasmide ainsi qu'à ceux observés en absence de transformation.

Les bactéries indigènes, extraites du sol de Rothamsted, étaient au nombre de ≈ 2.10^8cellules viables par mL de sucrose dont 2.68% de cultivables sur TSA-C (Tableau 8). Nous avons établi le niveau de résistance naturelle aux antibiotiques utilisés comme marqueurs dans notre procédure expérimentale et aucune bactérie naturellement résistante à la tétracycline et à la kanamycine n'a été isolée sur le milieu TSA-CTK. En revanche, certains halos de dégradation étaient détectés sur le milieu TSA-L suggérant un potentiel naturel de dégradation du lindane chez certaines bactéries de ce sol.

Le témoin de transformation naturelle ne contenait pas non plus de bactéries capables de résister aux deux antibiotiques mais contenait certaines bactéries dégradant le lindane. Quarante-trois pourcent des bactéries étaient capables de survivre au choc électrique et 20 UFC. mL^{-1} (en moyenne) étaient capables de résister aux deux antibiotiques sur TSA-CTK avec en plus, une augmentation de la taille des halos de dégradation du lindane sur TSA-L confirmant ainsi le potentiel d'électrotransformation des bactéries indigènes du sol Roth.

Potentiel de bioremédiation in situ des bactéries indigènes du sol électrotransformées in vitro

Les bactéries extraites du sol Roth et soumises à une impulsion électrique *in vitro* ont ensuite été replacées dans le sol afin d'évaluer si ce processus pourrait permettre la bioremédiation *in situ* et/ou être plus efficace que l'expérience uniquement *in situ* (cf. « Suivi de dégradation du lindane *via* électrotransformation sur une période de deux mois »). Comme au préalable, seuls le lindane, le TCB et le γ-PCCH ont pu être mesurés au cours du temps dans les différents échantillons. Aucun autre produit de dégradation n'a été détecté.

Après un mois d'incubation, aucun des échantillons n'a montré de diminution significative de la concentration en lindane comparé au témoin négatif contenant seulement le sol avec lindane. Après deux mois, tous les échantillons ont montré une diminution similaire

suggestive de l'adsorption du lindane sur les particules de sol, comme dans l'expérience *in situ* sur le sol Roth, empêchant de tirer des conclusions en ce qui concerne la dégradation du lindane. De même, il n'y avait pas de différence significative d'abondance relative de TCB entre les différents échantillons après un mois. Après deux mois, aucun des échantillons n'a montré d'augmentation significative du produit de dégradation TCB comparé au témoin négatif contenant seulement le sol avec lindane. Aucun des échantillons n'a montré d'augmentation significative de γ-PCCH en comparaison du témoin négatif contenant seulement le sol avec lindane, après un ou deux mois.

Bien que l'expérience d'électrotransformation *in vitro* ait donné de meilleurs résultats, elle n'a pas été efficace lorsque les bactéries ont été réinoculées dans les microcosmes de sol, ce qui est communément expliqué dans la littérature par la nature fondamentalement complexe des sols (Desaint *et al.*, 2003). De plus, le manque de pression de sélection *in situ* pourrait expliquer pourquoi les expériences d'électrotransformation liquide donnaient de meilleurs résultats que les expériences d'électrotransformation *in situ* des sols. L'explication la plus plausible est que pBLN ne fournirait pas d'avantage sélectif *in situ* aux bactéries exprimant les gènes qu'il code car ne permettant pas la formation de nouvelles sources de carbone utilisables par les bactéries l'hébergeant. De plus, le maintien du plasmide ainsi que l'acidification de l'environnement local due à la déchlorination du lindane représentent un fardeau métabolique et entraînent probablement la perte du plasmide.

Conclusion

Notre étude a montré que la bioaugmentation génétique liée à l'électrotransformation *in situ* ne pouvait pas servir d'outil de bioremédiation pour la dégradation du lindane dans ces conditions. L'adsorption sur les particules de sol semble constituer une difficulté majeure pour l'addition de pBLN et de lindane au cours du temps. Plusieurs améliorations pourraient être proposées : (i) la désorption des molécules liées aux particules de sol avant extraction de lindane et traitement DNase. En effet, les DNases (comme les acides nucléiques) peuvent être liées aux particules de sol (Demanèche *et al.*, 2001a), réduisant ainsi leur biodisponibilité ; (ii) l'amélioration de l'extraction de lindane des sols par étape d'extraction supplémentaire (Rigas *et al.*, 2007; Benimeli *et al.*, 2008; Calvelo Pereira *et al.*, 2008) ; (iii) l'extraction améliorée des seuls plasmides incorporés, (iv) l'utilisation de liposomes ou de cellules contenant de l'ADN pour améliorer la délivrance des gènes (Sato *et al.*, 2005).

Le système choisi dans cette étude, la dégradation du lindane *via* l'acquisition du plasmide catabolique pBLN, avait pour but de démontrer la faisabilité de la méthode générale

de bioremédiation mais s'est avéré inapproprié car ne contenant que le gène *linA* qui code une protéine réalisant seulement les deux premières étapes de déchlorination du lindane. Dans de futurs essais, les EGM choisis devraient conférer un avantage sélectif aux bactéries portant et exprimant l'ensenble des gènes codant une voie de dégradation du polluant afin d'améliorer leur persistance *in situ* dans la communauté bactérienne. Par exemple le plasmide pADP-1, qui contient les séquences cataboliques complètes (Martinez *et al.*, 2001) d'un autre pesticide dangereux largement utilisé, l'atrazine (Gammon *et al.*, 2005), pourrait conférer un avantage sélectif aux bactéries électrotransformées puisqu'elles pourraient utiliser l'atrazine comme source de carbone tout en étant moins affectées par l'acidification induite dans l'environnement local comme cela se produit durant la déchlorination des composés polychlorés comme le lindane.

Même si nous parvenions à surmonter ces difficultés avec les modifications proposées et à accomplir une bioremédiation efficace par électrotransformation *in situ*, un autre obstacle devrait être affronté – l'application actuelle de cette technologie sur le terrain. Deux méthodes peuvent être proposées : premièrement, la génération du courant *in situ* ou son application à des échantillons de sol plus petits ensuite replacés en champ. La seconde proposition, l'utilisation de microcosmes pour réinoculer le sol, serait plus faisable avec les technologies existantes. Les bactéries du sol pourraient être extraites du site à remédier, électrotransformées avec des gènes de dégradation d'intérêt avantageux et ensuite réinoculées sur le site. Ceci présente un avantage par rapport à la bioaugmentation, puisque les bactéries indigènes sont plus adaptées au site que les inocula étrangers. Cependant, de telles approches soulèveraient des questions légales et éthiques de formation *in situ* ou libération dans la nature d'organismes génétiquement modifiés.

CONCLUSION ET PERSPECTIVES

La présence dans les nuages de bactéries ayant la capacité de former des noyaux glaçogènes qui entraînent des précipitations (Morris *et al.*, 2004) et étant impliquées dans le déclenchement de la foudre (Gonçalves *et al.*, 2012) associées à des impulsions électriques compatibles (quelques kV.cm^{-1})(Nucci *et al.*, 1988), nous a amené à postuler que l'électrotransformation naturelle dans les nuages pourrait affecter les bactéries de la même façon que ce qui a été observé dans les sols (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006). Nous avons émis l'hypothèse que la bactérie glaçogène et phytopathogène *Pseudomonas syringae* tire avantage de son potentiel glaçogène et induit le déclenchement de phénomènes pouvant contribuer à augmenter son potentiel adaptatif, améliorant ainsi sa dissémination et son pouvoir pathogène.

Nous avons donc tout d'abord déterminé si une bactérie glaçogène pouvait survivre et acquérir de nouveaux gènes dans les nuages en électroporant *in vitro P. syringae* CC0094 afin de simuler les décharges de foudre. Comparé à deux autres bactéries (*Pseudomonas* sp.N3 et *Escherichia coli* TOP10), *P. syringae* semblait être le meilleur candidat pour survivre et être génétiquement électrotransformé dans les nuages, ce qui suggère sa capacité à survivre et d'évoluer tout en étant transporté dans les nuages. De plus, il se peut que *P. syringae* soit impliquée dans le déclenchement de la foudre dans les nuages (Gonçalves *et al.*, 2012), ce qui aiderait les bactéries aérosolisées à développer de nouvelles compétences comme la phytopathogénie, à améliorer leur fitness, à s'adapter à de nouvelles niches et influencer les communautés microbiennes indigènes.

Ensuite, nous avons évalué l'impact de la foudre sur la survie, le potentiel d'électrotransformation et la diversité des bactéries de la pluie. Elles ont montré une meilleure résistance à la foudre que les souches de laboratoire, peut-être parce qu'elles ont été positivement sélectionnées lors de leur séjour atmosphérique en conditions rigoureuses. Au contraire, il se peut que les conditions de laboratoire aient, d'une certaine manière « contre sélectionnées » les souches de laboratoire. *P. rhizosphaerae* était la seule espèce à être récupérée seulement après électrotransformation. *P. syringae* était relativement peu représentée parmi les isolats de pluie qui contenaient différents origines et mode de vies, notamment des bactéries adaptées au froid, glaçogènes ainsi que des pathogènes mammifères et végétaux, des saprophytes ou des promoteurs de croissance des plantes et des bactéries avec des capacités de biodégradation, représentatifs des différentes sources d'émission

terrestre de bactéries. Bien que résistante aux décharges électriques, *P. syringae* n'a pas été isolée parmi les bactéries naturellement ou électriquement transformées, contrairement à notre étude *in vitro* d'électrotransformation de *P. syringae* CC0094 en conditions de simulation des nuages. Cette étude, la première à notre connaissance à aborder ces questions, suggère l'aérosolisation des bactéries de différents écosystèmes terrestres et leur capacité à se disséminer vers de nouveaux habitats grâce aux nuages. La plupart de ces isolats de la pluie semblaient provenir, sans étonnement, de la végétation, comme constaté par Šantl-Temkiv *et al.* (2013), qui ont suggéré le fait que les nuages orageux sélectionnaient les bactéries des plantes en raison des facteurs de stress partagés entre la phyllosphère et l'atmosphère. Les bactéries associées aux plantes semblent être mieux adaptées que celles du sol à la survie et la croissance en conditions atmosphériques. La matière organique, quant à elle, est préférentiellement aérosolisée des sols et pourrait supporter la vie et la croissance des bactéries dans les nuages. De plus, la poussière du sol est un moteur majeur de la nucléation de la glace dans les nuages entraînant des précipitations (Conen *et al.*, 2011). La matière organique du sol pourrait agir de manière synergique avec les bactéries associées aux plantes afin d'aider ces organismes à augmenter leur potentiel adaptatif par l'intermédiaire de la formation de la foudre à l'intérieur des nuages et à se disséminer grâce aux précipitations.

Ces deux études suggèrent que, tout en se disséminant par l'intermédiaire des nuages et en induisant passivement le déclenchement de la foudre, les bactéries glaçogènes pourraient acquérir de nouveaux gènes dans les nuages par électrotransformation naturelle due à la foudre, pouvant ainsi augmenter leur diversité génétique. En plus des conditions rigoureuses que les bactéries rencontrent dans l'atmosphère, il se peut que la protection contre la foudre ainsi que la transformation naturelle ou l'électrotransformation contribue à l'évolution microbienne. Nos résultats pourraient expliquer pourquoi les *Pseudomonas* sont parmi les bactéries les plus ubiquitaires et les plus glaçogènes rencontrées dans la nature, comment elles s'adaptent à différents habitats et comment elles provoquent des maladies (de plantes) importantes d'un point de vue économique. De plus, ces bactéries pourraient être des agents responsables des précipitations et leur éradication pourrait menacer une source générative de pluie (Cohen, 2012).

Des travaux plus poussés devraient inclure des mesures d'activité de nucléation de la glace et de pathogénie sur les isolats de la pluie, et plus particulièrement sur *P. syringae* et sur les *P. rhizosphaerae* électrotransformés. Pour ces organismes, des études supplémentaires devraient être effectuées dans le but d'évaluer leur capacité à survivre et à être électrotransformés dans les conditions de nuages simulées utilisées dans la première partie de

ce travail (Chapitre I). Une autre voie de recherche serait de confirmer l'effet de protection contre la foudre en conditions glacées sûrement dû à l'activité glaçogène en testant les mutants *ina-* de la même souche de *P. syringae* (CC0094) qui ont perdu leur capacité glaçogène. D'avantage de confirmations des résultats devraient être recherchées en réalisant des études similaires en conditions expérimentales améliorées, les plus proches possibles de l'environnement *in situ* du nuage, par exemple en utilisant la chambre à nuage disponible dans l'établissement AIDA (Aerosol Interaction and Dynamics in the Atmosphere) à Forschungszentrum Karlsruhe (Möhler *et al.*, 2008). En effet, AIDA peut être mis en marche comme une chambre à expansion pour étudier les processus de formation des aérosols nuageux dans les conditions simulées de température et d'humidité des nuages atmosphériques.

Au-delà des études sur les nuages, il se peut que d'autres bactéries environnementales soient soumises à l'électrotransformation grâce à la foudre, plus particulièrement les bactéries du sol. Des expériences préliminaires rapportant l'isolement de transformants à partir de sol soumis à des décharges de foudre simulées ont permis de confirmer l'implication potentielle de tels phénomènes météorologiques dans le déclenchement d'échanges de gènes entre bactéries environnementales (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006). Le fait que l'électrotransformation naturelle due à la foudre contribue aux THG chez les bactéries telluriques nous a permis d'envisager l'application de l'électrotransformation pour l'ingénierie des bactéries indigènes du sol. La dernière partie de ce travail a ainsi porté sur le transfert d'ADN plasmidique libre directement dans les bactéries sur le terrain en appliquant une décharge électrique. Nous avons évalué si cette méthode pouvait affecter un nombre plus grand de bactéries et plus d'espèces bactériennes. Cette étude a été réalisée dans le but de développer un outil pour la bioremédiation du lindane, un pesticide largement utilisé avant d'être interdit d'utilisation en agriculture en 2006.

- Un pilote expérimental publié en 2010 dans 'Bioremediation Journal' (Lyon *et al.*, 2010) a permis de démontrer le potentiel d'utilisation de l'electrotransformation *in situ* entraînant la bioaugmentation génétique des sols comme outil de bioremédiation. Cependant, les expériences d'électrotransformation en milieu liquide se sont montrées plus efficaces que celles *in situ*.

- L'optimisation de la procédure *in situ* impliquant le contrôle méticuleux des conditions expérimentales (ajustement de l'humidité et des paramètres électriques, amélioration des étapes de préparation de biologie moléculaire et de chimie, essais sur des

sols avec différentes caractéristiques physico-chimiquess et suivis sur différentes périodes de temps) a révélé que les deux types de transformation, naturelle et électrique, contribuaient à l'incorporation par les bactéries indigènes du sol d'un plasmide portant un gène codant les premières étapes de déchlorination. La structure de la diversité bactérienne semblait être affectée par les différents traitements, le plus grand étant pour les électrotransformants. Il se peut que la proportion des bactéries capables de résister à des impulsions électriques répétées, surtout après électrotransformation, ait augmenté au sein de la communauté bactérienne.

- Finalement, les essais de bioaugmentation génétique basés sur l'électrotransformation *in vitro* de bactéries telluriques extraites avant d'être ré-inoculées dans leur sol d'origine, n'a pas montré d'effet significatif lorsqu'elles ont été remises dans le sol. Ceci suggère l'absence de potentiel de bioremédiation *in situ* des bactéries indigènes électrotransformées malgré leur capacité de bioremédiation *in vitro*.

Outre la possibilité de phénomènes d'adsorption sur les particules de sol au cours du temps (pour le plasmide et/ou le polluant, ce qui mériterait des essais plus poussés), il se peut que la faible bioremédiation basée sur l'électrotransformation résulte du fardeau métabolique imposé aux bactéries qui ont incorporé le plasmide. Par conséquent, des expériences plus approfondies devraient utiliser des éléments génétiques mobiles qui confèreraient aux bactéries de nouveaux traits, améliorant leur compétitivité dans la communauté bactérienne. La meilleure façon pour appliquer cette technologie sur le terrain devrait être néanmoins l'électrotransformation *in vitro* de bactéries extraites de sol avant leur ré-inoculation dans le sol. De nombreuses modifications et ajustements sont encore nécessaires pour optimiser le développement de l'outil avec pour objectif la bioremédiation.

Nous avons donc montré que l'électrotransformation naturelle due aux décharges électriques telles que celles ayant lieu dans les nuages et atteignant le sol, peut être impliquée dans le processus de THG chez les bactéries. Considérant l'importance de la foudre à travers le monde, ce phénomène pourrait jouer un rôle dans l'adaptation et l'évolution de ces organismes. Reste maintenant à démontrer l'existence de ce phénomène dans la nature et d'étudier jusqu'à quel point il peut constituer un mécanisme évolutif conséquent.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

For more than 3.5 billion years, bacteria have evolved on Earth with a world abundance now estimated at five nonillions of cells (5×10^{30}) (Whitman *et al.*, 1998). They adapt constantly to continuous disturbance of the environment, which has allowed them to colonize the most diverse ecosystems (Whitman *et al.*, 1998), including soils (Torsvik *et al.*, 1990), water (LeChevallier *et al.*, 1980), air (Imshenetsky *et al.*, 1978), rain (Ahern *et al.*, 2007), snow (Yan *et al.*, 2012) and clouds (Amato *et al.*, 2005). Bacteria are also encountered in extreme environments, such as in acid mine drainage (Baker and Banfield, 2003), deserts (Chanal *et al.*, 2006), under high temperature conditions (Stetter, 1999), in hyper-saline environments (Sorokin *et al.*, 2006), paleolithic caves (Urzì *et al.*, 2010), Earth polar ice (Staley and Gosink, 1999), deep-sea sediments (Quigley and Colwell, 1968), polluted sites (Hassen *et al.*, 1998), volcanoes (Lösekann *et al.*, 2007) and radioactive waste (Fredrickson *et al.*, 2004). They have considerable importance in ecosystems, where they actively participate in the biogeochemical cycles of many elements such as carbon, nitrogen, phosphorous, sulfur and magnesium (Evans, 1976; Falkowski *et al.*, 2008; Godfrey and Glass, 2011). They are capable of degradation and mineralization of the incoming and sometimes recalcitrant organic matter in many habitats (Amon and Benner, 1996; Štursová *et al.*, 2012). We also owe them "the invention" of photosynthesis, which, in the beginnings of life on Earth allowed atmospheric enrichment with oxygen (Edwards, 2004). They can also interact with humans, plants, animals, insects and other microorganisms (Slater, 1984; Lerouge and Vanderleyden, 2002; O'Hara and Shanahan, 2006; Douglas, 2009; Grice *et al.*, 2009; Mansfield *et al.*, 2012; Vorholt, 2012). In addition, microorganisms have a substantial impact in several economical sectors. For instance, bacteria play a huge role in sewage treatment and oil spill breakdown, and in agribusiness like cheese and yogurt production through fermentation (Leroy and De Vuyst, 2004). They are also important for the recovery of many metals in the mining sector (Rawlings, 2002), as well as in biotechnology and in the industrial production of many chemicals and antibiotics (Ishige *et al.*, 2005). They are strongly involved in agriculture through soil fertilization processes and maintenance (Babalola, 2010), plant growth promotion (Lucy *et al.*, 2004), and in pest population control (Raaijmakers *et al.*, 2002). They have important roles in human and animal health, living on the skin and within the digestive tract as a natural defense against pathogens (Thompson, 1978) and participating in the digestion and assimilation of some aliments (Tappenden and Deutsch, 2007). Unfortunately, they are also responsible for many infectious diseases, affecting humans (World Health Organization, 2013) and animals and are responsible for huge economical losses in agriculture by infecting many plant species (Mansfield *et al.*, 2012).

GENERAL INTRODUCTION

Contrary to the "traditional" vertical transmission of genetic characters from both parents to descendants allowing diversity in superior organisms during the sexual reproduction meiosis, the bacterial division by fission leads to only few genetic variations, resulting in a low degree of diversity between cells. To diversify their genetic material available and to adapt to environmental disturbances or new ecological niches (Wiedenbeck and Cohan, 2011), the evolutionary processes of bacteria have included evolution by mutations occurring in the genome through modifications in the nucleic sequence. Several factors are known to induce mutations such as UV light exposure and oxidative stress conditions (Demple, 1991) but also by cellular enzymes such as DNA polymerases involved in the maintenance, replication and repair of DNA molecules, and these are known to commit errors in the DNA sequence with a rate of 1 wrong base in every 10 000 000 bases processed (Voliotis *et al.*, 2012). In most cases, these mutations result in deleterious effects on cells by key gene modifications leading to their inactivation or by the production of altered or non-functional proteins. In these cases, the bacteria harboring such mutations would be less suitable for passing through the natural selection pressure from the environment (Loewe *et al.*, 2003). Nevertheless, mutations can sometimes result in direct or indirect positive effects that allow bacteria to quickly adapt to fluctuating environmental conditions, by increasing their adaptive value in terms of survival and reproduction, known as "fitness" (Matic *et al.*, 1997; Gordo *et al.*, 2011). However, adaptation through mutations is a slow and steady phenomenon. Cell endogenous processes such as the displacement of mobile genetic elements (including transposons and insertion sequences) from place to place in the genome are also involved to generate genetic variability (Mahillon and Chandler, 1998; Fehér *et al.*, 2012). However, mutations and endogenous processes cannot alone explain the incredible potential of bacteria to evolve and adapt to their environment.

Another driving force that has been suggested to lead to radical changes in prokaryote genomes is by acquisition of new genetic information: the Horizontal Gene Transfer (HGT) (Ochiai *et al.*, 1959; Syvanen, 1985; Jain *et al.*, 2002; Aminov, 2011; Zhaxybayeva and Doolittle, 2011). The mechanism of horizontal gene transfer is described as a process in which an organism incorporates foreign genetic material. These transfers occur rapidly and constantly compared with mutations, at relatively high frequency rates. Among closely related species, these transfers can counteract the deleterious effects of genome instability in the core genome (Fall *et al.*, 2007; Treangen *et al.*, 2008). HGT often cause drastic changes in the ecological character of bacterial species through the introduction of novel physiological traits originating from distantly related organisms, thereby promoting microbial diversification and

speciation (Dutta and Pan, 2002; Gogarten *et al.*, 2002). These transfers allow the spread of the appropriate genes among bacterial populations leading to their fast adaptation to fluctuating environments as well as harsh conditions (Koonin *et al.*, 2001). Horizontal gene transfer was recognized as the main force that permitted bacteria to succeed in colonizing most of the ecological niches available on Earth (Wiedenbeck and Cohan, 2011). The evolution of studies on genes and genomes indicate that many horizontal transfers occurred between prokaryotes and that these transfers have played a major role in the evolution of bacterial genomes (Lan and Reeves, 1996; Ochman *et al.*, 2000; Koonin *et al.*, 2001; Zaneveld *et al.*, 2008). Several types of nucleic acid-based material can potentially be transferred horizontally between bacterial cells. These comprise all types of DNA molecules and mainly mobile genetic elements (MGEs) known as the mobilome, including transposons, integrons, insertion sequences, genomic islands, bacteriophage-related elements, plasmids… but these are not involved to the same extent in the different types of gene transfer (Top *et al.*, 2002; Nojiri *et al.*, 2004; Popa and Dagan, 2011). Plasmids represent important elements among the mobilome, as they can be key vectors for transfer of advantageous genes (but not usually housekeeping ones) and they possess the advantage of being self-replicated. Some plasmids can be integrated into the bacterial chromosome (Demanèche *et al.*, 2002). They code for key proteins involved in many aspects of microbial biology, including detoxification, virulence, fertility, ecological interactions, bacteriocin production and antibiotic resistance (Smillie *et al.*, 2010; Andersson and Hughes, 2011; Liang *et al.*, 2012). There are three types of mechanisms for acquisition of genetic information through horizontal gene transfer (Figure 1) (Popa and Dagan, 2011):

- **Conjugation:** which involves a direct exchange of DNA between two bacteria *via* the constitution of a mating bridge called a "sexual *pilus*" (Lederberg and Tatum, 1953). This mode of transfer occurs between two specific types of bacteria: the male or donor ones that are equipped with the conjugative-plasmid that encodes the proteins responsible for the pilus synthesis and, the female or recipient bacteria that receive DNA from the donor. An intermediate class of plasmids exists: the mobilizable ones, which carry only a subset of the genes required for the transfer and need the help of a conjugative plasmid to be transferred by conjugation. Some bacteria can integrate the plasmid genes into their chromosome, giving them the ability to transfer at high frequency some of their chromosomal DNA with the plasmid during another conjugative event.

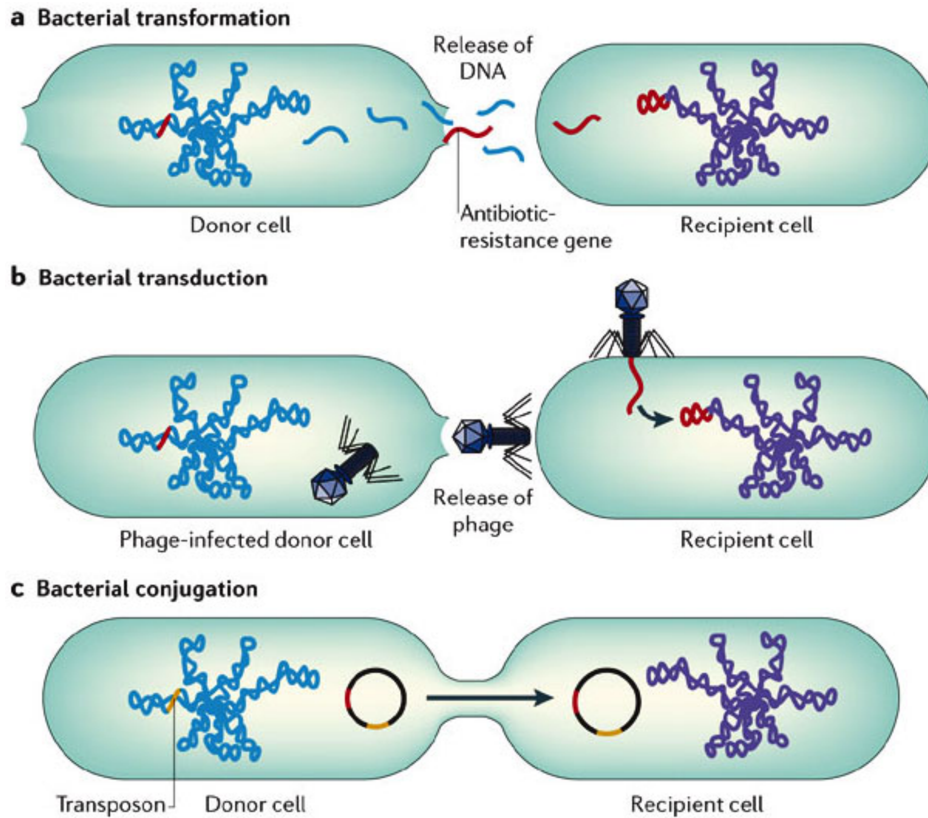


Figure 1: Horizontal gene transfers between bacteria

a) Transformation occurs when exogenous DNA is released after lysis of an organism and is taken up by another one. The DNA sequence can be integrated into the chromosome or plasmid of the recipient cell. b) In transduction, genes are transferred from one bacterium to another by bacteriophages and can be integrated into the chromosome of the recipient cell (lysogeny). c) Conjugation is the transfer of plasmids between bacteria having established a temporary physical contact, which can result in the acquisition of antibiotic resistance genes by the recipient cell. Transposons are sequences of DNA that carry their own recombination enzymes that allow transposition from one location to another; transposons can also carry antibiotic resistance genes (Furuya and Lowy, 2006).

The efficiency of this mechanism relies on the accomplishment of a stable contact between two cells involving a high bacterial density in the medium. The host range being specific, the accomplishment of such a process also relies on the ability for cells to be metabolically active and to express the genes required for the conjugative transfer (Thomas and Nielsen, 2005). However, conjugation has been described to occur frequently between environmental bacteria from soil and especially in the rhizosphere of plants, directly impacting their adaptation capacities (Ding and Hynes, 2009).

- **Transduction:** This mechanism involves bacterial viruses known as bacteriophages. They act as vectors of the bacterial genetic material from an infected bacterium to another one. During the infection cycle, viruses integrate their own genetic material into the bacterial chromosome. Two transduction phenomena exist: (i) the specialized transduction, which involves only one site of insertion of the viral genetic material within the bacterial chromosome, (Sato and Campbell, 1970) and (ii) the generalized transduction where random and multiple insertions exist (Ebel-Tsipis *et al.*, 1972). Two types of phages can also be differentiated: (i) the virulent phages which undertake a lytic stage *via* the massive production of viral particles using the host cell molecular machinery and resulting in its lysis and (ii) the temperate phages that undertake a lysogenetic cycle allowing the infected cell to live and divide normally with the dormant phage DNA inside their chromosome until the activation of phage DNA by environmental factors leading to the lytic stage. During the lytic stage of a generalized transduction, some pieces of the infected bacterial genome can be packed in the bacteriophages released. During the lysogenetic phase, the infected bacteria can eventually use the genetic material coming from a previously infected cell. This was demonstrated to result, in several cases, to ecological adaptations of the bacterial cells to harsh conditions encountered in soil (Schuch and Fischetti, 2009), and development of antibiotic resistance (Młynarczyk *et al.*, 1997). However, some environmental parameters such as the bacterial and viral density can affect the efficiency of such interactions. A tight relation of "co-evolution" exists between bacterial species and their related phage, involving a high specificity due to the constant adaptation of each partner, shaping their genomes to each other's (Gómez and Buckling, 2011).

- **Natural transformation** is the bacterial uptake of naked DNA from the environment. This process involves four principal steps: (i) the liaison between exogenous DNA and the cell surface, (ii) the uptake by the cell, (iii) the integration in the bacterial genome (or autonomous replication in the case of a plasmid acquisition) and (iv) the expression of the acquired genes (Claverys *et al.*, 2009). Full integration of the incoming DNA in existing

genetic structures such as chromosome or plasmids requires homologous or illegitimate recombination processes (Lawrence, 2002; Thomas and Nielsen, 2005). Approximately 1% of the described bacterial species are known to be naturally transformable (Thomas and Nielsen, 2005). Natural transformation requires expression of specific genes to develop a physiological step known as competence that promotes the uptake of DNA. Competence development is induced by specific signals and needs regulatory machinery not found in all bacteria (Claverys *et al.*, 2009). It is difficult to predict competence development in a complex and heterogeneous system like soil (Johnsborg *et al.*, 2007; Levy-Booth *et al.*, 2007). In addition to the specificity of cells required for natural transformation, another hurdle is the availability of exogenous DNA in the environment (Paget *et al.*, 1992; Paget and Simonet, 1994). This DNA is most likely released in the environment from decomposing materials or disrupted cells (plants, fungi, insects, bacteria, viral particles…), or through active excretion from living cells (Thomas and Nielsen, 2005). When extracellular DNA is released in soil, a significant part is rapidly degraded but another part is adsorbed onto soil particles and can persist in the environment for weeks and even months after its release from cells (Romanowski *et al.*, 1993). It was demonstrated that adsorbed DNA was partially available for bacterial transformation but also accessible to nucleases. Therefore, only a fraction of DNA can be incorporated into bacterial genome as a possible source of genetic information (Demanèche *et al.*, 2001b).

These three natural mechanisms can be used in laboratories to various research purposes. But their efficiency for transferring DNA depends on several parameters (cited above) such as expression of a set of genetically-regulated functions or host specificity (Thomas and Nielsen, 2005; Johnsborg *et al.*, 2007; Levy-Booth *et al.*, 2007; Claverys *et al.*, 2009; Popa and Dagan, 2011). In addition, when taken up into the cell, whatever the type of transfer, the incoming DNA has to resist numerous restriction and modification mechanisms (Thomas and Nielsen, 2005). Due to these limits, other methods for introducing genetic material into cells (prokaryotic and eukaryotic) have been developed. Indeed, DNA entry in cells can be stimulated by diverse factors such as thermal, chemical (e.g., salts) or electrical shocks, resulting in formation of holes called “pores” in bacterial membranes (Aune and Aachmann, 2010). Among these techniques, electroporation, developed in the 1960s, is the application of an electric field leading to the electrical permeabilization of membranes (Maniatis *et al.*, 1982). The basic effects of an electric field on a cell can be described by considering the cell as a conductive body (cytoplasm) surrounded by a dielectric layer (surface membrane) (Deng

et al., 2003). The application of an electric field leads to the accumulation of electric charges of different polarities on each side of the membrane thus creating a voltage drop in it. If this membrane voltage is not excessive and the pulse duration limited, the consequent important structural changes of the membrane are reversible. Therefore, electric fields over a threshold value permeabilize bacterial membranes, and the current allows migration of exogenous DNA through the cell membrane: this is called electrotransformation. While many studies show the effectiveness of electroporation on many bacterial taxa, as well as on fungal, plant or animal cells (Maniatis *et al.*, 1982; Dower *et al.*, 1988; Chakraborty and Kapoor, 1990; Gilchrist and Smit, 1991; Drury, 1996; Weaver and Chizmadzhev, 1996; Lurquin, 1997; Newell, 2000; Villemejane and Mir, 2009), electrotransformation efficiency often differs among species, generally requiring some cell-specific preparations for maximum transformation efficiency (Drury, 1996).

These laboratory methods for introducing DNA by electric discharges in bacteria mimic what can naturally occur in the environment during lightning discharges during thunderstorms. A thunderstorm is a form of turbulent weather and its process includes a progressive development of storm clouds (from cumulus to cumulonimbus) followed by lightning discharges and acoustic effects in the Earth's atmosphere known as thunder and these are usually accompanied by strong winds and precipitation (Gary, 1999). The electrical parameters related to this process have been extensively investigated step-by-step and are now well characterized (Rhouma and Auriol, 1997).

Rapid airflow in stormy clouds causes ice crystals to collide and cleave, inducing electrical charge separation. This charge separation builds up a net electric charge at the bottom and the top of the cloud, with opposite charges resulting in accumulation on the ground. For instance, in temperate climates, clouds are positively charged in the upper zone and negatively charged at the bottom, resulting in modifications of charge (positive) and electrical field distribution on the ground. The resulting high electrical fields due to electrical charge accumulation inside clouds or between clouds and Earth induce the formation of ionized channels. Indeed, the voltage difference between the cloud and the ground can reach up to 100 MV, corresponding approximately to 16 $kV.m^{-1}$ for flat ground surfaces, and up to 700 $kV.m^{-1}$ locally, due to a tip effect. Lightning (flash and noise) is due to the high potential electrical current flow into an ionized channel, which connects the ground to the cloud. Several high-intensity pulses of current can be delivered corresponding to the lightning flashes (Gary, 1999; Aguet and Ianoz, 2001).

At the ground level, these currents penetrate the soil from a small surface area (a few square centimeters) with a peak current intensity of the flashes varying from 10 to 200 kA (Rhouma and Auriol, 1997). Very near the impact point of the lightning, the bacterial strains may be destroyed but lightning creates an electrical field whose value is in the same range as this associated with electroporation (6 $kV.cm^{-1}$ *versus* 12.5 $kV.cm^{-1}$) with current injection considered to flow through about 2 m^2 of soil surface, resulting in very similar values as well (12 $kA.m^{-2}$ *versus* 5 $kA.m^{-2}$), suggesting that lightning could act naturally as an *in situ* "electroporator" for mediating gene transfer among environmental bacteria (Demanèche *et al.*, 2001c). With the environment being subjected to regular thunderstorms and lightning discharges that induce enormous electrical perturbations, the possibility of a "natural electrotransformation" mechanism of bacteria could explain the clear discrepancy between HGT frequencies deduced from *in silico* analysis and those resulting from *in situ* experiments (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994; Davison, 1999). Based on this hypothesis, Demanèche *et al.* (2001c) tested the electrotransformation potential of *Escherichia coli* cells inoculated into soil microcosms *via* simulated lightning-mediated current injection and storm cloud electrical fields. The high voltage generator used to simulate lightning delivered an electrical field with electrical parameters (voltage 7 $kV.cm^{-1}$, current density 2.5 $kA.m^{-2}$) similar to those of full-scale lightning (6 $kV.cm^{-1}$ and 12 $kA.m^{-2}$). These experiments were successful to demonstrate the occurrence of transfer events in soils by electrotransformation mediated by lightning simulating electrical parameters.

The current injected into the soil by lightning spreads more or less uniformly depending on the soil homogeneity (Cérémonie *et al.*, 2006a), its intensity decreasing with distance from the lightning impact point, leading to estimate that the volume of soil affected by electrotransformation compatible electrical parameters ranges from 2 to 950 m^3 per lightning strike. Electrotransformation efficiency also depends *in situ* on density and localization of potential recipients in internal or external soil compartments, as well as on concentration and availability of extracellular DNA free in the soil solution or adsorbed onto soil particles. It should also be considered that lightning could increase the pool of free-DNA by killing the bacteria present in the impact zone thus releasing their genomic content. Lightning discharges can also contribute to the desorption of DNA from soil particles (Cérémonie *et al.*, 2008).

Considering these different data, as well as the twenty lightning shocks reaching the soil every second worldwide, electrotransformation has been proposed as a fourth potential mechanism for gene transfer among bacteria in soils (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006a).

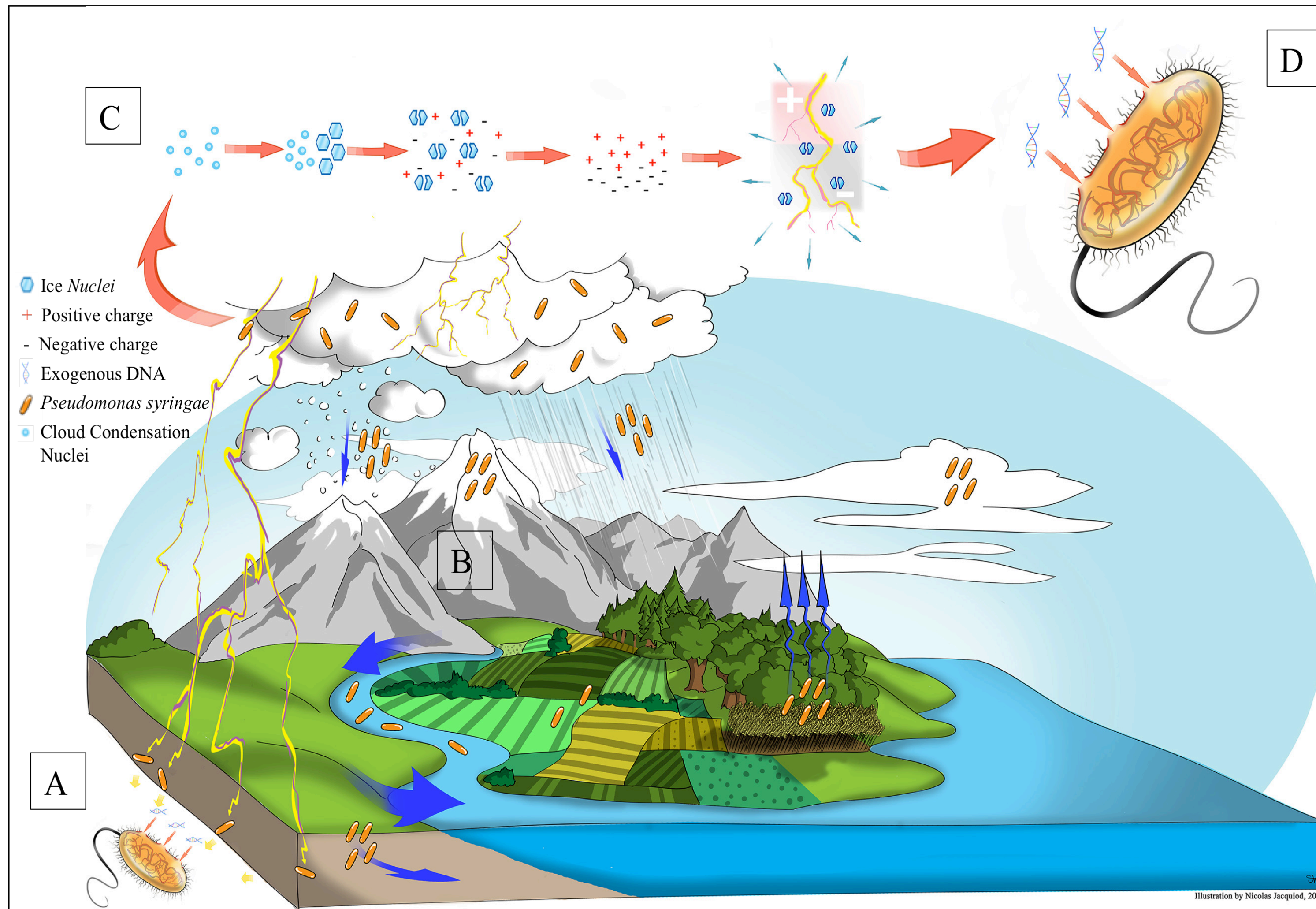


Figure 2: Lightning-mediated electrotransformation cycle of bacteria on Earth.

(A) Soil bacteria can be subjected to lightning-associated electric fields compatible with *in situ* electrotransformation. (B) *Pseudomonas syringae* is found in freshwater, on plants in fields and forests, and in clouds as part of its life history associated with the water cycle. (C) Ice nucleation activity of *P. syringae* in clouds can provoke collisions and cleavages of ice, leading to electrical charges separation and accumulation that can cause lightning. (D) Electrical field pulses formed before and during lightning strikes may potentially electrotransform cloud bacteria with exogenous DNA.

Cérémonie *et al.* (2004) isolated two lightning-competent soil bacteria identified as *Pseudomonas sp.* exhibiting an electrotransformation frequency in soil several orders of magnitude higher than *E. coli* (10^{-4} to 10^{-5} *versus* 10^{-5} to 10^{-8} electrotransformants per recipient cells) and this confirmed the potential role of natural electrotransformation as a significant adaptive mechanism for soil bacteria (Figure 2A). This lightning-mediated natural electrotransformation potential could thus be used for various applications especially to bioremediate polluted soils by adding DNA containing catabolic genes.

Additionally, while the soils are subjected to only a fifth of world lightning strikes, clouds are exposed to substantially more lightning strikes, with one hundred lightning channels formed inside them every second worldwide (Gary, 1999). Given the high temperature (about 2000°C), intensity (>200 A) and voltage (>100 kV) encountered in the lightning channel, survival and thus electroporation should not be possible (Gary, 1999; Aguet and Ianoz, 2001). However, excepting the heart of the lightning channel, many bacteria are able to survive in clouds (Amato *et al.*, 2005). Among them, some can act as Cloud Condensation Nuclei (CCN) by inducing the formation of small droplets from water vapor on their surface (Sun and Ariya, 2006; Möhler *et al.*, 2007) and as heterogeneous Ice Nuclei (IN) catalyzing freezing of supercooled water at temperatures above the activity of mineral ice nuclei (Maki and Willoughby, 1978; Vali, 1996; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Möhler *et al.*, 2008). Both of these nuclei production enable cloud formation and snow and rain precipitations (Mossop and Hallett, 1974; Vali *et al.*, 1976; Morris *et al.*, 2004; Möhler *et al.*, 2007; Christner *et al.*, 2008a). *Pseudomonas syringae*, for example, is one of the most well known and ice nucleating active bacterium and is also one of the most phytopathogenic species complex causing significant economic losses on a wide range of fruit and vegetable crops (Maki *et al.*, 1974; Arny *et al.*, 1976; Morris *et al.*, 2010; Mansfield *et al.*, 2012). Some authors have supposed that *P. syringae* could be spread by rain in host fields because of its presence in almost every stage of the water cycle (Morris *et al.*, 2008; Morris *et al.*, 2010; Morris *et al.*, 2011) (Figure 2B).

Ice nucleate active bacteria including *P. syringae* may thus be involved in the triggering of lightning in clouds (Figure 2C), the electric field pulses (few $kV.cm^{-1}$) associated with lightning currents far from the heart of the lightning channel potentially able to produce a genetic electrotransformation of bacteria (Nucci *et al.*, 1988; Gonçalves *et al.*, 2012) (Figure 2D). This leads us to hypothesize that a plant pathogen such as *P. syringae* could use its ice nucleate ability to induce the triggering of phenomena that could contribute to the increase in its adaptive potential by the acquisition of new genes *via* electrotransformation,

assuming that free-DNA is present in clouds, and thus improve its phytopathogen abilities and other competences.

Combined with a strong dissemination potential through precipitations from clouds, *P. syringae* could stand as a model for particularly adaptable microorganism for colonizing new niches (Jackson, 2009).

It is within this context that my PhD thesis takes place, funded by the French Ministry of Education. This project was developed with the objective of contributing to a better understanding of the mechanisms that govern evolution of bacteria including the impact of lightning for promoting gene transfer among environmental bacteria.

My PhD work is organized around three major research axes:

I: *In vitro* study of electroporation parameters on bacteria potentially subjected to lightning in clouds and/or soils.

II: Investigations of the impact of lightning on survival and electrotransformation of rain bacteria.

We have therefore conducted *in vitro* experiments to determine if *Escherichia coli*, *Pseudomonas* N3, *P. syringae* (Chapter I) and rain bacteria (Chapter II) would be able to survive the harsh conditions encountered during transport and residence in clouds, including electric field pulses related to lightning discharges and if they might take advantage of electric field pulses associated with lightning currents to acquire new genes.

III: The development of an electrotransformation-based tool for bioremediation of soil contaminated with lindane, a widely used pesticide, including a study of the impact of lightning-simulated electric discharges on bacterial community structures.

A global conclusion ends this study, where perspectives are evocated.

I. STUDY OF ELECTROPORATION PARAMETERS ON BACTERIA SUBJECTED TO LIGHTNING IN CLOUDS AND ON EARTH

INTRODUCTION:

For billions of years, bacteria have evolved on Earth adapting constantly to continuous disturbance of the environment. Their adaptation potential, having allowed them to colonize all ecosystems on the planet (Whitman *et al.*, 1998; Wiedenbeck and Cohan, 2011) is related to their ability for diversifying their genetic material, either by point mutations or endogenous genetic rearrangements leading to a slow and steady evolution or by horizontal gene transfers (HGT) mechanisms mediating the acquisition of new genes. This irregular acquisition of new genetic information can lead to radical changes in the genomes (Lan and Reeves, 1996; Ochman *et al.*, 2000; Koonin *et al.*, 2001; Zhaxybayeva and Doolittle, 2011) that could favor adaptation to new environmental conditions. HGT involves three main mechanisms: i) direct transfer of DNA between two bacteria having established a temporary physical contact (conjugation), ii) transport of bacterial genetic material by bacteriophages (transduction), and iii) transfer of free, naked DNA (transformation). In addition, another genetic transformation process called electroporation was developed in laboratories to be efficiently applied to a wide range of bacteria. Electroporation is based on the application of an electric shock that leads to the electrical breakdown of membranes, allowing a passive diffusion of exogenous DNA through bacterial cell envelopes more or less independently from the physiological state of the cells (Sambrook *et al.*, 1989; Aune and Aachmann, 2010). This "artificial" transformation method, which is used in almost any microbiological laboratory, simulates a natural process occurring in the environment during lightning discharges, which generate an electrical field associated with a current injection. This has led researchers to wonder if lightning discharges could act as natural "electroporators" for increasing the rate of HGT among environmental bacteria. Interestingly, the electrical parameters measured during lightning discharges (6 $kV.cm^{-1}$ and 12 $kA.m^{-2}$) are of the same order of magnitude as those governing the electroporation process *in vitro* (12.5 $kV.cm^{-1}$ and 5 $kA.m^{-2}$) (Demanèche *et al.*, 2001c). By using a high-voltage generator simulating lightning discharges (voltage: 7 $kV.cm^{-1}$ and current density 2.5 $kA.m^{-2}$) applied onto soil microcosms, Demanèche *et al.* (2001c) demonstrated that previously inoculated *Escherichia coli* cells could be efficiently "electrotransformed" by a plasmid added with bacteria. The environment being subjected to regular thunderstorms and lightning discharges that induce enormous electrical perturbations, the possibility of a "natural electrotransformation" mechanism of bacteria has been suggested and proposed as the fourth potential mechanism of HGT (Demanèche *et al.*, 2001c;

Cérémonie *et al.*, 2004, 2006a). This hypothesis was strengthened by the isolation of two soil bacteria identified as *Pseudomonas* sp. exhibiting a capacity for electroporation (*in vitro*) and electrotransformation (*in situ*) several orders of magnitude higher than *E. coli,* indicating that this property could have been positively selected by evolution (Cérémonie *et al.*, 2004).

Initial experimental studies were carried out on soils but bacteria colonizing environments other than soils could also be subjected to natural electrotransformation-mediated HGT. This includes the bacterial communities which persist and/or develop in clouds (Whitman *et al.*, 1998; Sattler *et al.*, 2001; Amato *et al.*, 2005; Amato *et al.*, 2007a; Amato *et al.*, 2007b; Vaïtilingom *et al.*, 2013), which are ecosystems that are subjected to five times the number of lightning discharges than soils (Gary, 1999), leading us to hypothesize that bacteria could be electrotransformed in clouds in the same way as bacteria in soils (Figure 2 in the General Introduction). In addition, some of the cloud bacteria are able to act as heterogeneous Ice Nuclei (IN), catalyzing the freezing of supercooled water at temperatures above the activity of mineral ice nuclei (Vali, 1996; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Möhler *et al.*, 2008), the presence of ice nucleating active bacteria in clouds multiplying by threefold the number of lightning flashes occurring (Gonçalves *et al.*, 2012). Among these bacteria, the case of *Pseudomonas syringae* as one of the most ice nucleate-active bacteria is particularly intriguing. *P. syringae* is also one of the most phytopathogenic species complexes, causing significant economic losses in a wide range of fruit and vegetable crops (Morris *et al.*, 2010; Mansfield *et al.*, 2012). Conjunction of these two properties in the same organism leads us to hypothesize that a plant pathogen could use its ice nucleate potential to induce the triggering of phenomena that would contribute to an increase in its adaptive potential (*i.e.*, the acquisition of new genes by electrotransformation-mediated HGT) for improving its phytopathogenic abilities and other properties. However, the use of these ice nucleate properties as adaptive mechanisms requires these bacteria to survive lightning discharges and to exhibit some electrotransformation ability for taking advantage of electric field pulses associated with lightning currents.

In this chapter, our goal was to carry out *in vitro* experiments to determine the fate of the ice nucleator *P. syringae* CC0094 used as model when submitted to different conditions of electroporation (simulating a range of electrical parameters encountered during lightning discharges affecting clouds) in terms of survival and ability to be genetically transformed in comparison to the highly electrocompetent bacterial strain *Pseudomonas* sp. N3 isolated from soil (Cérémonie *et al.*, 2004, 2006a) and to the laboratory type strain *E. coli* TOP10.

CHAPTER I : STUDY OF ELECTROPORATION PARAMETERS ON BACTERIA

Given the physical and chemical conditions encountered by bacteria in clouds, the electroporation tests were developed *in vitro* with the objective of simulating the cloud environment, including temperature and medium state (tests in ice), the possibility of being electrically shocked several times (application of two to five successive electric shocks), at different voltages (9.5 *versus* 12.5 $kV.cm^{-1}$), of a delayed contact between bacteria and DNA (addition of plasmid from 30 s to 30 min after the electrical shock[s]), of a variable density of bacteria (considered from 10^4 to 10^8 CFU per reaction), of their physiological stage (stationary *versus* active growth), of an exogenous origin of the transforming DNA (*E. coli versus P. syringae*-extracted plasmid). Parameters such as the survival and transformation frequency were considered after the bacteria were subjected to these various electroporation tests. Additionally, the genome integrity of *P. syringae* was monitored.

EXPERIMENTAL PROCEDURES:

1. Bacterial strains, plasmids and culture conditions

Escherichia coli TOP10

Electrocompetent cells of *Escherichia coli* strain TOP10 (Invitrogen – Life technologies, Paisley, UK) were prepared with a protocol adapted from Drury (1996). A single colony from LB (Lysogeny Broth) agar low salt medium (LBa – Duchefa Biochemie, Haarlem, The Netherlands) was grown in 1 mL of LB at 37°C under agitation, to be subsequently used as an inoculum in 100 mL culture medium incubated under the same conditions until the culture reaches an OD_{600} of 0.8-1.0. Cells were maintained on ice for 20 min before centrifugation at 5500×*g* for 10 min at 4°C and four successive washes with decreasing volumes of cold glycerol (10% w/v) to be finally concentrated 100-fold in cold glycerol (10% w/v) and stored at -80°C.

Pseudomonas syringae CC0094

Electrocompetent *Pseudomonas syringae* CC0094 cells were prepared using a protocol adapted from Bassett & Janisiewicz (2003). A single colony originally grown on King's B medium (Duchefa Biochemie, Haarlem, The Netherlands) was inoculated into 1 mL of Nutrient Broth (Difco™ Becton, Dickinson and Co., Sparks, USA) with 2.5% (w/v) glycerol (NBG) and incubated overnight at 22°C with shaking. This was used to inoculate 100 mL NBG for incubation at 22°C with shaking until reaching an OD_{640} of 0.5-0.6. Cells were maintained on ice for 20 min, before centrifugation at 5500×*g* for 10 min at 4°C and four successive washes with decreasing volumes of cold sucrose (0.5 M), before being finally concentrated 100-fold in cold sucrose (0.5 M) and stored at -80°C.

Pseudomonas sp. *N3*

Electrocompetent *Pseudomonas* sp. N3 cells were prepared as described by Cérémonie *et al.* (2004). A single colony from LBa was grown in 1 mL of LB at 29°C under agitation and subsequently used as an inoculum in 100 mL culture medium incubated under the same conditions until the culture reached an OD_{600} of 0.6 - 0.7. Cells were maintained on ice for 20 min before centrifugation at 5500×*g* for 10 min at 4°C and four successive washes with

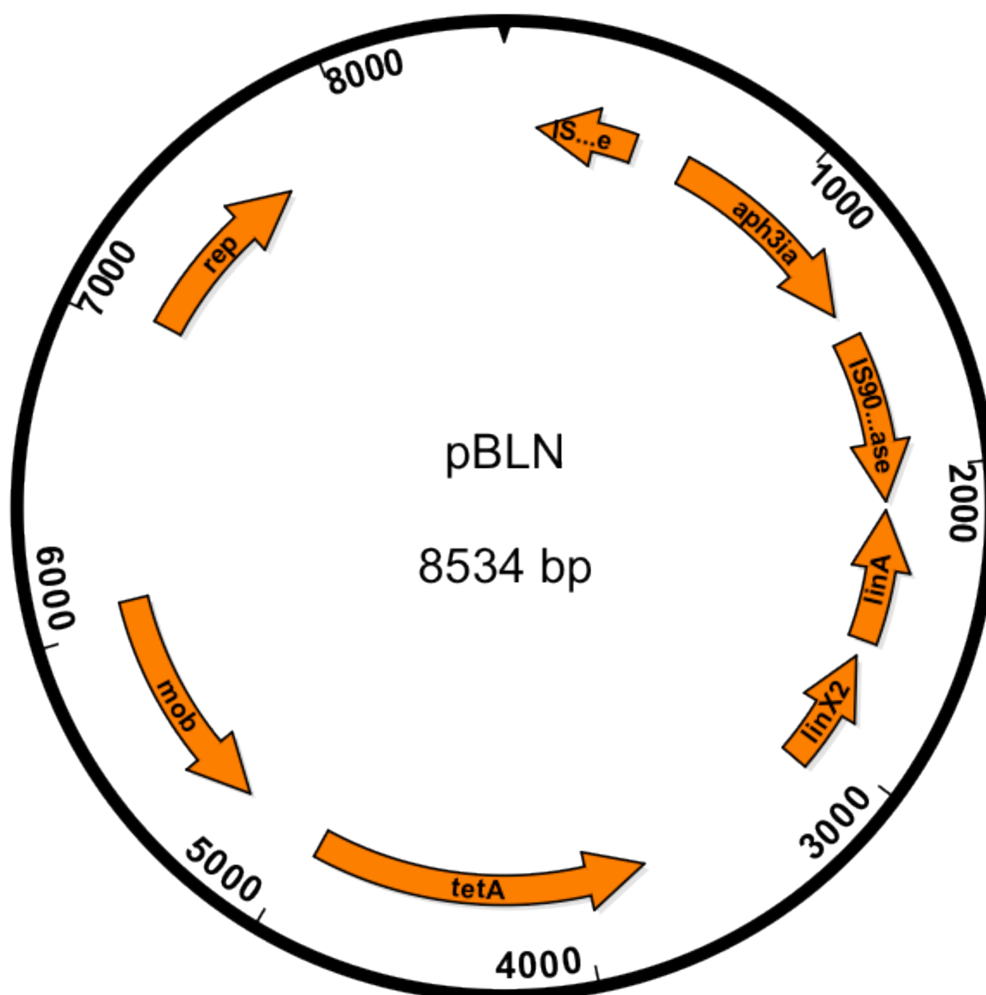


Figure I-1: pBLN map (8534 bp) obtained with Seqbuilder™ (Lasergene® DNASTAR, Inc., Madison, USA)

rep: replication gene, IS…e = IS903 transposase: insertion sequence with transposase, *aph3ia*: Aminoglycoside O-phosphotransferase gene, conferring resistance to kanamycine, IS90…ase = IS903 transposase: insertion sequence with transposase (Both IS903 transposases and *aph3ia* gene form the Tn903 transposon), *linA*: dehydrochlorinase, *linX2*: dehydrogenase genetically linked to *linA*, *tetA*: tetracycline efflux pump conferring resistance to tetracycline, *mob*: mobilization gene.

decreasing volumes of cold sucrose (0.5 M) before being finally concentrated 100-fold in cold sucrose (0.5 M) and stored at -80°C.

***E. coli* TOP10 - pBLN, *P.* sp N3 - pBLN and *P. syringae* CC0094 - pBLN**

Strains of *E. coli* TOP10, *Pseudomonas* sp. N3 and *P. syringae* CC0094 containing the pBLN plasmid (obtained by the standard electroporation procedure, see 3.a in this section) were prepared as indicated before, with addition of tetracycline and kanamycin (25 $\mu g.mL^{-1}$).

PBLN plasmid

The 8.5-kb plasmid pBLN used in this study (Figure I-1) was constructed in the course of a previous study (Lyon *et al.*, 2010) from the broad-host-range plasmid pBBR1-MCS3 (GeneBank ref.: XXU25059) and is able to replicate in many different types of bacteria (Kovach *et al.*, 1995), harbouring the *tetA* gene encoding resistance to tetracycline. Genes from the plasmid pCEAlinA-nptII conferring kanamycin resistance (*aph3ia*) and involved in the two first-steps of lindane degradation by *Sphingobium francense sp+* (*linA*) (Cérémonie *et al.*, 2006b) were cloned into pBBR1-MCS3. The pBLN plasmid was maintained in *Escherichia coli* TOP10 in LB broth with 25 $\mu g.mL^{-1}$ kanamycin monosulfate and 25 $\mu g.mL^{-1}$ tetracycline hydrochloride (Duchefa Biochemie, Haarlem, The Netherlands) and extracted when necessary using the NucleoSpin Plasmid Extraction kit (Macherey Nagel, Bethlehem, USA).

pBLN has also been isolated from *P. syringae* CC0094 containing pBLN with different methods: (i) with the NucleoSpin Plasmid purification kit used for *E. coli*, (ii) with the NucleoSpin Plasmid purification kit – Endotoxin-free, according to the manufacturer's instructions (Macherey Nagel, Bethlehem, USA), (iii) with a protocol for cell lysis coupled with the extraction of covalently closed circular (CCC) DNA by Cesium Chloride/Ethidium Bromide gradient separation after ultracentrifugation, according to the method described by Schwinghamer (1980) with the following modifications: 50 mL of a two-day culture (OD_{640} 5.19) of *P. syringae* CC0094-pBLN in NBGTK were centrifuged at 10,000×*g* for 15 min at 0°C and resuspended in cold TS (Tris 0.05 M, NaCl 0.05 M, pH 8) buffer. Cells were lysed using 0.1% (v/v) Triton-X100 followed by an osmotic shock using concentrated sucrose (1.6 M), followed by enzymatic lysis with 5mg.mL^{-1} lysosyme. Isolation of CCC plasmid DNA forms was performed by dye-buoyant equilibrium centrifugation at 20°C for 12 h at (73,0000×*g*) in an MLA-55 rotortype, (Beckman-Coulter, USA) in a solution containing

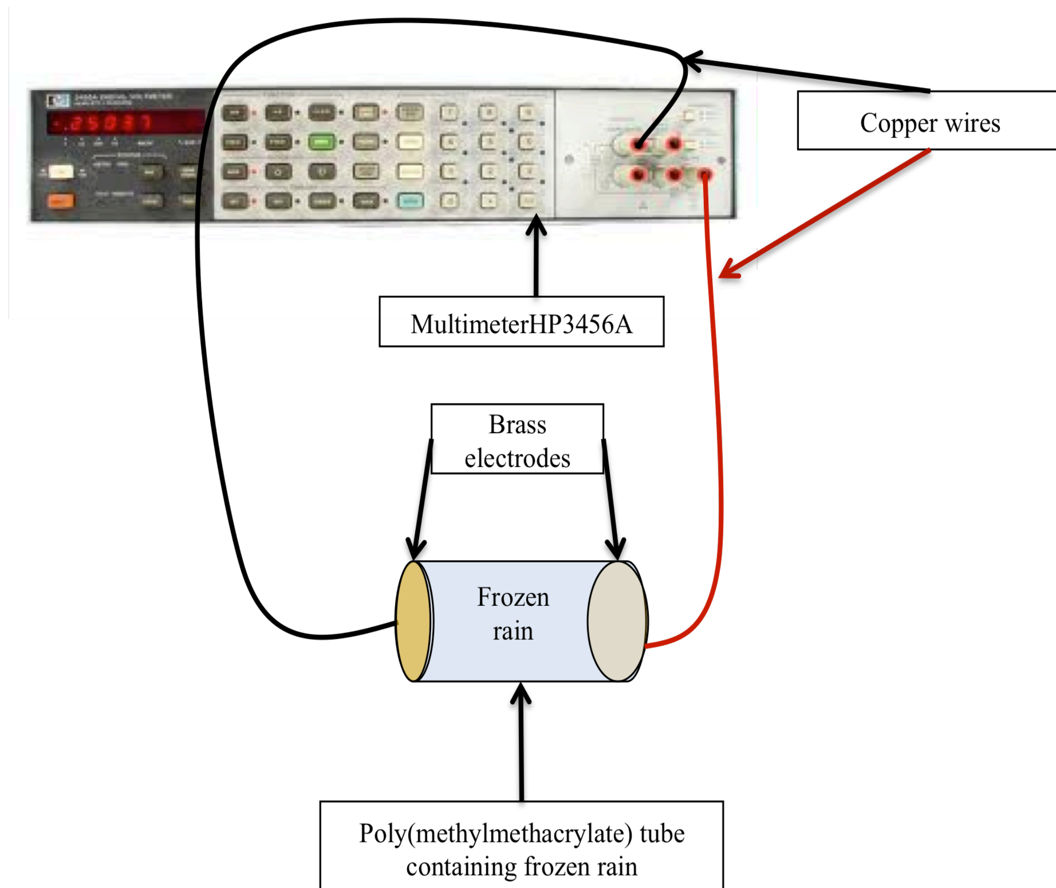


Figure I-2: System for measuring frozen rain resistance

49.35% (w/w) CsCl salt and 275 μg.mL^{-1} EtBr for constituting a density gradient. Gradients were examined under UV-light for visual observation of CCC DNA bands by EtBr staining.

The CCC DNA band was then extracted with a syringe and EtBr was removed by repeated butan-1-ol extraction (approximately 3-4 times, until the pinkish color in the aqueous solution had faded) and DNA was purified by ethanol precipitation.

Plasmid concentration was evaluated by two methods: (i) spectrophotometry (Nanophotometer, Implen, München Germany) (ii) fluorometry (Qubit fluorometer, Invitrogen – Life Technologies, Paisley, UK) and DNA quality checked by electrophoresis on a 0.8% agarose gel containing ethidium bromide (2.5 μL for 100 mL of gel) in 45 mM Tris-borate-1 mM EDTA buffer (TBE 0.5 X) in the presence of the 1kb+ ladder for size estimation (Fisher Scientific, Illkirch, FRANCE).

2. Measure of ice conductivity

Rain was directly collected in a 100 mL plastic bottle (Dutscher, Brumath, France and incubated at -20°C for 30 min in a Poly(methyl methacrylate) tube corked with brass electrodes at both ends connected with copper wires. It was then connected to a multimeter HP3456A (Hewlett-Packard, Palo Alto, California, USA) to measure frozen rain resistance, as indicated in Figure I-2.

Conductivity was calculated according to the follow equation.

$$\sigma = \frac{1}{R \times A}$$

σ = Conductivity in Siemens per meter (S.m^{-1})

l = Length in meters (m)

R = Resistance in ohm (Ω)

A = Cross-section area of the conductor measured in square meters (m²)

3. Electroporation conditions

Several experiments were performed to test the electrotransformation potential of the different strains with controls in which the bacteria were not submitted to the electric shocks (potential detection of natural transformation-mediated transfer events). For each condition, the mean counts from three agar plates were considered, with all experiments being carried out in triplicate.

Table I-1: Incubation conditions for *E. coli* Top10, *Pseudomonas* sp. N3 and *P. syringae* CC0094

Incubation conditions		*E. coli* TOP10	*P.* sp. N3	*P. syringae* CC0094
1. Broth:	Broth media	LB	LB	NBG
	Incubation time after shock	1 h	2 h	2 h
	Incubation temperature	37°C	29°C	22°C
2. Agar:	Agar plates	LB and LBTK	LB and LBTK	KB and KBTK
	Incubation time	24 h	48 h	72 h
	Incubation temperature	37°C	29°C	22°C

Standard electroporation

Fifty microliters of bacterial suspensions were incubated with 1 µL of pBLN (70 ng.µL^{-1}) isolated from *E. coli* TOP10 and submitted to an electrical shock with the Gene Pulser II electroporation system (Biorad, Hercules, USA) at 12.5 kV.cm^{-1}, 25 µF, 200 Ω in a 2 mm cuvette for 5 ms (Cells Projects, Harrietsham, United Kingdom). For *P. syringae* CC0094 only, the electroporation was also performed at 9.5 kV.cm^{-1} according to Bassett & Janisiewicz (2003). After incubation in 1 mL broth (Table I-1) under agitation at 225 rpm, bacterial suspensions were diluted and spread on three agar plates for total bacterial counts and onto media containing Tetracycline and Kanamycin (25 µg.mL^{-1} – LBTK and KBTK) to assess the survival and electrotransformation rate of the three studied bacteria. Agar plates were then incubated as indicated in Table I-1.

Multiple shock experiments

Multiple shocks were performed on the three bacteria to assess the effects on survival and electrotransformation rates. Electroporation was performed under the same conditions as for the standard electroporation. Fifty microliters of bacterial suspensions were incubated with 1 µL of pBLN (70 ng.µL^{-1}) and submitted to three or five successive electric shocks at 12.5 kV.cm^{-1}. Subsequent incubations and spreadings were done as before.

Multiple shocks were also conducted on the three bacteria in which the pBLN was introduced in order to assess their cumulative effect on survival and plasmid loss in these bacterial species. Fifty microliters of bacterial suspensions were submitted to 1, 2, 3, 4 or 5 electric shocks at 12.5 kV.cm^{-1}. Subsequent incubations and spreading were done as before except that bacterial suspensions were spread on media with and without antibiotics.

Results were statistically analyzed using the Grubbs (1969) test adapted and performed by a homemade computer program (Riccardo Scorretti, personal communication) detecting outlying observations ("outliers") among collected data. The main idea of this kind of test is to compute a statistic T_i for each point x_i, and to compare it to a critical value, which is computed according to: i) the required significance level, and ii) the distribution of the points. In our case, we computed the following T statistic:

$$T_i = \frac{|x_i - \bar{x}|}{s}$$

where: $\bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$ is the experimental average, and $s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2}$ is the unbiased estimate of the standard deviation.

The condition for identifying a point as an outlier is:

$$T_i \geq T_{crit}(N, \alpha)$$

where the critical value $T_{crit}(N, \alpha)$ has been computed under the assumption that the points follow a Gaussian distribution, for a significance level α=0.05 – that is the probability of identifying as outlier a "regular" point (less than 5%, type I error).

When a point is identified as an outlier, it is removed from the pool of experimental points, and the test is repeated to identify more outliers. Iterations are stopped when the number of points in the pool decreases to 6, after removal of the outliers.

Electroporation in icy conditions

Fifty microliters of bacterial suspensions were incubated with 1 µL of pBLN (70 $ng.\mu L^{-1}$) as for the standard electroporation but this mix was frozen for 10 min at -20°C before being submitted to a 12.5 $kV.cm^{-1}$ electrical shock. Subsequent incubations and spreading were done as for the standard electroporation.

Time of pore resealing

In order to determine the time the pores remain open after an electrical shock, experiments were developed with the three recipient bacteria in which 50 µL of each strain suspension were submitted to a 12.5 $kV.cm^{-1}$ electrical shock as for standard electroporation but with the addition of 1 µL of pBLN (70 $ng.\mu L^{-1}$) delayed for 30 s (in triplicate) and in addition for 45 s, 1.15 min, 3 min, and 30 min with *E. coli* TOP10 (without replicates) and for 54 s and 3 min with *P. syringae* CC0094 (without replicates) after the electric shock. Additionally, each of the three bacteria was also submitted (without replicates) to five shocks prior to the addition of the plasmid thirty seconds after the last shock or to one shock and a thirty second delay in icy conditions.

In all conditions tested, the pBLN plasmid was mixed with cells before broth addition, and subsequent incubations (Table I-1) and spreading on three agar plates were as indicated for the standard electroporation.

Effect of cell density on electroporation efficiency

The impact of cell concentration on cell survival and electrotransformation rates after electroporation was determined with each of the bacteria in a cell density range of 10^2 to 10^8 cells per 50 µL electroporation test, after suspensions were diluted 10-fold in glycerol (10% w/v) (for *E. coli* TOP10) or sucrose 0.5 M (for both *Pseudomonas* sp.). Electroporation tests were performed as indicated before with 50 µL of each diluted suspension incubated with 1 µL of pBLN (70 ng.µL^{-1}) and submitted to a 12.5 kV.cm^{-1} electrical shock. Subsequent incubations and spreading were done as indicated above.

Electroporation of P. syringae CC0094 in a stationary phase

The influences of the growth stage on survival and electrotransformation rates after electroporation were investigated by submitting *P. syringae* CC0094 to standard electroporation after the culture had reached the stationary phase. These cells were cultivated in the same way as previously described, except that the 100 mL culture was incubated at 22°C under agitation for 60 h. After four washes with decreasing volumes of cold sucrose (0.5 M) and 100-fold concentration, the cell density was adjusted to the standard OD_{640} used routinely (of 1.886) before cells were stored at -80°C.

Effect of plasmid origin on the electrotransformation frequency of P. syringae CC0094

Electrotransformation tests on *P. syringae* CC0094 strain were also carried out with the same plasmid isolated either from *E. coli* (1 µL of 71.5 ng.µL^{-1} pBLN) or from *P. syringae* CC0094 (2.33 µL of 30 ng.µL^{-1} pBLN). Electrotransformation tests were performed as described before, the only difference being the bacterial source of the plasmid DNA solution.

4. Survival, plasmid acquisition and loss calculations

- The survival percentage after electrical shock was calculated as a ratio of the number of surviving bacteria *versus* the number of unshocked bacteria:

$$\%Survival = \frac{b}{a} \times 100$$

a = Number of total culturable bacteria on LB or KB without shock (CFU.mL^{-1})

b = Number of surviving bacteria on LB or KB aftershock (CFU.mL^{-1})

For electroporation in icy media, survival rate was calculated as the ratio of the number of surviving bacteria *versus* the number of frozen bacteria without shock, to account for mortality due to freezing.

- The electrotransformation rates were determined as the number of tetracycline and kanamycin resistant colonies divided by the number of surviving cells:

$$Electrotransformation\ rate = \frac{c}{b}$$

c = Number of bacteria on LBTK or KBTK after shock (CFU.mL^{-1})

- The plasmid loss percentages were determined as the proportions of surviving bacteria sensitive to tetracycline and kanamycin relative to the total number of surviving cells after incubation:

$$\%Plasmid\ loss = \left[\frac{b-c}{b}\right] \times 100$$

5. Statistical analyses

Hereafter, we consider that two survival, electrotransformation or loss rates S_1 and S_2 are significantly different when their confidence intervals (CI) do not intersect: for instance, for a significance level α=0.05, S_1 and S_2 are considered significantly different if:

$$|S1 - S2| \geq 1.96\sigma\, 2N$$

where it is implicitly assumed that:

- both S_1 and S_2 follow a Gaussian distribution with the same variance s^2
- the experiments have been repeated *N* times.

This corresponds to the intuitive idea that the intersection of the intervals where S_1 and S_2 lay with a probability of 95% is empty. For the sake of simplicity (and with an abuse of notation), in this and in the following expressions we use the same symbol S_1 to mean the rate and its experimental average $\bar{S_1}$:

$$\bar{S_1} = \frac{1}{N}\sum_{i=1}^{N} S_1^{(i)}$$

where $S_1^{(i)}$ mean is the i-th realization of S_1 over N repetitions of the experiment.

6. BOX-PCR

Genomic structure stability profiles were followed by BOX-PCR after one shock at 9.5 $kV.cm^{-1}$, one, three or five shocks at 12.5 $kV.cm^{-1}$ and one shock at 12.5 $kV.cm^{-1}$ in icy media for *P. syringae* CC0094. After 2 h of post-electroporation incubation, DNA from bacterial cells was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel, Bethlehem, USA), according to the manufacturer's instructions. Repetitive Extragenic Palindromic sequences were amplified by PCR using the primer BOXA1R (3'-CTACGGCAAGGCGACGCTGACG-5') and the hot start mix RTG (Illustra™ - GE Healthcare, Buckinghamshire, UK) (Versalovic *et al.*, 1998). PCR conditions included an initial denaturation step at 96°C for 15 min, 30 cycles at 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min with a final elongation step at 65°C for 10 min. An aliquot of the PCR products was electrophoresed through a chip of the Agilent DNA 7500 kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

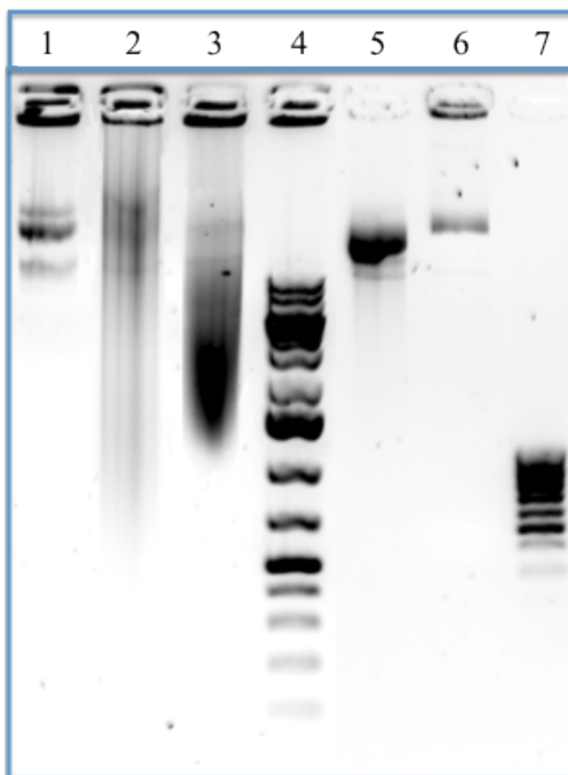


Figure I-3: Agarose gel electrophoresis of pBLN extracted from *E. coli* TOP10 or *P. syringae* CC0094.

Lane 1: pBLN plasmid isolated from *E. coli* TOP10 using the NucleoSpin Plasmid purification kit; **Lane 2:** pBLN plasmid isolated from *P. syringae* CC0094 using the NucleoSpin Plasmid purification kit; **Lane 3:** pBLN plasmid isolated from *P. syringae* CC0094 using the NucleoSpin Plasmid purification kit – Endotoxin-free; **Lane 4:** 1kb+ladder; **Lane 5:** CCC DNA band of pBLN plasmid obtained from *P. syringae* CC0094 after ultracentrifugation in CsCl-EtBr gradients, before EtBr removal and purification; **Lane 6:** CCC DNA band of pBLN plasmid obtained from *P. syringae* CC0094 after ultracentrifugation in CsCl-BEt gradients, after EtBr removal and purification; **Lane 7:** low range ladder (Fisher Scientific, Illkirch, FRANCE).

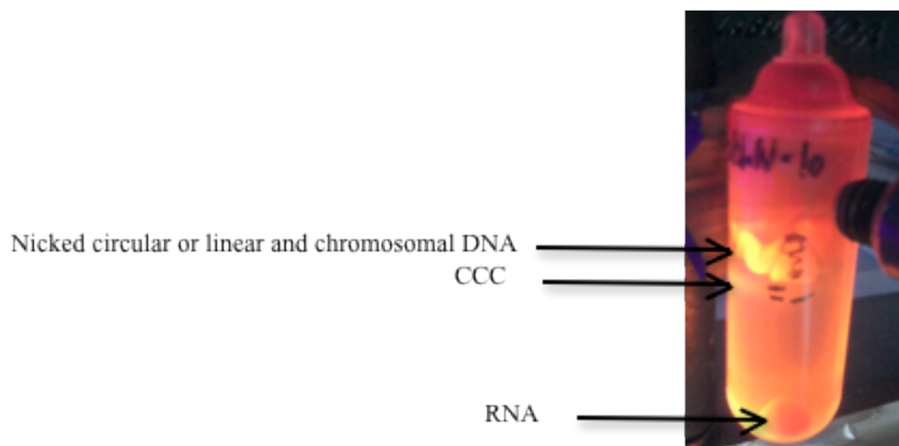


Figure I-4: Visual examination of gradients under UV-light.

Nicked circular or linear and chromosomal DNA are above the CCC DNA band and RNA pellet is at the bottom of the tube. Proteins, supposed to be above chromosomal DNA, are not visible.

RESULTS:

1. Purification of pBLN plasmid

Plasmid pBLN was isolated from *E. coli* TOP10 according to the NucleoSpin Plasmid purification kit, spectrophotometrically quantified and diluted to 71.5 ng.μL^{-1} and the concentration confirmed by fluorometry (68.2 ng.μL^{-1}). The three bands corresponding to the linear, open circular (OC), and covalently closed circular (CCC) forms could be detected after electrophoresis in agarose gel (Figure I-3, lane 1).

The same plasmid was also isolated using the NucleoSpin Plasmid purification kit from *P. syringae* CC0094 in which it had been transferred, then diluted to the 71.5 ng.μL^{-1} after quantification by spectrophotometry. Fluorometric quantification resulted in a significantly lower concentration (28.1 ng.μL^{-1}). This result could be explained by degradation of the plasmid, as observed by a smear in the agarose gel (Figure I-3, lane 2), a problem which was not solved by the use of the NucleoSpin Plasmid purification kit – Endotoxin-free (Figure I-3, lane 3). The only technique that allowed isolation of a non-degraded pBLN plasmid was its transfer into *P. syringae* CC0094 and its extraction by a method adapted from Schwinghamer (1980) in which *P. syringae* CC0094 cells were lysed *in situ* in the centrifugation tube prior to isopycnic ultracentrifugation in CsCl-BEt gradients to separate CCC from linear and OC forms (Figure I-4).

After specific recovery of the CCC DNA band, EtBr removal and purification, concentration was determined by spectrophotometry and fluorometry (30 ng.μL^{-1}). Presence of a single band on the gel (Figure I-3, lane 6) confirmed the isolation of the CCC form of the plasmid that switched progressively to OC and linear forms after repeated freeze-thaw cycles (data not shown), leading us to aliquot the initial solution to avoid the loss of intact plasmids for subsequent experiments.

Table I-2: Survival percentage of the three studied bacteria after different treatments.

Survival rate	*P. syringae* CC0094	*Pseudomonas* sp. N3	*E. coli* TOP10
Liquid (control condition)	15.00 ± 8.62%	11.55 ± 9.36%	19.25 ± 9.12%
9.5 $kV.cm^{-1}$	17.77 ± 4.34%	/	/
Icy media	60.21 ± 7.72%*	33.44 ± 1.64%	18.52 ± 10.65%
3 shocks	1.60 ± 0.73%	5.25 ± 3.87%	6.45 ± 3.54%
5 shocks	1.53 ± 0.80%	3.16 ± 2.08%	3.34 ± 1.37%
Addition of plasmid 30 seconds after shock	16.55 ± 9.98%	4.84 ± 2.09%	11.19 ± 2.91%
10^6 bacteria	/	0.88 ± 0.18%	/
10^5 bacteria	/	2.16 ± 1.18%	/
10^4 bacteria	4.09 ± 0.38%	1.16 ± 0.49%	33.86 ± 13.44%
10^3 bacteria	3.41 ± 1.19%	20.46 ± 24.02%	22.84 ± 2.24%
10^2 bacteria	5.13 ± 0.45%	1.39 ± 1.96%	9.80 ± 6.06%
Stationary phase	37.29 ± 5.05%	/	/
pBLN from *P. syringae* CC0094	33.56 ± 9.14%	/	/

Values deal with average and standard deviation of survival rates from minimum three experiment repetitions. Asterisk shows the values that are significantly different ($p<0.05$) from the control condition.

Table I-3: Survival percentage of the three studied bacteria containing pBLN with zero to five shocks.

	P. syringae CC0094 + pBLN	*Pseudomonas* sp. N3 + pBLN	*E. coli* TOP10 + pBLN
Without shock	100.00 ± 0.00%*	100.00 ± 0.00%*	100.00 ± 0.00%*
1 shock (control condition)	26.48 ± 15.20%	19.98 ± 18.86%	87.94 ± 97.06%
2 shocks	14.41 ± 9.00%	16.96 ± 8.43%	63.13 ± 70.50%
3 shocks	14.40 ± 10.64%	15.31 ± 11.82%	59.02 ± 71.33%
4 shocks	5.92 ± 5.11%	20.56 ± 18.04%	49.99 ± 63.62%
5 shocks	5.47 ± 6.90%	2.59 ± 1.63%	16.24 ± 11.39%

Values deal with averages and standard deviations of survival rates from a minimum of three experiment repetitions. Asterisks show those values that are significantly different ($p<0.05$) from the control condition with one shock.

2. Electrical shock survival

Control condition

P. syringae CC0094, *Pseudomonas* sp. N3 and *E. coli* TOP10 survived a 5 ms and 12.5 $kV.cm^{-1}$ electrical shock at rates of 15.00 ± 8.62%, 11.55 ± 9.36% and 19.25 ±9.12%, respectively (Table I-2) with no significant differences (up to a significance level of $p<0.05$) if the strains harbor the pBLN plasmid (26.48 ± 15.20% for *P. syringae* CC0094, 19.98 ± 18.86% for *Pseudomonas* sp. N3 and 87.94 ± 97.06% for *E. coli* TOP10) (Table I-3).

Other parameters

Pseudomonas sp. N3 and *E. coli* TOP10 exhibited similar survival rates to the same parameters (up to a significance level $p<0.05$) whatever the conditions tested (icy media and several successive shocks, for example) (Table I-2, Figure I-5). *P. syringae* CC0094 increased its survival rate to electroporation from 15.00% in liquid medium to 60.21% in icy medium (Table I-2, Figure I-5) while a strong heterogeneity of survival was detected in the other strains containing the plasmid pBLN without any clear biological explanation (Table I-3). The use of the Grubbs statistical test to remove the outliers shows that the survival rate of the three bacteria was not statistically modified (up to a significance level of $p<0.05$) by plasmid presence however many shocks the cells were submitted to (Figure I-6).

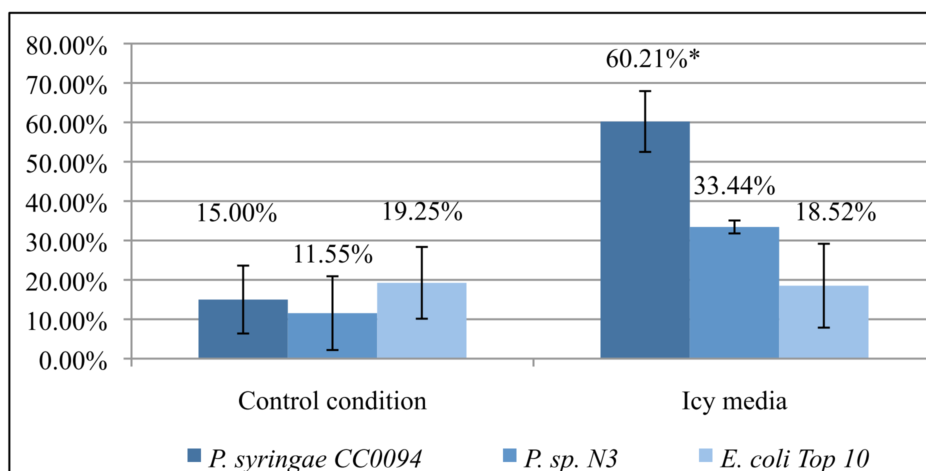


Figure I-5: Survival percentage of *P. syringae* CC0094, *Pseudomonas* sp. N3 and *E. coli* Top 10 after a 12.5 $kV.cm^{-1}$ electrical pulse in liquid and in ice.

Results are expressed as mean values with standard deviation as error bars. Asterisks indicate the values that are significantly different ($p<0.05$) from the control condition.

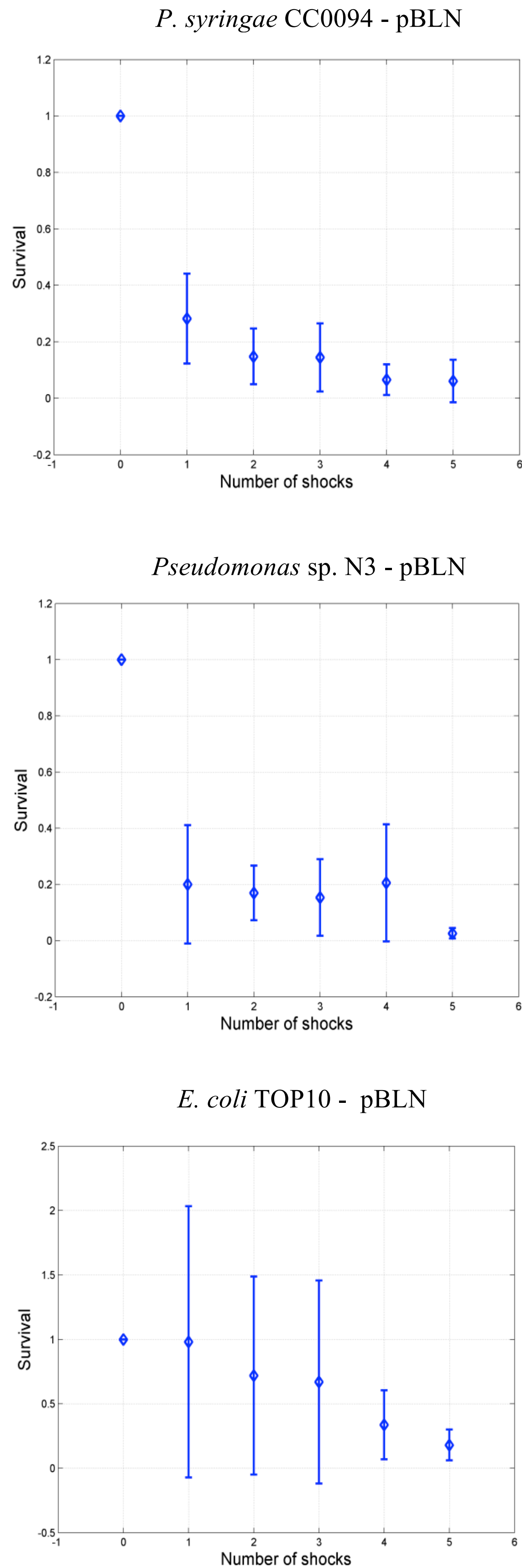


Figure I-6: Survival rates of *P. syringae* CC0094 - pBLN, *Pseudomonas* sp. N3 - pBLN and *E. coli* Top 10 - pBLN with up to five 12.5 $kV.cm^{-1}$ electrical pulses.

Results are expressed as mean values with standard deviations as error bars

3. Electrotransformation rates

P. syringae CC0094, *Pseudomonas* sp. N3 and *E. coli* TOP10 electrotransformation rates reached $2.55\times10^{-3} \pm 3.34\times10^{-3}$, $5.83\times10^{-3} \pm 6.87\times10^{-3}$ and $1.68\times10^{-3} \pm 2.38\times10^{-3}$, respectively (Table I-4). These rates were measured as the number of cells able to take up the pBLN plasmid divided by the number of surviving cells and were not significantly different (up to a significance level of $p<0.05$).

Different voltages

Initially, *P. syringae* CC0094 was electrotransformed with a 9.5 $kV.cm^{-1}$ electrical pulse according to the previous reports (Bassett and Janisiewicz, 2003). No transformants were recovered. The conventional 12.5 $kV.cm^{-1}$ electrical pulse yielded an electrotransformation rate in the same range as for the two other strains (Table I-4) (2.55×10^{-3} for *P. syringae* CC0094 *versus* 5.83×10^{-3} and 1.68×10^{-3} for *Pseudomonas* sp. N3 and *E. coli* TOP10, respectively).

Icy condition

A comparison of electrotransformation frequencies between the three studied bacteria in an icy medium was performed (Table I-4, Figure I-7). The two *Pseudomonas* species yielded values that were significantly ($p<0.05$) different from the liquid control. *P. syringae* CC0094 and *Pseudomonas* sp. N3 electrotransformation rates decreased from 2.55×10^{-3} and 5.83×10^{-3} to 7.93×10^{-6} and 3.94×10^{-6} respectively.

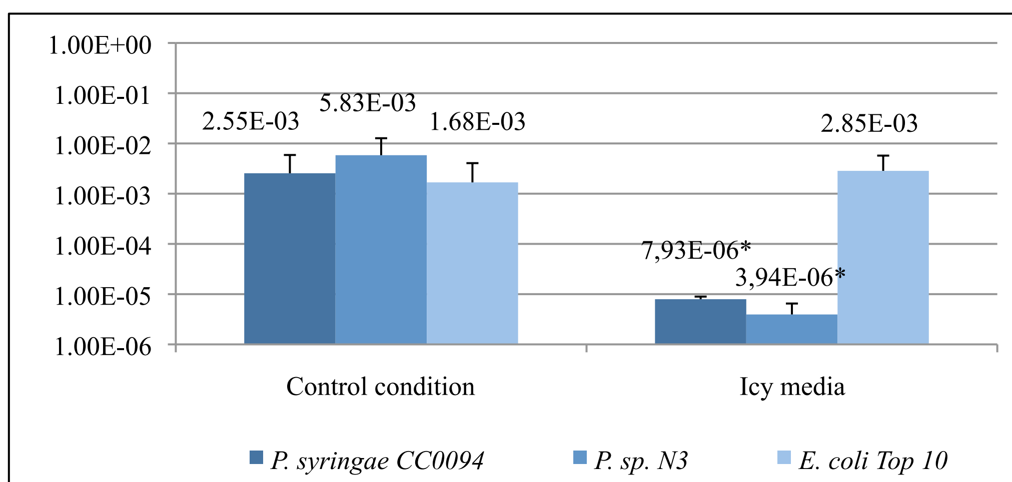


Figure I-7: Electrotransformation rate of *P. syringae* CC0094, *Pseudomonas* sp. N3 and *E. coli* Top 10 after a 12.5 $kV.cm^{-1}$ electrical pulse in liquid and in ice.

Results are expressed as mean values with standard deviations as error bars. Asterisks indicate the values that are significantly different ($p<0.05$) from the control condition.

Table I-4: Electrotransformation rate of the three studied bacteria after different treatments.

Electrotransformation rate	*P. syringae* CC0094	*Pseudomonas* sp. N3	*E. coli* TOP10
Liquid (control condition)	$2.55\times10^{-3} \pm 3.34\times10^{-3}$	$5.83\times10^{-3} \pm 6.87\times10^{-3}$	$1.68\times10^{-3} \pm 2.38\times10^{-3}$
9.5 kV.cm^{-1}	$0.00\times10^{0} \pm 0.00\times10^{0}$*	/	/
Icy media	$7.93\times10^{-6} \pm 1.01\times10^{-6}$*	$3.94\times10^{-6} \pm 2.57\times10^{-6}$*	$2.85\times10^{-3} \pm 2.88\times10^{-3}$
3 shocks	$3.20\times10^{-3} \pm 2.07\times10^{-3}$	$9.32\times10^{-5} \pm 8.94\times10^{-5}$*	$5.10\times10^{-3} \pm 2.89\times10^{-3}$
5 shocks	$9.82\times10^{-4} \pm 7.59\times10^{-4}$	$1.36\times10^{-4} \pm 1.40\times10^{-4}$*	$2.74\times10^{-3} \pm 1.41\times10^{-3}$
Addition of plasmid 30 min, 3 min, 75 or 45 s after shock	/	/	0.00×10^{0}*
Addition of plasmid 3 min or 54 s after shock	0.00×10^{0}*	/	/
Addition of plasmid 30 s after 5 shocks	0.00×10^{0}*	0.00×10^{0}*	0.00×10^{0}*
Addition of plasmid 30 s after shock in icy medium	0.00×10^{0}*	0.00×10^{0}*	0.00×10^{0}*
Addition of plasmid 30 s after shock	$2.01\times10^{-7} \pm 1.57\times10^{-7}$*	$0.00\times10^{0} \pm 0.00\times10^{0}$*	$1.32\times10^{-7} \pm 1.14\times10^{-7}$*
10^{6} bacteria	/	$4.39\times10^{-2} \pm 1.80\times10^{-2}$	/
10^{5} bacteria	/	$0.00\times10^{0} \pm 0.00\times10^{0}$*	/
10^{4} bacteria	$8.95\times10^{-4} \pm 4.78\times10^{-4}$	$0.00\times10^{0} \pm 0.00\times10^{0}$*	$8.07\times10^{-7} \pm 1.14\times10^{-6}$*
10^{3} bacteria	$0.00\times10^{0} \pm 0.00\times10^{0}$*	$0.00\times10^{0} \pm 0.00\times10^{0}$*	$0.00\times10^{0} \pm 0.00\times10^{0}$*
10^{2} bacteria	$0.00\times10^{0} \pm 0.00\times10^{0}$*	$0.00\times10^{0} \pm 0.00\times10^{0}$*	$0.00\times10^{0} \pm 0.00\times10^{0}$*
Stationary phase	$5.42\times10^{-6} \pm 8.95\times10^{-7}$*	/	/
pBLN from *P. syringae* CC0094	$1.18\times10^{-2} \pm 9.91\times10^{-3}$	/	/

Values deal with averages and standard deviations of electrotransformation rates from a minimum of three repetitions of experiments. Asterisks show the values that are significantly different ($p<0.05$) from the control condition.

Multiple shocks

The electrotransformation rates of the three bacteria were determined after three or five repeated pulses (Table I-4, Figure I-8). Values were significantly different from the one shock control condition only for *Pseudomonas* sp. N3 (9.32×10^{-5} and 1.36×10^{-4} for three and five shocks, respectively *versus* 5.83×10^{-3} for the control condition).

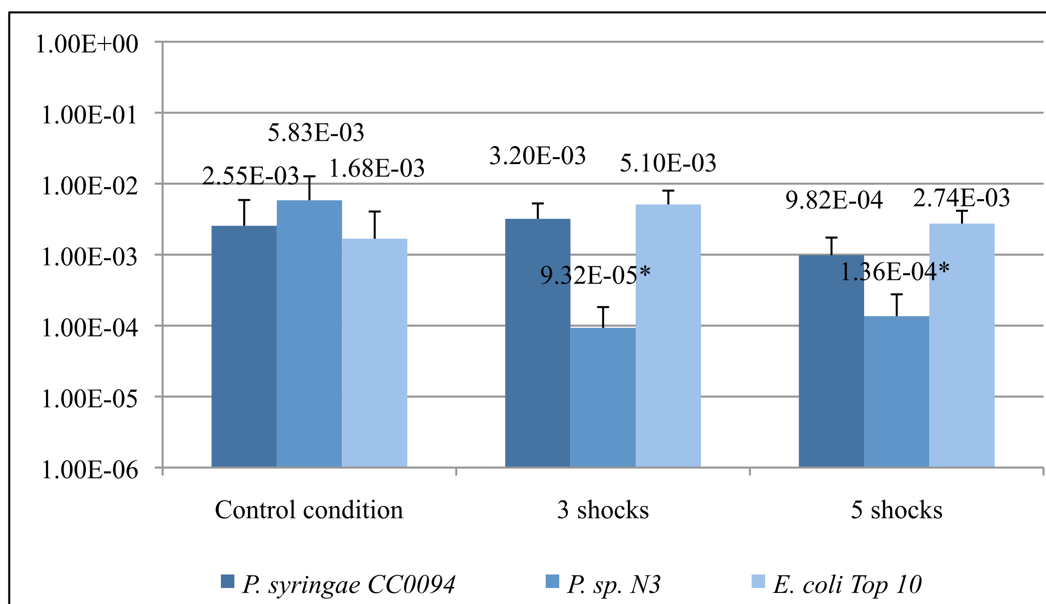


Figure I-8: Electrotransformation rate of *P. syringae* CC0094, *Pseudomonas* sp. N3 and *E. coli* Top 10 after one, three or five 12.5 kV.cm^{-1} electrical pulses.

Results are expressed as mean values with standard deviations as error bars. Asterisks indicate the values that are significantly different ($p<0.05$) from the control condition.

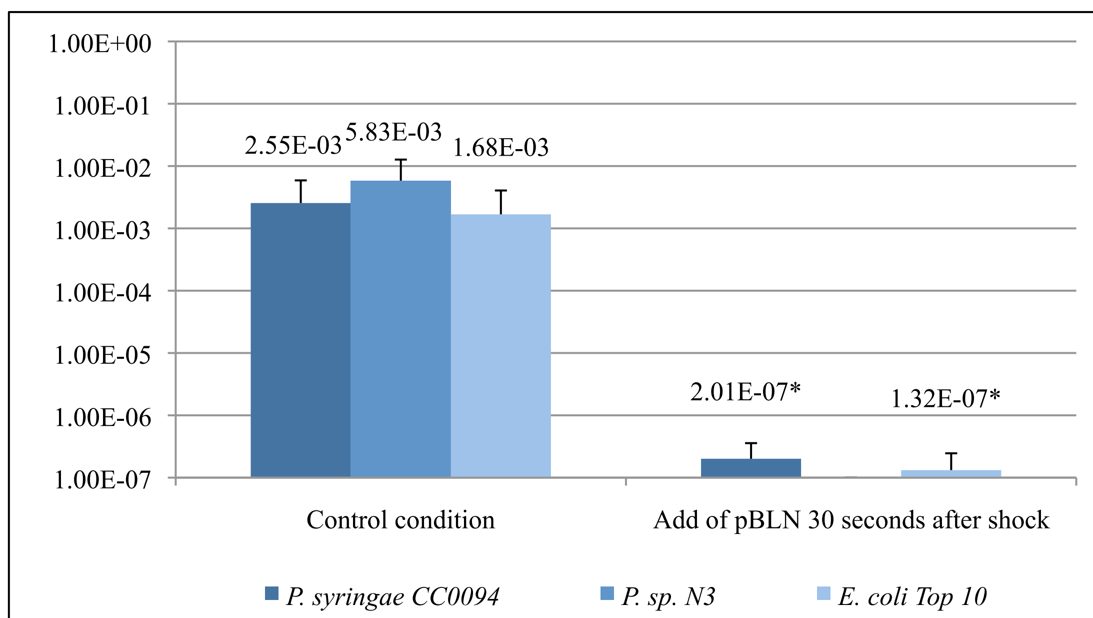


Figure I-9: Electrotransformation rate of *P. syringae* CC0094, *Pseudomonas* sp. N3 and *E. coli* Top 10 when pBLN is added 30 seconds after a 12.5 kV.cm^{-1} electrical pulse.

Results are expressed as mean values with standard deviations as error bars. Asterisks indicate the values that are significantly different ($p<0.05$) from the control condition.

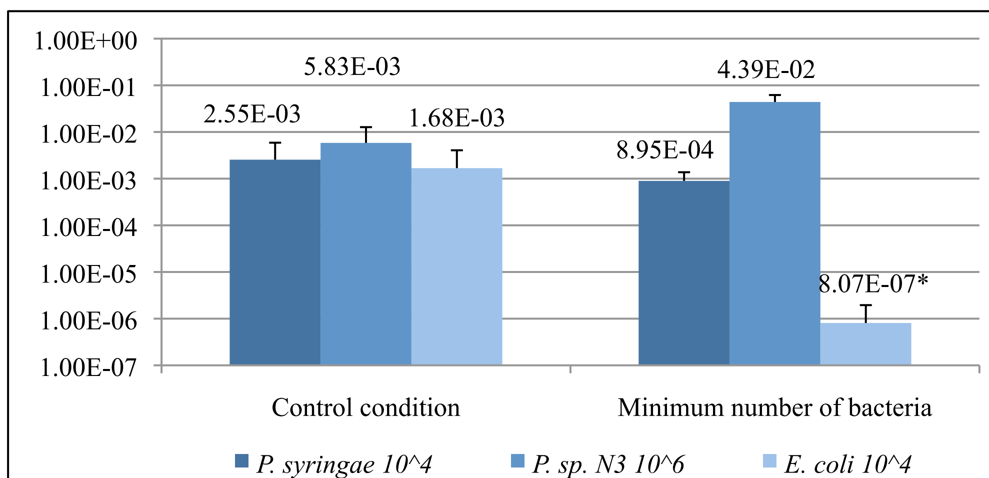


Figure I-10: Electrotransformation rate of minimal number of *P. syringae* CC0094, *Pseudomonas* sp. N3 and *E. coli* Top10 after a 12.5 kV.cm^{-1} electrical pulse.

Results are expressed as mean values with standard deviations as error bars. Asterisks indicate which values are significantly different ($p<0.05$) to the control condition.

Time of pore resealing

The pBLN plasmid was added 30 min, 3 min, 75 or 45 s after the pulse for *E. coli* TOP10, and 3 min or 54 s after the pulse for *P. syringae* CC0094 and no transformants were recovered (Table I-4). Adding of the pBLN plasmid 30 s after five shocks or after a single electrical pulse in icy conditions were performed. In both cases, no transformants were recovered for either one of the studied bacteria (Table I-4). Plasmid pBLN was also added 30 s after the 12.5 $kV.cm^{-1}$ electrical pulse and the average electrotransformation rates of the three studied bacteria are shown in the Table I-4 and Figure I-9. Values were significantly different ($p<0.05$) from the control condition for the three bacteria with *Pseudomonas* sp. N3 yielding no transformants, while other strains yielded results significantly ($p<0.05$) lower than the control (2.01×10^{-7} and 1.32×10^{-7} for *P. syringae* CC0094 and *E. coli* TOP10 *versus* 2.55×10^{-3} and 1.68×10^{-3} for the control condition, respectively).

Cell density

The minimum number of bacteria required to obtain transformants was 10^4, 10^6 and 10^4 bacteria for *P. syringae* CC0094, *Pseudomonas* sp. N3 and *E. coli* TOP10, respectively (Table I-4). The electrotransformation rates of the three studied bacteria minimum number of cells are shown in Figure I-10, with this showing a significantly different ($p<0.05$) frequency from the control with only *E. coli* TOP10 (8.07×10^{-7} *versus* 1.68×10^{-3} for the control condition). The plasmid acquisition rate in *Pseudomonas* sp. N3 was increased but not significantly ($p<0.05$) (4.39×10^{-2} *versus* 5.83×10^{-3} for the control condition).

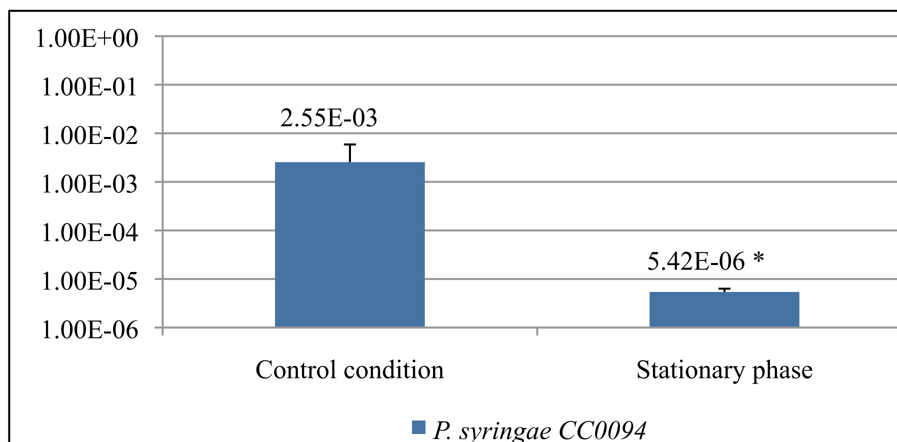


Figure I-11: Electrotransformation rate of stationary phase *P. syringae* CC0094 after a 12.5 kV.cm^{-1} electrical pulse.

Results are expressed as mean values with standard deviations as error bars. Asterisks indicate which values are significantly different ($p<0.05$) to the control condition.

Table I-5: Plasmid loss rate of the three studied bacteria containing pBLN with zero to five shocks.

	***P. syringae* CC0094-pBLN**	***Pseudomonas* sp. N3-pBLN**	***E. coli* TOP10-pBLN**
Without shock	-20.32 ± 24.50%	-35.74 ± 40.22%	-21.38 ±29.53%
1 shock	18.89 ± 22.23%	-14.39 ± 36.99%	-10.32 ± 19.20%
2 shocks	5.65 ± 38.78%	-52.47 ± 66.10%	20.49 ± 23.14%
3 shocks	17.07 ± 59.06%	-55.74 ± 74.54%	-9.52 ± 46.05%
4 shocks	18.39 ± 30.76%	-6.46 ± 83.03%	-3.10 ± 29.94%
5 shocks	5.10 ± 36.76%	-32.72 ± 101.83%	12.76 ± 27.53%

Values deal with average and standard deviations of loss rates from a minimum of four experimental repetitions.

Stationary phase P. syringae CC0094

Electrotransformation rate of *P. syringae* tested under a stationary phase was significantly ($p<0.05$) decreased in comparison to the control condition (5.42×10^{-6} *versus* 2.55×10^{-3} for the control condition) (Table I-4, Figure I-11).

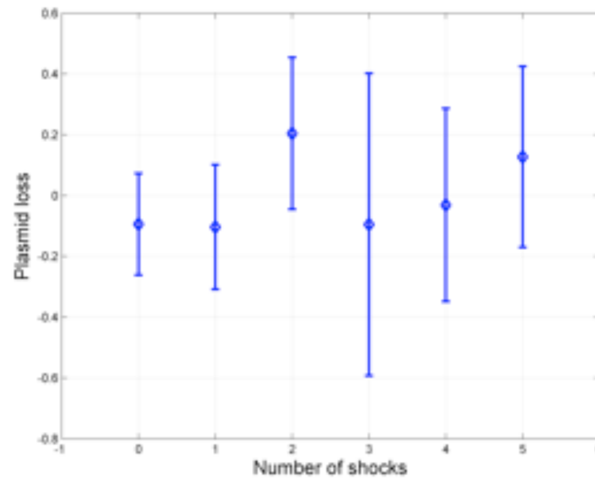
Plasmid origin

Electrotransformation rates of *P. syringae* CC0094, using the plasmid isolated from the strain CC0094 as donor DNA, was not significantly ($p<0.05$) increased in comparison to experiments with the same plasmid isolated from *E. coli* TOP10 (1.18×10^{-2} with pBLN from *P.syringae* CC0094 *versus* 2.55×10^{-3} for the control condition with the plasmid isolated form *E. coli* TOP10) (Table I-4).

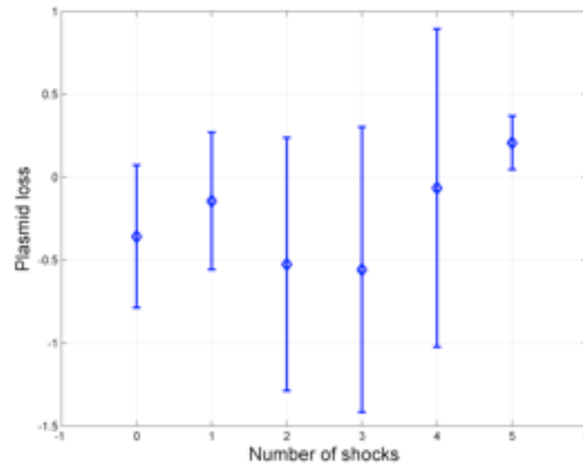
4. Plasmid loss percentages

Plasmid loss experiments have been performed at least four times on the three studied bacteria. The plasmid loss rates of the three studied bacteria containing pBLN with zero to five shocks are given in the Table I-5. The loss rates after one shock are not significantly different between the three strains (-20.32±24.50% for *P. syringae* CC0094, -35.74 ±40.22% for *P. sp.* N3 -21.38±29.53% for *E. coli* Top 10). Multiple shocks did not significantly (up to a significance level of $p<0.05$) change the plasmid loss compared to the controls without shock. The results exhibit a large variability. In the absence of any simple explanation, we applied the Grubbs statistical test that revealed outliers within the collected data. The figure I-12 presents the plasmid loss results after the removal of the outliers.

E. coli Top10 - pBLN



Pseudomonas sp. N3 - pBLN



P. syringae CC0094 - pBLN

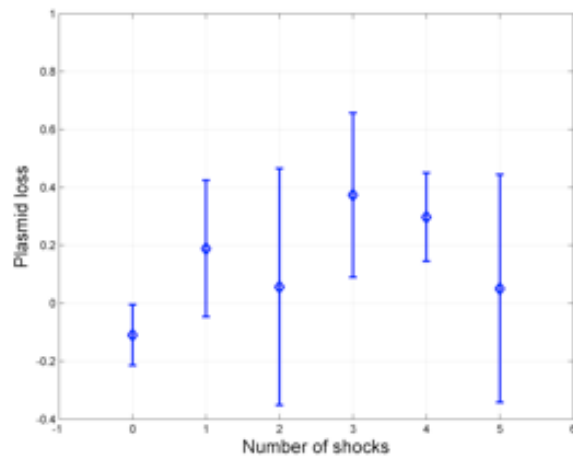


Figure I-12: Plasmid loss rates of E. coli Top 10 - pBLN, Pseudomonas sp. N3 - pBLN and P. syringae CC0094 - pBLN with up to five 12.5 kV.cm-1 electrical pulses.
Results are expressed as mean values with standard deviations as error bars

Even after removal of outliers by the Grubbs statistical test, no significant differences of plasmid loss rates could be detected between the three bacteria containing pBLN with zero to five shocks.

5. Genomic stability of *P. syringae* CC0094

BOX-PCR genomic structure profiles of *P. syringae* CC0094 under variable conditions are shown in Figure I-12. None of the conditions tested, (including a unique 9.5 $kV.cm^{-1}$ electroporation shock, multiple 12.5 $kV.cm^{-1}$ shocks, as well as a single electroporation shock in icy or liquid media), yielded a shift of the genomic structure profile of *P. syringae* CC0094 when compared with the non-shocked profile.

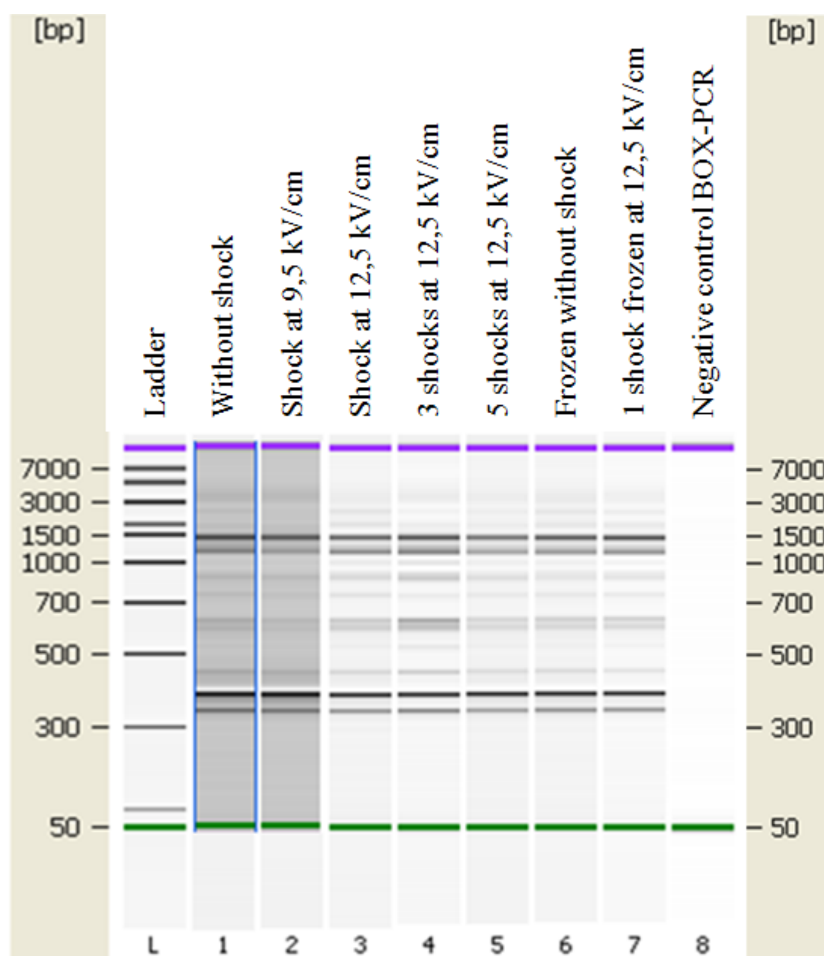


Figure I-13: BOX PCR (rep-PCR) on DNA extracted from *P. syringae* CC0094.
Control without shock (lane 1), with one shock at 9.5 $kV.cm^{-1}$ (lane 2), one, three and five shocks at 12.5 $kV.cm^{-1}$ (lane 3, 4 and 5), frozen without shock (lane 6) and one shocked frozen (lane 7); ladder (lane L); BOX-PCR negative control (lane 8)

DISCUSSION:

Electroporation is an efficient method for DNA transfer into cells. It can be applied to a large diversity of microorganisms (among which includes bacteria), as well as to plant or animal cells (Maniatis *et al.*, 1982; Dower *et al.*, 1988; Chakraborty and Kapoor, 1990; Gilchrist and Smit, 1991; Drury, 1996; Weaver and Chizmadzhev, 1996; Lurquin, 1997; Newell, 2000; Villemejane and Mir, 2009). Another advantage of electroporation is that the mechanism is much less affected than HGT by the physiological state of the cell, even if the *in vitro* efficiency varies with the cell growth stage (Drury, 1996; Cérémonie *et al.*, 2004). The tests focusing on *E. coli* (Demanèche *et al.*, 2001c) and *Pseudomonas* sp. N3 (Cérémonie *et al.*, 2004, 2006a) submitted to lightning-simulated electrical discharges in soil microcosms indicate that electrotransformation efficiency of bacteria under natural conditions remains high enough to allow gene transfer to occur *in situ* at a detectable level, whereas natural transformation of inoculated bacteria in soil did not yield any transformants. These results led us to investigate whether bacteria living in ecosystems subjected to more lightning discharges than soils, such as clouds (Gary, 1999), had developed specific adaptive capacities relying on natural electrotransformation. This question is particularly relevant for bacteria acting as heterogeneous Ice Nuclei (IN) that catalyze freezing of supercooled water at temperatures above that of mineral ice nuclei activity. Presence of IN in clouds was reported to trigger lightning, leading us to speculate on the selection of such an activity by bacteria resulting in the increasing of the frequency of electrotransformation-mediated HGT and the enhancing of their adaptive potential. Among IN cloud bacteria, *Pseudomonas syringae*, known for its extensive occurrence in clouds, its potential to adapt a wide range of ecosystems and its pathogenicity potential on plants, constitutes one of the best models to investigate a putative link between IN activity (lightning-triggering) related HGT potential and adaptive capacity. To evaluate the potential specific capacities of IN bacteria, *P. syringae* response to electroporation was compared to *E. coli* TOP10, the laboratory model for electroporation, and *Pseudomonas* sp. N3, a highly electrocompetent bacteria isolated from soil (Cérémonie *et al.*, 2004, 2006a) in a series of *in vitro* experiments.

Table I-6: Correspondence between laboratory tested conditions and literature

	Laboratory experiments (*in vitro*)	**Current state of the literature**
a-	9.5 *versus* 12.5 $kV.cm^{-1}$	Few $kV.cm^{-1}$ (Nucci *et al.*, 1988; Wendt-Potthoff *et al.*, 1992; Bassett and Janisiewicz, 2003)
b-	Icy conditions	Ice Nuclei (Morris *et al.*, 2004)
c-	One to five shocks	Several pulses (Gonçalves *et al.*, 2012)
d-	Exponential *versus* Stationary phase	Actively growing bacteria (Sattler *et al.*, 2001)
e-	10^4 to 10^8 bacteria per experiment	10^4 viable cells per m^3 of cloud volume (Amato *et al.*, 2005)
f-	Addition of plasmid 30 s to 30 min after shock	Metastable pores (Smith *et al.*, 2004)
g-	Plasmid from *P. syringae versus E. coli*	Provenance of plasmid (Bassett and Janisiewicz, 2003)

Adaptation to cloud ecosystems to benefit from lightning-related electrical parameters to transfer genes, requires physiological properties from bacteria lying far beyond the electrotransformability that can be expressed *in vitro*. These include a good resistance level of bacterial cells to the extreme physical and chemical conditions (low temperature, electric fields, pollutants, low nutrient content) encountered to maintain a high survival rate of the population and their ability to be efficiently electroporated under these conditions. Our experimental procedure was thus designed to match the observed *in situ* parameters (Table I-6).

1. Survival potential

The survival potential of bacteria after a lightning discharge is one of the most important parameters necessary for adaption to the cloud environment. According to Sambrook and Russell (2001), the electrical factors fatal to bacteria are the strength of the electrical field and duration of electroporation. Above a certain limit, each factor alone or in combination, kills the cell by membrane disruption and efflux of cellular components. Our results demonstrate that the three bacteria exhibited the same survival rate (ranging from 11 to 20%) when similar numbers of cells suspended in an identical volume were submitted to standard electroporation conditions (Drury, 1996), including the use of 2 mm cuvettes for the delivery of 9.5 or 12.5 $kV.cm^{-1}$ (Wendt-Potthoff *et al.*, 1992; Bassett and Janisiewicz, 2003), 25 µF, 200 Ω and an immediate incubation of electroporated cells in growth medium to favor their recovery. IN bacteria do not appear to have an advantage over non-IN bacteria when subjected to a single electric discharge.

Since Gonçalves *et al.* (2012) demonstrated that the presence of IN active bacteria in clouds significantly increased the number of lightning pulses, we next evaluated if our bacteria would respond in a likewise manner to multiple electric shocks. The survival rate of *E. coli* TOP10, *Pseudomonas* sp. N3, *P. syringae* CC0094 (whether containing the plasmid or not) did not decrease significantly after multiple shocks when compared with the single shock condition. The standard deviations when bacterial strains harbored the pBLN plasmid (most notably *E. coli*) were however larger. In the absence of any known reason that could explain such variability, we used the Grubbs statistical test (1969) to spot outliers among the collected data. The results provided must however be critically considered because: (i) our test is based upon the hypothesis that all the points follow a Gaussian distribution: this hypothesis cannot be verified in a reliable way due to the reduced size of the available data, (ii) the experimental

average and standard deviation are *estimates* of the expected values and of the true standard deviation, (iii) the test of Grubbs is suited for identifying one outlier, therefore, the application of the test in an iterative way is questionable, because of the reduced size of our samples (at most 10 points). The variability remained large even after Grubbs statistical analyses and the only significant difference observed in these analyses was the survival rate between shocked and non-shocked bacteria. Therefore the three bacteria could survive at least five shocks, with IN *P. syringae* not having a specific advantage.

When shocked in ice, *P. syringae* CC0094 survival rate increased significantly to reach 60.21% whereas *E. coli* TOP10 survival was identical in both liquid or icy media and *Pseudomonas* sp. N3 showed an intermediate behavior (moderately increased survival in icy medium although not significantly different from that in the liquid condition). Given that *P. syringae* is able to form ice nuclei at temperatures down to -6°C (Vali, 1996; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Möhler *et al.*, 2008), while the two others are not (data not shown), we hypothesized that the ice crystals formed could protect *P. syringae* cells from the lightning discharge. This phenomenon may be explained by the low conductivity in ice since frozen rain resistance was measured at 45 MΩ corresponding to a conductivity (1.26 $\mu S.m^{-1}$) lower than that of liquid water. Groundwater and deionized water conductivities reach about 5 $mS.m^{-1}$ and 10 $\mu S.m^{-1}$, respectively, whereas seawater is very conductive (4.80 $S.m^{-1}$) due to the presence of salt (Cox *et al.*, 1967; Maxwell *et al.*, 1971; Saxena and Ahmed, 2001; Pashley *et al.*, 2005). Ice protection to electrical discharge could also be explained by the lower availability of liquid water molecules since reversible formation of pores is assumed to be due to the electric gradient at the interface between lipid and water layers and to the related displacement of water molecules in the gradient and probably through the membrane that accelerate the process of pore formation (Tieleman, 2004). Thus bacteria capable of ice nucleation do seem to possess an advantage for surviving in cold environments such as clouds. Confirming this hypothesis of ice protection-related survival should focus on evaluating the survival rate of an *ina*- mutant of the *P. syringae strain* CC0094 (which has lost the IN property) as well as testing additional environmental strains.

Another factor to consider is the growth state of bacteria. Although actively growing bacteria had been isolated from clouds (Sattler *et al.*, 2001; Amato *et al.*, 2007a; Amato *et al.*, 2007b; Vaïtilingom *et al.*, 2013), all bacteria are presumably not in exponential growth phase. Thus, we wondered whether *P. syringae* CC0094 cells in stationary phase were still able to

survive when submitted to an electric chock. Calvin and Hanawalt (1988) determined that cells were more affected by electrical pulse at the beginning of growth but then survival rates stabilized when cells reached the end of exponential growth. This was also the case for *P. syringae* CC0094. The survival rate of stationary phase *P. syringae* CC0094 cells was not significantly different compared to the control condition, indicating that whatever the growth phase of this bacterium in clouds, it would survive lightning discharges.

Finally, we verified the impact of bacterial density on survival. Total bacterial density in clouds being lower than the bacterial loads used in laboratory electroporations (Amato *et al.*, 2005), we tested if bacteria were more sensitive to an electrical shock when fewer in number, due to the lack of protection from surrounding cells. However, survival rates were not significantly different between the different cell concentrations for any of the three studied strains indicating that a low density of bacteria in clouds does not impact their ability to survive electric shocks.

2. Electrotransformation potential

Secondly we studied the ability of bacteria to acquire plasmid DNA by electroporation. Our aim was to compare the transfer frequency between the three species by simulating the conditions likely to be encountered by bacteria *in situ* (in clouds) (Table I-6). Before and during lightning, electrical pulses of few $kV.cm^{-1}$ are involved in clouds (Nucci *et al.*, 1988), which correspond to values allowing electrotransformation. Since no *P. syringae* CC0094 transformants were obtained when electroporations were conducted at 9.5 $kV.cm^{-1}$, the voltage advised by Bassett and Janiesiewicz (2003), this was increased to 12.5 $kV.cm^{-1}$ (Calvin and Hanawalt, 1988; Wendt-Potthoff *et al.*, 1992) to raise transformation efficiency and this successfully produced *P. syringae* CC0094 transformants. The electrical settings (12.5 $kV.cm^{-1}$, 25 µF, 200 Ω, 5 ms and 2 mm cuvettes) applied to the three bacterial strains produced similar electrotransformation rates (from 1×10^{-3} to 6×10^{-3}) in the single-shock liquid condition.

Since the bacterial strains were able to survive at least five shocks, we tested if several shocks could influence the amount of DNA that bacteria were able to acquire. Electroporation frequencies were not significantly increased when *E. coli* and *P. syringae* cells were subjected to one or multiple shocks, consistently to the work by Calvin and Hanawalt (1988) who tried to apply repeated shocks to improve transformation rates in *E. coli* but concluded that a single

pulse was optimal for transformation efficiency. Surprisingly the electrotransformation rate for *Pseudomonas* sp. N3 was significantly reduced after three and five shocks. However, in mammalian cells, multishocks lead to higher efficiency of transformation but also to drastic decline in cell survival (Baron *et al.*, 2000; Song *et al.*, 2010), contrary to our results showing conserved survival rate after multiple electrical pulse treatment for the three strains. More complex phenomena may be at play, as seen in yeasts for which variable outcomes are reported when multiple pulses are applied, depending on macromolecules involved and electric fields (Brown *et al.*, 1992).

Given that exogenous DNA can enter cells after electrical discharges due to cell membrane permeabilization, we wondered if successive lightning strikes in clouds could lead to plasmid loss. The three studied strains harboring pBLN and submitted to multiple electrical discharges did not show a rate of plasmid loss significantly different from the controls (no shock) even after the removal of outliers identified through Grubbs statistical analyses. Successive lightning strikes as can happen in clouds (Gonçalves *et al.*, 2012) are thus unlikely to lead to plasmid loss, in contrast to the results obtained by Heery *et al.* (1989) who applied high voltage electroporations that led to a plasmid cure from *E. coli* cells at an 80-90% frequency. This discrepancy may be explained by the fact that some of the broad-host range plasmids such as pBLN can be very stably resident in bacteria (Eynard and Teissie, 2000). The use of a single-copy plasmid could improve the detection of plasmid loss in cultured bacteria as pBLN is a low-copy plasmid, implying that, as for high-copy plasmids, all the copies would have to be lost for a decrease of CFU to be detected on media with antibiotics. Bacteria are thus likely to be able to electrotransform exogenous DNA without plasmid loss in clouds when successive lightning discharges are delivered.

In icy conditions, the two *Pseudomonas* strains exhibited significantly reduced rates of plasmid acquisition, as was expected because of the lower conductivity of ice. Surprisingly, *E. coli* electrotransformation rates in icy medium were the same as in liquid condition. This latter result parallels the conservation of *E. coli* survival rate irrespectively of the icy or liquid electroporation condition, whereas the *Pseudomonas* strains show opposite trends of survival and electrotransformation. Fewer pores might have been created in the icy conditions, which may have led to a reduced ability of these bacteria to acquire the plasmid. Moreover, the difference of membrane composition and mucus presence between *Escherichia* and *Pseudomonas* species could take part in the comportment faced to shock in icy conditions. Further exploration, *e.g.*, microscopy analyses, is required to understand whether these

inversely related phenomena (increased survival and lower electrotransformation success) rely on common mechanisms. Nevertheless our results, to our knowledge the first on electroporation in frozen conditions, support the hypothesis of survival and electrotransformation of bacteria in cloud ice nuclei. Moreover, the low temperatures of supercooled water droplets observed in clouds (Delort *et al.*, 2010) could increase the electrotransformation rates (Shi *et al.*, 2003).

Although with a reduced electrotransformation efficiency as previously observed for *E. coli* (Calvin and Hanawalt, 1988), *P. syringae* CC0094 was still able to acquire the plasmid when in stationary phase, even after 60 hours of growth without medium supply. In turn, the electrotransformation mean rate of stationary phase *Pseudomonas* sp. N3 was maintained (not significantly increased) in comparison to exponential phase cells (Cérémonie *et al.*, 2004). Similarly, *Pseudomonas* sp. N3 stood out with regard to the minimum number of cells necessary for transformation (10^6). Indeed, transformants of *E. coli* TOP10 and *P. syringae* CC0094 were still recovered at 10^4 bacterial cell concentrations, at a rate lower (*E. coli* TOP10) or similar (*P. syringae* CC0094) to the control condition (10^8 bacteria). *Pseudomonas* sp. N3 might thus have to reach a sufficient bacterial concentration before being electrotransformed successfully, possibly due to a minimal quantity of mucus needed. The significantly reduced electrotransformation rates at 10^4 bacterial cell density observed for *E. coli* TOP10 (one of the best commercial cells for the electrotransformation *in vitro*) is most easily explained by the very high concentrations (10^8-10^9 CFU.mL^{-1}), not usually encountered in the environment, which were shown to improve the transformation efficiency of this bacterium (Drury, 1996) and correspond to standard laboratory practice. Yet, in clouds total bacterial counts reach about 10^4 cells per m^3 of cloud volume (Amato *et al.*, 2005). *P. syringae* CC0094 would be alone among the three strains we tested to be transformed at low cell densities in clouds. Thus, *P. syringae* CC0094 capable of acquiring exogenous DNA, whatever its growth phase and density, appears to be the most adapted strain among the three studied bacteria for electrotransformation in cloud-like conditions.

No transformants were recovered when adding the plasmid 30 min, 3 min, 75 or 45 s after the pulse for *E. coli* TOP10, and 3 min or 54 s after the pulse for *P. syringae* CC0094. DNA exchange is thus not occurring beyond the time of lightning striking although pores created by lightning shocks might stay open for a sufficient time to allow transformation to occur later under other conditions (Smith *et al.*, 2004). Indeed, several studies involving

mammalian or bacterial cells showed that some of the hydrophobic pores could be converted to metastable hydrophilic ones, requiring less energy to form and maintain and leading to pores staying open during several minutes or hours - or even never resealing. These long lifetime pores were expected to allow DNA to enter long after the transmembrane voltage has returned to low values while not being large enough to lead to cytoplasmic release and cell death (Benz and Zimmermann, 1980; Kwee *et al.*, 1992; Saulis, 1997; Ryttsén *et al.*, 2000; Sambrook and Russell, 2001; Rae and Levis, 2002; Langham, 2004; Smith *et al.*, 2004; Schmeer, 2009). The two time lapses tested for *P. syringae* CC0094 (3 min or 54 s) represent the time for the rain to fall in flat zones, calculated using the cloud distance from Earth (between three and five km above sea level) and the rainfall speed (between 100 and 200 $km.h^{-1}$). In such conditions, rain falling on these flat zones, *P. syringae* would not be able to uptake DNA after lightning shocks. The possibility that the time for pore reclosing may influence the DNA uptake was further tested by adding the DNA 30 s after the electrical pulse using repeated shocks known to increase that time (Ryttsén *et al.*, 2000; Smith *et al.*, 2004) while not significantly reducing the survival of the three studied strains, or after a single electrical pulse in icy conditions since cooling the cells to 0°C can delay the stochastic reclosing of pores (Saulis, 1997). In both cases, no transformants were recovered for either one of the studied bacteria.

In turn, the addition of plasmid thirty seconds after a single electrical discharge led to different results depending on the bacterial strain: *Pseudomonas* sp. N3 was not able to uptake plasmid whereas both *E. coli* TOP10 and *P. syringae* CC0094 were, although with a four-fold log reduction in efficiency. This suggests that *P. syringae* could still electrotransform DNA after being deposited by rainfall from low-lying storm clouds (because of delayed pore resealing) over mountains that constitute a propitious environment for gene uptake. The population densities of this bacterium in the surface litter in alpine prairies are as high as 10^9 $bacteria.m^{-2}$ and those of total bacteria are 100-fold richer (Monteil *et al.*, 2012).

P. syringae CC0094 transformants harboring the pBLN plasmid were used to re-isolate pBLN to evaluate the efficiency of electrotransformation depending on the provenance and topology of plasmid. Extractions performed using both Nucleospin plasmid purification kits (classic and endotoxin-free) returned degraded plasmids, suggesting that *P. syringae* CC0094 released nucleases that degrade DNA during plasmid isolation (Casse *et al.*, 1979). To overcome this problem, the Schwinghamer protocol (1980) that inactivates potential nucleases by preparation on ice, followed by the immediate separation of nucleases from DNA by

gradient separation was applied successfully. The pBLN plasmid thus extracted from *P. syringae* CC0094 produced only the covalently closed circular (CCC) form whereas the three forms (linear, open circular and a majority of CCC) of the same plasmid were isolated from *E. coli* TOP10 using the NucleoSpin Plasmid purification kit. The rate of *P. syringae* CC0094 electrotransformation using the *P. syringae*-reisolated plasmid did not significantly increase, in contrast to previous reports of *P. syringae* L-59-66 where a two- to three-fold log incorporation increase was noticed with plasmid isolated from the same species and from a methylation deficient *E. coli* (Bassett and Janisiewicz, 2003). Plasmid topology and differences in methylation systems (*P. syringae versus E. coli*) do not appear to influence the transformation efficiencies of *P. syringae* CC0094, although topologies of DNA molecules are known to affect bacterial transformation (Drury, 1996). The linear plasmid form is less efficiently transformed than OC or CCC forms when autonomous replication plasmids are used (Demanèche *et al.*, 2002) possibly because it is more susceptible to nucleases (Sambrook *et al.*, 1989). This result confirms and supports the fact that *P. syringae* CC0094 is well adapted to exogenous DNA acquisition.

Parameters related to membrane integrity (such as nutritional stresses, the presence of cations and surfactants, as well as cooling of the medium) known to increase electrotransformation rates (Shi *et al.*, 2003), were not tested in this study but should subsequently be considered to gain further insight into the mechanisms involved in efficient electrotransformation in clouds. Indeed, in this environment, the temperatures are low (Delort *et al.*, 2010), cations can be present (Amato *et al.*, 2007b; Vaïtilingom *et al.*, 2013) and some bacteria produce surfactants that facilitate CCN formation (Ahern *et al.*, 2007).

3. Genomic stability of *P. syringae* CC0094

Finally, we verified that electroporation in the various conditions tested (one pulse in liquid or ice or even after five electrical discharges), did not affect the genome structure of *P. syringae* CC0094. Based on BOX-PCR profiles (Louws *et al.*, 1994), no noticeable modifications could be detected, attesting to the maintenance of the repetitive extragenic palindromic sequence distances, hence of the overall genomic structural stability of this bacterium.

CONCLUSION:

Overall, our results provide a proof of concept that electrotransformation-based DNA acquisition by bacteria could occur in cloud environment through the mediation of lightning-related electrical discharges.

We conducted *in vitro* experiments to determine if *P. syringae* CC0094 was able to acquire new genetic material when in clouds using simulated lightning shocks. Firstly, *P. syringae* CC0094 submitted to a 12.5 $kV.cm^{-1}$ simulated lightning shock behaves in a likewise manner to *Pseudomonas* sp. N3 and *E. coli* TOP10 in terms of survival and electrotransformation rate under standard conditions. In turn, only *P. syringae* CC0094 exhibited a high resistance to simulated lightning shocks in icy conditions, most probably in relation to its ability to form ice nuclei. Moreover, it was still able to acquire plasmid DNA in icy conditions although with a reduced efficiency. *P. syringae* CC0094 was also able to resist several shocks as might occur in clouds, especially in the presence of IN bacteria, without either genomic structure modification or a change in rates of plasmid acquisition or plasmid loss. Its electrotransformation ability was maintained although decreased when in stationary phase. *P. syringae* CC0094 was also able to acquire DNA at low cell density. *Pseudomonas* sp. N3, a naturally electrotransformable bacterium isolated from soil, is less adapted to cloud-like environment, particularly because of its low survival in icy condition, its quick resealing of pores and the minimal bacterial density needed for its electrotransformation. *Escherichia coli*, although known to be the most electrotransformable strain, is not protected by ice nucleating activity and is less electrotransformed under low cell densities, in contrast to *P. syringae.*

Because of the harsh conditions encountered in clouds, bacterial DNA may be released as a result of cell lyses. Although the bacterial density is probably too low in a frozen cloud droplet for direct gene exchange to occur, membrane permeabilization by lightning shocks seems to last for up to 30 seconds after shocks, allowing subsequent transformations to occur, possibly in DNA-rich environments where bacteria may be transported by rainfall from storms.

To conclude, *P. syringae* CC0094 shows higher survival and electrotransformation potentials compared to *E. coli* TOP10 and *Pseudomonas* sp. N3 for the different parameters studied. All our observations support the hypothesis that a plant pathogen such as *P. syringae* is a good candidate for survival and electrotransformation in clouds. Its ability to use its

nucleation potential to increase its fitness in phytopathogenicity and colonization of new ecosystems still needs to be confirmed by a mutational approach. However, the ability of *P. syringae* to survive and evolve while being transported in clouds could explain how this ubiquitous ice-nucleating active bacterium is also one of the most significant phytopathogens, causing huge economic losses worldwide.

II. IMPACT OF LIGHTNING ON DIVERSITY, SURVIVAL AND ELECTROTRANSFORMATION POTENTIAL OF RAIN BACTERIA

INTRODUCTION

Bacteria are detectable almost everywhere in the biosphere from deep in the Earth's crust (Edwards *et al.*, 2003) to high up in the stratosphere, their aerosolization at altitudes of 40 km and above (Imshenetsky *et al.*, 1978) being due to convective updrafts from soil, vegetation and also bubbles bursting from water surfaces (Lighthart, 1997; Delort *et al.*, 2010; Morris *et al.*, 2011). Microorganisms or charged ice particles that may contain microorganisms can also be uplifted into the stratosphere by electric fields related to the formation of thunderstorms (Dehel *et al.*, 2008). Their total number in clouds reaches 10^{19} on a global scale, sufficient to affect biogeochemical processes (Whitman *et al.*, 1998; Bauer *et al.*, 2002). Clouds could thus play a major role in disseminating transient microbes over long distances then bringing moving airborne bacteria back to ground by wet deposition (Vali *et al.*, 1976; Delort *et al.*, 2010). Airborne microorganisms can also be transported across long distances whilst being fed on Saharan or Asian dust (Griffin *et al.*, 2001; Griffin *et al.*, 2002; Smith *et al.*, 2011; Hara and Zhang, 2012; Smith *et al.*, 2012; Yamaguchi *et al.*, 2012; Creamean *et al.*, 2013), as well as from the Black Sea to Sweden during a sandstorm (Bovallius *et al.*, 1978). Microorganisms have also been found functionally stable in hailstones (Šantl-Temkiv *et al.*, 2012; Šantl-Temkiv *et al.*, 2013), in fog droplets that act as culture medium (Fuzzi *et al.*, 1997), as well as actively growing in cloud water and rain and snow precipitations (Casareto *et al.*, 1996; Carpenter *et al.*, 2000; Sattler *et al.*, 2001; Amato *et al.*, 2007a; Amato *et al.*, 2007b; Vaïtilingom *et al.*, 2013).

To disseminate through the high atmosphere, bacteria have to survive stressful conditions such as lack of nutrients, wind, high oxidative conditions, desiccation, low temperature, low pH, UV radiation, repeated freeze-thaw phases and osmotic shocks (Deguillaume *et al.*, 2008; Delort *et al.*, 2010; Wilson *et al.*, 2012). Some bacteria exploit condensation process properties so as to be protected against desiccation, repeated freeze-thaw phases and UV radiation. They can induce the formation of small droplets from water vapor on their surface by production of biosurfactants, thereby acting as Cloud Condensation Nuclei (CCN) (Bauer *et al.*, 2003; Sun and Ariya, 2006; Möhler *et al.*, 2007). In addition, as a result of a membrane protein triggered by low temperatures and nutrient limitations, some bacteria can act as heterogeneous Ice Nuclei (IN) catalyzing freezing of supercooled water at temperatures above the activity of mineral IN, which are much more abundant in the atmosphere (up to -2°C *versus* -10°C) (Vali *et al.*, 1976; Vali, 1996;

Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Christner *et al.*, 2008a; Möhler *et al.*, 2008). Both of these mechanisms of nucleus production enable cloud formation, and snow and rain precipitation (Morris *et al.*, 2004; Möhler *et al.*, 2007; Christner *et al.*, 2008b; Christner *et al.*, 2008a) through the Wegener-Bergeron-Findeisen (Wegener, 1911; Bergeron, 1935; Findeisen, 1938) and Hallett-Mossop (Mossop and Hallett, 1974) processes, by which ice crystals grow and multiply within the cloud, respectively.

Under stormy conditions, rapid airflow in clouds causes ice crystals to collide and cleave, inducing electrical charge separation (Gary, 1999; Aguet and Ianoz, 2001). The resulting high electrical fields (due to electrical charge accumulation inside clouds or between clouds and Earth) induce the formation of ionized channels where several high-intensity pulses of currents can be delivered, corresponding to the lightning flashes. Gonçalves *et al.* (2012) demonstrated through modeling that the presence of ice nucleation active bacteria in clouds significantly increases the numbers of lightning pulses.

Some of the bacteria frequently isolated on harsh environments, such as plant surfaces or permafrost, are found in the high atmosphere (Lighthart, 1997; Amato *et al.*, 2005; Amato *et al.*, 2007d; Amato *et al.*, 2010; Delort *et al.*, 2010), among which *Pseudomonas syringae*, one of the most phytopathogenic species complexes, causing significant economic losses on a wide range of fruit and vegetable crops (Morris *et al.*, 2010; Mansfield *et al.*, 2012). Interestingly, *P. syringae* is one of the most ice nucleate active (INA) bacterium, hence being responsible for frost development at temperature above the activity of mineral ice nuclei, contributing to freezing plant buds and destroying crops (Maki *et al.*, 1974; Arny *et al.*, 1976; Maki and Willoughby, 1978; Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Kennelly *et al.*, 2007; Möhler *et al.*, 2008). Due to its presence in almost every stage of the water cycle such as river epilithon, streams, lakes, irrigation ditches, and snowpack as well as in ascending dry aerosols, in clouds with a shorter time of residence than *ina-* bacteria and in precipitation, *P. syringae* is postulated to be disseminated *via* the water cycle and could be spread by rain to cropfields (Figure 2B in the General Introduction) (Maki and Willoughby, 1978; Constantinidou *et al.*, 1990; Morris *et al.*, 2004; Morris *et al.*, 2008; Morris *et al.*, 2010; Morris *et al.*, 2011; Monteil *et al.*, 2012; Morris and Sands, 2012). Furthermore, INA bacteria including *P. syringae* can be involved in the triggering of lightning in clouds (Figure 2C in the General Introduction), the electric field pulses (few $kV.cm^{-1}$) associated with lightning currents (Nucci *et al.*, 1988) being susceptible to produce a genetic electrotransformation of bacteria, similarly to what has been described in soil (Figure 2D in the General Introduction) (Demanèche *et al.*, 2001c).

This leads us to hypothesize that a plant pathogen such as *P. syringae* could use its ice nucleate potential to induce the triggering of phenomena that could contribute to increase its adaptive potential (i.e. acquisition of new genes by lightning-mediated electrotransformation) for improving phytopathogenic abilities and other properties. Combined with a strong dissemination potential through precipitations from clouds, *P. syringae* thus stands as a model of a particularly adaptable microorganism for the colonizing of new niches, among which includes snow, soils, lakes and oceans. However, such adaptive properties imply that these bacteria are able to resist the harsh conditions that they undergo during their transport and residence in clouds, (including electric field pulses related to lightning discharges) and that they exhibit some electrotransformation abilities for taking advantage of electric field pulses associated with lightning currents.

In order to explore these phenomena, we isolated *in vitro* bacterial communities from five rain events as a proxy to cloud bacteria triggering precipitation. We characterized their adaptive properties, including survival to lightning, natural resistance to two antibiotics and ability to be naturally and electro-transformed and determined the proportion of *Pseudomonas* sp., in particular *P. syringae*.

EXPERIMENTAL PROCEDURES:

1. Rain collection

Five rain episodes were sampled in Ecully (France - 45.784921 N 4.767873 E) between April 10th and 24th 2012. Rainwater was collected using a conic polyan canvas cover (about 1.875 m² in surface) (Figure 1 in Appendix II-1) that had previously been sterilized with 70% (v/v) ethanol and rinsed with sterile water. Rainwater flowed through a 0.5 mm nylon filter (Nitex, Dutscher, Brumath, France) in a 150 mL Büshner funnel (Nalgene, Rochester, USA) for removal of large particles, and was recovered into 2 L polyethylene sterile containers (Dutscher, Brumath, France). These containers were stored at +4°C until processing.

2. Bacterial recovery

Particles above 5 µm were removed by vacuum filtration using 5 µm TMTP Isopore™ membrane filters (Millipore, Billerica, USA). Bacteria were then recovered by rainwater filtration on 0.2 µm GTTP filters (Millipore, Billerica, USA). Filters were incubated in 25 mL of 0.5 M sucrose (except for the fourth rain event, when the filter was placed in 10% [w/v] glycerol) and stored overnight at +4°C. Bacteria were removed from filters by vortex agitation for 1 h. Resulting suspensions were then centrifuged at 6000×*g* for 10 minutes at 4°C and washed four times with decreasing volumes of 0.5 M sucrose (or 10% glycerol for rain 4) to a final volume corresponding to a 5000-fold concentration (except for the third rain event that was 4000-fold concentrated).

3. Isolation and enumeration of culturable bacteria in rain

For the five rain events, 50 µL of concentrated bacterial suspensions were incubated in 1 mL of Lysogeny Broth low salt (LB) (Duchefa Biochemie, Haarlem, The Netherlands) for 2 h at 22°C under agitation (225 rpm). To estimate the total culturable bacteria, bacterial suspensions were spread on three LB agar plates supplemented with 200 $\mu g.mL^{-1}$ cycloheximide (Duchefa Biochemie, Haarlem, The Netherlands) to avoid eukaryote growth (LBC medium). To determine the rainwater content in putative *Pseudomonas* spp., suspensions were plated onto KBC medium (Mohan and Schaad, 1987). Three KBC agar plates were used for rains 1, 2 and 5 samples whereas only one plate was used for rain events 3 and 4. Numbers of total culturable bacteria were estimated for the five rain events,

whereas subsequent investigations were performed on the three selected biological triplicates: rain events 1, 2 and 5.

Counts of naturally antibiotic-resistant bacteria were also determined using both medium (LBC and KBC) supplemented with 25 µg.mL^{-1} tetracycline and 25 µg.mL^{-1} kanamycin (LBCTK and KBCTK, respectively). All plates were incubated for 5 d at 22°C and these samples were named "Sc" (Sample control). The number of bacteria naturally resistant to tetracycline and kanamycin (R) was calculated as follows for either total culturable bacteria or putative *Pseudomonas*:

$$R = \frac{c}{a}$$

a = Number of total culturable bacteria growing on LBC or KBC without shock (CFU.mL^{-1})

c = Number of bacteria growing on LBCTK or KBCTK for the sample "Sc" (CFU.mL^{-1})

4. Plasmid description and preparation

The 8.5-kb plasmid pBLN used in this study (Figure 1 in Chapter I) was constructed in the course of a previous study (Lyon *et al.*, 2010) from the broad-host-range plasmid pBBR1-MCS3 (GenBank accession XXU25059) able to replicate in many different types of bacteria (Kovach *et al.*, 1995) and harbouring the *tetA* gene encoding resistance to tetracycline. Genes from the plasmid pCEAlinA-nptII conferring kanamycin resistance (*aph3ia*) and involved in the two first-steps of lindane degradation by *Sphingobium francense sp*+ (*linA*) (Cérémonie *et al.*, 2006b) were cloned in pBBR1-MCS3. The pBLN plasmid was maintained in *Escherichia coli* Top10 in LB broth with 25 µg.mL^{-1} kanamycin monosulfate and 25 µg.mL^{-1} tetracycline hydrochloride (Duchefa Biochemie, Haarlem, The Netherlands) and extracted when necessary using the NucleoSpin Plasmid Extraction kit (Macherey Nagel, Bethlehem, USA).

5. Natural transformation potential of rain bacteria

Seventy nanograms of the plasmid pBLN were added to 50 µL of bacterial suspension before incubation in 1 mL of LB for 2 hours at 22°C and 225 rpm in an orbital shaker. Bacterial suspensions were then spread on three LBC agar plates for total culturable bacteria counts, and on three KBC agar plates for putative *Pseudomonas* spp. counts, as well as on

both media supplemented with 25 µg.mL^{-1} tetracycline and 25 µg.mL^{-1} kanamycin (LBCTK and KBCTK) to assess the natural transformation ability of rain bacteria. Plates were incubated for 5 days at 22°C. These samples were named "Sp" (Sample pBLN).

6. Electro-transformation potential of rain bacteria

Seventy nanogrammes of the plasmid pBLN were added to 50 µL of bacterial suspensions and submitted to a lightning-simulated electrical shock at 12.5 kV.cm^{-1}, 25 µF, 200 Ω in a 2 mm cuvette (Cells projects, Harrietsham, United Kingdom) using the Gene Pulser II electroporation system (Biorad, Hercules, USA). Immediately after the shock, LB broth (*qs* 1 mL) was added. After 2 h of incubation at 22°C and 225 rpm agitation, bacterial suspensions were spread onto three LBC agar plates for total culturable bacteria counts and on three KBC agar plates for putative *Pseudomonas* spp. counts, as well as on LBCTK and KBCTK to assess the electrotransformation potential of rain bacteria. Plates were incubated for 5 d at 22°C. These samples were named "Ss" (Sample Shock + pBLN).

The survival percentage after electrical shock (S) was calculated for either total culturable bacteria or putative *Pseudomonas* as follows:

$$S = \frac{b}{a} \times 100$$

a = Number of total culturable bacteria growing on LBC or KBC without shock (CFU.mL^{-1})

b = Number of total culturable bacteria growing on LBC or KBC after shock (CFU.mL^{-1})

7. Isolate characterization and nomenclature

Macroscopic descriptions, based on morphology and particularly color of colonies, were used to differentiate between isolates and three colonies representative of each phenotype were selected for further characterization. For colonies grown on KBC (putative *Pseudomonas* spp.), the oxidase test (Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was performed according to the manufacturer's instructions, in triplicat for each phenotype when possible. These characteristics are provided in Table 1 of Appendix II-2. This table also contains the complete list of isolates and the accession number of their *rrs* sequences. Isolates were named to reflect their detailed origin and obtention:

- Rain event: Ri (i = 1 to 5)

- Sample treatment: Sc = Control / Sp = pBLN / Ss = Shock + pBLN
- Growth Medium: M1 = LBCTK, M2 = KBCTK, M3 = KBC
- Agar Plate number: P*i* (*i* = 1 to 3)
- CFU number: C*i* (*i* = 1 to 25)

8. *linA* amplification

For rain samples 1, 2 and 5, colonies were grown on LBCTK and KBCTK and PCR amplification of *linA* was performed on bacterial lysates of these, with template DNA obtained after incubation of cultures at 95°C for 10 min in sterile distilled water, to verify the presence of plasmid pBLN. Positive controls were conducted with 70 ng of plasmid pBLN and on a single colony of *Sphingobium francence sp+*, which naturally harbours the *linA* gene, as respective templates. Checking for the absence of *linA* amplification was performed on 48 h LB broth cultures of three colonies of each phenotype grown on KBC, isolated from rain events 1 and 2 and stored at -80°C in 12.5% (w/v) glycerol as well as in the negative control with water.

Amplifications were performed with a PCR hot start mix, RTG Illustra™ (GE Healthcare, Little Chalfont, United Kingdom) on 2 µL of different samples. A final concentration of four hundred nanomolar of primers (nested-linA-F1 (forward): 5'-GCTCATTGCCGTAGACAA-3') (nested-linA-R1 (reverse): 5'-GCTCATACTCATCCGTGAAG-3') was used, providing a 296 bp product (Lyon *et al.*, 2010). Temperature cycling was performed in Biometra thermocycler (Goettingen, Germany) starting with an initial denaturation step at 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s and a final elongation at 72°C for 5 min. An aliquot (1 µL) of each amplification reaction product was visualized by electrophoresis separation in 2% (w/v) agarose gels containing ethidium bromide (2.5 µL for 100 mL of gel) in 45 mM Tris-borate-1 mM EDTA buffer (TBE 0.5X). Five microlitres of low range ladder (*i.e.,* 80 to 1031 bp) (Fisher Scientific, Illkirch, France) was used as reference for PCR product size estimation.

9. Transformation rate calculations

- The natural transformation rate (N) was calculated as follow for either total culturable bacteria or putative *Pseudomonas*:

$$N = \frac{(d \times e)}{a}$$

a = Number of total culturable bacteria growing on LBC or KBC without shock (CFU.mL^{-1})
d = Number of bacteria growing on LBCTK or KBCTK for the sample Sp (CFU.mL^{-1})
e = Percentage of colonies harbouring the *linA* gene

- The electrotransformation rate (E) was calculated as follows, for either total culturable bacteria or putative *Pseudomonas,* as:

$$E = \frac{(f \times e)}{b}$$

b = Number of total culturable bacteria growing on LBC or KBC after shock (CFU.mL^{-1})
f = Number of bacteria growing on LBCTK or KBCTK for the sample Ss (CFU.mL^{-1})

This electrotransformation rate was also calculated on total culturable bacteria without shock (E') to be compared with the other rates, as:

$$E' = \frac{(f \times e)}{a}$$

- The percentage of naturally resistant bacteria among all resistant bacteria was calculated as follows for each sample:

$$\%R = \frac{R}{(R + N + E')} \times 100$$

- The percentage of natural transformed bacteria among all resistant bacteria was calculated as follows for each sample:

$$\%N = \frac{N}{(R + N + E')} \times 100$$

- The percentage of electro-transformed bacteria among all resistant bacteria was calculated as follows for each sample:

$$\%E' = \frac{E'}{(R + N + E')} \times 100$$

10. SSU rDNA amplification and sequencing

Some of the rain culturable bacteria and bacteria able to grow on KBC were identified by SSU rDNA amplification and sequencing. Each phenotype of colonies growing on KBC, LBCTK and KBCTK for each sample was analyzed in triplicate when possible. Each colony sampled was cultivated 48 h in LB broth then supplemented with 25% (w/v) glycerol for -80°C storage.

Amplifications were performed by PCR (Platinium® PCR supermix 96, Invitrogen™, Carlsbad, USA) on 2 µL of 48 h cultures as templates. A final concentraion of four hundred nanomolar of primers (pA forward: 5'-AGAGTTTGATCCTGGCTCAG-3') (pH reverse primer: 5'-AAGGAGGTGATCCAGCCGCA-3') was used to amplify 1.6 kb of the SSU rDNA gene (Edwards *et al.*, 1989). The positive and negative controls were 70 ng of previously amplified SSU rDNA of *Sphingobium francence sp+* electrophoresed and extracted from gel using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE healthcare, Little Chalfont, United Kingdom) and sterile distilled water, respectively. Temperature cycling was the same as for *linA* amplification except for the annealing temperature (55°C) and the elongation step (2 min). An aliquot (1 µL) of each amplification reaction product was visualized by electrophoresis separation in 0.8% (w/v) agarose gels containing ethidium bromide (2.5 µL for 100 mL of gel) in 45 mM Tris-borate-1 mM EDTA buffer (TBE 0.5X). Estimation of the amplified fragment size was performed with reference to 6 µL of the 1kb+ ladder (Fisher Scientific, Illkirch, FRANCE). The remaining reaction volumes were sent to Beckman Coulter Genomics (Brea, USA) for Sanger sequencing in both directions of the SSU rDNA fragment using the same primers.

Fragment sequences were assembled into near-full length SSU rDNA using Seqman™ software (Lasergene® Madison, USA).

11. Taxonomical characterization of rain isolates

Consensuses were first identified using the *National Center for Biotechnology Information* BLAST service (NCBI - version 2.2.27 - http://blast.ncbi.nlm.nih.gov -

(Altschul *et al.*, 1990) and confirmed using the SeqMatch function of Ribosomal Database Project (RDP - http://rdp.cme.msu.edu), since the first database is more complete with a sequence database derived from all organisms, whereas the second one is specific for prokaryote SSU gene sequences only. BLAST analysis of SSU rDNA is a rapid and efficient way to identify the most similar sequence (best hit). However, the closest BLAST hit is often not the closest phylogenetic relative (Koski and Golding, 2001). That's why we decided to perform the taxonomic identification with phylogenetic analyses.

SSU rDNAs of rain isolates were classified using the Ribosomal Database Project online services (Cole *et al.*, 2009) using the naïve Bayesian classifier (Wang *et al.*, 2007b). SSU rDNA sequences of the type strains belonging to the bacterial groups thus identified were downloaded from the RDP website (version 10.30) and completed with sequences from Genbank (release 193.0). The complete lists of SSU-rDNAs sequences used in the analyses are given in Tables 1 to 3 of Appendix II-3. In one case (*E. coli*), SSU rDNA of completely sequenced genomes were added to the type species sequences to refine the rain isolate identification (Appendix II-3). Finally, a number of outgroup SSU rDNA sequences were added to the datasets (Appendix II-3).

All near full-length SSU rDNA sequences were handled using Seaview (Gouy *et al.*, 2010). Sequences were aligned using ClustalW (Larkin *et al.*, 2007) and sites of the aligned sequences corresponding to nucleotides 39 to 1438 in *Escherichia coli* type strain SSU rDNA (X80725) were used for phylogeny inference. After processing of the sequence alignments by Gblocks (Castresana, 2000), phylogenies were reconstructed using the maximum likelihood method implemented in PhyML (Guindon and Gascuel, 2003). Analyses were run under the Generalized Time Reversible model of evolution (Tavaré, 1986) with a gamma distribution of evolutionary rates across sites (Yang, 1996). Internal branch supports were evaluated using the approximate Likelihood Ratio Test (Anisimova and Gascuel, 2006). Trees were manipulated using Figtree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

12. Taxonomical distribution of rain isolates

All pie charts and histograms were produced using Microsoft Excel spreadsheet software (Version 2008 for Mac, 12.3.6). Distributions of the isolates across the phylogenetic lineages were calculated after sample size correction of absolute counts per rain event, treatment and growth medium as follows:

$$L_i = \frac{l_i}{C_i} \times \sum_{i=1}^{n} C_i$$

L_i = normalized number of isolates represented by SSU rDNA sequences belonging to a given phylogenetic lineage for a given sample

l_i = number of isolates represented by SSU rDNA sequences belonging to a given phylogenetic lineage for a given sample

C_i = total number of colonies for a given sample

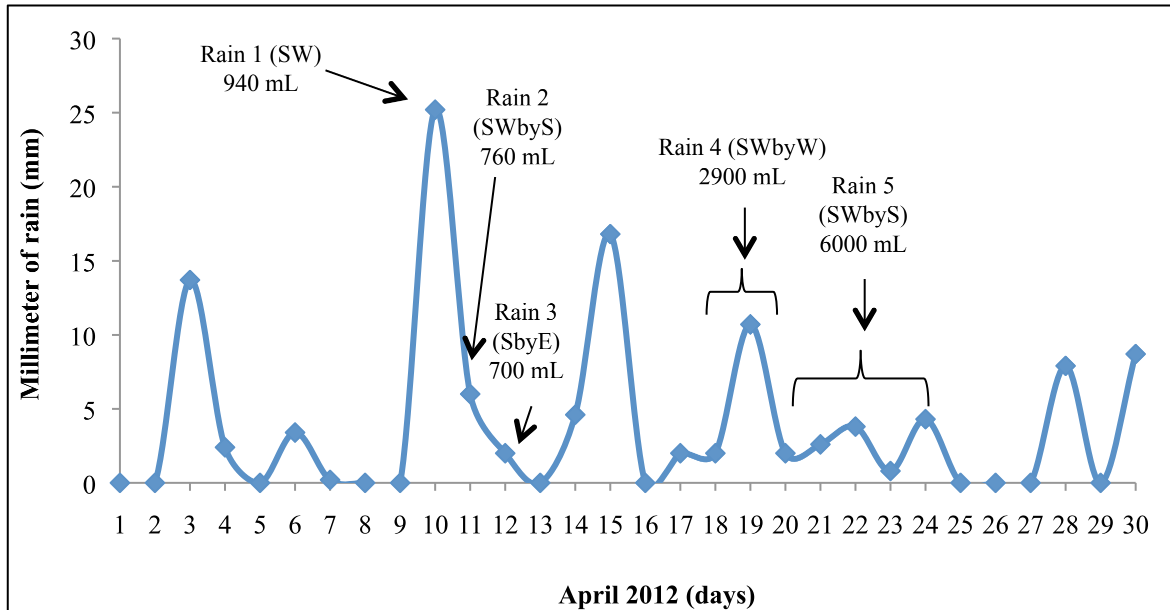


Figure II-1: Daily precipitation amount collected at Ecully (45.784921 N 4.767873 E) during the month of April 2012 (METEO FRANCE nearest station, Bron).

The five rain events sampled are indicated by arrows, together with other parameters such as wind direction (in brackets) and volume collected (mL). SW: SouthWest; SWbyS: SouthWest by South; SbyE: South by East; SWbyW: SouthWest by West.

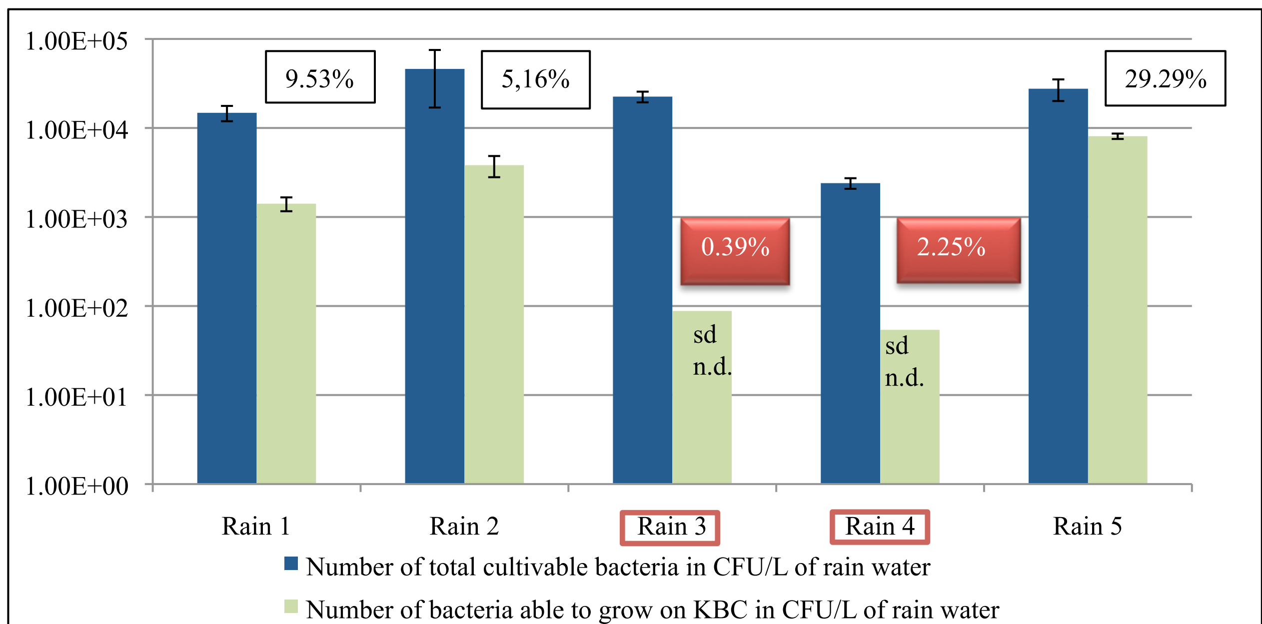


Figure II-2: Number of total culturable bacteria on LBC and putative *Pseudomonas* spp. growing on KBC in five different rain events.

The standard deviations were determined using three Plate counts except for rain 3 and 4 KBC counts where standard deviations were not determined (sd n.d.). The percentages of putative *Pseudomonas* spp. in each rain event are indicated in boxes.

RESULTS:

1. Rain collection and processing

Five rain events were sampled. Daily precipitation volumes for the month of April are given in Figure II-1.

Rain bacteria were recovered by filtration followed by a 5000-fold or 4000-fold (for rain event 3) concentration. Sample final volumes were 188 µL, 152 µL, 175 µL, 580 µL and 1200 µL for rain events 1, 2, 3, 4, and 5, respectively. Fractions (50 µL) of the concentrated rain bacteria solutions were used in the different experiments.

2. Isolation of rain culturable bacteria

Total culturable bacteria estimation

For each rain event, the number of total culturable bacteria grown on LBC medium was estimated by colony counting (Figure 1a, b, c, d & e in Appendix II-4) and varied from 2.40×10^{3} (fourth rain event) to 4.61×10^{4} $CFU.L^{-1}$ of rainwater (second rain event). The overall average of the five rain events total culturable bacterial concentration reached 2.27×10^{4} $CFU.L^{-1}$ of rainwater with a standard deviation of 1.45×10^{4} $CFU.L^{-1}$ of rainwater (Figure II-2, bar "Number of total cultivable bacteria in $CFU.L^{-1}$ of rain water").

Putative Pseudomonas spp. estimation

For each rain event, the number of putative *Pseudomonas* spp. was evaluated as the bacteria growing on KBC medium (Figure 2a, b, c, d & e in Appendix II-4) and ranged from 5.40×10^{1} (fourth rain event) to 8.09×10^{3} $CFU.L^{-1}$ of rainwater (fifth rain event) with an average over the five-rain events reaching 2.69×10^{3} $CFU.L^{-1}$ of rainwater with a standard deviation of 3.02×10^{3} $CFU.L^{-1}$ of rainwater (Figure II-2, bar "Number of bacteria able to grow on KBC in CFU L^{-1} of rain water").

The number of putative *Pseudomonas* spp. relative to all culturable bacteria varied across the rain events, with CFU numbers recovered on KBC for rain events 3 and 4 being noticeably low in comparison to rain events 1, 2 and 5 (Figure II-2, bar "Number of bacteria able to grow on KBC in $CFU.L^{-1}$ of rain water"). This was in part related to an overall lower count of total culturable bacteria in the case of rain episode 4. Average putative

Pseudomonas spp. percentages in rain amounted to 9.33±10.45% for all five rain events and to 14.66±10.50% for rain events 1, 2 and 5.

The objective of this study, being mainly focused on *Pseudomonas,* led us to consider exclusively rain events 1, 2 and 5 as containing the strongest proportion of putative *Pseudomonas,* while their numbers in rain events 3 and 4 led us to exclude them of further analyses.

3. Natural resistance to selected antibiotics of rain bacteria

Natural resistance to both tetracycline and kanamycin of rain bacteria was investigated for total culturable bacteria (LBCTK medium) as well as for putative *Pseudomonas* spp. (KBCTK medium) of the three selected biological triplicates (1, 2 and 5) in the samples named "Sc" (*i.e.* without electrical shock and without plasmid). Single resistance to either antibiotic was not tested.

Table II-1: Concentrations of total rain bacteria and putative *Pseudomonas* spp. in the "Sc" sample

Rain episode	$CFU.L^{-1}$ of rain on LBCTK	$CFU.L^{-1}$ of rain on KBCTK
R1	4.00×10^0	0.00×10^0
R2	0.00×10^0	0.00×10^0
R5	0.00×10^0	0.00×10^0

Bacteria naturally resistant to both antibiotics were isolated on LBCTK from rain 1 only (corresponding to 4 $CFU.L^{-1}$) leading to a mean concentration, for the three rain events, of 1.33 CFU of antibiotic-resistant bacteria per liter of rainwater whereas no TK-resistant colony-forming units were recovered on KBCTK medium (Table II-1). The natural resistance rate for the first rain event culturable bacteria calculated reached 2.70×10^{-4} (Table II-5).

The only colony recovered from direct plating on LBCTK (rain 1) was negative for *linA* presence (Table II-3 – lane "Sc").

Table II-2: Concentrations of total rain bacteria and putative *Pseudomonas* spp. incubated with pBLN ("Sp" sample)

Rain episode	$CFU.L^{-1}$ of rain on LBCTK	$CFU.L^{-1}$ of rain on KBCTK
R1	4.00×10^{0}	0.00×10^{0}
R2	0.00×10^{0}	0.00×10^{0}
R5	0.00×10^{0}	0.00×10^{0}

Table II-3: Proportions of colonies sampled and percentage of *linA*+ isolates for the two first rain events

Rain episode[a]	Sample [b]	Medium[c]	Total CFU number	Number of tested isolates	% of *linA* + isolates
R1[c]	Sc	LBCTK	1	1	0%
	Sp	LBCTK	21	7	100%
		KBC	2116	7	0%
	Ss	LBCTK	11	7	100%
R2[d]	Sp	LBCTK	52	15	93%
		KBC	5730	10	0%
	Ss	LBCTK	34	16	100%
		KBCTK	1	1	100%

[a] No electrotransformant were recovered in the case of rain event 5 (Tables II-1, II-2 and II-4)

[b] "Sc": rain bacteria; "Sp": rain bacteria incubated with pBLN; "Ss": rain bacteria incubated with pBLN and submitted to a 12.5 $kV.cm^{-1}$ electrical pulse.

[c] No electrotransformant *Pseudomonas* spp. were recovered (KBCTK medium) for rain event 1 (Tables II-1, II-2 and II-4).

[d] No culturable bacteria were recovered by direct plating ("Sc" sample) of rain event 2 (Table II-1).

4. Natural transformation potential of rain bacteria

Before plating, rain bacteria were incubated in the presence of plasmid pBLN to determine whether any was able to naturally acquire DNA (“Sp” samples *i.e.,* without electrical shock and with plasmid).

Putative naturally transformed bacteria, *i.e.,* bacteria growing on TK supplemented medium after incubation with pBLN, were identified among total culturable bacteria from rain events 1 (16.80 $CFU.L^{-1}$ of rainwater) and 2 (41.60 $CFU.L^{-1}$ of rainwater), leading to a mean concentration, for the three rain events, of 19.50 CFU of potentially naturally-transformed bacteria per liter of rainwater (Table II-2; Figure 3a, b & c in Appendix II-4). However, no growth on TK-supplemented KBC medium was observed, which suggests that *Pseudomonas*-like bacteria were unable to internalize the plasmid by natural transformation.

For both rain events 1 and 2 and whenever possible, triplicate colonies according to phenotype grown on LBCTK were sampled and *linA* was amplified by PCR to ascertain the presence of pBLN. The proportions of colonies sampled as well as the percentages of positive strains for *linA* amplification are listed in Table II-3 (Lanes “Sp”); 100% (7/7) and 93% (14/15) of the checked colonies isolated from rain events 1 and 2, respectively, on LBCTK tested positives for *linA* presence. The *linA* gene could not be amplified from one isolate (**R2SpM1P1C14** -Appendix II-1) of three sampled colonies presenting a similar phenotype (**R2SpM1P1C13/C14/C15**).

The natural transformation rates then calculated using the proportion of *linA*+ isolates (according to the experimental procedure calculations) amounted to 1.14×10^{-3} and 8.43×10^{-4} for rain event 1 and 2, respectively (Table II-5).

For colonies grown on KBC, three colonies of each phenotype were checked for linA absence and no colonies harbored the pBLN plasmid. The proportions of colonies sampled on KBC are indicated in the Table II-3 (Lanes “Sp”).

Table II-4: Concentrations of rain bacteria incubated with pBLN and submitted to a 12.5 kV.cm^{-1} electrical pulse ("Ss" sample) growing on LBC, KBC, LBCTK or KBCTK.

Rain episode	CFU.L^{-1} of rain on LBC	CFU.L^{-1} of rain on LBCTK	CFU.L^{-1} of rain on KBC	CFU.L^{-1} of rain on KBCTK
R1	4.40×10^{3}	8.80×10^{0}	5.75×10^{2}	0.00×10^{0}
R2	2.77×10^{4}	2.72×10^{1}	2.24×10^{3}	8.00×10^{-1}
R5	9.01×10^{3}	0.00×10^{0}	2.14×10^{3}	0.00×10^{0}

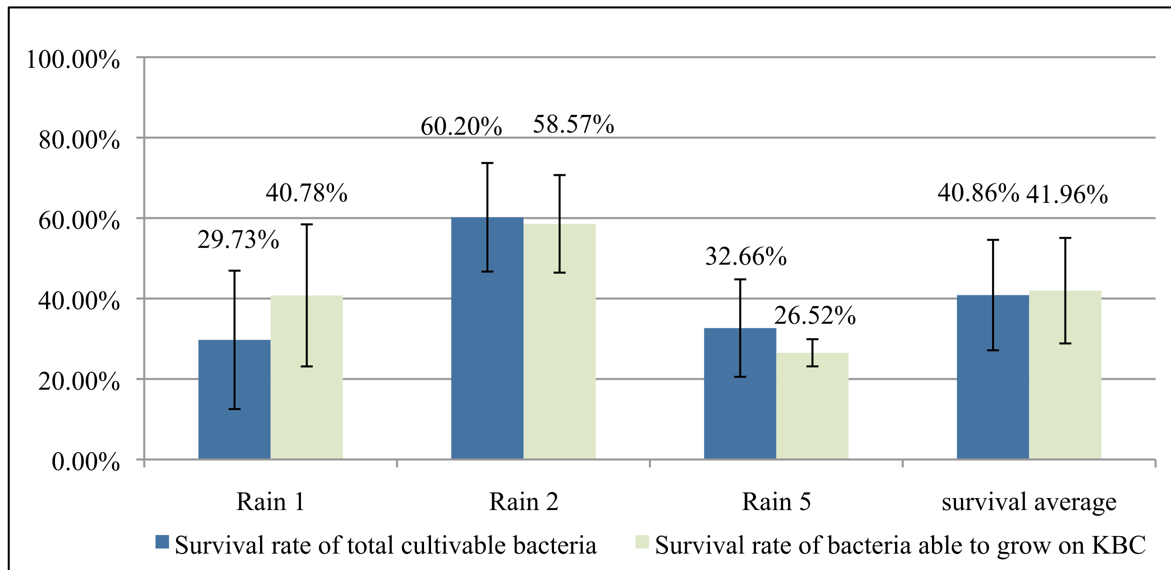


Figure II-3: Survival percentage of total culturable bacteria grown on LBC and putative *Pseudomonas* grown on KBC from different rain episodes after a 12.5 kV.cm^{-1} electrical pulse

The standard deviations for each rain event correspond to the three LBC or KBC Plate counts for total culturable bacteria or putative *Pseudomonas* spp. aftershock, respectively. Average of biological triplicate survival rates are also shown with standard deviations as error bars.

5. Survival and electro-transformation rates of rain bacteria

To simulate lighting discharges, 12.5 $kV.cm^{-1}$ electroporations were conducted on aliquots of concentrated rainwater before plating on LBC and KBC, as well as LBCTK and KBCTK. This aimed to evaluate their survival potential on one hand and their potential to acquire DNA by electrotransformation on the other (“Ss” samples *i.e.* with electrical shock and with plasmid).

Survival rate

The number of cultivable bacteria growing on LBC after the electrical discharge ranged from 4.40×10^3 (first rain event) to 2.77×10^4 $CFU.L^{-1}$ of rainwater (second rain event) whereas the number of putative *Pseudomonas* spp. varied from 5.75×10^2 (first rain event) to 2.24×10^3 $CFU.L^{-1}$ of rainwater (second rain event) (Table II-4; Figures 4a, b & c and 5a, b & c in Appendix II-4).

Survival percentages were calculated as indicated in the Experimental procedures section. Those of total culturable rain bacteria in rains 1, 2 and 5, after application of the electrical discharge, amounted to 29.73%, 60.20% and 32.66% respectively, corresponding to an average of 40.86% with a standard deviation of 13.73% (Figure II-3 bar “Survival rate of total cultivable bacteria”).

Putative *Pseudomonas* spp. survival rates were 40.78%, 58.57% and 26.52% for rains 1, 2 and 5, respectively, corresponding to an average of 41.96% with a standard deviation of 13.11% (Figure II-3, bar “Survival rate of bacteria able to grow on KBC”).

Electrotransformation rate

Total bacteria from the shocked samples with pBLN (“Ss”) were isolated on LBCTK from rain 1 (8.80 $CFU.L^{-1}$) and rain 2 (27.20 $CFU.L^{-1}$), leading to an average concentration for the three rain events, of 12 $CFU.L^{-1}$ of rainwater (Table II-4; Figure 6a, b & c in Appendix II-4).

No *Pseudomonas*-like bacteria from rain events 1 and 5 were able to grow on KBCTK after being shocked with pBLN, whereas 4 $CFU.mL^{-1}$ of 5000 fold concentrated rainwater were detected in the rain episode 2, corresponding to a final concentration of 0.8 $CFU.L^{-1}$ of rain 2 (Table II-4; Figure 7a, b & c in Appendix II-4). The only colony isolated corresponds to bacterium **R2SsM2P1C1** (Appendix II-1).

Table II-5: Rates of total culturable bacteria and putative *Pseudomonas* naturally resistants to tetracycline and kanamycin, naturally transformed and electro-transformed by pBLN for each rain events

Rain episode	Growth medium	Natural resistance rate (R)	Natural transformation rate (N)	Electrotransformation rate (E)
R1	LBCTK	2.70×10^{-4}	1.14×10^{-3}	2.00×10^{-3}
	KBCTK	0.00×10^{0}	0.00×10^{0}	0.00×10^{0}
R2	LBCTK	0.00×10^{0}	8.43×10^{-4}	9.81×10^{-4}
	KBCTK	0.00×10^{0}	0.00×10^{0}	3.58×10^{-4}
R5	LBCTK	0.00×10^{0}	0.00×10^{0}	0.00×10^{0}
	KBCTK	0.00×10^{0}	0.00×10^{0}	0.00×10^{0}

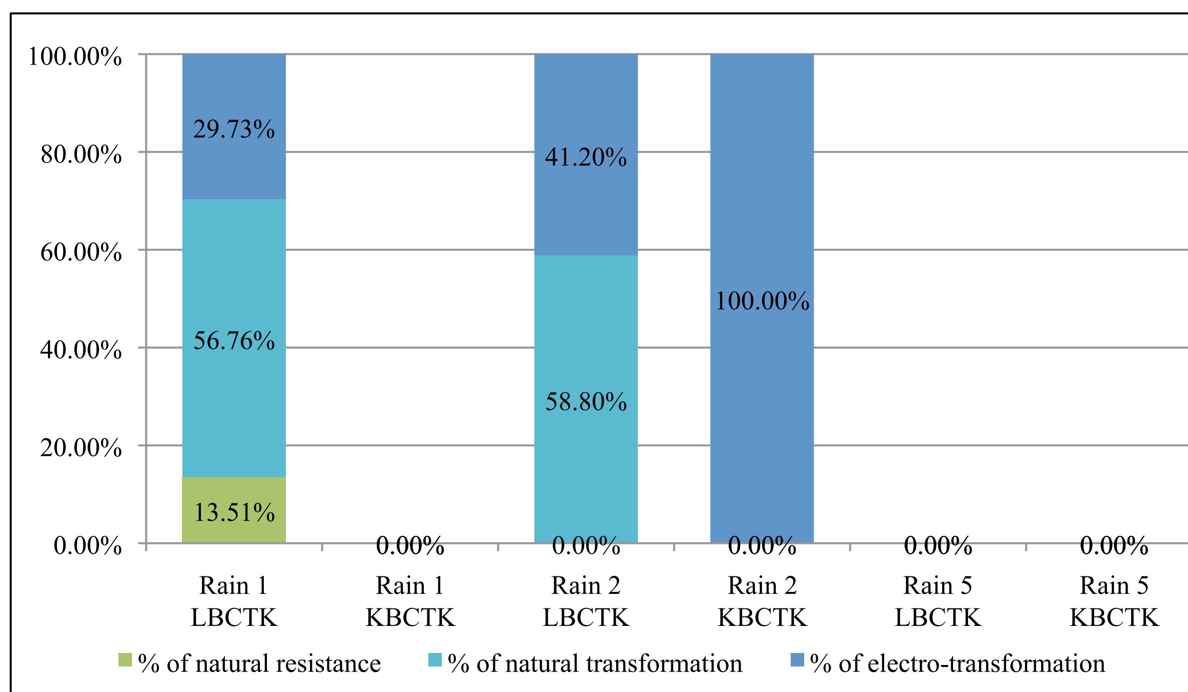


Figure II-4: Distribution in percentages of the three types of resistance toward tetracycline and kanamycin among culturable bacteria and putative *Pseudomonas* of the rain events 1, 2 and 5 isolated on LBCTK and KBCTK.

After electroporation and whenever possible, triplicate colonies according to phenotype of both rain event 1 and 2 were sampled and *linA* was amplified by PCR to ascertain the presence of pBLN. The proportions of colonies sampled as well as percentages of positive strains for *linA* amplification are listed in the Table II-3 (Lanes "Ss"). All isolates tested from either total culturable bacteria (LBCTK medium) or putative *Pseudomonas* spp. (KBCTK medium) were *linA*+ and appeared to be able to acquire pBLN.

The electrotransformation rates of culturable bacteria isolated on LBCTK then calculated with the proportion of *linA*+ isolates (according to the experimental procedure calculations) reached 2.00×10^{-3} and 9.81×10^{-4} for the first and second rain events, respectively (Table II-5).

The corresponding electrotransformation rate of the only colony isolated on KBCTK from rain 2 reached 3.58×10^{-4} (Table II-5, Figure II-4).

6. Contribution of electrotransformation to rain bacteria antibiotic-resistance

To be able to compare the relative contribution of each antibiotic-resistance mechanism within the three studied rain events bacteria, the electrotransformation rate was calculated anew using the total number of unshocked culturable bacteria as indicated in Experimental procedures section. Percentages of each type of resistance toward tetracycline and kanamycin were then calculated among the culturable bacteria isolated on LBCTK for the two first rain events (Figure II-4).

Bacteria sampled from either the first or second rain event, and able to grow on LBCTK comprised a majority of naturally transformed bacteria (56.76% and 58.80% respectively) whereas natural resistant bacteria were considerably less represented and only among rain 1 LBCTK isolates (13.51%). The rest of resistant bacteria correspond to electrotransformation: 29.73% of rain event 1 and 41.20% of rain event 2 LBCTK isolates acquired pBLN electrically while 100% did on KBCTK (rain episode 2 only). As already shown, none of the bacteria isolated from the fifth rain event appeared to be able to possess or acquire TK resistance.

Table II-6: Number of colonies sampled and SSU rDNA sequences obtained among rain events, sample treatments and growth media

Rain episode	Sample[a]	Medium	Number of colonies sampled/ Total CFU number	Number of sequences obtained	% of sequenced colonies
R1	Sp	LBCTK	7/21	5	23.81%
		KBC	18/2116	13	0.61%
	Ss	LBCTK	7/11	6	54.55%
		KBC	34/863	24	2.78%
R2	Sp	LBCTK	15/52	13	25.00%
		KBC	25/5730	19	0.33%
	Ss	LBCTK	16/34	13	38.24%
		KBC	30/3356	19	0.57%
		KBCTK	1/1	1	100%
R3	Sc	KBC	26/176	20	11.36%
R4	Sc	KBC	9/27	3	11.11%
R5	Sp	KBC	15/3032	11	0.36%
	Ss	KBC	27/804	10	1.24%

[a] "Sc": rain bacteria; "Sp": rain bacteria incubated with pBLN; "Ss": rain bacteria incubated with pBLN and submitted to a 12.5kV.cm^{-1} electrical discharge

7. Taxonomic characterization of rain isolates

SSU rDNA sequencing

When possible, three colonies per phenotype growing on KBC, LBCTK and KBCTK for all rain events were analyzed. Of the 230 colonies sampled for SSU rDNA gene amplification and sequencing, only 157 good quality near full-length sequences were obtained (68.26% of amplifications sent to the sequencing compagny). Numbers of colonies sampled and sequences obtained per condition are listed in the Table II-6 together with the resulting percentages of colonies sequenced.

Between 23.81% (first rain event, sample "Sp") and 54.55% (first rain event, sample "Sp") of colonies grown on LBCTK were sequenced (Table II-6). The SSU rDNA sequence of the only colony grown on KBCTK after being shocked with pBLN for the rain 2 was sequenced. For colonies grown on KBC in the "Sp" and "Ss" samples (rain 1, 2 and 5), triplicate phenotypes sampled represented between 0.33% (second rain event, sample "Sp") and 2.78% (first rain event, sample "Sp") of the total colonies grown on agar plates (Table II-6). Between 11.11 and 11.36% of colonies grown on KBC for the "Sc" sample (rain 3 and 4 respectively) were sequenced (Table II-6).

Taxonomic analysis

As one of the primary aims of this work was to study bacteria able to uptake exogenous DNA, the *linA* negative isolate (**R2SpM1P1C14**) was not included in the phylogenetic analysis. The RDP online SSU rDNA classifier identified three major taxonomic groups among the 156 sequenced genes. Eight isolates clustered within the *Escherichia-Shigella* lineage in the *Enterobacteriaceae* (*Gamma-proteobacteria*), another eight isolates grouped with *Microbacterium* and *Plantibacter* spp. within the *Microbacteriaceae* (*Actinobacteria*), and as expected considering our methodological procedure, most isolates (140) belonged to the *Pseudomonas* genus within the *Pseudomonadales* (*Gamma-proteobacteria*).

BLAST on NCBI as well as the SeqMatch function on RDP identified all of the assembled contigs with a minimum similarity score of 98% on both databases. Further taxonomic classification was based on phylogenetic analyses of the SSU rDNA sequences using reference type species belonging to each of the three lineages characterized in our

isolate samples (Tables 1 to 3 in Appendix II-3). Reference species closest to each rain isolate are listed in Table 1 of Appendix II-5.

***Escherichia-Shigella* rain isolates**

The phylogenetic comparison of the eight isolates belonging to the *Escherichia-Shigella* lineage to 106 *Enterobacteriaceae* reference species (Table 2 in Appendix II-3) showed that the rain isolates are *E. coli* strains most closely related to strains SE11, SMS-3-5 and UMN026 in our analysis (Figure II-5; Table 1 in Appendix II-5).

All strains but one were isolated from rain episode 1 (orange/red-coded), the other one being recovered from rain event 2 (green-coded). All are able to uptake the pBLN plasmid directly or after electroporation (growing on LBCTK medium).

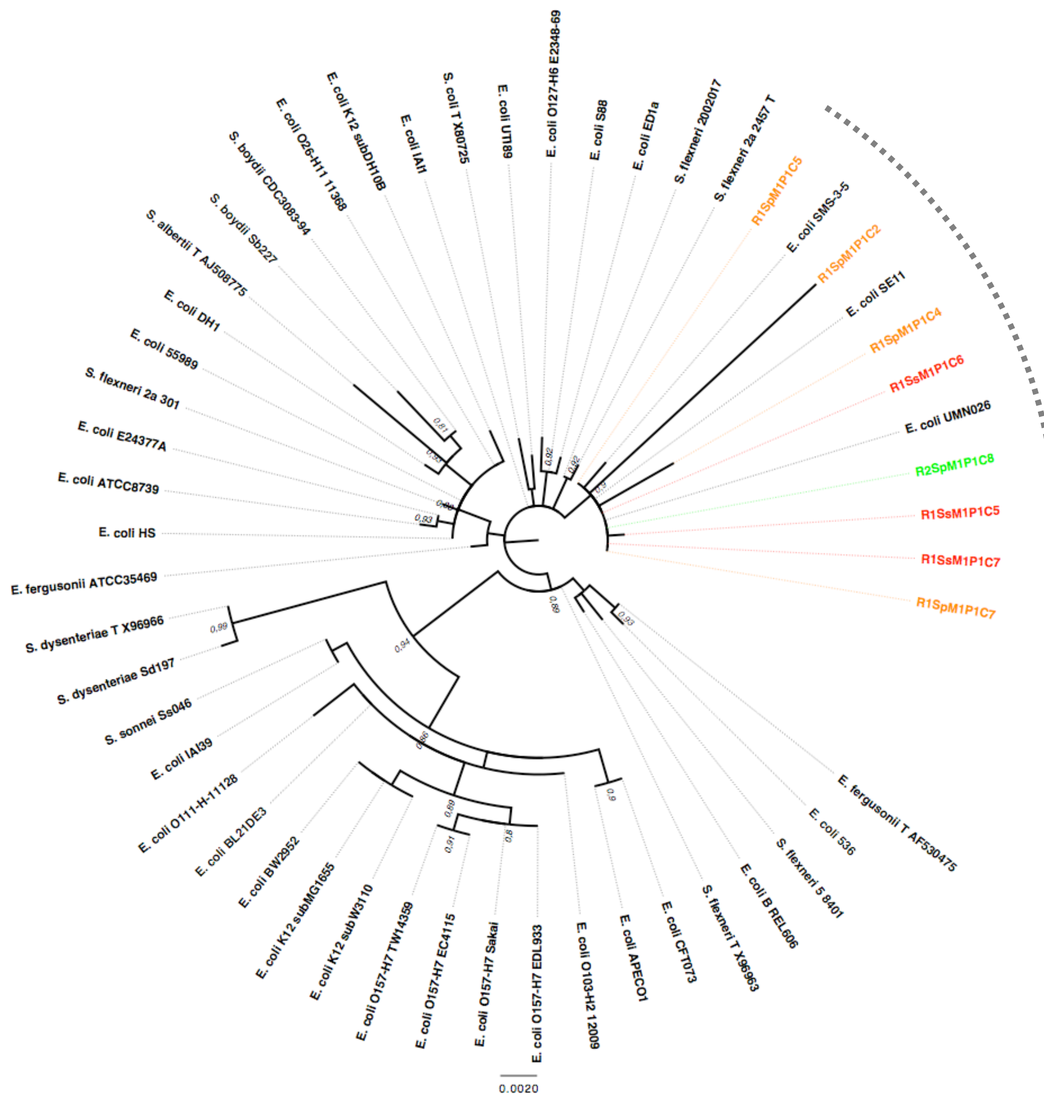


Figure II-5: Phylogeny of rain isolates belonging to the *Escherichia* genus.
Ln Likelihood = -6158.070607. *Enterobacteriaceae* used as outgroups are not shown for the sake of clarity. Branch supports >0.80 (used as significance threshold) are indicated. The scale bar corresponds to the number of substitutions per nucleotidic site.

***Microbacteriaceae* rain isolates**

Among the *Microbacteriaceae* rain isolates, one belonged to the *Plantibacter* genus whereas the other seven belonged to the *Microbacterium* genus (Figure II-6; Table 1 in Appendix II-5). No single reference species could be identified as the closest to any of the rain isolates.

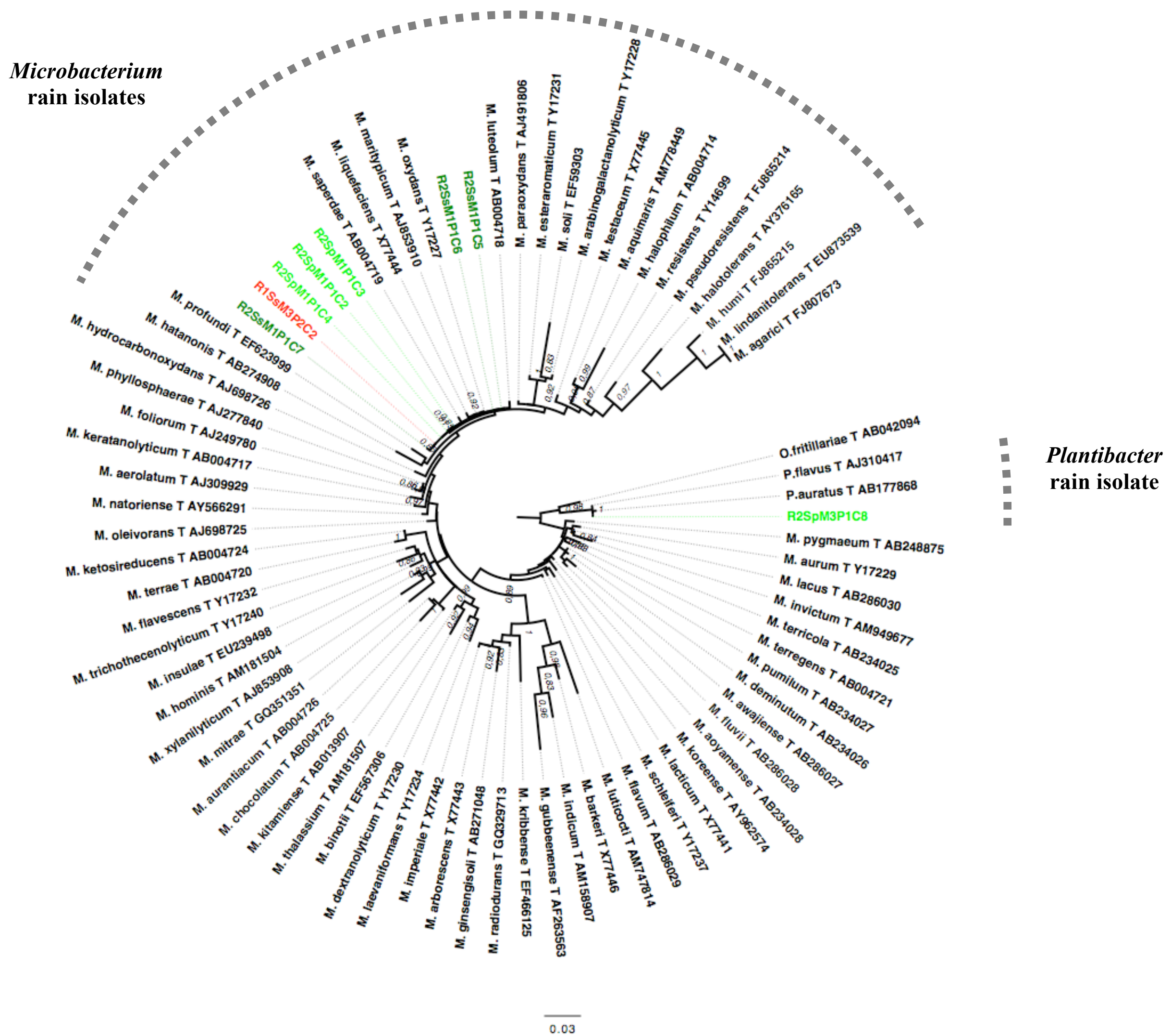


Figure II-6: Phylogeny of *Microbacter* and *Plantibacter* rain isolates.
Ln Likelihood = -18416.203350. The *Microbacteriaceae* used as outgroups are not shown for the sake of clarity. Branch supports >0.80 (used as significance threshold) are indicated. The scale bar corresponds to the number of substitutions per nucleotidic site.

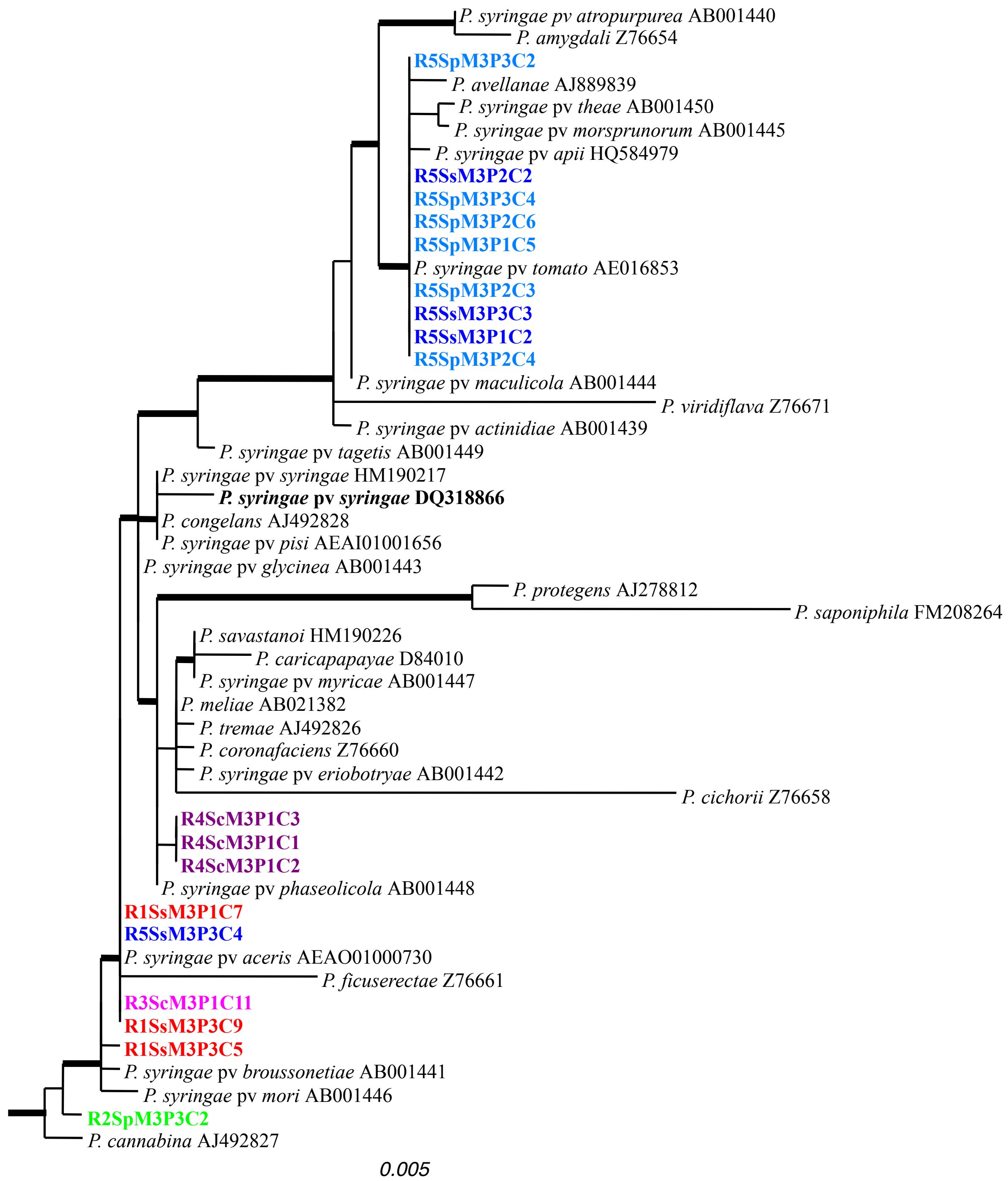


Figure II-7: Phylogeny of the rain isolates belonging to the *Pseudomonas syringae* group, extracted from the global phylogeny (Appendix 6).

Thick lines indicate branch supports > 0.80 (used as significance threshold). The type species of the group appears in bold. The scale bar corresponds to the number of substitutions per nucleotidic site.

All but one *Microbacteriaceae* strains were recovered from rain episodes 2 (green-coded). One isolates of each rain event (1 and 2) grew on putative *Pseudomonas*-specific KBC medium (**R1SsM3P2C2** and **R2SpM3P1C8**, respectively). All other strains isolated from rain event 2 were able to uptake the pBLN plasmid directly or after electroporation (growing on LBCTK medium).

***Pseudomonas* rain isolates**

Most of the rain bacteria isolated from the five rain events (LBCTK, KBCTK or KBC isolates), grown directly or after incubation with pBLN with or without electrical discharge (Samples “Sc”, Sp” or “Ss”) were representatives of the genus *Pseudomonas*. Four groups (defined by Mulet *et al.*, 2010) dominated the rain isolates (Figure 1 in Appendix II-6). Indeed, all but two isolates distributed across *P. syringae* group (Figure II-7), *P. lutea* and *P. rhizosphaerae* groups (Figure II-8), and *P. fluorescens* group (Figure II-9). The reference species closest to each of the rain isolates are listed in Table 1 in Appendix II-4.

The *P. syringae* phylogenetic tree (Figure II-7) contains bacteria from the five rain events isolated on KBC medium with or without electrical shock (Samples “Sc”, “Sp” and “Ss”). *P. syringae* was the sole taxon represented among rain 4 isolates (Figure II-7), whereas rain 5 isolates belonged to *P. syringae* and *P. lutea* group (Figures II-7 and II-8).

The rain isolates represented in the *P. lutea* and *P.rhizosphaerae* groups (Figure II-8) were isolated from rain episodes 1, 2, 3 and 5 on LBCTK, KBCTK or KBC media.

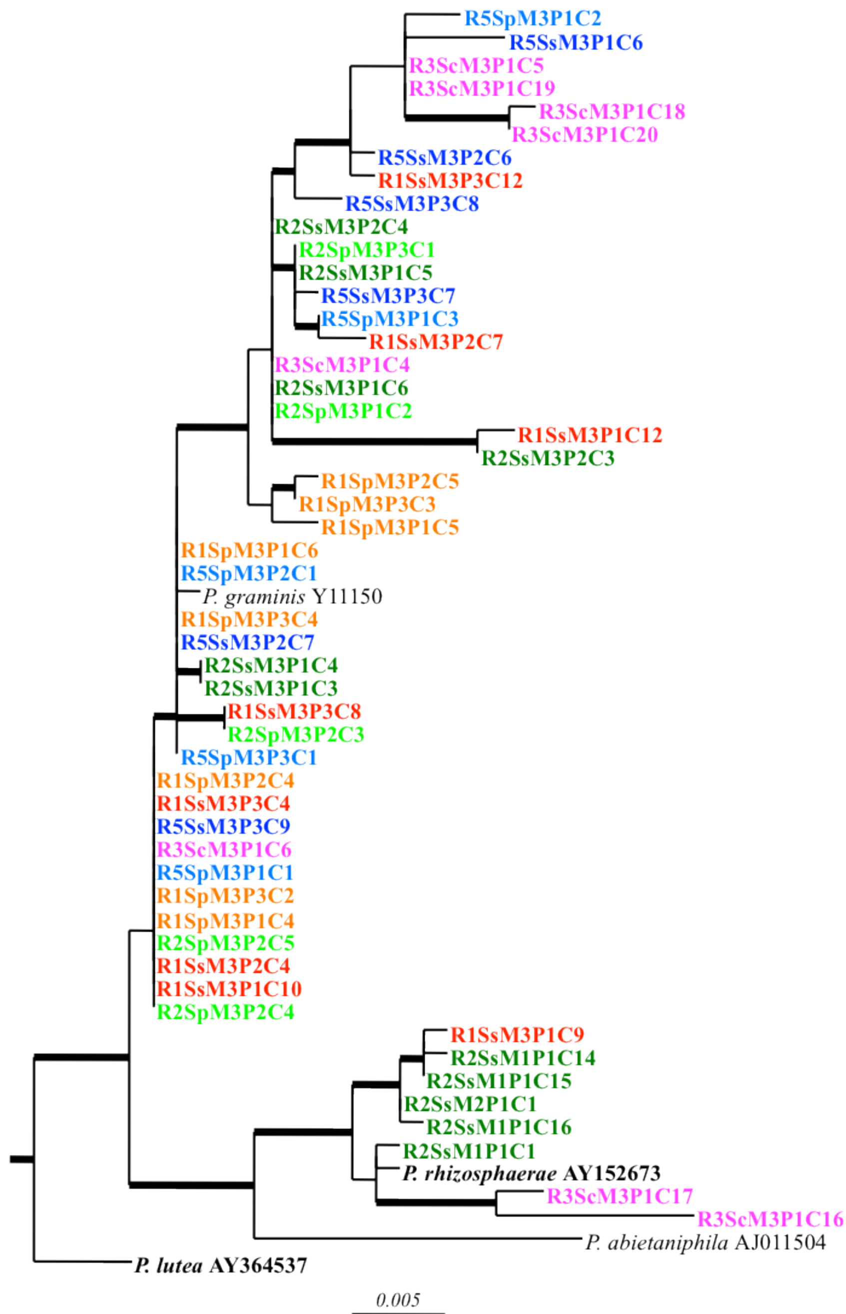


Figure II-8: Phylogeny of the rain isolates belonging to the *Pseudomonas lutea* / *P. rhizosphaerae* groups, extracted from the global phylogeny (Appendix 6).

Thick lines indicate branch supports > 0.80 (used as significance threshold). The type species of each group appears in bold. The scale bar corresponds to the number of substitutions per nucleotidic site.

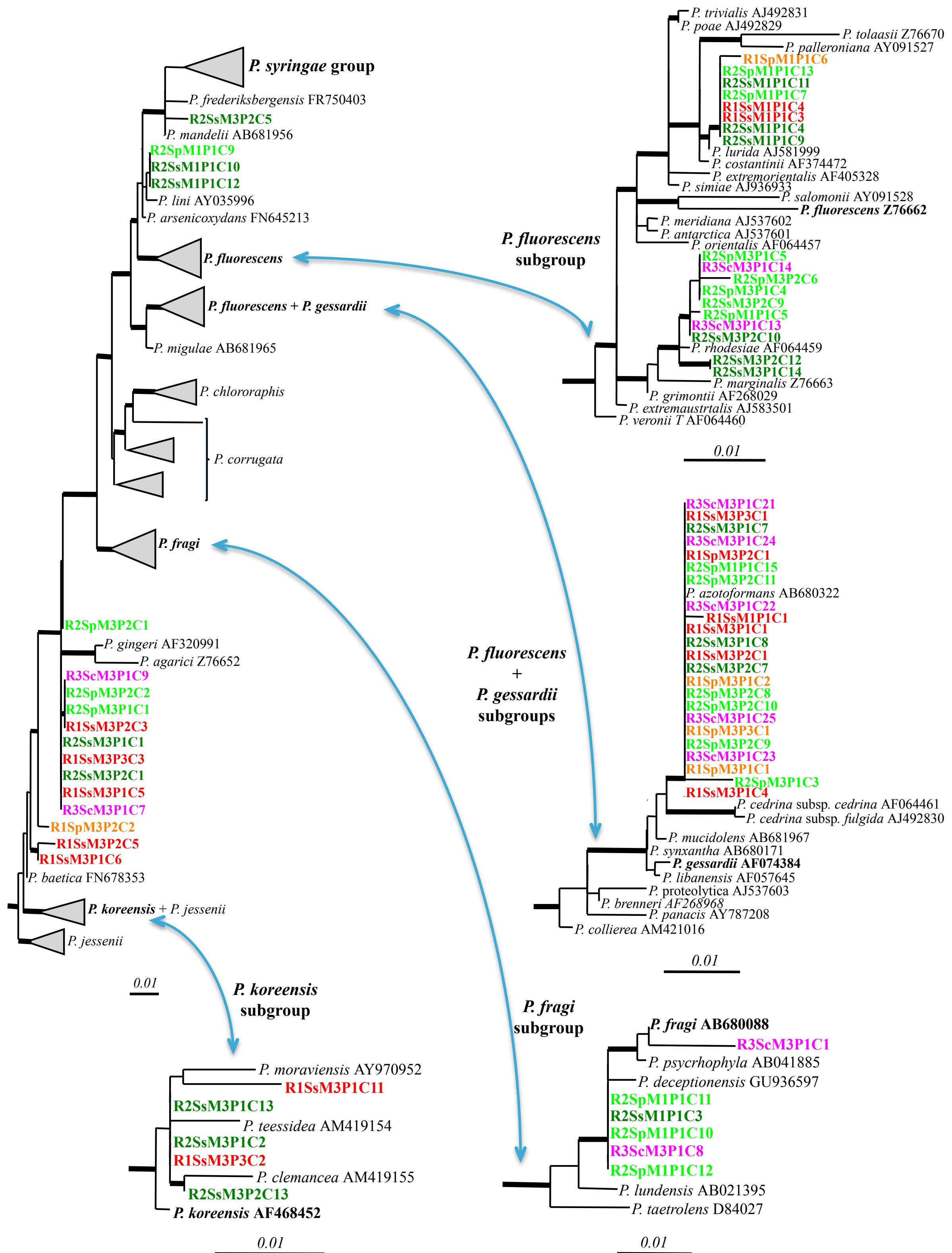


Figure II-9: Phylogeny of the rain isolates belonging to the *Pseudomonas fluorescens* group, extracted from the global phylogeny (Appendix 6).

Subtrees of each *P. fluorescens* subgroups containing rain isolates are shown. Thick lines indicate branch supports > 0.80 (used as significance threshold). The type species of each group/subgroup appears in bold. The scale bar corresponds to the number of substitutions per nucleotidic site.

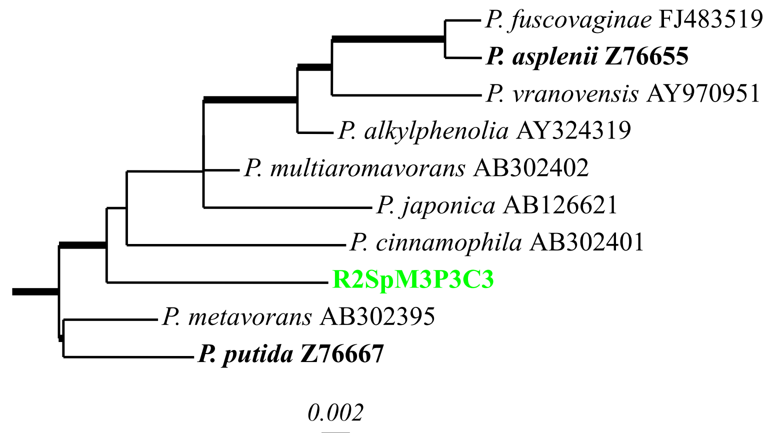


Figure II-10: Phylogeny of the rain isolates belonging to the *Pseudomonas putida* group / *P. fluorescens* group (*P. asplenii* subgroup).
Thick lines indicate branch supports > 0.80 (used as significance threshold). The type species of each group/subgroup appears in bold. The scale bar corresponds to the number of substitutions per nucleotidic site.

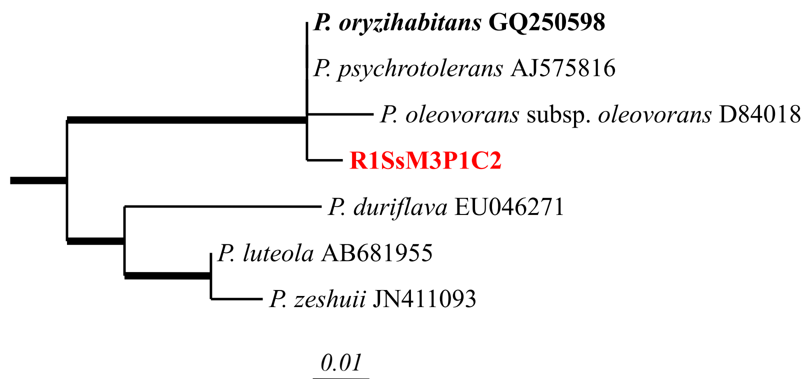


Figure II-11: Phylogeny of the rain isolates belonging to the *Pseudomonas orizyhabitans* group.
Thick lines indicate branch supports > 0.80 (used as significance threshold). The type species of each group/subgroup appears in bold. The scale bar corresponds to the number of substitutions per nucleotidic site.

Most of the bacteria from rain episodes 1, 2 and 3 isolated on LBCTK or KBC media directly or after an electrical shock (Samples “Sc”, “Sp” and “Ss”) clustered within the *P. fluorescens* groups and subgroups (Figures II-9 and II-10).

Furthermore, the two additional lineages, *P. putida* group / *P. asplenii* subgroup within *P. fluorescens* group (Figure II-10) and *P. oryzihabitans* group (Figure II-11), were represented by one isolate each, from rain 2 and 1 respectively.

Natural and electrically induced transformants (LBCTK or KBCTK media / “Sp” or “Ss” isolates) were among the *P. fragi, P. fluorescens and P. gessardi* subgroups within the *P. fluorescens* group (Figure II-9), whereas the *P. rhizosphaerae* group (LBCTK or KBCTK media / “Ss” isolates) contained only electrically induced transformants (Figure II-8).

However, no transformants (only KBC growing bacteria) were among the recovered *P. syringae* isolates (Figure II-7), the isolates in *P. koreensis* subgroup within the *P. fluorescens* group (Figure II-9), *P. putida* group / *P. asplenii* subgroup within *P. fluorescens* group (Figure II-10, one isolate) and the *P. oryzihabitans* group (Figure II-11, one isolate).

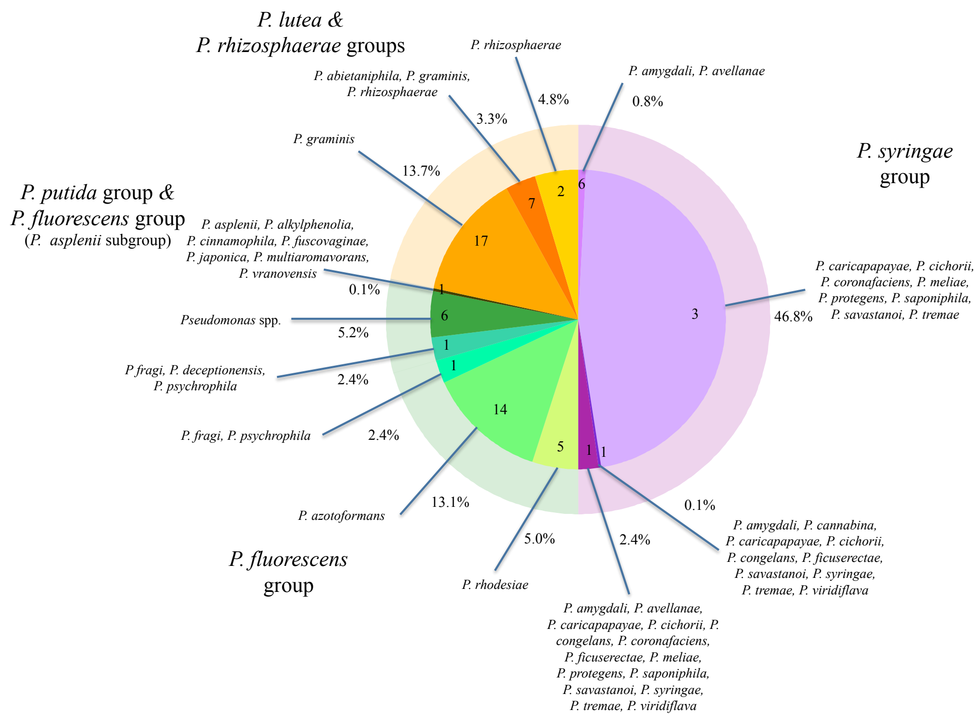


Figure II-12: Representation of *Pseudomonas* species among combined rain isolates.

Isolates grown on KBC medium ("Sc"sample for rain events 3 and 4 and "Sp"sample for rain episodes 1, 2 and 5). Number of isolates for each phylogenetic lineage are given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

8. Taxonomic distribution of bacteria in rain samples

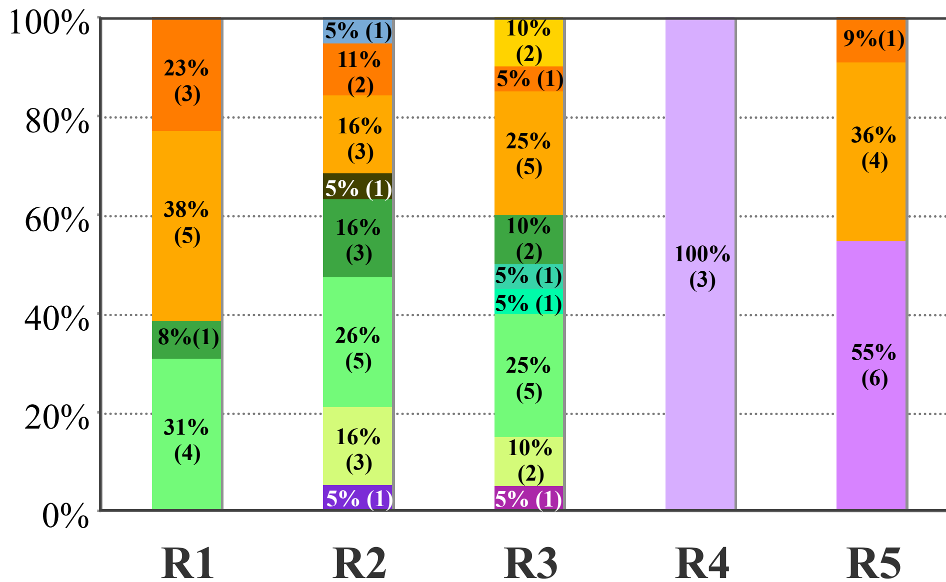
The taxonomical distribution of bacteria has been studied for each rain event, before and after shocks for bacteria that have acquired the pBLN plasmid by natural or electrotransformation and putative *Pseudomonas* isolated on KBC. Distributions of the isolates across the phylogenetic lineages were calculated after sample size correction of absolute counts per rain event, treatment and growth medium as described in the experimental procedure. These distributions are described and illustrated by graphs in the following section.

Diversity of rain putative <u>Pseudomonas</u>

Ninety-eight per cent (65/66) of the bacterial strains isolated on KBC medium ("Sc" for rain episodes 3 and 4, and "Sp" for rain episodes 1, 2 and 5), *i.e.,* all but one (*Plantibacter sp.* - Table 1 in Appendix II-5), belonged to the *Pseudomonas* genus (Figure II-12). After normalization to correct for the differing sizes of the samples, half of the isolates belonged to *P. syringae* lineage (50.1%) whilst *P. fluorescens* and *P. lutea* lineages each were represented by about one fourth of the isolates (28.1% and 21.9% respectively).

This distribution may however be biased toward *P. syringae* through a sampling effect related to the very small size of the sample recovered from rain event 4 (3 isolates, all of which belong to the *P. syringae* lineage) (Figure II-13).

Indeed, when the two smaller samples, rain episodes 3 and 4, are omitted, the *P. syringae* is the less represented lineage (16.7%) wheras *P. lutea*/*P. rhizosphaerae* lineages becomes the most abundant (48.8%) with *P. fluorescens* lineage being in the same range (33.2%) (Figure II-14).



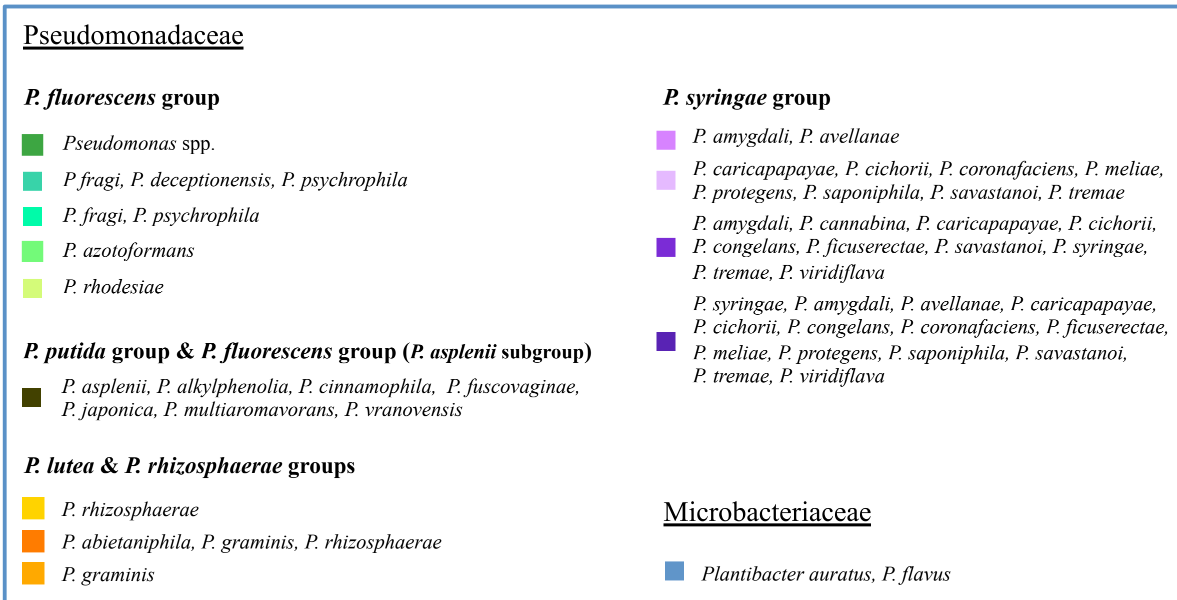


Figure II-13: Representation of *Pseudomonas* species among isolates from each rain episode.
Isolates grown on KBC medium ("Sc" sample for rain events 3 and 4 and "Sp" sample for rain events 1, 2 and 5). Lineages are colored according to phylogenetic clustering data. Percentages and number of isolates (into brackets) for each phylogenetic lineage are given.

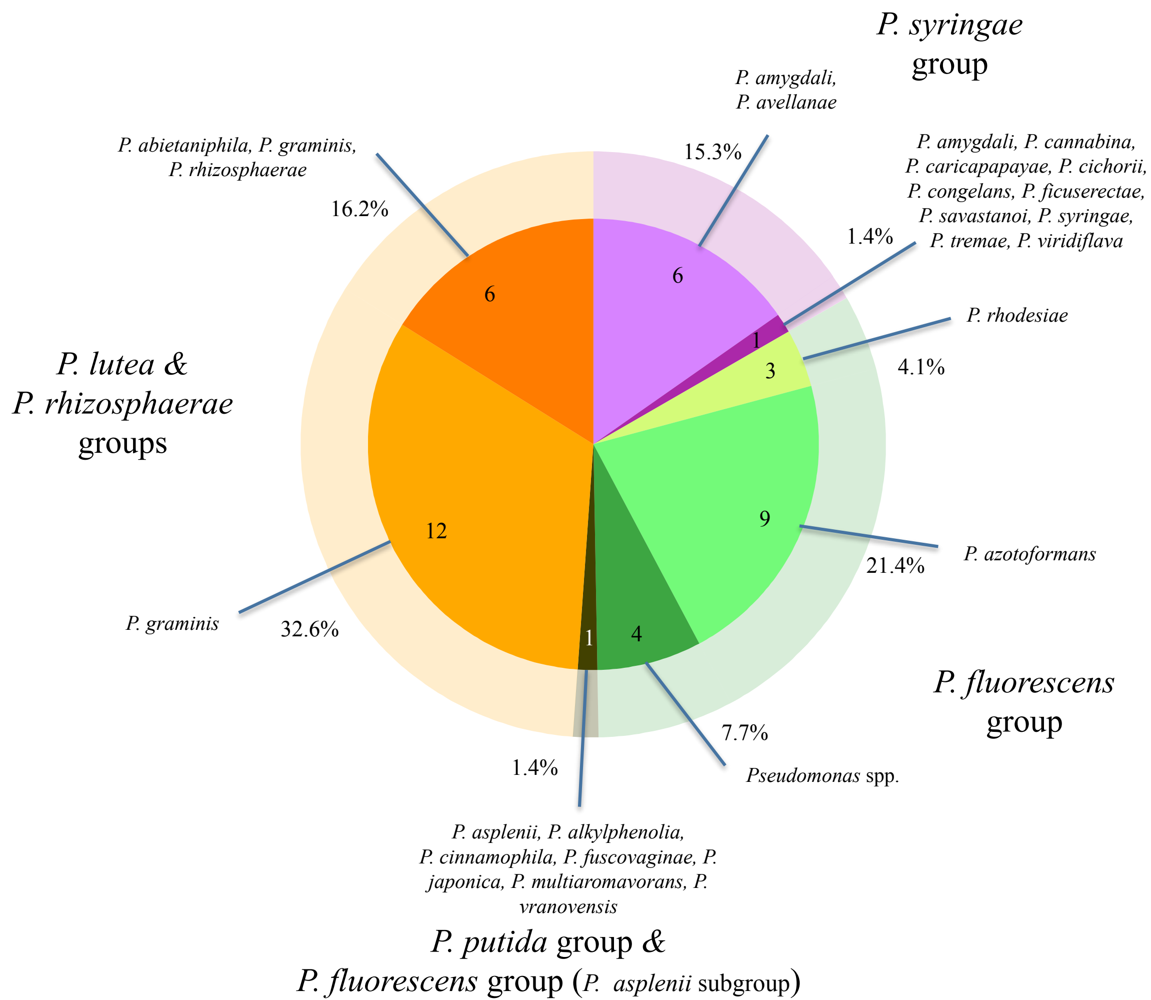


Figure II-14: Representation of *Pseudomonas* species among isolates from combined rain episodes 1, 2 and 5.
Isolates grown on KBC medium ("Sp" samples). Number of isolates for each phylogenetic lineage are given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

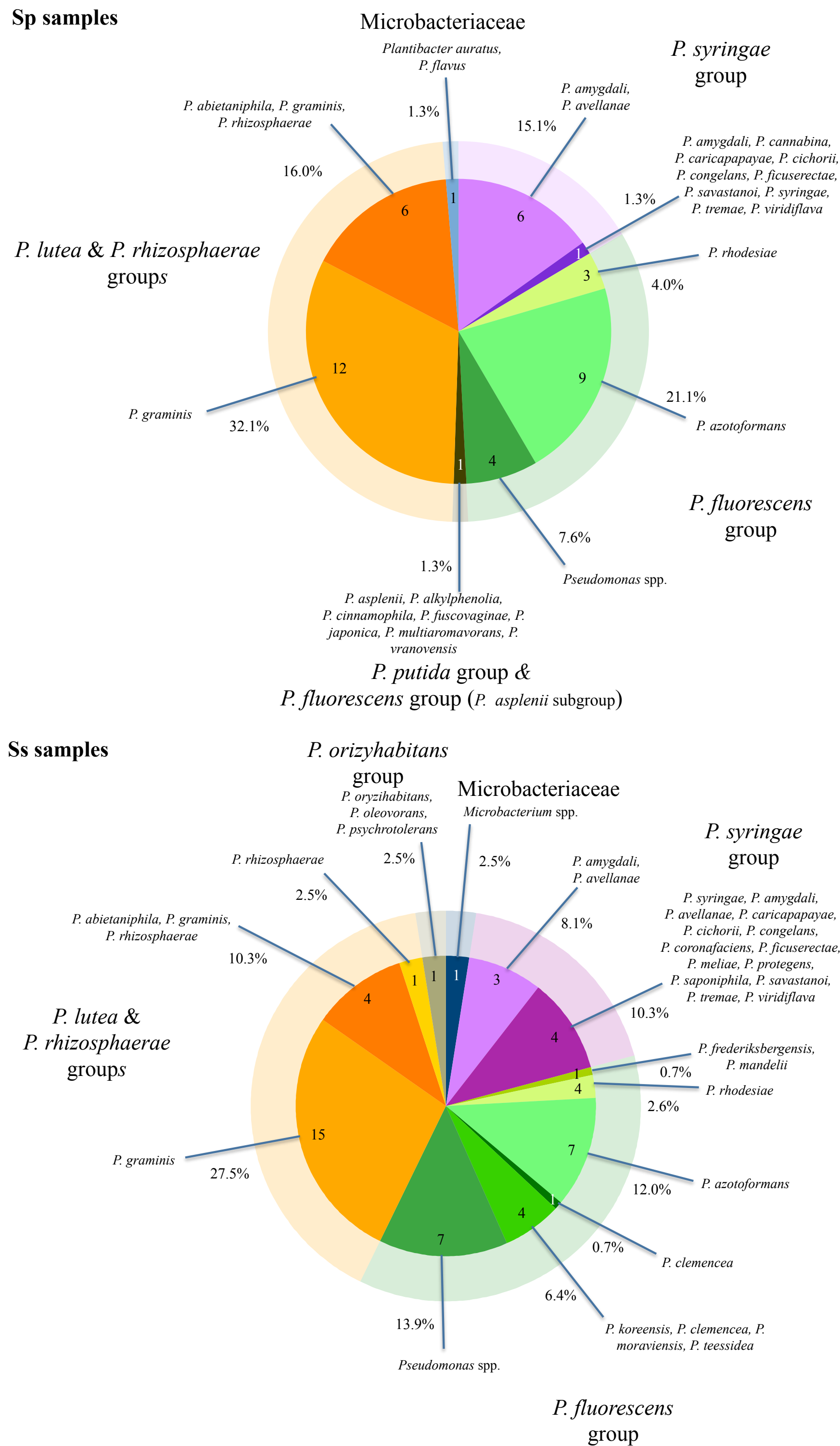


Figure II-15: Overall representation of the different lineages identified among isolates from rain episodes 1, 2 and 5 incubated with pBLN without shock ("Sp" samples) and those submitted to an electric pulse ("Ss" samples).

Isolates grew on KBC medium. Number of isolates for each phylogenetic lineage are given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

The comparison of the KBC-bacterial distribution of rain episodes 1, 2 and 5 incubated with pBLN without electric shock ("Sp" samples) and those submitted to an electric pulse ("Ss" samples) is presented in Figure II-15. From the 53 bacteria isolated on KBC from the three rain events ("Ss" samples), only one did not belong to the *Pseudomonas* genus and was instead affiliated with *Microbacterium* spp. (Table 1 in Annexe 4).

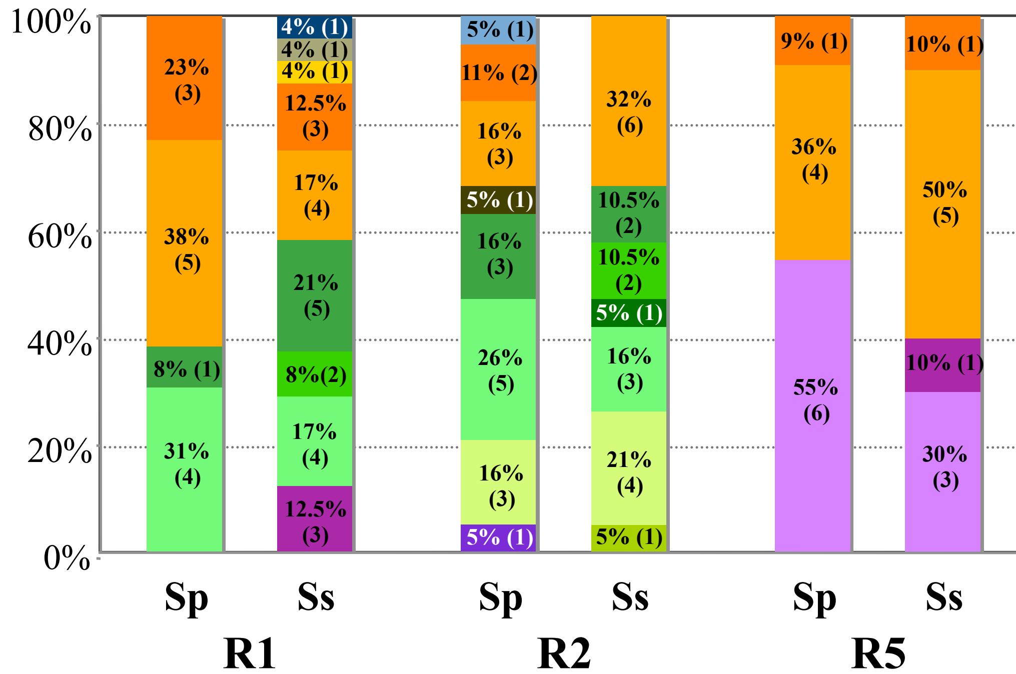
The distribution of the isolates across the main phylogenetic lineages varied little from that observed in the absence of such treatment, with the proportions of *P. syringae*, *P. fluorescens*, and *P. lutea/rhizosphaerae* groups changing from 16% to 18.4%, 31.9% to 36%, 46.9% to 40.3%, respectively. However, a higher taxonomic diversity was revealed following the electric shock and is most noticeable within the *P. fluorescens* and *P. lutea* & *P. rhizosphaerae* lineages.

The same analyses of distribution have been calculated for rain events with or without shock separately to see whether the distribution and response to electrical shock differed between rain events. These data are shown in Figure II-16. The electric shock revealed a higher taxonomic diversity in rain events 1 and 5 while the number of lineages identified decreased in the rain episode 2 aftershock.

Diversity of naturally and electro-transformed antibiotic-resistant bacteria

The distribution of naturally transformed culturable bacteria was analyzed according to phylogenetic results (Figure II-17). *E. coli* was as equally represented as *Pseudomonas* spp. among the natural transformants (45% *versus* 43%, respectively) (Figure II-17) and a lower but significant part of the transformants recovered after electroporation (Figure II-18). In both cases, *Microbacterium* spp. represented about 10% of the isolates. Interestingly, members of the *P. lurida* lineage were recovered here despite their absence from KBC-grown samples distribution.

The same analyses of distribution have been assessed for rain events with or without shock separately (Figure II-19). The main difference between natural and electroporated transformants was characterized by an apparent enrichment in *Pseudomonas* strains. As for KBC-growing samples, electroporation resulted in a somewhat higher *Pseudomonas* phylogenetic diversity characterized by the presence of *P. rhizosphaerae* (Figures 18 and 19).



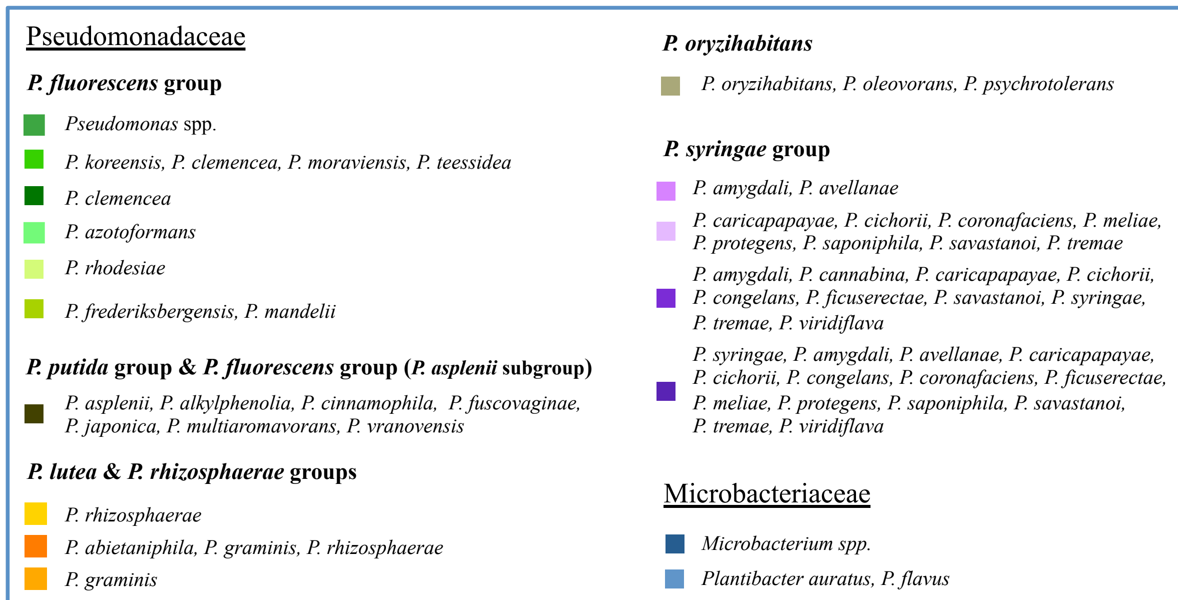


Figure II-16: Representation of the different lineages identified among isolates from rain episodes 1, 2 and 5, respectively, incubated with pBLN only ("Sp" sample) or submitted to an electric pulse ("Ss" samples).

Isolates were grown on KBC medium. Number of isolates for each phylogenetic lineage is given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

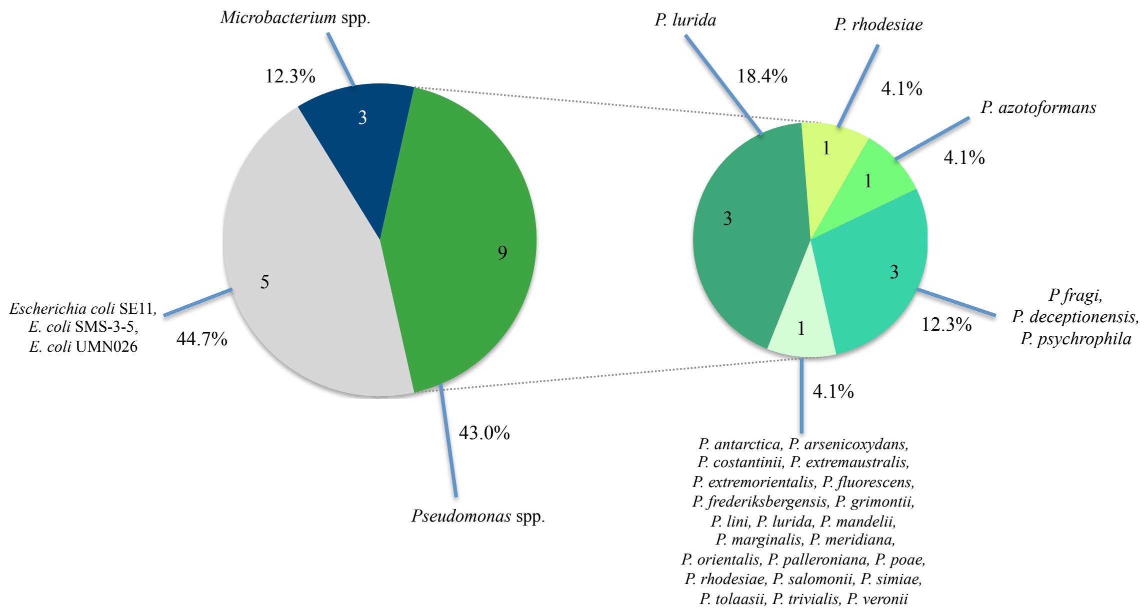


Figure II-17: Distribution of combined rain 1 and 2 natural transformants ("Sp" samples) isolated on LBCTK medium.

Isolates grew on LBCTK medium. Number of isolates for each phylogenetic lineage is given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

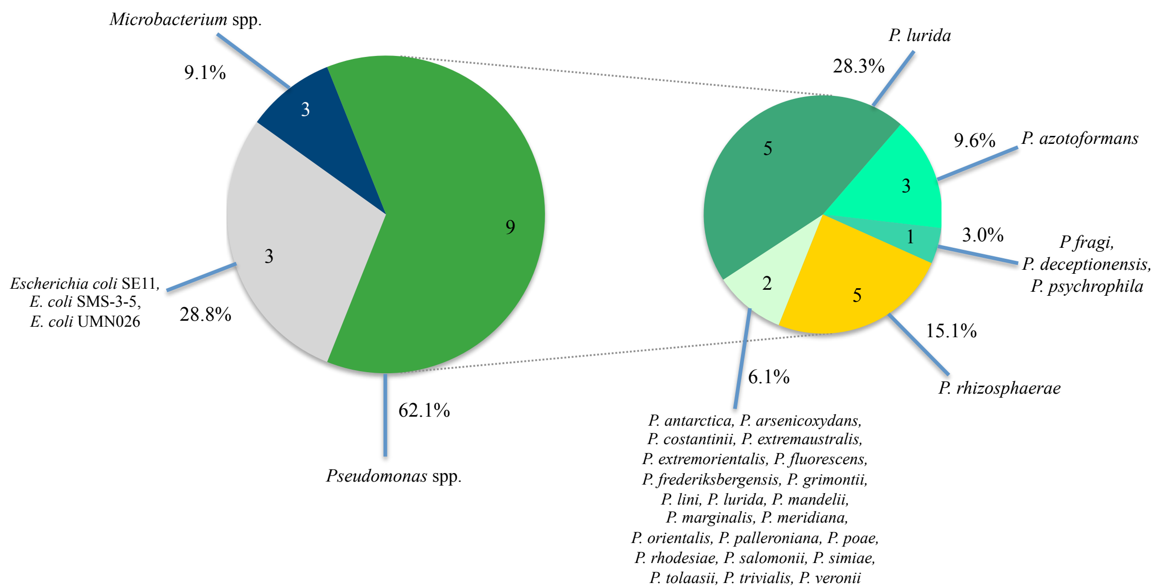


Figure II-18: Distribution of combined rain 1 and 2 electroporated transformants ("Ss" samples) isolated on LBCTK and KBCTK media.

Isolates grown on LBCTK medium. Number of isolates for each phylogenetic lineage is given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

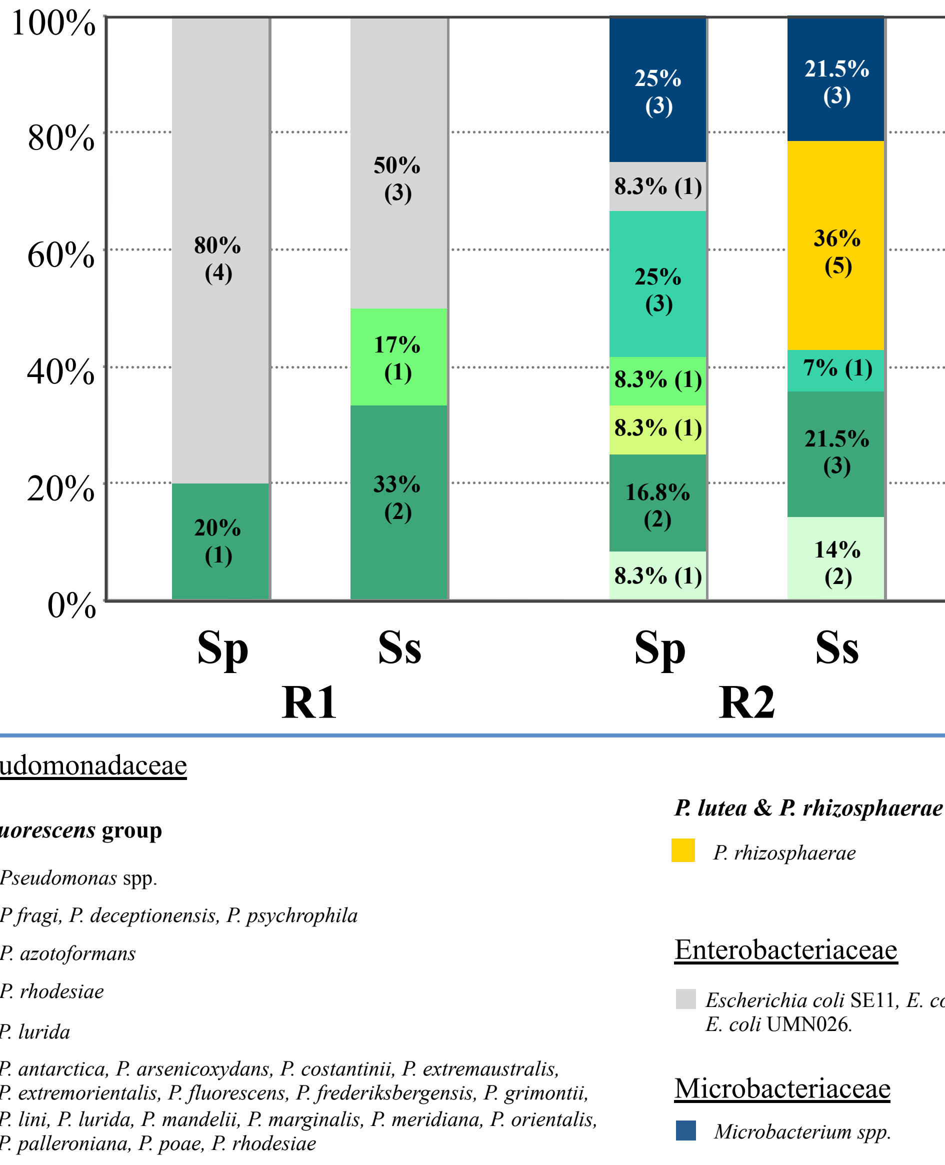


Figure II-19: Representation of the different lineages identified among transformants from rain episodes 1 and 2, respectively, incubated with pBLN without shock ("Sp" samples) or submitted to an electric pulse ("Ss" samples).
Isolates grown on LBCTK or KBCTK media. Number of isolates for each phylogenetic lineage is given, as well as the percentages calculated after sample size correction. Lineages are colored according to phylogenetic clustering data.

DISCUSSION:

Fluxes of bacteria from Earth surfaces (including soil, water and plants) to clouds result from aerosolization (Lighthart, 1997; Dehel *et al.*, 2008; Delort *et al.*, 2010; Morris *et al.*, 2011). The mean global emission of bacteria from terrestrial surfaces ranges from 140 to 380 CFU per square meter per second (Burrows *et al.*, 2009), only a small fraction of which reach the free atmosphere alive (Kellogg and Griffin, 2006; Šantl-Temkiv *et al.*, 2012). So far, most studies have focused on the relationship between the presence of bacteria in clouds and geochemical processes; few have addressed the impact of cloud journeying on bacteria life cycles. Cloud bacteria are able to resist UV radiation, desiccation and high oxidative conditions or even to grow actively (Sattler *et al.*, 2001) and to degrade chemical compounds in clouds (Delort *et al.*, 2010; Vaïtilingom *et al.*, 2010; Vaïtilingom *et al.*, 2011; Vaïtilingom *et al.*, 2013). They are also submitted to electrical field pulses (fews $kV.cm^{-1}$) associated with lightning currents during lightning flashes (Nucci *et al.*, 1988). Furthermore, some bacteria that possess ice nucleation abilities are involved in the triggering of lightning (Gonçalves *et al.*, 2012). The bacteria able to survive such a harsh physical and chemical environment may thus possess an important adaptive potential. For example, antibiotic-resistant bacteria could reach clouds and be selected for either by passing through the harsh conditions of aerosolization or by the presence of chemical compound (Baker-Austin *et al.*, 2006). Interestingly, despite leading to a decreased survival of bacteria, electric field pulses are also known to permeabilize bacterial membranes and thus to facilitate the penetration of naked DNA in the surviving cells and their acquiring of new properties (Demanèche *et al.*, 2001c).

In order to explore the hypothesis that rain bacteria have the potential to resist electric shocks related to lightning in clouds and develop adaptive strategies for living in this environment, we studied the transformation ability of bacterial communities isolated from five rain episodes, with a special emphasis on *Pseudomonas syringae,* a major plant pathogen and one of the most ice nucleate active bacterium (Morris *et al.*, 2008). We used rain bacterial communities as a proxy for cloud bacteria triggering precipitation. Indeed, the rain bacteria are most likely to be representative of the cloud communities since the rain scavenging of aerosols below clouds is very inefficient for particles smaller than several microns in diameter (McDonald, 1962; Respondek *et al.*, 1995). We assessed the ability of these rain bacteria to survive electrical shocks such as those encountered in clouds, and to

internalize and express a plasmid harboring two antibiotic resistance genes after electrotransformation. To ensure that laboratory-adapted strains would not be selected, all tests were performed prior to cultivation, directly on fractions of the bacterial communities collected during the rain episodes and concentrated. Cycloheximide-supplemented media were used in all culturing to avoid fungi and yeasts contaminations, since these are frequently isolated from airborne microbial communities (Lin *et al.*, 1999; Amato *et al.*, 2007a).

1. Concentration of culturable bacteria in rain

Culturable bacteria recovered from the five rain samples that were collected in April 2012, averaged to 2.27×10^4 $CFU.L^{-1}$. Considering the accepted 1% rate of culturable bacteria in atmosphere environments (Lighthart, 1997; Bauer *et al.*, 2002; Amato *et al.*, 2005; Amato *et al.*, 2007b), the total bacterial concentration would be 100 times higher, about 2×10^6 $cells.L^{-1}$, which is in the range of the observed 10^6 to 10^7 total bacterial cells per liter of rainwater or snow precipitations (Casareto *et al.*, 1996; Bauer *et al.*, 2002), 2.10^5 to 5.10^6 bacterial cells per liter in the case of snowmelt in the upper surface snow at the South Pole (Carpenter *et al.*, 2000), as well as estimate of about 10^6 to 10^8 bacteria per liter of cloud water (Sattler *et al.*, 2001; Bauer *et al.*, 2002; Amato *et al.*, 2005; Amato *et al.*, 2007b; Hill *et al.*, 2007; Vaïtilingom *et al.*, 2013). The difference in bacterial concentrations between cloud water and rain or snow precipitations (10^5 to $10^6.L^{-1}$), although negligible, is likely to be related to the ability of only part of the cloud droplets containing precipitation-triggering bacteria (*i.e.,* those presenting an ice nucleating activity) to fall (Szyrmer and Zawadzki, 1997; Morris *et al.*, 2004; Morris *et al.*, 2011). This uniform bacterial concentration across distant precipitations is suggestive of similar overall constraints in those environments. Seasonal and diurnal variations such as were observed in the concentration of total culturable microorganisms of the atmosphere (Amato *et al.*, 2007b), in aerosol samples (Bowers *et al.*, 2012) and around midday (Lighthart and Shaffer, 1995; Lighthart, 1999), are not relevant in the case of the bacterial concentrations recovered, since the rain episodes were sampled within the same month over a 24 hour-interval.

Concentration of atmospheric microbes is strongly influenced by wind orientation, sampling location, salinity and pollution, which led to identify three types of air masses, maritime, continental and urban depending on the West, Southwest and Northeast wind orientation in the center of France (Marinoni *et al.*, 2004; Amato *et al.*, 2005; Vaïtilingom *et al.*, 2013). Four of our five rain samples were collected from South- or West-originating air

masses, expected to carry highest microorganism concentrations in relation to a strong emission from the Atlantic Ocean. The other sample (rain episode 3) was collected from a rain released by SouthEast-originating clouds deprived of an ocean contribution and potentially submitted to pollution including SO_2 and NOx that affects the multiplication and survival of microorganisms (Marquardt *et al.*, 2001; Amato *et al.*, 2007b; Delort *et al.*, 2010). However, concentrations of total culturable bacteria were in the same range of magnitude for the five rain episodes.

Among rain borne culturable bacteria, specific attention was paid to *Pseudomonas syringae*, a major plant pathogen and one of the most active ice nucleate bacteria, whose growth is favored by the use of a semi-selective culture medium for isolating pure cultures of *Pseudomonas syringae* pathovars (King *et al.*, 1954; Mohan and Schaad, 1987). Numeration results confirmed the importance of this bacterium in rain (10^2-10^4 *P. syringae*.L^{-1} of rain) (Morris *et al.*, 2008) since it represents almost one tenth (10^3 CFU.L^{-1} of rainwater) of all cultivated bacteria (2.27×10^4 CFU.L^{-1}) in the three rain samples collected under South by West winds that were studied in detail. Unsurprisingly, the number of putative *P. syringae* dropped by a one hundred-fold factor (10^1 CFU.L^{-1} of rainwater) when wind was blowing from South by East. This may be related to either a much higher urban emission compared to plant or marine surfaces or a particularly marked sensitivity to chemical compounds present in polluted clouds.

The fourth rain event was sampled, as with the other rain events, when winds blew from SouthWest, but could not be processed in the same manner as the other rain samples due to a temporary sucrose shortage at the time of collection. The bacteria were then washed and concentrated with 10% (w/v) glycerol instead of 0.5 M-sucrose. Glycerol is frequently used as an osmoticum for washing bacterial cells, especially for *E. coli*, but 0.5 M sucrose is preferred for *Pseudomonas* preparation (Bassett and Janisiewicz, 2003). This study being foremost focused on *Pseudomonas* spp*.,* rain events 1, 2 and 5 containing the strongest proportion of putative *Pseudomonas* were studied in more detail while the lower bacterial counts in rain events 3 and 4 led us to exclude them from further analyses.

2. Survival of rain bacteria to lightning

Adaptation to the cloud environment requires aerosolized bacteria to be able to survive lightning discharges. We evaluated the survival potential of rain bacterial communities by comparing estimates of culturable bacteria directly with concentrated rain bacteria or after electroporation. The electrical parameters of the *in vitro* electroporation (12.5 kV.cm^{-1} and 5

$kA.m^{-2}$) are of the same order of magnitude as those measured during lightning discharges (6 $kV.cm^{-1}$ and 12 $kA.m^{-2}$) (Demanèche *et al.*, 2001c). We thus simulated the electrical phenomena occurring in cloud using electroporation.

Survival rates that we observed for rain bacteria far exceeded the 5% survival rate of laboratory bacteria such as *E. coli* K12 (Calvin and Hanawalt, 1988). All our results indicated a remarkable tolerance of bacteria (including *P. syringae*) collected from fresh rainfall to electric shocks, since on average 40% of the isolated bacteria survived the shock. This discrepancy may reflect the difference between laboratory-habituated and cloud bacteria with the potential to resist electric shocks which may have been modified through the conditions encountered within clouds, including nutrient limitation, highly oxidative environment, ice nucleation, desiccation, temperature, pH, UV radiations, repeated freeze-thaw cycles and osmotic shocks that could act separately, or synergistically. Furthermore, identical trends were found for either total culturable bacteria and putative *Pseudomonas* counts in each sample. This in turn suggests that *Pseudomonas* isolated on KBC and expected to contain IN bacteria may not be more advantaged than other cloud bacteria as far as electric stress is concerned.

Electrical factors involved in bacterial survival include the strength of the electrical field and the duration of the electrical pulses that, alone or in combination and when above a limit, lead to membrane disruption and the efflux of cellular components (Sambrook and Russell, 2001). Some bacteria can survive electric field pulses even stronger than those commonly applied, while others are extremely sensitive, most likely by experiencing irreversible damages to their cell membrane. The rain bacteria in our samples may thus be better able to survive lightning-simulated electrical shocks by having been selected for by atmospheric transport (Šantl-Temkiv *et al.*, 2012).

3. Impact of lightning on bacterial diversity

We also investigated the taxonomical structure of rain putative *Pseudomonas* bacterial communities before and after lightning-simulating pulse to search for a possible effect of lightning-like electrical stresses on bacterial diversity.

Rain putative Pseudomonas spp. control distribution

A diversity of *Pseudomonas* spp. with reference to Mulet *et al.* (2010) was identified among the KBC-grown isolates from the five rain events. This culture medium is expected

to be specific to *Pseudomonas syringae* pathovars (Mohan and Schaad, 1987), and we did recover some bacteria related to that species. However, our isolates represented a much wider taxonomic sampling of the *Pseudomonas* genus. More surprisingly, one actinobacterium (rain event 2), related to *Plantibacter* sp. (*Microbacteriaceae*), was recovered as well, highlighting a limitation of King's medium as far as *Pseudomonas* specificity is concerned.

Among the *Pseudomonas* isolates identified from the five rain episodes, the *P. syringae* lineage appeared to be the most abundant (half of the isolates overall, all rain episodes but rain 1). Culturable species identified as *P. syringae* were always oxidase negative (C. E. Morris, personal communication), but other *Pseudomonas* species that we identified were also oxidase negative. *P. syringae* colony color varied from beige to white and orange but didn't show fluorescence systematically. This species and others sampled in our rain events were pigmented and some of these pigments may belong to the carotenoid family protecting bacteria from low temperatures, UV radiation and low salt exposures (Imshenetsky *et al.*, 1978; Fong *et al.*, 2001). Pigmented bacteria are also frequently encountered in Arctic ice (Fong *et al.*, 2001; Amato *et al.*, 2005).

P. syringae was not found to be the most abundant microorganism in the atmosphere (Lighthart, 1997), and constituted no more than 5-9% of *P. syringae*-like bacteria (Constantinidou *et al.*, 1990). Similarly, no culturable *P. syringae* populations were obtained in fresh snow samples, although significant levels of IN bacteria were detected (Christner *et al.*, 2008a). However, when the two smaller samples (rains 3 and 4, amounting to only 1.25% of the isolates) are omitted, that lineage is only the third most represented *Pseudomonas* group (16.7%), behind the combined *P. lutea* and *P. rhizosphaerae* lineages (close to half of the isolates) and *P. fluorescens* group (slightly more than 30 %). Interestingly, *P. graminis*, *P. rhizosphaerae,* as well as *P. syringae* bacteria, were isolated from clouds by Amato *et al.* (2007a), although they did not use *Pseudomonas*-specific culture medium.

Within *P. syringae* group, the closest relatives to our rain isolates were phytopathogens that have a wide host range and can have an important economical impact on different crops (O'Brien *et al.*, 2011; Mansfield *et al.*, 2012); for example, they can lead to olive knot (*P. savastanoi*) or canker of hazelnut (*P. avellanae*). The sole *P. syringae* species has been subdivided into more than 50 pathovars and is responsible for many different plant diseases, including the brown spot of beans decreasing product marketability, plant necrosis by the production of phytotoxins, speck of tomato (*P. syringae* pv. *tomato*), brown spot as well as

apical necrosis of mango (*P. syringae pv. syringae*), halo blight of bean (*P. syringae* pv. *phaseolicola*) and canker of stone and pome fruits (*P. syringae* pvs. *morsprunorum* and *syringae*) (Beattie, 2006; Kennelly *et al.*, 2007).

Most of our *Pseudomonas* isolates (all rain episodes but episode 4) are more closely related to *P. rhizosphaerae, P. abietaniphila* and/or *P. graminis* species. They may thus originate from aerosolization from vegetation (Lindemann *et al.*, 1982; Lighthart and Shaffer, 1995; Morris *et al.*, 2008), since *P. graminis* and *P. rhizosphaerae* have been isolated from the phyllosphere and the rhizosphere of grasses, respectively (Behrendt *et al.*, 1999; Peix *et al.*, 2003). *P. graminis* has been also identified as a biocontrol agent of fire blight (Mikiciński *et al.*, 2011). The identification of *P. abietaniphila*, a resin acid degrader isolated from pulp mill wastewater (Muttray *et al.*, 2001), is compatible with a plant-related provenance as well.

Other *Pseudomonas* species identified were affiliated to the *P. fluorescens* lineage, which contains a wide diversity of subgroups and species with different lifestyles. The most represented in our survey were the pathogenic and plant growth promoter *P. azotoformans* (Levenfors *et al.*, 2011) that was identified among the isolates from rain events 1, 2 and 3, and the saprophyte *P. rhodesiae* that we isolated from rain events 2 and 3 (Coroler *et al.*, 1996). Our other isolates belong to the *P. fragi* and *P. asplenii* subgroups and have closest relatives with biodegradation capacities (*P. vranovensis, P. asplenii, P. japonica* and *P. alkylphenolia)* (Tvrzová *et al.*, 2006; Pungrasmi *et al.*, 2008; Cho *et al.*, 2011), psychrophilic (*P. fragi, P. deceptionensis* and *P. psychrophila*) (Eichholz, 1902; Yumoto *et al.*, 2001; Carrión *et al.*, 2011) or phytopathogen (*P. asplenii* and *P. fuscovaginae*) abilities (Miyajima *et al.*, 1983; Beattie, 2006).

Overall, besides potential IN *Pseudomonas*, cold-adapted bacteria, saprophytes or plant growth promoters and bacteria with biodegradation capacities, as well as diverse phytopathogens that may constitute a threat to ecosystems, may also be present among rain bacteria (Kellogg and Griffin, 2006; Brodie *et al.*, 2007).

Distribution of rain putative Pseudomonas after lightning-simulating pulse

The distribution of the isolates submitted to an electrical discharge across the main phylogenetic lineages of *Pseudomonas* varied little from that observed in the absence of an electrical pulse. However, this treatment revealed a higher taxonomic diversity that was most noticeable within the *P. fluorescens* and *P. lutea* & *P. rhizosphaerae* lineages. This

may be related to a decrease of the total number of bacteria, which may thus reveal the formerly less easily detectable minor groups. Alternatively, some lineages may be more resistant to the electrical stress and could have been selected during the electroporation. The *P. putida/P. fluorescens asplenii* subgroup (a single isolate in the previous distribution) was no longer represented when rain bacteria were submitted to an electrical discharge, which suggests that these species may lack resistance against lightning. In turn, the *P. oryzihabitans* lineage (a single isolate), which contains opportunistic human pathogens and psychrotolerant species (Kodama *et al.*, 1985; Freney *et al.*, 1988; Hauser *et al.*, 2004), as well as the *P. koreensis* and *P. mandelii* subgroups within the *P. fluorescens* group distribution, were newly recovered after shock. These latter closest relatives are species with biocontrol (*P. koreensis*) (Kwon *et al.*, 2003; Hultberg *et al.*, 2010b; Hultberg *et al.*, 2010a) and biodegradation capacities *(P. frederiksbergensis* and *P. moraviensis*) (Andersen *et al.*, 2000; Tvrzová *et al.*, 2006) as well as psychrophilic abilities (*P. mandelii*) (Verhille *et al.*, 1999; Jang *et al.*, 2012).

4. Electrotransformation potential of rain isolates

So far, lightning electrotransformation has been shown to occur on soil bacteria (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006a). Since clouds are five times more likely to be subjected to lightning discharges than soil (Gary, 1999), we hypothesized that bacteria could be electrotransformed in clouds as well. Electrotransformation of rain isolates was thus the second parameter that we investigated to determine whether electric conditions encountered by bacteria in clouds could contribute to their adaptive potential and their specific selection.

Two ways to perform and confirm transformation could have been determined: (i) the use of a gene, which presence is rare in the environment and that can be difficult to find, or (ii) the use of synthetic DNA. We chose the first way as presence of the gene *linA* (a mosaic and specific gene whose occurrence in environmental bacteria is extremely low, being restricted to highly lindane-contaminated soil bacteria) (Boubakri *et al.*, 2006) on the donor plasmid (pBLN) (Lyon *et al.*, 2010) was used for PCR confirmation of plasmid presence in isolates exhibiting resistance to the two antibiotics. This was used to distinguish these events from spontaneous mutations and natural resistance to multiple (eighteen) antibiotics frequently encountered in the environment (Dantas *et al.*, 2008). Electrotransformation was thus ascertained by comparing the resistance level of the electrotransformed isolates to that

obtained after natural transformation of the plasmid as well as to that observed in the absence of transformation.

Control conditions of electrotransformation

We first established the level of natural resistance to the antibiotics used as markers in our experimental procedure and found just one naturally tetracycline and kanamycin-resistant isolate (rain 1 sample). All others, from rain samples 1 and 2 (none were isolated from rain 5), were obtained through either natural transformation (the majority) or electrotransformation.

The natural transformation rates found for rain 1 and 2 samples were in the same order of magnitude and surprisingly large (1.10×10^{-3} and 8.43×10^{-4} respectively) with respect to known transformation rates obtained with given transformable bacteria (from 1.90×10^{-9} for *Vibrio parahaemolyticus* to 9.50×10^{-2} for *Azotobacter vinelandii*) (Lorenz and Wackernagel, 1994). Genetic potential for natural transformation was detected in approximately 1% of the described bacterial species (Thomas and Nielsen, 2005) with varying proportions between species (Lorenz and Wackernagel, 1994; Snyder and Champness, 1997). Naturally competent bacteria are found in several Gram negative or positive genera as *Bacillus, Neisseria, Streptococcus, Haemophilus, Acinetobacter* and *Pseudomonas* for the most studied (Snyder and Champness, 1997; Claverys and Martin, 2003). The rate we detected corresponds to the proportion of transformable bacteria from the whole rain bacterial community and may vary individually from one isolated species to another. The laboratory conditions we used correspond to the standard conditions applied to the development of *Acinetobacter* competence (Demanèche *et al.*, 2002). We did not take into account the specific requirements for the competence development of other bacteria, *e.g.*, specific growth phase, nutritional demand, adverse conditions or need of competence factors presence (Lorenz and Wackernagel, 1994; Solomon and Grossman, 1996). Our estimation (10^{-3} to 10^{-4}) may thus represent an underestimation of the actual natural transformability potential of the rain isolates. One putative natural transformant ("Sp" isolate from rain episode 2 growing on antibiotic-supplemented medium) was negative for *linA* presence, whereas no colonies could be detected in the control ("Sc") plate of the same rain episode. This strain may thus be naturally resistant or the *linA* gene that was used as a marker of the plasmid presence in the cell was somehow lost or damaged, possibly by

recombination after internalization of the plasmid by natural transformation (Thomas and Nielsen, 2005; Fall *et al.*, 2007).

Concerning putative *Pseudomonas* spp. transformation potential, no natural transformants were detected within the three rain events. This may not be surprising given that most bacteria will not accept exogenous DNA without being subjected to a physical or chemical treatment leading to a competent state. However, a number of *Pseudomonas* species have been shown to undergo natural transformation, *e.g.*, *P. fluorescens* in soil microcosms (Demanèche *et al.*, 2001a), *P. stutzeri, P. mendocina, P. alcaligenes,* and *P. pseudoalcaligenes in vitro* (as well as in soils for *P. stutzeri* that exhibit selective DNA binding and uptake properties as well as a specific growth phase and nutritional requirements) (Carlson *et al.*, 1983; Stewart and Sinigalliano, 1989; Lorenz and Wackernagel, 1990; Lorenz and Wackernagel, 1991; Lorenz and Wackernagel, 1994).

Electrotransformation rate of rain isolates

The electrotransformation efficiency reached 10^{-3} for the total culturable bacteria. Electrotransformation can be impacted by factors such as DNA quality, conditions of electroporation, number of bacteria and their growing phase, higher rates being obtained when bacteria are concentrated and exponentially grown (Cérémonie *et al.*, 2004). Furthermore, parameters related to membrane integrity such as nutritional stresses, the presence of cations and surfactants, as well as the cooling of the medium, are known to increase electrotransformation rates (Shi *et al.*, 2003), and could lead to a better electrotransformation efficiency in clouds. Indeed, in this environment, temperature is low (Delort *et al.*, 2010), cations may be present when the cloud chemical composition is linked to oceanic influence (Amato *et al.*, 2007b; Vaïtilingom *et al.*, 2013) and some bacteria produce biosurfactants that facilitate CCN formation (Ahern *et al.*, 2007). Thus our finding that 30 to 40% of antibiotic-resistant culturable rain bacteria are able to uptake and express plasmid DNA upon electric stress supports the hypothesis that rain bacteria possess an increased electrotransformation potential. Such efficiency of exogenous DNA acquisition by associated electric field pulses with lightning currents during lightning flashes in clouds could have considerable consequences on the adaptation and evolution of disseminated bacteria.

Only one putative *Pseudomonas* spp. isolate (rain episode 2) was recovered after electroporation although the method we used to prepare electrocompetent cells, including

the washing of cells in 0.5 M sucrose, is expected to be particularly efficient for *Pseudomonas* species (Bassett and Janisiewicz, 2003). This translates into a 10^{-4} electrotransformation efficiency, a somewhat low level with respect to the electrotransformation potential of commercial electro-competent cells that are used at about 10^{9} concentrations and can reach rates as high as 80% of surviving cells (Drury, 1996) and to some soil isolated *Pseudomonas* strains exhibiting very high electrotransformation rates (10^{-2}), two orders of magnitude higher than commercial electro-competent *E. coli* strain (Cérémonie *et al.*, 2006a). In contrast, this value is higher than the best electrotransfer efficiency of 10^{-5} known for *P. syringae* (Wendt-Potthoff *et al.*, 1992; Bassett and Janisiewicz, 2003).

Electrotransformed rain bacterial diversity

Here, only strains containing the *linA* gene were analyzed. However, we cannot clearly identify bacteria able to naturally transform pBLN or due to electroporation but we can compare the distribution of bacterial species isolated on media with antibiotics with and without electrical shock. The bacterial taxa identified among the total culturable bacteria (LBCTK medium) differed significantly from those characterized from the KBC-grown isolates. Members of the two bacterial phyla *Actinobacteria* and *Proteobacteria* constituted the naturally or electrically transformed bacterial communities (*i.e.*, growing on media supplemented with antibiotics that we isolated for rain episodes 1 and 2), in accordance with previous reports on bacterial diversity found in the atmosphere, clouds, rain, hailstones or snow (Amato *et al.*, 2005; Maron *et al.*, 2005; Amato *et al.*, 2007a; Brodie *et al.*, 2007; Jones *et al.*, 2008; Bowers *et al.*, 2011; Vaïtilingom *et al.*, 2012). In contrast to those studies, we did not recover any *Firmicutes* or *Bacteroidetes* (Fuzzi *et al.*, 1997; Amato *et al.*, 2007a; Šantl-Temkiv *et al.*, 2012), nor did we retrieve any *Burkholderiales* or *Moraxellaceae* and more generally *Beta-proteobacteria* that had been found to be major component of airborne bacterial communities (Bowers *et al.*, 2011), or any *Alpha-proteobacteria* (Amato *et al.*, 2007d; Šantl-Temkiv *et al.*, 2012) among the *Proteobacteria*. The absence of *Firmicutes, Bacteroidetes* and other *Proteobacteria* from our samples may simply result from their inability to accept exogenous DNA in the conditions we used.

Whilst the Gram-positive fraction of culturable bacteria in air, cloud water, precipitations or glacier ice is generally the most abundant (Shaffer and Lighthart, 1997; Bauer *et al.*, 2002; Zhang *et al.*, 2002; Kellogg and Griffin, 2006; Morris *et al.*, 2011),

possibly because Gram-positive bacteria include spore-formers that are more resistant, we retrieved approximately 10% of Gram positive bacteria, all belonging to the *Microbacterium* genus in the *Microbacteriaceae* family within *Actinobacteria*. We did not identify any known spore-formers in our rainwater samples, most likely as a result of our experimental procedure. Indeed, no germination step was applied previous to isolating the strains. *Microbacterium* spp. were only isolated during the second rain event and represented 21% (electroporated sample) to 25% (natural transformants) of the transformed bacteria, which is unusual for this genus. The closest species related to our isolates included strains encompassing a diversity of life modes and habitats such as plants (rice) (*M. testaceum*), rhizosphere (ginseng and mangrove) (*M. soli* and *M. halophilum*) (Takeuchi and Hatano, 1998a; Srinivasan *et al.*, 2010), mushroom (*M. agarici*) (Young *et al.*, 2010), insect (*M. saperdae*) (*Saperda carcharias*), hamster (*M. oxydans*) (Ushakova *et al.*, 2004) as well as alkaline lakes (Wu *et al.*, 2011), air and clouds (*M. oxydans*) (Amato *et al.*, 2005), soils as saprophyte (*M. luteolum*), seawater and marine-muds (*M. maritypicum*) and deep-sediments (*M. profundi*) (Wu *et al.*, 2008); some have been identified as animal or human pathogens (*M. oxydans* and *M. paraoxydans*) (Laffineur *et al.*, 2003; Buczolits *et al.*, 2008; Woo *et al.*, 2010; Yasuma *et al.*, 2011) or hairspray contaminants (*M. hatanonis*) (Bakir *et al.*, 2008); others exhibit properties such as insecticide degradation (*M. esteraromaticum*) (Cáceres *et al.*, 2009; Cabrera *et al.*, 2010) or vancomycin resistance (*M. resistens*) (Behrendt *et al.*, 2001). Among these related species, M. *oxydans* has been reported in the last few years for its increasing pathogenic potential, which emphasizes the risk that can be encountered by its dissemination in rain and airborne bacteria (Woo *et al.*, 2010; Yasuma *et al.*, 2011).

Gram-negative bacteria dominated the species distribution of bacteria able to acquire DNA, as already found in aerosols by Jones *et al.* (2008) or cloud water (Lighthart, 1997), and constituted about 90% of either natural or electrically induced transformant communities, comparable with the 80% proportion of airborne bacteria (Bauer *et al.*, 2003). This conclusion must however be taken with caution: since we focused on the bacteria able to uptake exogenous DNA, we can only conclude with confidence that Gram-negative bacteria from rainwater seemed to be more transformation-capable than Gram-positive. All our Gram-negative isolates are *Gamma-proteobacteria*, with at least half of them belonging to *Pseudomonadaceae*, in contrast to the low retrieval percentage observed in snow (1%) or air (3%) samples by Bowers *et al.* (2009), but in agreement with *Pseudomonas* bacteria abundance described in cloud droplets (Amato *et al.*, 2007a), air samples (Shaffer and Lighthart, 1997) and fog water (Fuzzi *et al.*, 1997). Besides *Pseudomonadaceae*, a single

other gamma-proteobacterium, *E. coli*, was retrieved and constitutes the second most abundant taxon in the rain samples. This enterobacterium is not usually found in airborne communities. The *Escherichia coli* isolates dominated the rain bacterial community in the first rain event: they represented from 50% (electroporated sample) to 80% (natural transformants) of the transformed bacteria. In turn, a single isolate was retrieved among the natural transformants of rain episode 2. The occurrence of this bacterial species in rain is unrecorded. Because of its ecology, we can imagine that it could result from bird fecal contaminations (Kaper *et al.*, 2004). Such particles would be large enough (more than several microns in diameter) to be scavenged by raindrop fall (McDonald, 1962; Respondek *et al.*, 1995); they thus may not be subjected to lightning in clouds. However, albeit in a smaller proportion, some were recovered in the lightning-like treated sample. Interestingly, the *E. coli* isolates that we recovered after both natural and electro-transformation are most closely related to virulent and multiresistant strains (SMS 3-5 and UMN026) (Fricke *et al.*, 2008; Touchon *et al.*, 2009). The dissemination through aerosolization and rain of such bacteria may thus constitute a threat for human health. The observed high proportions of Gram-negative bacteria (foremost those of *E. coli*) may also result from the experimental conditions we applied. Indeed, these correspond to *Acinetobacter* competence development and, as such, may be more favourable to gamma-proteobacteria.

In contrast to *Microbacterium* spp. and *E. coli*, the *Pseudomonadaceae* portion of the transformant distribution increased from 43% to 62% when an electrical discharge was applied to the rain samples, suggesting higher electrotransformation abilities. Most isolates belonged to the *P. fluorescens* group, which may not be unexpected, since *P. fluorescens* has been identified among ice-nucleating bacteria (Beattie, 2006; Bowers *et al.*, 2009). *P. lurida* was the sole species to be identified in all samples, rain episodes 1 and 2 as well as both types of transformation. This bacterium, not retrieved when putative *Pseudomonas*-specific growth medium was used, is a psychrotolerant plant growth promoter that was isolated from the phyllosphere of grasses as well as in a high altitude rhizospheric soil (Behrendt *et al.*, 2007; Selvakumar *et al.*, 2011). Its proportion increased in the electroporated samples. A similar trend was observed in the case of rain event 2 isolates affiliated to the *P. fluorescens* subgroup. Members of this lineage are mostly nonpathogenic saprophytes that produce a fluorescent pigment. The best known among them, *P. fluorescens*, is ubiquitous and can be isolated from soils, water, in food as a spoilage agent and in humans as an opportunistic pathogen (Baggi *et al.*, 1983; Gershman *et al.*, 2008). Similarly to *P. trivialis* (Behrendt *et al.*, 2003; Mejri *et al.*, 2012), it may present biodegradation (Beattie, 2006) and biocontrol

properties, preventing plant disease and promoting their growth (Baggi *et al.*, 1983; Haas and Défago, 2005). Within this group, the species most closely related to our isolates correspond to psychrophilic or psychrotolerant bacteria isolated from Antarctica, cold rooms for food storage and food spoilage isolates (*P. antarctica, P. extremaustralis, P. meridiana, P. mandelii, P. fragi, P. deceptionensis, P. psychrophila*) (Reddy *et al.*, 2004; López *et al.*, 2009; Carrión *et al.*, 2011; Jang *et al.*, 2012; Tribelli *et al.*, 2012; Ismail, 1998; Yumoto *et al.*, 2001; Broekaert *et al.*, 2011); all species that are most likely adapted to harsh environmental conditions. In turn, the fraction of the latter was larger in the natural transformation sample. Isolates corresponding to *P. azotoformans* species, a phytopathogen of cereal grain and also able to fix nitrogen as a plant growth promoter, were found among both the electro-transformants of rain 2 episode and the natural transformants of rain episode 1. Similarly, the natural mineral water saprophyte *P. rhodesiae* were found in one sample only.

Most significantly, the only other *Pseudomonadaceae* that we identified belonged to the *P. rhizosphaerae* species lineage. This species is a motile and aerobic bacterium that was isolated from rhizospheric soil of grasses in Spain and that actively solubilizes phosphate *in vitro*, thus possibly acting as a plant growth promoter. As for rain 1 sample grown on *Pseudomonas*-specific medium after electroporation, *P. rhizosphaerae* isolates from rain episode 2 were only retrieved from electroporated samples on both media supplemented with antibiotics.

Contrary to what was expected for this highly adaptive bacterium, no *P. syringae* were transformed either naturally or electrically probably because they were not predominant in our rain overall distribution.

5. Relevance for bacterial dissemination

The ability of bacteria to exchange genetic material is a major evolutionary force that has permitted bacteria to adapt to new niches and to colonize most of our biosphere ecosystems, including those characterized by harsh physical and chemical conditions. Several mechanisms can be involved for promoting HGT including conjugation for plasmid exchange between two cells, bacteriophage-mediated transduction and natural transformation characterized by the uptake of naked and extracellular DNA or electrically-induced transformation. Aerosolized bacteria reaching the free atmosphere alive may thus benefit from conditions markedly different from their original marine or terrestrial ecosystems, the physico-chemical cloud conditions possibly triggering the genetic potential

of bacterial competence. Our results support the hypothesis that some of the cloud bacteria can experience an increased potential for transformation in comparison to their original environments, with the possible consequence of acquiring new genes.

The presence of naked and sufficiently preserved DNA in the surrounding of cells is required for either natural or electrically induced transformation and as dissolved DNA of different origin has been detected in marine water, freshwater, sediments and soils at significant concentrations (Ogram *et al.*, 1987; Selenska and Klingmüller, 1992; Nielsen *et al.*, 2007), these data are lacking for clouds, rain or outdoor air. To our knowledge, the only studies aimed at detecting DNA in air have focused on human pathogenic virus and fungus DNA in indoor air from infected patients' homes and hospital rooms (Sawyer *et al.*, 1994; Bartlett *et al.*, 1997). Concentrations of naked DNA are probably low in the atmosphere but dust-bound protected DNA molecules could act as a source of transforming DNA, as clay-bound DNA does in soil (Paget *et al.*, 1992; Demanèche *et al.*, 2001b). In addition, the strong emission of bacteria from different surfaces as plant, soil and water can implicate exogenous DNA as well, and the DNA released by dying bacteria unable to adapt to the harsh conditions encountered in clouds can still enrich this potential atmospheric reservoir. Therefore, future works should evaluate the presence of naked DNA in atmospheric, cloud and rain samples.

The diversity of atmospheric bacteria varies depending on types of air masses (Amato *et al.*, 2005; Amato *et al.*, 2007b; Delort *et al.*, 2010; Vaïtilingom *et al.*, 2013). Gram-negative bacteria are increased in urban atmosphere (di Giorgio *et al.*, 1996), whereas IN bacteria have been isolated from the atmosphere above plant canopies (Lindemann *et al.*, 1982). The structure of culturable bacterial communities differed markedly for the third rain event with wind coming from South by East. However, this would require further testing because of the much smaller size of rain event 3 (hardly more than 1% of all isolates).

Atmospheric pollution, also characterized by elevated concentrations of carboxilic compounds such as methanol, formaldehyde, succinate, acetate, formate and lactate, could contribute to a selection of adapted bacteria able to metabolize these compounds and therefore creating other sources of organic carbon for a potential selection of additional adapted bacteria (Herlihy *et al.*, 1987; Amato *et al.*, 2005; Amato *et al.*, 2007c) (Deguillaume *et al.*, 2008; Husárová *et al.*, 2011; Šantl-Temkiv *et al.*, 2012; Vaïtilingom *et al.*, 2013). It is now well recognized that the concentration and diversity of microorganisms in clouds are significantly affected by anthropogenic activities, as confirmed by the isolation of greater numbers of cloud bacteria with increased capacity for degradation of chemical

compounds (Amato *et al.*, 2007b; Vaïtilingom *et al.*, 2010; Vaïtilingom *et al.*, 2011; Vaïtilingom *et al.*, 2013). Human activities are also known to increase bacterial resistance to antibiotics and the occurrence of resistance genes in environmental bacteria as a result of increased antibiotic use, selection of resistant strains leading to horizontal transfer of resistance genes, conferring to bacteria isolated from the environment an anthropization level indicator status of antibiotic resistance (Patterson *et al.*, 2007; Dantas *et al.*, 2008; Srinivasan *et al.*, 2008; Wright, 2010). Although limited to two antibiotics (kanamycin and tetracyclin), our results do not show a strong occurrence of natural antibiotic resistance mechanisms in strains isolated from rain samples under western winds, with only a single resistant strain detected only in the first rain event. These results can be explained by their putative oceanic origin characterized by a very limited anthropization level in comparison to terrestrial surfaces. Indeed, multiple-resistances are more frequently encountered in anthropogenic areas (Malik *et al.*, 2008) and agricultural environments where some sites contain more resistant bacteria than others (Macauley *et al.*, 2007; Patterson *et al.*, 2007; Srinivasan *et al.*, 2008). The difference between the antibiotic-resistance comportments of the three rain events could be explained by the potentially different surfaces of emission and localization of bacteria to these different rain events. Unfortunately, antibiotic resistance having not been studied on the rain event under South-East winds, we cannot conclude on the level of resistance depending on potential soil or ocean origin. Most antibiotic resistance genes detected in human pathogens have their origins in environmental bacteria (Dantas *et al.*, 2008; Martínez, 2008; Wright, 2010). This type of resistance, as yet unexplored in the atmosphere, is thus important to further investigate the potential role of bacteria in disseminating human and animal diseases at large geographical scales.

CONCLUSION AND FURTHER WORK:

To our knowledge, this work is the first one reporting the behavior of bacterial communities to lightning. We showed that rain bacteria are more resistant to lightning than laboratory bacteria and that they are able to acquire DNA by natural or electro-transformation. Furthermore, some exhibit natural resistance to antibiotics. Concentrations of culturable rain bacteria were similar to those in other aerosol or precipitation studies but the bacterial community structures were different and could not be readily compared, considering that we only looked for the diversity of bacteria able to take up exogenous DNA with a special focus on *Pseudomonas* and particularly *P. syringae*. The bacteria we isolated most likely differ in origin and life mode: in addition to being IN bacteria, they may be cold-adapted bacteria as well as saprophytes, plant growth promoters and bacteria with biodegradation capacities but also plant and mammal pathogens, including *P. syringae*. In addition to nutrient releases from plants *via* the wounds caused during frosts, and to raindrop creations that serve as dissemination vectors for the descent back to the Earth's surface, catalysis of ice formation in clouds mediated by ice nucleation activity may enhance the bacterium genetic diversity, despite no *P. syringae* transformants being recovered. These different uses of ice nucleation activity could explain why *P. syringae* is one of the most ubiquitous and highly ice nucleation active bacteria found in nature, and may explain how it adapts to different habitats and causes economically important plant diseases. Even if *P. syringae* has huge potential and is already used for snowmaking and cloud seeding, as well as food texturing (Margaritis and Bassi, 1991; Cochet and Widehem, 2000; Christner, 2010), it is now one of the pathogen most frequently encountered and its distribution by rainwater may threaten crop fields.

Sequencing and phylogenetic analyses based on SSU rDNA sequences are not sufficient to identify strains at the pathovar level. The next step will be to evaluate the pathogenicity of strains; aggressiveness should be tested on tobacco or tomato plants with the hypersensitivity reaction test (Lindgren *et al.*, 1986). Confirmation of *P. syringae* pathovars should be made by identification of *cts* haplotypes. Indeed, the *cts* gene is a housekeeping gene, part of the *P. syringae* core-genome, encoding a citrate synthase and used in Multi-Locus Sequence Typing (MLST) analyses, most notably for *Pseudomonas* strains. Morris *et al.* (2010) proved that this gene was a good indicator of the genetic diversity of *P. syringae* populations clustering aggressive haplotypes. Moreover, ice nucleation activity should be

confirmed by *in vitro* tests of the isolated strains and search of the homologous *ina* gene. Given that identification of species cannot rely only on *rrs* analyses (Fox *et al.*, 1992), isolates identities could be confirmed by DNA profiling or microarrays or by the sequencing of housekeeping genes as MLST analyses (Stackebrandt *et al.*, 2002) and sequences compared against pathogens detailed in the Plant Associated and Environmental Microbes Database (PAMDB –(Almeida *et al.*, 2010) and the *PseudoMLSA* Database (Bennasar *et al.*, 2010).

This study confirms that bacteria are aerosolized from diverse terrestrial ecosystems and spread to new habitats by rainfall. We wish to draw attention to the fact that dispersion of pathogenic microorganisms may pose a risk to ecosystems and human health and could also increase the economic impact from plant damages. Besides the harsh conditions that bacteria encounter in the atmosphere, protection against lightning as well as natural or chemical transformation and electrotransformation probably drive microbial evolution.

APPENDICES:

Appendix II-1: Rain collector

Figure 1: Rain collector constituted a conic polyan canvas cover (about 1.875 m² in surface area), allowing rainwater to flow through a 0.5 mm nylon filter in a 150 mL Büshner funnel to be collected in a 2 L polyethylene sterile container.

Appendix II-2: Characteristics of the rain isolates.

Table 1: Characteristics of the rain isolates.
Isolate names constitute the detailed origin of each isolate (as sample and medium used to obtain this isolate) as well as rain event, agar plate and colony number. For example, the first line isolate (R1SpM1P1C2) is the second colony (C2) sampled from the first agar plate (P1) containing LBCTK (M1) from the Control + pBLN (Sp) of the first rain event (1). Isolate colors are those used in phylogenetic trees. This table also contains the CFU color of each isolate as well as their oxidase test result for colonies grown on KBC and their SSU rDNA sequence accession number. n.d.: not determined.

Isolate	Rain episode n°	Sample treatment	Culture Medium	Agar Plate n°	CFU n°	CFU color[1]	Oxidase test	SSU rDNAsequence accession n°
R1SpM1P1C2	1	p	1	1	2	beige	/	KF147131
R1SpM1P1C4	1	p	1	1	4	beige	/	KF147127
R1SpM1P1C5	1	p	1	1	5	beige	/	KF147128
R1SpM1P1C6	1	p	1	1	6	white	/	KF147006
R1SpM1P1C7	1	p	1	1	7	beige	/	KF147129
R1SpM3P1C1	1	p	3	1	1	fluorescent beige	-	KF147038
R1SpM3P1C2	1	p	3	1	2	fluorescent beige	-	KF147040
R1SpM3P1C4	1	p	3	1	4	fluorescent yellow	-	KF147103
R1SpM3P1C5	1	p	3	1	5	pale yellow	-	KF147066
R1SpM3P1C6	1	p	3	1	6	pale yellow	-	KF147089
R1SpM3P2C1	1	p	3	2	1	fluorescent beige	-	KF147028
R1SpM3P2C2	1	p	3	2	2	fluorescent beige	+	KF147061
R1SpM3P2C4	1	p	3	2	4	pale yellow	-	KF147099
R1SpM3P2C5	1	p	3	2	5	pale yellow	-	KF147063
R1SpM3P3C1	1	p	3	3	1	fluorescent beige	-	KF147029
R1SpM3P3C2	1	p	3	3	2	fluorescent yellow	-	KF147101
R1SpM3P3C3	1	p	3	3	3	pale yellow	-	KF147064
R1SpM3P3C4	1	p	3	3	4	pale yellow	n.d.	KF147090
R1SsM1P1C1	1	s	1	1	1	off-white beige	/	KF147045
R1SsM1P1C3	1	s	1	1	3	off-white beige	/	KF147009

Isolate	Rain episode n°	Sample treatment	Culture Medium	Agar Plate n°	CFU n°	CFU color[1]	Oxidase test	SSU rDNAsequence accession n°
R1SsM1P1C4	1	s	1	1	4	off-white beige	/	KF147010
R1SsM1P1C5	1	s	1	1	5	white	/	KF147132
R1SsM1P1C6	1	s	1	1	6	white	/	KF147133
R1SsM1P1C7	1	s	1	1	7	white	/	KF147134
R1SsM3P1C1	1	s	3	1	1	yellow	-	KF147023
R1SsM3P1C2	1	s	3	1	2	yellow	-	KF147126
R1SsM3P1C4	1	s	3	1	4	beige	-	KF147030
R1SsM3P1C5	1	s	3	1	5	beige - white ring	+	KF147118
R1SsM3P1C6	1	s	3	1	6	off-white beige	-	KF147062
R1SsM3P1C7	1	s	3	1	7	off-white beige	-	KF147000
R1SsM3P1C9	1	s	3	1	9	yellow	-	KF147112
R1SsM3P1C10	1	s	3	1	10	pale yellow	-	KF147096
R1SsM3P1C11	1	s	3	1	11	white	-	KF147056
R1SsM3P1C12	1	s	3	1	12	fluorescent yellow	+	KF147085
R1SsM3P2C1	1	s	3	2	1	beige	+	KF147039
R1SsM3P2C2	1	s	3	2	2	beige - white ring	-	KF147141
R1SsM3P2C3	1	s	3	2	3	off-white beige	-	KF147121
R1SsM3P2C4	1	s	3	2	4	pale yellow	-	KF147100
R1SsM3P2C5	1	s	3	2	5	white	-	KF147114
R1SsM3P2C7	1	s	3	2	7	fluorescent yellow	-	KF147076
R1SsM3P3C1	1	s	3	3	1	fluorescent beige	-	KF147037
R1SsM3P3C2	1	s	3	3	2	beige - white ring	-	KF147060
R1SsM3P3C3	1	s	3	3	3	off-white beige	-	KF147116
R1SsM3P3C4	1	s	3	3	4	pale yellow	-	KF147102
R1SsM3P3C5	1	s	3	3	5	white	-	KF147004
R1SsM3P3C8	1	s	3	3	8	yellow	-	KF147087
R1SsM3P3C9	1	s	3	3	9	white	-	KF147002

Isolate	Rain episode n°	Sample treatment	Culture Medium	Agar Plate n°	CFU n°	CFU color[1]	Oxidase test	SSU rDNAsequence accession n°
R1SsM3P3C12	1	s	3	3	12	fluorescent yellow	-	KF147073
R2SpM1P1C2	2	p	1	1	2	yellow	/	KF147135
R2SpM1P1C3	2	p	1	1	3	yellow	/	KF147136
R2SpM1P1C4	2	p	1	1	4	yellow	/	KF147137
R2SpM1P1C5	2	p	1	1	5	yellow	/	KF147013
R2SpM1P1C7	2	p	1	1	7	white	/	KF147008
R2SpM1P1C8	2	p	1	1	8	white	/	KF147130
R2SpM1P1C9	2	p	1	1	9	white	/	KF147049
R2SpM1P1C10	2	p	1	1	10	beige	/	KF147053
R2SpM1P1C11	2	p	1	1	11	beige	/	KF147050
R2SpM1P1C12	2	p	1	1	12	beige	/	KF147054
R2SpM1P1C13	2	p	1	1	13	beige	/	KF147012
R2SpM1P1C14	2	p	1	1	14	beige	/	/
R2SpM1P1C15	2	p	1	1	15	beige	/	KF147024
R2SpM3P1C1	2	p	3	1	1	beige - white ring	-	KF147124
R2SpM3P1C2	2	p	3	1	2	pale yellow	-	KF147081
R2SpM3P1C3	2	p	3	1	3	fluorescent yellow	+	KF147044
R2SpM3P1C4	2	p	3	1	4	beige	+	KF147016
R2SpM3P1C5	2	p	3	1	5	beige	-	KF147019
R2SpM3P1C8	2	p	3	1	8	yellow	-	KF147142
R2SpM3P2C1	2	p	3	2	1	beige - white ring	-	KF147120
R2SpM3P2C2	2	p	3	2	2	beige - white ring	+	KF147123
R2SpM3P2C3	2	p	3	2	3	pale yellow	-	KF147086
R2SpM3P2C4	2	p	3	2	4	fluorescent yellow	-	KF147104
R2SpM3P2C5	2	p	3	2	5	fluorescent yellow	-	KF147097
R2SpM3P2C6	2	p	3	2	6	beige	+	KF147020
R2SpM3P2C8	2	p	3	2	8	white	-	KF147041
R2SpM3P2C9	2	p	3	2	9	white	-	KF147027

Isolate	Rain episode n°	Sample treatment	Culture Medium	Agar Plate n°	CFU n°	CFU color[1]	Oxidase test	SSU rDNAsequence accession n°
R2SpM3P2C10	2	p	3	2	10	white	-	KF147031
R2SpM3P2C11	2	p	3	2	11	white	-	KF147026
R2SpM3P3C1	2	p	3	3	1	pale yellow	-	KF147077
R2SpM3P3C2	2	p	3	3	2	white	-	KF146996
R2SpM3P3C3	2	p	3	3	3	yellow	-	KF147125
R2SsM1P1C1	2	s	1	1	1	yellow	/	KF147108
R2SsM1P1C3	2	s	1	1	3	beige	/	KF147052
R2SsM1P1C4	2	s	1	1	4	beige	/	KF147011
R2SsM1P1C5	2	s	1	1	5	yellow	/	KF147138
R2SsM1P1C6	2	s	1	1	6	yellow	/	KF147139
R2SsM1P1C7	2	s	1	1	7	yellow	/	KF147140
R2SsM1P1C9	2	s	1	1	9	white	/	KF147007
R2SsM1P1C10	2	s	1	1	10	white	/	KF147047
R2SsM1P1C11	2	s	1	1	11	transparent	/	KF147005
R2SsM1P1C12	2	s	1	1	12	beige	/	KF147048
R2SsM1P1C14	2	s	1	1	14	deep yellow	/	KF147113
R2SsM1P1C15	2	s	1	1	15	deep yellow	/	KF147111
R2SsM1P1C16	2	s	1	1	16	deep yellow	/	KF147110
R2SsM2P1C1	2	s	2	1	1	beige	-	KF147109
R2SsM3P1C1	2	s	3	1	1	beige - white ring	-	KF147119
R2SsM3P1C2	2	s	3	1	2	beige - white ring	-	KF147058
R2SsM3P1C3	2	s	3	1	3	fluorescent yellow	-	KF147093
R2SsM3P1C4	2	s	3	1	4	fluorescent yellow	-	KF147094
R2SsM3P1C5	2	s	3	1	5	yellow	-	KF147079
R2SsM3P1C6	2	s	3	1	6	pale yellow	-	KF147083
R2SsM3P1C7	2	s	3	1	7	white	-	KF147043
R2SsM3P1C8	2	s	3	1	8	white	-	KF147025
R2SsM3P1C13	2	s	3	1	13	white	-	KF147057
R2SsM3P1C14	2	s	3	1	14	white	-	KF147021

Isolate	Rain episode n°	Sample treatment	Culture Medium	Agar Plate n°	CFU n°	CFU color[1]	Oxidase test	SSU rDNAsequence accession n°
R2SsM3P2C1	2	s	3	2	1	beige - white ring	-	KF147115
R2SsM3P2C3	2	s	3	2	3	yellow	+	KF147084
R2SsM3P2C4	2	s	3	2	4	yellow	-	KF147080
R2SsM3P2C5	2	s	3	2	5	pale yellow	-	KF147046
R2SsM3P2C7	2	s	3	2	7	white	-	KF147042
R2SsM3P2C9	2	s	3	2	9	beige	+	KF147014
R2SsM3P2C10	2	s	3	2	10	beige	+	KF147015
R2SsM3P2C12	2	s	3	2	12	white	-	KF147022
R2SsM3P2C13	2	s	3	2	13	transparent white	-	KF147059
R3ScM3P1C1	3	c	3	1	1	beige	+	KF147055
R3ScM3P1C4	3	c	3	1	4	yellow	-	KF147078
R3ScM3P1C5	3	c	3	1	5	yellow	-	KF147065
R3ScM3P1C6	3	c	3	1	6	yellow	-	KF147095
R3ScM3P1C7	3	c	3	1	7	beige	-	KF147117
R3ScM3P1C8	3	c	3	1	8	beige	+	KF147051
R3ScM3P1C9	3	c	3	1	9	beige	+	KF147122
R3ScM3P1C11	3	c	3	1	11	fluorescent white	-	KF147001
R3ScM3P1C13	3	c	3	1	13	beige	-	KF147018
R3ScM3P1C14	3	c	3	1	14	beige	-	KF147017
R3ScM3P1C16	3	c	3	1	16	orange	-	KF147107
R3ScM3P1C17	3	c	3	1	17	orange	-	KF147106
R3ScM3P1C18	3	c	3	1	18	yellow	-	KF147068
R3ScM3P1C19	3	c	3	1	19	yellow	-	KF147067
R3ScM3P1C20	3	c	3	1	20	yellow	-	KF147069
R3ScM3P1C21	3	c	3	1	21	beige	-	KF147033
R3ScM3P1C22	3	c	3	1	22	beige	-	KF147032
R3ScM3P1C23	3	c	3	1	23	beige	-	KF147036
R3ScM3P1C24	3	c	3	1	24	white	-	KF147035
R3ScM3P1C25	3	c	3	1	25	white	-	KF147034
R4ScM3P1C1	4	c	3	1	1	fluorescent beige	-	KF146999
R4ScM3P1C2	4	c	3	1	2	fluorescent beige	-	KF146998

Isolate	Rain episode n°	Sample treatment	Culture Medium	Agar Plate n°	CFU n°	CFU color[1]	Oxidase test	SSU rDNAsequence accession n°
R4ScM3P1C3	4	c	3	1	3	fluorescent beige	-	KF146997
R5SpM3P1C1	5	p	3	1	1	yellow	-	KF147105
R5SpM3P1C2	5	p	3	1	2	pale yellow	-	KF147071
R5SpM3P1C3	5	p	3	1	3	pale yellow	-	KF147075
R5SpM3P1C5	5	p	3	1	5	transparent white	-	KF146990
R5SpM3P2C1	5	p	3	2	1	pale yellow	-	KF147092
R5SpM3P2C3	5	p	3	2	3	transparent	-	KF146987
R5SpM3P2C4	5	p	3	2	4	transparent	-	KF146994
R5SpM3P2C6	5	p	3	2	6	transparent white	-	KF146995
R5SpM3P3C1	5	p	3	3	1	pale yellow	-	KF147088
R5SpM3P3C2	5	p	3	3	2	transparent	-	KF146992
R5SpM3P3C4	5	p	3	3	4	transparent white	-	KF146993
R5SsM3P1C2	5	s	3	1	2	transparent white	-	KF146989
R5SsM3P1C6	5	s	3	1	6	yellow	-	KF147070
R5SsM3P2C2	5	s	3	2	2	white	-	KF146991
R5SsM3P2C6	5	s	3	2	6	yellow	-	KF147072
R5SsM3P2C7	5	s	3	2	7	pale yellow	+	KF147091
R5SsM3P3C3	5	s	3	3	3	transparent	-	KF146988
R5SsM3P3C4	5	s	3	3	4	white	-	KF147003
R5SsM3P3C7	5	s	3	3	7	yellow	-	KF147082
R5SsM3P3C8	5	s	3	3	8	beige	-	KF147074
R5SsM3P3C9	5	s	3	3	9	white	-	KF147098

[1] Colour of colonies and fluorescence visible to the naked eye

Appendix II-3: SSU rDNA sequences used for phylogenetic characterization of rain isolates.

Table 1: List of *Pseudomonadaceae* SSU rDNA sequences used for phylogenetic characterization of rain isolates.

Species	Strain	Accession n° (NCBI GenBank database)
Acinetobacter baumannii	DSM30007 T	X81660
Acinetobacter baylyi	B2 T	AF509820
Acinetobacter beijerinckii	LUH4759 T	AJ626712
Acinetobacter bereziniae	ATCC17924 T	Z93443
Acinetobacter bouvetii	4B02 T	AF509827
Acinetobacter calcoaceticus	NCCB22016 T	AJ888983
Acinetobacter gerneri	9A01 T	AF509829
Acinetobacter guillouiae	DSM590 T	X81659
Acinetobacter gyllenbergii	RUH422 T	AJ293694
Acinetobacter haemolyticus	DSM6962 T	X81662
Acinetobacter johnsonii	ATCC17909 T	Z93440
Acinetobacter junii	DSM6964 T	X81664
Acinetobacter lwoffii	DSM2403 T	X81665
Acinetobacter parvus	LUH4616 T	AJ293691
Acinetobacter radioresistens	DSM6976 T	X81666
Acinetobacter schindleri	LUH5832 T	AJ278311
Acinetobacter soli	B1 T	EU290155
Acinetobacter tandoii	4N13 T	AF509830
Acinetobacter tjernbergiae	7N16 T	AF509825
Acinetobacter towneri	AB1110 T	AF509823
Acinetobacter ursingii	LUH3792 T	AJ275038
Acinetobacter venetianus	ATCC31012 T	AJ295007
Alkanindiges illinoisensis	MVAB-Hex1 T	AF513979
Cellvibrio japonicus	NCIMB10462	AF452103
Dasania marina	KOPRI 20902 T	AY771747
Enhydrobacter aerosaccus	LMG21877 T	AJ550856
Moraxella atlantae	CCUG6415 T	HM161851
Moraxella boevrei	ATCC700022 T	DQ156147
Moraxella bovis	ATCC10900 T	AF005182
Moraxella bovoculi	237 T	DQ153089
Moraxella canis	N7 T	AJ269511
Moraxella caprae	ATCC700019 T	DQ156148
Moraxella catarrhalis	ATCC25238 T	AF005185
Moraxella caviae	CCUG355 T	AF005187
Moraxella cuniculi	CCUG2154 T	AF005188
Moraxella equi	327-72 T	AF005184
Moraxella lacunata	ATCC17967 T	D64049
Moraxella nonliquefaciens	CCUG348 T	HM152563
Moraxella ovis	ATCC33078 T	AF005186
Moraxella pluranimalium	248-01 T	AM884564
Moraxella porci	SN9-4M T	FM872292

Species	Strain	Accession n° (NCBI GenBank database)
Perlucidibaca piscinae	IMCC1704 T	DQ664237
Pseudomonas coronafaciens	LMG 13190 T	Z76660
Pseudomonas corrugata	CFBP2431 T	HM190230
Pseudomonas costantinii	CFBP 5705 T	AF374472
Pseudomonas cremoricolorata	NBRC16634 T	AB681090
Pseudomonas cuatrocienegasensis	1N T	EU791281
Pseudomonas deceptionensis	M1 T	GU936597
Pseudomonas delhiensis	RLD-1 T	DQ339153
Pseudomonas duriflava	HR2 T	EU046271
Pseudomonas entomophila	CECT 7985 T	AY907566
Pseudomonas extremaustralis	DSM 17835 T	AJ583501
Pseudomonas extremorientalis	LMG 19695 T	AF405328
Pseudomonas ficuserectae	CCUG 32779 T	Z76661
Pseudomonas flavescens	NBRC103044 T	AB681924
Pseudomonas fluorescens	DSM 50090 T	Z76662
Pseudomonas fragi	NBRC3458 T	AB680088
Pseudomonas frederiksbergensis	DSM13022 T	FR750403
Pseudomonas fulva	NBRC16637 T	AB681093
Pseudomonas fuscovaginae	DSM7231 T	FJ483519
Pseudomonas gessardii	CIP 105469 T	AF074384
Pseudomonas gingeri	NCPPB 3147 T	AF320991
Pseudomonas graminis	DSM 11363 T	Y11150
Pseudomonas grimontii	CFML 97-514 T	AF268029
Pseudomonas guezennei	RA26 T	AJ876736
Pseudomonas guineae	LMG2401 T	AM491810
Pseudomonas indica	NBRC103045 T	AB681925
Pseudomonas japonica	IAM 15071 T	AB126621
Pseudomonas jessenii	1515 T	AF068259
Pseudomonas jinjuensis	NBRC103047 T	AB681927
Pseudomonas kilonensis	520-20 T	AJ292426
Pseudomonas knackmussii	B13 T	AF039489
Pseudomonas koreensis	LMG21318 T	AF468452
Pseudomonas kuykendallii	H2 T	JF749828
Pseudomonas libanensis	CIP105460 T	AF057645
Pseudomonas lindanilytica	IPL-1 T	DQ916277
Pseudomonas lini	CFBP5737 T	AY035996
Pseudomonas litoralis	CECT 7670 T	FN908483
Pseudomonas lunatum	NBRC13958 T	AB680536
Pseudomonas lundensis	ATCC 49968 T	AB021395
Pseudomonas lurida	DSM15835 T	AJ581999
Pseudomonas lutea	OK2 T	AY364537
Pseudomonas luteola	NBRC103146 T	AB681955
Pseudomonas mandelii	NBRC103147 T	AB681956
Pseudomonas marginalis	LMG 2210 T	Z76663
Pseudomonas marincola	KMM 3042 T	AB301071
Pseudomonas mediterranea	CFBP 5447 T	AF386080

Species	Strain	Accession n° (NCBI GenBank database)
Pseudomonas meliae	MAFF 301463 T	AB021382
Pseudomonas mendocina	NCIB 10541 T	D84016
Pseudomonas meridiana	CMS38 T	AJ537602
Pseudomonas metavorans	F-1 T	AB302395
Pseudomonas migulae	NBRC103157 T	AB681965
Pseudomonas mohnii	IpA-2 T	AM293567
Pseudomonas monteilii	NBRC103158 T	AB681966
Pseudomonas moorei	RW10 T	AM293566
Pseudomonas moraviensis	CCM 7280 T	AY970952
Pseudomonas mosselii	CIP105259 T	AF072688
Pseudomonas mucidolens	NBRC103159 T	AB681967
Pseudomonas multiaromavorans	C-18 T	AB302402
Psuedomonas multiresinivorans	ATCC 700690 T	X96787
Pseudomonas nitroreducens	ATCC700690 T	X96787
Pseudomonas oleovorans subsp. *oleovorans*	NBRC13583 T	D84018
Pseudomonas oleovorans subsp. *lubricantis*	RS1 T	DQ842018
Pseudomonas orientalis	DSM 17489 T	AF064457
Pseudomonas oryzihabitans	LMG7040 T	GQ250598
Pseudomonas otitidis	MCC10330 T	AY953147
Pseudomonas pachastrellae	KMM330 T	AB125366
Pseudomonas palleroniana	CFBP 4389 T	AY091527
Pseudomonas panacis	CG20106 T	AY787208
Pseudomonas parafulva	NBRC16636	AB681092
Pseudomonas pelagia	CL-AP6 T	EU888911
Pseudomonas peli	R-20805 T	AM114534
Pseudomonas pertucinogena	NBRC14163 T	EF673695
Pseudomonas plecoglossicida	NBRC103162 T	AB681970
Pseudomonas poae	DSM14936 T	AJ492829
Pseudomonas pohangensis	H3-R18 T	DQ339144
Pseudomonas protegens	CHA0 T	AJ278812
Pseudomonas proteolytica	CMS64 T	AJ537603
Pseudomonas psychrophyla	DSM 17535 T	AB041885
Pseudomonas psychrotolerans	LMG 21977 T	AJ575816
Pseudomonas putida	NBRC14164 T	Z76667
Pseudomonas reinekei	MT1 T	AM293565
Pseudomonas resinovorans	ICMP13541 T	Z76668
Pseudomonas rhizosphaerae	LMG 21640 T	AY152673
Pseudomonas rhodesiae	LMG 17764 T	AF064459
Pseudomonas sabulinigri	J64 T	EU143352
Pseudomonas salomonii	LMG 22120 T	AY091528
Pseudomonas saponiphila	DSM9751 T	FM208264
Pseudomonas savastanoi	CFBP1670 T	HM190226
Pseudomonas segetis	FR1439 T	AY770691
Pseudomonas seleniipraecipitans	CA5 T	FJ422810
Pseudomonas simiae	OLi T	AJ936933
Pseudomonas straminea	NBRC16665	AB681101

Species	Strain	Accession n° (NCBI GenBank database)
Pseudomonas stutzeri	NBRC14165 T	AB680573
Pseudomonas synxantha	NBRC3913 T	AB680171
Pseudomonas syringae	LMG 1247 T	Z76669
Pseudomonas syringae	ICMP3023 T	AJ308316
Pseudomonas syringae pv. *aceris*	M302273	AEAO01000730
Pseudomonas syringae pv. *actinidiae*	Kw11	AB001439
Pseudomonas syringae pv. *apii*	BS463	HQ584979
Pseudomonas syringae pv. *atropurpurea*	MAFF 301017	AB001440
Pseudomonas syringae pv. *broussonetiae*	KOZ 8101	AB001441
Pseudomonas syringae pv. *eriobotryae*	MAFF 302259	AB001442
Pseudomonas syringae pv. *glycinea*	MAFF 302260	AB001443
Pseudomonas syringae pv. *maculicola*	MAFF 302264	AB001444
Pseudomonas syringae pv. *mori*	MAFF 302279	AB001446
Pseudomonas syringae pv. *morsprunorum*	MAFF 302280	AB001445
Pseudomonas syringae pv. *myricae*	MAFF 301464	AB001447
Pseudomonas syringae pv. *phaseolicola*	MAFF 302282	AB001448
Pseudomonas syringae pv. *pisi*	1704B	AEAI01001656
Pseudomonas syringae pv. *syringae*	NCPPB 281 T	DQ318866
Pseudomonas syringae pv. *syringae*	ICMP3023 T	HM190217
Pseudomonas syringae pv. *tagetis*	MAFF 302271	AB001449
Pseudomonas syringae pv. *theae*	PT1	AB001450
Pseudomonas syringae pv. *tomato*	DC3000	AE016853
Pseudomonas taeanensis	MS-3 T	FJ424813
Pseudomonas taetrolens	IAM1653 T	D84027
Pseudomonas taiwanensis	BCRC17751 T	EU103629
Pseudomonas teessidea	PR65 T	AM419154
Pseudomonas thermotolerans	CM3 T	AJ311980
Pseudomonas thivervalensis	CFBP 11261 T	AF100323
Pseudomonas tolaasii	LMG 2342 T	Z76670
Pseudomonas toyotomiensis	HT-3 T	AB453701
Pseudomonas tremae	CFBP6111	AJ492826
Pseudomonas trivialis	DSM14937 T	AJ492831
Pseudomonas tuomuerensis	78-123 T	DQ868767
Pseudomonas umsongensis	LMG 21317 T	AF468450
Pseudomonas vancouverensis	DhA-51 T	AJ011507
Pseudomonas veronii	CIP104663 T	AF064460
Pseudomonas viridiflava	LMG2352 T	Z76671
Pseudomonas vranovensis	CCM 7279 T	AY970951
Pseudomonas xanthomarina	KMM1447 T	AB176954
Pseudomonas xiamenensis	JCM 13530 T	DQ088664
Pseudomonas xinjiangensis	S3-3 T	EU286805
Pseudomonas zeshuii	BY-1 T	JN411093
Psychrobacter aestuarii	SC35 T	EU939718
Psychrobacter alimentarius	JG-100 T	AY513645
Psychrobacter aquaticus	CMS56 T	AJ584833
Psychrobacter aquimaris	SW-210 T	AY722804

Species	Strain	Accession n° (NCBI GenBank database)
Psychrobacter arcticus	273-4 T	AY444822
Psychrobacter arenosus	KMM3659 T	AJ609273
Psychrobacter celer	SW-238 T	AY842259
Psychrobacter cibarius	JG-219 T	AY639871
Psychrobacter cryohalolentis	K5 T	AY660685
Psychrobacter faecalis	Iso-46 T	AJ421528
Psychrobacter fozii	NF23 T	AJ430827
Psychrobacter frigidicola	DSM12411 T	AJ609556
Psychrobacter fulvigenes	KMM3954 T	AB438958
Psychrobacter glacincola	DSM12194 T	AJ312213
Psychrobacter jeotgali	YKJ-103 T	AF441201
Psychrobacter luti	NF11 T	AJ430828
Psychrobacter lutiphocae	IMMIBL-1110 T	FM165580
Psychrobacter marincola	KMM277 T	AJ309941
Psychrobacter maritimus	KMM3646 T	AJ609272
Psychrobacter namhaensis	SW-242 T	AY722805
Psychrobacter nivimaris	88/2-7 T	AJ313425
Psychrobacter okhotskensis	MD17 T	AB094794
Psychrobacter pacificensis	IFO16279 T	AB016057
Psychrobacter piscatorii	T-3-2 T	AB453700
Psychrobacter proteolyticus	116 T	AJ272303
Psychrobacter pulmonis	CECT5989 T	AJ437696
Psychrobacter salsus	DD48 T	AJ539104
Psychrobacter submarinus	KMM225 T	AJ309940
Psychrobacter urativorans	DSM14009 T	AJ609555
Psychrobacter vallis	CMS39 T	AJ584832
'Pseudomonas' boreopolis	ATCC 33662T	AB021391
'Pseudomonas' genuculata	ATCC 19374T	AB021404
'Pseudomonas' halophila	DSM3050	FR746108

Table 2: List of *Enterobacteriaceae* SSU rDNA sequences used for rain isolate phylogeny characterization.

Species	Strain	Accession n° (NCBI GenBank database)
Citrobacter braakii	CDC 080-58 T	AF025368
Citrobacter farmeri	CDC 2991-81 T	AF025371
Citrobacter freundii	DSM 30039 T	AJ233408
Citrobacter gillenii	CDC 4693-86 T	AF025367
Citrobacter koseri	ATCC BAA-895	CP000822
Citrobacter murliniae	CDC 2970-59 T	AF025369
Citrobacter rodentium	CDC 1843-73 T	AF025363
Citrobacter sedlakii	CDC 4696-86 T	AF025364
Citrobacter werkmanii	CDC 0876-58 T	AF025373
Citrobacter youngae	GTC 1314 T	AB273741
Cronobacter dublinensis	E464 T	EF059838
Cronobacter dublinensis	E515 T	EF059841
Cronobacter dublinensis	DES187 T	EF059892
Cronobacter malonaticus	E825 T	EF059881
Cronobacter muytjensii	E603 T	EF059845
Cronobacter sakazakii	ATCC 29544 T	EF088379
Cronobacter turicensis	z3032 T	EF059891
Enterobacter aerogenes	JCM 1235 T	AB004750
Enterobacter amnigenus	JCM 1237 T	AB004749
Enterobacter asburiae	JCM 6051 T	AB004744
Enterobacter cancerogenus	LMG 2693 T	Z96078
Enterobacter cloacae	ATCC 13047 T	AJ251469
Enterobacter cloacae	LMG 2683 T	Z96079 R
Enterobacter cowanii	CIP 107300 T	AJ508303
Enterobacter gergoviae	JCM 1234 T	AB004748
Enterobacter hormaechei	CIP 103441 T	AJ508302
Enterobacter kobei	CIP 105566 T	AJ508301
Enterobacter ludwigii	EN-119 T	AJ853891
Enterobacter nimipressuralis	LMG 10245 T	Z96077 R
Enterobacter oryzae	Ola 51 T	EF488759
Enterobacter pulveris	601/05 T	DQ273684
Enterobacter pyrinus	KCTC 2520 T	AJ010486
Enterobacter radicincitans	DSM 16656 T	AY563134
Escherichia/Shigella albertii	LMG 20976 T	AJ508775
Escherichia/Shigella boydii	CDC 3083-94	CP001063
Escherichia/Shigella boydii	Sb227	CP000036
Escherichia/Shigella coli	ATCC 11775 T	X80725
Escherichia/Shigella coli	S88	CU928161
Escherichia/Shigella coli	SE11	AP009240
Escherichia/Shigella coli	SMS-3-5	CP000970
Escherichia/Shigella coli	UMN026	CU928163
Escherichia/Shigella coli	UTI89	CP000243
Escherichia/Shigella coli	536	CP000247

Species	Strain	Accession n° (NCBI GenBank database)
Escherichia/Shigella coli	55989	CU928145
Escherichia/Shigella coli serovar O1:K1	APEC O1	CP000468
Escherichia/Shigella coli	ATCC 8739	CP000946
Escherichia/Shigella coli serovar B	REL606	CP000819
Escherichia/Shigella coli	BL21DE3	AM946981
Escherichia/Shigella coli	BW2952	CP001396
Escherichia/Shigella coli	CFT073	AE014075
Escherichia/Shigella coli	DH1	CP001637
Escherichia/Shigella coli	E24377A	CP000800
Escherichia/Shigella coli	ED1a	CU928162
Escherichia/Shigella coli	HS	CP000802
Escherichia/Shigella coli	IAI1	CU928160
Escherichia/Shigella coli	IAI39	CU928162
Escherichia/Shigella coli	K-12 substr. DH10B	CP000948
Escherichia/Shigella coli	K-12 substr. MG1655	U00096
Escherichia/Shigella coli	K-12 substr. W3110	AP009048
Escherichia/Shigella coli serovar O103-H2	12009	AP010958
Escherichia/Shigella coli serovar O111-H-	11128	AP010960
Escherichia/Shigella coli serovar O127-H6	E2348-69	FM180568
Escherichia/Shigella coli serovar O157-H7	EC4115	CP001164
Escherichia/Shigella coli serovar O157-H7	EDL933	AE005174
Escherichia/Shigella coli serovar O157-H7	Sakai	BA000007
Escherichia/Shigella coli serovar O157-H7	TW14359	CP001368
Escherichia/Shigella coli serovar O26-H11	11368	AP010953
Escherichia/Shigella dysenteriae	ATCC 13313 T	X96966
Escherichia/Shigella dysenteriae	Sd197	CP000034
Escherichia/Shigella fergusonii	ATCC 35469 T	AF530475
Escherichia/Shigella flexneri	ATCC 29903 T	X96963
Escherichia/Shigella flexneri	2002017	CP001383
Escherichia/Shigella flexneri serotype 2a	2457T	AE014073
Escherichia/Shigella flexneri serotype 2a	301	AE005674
Escherichia/Shigella flexneri serotype 5	8401	CP000266
Escherichia/Shigella sonnei	Ss046	CP000038
Klebsiella pneumoniae	DSM 30104 T	X87276 R
Klebsiella pneumoniae	ATCC 13884T T	Y17657 R
Klebsiella pneumoniae	ATCC 11296 T	AF130982
Klebsiella singaporensis	LX3 T	AF250285
Klebsiella variicola	F2R9 T	AJ783916
Leclercia adecarboxylata	LMG 2803 T	GQ856082
Salmonella bongori	BR 1859 T	AF029227
Salmonella enterica subsp. *arizonae*	ATCC 13314	AF008580
Salmonella enterica subsp. *diarizonae*	DSM 14847	EU014688
Salmonella enterica subsp. *enterica* serovar Typhimurium	LT2 T	AE006468
Salmonella enterica subsp. *houtenae*	DSM 9221	EU014684
Salmonella enterica subsp. indica	DSM 14848	EU014680

Species	Strain	Accession n° (NCBI GenBank database)
Salmonella enterica subsp. salamae	DSM 9220	EU014685
Salmonella subterranea	FRC1 T	AY373829
Yokenella regensburgei	JCM 2403 T	AB519796

Table 3: List of *Microbacteriaceae* SSU rDNA sequences used for rain isolate phylogenetic characterization.

Species	Strain	Accession n° (NCBI GenBank database)
Agreia bicolorata	VKM Ac-1804 T	AF159363
Agreia pratensis	P229-10 T	AJ310412
Agrococcus baldri	IAM 15147 T	AB279548
Agrococcus casei	LMG 22410 T	DQ168427
Agrococcus citreus	IAM 15145 T	AB279547
Agrococcus jejuensis	SSW1-48 T	AM396260
Agrococcus jenensis	DSM 9580 T	X92492
Agrococcus lahaulensis	K22-21 T	DQ156908
Agrococcus terreus	DNG5 T	FJ423764
Agrococcus versicolor	DSM 19812 T	AM940157
Agromyces albus	VKM Ac-1800 T	AF503917
Agromyces allii	UMS-62 T	DQ673873
Agromyces atrinae	P27 T	FJ607310
Agromyces aurantiacus	YIM21741 T	AF389342
Agromyces bauzanensis	BZ41 T	FJ972171
Agromyces bracchium	IFO 16238 T	AB023359
Agromyces cerinus	JCM 9083 T	D45060
Agromyces cerinus	IMET 11532 T	AY277619
Agromyces hippuratus	JCM 9086 T	D45061
Agromyces humatus	CD5 T	AY618216
Agromyces italicus	CD1 T	AY618215
Agromyces lapidis	CD55 T	AY618217
Agromyces luteolus	IFO 16235 T	AB023356
Agromyces mediolanus	DSM 20152 T	X77449
Agromyces neolithicus	23-23 T	AY507128
Agromyces ramosus	DSM 43045 T	X77447
Agromyces rhizosphaerae	IFO 16236 T	AB023357
Agromyces salentinus	20-5 T	AY507129
Agromyces subbeticus	Z33 T	AY737778
Agromyces terreus	DS-10 T	EF363711
Agromyces tropicus	CM9-9 T	AB454378
Agromyces ulmi	XIL01 T	AY427830
Amnibacterium kyonggiense	KSL51201-037 T	FJ527819
Chryseoglobus frigidaquae	CW1 T	EF373534
Clavibacter michiganensis	ATCC 33566 T	U30254
Cryobacterium mesophilum	MSL-15 T	EF466127
Cryobacterium psychrophilum	DSM 4854 T	AJ544063
Cryobacterium psychrotolerans	0549 T	DQ515963
Cryobacterium roopkundense	RuGl 7 T	EF467640
Curtobacterium albidum	IFO 15078T T	AB046363
Curtobacterium ammoniigenes	NBRC 101786 T	AB266597
Curtobacterium citreum	DSM 20528 T	X77436
Curtobacterium flaccumfaciens	LMG 3645 T	AJ312209

Species	Strain	Accession n° (NCBI GenBank database)
Curtobacterium ginsengisoli	DCY26 T	EF587758
Curtobacterium herbarum	P 420/07 T	AJ310413
Curtobacterium luteum	DSM 20542 T	X77437
Curtobacterium pusillum	DSM 20527 T	AJ784400
Frigoribacterium faeni	801 T	Y18807
Frigoribacterium mesophilum	MSL08 T	EF466126
Frondihabitans australicus	E1HC-02 T	DQ525859
Frondihabitans peucedani	RS-15 T	FM998017
Glaciibacter superstes	AHU1791 T	AB378301
Gulosibacter molinativorax	ON4 T	AJ306835
Herbiconiux ginsengi	wged11 T	DQ473536
Herbiconiux solani	K 134-01 T	FN432340
Humibacter albus	SC-083 T	AM494541
Klugiella xanthotipulae	44C3 T	AY372075
Labedella gwakjiensis	KSW2-17 T	DQ533552
Leifsonia antarctica	SPC20 T	AM931710
Leifsonia aquatica	JCM 1368 T	D45057
Leifsonia bigeumensis	MSL27 T	EF466124
Leifsonia kafniensis	KFC-22 T	AM889135
Leifsonia kribbensis	MSL13 T	EF466129
Leifsonia lichenia	2Sb T	AB278552
Leifsonia naganoensis	DB103 T	DQ232612
Leifsonia pindariensis	PON10 T	AM900767
Leifsonia poae	VKM Ac-1401 T	AF116342
Leifsonia rubra	CMS 76r T	AJ438585
Leifsonia shinshuensis	DB10 T	DQ232614
Leifsonia soli	TG-S248 T	EU912483
Leifsonia xyli	JCM 9733 T	AB016985
Leucobacter aerolatus	Sj10 T	FN597581
Leucobacter albus	IAM 4851 T	AB012594
Leucobacter alluvii	RB10 T	AM072820
Leucobacter aridicollis	L9 T	AJ781047
Leucobacter chironomi	MM2LB T	EU346911
Leucobacter chromiireducens	L-1 T	AJ781046
Leucobacter chromiireducens	TAN31504 T	DQ845457
Leucobacter chromiiresistens	JG31 T	GU390657
Leucobacter iarius	40 T	AM040493
Leucobacter komagatae	JCM 9414 T	D45063
Leucobacter luti	RF6 T	AM072819
Leucobacter salsicius	M1-8 T	GQ352403
Leucobacter tardus	DSM19811 T	AM940158
Marisediminicola antarctica	ZS314 T	GQ496083
Microbacterium aerolatum	V-73 T	AJ309929
Microbacterium agarici	CC-SBCK-209 T	FJ807673
Microbacterium aoyamense	KV-492 T	AB234028
Microbacterium aquimaris	JS54-2 T	AM778449

Species	Strain	Accession n° (NCBI GenBank database)
Microbacterium arabinogalactanolyticus	DSM 8611 T	Y17228
Microbacterium arborescens	DSM 20754 T	X77443
Microbacterium aurantiacum	IFO 15234 T	AB004726
Microbacterium aurum	DSM 8600 T	Y17229
Microbacterium awajiense	YM13-414 T	AB286027
Microbacterium barkeri	DSM 20145 T	X77446
Microbacterium binotii	CIP 101303 T	EF567306
Microbacterium chocolatum	IFO 3758 T	AB004725
Microbacterium deminutum	KV-483 T	AB234026
Microbacterium dextranolyticum	DSM 8607 T	Y17230
Microbacterium esteraromaticum	DSM 8609 T	Y17231
Microbacterium flavescens	DSM 20643 T	Y17232
Microbacterium flavum	YM18-098 T	AB286029
Microbacterium fluvii	YSL3-15 T	AB286028
Microbacterium foliorum	DSM 12966 T	AJ249780
Microbacterium ginsengisoli	Gsoil259 T	AB271048
Microbacterium gubbeenense	LMG S-19263 T	AF263563
Microbacterium halophilum	IFO 16062 T	AB004714
Microbacterium halotolerans	YIM70130 T	AY376165
Microbacterium hatanonis	JCM 14558 T	AB274908
Microbacterium hominis	DSM 12509 T	AM181504
Microbacterium humi	CC012309 T	FJ865215
Microbacterium hydrocarbonoxydans	DSM 16089 T	AJ698726
Microbacterium imperiale	DSM 20530 T	X77442
Microbacterium indicum	BBH6 T	AM158907
Microbacterium insulae	DS-66 T	EU239498
Microbacterium invictum	DC-200 T	AM949677
Microbacterium keratanolyticum	IFO 13309 T	AB004717
Microbacterium ketosireducens	IFO 14548 T	AB004724
Microbacterium kitamiense	kitami C2 T	AB013907
Microbacterium koreense	JS53-2 T	AY962574
Microbacterium kribbense	MSL 04 T	EF466125
Microbacterium lacticum	DSM 20427 T	X77441
Microbacterium lacus	A5E-52 T	AB286030
Microbacterium laevaniformans	DSM 20140 T	Y17234
Microbacterium lindanitolerans	MNA2 T	EU873539
Microbacterium liquefaciens	DSM 20638 T	X77444
Microbacterium luteolum	IFO 15074 T	AB004718
Microbacterium luticocti	SC-087B T	AM747814
Microbacterium maritypicum	DSM 12512 T	AJ853910
Microbacterium mitrae	M4-8 T	GQ351351
Microbacterium natoriense	TNJL143-2 T	AY566291
Microbacterium oleivorans	DSM 16091 T	AJ698725
Microbacterium oxydans	DSM 20578 T	Y17227
Microbacterium paraoxydans	CF36 T	AJ491806

Species	Strain	Accession n° (NCBI GenBank database)
Microbacterium phyllosphaerae	DSM 13468 T	AJ277840
Microbacterium profundi	Shh49 T	EF623999
Microbacterium pseudoresistens	CC005209 T	FJ865214
Microbacterium pumilum	KV-488 T	AB234027
Microbacterium pygmaeum	KV-490 T	AB248875
Microbacterium radiodurans	GIMN1002 T	GQ329713
Microbacterium resistens	DMMZ1710 T	Y14699
Microbacterium saperdae	IFO 15038 T	AB004719
Microbacterium schleiferi	DSM 20489 T	Y17237
Microbacterium soli	DCY17 T	EF593038
Microbacterium terrae	IFO 15300 T	AB004720
Microbacterium terregens	IFO 12961 T	AB004721
Microbacterium terricola	KV-448 T	AB234025
Microbacterium testaceum	DSM 20166 T	X77445
Microbacterium thalassium	DSM 12511 T	AM181507
Microbacterium trichothecenolyticum	DSM 8608 T	Y17240
Microbacterium xylanilyticum	S3-E T	AJ853908
Microcella alkaliphila	AC4r T	AJ717385
Microcella putealis	CV2 T	AJ717388
Microterricola viridarii	KV-677 T	AB282862
Mycetocola lacteus	CM-10 T	AB012648
Mycetocola reblochoni	LMG 22367 T	DQ062097
Mycetocola saprophilus	CM-01 T	AB012647
Mycetocola tolaasinivorans	CM-05 T	AB012646
Okibacterium fritillariae	VKM Ac-2059 T	AB042094
Phycicola gilvus	SSWW-21 T	AM286414
Plantibacter auratus	IAM 18417 T	AB177868
Plantibacter flavus	P 297-02 T	AJ310417
Pseudoclavibacter chungangensis	CAU59 T	FJ514934
Pseudoclavibacter helvolus	DSM 20419 T	X77440
Pseudoclavibacter soli	KP02 T	AB329630
Rathayibacter caricis	VKM Ac-1799 T	AF159364
Rathayibacter festucae	DSM 15932 T	AM410683
Rathayibacter iranicus	DSM 7484 T	AM410684
Rathayibacter rathayi	DSM 7485 T	X77439
Rathayibacter toxicus	JCM 9669 T	D84127
Rathayibacter tritici	DSM 7486 T	X77438
Rhodoglobus aureus	CMS81 T	AJ438586
Rhodoglobus vestalii	LV3 T	AJ459101
Salinibacterium amurskyense	KMM3673 T	AF539697
Salinibacterium xinjiangense	0543 T	DQ515964
Schumannella luteola	KHIA T	AB362159
Subtercola boreus	**K300** T	AF224722
Subtercola frigoramans	**K265** T	AF224723
Yonghaparkia alkaliphila	KSL-113 T	DQ256087
Zimmermannella alba	IFO 15616 T	AB012590

Species	Strain	Accession n° (NCBI GenBank database)
Zimmermannella bifida	IAM 14848 T	AB012595
Zimmermannella faecalis	ATCC 13722 T	AF300651

Appendix II-4: Photographs of selection and counting of rain bacterial isolates

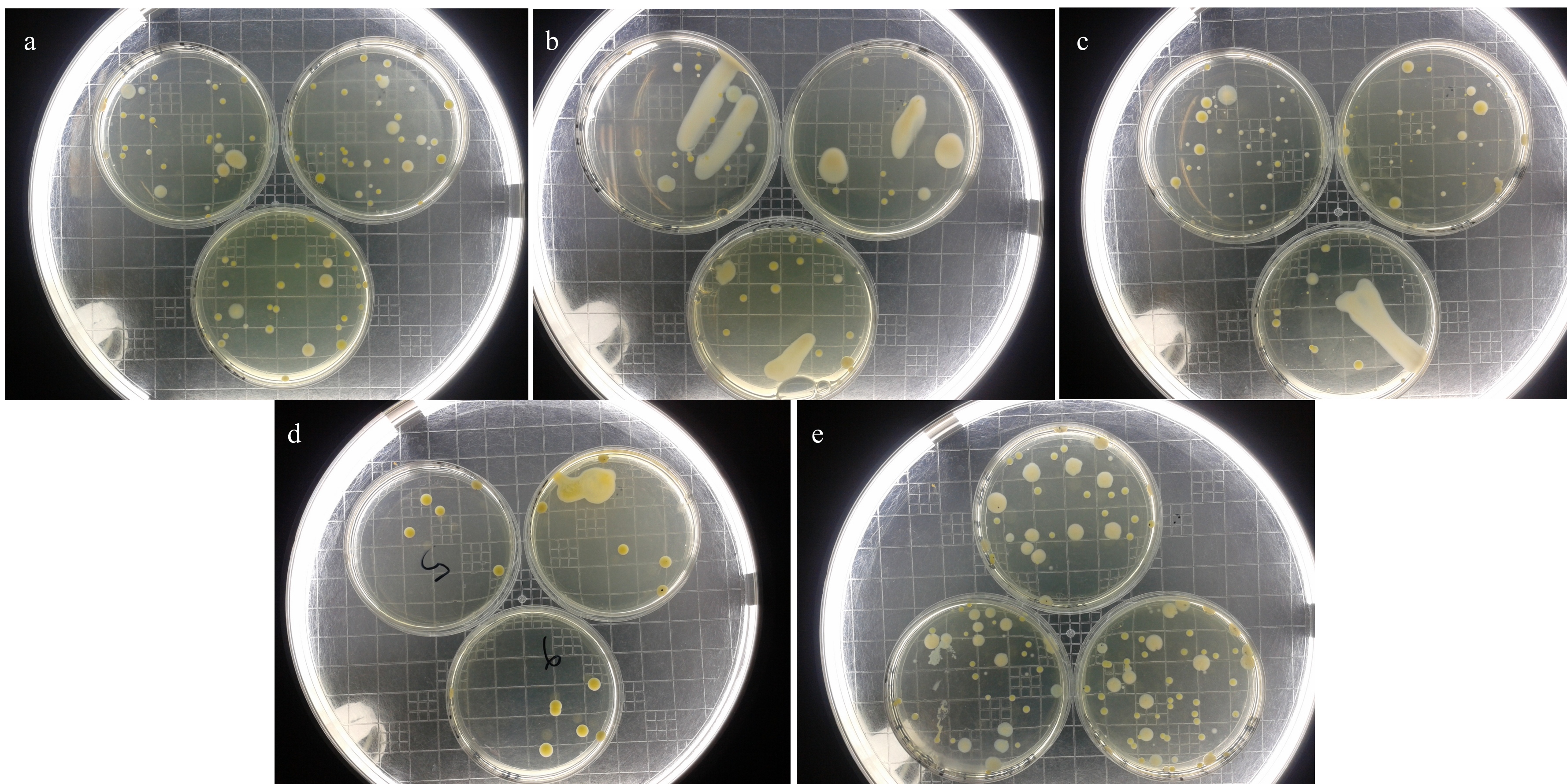


Figure 1: LBC medium agar plates of the 5 rain isolates. (a) Rain event 1 spread-plated at 10^{-2} dilution (b) Rain event 2 spread-plated at 10^{-3}. (c) Rain event 3 spread-plated at 10^{-2}. (d) Rain event 4 spread-plated at 10^{-2}. (e) Rain event 5 spread-plated at 10^{-2}.

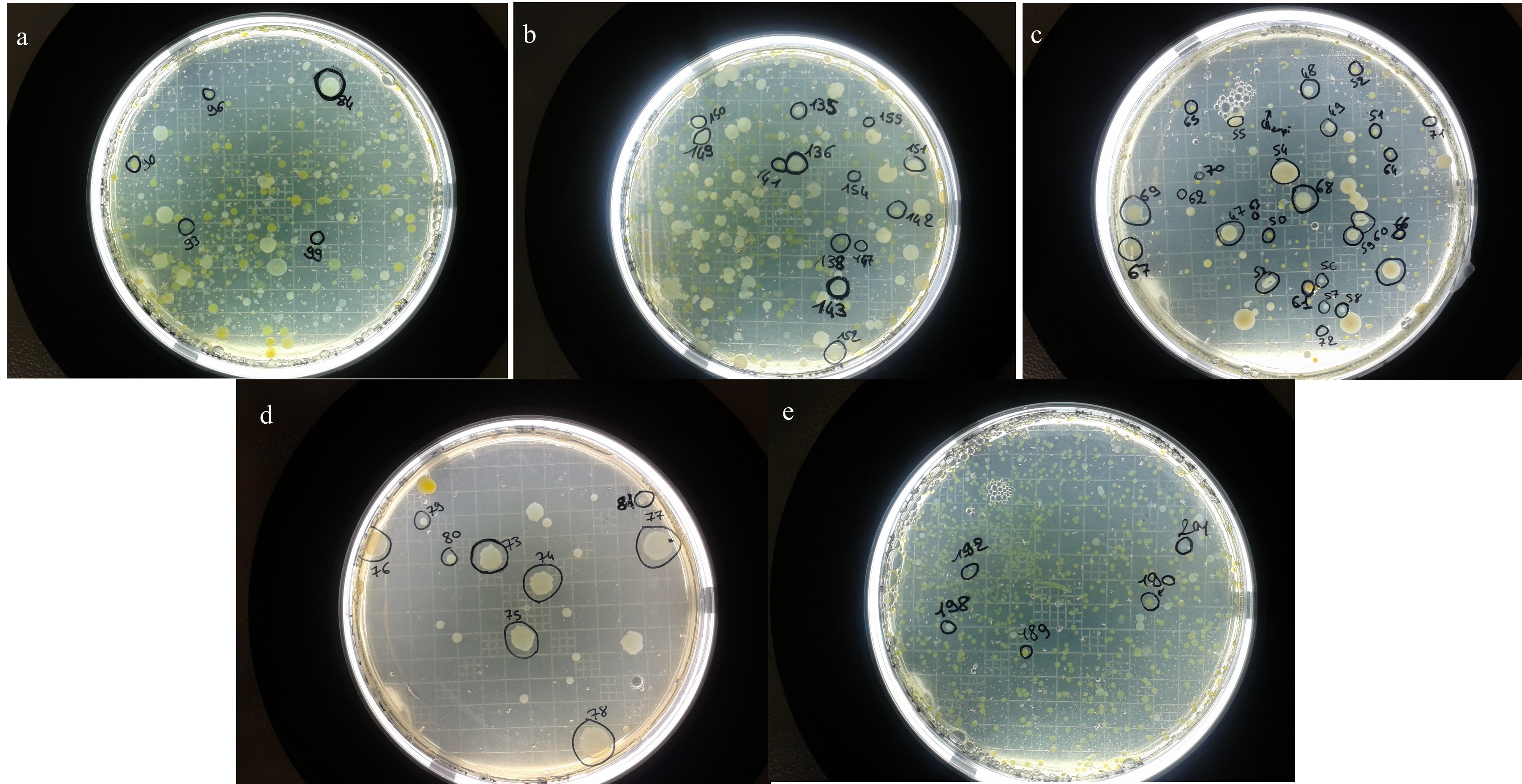


Figure 2: KBC medium agar plates of the 5 rain isolates. (a) Rain event 1 spread-plated at 10^0 (one of 3 Petri dishes). (b) Rain event 2 spread-plated at 10^0 (one of 3 Petri dishes). (c) Rain event 3 spread-plated at 10^0. (d) Rain event 4 spread-plated at 10^0. (e) Rain event 5 spread-plated at 10^0 (one of 3 Petri dishes). ("Sc" sample for rain events 3 and 4 and "Sp" sample for rain events 1, 2 and 5).

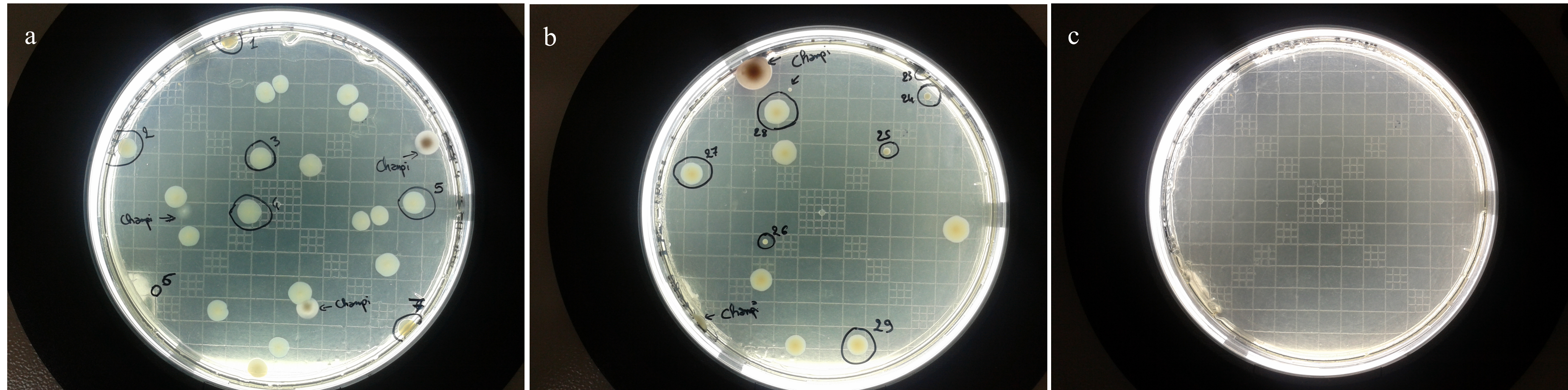


Figure 3: LBCTK medium agar plates of the three selected rain event isolates after incubation with pBLN ("Sp" sample). (a) Rain event 1 spread-plated at 10^0. (b) Rain event 2 spread-plated at 10^0. (c) Rain event 5 spread-plated at 10^0.

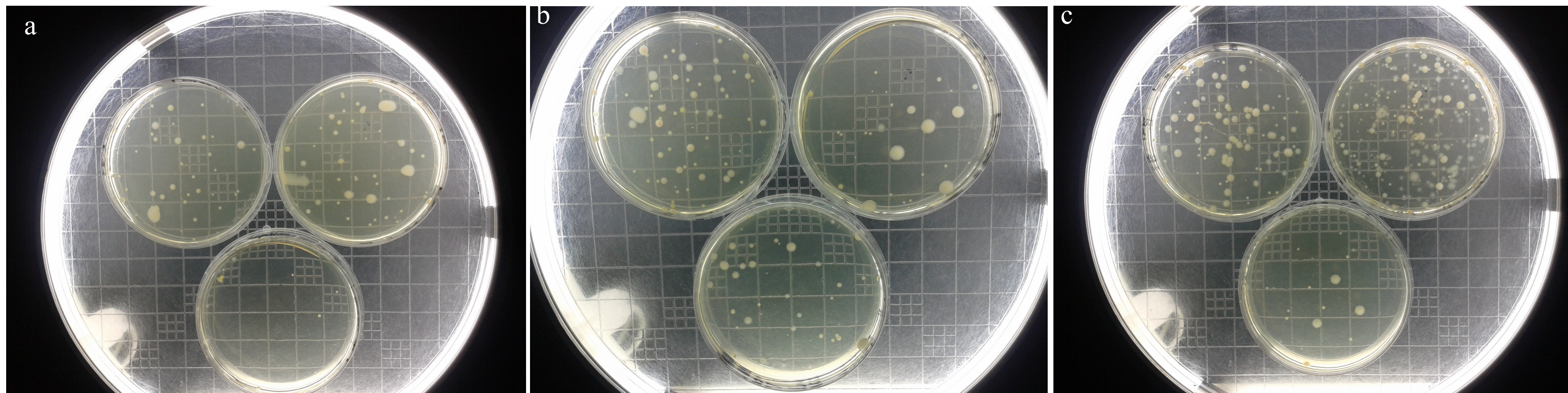


Figure 4: LBC medium agar plates of the three selected rain event isolates after electroporation with pBLN ("Ss" sample). (a) Rain event 1 spread-plated at 10^{-2}. (b) Rain event 2 spread-plated at 10^{-2}. (c) Rain event 5 spread-plated at 10^{-2}.

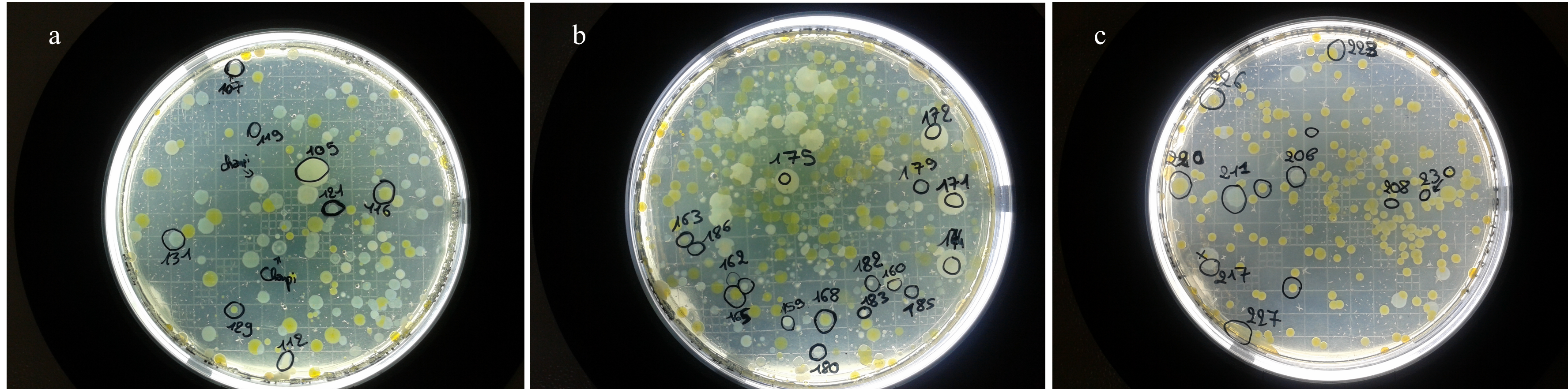


Figure 5: KBC medium agar plates of the three selected rain event isolates after electroporation with pBLN ("Ss" sample). (a) Rain event 1 spread-plated at 10^0. (b) Rain event 2 spread-plated at 10^0. (c) Rain event 5 spread-plated at 10^0.

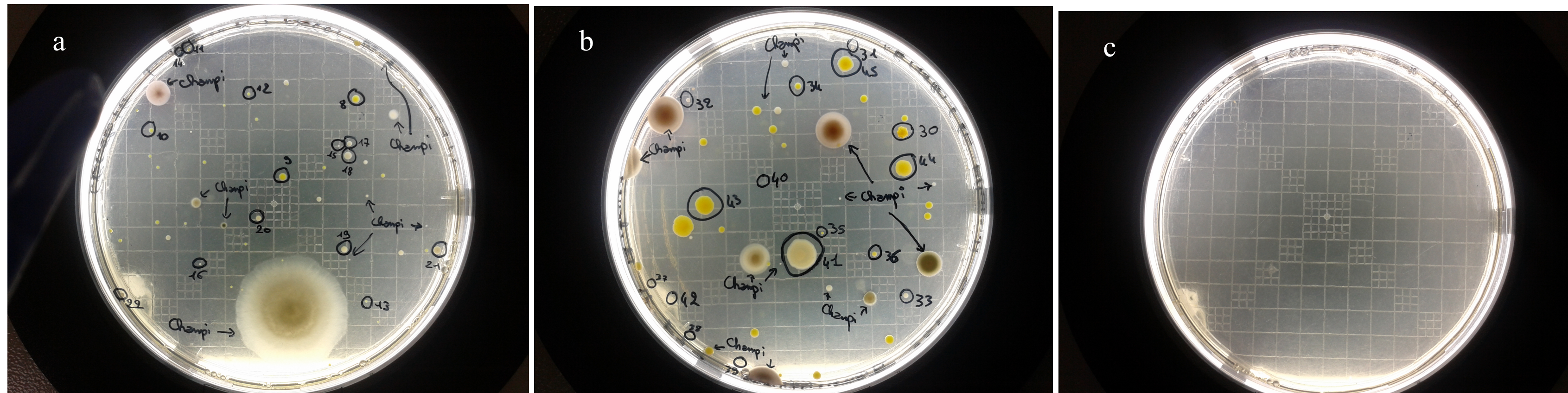


Figure 6: LBCTK medium agar plates of the three selected rain event isolates after electroporation with pBLN ("Ss" sample). (a) Rain event 1 spread-plated at 10^0. (b) Rain event 2 spread-plated at 10^0. (c) Rain event 5 spread-plated at 10^0.

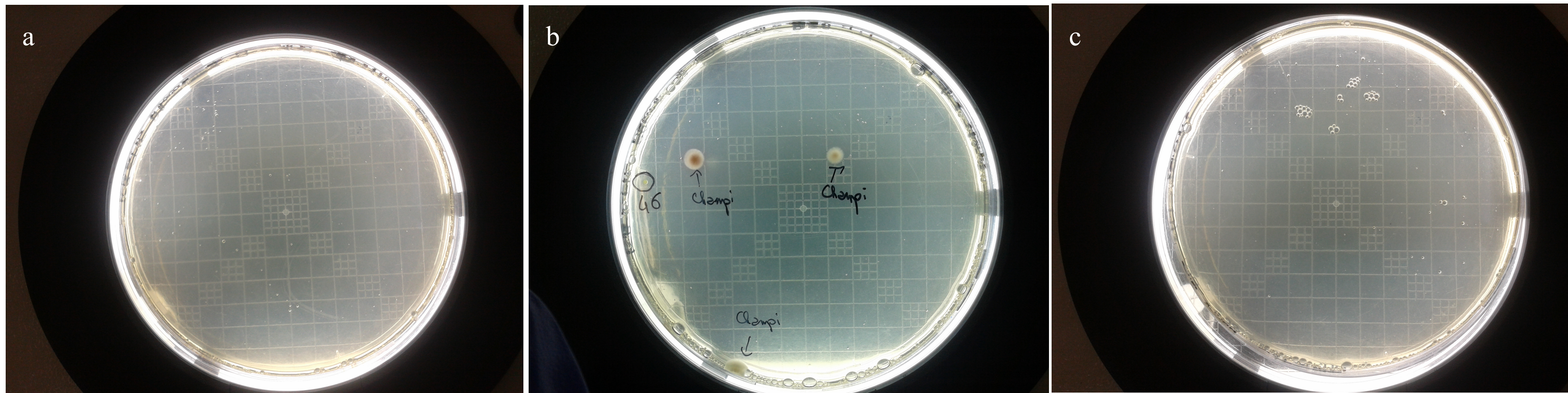


Figure 7: KBCTK medium agar plates of the three selected rain event isolates after electroporation with pBLN ("Ss" sample). (a) Rain event 1 spread-plated at 10^0. (b) Rain event 2 spread-plated at 10^0. (c) Rain event 5 spread-plated at 10^0.

Appendix II-5: Identification of isolates from the five rains based on phylogenetic analyses.

Table 1: Identification of isolates from the five rains based on phylogenies reconstructed using maximum likelihood method implemented in PhyML.
The closest reference species, their accession numbers as well as the origin of isolation and the authors having isolated this strain are indicated in the table. (T) indicates typ species n.a.: not applicable.

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R1SpM1P1C2 R1SpM1P1C4 R1SpM1P1C5 R1SpM1P1C7	Enterobacteriaceae (Gamma-Proteobacteria)	*Escherichia*	n.a.	- *E. coli* SE11 - *E. coli* SMS-3-5 - *E. coli* UMN026	- Human feces - Shipyard Creek, USA - Urinary tract	- (Oshima *et al.*, 2008) - (Fricke *et al.*, 2008) - (Touchon *et al.*, 2009)	AP009240 CP000970 CU928163
R1SpM1P1C6	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens (fluorescens)*	- *P. lurida* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 2007)	AJ581999
R1SpM3P1C1 R1SpM3P1C2 R1SpM3P2C1 R1SpM3P3C1	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens (fluorescens)*	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009
R1SpM3P1C4 R1SpM3P2C4 R1SpM3P3C2	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea / P. rhizosphaerae*	- *P. abietaniphila* (T) - *P. graminis* (T) - *P. rhizosphaerae* (T)	- Pulpmill effluent - Phyllosphere of grasses - Rhizosphere of grasses	- (Mohn *et al.*, 1999) - (Behrendt *et al.*, 1999) - (Peix *et al.*, 2003)	AJ011504 Y11150 AY152673
R1SpM3P1C5 R1SpM3P1C6 R1SpM3P2C5 R1SpM3P3C3 R1SpM3P3C4	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150
R1SpM3P2C2	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens*	/	/	/	/
R1SsM1P1C1	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens (fluorescens)*	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R1SsM1P1C3 **R1SsM1P1C4**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. lurida* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 2007)	AJ581999
R1SsM1P1C5 **R1SsM1P1C6** **R1SsM1P1C7**	Enterobacteriaceae (Gamma-Proteobacteria)	*Escherichia*	n.a.	- *E. coli* SE11 - *E. coli* SMS-3-5 - *E. coli* UMN026	- Human feces - Shipyard Creek, USA - Urinary tract	- (Oshima *et al.*, 2008) - (Fricke *et al.*, 2008) - (Touchon *et al.*, 2009)	AP009240 CP000970 CU928163
R1SsM3P1C1 **R1SsM3P1C4** **R1SsM3P2C1** **R1SsM3P3C1**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009
R1SsM3P1C2	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. oryzihabitans*	- *P. oleovorans* (T) - *P. oryzihabitans* (T) - *P. psychrotolerans* (T)	- Water-oil emulsions - Rice paddy - Water under a dog's cage (Austria)	- (Lee and Chandler, 1941) - (Kodama *et al.*, 1985) - (Hauser *et al.*, 2004)	D84018 GQ250598 AJ575816
R1SsM3P1C5 **R1SsM3P1C6** **R1SsM3P2C3** **R1SsM3P2C5** **R1SsM3P3C3**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens*	/	/	/	/
R1SsM3P1C7 **R1SsM3P3C5** **R1SsM3P3C9**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. amygdali* (T) - *P. avellanae* (T) - *P. caricapapayae* (T) - *P. cichorii* (T) - *P. congelans* (T) - *P. coronafaciens* (T) - *P. ficuserectae* (T) - *P. meliae* (T) - *P. protegens* (T) - *P. saponiphila* (T) - *P. savastanoi* (T) - *P. syringae* (T) - *P. tremae* (T) - *P. viridiflava* (T)	- Almond - *Corylus avellana* (bacterial twig dieback) - *Carica papaya* - *Cichorium endivia* (endive) - Phyllosphere of grasses - Halo blight of oats - *Ficus erectae* - Bacterial gall of chinaberry (*Melia azedarach*) - Soil suppressing black root rot of tobacco - Xenobiotic degrader - *Olea europaea* (olive tree) - Lilac tree - *Trema orientalis* (charcoal-tree) - Bean	- (Psallidas and Panagopoulos, 1975) - (Janse *et al.*, 1996) - (Robbs, 1956) - (Stapp, 1928) - (Behrendt *et al.*, 2003) - (Ait Tayeb *et al.*, 2005) - (Goto, 1983) - (Ogimi, 1977) - (Ramette *et al.*, 2011) - (Lang *et al.*, 2010) - (Gardan *et al.*, 1992) - (van Hall, 1902) - (Gardan *et al.*, 1999) - (Dowson, 1939)	Z76654 AJ889839 D84010 Z76658 AJ492828 Z76660 AB021378 AB021382 AJ278812 FM208264 AB021402 DQ318866 AJ492826 AY180972

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R1SsM3P1C9	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. rhizosphaerae*	- *P. rhizosphaerae* (T)	- Rhizosphere of grasses	- (Peix *et al.*, 2003)	AY152673
R1SsM3P1C10 **R1SsM3P2C4** **R1SsM3P3C4**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea* / *P. rhizosphaerae*	- *P. abietaniphila* (T) - *P. graminis* (T) - *P. rhizosphaerae* (T)	- Pulpmill effluent - Phyllosphere of grasses - Rhizosphere of grasses	- (Mohn *et al.*, 1999) - (Behrendt *et al.*, 1999) - (Peix *et al.*, 2003)	AJ011504 Y11150 AY152673
R1SsM3P1C11 **R1SsM3P3C2**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*koreensis*)	- *P. clemencea* (T) - *P. koreensis* (T) - *P. moraviensis* (T) - *P. teessidea* (T)	- Northeast England soil - Farm soils (Korea) - Soil besides highway (Czech Republic) - Northeast England soil	- (Rahman *et al.*, 2009) - (Kwon *et al.*, 2003) - (Tvrzová *et al.*, 2006) - (Rahman *et al.*, 2009)	AM419155 AF468452 AY970952 AM419154
R1SsM3P1C12 **R1SsM3P2C7** **R1SsM3P3C8** **R1SsM3P3C12**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150
R1SsM3P2C2	Microbacteriaceae (Actinobacteria)	*Microbacterium*	n.a.	- *M. agarici* (T) - *M. arabinogalactanolyticum* (T) - *M. aquimaris* (T) - *M. esteraromaticum* (T) - *M. halophilum* (T) - *M. hatanonis* (T) - *M. humi* (T) - *M. lindanitolerans* (T) - *M. liquefaciens* (T) - *M. luteolum* (T) - *M. maritypicum* (T) - *M. profundi* (T) - *M. oxydans* (T) - *M. paraoxydans* (T) - *M. pseudoresistens* (T) - *M. resistens* (T) - *M. saperdae* (T) - *M. soli* (T) - *M. testaceum* (T)	- *Agaricusblazei* stalk (mushroom, Taiwan) - Soil - Seawater (Korea) - Unknown - Mangrove rhizosphere - Hairspray (Japan) - *Agaricusblazei* stalk (mushroom, Taiwan) - Lindane-contaminated soil (India) - Milk - Soil - Sea water and marine mud - Deep-sea sediment (East Pacific Ocean polymetallic nodule region) - Air, contaminated hospital material and growth medium compounds - Leukemic child blood and mouse liver - *Agaricusblazei* stalk (mushroom, Taiwan) - Corneal ulcer - *Saperda carcharias* (insect) - Ginseng field soil (Korea) - Rice	- (Young *et al.*, 2010) - (Takeuchi and Hatano, 1998b) - (Kim *et al.*, 2008) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998a) - (Bakir *et al.*, 2008) - (Young *et al.*, 2010) - (Lal *et al.*, 2010) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998a) - (Wu *et al.*, 2008) - (Schumann *et al.*, 1999) - (Buczolits *et al.*, 2008) - (Young *et al.*, 2010) - (Behrendt *et al.*, 2001) - (Takeuchi and Hatano, 1998b) - (Srinivasan *et al.*, 2010) - (Takeuchi and Hatano, 1998b)	FJ807673 Y17228 AM778449 KC422704 AB004714 AB274908 FJ865215 EU873539 X77444 AB004718 AJ853910 EF623999 Y17227 AJ491806 FJ865214 Y14699 AB004719 EF593042 X77445

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R2SpM1P1C2 **R2SpM1P1C3** **R2SpM1P1C4**	Microbacteriaceae (Actinobacteria)	*Microbacterium*	n.a.	- *M. agarici* (T) - *M. arabinogalactanolyticum* (T) - *M. aquimaris* (T) - *M. esteraromaticum* (T) - *M. halophilum* (T) - *M. hatanonis* (T) - *M. humi* (T) - *M. lindanitolerans* (T) - *M. liquefaciens* (T) - *M. luteolum* (T) - *M. maritypicum* (T) - *M. profundi* (T) - *M. oxydans* (T) - *M. paraoxydans* (T) - *M. pseudoresistens* (T) - *M. resistens* (T) - *M. saperdae* (T) - *M. soli* (T) - *M. testaceum* (T)	- *Agaricusblazei* stalk (mushroom, Taiwan) - Soil - Seawater (Korea) - Unknown - Mangrove rhizosphere - Hairspray (Japan) - *Agaricusblazei* stalk (mushroom, Taiwan) - Lindane-contaminated soil (India) - Milk - Soil - Sea water and marine mud - Deep-sea sediment (East Pacific Ocean polymetallic nodule region) - Air, contaminated hospital material and growth medium compounds - Leukemic child blood and mouse liver - *Agaricusblazei* stalk (mushroom, Taiwan) - Corneal ulcer - *Saperda carcharias* (insect) - Ginseng field soil (Korea) - Rice	- (Young *et al.*, 2010) - (Takeuchi and Hatano, 1998b) - (Kim *et al.*, 2008) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998a) - (Bakir *et al.*, 2008) - (Young *et al.*, 2010) - (Lal *et al.*, 2010) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998a) - (Wu *et al.*, 2008) - (Schumann *et al.*, 1999) - (Buczolits *et al.*, 2008) - (Young *et al.*, 2010) - (Behrendt *et al.*, 2001) - (Takeuchi and Hatano, 1998b) - (Srinivasan *et al.*, 2010) - (Takeuchi and Hatano, 1998b)	FJ807673 Y17228 AM778449 KC422704 AB004714 AB274908 FJ865215 EU873539 X77444 AB004718 AJ853910 EF623999 Y17227 AJ491806 FJ865214 Y14699 AB004719 EF593042 X77445
R2SpM1P1C5	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. rhodesiae* (T)	- Natural mineral water (France)	- (Coroler *et al.*, 1996)	AF064459
R2SpM1P1C7 **R2SpM1P1C13**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. lurida* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 2007)	AJ581999
R2SpM1P1C8	Enterobacteriaceae (Gamma-Proteobacteria)	*Escherichia*	n.a.	- *E. coli* SE11 - *E. coli* SMS-3-5 - *E. coli* UMN026	- Human feces - Shipyard Creek, USA - Urinary tract	- (Oshima *et al.*, 2008) - (Fricke *et al.*, 2008) - (Touchon *et al.*, 2009)	AP009240 CP000970 CU928163

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R2SpM1P1C9	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. antarctica* (T) - *P. arsenicoxydans* (T) - *P. costantinii* (T) - *P. extremaustralis* (T) - *P. extremorientalis* (T) - *P. fluorescens* (T) - *P. frederiksbergensis* (T) - *P. grimontii* (T) - *P. lini* (T) - *P. lurida* (T) - *P. mandelii* (T) - *P. marginalis* (T) - *P. meridiana* (T) - *P. orientalis* (T) - *P. palleroniana* (T) - *P. poae* (T) - *P. rhodesiae* (T) - *P. salomonii* (T) - *P. simiae* (T) - *P. tolaasii* (T) - *P. trivialis* (T) - *P. veronii* (T)	- Cyanobacterial mat sample (Antarctica) - Sediment (Atacama desert, Chile) - Roots of *Arabidopsis thaliana* (Germany) - Temporary pond (Antarctica) - Drinking water reservoir (Russia) - Soil, water - Coal gasification site (Denmark) - Natural mineral waters (France) - Rhizospheric soil of Linum (flax) - Phyllosphere of grasses - Mineral waters - Infectedchick-pea and soft rot of potato tuber - Cyanobacterial mat sample (Antarctica) - Spring water (Lebanon) - *Oryza sativa* (rice - Cameroon) - Phyllosphere of grasses - Natural mineral water (France) - Garlic - Liver of a White-headed marmoset - *Agaricus bisporus* (common mushroom) - Phyllosphere of grasses - Natural mineral waters (France)	- (Reddy *et al.*, 2004) - (Campos *et al.*, 2010) - (Munsch *et al.*, 2002) - (López *et al.*, 2009) - (Ivanova *et al.*, 2002) - (Migula, 1895) - (Andersen *et al.*, 2000) - (Baïda *et al.*, 2002) - (Delorme *et al.*, 2002) - (Behrendt *et al.*, 2007) - (Verhille *et al.*, 1999) - (Stevens, 1925) - (Reddy *et al.*, 2004) - (Dabboussi *et al.*, 1999) - (Gardan *et al.*, 2002) - (Behrendt *et al.*, 2003) - (Coroler *et al.*, 1996) - (Gardan *et al.*, 2002) - (Vela *et al.*, 2006) - (Paine, 1919) - (Behrendt *et al.*, 2003) - (Elomari *et al.*, 1996)	AJ537601 FN645213 JX979126 AJ583501 AF405328 D84013 AJ249382 AF268029 AY035996 AJ581999 AF058286 Z76663 AJ537602 AF064457 AY091527 AJ492829 AF064459 AY091528 AJ936933 AF255336 AJ492831 AF064460
R2SpM1P1C10 **R2SpM1P1C11** **R2SpM1P1C12**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fragi*)	- *P. fragi* (T) - *P. deceptionensis* (T) - *P. psychrophila* (T)	- Milk - Antarctic sediment sample - Cold room for food storage	- (Griiber, 1905) - (Carrión *et al.*, 2011) - (Yumoto *et al.*, 2001)	AF094733 GU936597 AB041885
R2SpM1P1C15	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009
R2SpM3P1C1 **R2SpM3P2C1** **R2SpM3P2C2**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens*	/	/	/	/
R2SpM3P1C2 **R2SpM3P2C3** **R2SpM3P3C1**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R2SpM3P1C3 **R2SpM3P2C8** **R2SpM3P2C9** **R2SpM3P2C10** **R2SpM3P2C11**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009
R2SpM3P1C4 **R2SpM3P1C5** **R2SpM3P2C6**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. rhodesiae* (T)	- Natural mineral water (France)	- (Coroler *et al.*, 1996)	AF064459
R2SpM3P1C8	Microbacteriaceae (Actinobacteria)	*Plantibacter*	n.a.	- *P. auratus* (T) - *P. flavus* (T)	- Unknown - Phyllosphere of grasses	- (Lin and Yokota, 2006) - (Behrendt *et al.*, 2002)	AB177868 AJ310417
R2SpM3P2C4 **R2SpM3P2C5**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea* / *P. rhizosphaerae*	- *P. abietaniphila* (T) - *P. graminis* (T) - *P. rhizosphaerae* (T)	- Pulpmill effluent - Phyllosphere of grasses - Rhizosphere of grasses	- (Mohn *et al.*, 1999) - (Behrendt *et al.*, 1999) - (Peix *et al.*, 2003)	AJ011504 Y11150 AY152673
R2SpM3P3C2	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. amygdali* (T) - *P. cannabina* (T) - *P. caricapapayae* (T) - *P. cichorii* (T) - *P. congelans* (T) - *P. ficuserectae* (T) - *P. savastanoi* (T) - *P. syringae* (T) - *P. tremae* (T) - *P. viridiflava* (T)	- Almond - *Cannabis Sativa* - *Carica papaya* - *Cichorium endivia* (endive) - Phyllosphere of grasses - *Ficus erectae* - *Olea europaea* (olive tree) - Lilac tree - *Trema orientalis* (charcoal-tree) - Bean	- (Psallidas and Panagopoulos, 1975) - (Gardan *et al.*, 1999) - (Robbs, 1956) - (Stapp, 1928) - (Behrendt *et al.*, 2003) - (Goto, 1983) - (Gardan *et al.*, 1992) - (van Hall, 1902) - (Gardan *et al.*, 1999) - (Dowson, 1939)	Z76654 AJ492827 D84010 Z76658 AJ492828 AB021378 AB021402 DQ318866 AJ492826 AY180972
R2SpM3P3C3	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. putida* / *P. fluorescens* (*asplenii*)	- *P. alkylphenolia* (T) - *P. asplenii* (T) - *P. cinnamophila* (T) - *P. fuscovaginae* (T) - *P. japonica* (T) - *P. multiaromavorans* (T) - *P. vranovensis* (T)	- Industrial complex soils in Changwon, Korea - *Asplenium nidus* - Soil - *Oriza sativa* - Activated sludge sample - Soil - Soil in South Moravia	- (Jeong *et al.*, 2003) - (Tvrzová *et al.*, 2006) - (Nonaka *et al.*, 2008) - (Miyajima *et al.*, 1983) - (Pungrasmi *et al.*, 2008) - (Nonaka *et al.*, 2008) - (Tvrzová *et al.*, 2006)	AY324319 Z76655 AB302401 FJ483519 AB126621 AB302402 AY970951

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R2SsM1P1C1 **R2SsM1P1C14** **R2SsM1P1C15** **R2SsM1P1C16**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. rhizosphaerae*	- *P. rhizosphaerae* (T)	- Rhizosphere of grasses	- (Peix *et al.*, 2003)	AY152673
R2SsM1P1C3	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fragi*)	- *P. fragi* (T) - *P. deceptionensis* (T) - *P. psychrophila* (T)	- Milk - Antarctic sediment sample - Cold room for food storage	- (Griiber, 1905) - (Carrión *et al.*, 2011) - (Yumoto *et al.*, 2001)	AF094733 GU936597 AB041885
R2SsM1P1C4 **R2SsM1P1C9** **R2SsM1P1C11**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. lurida* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 2007)	AJ581999
R2SsM1P1C5 **R2SsM1P1C6** **R2SsM1P1C7**	Microbacteriaceae (Actinobacteria)	*Microbacterium*	n.a.	- *M. agarici* (T) - *M. arabinogalactanolyticum* (T) - *M. aquimaris* (T) - *M. esteraromaticum* (T) - *M. halophilum* (T) - *M. hatanonis* (T) - *M. humi* (T) - *M. lindanitolerans* (T) - *M. liquefaciens* (T) - *M. luteolum* (T) - *M. maritypicum* (T) - *M. profundi* (T) - *M. oxydans* (T) - *M. paraoxydans* (T) - *M. pseudoresistens* (T) - *M. resistens* (T) - *M. saperdae* (T) - *M. soli* (T) - *M. testaceum* (T)	- *Agaricusblazei* stalk (mushroom, Taiwan) - Soil - Seawater (Korea) - Unknown - Mangrove rhizosphere - Hairspray (Japan) - *Agaricusblazei* stalk (mushroom, Taiwan) - Lindane-contaminated soil (India) - Milk - Soil - Sea water and marine mud - Deep-sea sediment (East Pacific Ocean polymetallic nodule region) - Air, contaminated hospital material and growth medium compounds - Leukemic child blood and mouse liver - *Agaricusblazei* stalk (mushroom, Taiwan) - Corneal ulcer - *Saperda carcharias* (insect) - Ginseng field soil (Korea) - Rice	- (Young *et al.*, 2010) - (Takeuchi and Hatano, 1998b) - (Kim *et al.*, 2008) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998a) - (Bakir *et al.*, 2008) - (Young *et al.*, 2010) - (Lal *et al.*, 2010) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998b) - (Takeuchi and Hatano, 1998a) - (Wu *et al.*, 2008) - (Schumann *et al.*, 1999) - (Buczolits *et al.*, 2008) - (Young *et al.*, 2010) - (Behrendt *et al.*, 2001) - (Takeuchi and Hatano, 1998b) - (Srinivasan *et al.*, 2010) - (Takeuchi and Hatano, 1998b)	FJ807673 Y17228 AM778449 KC422704 AB004714 AB274908 FJ865215 EU873539 X77444 AB004718 AJ853910 EF623999 Y17227 AJ491806 FJ865214 Y14699 AB004719 EF593042 X77445

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R2SsM1P1C10 **R2SsM1P1C12**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. antarctica* (T) - *P. arsenicoxydans* (T) - *P. costantinii* (T) - *P. extremaustralis* (T) - *P. extremorientalis* (T) - *P. fluorescens* (T) - *P. frederiksbergensis* (T) - *P. grimontii* (T) - *P. lini* (T) - *P. lurida* (T) - *P. mandelii* (T) - *P. marginalis* (T) - *P. meridiana* (T) - *P. orientalis* (T) - *P. palleroniana* (T) - *P. poae* (T) - *P. rhodesiae* (T) - *P. salomonii* (T) - *P. simiae* (T) - *P. tolaasii* (T) - *P. trivialis* (T) - *P. veronii* (T)	- Cyanobacterial mat sample (Antarctica) - Sediment (Atacama desert, Chile) - Roots of *Arabidopsis thaliana* (Germany) - Temporary pond (Antarctica) - Drinking water reservoir (Russia) - Soil, water - Coal gasification site (Denmark) - Natural mineral waters (France) - Rhizospheric soil of Linum (flax) - Phyllosphere of grasses - Mineral waters - Infectedchick-pea and soft rot of potato tuber - Cyanobacterial mat sample (Antarctica) - Spring water (Lebanon) - *Oryza sativa* (rice - Cameroon) - Phyllosphere of grasses - Natural mineral water (France) - Garlic - Liver of a White-headed marmoset - *Agaricus bisporus* (common mushroom) - Phyllosphere of grasses - Natural mineral waters (France)	- (Reddy *et al.*, 2004) - (Campos *et al.*, 2010) - (Munsch *et al.*, 2002) - (López *et al.*, 2009) - (Ivanova *et al.*, 2002) - (Migula, 1895) - (Andersen *et al.*, 2000) - (Baïda *et al.*, 2002) - (Delorme *et al.*, 2002) - (Behrendt *et al.*, 2007) - (Verhille *et al.*, 1999) - (Stevens, 1925) - (Reddy *et al.*, 2004) - (Dabboussi *et al.*, 1999) - (Gardan *et al.*, 2002) - (Behrendt *et al.*, 2003) - (Coroler *et al.*, 1996) - (Gardan *et al.*, 2002) - (Vela *et al.*, 2006) - (Paine, 1919) - (Behrendt *et al.*, 2003) - (Elomari *et al.*, 1996)	AJ537601 FN645213 JX979126 AJ583501 AF405328 D84013 AJ249382 AF268029 AY035996 AJ581999 AF058286 Z76663 AJ537602 AF064457 AY091527 AJ492829 AF064459 AY091528 AJ936933 AF255336 AJ492831 AF064460
R2SsM2P1C1	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. rhizosphaerae*	- *P. rhizosphaerae* (T)	- Rhizosphere of grasses	- (Peix *et al.*, 2003)	AY152673
R2SsM3P1C1 **R2SsM3P2C1**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens*	/	/	/	/
R2SsM3P1C2 **R2SsM3P1C13**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*koreensis*)	- *P. clemencea* (T) - *P. koreensis* (T) - *P. moraviensis* (T) - *P. teessidea* (T)	- Northeast England soil - Farm soils (Korea) - Soil besides highway (Czech Republic) - Northeast England soil	- (Rahman *et al.*, 2009) - (Kwon *et al.*, 2003) - (Tvrzová *et al.*, 2006) - (Rahman *et al.*, 2009)	AM419155 AF468452 AY970952 AM419154

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R2SsM3P1C3 **R2SsM3P1C4** **R2SsM3P1C5** **R2SsM3P1C6** **R2SsM3P2C3** **R2SsM3P2C4**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150
R2SsM3P1C7 **R2SsM3P1C8** **R2SsM3P2C7**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009
R2SsM3P1C14 **R2SsM3P2C9** **R2SsM3P2C10** **R2SsM3P2C12**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. rhodesiae* (T)	- Natural mineral water (France)	- (Coroler *et al.*, 1996)	AF064459
R2SsM3P2C5	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. frederiksbergensis* (T) - *P. mandelii* (T)	- Coal gasification site (Denmark) - Mineral waters	- (Andersen *et al.*, 2000) - (Verhille *et al.*, 1999)	AJ249382 AF058286
R2SsM3P2C13	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*koreensis*)	- *P. clemencea* (T)	- Northeast England soil	- (Rahman *et al.*, 2009)	AM419155
R3ScM3P1C1	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fragi*)	- *P. fragi* (T) - *P. psychrophila* (T)	- Milk - Cold room for food storage	- (Griiber, 1905) - (Yumoto *et al.*, 2001)	AF094733 AB041885
R3ScM3P1C4 **R3ScM3P1C5** **R3ScM3P1C18** **R3ScM3P1C19** **R3ScM3P1C20**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150
R3ScM3P1C6	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea* / *P. rhizosphaerae*	- *P. abietaniphila* (T) - *P. graminis* (T) - *P. rhizosphaerae* (T)	- Pulpmill effluent - Phyllosphere of grasses - Rhizosphere of grasses	- (Mohn *et al.*, 1999) - (Behrendt *et al.*, 1999) - (Peix *et al.*, 2003)	AJ011504 Y11150 AY152673
R3ScM3P1C7 **R3ScM3P1C9**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens*	/	/	/	/

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R3ScM3P1C8	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fragi*)	- *P. fragi* (T) - *P. deceptionensis* (T) - *P. psychrophila* (T)	- Milk - Antarctic sediment sample - Cold room for food storage	- (Griiber, 1905) - (Carrión *et al.*, 2011) - (Yumoto *et al.*, 2001)	AF094733 GU936597 AB041885
R3ScM3P1C11	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. amygdali* (T) - *P. avellanae* (T) - *P. caricapapayae* (T) - *P. cichorii* (T) - *P. congelans* (T) - *P. coronafaciens* (T) - *P. ficuserectae* (T) - *P. meliae* (T) - *P. protegens* (T) - *P. saponiphila* (T) - *P. savastanoi* (T) - *P. syringae* (T) - *P. tremae* (T) - *P. viridiflava* (T)	- Almond - *Corylus avellana* (bacterial twig dieback) - *Carica papaya* - *Cichorium endivia* (endive) - Phyllosphere of grasses - Halo blight of oats - *Ficus erectae* - Bacterial gall of chinaberry (*Melia azedarach*) - Soil suppressing black root rot of tobacco - Xenobiotic degrader - *Olea europaea* (olive tree) - Lilac tree - *Trema orientalis* (charcoal-tree) - Bean	- (Psallidas and Panagopoulos, 1975) - (Janse *et al.*, 1996) - (Robbs, 1956) - (Stapp, 1928) - (Behrendt *et al.*, 2003) - (Ait Tayeb *et al.*, 2005) - (Goto, 1983) - (Ogimi, 1977) - (Ramette *et al.*, 2011) - (Lang *et al.*, 2010) - (Gardan *et al.*, 1992) - (van Hall, 1902) - (Gardan *et al.*, 1999) - (Dowson, 1939)	Z76654 AJ889839 D84010 Z76658 AJ492828 Z76660 AB021378 AB021382 AJ278812 FM208264 AB021402 DQ318866 AJ492826 AY180972
R3ScM3P1C13 **R3ScM3P1C14**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. rhodesiae* (T)	- Natural mineral water (France)	- (Coroler *et al.*, 1996)	AF064459
R3ScM3P1C16 **R3ScM3P1C17**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. rhizosphaerae*	- *P. rhizosphaerae* (T)	- Rhizosphere of grasses	- (Peix *et al.*, 2003)	AY152673
R3ScM3P1C21 **R3ScM3P1C22** **R3ScM3P1C23** **R3ScM3P1C24** **R3ScM3P1C25**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. fluorescens* (*fluorescens*)	- *P. azotoformans* (T)	- Cereal grains	- (Iizuka and Komagata, 1963)	D84009

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R4ScM3P1C1 **R4ScM3P1C2** **R4ScM3P1C3**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. caricapapayae* (T) - *P. cichorii* (T) - *P. coronafaciens* (T) - *P. meliae* (T) - *P. protegens* (T) - *P. saponiphila* (T) - *P. savastanoi* (T) - *P. tremae* (T)	- *Carica papaya* - *Cichorium endivia* (endive) - Halo blight of oats - Bacterial gall of chinaberry (*Melia azedarach*) - Soil suppressing black root rot of tobacco - Xenobiotic degrader - *Olea europaea* (olive tree) - *Trema orientalis* (charcoal-tree)	- (Robbs, 1956) - (Stapp, 1928) - (Ait Tayeb *et al.*, 2005) - (Ogimi, 1977) - (Ramette *et al.*, 2011) - (Lang *et al.*, 2010) - (Gardan *et al.*, 1992) - (Gardan *et al.*, 1999)	D84010 Z76658 Z76660 AB021382 AJ278812 FM208264 AB021402 AJ492826
R5SpM3P1C1	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea / P. rhizosphaerae*	- *P. abietaniphila* (T) - *P. graminis* (T) - *P. rhizosphaerae* (T)	- Pulpmill effluent - Phyllosphere of grasses - Rhizosphere of grasses	- (Mohn *et al.*, 1999) - (Behrendt *et al.*, 1999) - (Peix *et al.*, 2003)	AJ011504 Y11150 AY152673
R5SpM3P1C2 **R5SpM3P1C3** **R5SpM3P2C1** **R5SpM3P3C1**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150
R5SpM3P1C5 **R5SpM3P2C3** **R5SpM3P2C4** **R5SpM3P2C6** **R5SpM3P3C2** **R5SpM3P3C4**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. amygdali* (T) - *P. avellanae* (T)	- Almond - *Corylus avellana* (bacterial twig dieback)	- (Psallidas and Panagopoulos, 1975) - (Janse *et al.*, 1996)	Z76654 AJ889839
R5SsM3P1C2 **R5SsM3P2C2** **R5SsM3P3C3**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. amygdali* (T) - *P. avellanae* (T)	- Almond - *Corylus avellana* (bacterial twig dieback)	- (Psallidas and Panagopoulos, 1975) - (Janse *et al.*, 1996)	Z76654 AJ889839
R5SsM3P1C6 **R5SsM3P2C6** **R5SsM3P2C7** **R5SsM3P3C7** **R5SsM3P3C8**	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea*	- *P. graminis* (T)	- Phyllosphere of grasses	- (Behrendt *et al.*, 1999)	Y11150

Isolate	Family	Genus	Group (subgroups)	Closest species	Origin	References	SSU rDNA sequence accession n°
R5SsM3P3C4	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. syringae*	- *P. amygdali* (T) - *P. avellanae* (T) - *P. caricapapayae* (T) - *P. cichorii* (T) - *P. congelans* (T) - *P. coronafaciens* (T) - *P. ficuserectae* (T) - *P. meliae* (T) - *P. protegens* (T) - *P. saponiphila* (T) - *P. savastanoi* (T) - *P. syringae* (T) - *P. tremae* (T) - *P. viridiflava* (T)	- Almond - *Corylus avellana* (bacterial twig dieback) - *Carica papaya* - *Cichorium endivia* (endive) - Phyllosphere of grasses - Halo blight of oats - *Ficus erectae* - Bacterial gall of chinaberry (*Melia azedarach*) - Soil suppressing black root rot of tobacco - Xenobiotic degrader - *Olea europaea* (olive tree) - Lilac tree - *Trema orientalis* (charcoal-tree) - Bean	- (Psallidas and Panagopoulos, 1975) - (Janse *et al.*, 1996) - (Robbs, 1956) - (Stapp, 1928) - (Behrendt *et al.*, 2003) - (Ait Tayeb *et al.*, 2005) - (Goto, 1983) - (Ogimi, 1977) - (Ramette *et al.*, 2011) - (Lang *et al.*, 2010) - (Gardan *et al.*, 1992) - (van Hall, 1902) - (Gardan *et al.*, 1999) - (Dowson, 1939)	Z76654 AJ889839 D84010 Z76658 AJ492828 Z76660 AB021378 AB021382 AJ278812 FM208264 AB021402 DQ318866 AJ492826 AY180972
R5SsM3P3C9	Pseudomonadaceae (Gamma-Proteobacteria)	*Pseudomonas*	*P. lutea* / *P. rhizosphaerae*	- *P. abietaniphila* (T) - *P. graminis* (T) - *P. rhizosphaerae* (T)	- Pulpmill effluent - Phyllosphere of grasses - Rhizosphere of grasses	- (Mohn *et al.*, 1999) - (Behrendt *et al.*, 1999) - (Peix *et al.*, 2003)	AJ011504 Y11150 AY152673

Appendix II-6: Relationships of *Pseudomonas* rain isolates within the *Pseudomonas* genus

Figure 1: Cladogramme depicting the relationships of *Pseudomonas* rain isolates within the *Pseudomonas* genus.

Derived from the global phylogeny of the *Pseudomonas* genus, rooted using other *Pseudomonadaceae* (Ln Likelihood = -22834.873273). The *Pseudomonadaceae* used as outgroups are not shown for the sake of clarity.

III. ASSESSMENT OF ELECTROTRANSFORMATION-BASED GENE BIOAUGMENTATION FOR BIOREMEDIATION

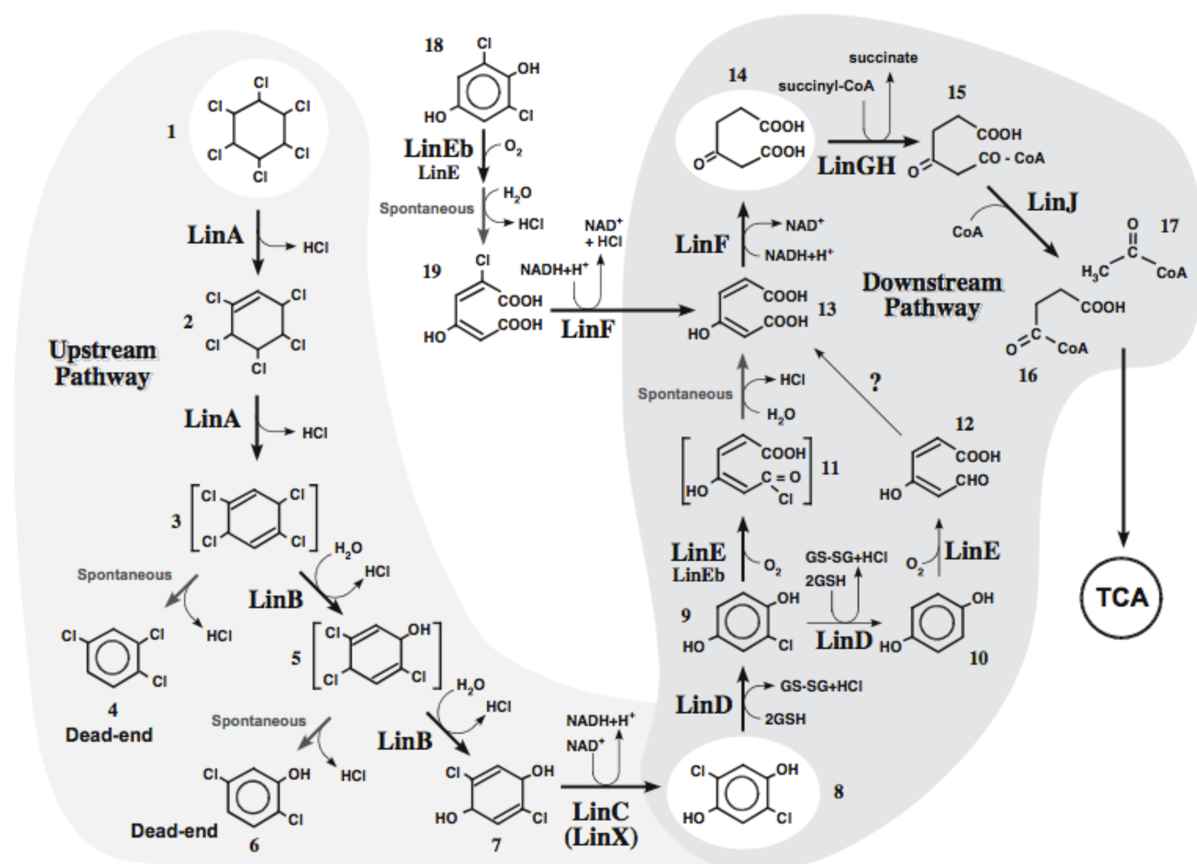
INTRODUCTION

Bioremediation relies on the ability of microorganisms to degrade or transform contaminants into less noxious chemicals and, in so doing, clean up polluted sites. Bacteria involved in bioremediation must harbor in their genomes the genes encoding the necessary degradation enzymes and be able to express them at the polluted site. One way to ensure that desired functionalities are available at the site is to add or increase the presence of degradation genes by "bioaugmentation", *i.e.*, by adding bacteria known to express the enzymes of interest (Roane *et al.*, 2001). The primary hurdle of bioaugmentation is survival of the inoculum *in situ* (Cases and de Lorenzo, 2005; Park *et al.*, 2008). Indeed, inocula are typically out-competed, predated, or starved in their "new" environment by the better-adapted indigenous bacteria, leading frequently to the inefficiency of the method (Vogel, 1996; El Fantroussi and Agathos, 2005).

To circumvent this difficulty, it was proposed to introduce the degradation genes themselves into indigenous bacteria, providing them with the enzyme function and a further competitive advantage. In nature, gene transfer occurs between bacterial cells and species *via* horizontal gene transfer (HGT) through different mechanisms. HGT has played a major role in the evolution of bacterial genomes (Lan and Reeves, 1996; Ochman *et al.*, 2000; Koonin *et al.*, 2001) and is also responsible for the spreading of antibiotic resistance genes (Summers, 2006; Zaneveld *et al.*, 2008). It is usually mediated by transmissible nucleic acid–based mobile genetic elements (MGEs), such as plasmids, transposons, bacteriophage-related elements, and genomic islands (Aminov, 2011; Popa and Dagan, 2011). Taking advantage of this natural process may thus increase the chance of bioremediation success. There is an abundance of HGT methods and MGEs that have been used for "gene bioaugmentation" (Top *et al.*, 2002; Nojiri *et al.*, 2004). Conjugation, or the transfer of plasmids between bacteria having established a temporary physical contact, has been successfully tested in the laboratory for the transfer of degradation genes (Top *et al.*, 1998; Pepper *et al.*, 2002; Desaint *et al.*, 2003; Bathe *et al.*, 2005; Nancharaiah *et al.*, 2008). However, bioremediation *via* conjugation methods still relies upon a bacterial inoculum to introduce the degradation genes into the soil, and because only certain types of bacteria can conjugate with each other, a limited number of bacteria would be involved. Transduction, another HGT mechanism, involving the use of bacteriophages infecting bacteria of their host specificity range, allows the transfer of DNA between two proximal bacteria only through generalized transduction (Ebel-Tsipis *et al.*,

1972) with temperate phages, and is thus of limited use for bioremediation purposes (Davison, 1999). The bacterial uptake of cell-free DNA from the environment, *i.e.*, transformation, can occur without external forces when the bacteria are naturally competent. Natural transformation has been successfully used to introduce an atrazine-degradation gene on a plasmid into biofilm bacteria (Perumbakkam *et al.*, 2006). However, natural competence is induced by specific signals and necessitates uptake and regulatory machineries not present in all bacteria (Claverys *et al.*, 2009). Given that approximately 1% of described bacterial species are naturally transformable (Thomas and Nielsen, 2005), the expression of competence genes is difficult to predict in a complex and heterogeneous system such as soil (Johnsborg *et al.*, 2007; Levy-Booth *et al.*, 2007).

To affect a larger number and variety of bacteria, the use of electrotransformation to transfer cell-free plasmid DNA directly into bacteria *in situ* at a field site by electric exponential discharge application was proposed (Lyon *et al.*, 2010). Electrotransformation uses a pulsed electric field (PEF) to induce bacterial uptake of exogenous naked or bacterial contained DNA. The brief application of an exponentially decreasing electrical field causes electroporation, which is the formation of transmembrane “pores” (Weaver, 1995; Teissie *et al.*, 2005) in which extracellular DNA can enter the cell by migration due to current (Tieleman, 2004). Electrotransformation is amongst the more powerful of the transformation methods, since it is independent of the cell physiological state and can be applied to all types of cells (Prasanna and Panda, 1997; Yuan, 2007). Commercially available electroporation instruments are able to deliver high-voltage electric pulses to small amounts of liquid medium. Moreover, the application of such a pulse to a volume of soil by a high-voltage generator, in the context of lightning simulation, was shown to facilitate gene transfer in soil bacteria (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006a). Therefore, the addition of a PEF facilitates the entry of these elements into microorganisms that might not ordinarily participate in HGT. Advantageous DNA could thus be introduced in soil and integrated into bacteria by electrotransformation in order to give them essential functions. This method might be particularly useful when attempting to remediate xenobiotics such as relatively newly manufactured compounds with entirely novel chemical structures, for which the degradation genes are not widespread or even available (Leisinger, 1983; Timmis *et al.*, 1994).



<u>Figure III-1: Proposed degradation pathways of γ-HCH in *Sphingobium japonicum* UT26 (From Nagata *et al.*, 2007)</u>

Compounds: **1** γ-Hexachlorocyclohexane (γ-HCH), **2** pentachlorocyclohexene (γ-PCCH), **3** 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN), **4** 1,2,4-trichlorobenzene (1,2,4-TCB), **5** 2,4,5-trichloro- 2,5-cyclohexadiene-1-ol (2,4,5-DNOL), **6** 2,5-dichlorophenol (2,5- DCP), **7** 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL), **8** 2,5-dichlorohydroquinone (2,5-DCHQ), **9** chlorohydroquinone (CHQ), **10** hydroquinone (HQ), **11** acylchloride, **12** γ-hydroxymuconic semialdehyde, **13** maleylacetate (MA; 2-maleylacetate, 4-oxohex-2-enedioate), **14** β-ketoadipate (3-oxoadipate), **15** 3-oxoadipyl-CoA, **16** succinyl-CoA, **17** acetyl-CoA, **18** 2,6-dichlorohydroquinone (2,6- DCHQ), and **19** 2-chloromaleylacetate (2-CMA). TCA: citrate/tricarboxylic acid cycle; GSH: glutathione (reduced form); GS-SG: glutathione (oxidized form). Square brackets show unstable compounds that have yet to be detected. LinE and LinEb are mainly involved in the degradation of CHQ and 2,6-CHQ, respectively, in UT26 (Endo *et al.*, 2005). The light and dark shaded areas indicate upstream and downstream pathways, respectively.

In their pilot experiment, Lyon *et al.* (2010) (Appendix III-1) tested the feasibility of both *in vitro* (liquid media) and *in situ* (soil) electrotransformation of a plasmid encoding a pollutant-degrading gene into resident bacteria from two different soils (La Côte Saint André (CSA) and Montrond (MON)). In both *in vitro* and *in situ* experiments, the electrotransformed bacteria displayed an increase in the degradation of the pollutant (lindane) and in plasmid copy number, the lindane-degradation activity apparently functioning better in the CSA soil. Although liquid electrotransformation experiments were more successful than the *in situ* soil ones, Lyon *et al.* (2010) thus demonstrated that the use of *in situ* electrotransformation could improve pollutant degradation rates and potentially provide another tool for bioremediation.

Building on these preliminary results, we decided to explore this new avenue for bioremediation efforts and to continue evaluating the electrotransformation-based gene bioaugmentation of bacteria, using lindane contamination of soil as a case study. Lindane, or γ-hexachlorocyclohexane, was a widely used pesticide prior to the 2006 ban on its agricultural use. Lindane contamination of soils is a relatively common problem due to its widespread agricultural application and its chemical stability. Despite its relatively recent release into the environment (less than 60 years), several bacterial species capable of degrading and mineralizing lindane have been isolated (Lal *et al.*, 2006). One such species, *Sphingobium japonicum*, contains a gene, *linA*, which encodes a protein that performs the first two-dechlorination steps of lindane, possibly the most important steps in the compound's degradation (Nagata *et al.*, 1999; Nagata *et al.*, 2007) (Figure III-1). In this research, a broad-host-range plasmid (pBLN) containing *linA* and two antibiotic-resistance genes (*tetA* and *aph3ia*) was added to soil microbial communities that had not previously been exposed to lindane to evaluate the ability of bioremediation by electrotransformation-based gene bioaugmentation.

EXPERIMENTAL PROCEDURES

1. Bacterial strains, plasmid and culture conditions:

The 8.5-kb plasmid pBLN used in this study (Figure 1 in Chapter I) was constructed in the course of a previous study (Lyon *et al.*, 2010) from the broad-host-range plasmid pBBR1-MCS3 (GeneBank ref.: XXU25059) able to replicate in many different types of bacteria (Kovach *et al.*, 1995) and harboring the *tetA* gene encoding resistance to tetracycline. Genes from the plasmid pCEAlinA-nptII conferring kanamycin resistance (*aph3ia*) and involved in the two first-steps of lindane degradation by *Sphingobium francense* sp+ (*linA*) (Cérémonie *et al.*, 2006b) were cloned in pBBR1-MCS3. The pBLN plasmid was maintained in *Escherichia coli* Top 10 in LB broth with 25 µg.mL^{-1} kanamycin monosulfate and 25 µg.mL^{-1} tetracycline hydrochloride (Duchefa Biochemie, Haarlem, The Netherlands), and extracted using the NucleoSpin Plasmid Extraction kit (Macherey Nagel, Bethlehem, USA), according to the manufacturer's instructions. Plasmid was quantified using two methods: (i) spectrophotometry (Nanophotometer, Implen, München Germany) and (ii) fluorometry (Qubit fluorometer, Invitrogen – Life technologies, Paisley, UK). DNA integrity was checked by electrophoresis on 0.8% (w/v) agarose gel containing ethidium bromide (2.5 µL for 100 mL of gel) in 45 mMTris-borate-1 mM EDTA buffer (TBE 0.5X). Five hundred nanograms of the 1kb+ ladder (Thermo Fisher Scientific, Villebon sur Yvette, France) were used as size marker.

In situ electrotransformation experiments were carried out using the soil-isolated bacterium *Pseudomonas* sp. N3 (Cérémonie *et al.*, 2004). The strain harboring the pBLN plasmid (N+) incubated in LB medium supplemented with antibiotics at 29°C for 48 h was used as positive control for lindane degradation, whilst a strain devoid of the plasmid (N-) incubated in LB medium at 29°C for 48 h was used as positive control for electrotransformation. After centrifugation at 8228×*g* for 10 min, pellets were washed twice and resuspended in 150 µL of sterile water prior to OD_{600} determination.

For *in vitro* electrotransformation, *Pseudomonas* sp. N3 (N-) electrocompetent cells were prepared according to Cérémonie *et al.* (2004), with minor protocol modifications. A single colony from LBagar was grown in 1 mL of LB at 29°C under agitation (225 rpm) overnight prior to inoculating a 100 mL culture incubated at 29°C with shaking until the exponential

phase was reached (OD_{600}= 0.6-0.7). Cells were incubated on ice for 15 to 30 min, centrifuged at 5500×*g* for 10 min at 4°C and washed four times with decreasing volumes of cold sucrose (0.5 M) before final concentration 100-fold for storage at -80°C. Strains of *P.* sp. N3 containing pBLN (N+) were prepared as the strains without pBLN (N-), the only difference being the growth medium containing tetracycline and kanamycin (25 $\mu g.mL^{-1}$).

2. Soil samples:

Two types of non-contaminated soils were used. The first one, sampled in November 2009, is a sandy loam cultivated soil from La Côte Saint-André in France (CSA) with 50% sand, 41% silt, 9% clay, 40.6 g of organic matter per kg of dry soil with a neutral pH (6.8) (Demanèche *et al.*, 2001c). The second soil was sampled at Park Grass, Rothamsted (United Kingdom) in June 2010 (Roth 1) and in March 2012 (Roth 2). The Park Grass soil is an internationally recognized resource and is targeted as a reference soil for soil metagenomic studies (Vogel *et al.*, 2009). It is classified as chromic luvisol according to FAO guidelines (Food and Agricultural Organization of the United Nations, 2013) and is silty clay loam overlying clay with flints with a pH of 5.2 (measured in H_2O).

Soils were sampled between 0 and 5 cm depth, dried, sieved through 4 mm and 2 mm-mesh sieves and stored in plastic jars (22°C) until processing.

3. Soil processing:

Soil humidity measurement

Thirty grams (3 x 10 g) of each soil were dried overnight in an oven at 80-100°C. Soil humidity (H) was measured in triplicate for each soil samples by massing the difference between damp (a) and dry (b) soil as follows:

$$H = \frac{(a-b)}{b}$$

Soil bacterial extraction

Soil indigenous bacteria were extracted from the soil Roth 2 using a protocol adapted from Bertrand *et al.* (2005). These authors set up the extraction and purification of bacteria through a Nycodenz gradient with the preliminary step of soil particle dispersion with 0.2% sodium hexametaphosphate (HMP). They counted greater numbers of bacteria with acridine orange after the first step than after the Nycodenz gradient.

Table III-1: Different samples and analyses performed on soil samples

Soil	CSA (x repeat)	Roth 1 (x repeat)	Roth 2 (x repeat)
Samples	SL SPL (x2) SFP SLFP (x2) SFPLN- SLN+	S (x4) SF (x4) SPL (x4) SLFP (x4) SNaCl (x4)	S (x3) SSL (x1) SL (x4) SPL (x3) SFL (x3) SLFP (x3) SFPLN- (x3) SLN+ (x3)
Quantity of pBLN plasmid (µg/30g of soil)	10	30	30
Number of electric shocks	1	3 shocks 2 times a week during 3 weeks	1
Quantity of lindane (mg/30g of soil)	3	3	3
Humidity after shock	Upgraded to 18%	Not upgraded	Not upgraded
Incubation temperature (°C)	29	22	22
Sampling dates (D: day; W: week; M: month)	D0, D1, D3, D7, D14	W0, W1, W2, W3, W9	M0, M1, M2 Except for SL (x1) and SSL: D23
Analyses	- *linA* qPCR - *linA* RT-qPCR - Lindane GC-MS	-*linA* qPCR - RISA	- Lindane GC-MS

S: soil; SL: soil with lindane; SSL: sterile soil with lindane; SF: Soil subjected to PEF; SPL: Soil with pBLN and lindane; SFP: Soil subjected to PEF with pBLN; SFL: Soil subjected to PEF with lindane; SLFP: Soil subjected to PEF with pBLN and lindane; SFPLN-: Soil subjected to PEF with pBLN, lindane and *P.* sp. N3; SLN+: Soil with lindane and *P.* sp.N+; SNaCl: Soil with NaCl. D: Days; W: Weeks; M: Months. qPCR: quantitative polymerase chain reactions ; RT: Reverse Transcription; GC-MS: Gas Chromatography coupled with a Mass spectrometer; RISA: Ribosomal intergenic spacer analysis

Therefore, 18 g of soil were ground in 70 mL of sterile 0.2% (w/v) sodium hexametaphosphate (Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in a prechilled Waring blender for 3 min. The large soil particles were allowed to settle by incubation for 30 min at 4°C. Then, supernatant was centrifuged at 18514×*g* for 10 min and the resulting pellets resuspended in 2 mL 0.5 M sucrose. Viable cells were counted on a microscope with the LIVE/DEAD Cell Viability kit according to the manufacturer's instruction (Invitrogen, Carlsbad, USA).

Sample preparation

Soil samples were prepared by crushing, using a mortar and pestle to achieve a fine texture and then adjustment of the humidity percentage to deliver an optimum PEF.

4. *In situ* electrotransformation of soil bacteria:

Microcosm preparation

Different samples and analyses were prepared depending on soil (CSA, Roth 1 & 2) (Table III-1). Each sample consisted of 30 g of soil supplemented with 50 or 150 µL of 200 ng.µL^{-1} pBLN solution added homogeneously (to obtain 10 or 30 µg per sample depending on soil) where needed (SPL, SFP, SLFP and SFPLN-). The corresponding number of pBLN copies per soil bacteria (N) can be calculated as follows:

$$N = \frac{(Q \times A)}{(MW \times P \times B)}$$

Q = quantity of pBLN (g) added to 30g of soil

A = Avogadro constant (6.02 x 10^{23} constituent particle.mol^{-1})

MW = Molecular weight average of a base pair (660 g.mol^{-1}.bp^{-1})

P = plasmid length in bp (8534 bp for pBLN)

B = Number of bacteria per 30 g of soil (estimated to 3×10^{9}) (Hoorman and Islam, 2010)

150 µL of *Pseudomonas* sp. N- and N+ cultures were added homogeneously where needed (SFPLN- and SLN+ respectively) and 750 µL of 120 g.L^{-1} NaCl solution was added homogeneously where needed.

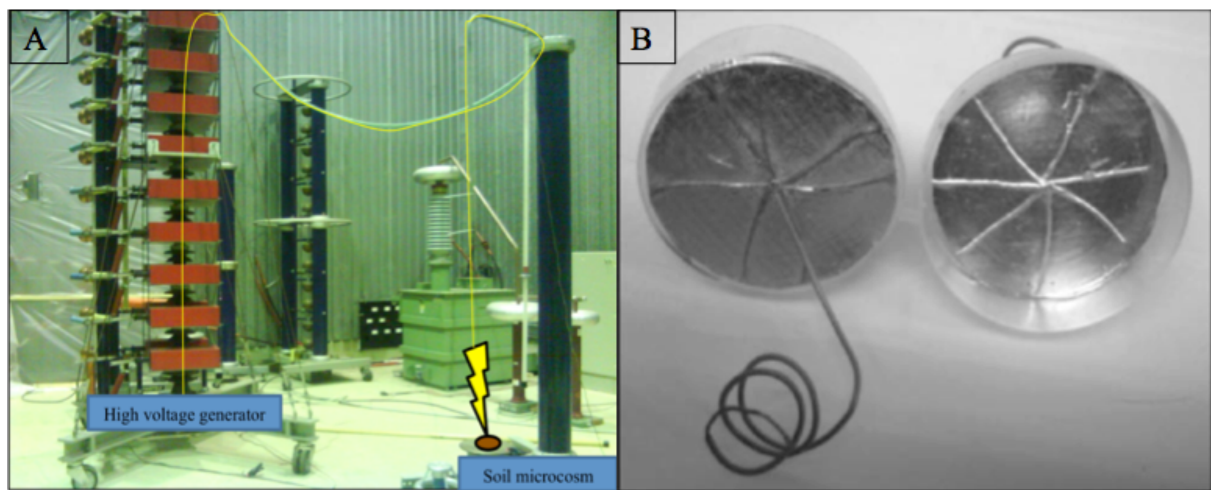


Figure III-2: (A) High-voltage generator and placement of the soil sample; (B) rear and front view of modified Petri dishes lined with aluminum for soil electrotransformation

Samples without lindane (S and SF) are the negative controls. Samples with *Pseudomonas* sp. N- (SFPLN-) and N+ (SLN+) as well as samples supplemented with NaCl (SNaCl) are considered as positive controls for electrotransformation, lindane degradation and bacterial diversity change, respectively. Soils were adjusted to their optimal humidity percentages while taking into account the water added along with pBLN and bacterial inocula.

Electrotransformation

Pulse electric field (PEF) assays were performed at different humidity percentages for each soil to reach optimal voltage and intensity (respectively between 4 to 8 kV and about 5 A).

The soil samples (30 g) were transferred and compacted into previously alcohol- and UV-sterilized Petri dishes (5 cm in diameter and 1 cm thick) lined with aluminum foil and wired to facilitate electricity transmission (Figure III-2B). For each PEF experiment, the Petri dish was sat directly on a solid bronze disk (0.5 m in diameter and 2 cm thick), itself grounded for the applied current. A high-voltage generator (Figure III-2A) was used to deliver an electric pulse between 4 and 10 kV with a time constant value equal to 6 ms *via* a brass cylindrical electrode (5 cm in diameter, 3.5 cm thick). The output voltage (V) was measured by an oscilloscope (NorthStar probe) dividing the tension by 10 000. The current (until 8 A) flowing from the electrode through the soil to the aluminum foil at the bottom of the Petri dish was measured by using a resistive shunt (5 Ω) inserted serially in the discharge circuit.

After exposures to the PEF, the soil sample was transferred into an autoclave-sterilized glass jar. Three mg of lindane were added homogeneously to each of the jars of soil for all samples, except the controls without lindane. The humidity of the CSA sample was upgraded to its initial humidity level and microcosms were incubated until processing with periodic shaking and air intake. Temperatures and incubation times, as well as numbers of electrical pulses and sampling times, are indicated in Table III-1. The samples were then analyzed for lindane degradation *via* lindane extraction and GC-MS analyses and their DNA was extracted to test for *linA* presence and expression *via* qPCR and RT-qPCR and for the distribution of bacterial communities *via* RISA.

5. *In vitro* electrotransformation of soil bacteria:

Test on agar plates

One microliter of pBLN (70 ng.µL^{-1}) was added to 50 µL of soil bacteria without electroporation to evaluate the level of natural transformation.

Fifty microliters of soil bacteria were submitted in triplicate to an electrical shock in the presence of 1 µL of pBLN (70 ng.µL^{-1}) with the Gene Pulser II Electroporation System (Biorad, Hercules, USA) at 12.5 kV.cm^{-1}, 25 µF, 200 Ω in a 2 mm cuvette for 5 ms (Cells projects, Harrietsham, United Kingdom) to assess their electrotransformation potential.

All suspensions were spread on:

- 1/10 Trypticase soy agar (TSA) plates with 200 µg.mL^{-1} cycloheximide (Duchefa Biochemie, Haarlem, The Netherlands) (TSA-C),
- 1/10 TSA with cycloheximide and 25 µg.mL^{-1} of tetracycline and kanamycin (TSA-CTK),
- 1/10 TSA with 1 mg.mL^{-1} lindane (from a stock of 50 mg.mL^{-1} lindane; DMSO - TSA-L).

Then, agar plates were incubated for 5 d at 22°C and cultivable bacteria were counted; antibiotic resistance, as well as lindane degradation potential and survival after electrical shock were evaluated.

The survival percentage after electrical shock was calculated as the number of surviving bacteria divided by the number of bacteria not submitted to electroporation:

$$\%Survival = \frac{b}{a} \times 100$$

a = Number of culturable soil bacteria on 1/10 TSA-C without shock (CFU.mL^{-1})

b = Number of surviving soil bacteria on 1/10 TSA-C aftershock (CFU.mL^{-1})

Soil microcosms

Each sample consisted of 10 g of soil with 50 µL of indigenous bacteria extracted from soil or *P.* sp. N3 electrocompetent cells (N- or N+). Where needed, 1 µL (70 ng.µL^{-1}) of pBLN solution was added homogeneously to the cells prior to addition to the soil. When needed, the electroporations were performed on 50 µL of bacteria with or without the pBLN plasmid in a 0.2 cm gap cuvette at 12.5 kV.cm^{-1}, 200 Ω and 25 µF. Suspensions were added to

each soil sample, and microcosms were transferred into autoclave-sterilized glass jars. One mg of lindane was added homogeneously to each sample except the negative control.

Samples included negative controls of soil bacteria added to soil (SB), soil bacteria added to soil with lindane (SBL), electroporated soil bacteria added to soil with lindane (SBEL), control for natural transformation of soil bacteria mixed with 1 µL of 70 $ng.\mu L^{-1}$ pBLN and added to soil with lindane (SBPL), assay of soil bacteria electroporated with 1 µL of 70 $ng.\mu L^{-1}$ pBLN and added to soil with lindane (SBPEL), and positive controls for electrotransformation and lindane degradation consisting respectively of (i) electrocompetent *P.* sp. N3 (N-) electroporated with 1 µL of 70 $ng.\mu L^{-1}$ pBLN and added to soil with lindane (SN-PEL) and (ii) electrocompetent *P.* sp. N3 (N+) with 1 µL of 70 $ng.\mu L^{-1}$ pBLN added to soil with lindane (SN+L). All samples were performed in triplicate.

Microcosms were incubated at 22°C until processing with periodic shaking and air intake. They were sampled at T0 and after 1 and 2 months for assessment of lindane degradation *via* lindane extraction and GC-MS analyses.

6. Detection of lindane degradation

Compound extraction

Lindane (γ-hexachlorocyclohexane) and degradation products (γ-pentachlorocyclohexene: γ-PCCH and 1,2,4-trichlorobenzene: TCB) were extracted from CSA and Roth 2 soils as well as from previously autoclaved sterile Roth 2 soil by agitation of 0.25 g of soil in 750 µL hexane for 30 min (CSA) to 1 h (Roth 2). Experiments also included negative controls containing only hexane and only soil. Rothamsted soil was also sent to Wessling (Saint-Quentin-Fallavier, France) to verify the absence of lindane with a gas-chromatographic method coupled with electron capture detection (ISO 10382 (A)).

GC-MS quantification

The samples were filtered using a syringe equipped with a 0.2 µm filter (Nalgene, Rochester, USA). One microliter of the different samples were analyzed using an Agilent Technologies 6850 Network GC System with a 5975 VL mass spectrometer equipped with a HP-5MS fused silica capillary column. The GC was kept at 50°C for 2 min, then increased at $15°C.min^{-1}$ to 325°C, and maintained at this temperature for 3 min. The injector was set at 250°C, and helium was used as the carrier gas at 0.5 $mL.min^{-1}$ constant flow. Both split and splitless analyses were performed on samples to quantify lindane and evaluate relative

concentrations of degradation products, respectively. Before quantification of lindane in samples, different known quantities of lindane were added to the two different soils, extracted and analyzed with the same split protocol for calibration. These calibrations, in addition to standard and sample quantifications and degradations were checked by a standard calibration with a standard powder (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) containing the different forms of hexachlorocyclohexane (α, β, δ et γ-) directly dissolved in hexane, and analyzed as before.

7. Estimation of *linA* copy number and expression

Extracellular DNA degradation in soil

For Roth 1 soil, DNase treatment was optimized. We tested different concentrations of DNase (3, 20 and 40 Units) for the treatment of 1g of soil containing 3 µg of pBLN plasmid. After having determined the best concentration for complete degradation of plasmids (verified by qPCR), different concentrations of EDTA (40, 100 and 300 mM) were tested in order to find the optimal one for inactivating the DNases (verified by qPCR after the addition of 3 µg of pBLN plasmid).

Triplicates of, 1.5 g and 1 g of CSA and Roth 1 soil sample, respectively, was incubated in 1 mL DNase buffer (40 mM Tris-HCl, 8 mM $MgCl_2$) with 30 (CSA) and 40 (Roth 1) units of DNase (Roche, Basel, Switzerland) at 37°C for 1 h. The reaction was stopped with 300 mM EDTA, and the soil samples were centrifuged at 12,000×*g* for 5 min.

RNA and DNA extraction

Total RNA and DNA were extracted from CSA and Roth 1 soils using the RNA Powersoil® Total RNA isolation kit and DNA elution accessory kit according to manufacturer's instructions (Mobio Carlsbad, California, USA). These kits are designed for the recovery of the total nucleic acid content of soil samples. The properties of the RNA isolation kit permit the consistent removal of humic substances, fulvic acids, and other PCR inhibitors from soil-purified nucleic acids. Then, total RNA and DNA are co-isolated and preferentially eluted into two separate fractions from the proprietary matrix of the capture column.

RNA and DNA quantification

Extracted RNA and DNA were quantified using two methods: (i) spectrophotometry for CSA DNA and RNA with baseline correction (Nanophotometer, Implen, München Germany) and (ii) fluorometry for Roth 1 DNA (Qubit fluorometer, Invitrogen – Life technologies, Paisley, UK). Then RNA and DNA samples were diluted to 10 ng.μL^{-1}.

qPCR and RT-qPCR

Real time PCR and RT-PCR were performed on a Rotor-Gene 6000 real-time rotary system (Corbett Life Science – Qiagen – Hombrechtikon, Switzerland) using the SensiMixPlus SYBR kit and the Sensimix One step kit, respectively, according to the manufacturer's instructions (Quantace, London, United Kingdom). These determined the initial concentration of templates in samples from a calibration curve generated using 10-fold dilutions of pBLN plasmid DNA, with concentrations ranging from 2.5×10^{0} to 2.5×10^{7} pBLN copies per µL (scale of pBLN concentration = 8 logs; cycle threshold ranging from 3.82-37.02). The corresponding number of pBLN copies in the calibration curve can be calculated as follows:

$$N = \frac{Q \times A}{MW \times P}$$

Q = quantity of pBLN (g)
A = Avogadro constant (6.02×10^{23} constituent particle/mol)
MW = Molecular weight average of a base pair (660 g/mol/bp)
P = plasmid length in bp (8534 bp for pBLN)

The cycle thresholds of all samples were higher than the lowest cycle threshold corresponding to the highest calibration point. For all of the qPCR assays, there was a linear relationship between the log of the plasmid DNA copy number and the calculated threshold cycle value across the specified concentration range. *lin*A amplifications were performed on 4 µL of different samples (40 ng of DNA or RNA) in a 20 µL reaction with 5 µM of initial primer concentration (forward nested-linA-F1: 5'-GCTCATTGCCGTAGACAA-3'; reverse nested-linA-R1: 5'-GCTCATACTCATCCGTGAAG-3'), providing a single DNA product of 296 bp identified by a uniform melting temperature (84.8 ± 0.5°C) (Lyon *et al.*, 2010). Temperature cycling was 42°C for 30 min corresponding to the reverse transcription step for RNA samples only, and 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 60°C for 20

s, and 72°C for 20 s, followed by a melting curve analysis from 50 to 99°C. To further identify the DNA amplicons, the products were sent to GATC Biotech (Konstanz, Germany) for a Sanger sequencing in both directions of the *linA* fragment using the same annealing primers.

To ensure that equal quantities of DNA were being analyzed, a qPCR of SSU rDNA was performed on CSA DNA. Initial concentration of templates in samples were determined from a calibration curve generated using 10-fold dilutions of a previously amplified SSU rDNA (Chapter II, Experimental procedures, SSU rDNA amplification and sequencing) of *Sphingobium francence* sp+, electrophoresed and extracted from gel using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, United Kingdom). Calibration curve concentrations ranged from 2.5×10^{1} to 2.5×10^{8} SSU rDNA copies per µL (scale of SSU rDNA concentration = 8 logs; cycle threshold rangingfrom 4.00 to 30.95). Copy numbers of SSU rDNA fragments in the calibration curve were calculated as before.

For all qPCR assays, there was a linear relationship between the log of the plasmid DNA copy number and the calculated threshold cycle value across the specified concentration range. Amplifications were performed on 4 µL of different samples (40 ng of DNA) in a 20 µL reaction with 5 µM initial primers concentration (forward Eub338: 5'-ACTCCTACGGGAGGCAGCAG-3'; reverse EUB518: 5'-ATTACCGCGGCTGCTGG-3') (Fierer *et al.*, 2005) for an expected amplicon of about 200 bp identified by a uniform melting temperature (85.8 ± 1.8°C). Temperature cycling was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 53°C for 30 s, and 72°C for 60 s, followed by the melting curve analysis.

Copy number of *linA* DNA and RNA per gram of soil were calculated as follows:

$$Copies/g\ of\ soil = \frac{N \times C_2 \times V_2}{C_1 \times V_1 \times S}$$

N = Copy number per qPCR reaction obtained by the Rotor-Gene 6000

V_1 = Volume of DNA or RNA per qPCR reaction (= 4 µL)

C_1 = Concentration of DNA or RNA added in the qPCR reaction (= 10 $ng.\mu L^{-1}$)

V_2 = Volume of DNA or DNA extracted (100 µL)

C_2 = Concentration of extracted DNA or RNA ($ng.\mu L^{-1}$)

S = Quantity of soil used for the extraction of DNA and RNA (g)

8. Ribosomal intergenic spacer analysis (RISA)

The intergenic spacer (IGS) region between the small (SSU) and the large (LSU) subunits of ribosomal sequences were amplified by PCR in a 25 µL reaction with 10 µM initial primers concentration (forward S-D-Bact-1522-b-S-20: 5'-TGCGGCTGGATCCCCTCCTT-3' and reverse L-D-Bact-132-a-A-18: 5'-CCGGGTTTCCCCATTCGG-3') (Ranjard *et al.*, 2000). Amplifications were performed on 40 ng of DNA from the different Roth 1 samples using PCR hot start mix RTG Illustra™ (GE Healthcare, Little Chalfont, United Kingdom). PCR cycles consisted of 95°C for 10 min and then 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min and 20 seconds, followed by 72°C for 15 min, with a Biometra thermocycler (Goettingen, Germany). One microliter of the PCR mix was then loaded into an Agilent DNA 7500 Lab on a Chip. Electrophoregrams were analyzed and data normalized using the Agilent 2100 Bioanalyzer expert software (version 2003). Principal component analyses (PCA) and between group analyses (BGA) were performed using R (version 2.15.2 – Ihaka and Gentleman, 1996).

9. Statistical analyses:

Hereafter, we consider that two concentrations, abundances, or copy number averages S_1 and S_2 are significantly different when their confidence intervals (CI) do not intersect. For instance, for a significance level α=0.05, S_1 and S_2 are considered significantly different if:

$$|S_1 - S_2| > 1.96\sigma \times \frac{2}{\sqrt{N}}$$

where it is implicitly assumed that:

- both S_1 and S_2 follow a Gaussian distribution with the same variance s^2
- the experiments have been repeated N times.

This corresponds to the intuitive idea that the intersection of the intervals where S_1 and S_2 lay with a probability of 95% is empty. For the sake of simplicity (and with an abuse of notation), in this and in the following expression we use the same symbol S_1 to mean data and its experimental average $\bar{S_1}$:

$$\bar{S_1} = \frac{1}{N}\sum_{i=1}^{N} S_1^{(i)}$$

where $S_1^{(i)}$ means the *i*-th realization of S_1 over N repetitions of the experiment.

RESULTS AND DISCUSSION

Soil bacteria can be efficiently electrotransformed by DNA that can extend their functional repertories (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006a). The goal of this research was to evaluate whether a biodegrading activity, such as lindane degradation, could be efficiently introduced into a soil microbial community *via* electrotransformation of the corresponding genes. This was tested with the introduction, by pulse electric field (PEF), of a plasmid (pBLN) fitted with a lindane degradation gene (*lin*A) into artificially contaminated soils. PEF was applied directly to soils as well as on extracted bacteria. Microcosms were then incubated and monitored for days, weeks or months, and lindane degradation was assessed by lindane extraction and GC-MS analyses. The presence and expression of the *linA* gene was verified and quantified using qPCR and RT-qPCR, respectively, on soil extracted DNA and RNA and the structure of the bacterial communities was monitored using RISA. The *lin*A gene encodes an enzyme that performs the first two steps of lindane (γ-hexachlorocyclohexane) dechlorination, including production of successively γ-pentachlorocyclohexene (γ-PCCH) and 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) spontaneously transformed into 1,2,4-trichlorobenzene (TCB) (Figure III-1). Presence and abundance of these various compounds in soil samples were analyzed by GC-MS as well.

1. Determination of optimal experimental conditions:

Baker *et al.* (2010) showed that pesticide degradations depend on microbial community composition but also on the physicochemical properties of soil. Because of its different composition in clay and organic matter, Rothamsted soil (Roth 1 and Roth 2 samples) could behave differently in comparison to the sandy loam soil from Côte Saint-André (CSA). Indeed, lindane-degradation activity had been shown to be more efficient in the CSA soil than in the Montrond prairie soil (MON) (Lyon *et al.*, 2010).

Another important parameter is the water content of soil, which can affect soil microbial activity (Butenschoen *et al.*, 2011) and the diffusion of electrical parameters (Cérémonie *et al.*, 2004). A dry soil leads to lightning breakdown, whereas a humid soil leads to a decrease in electrical tension. PEF assays were thus performed with various humidity percentages for each tested soil to reach optimal current voltage and intensity (respectively 4-8 kV and ≈5 A) (Data not shown). Table III-2 presents those optimum humidity percentages which led to the

best output voltages and intensity for the three soils used in our study: CSA, Roth 1 and Roth 2.

Table III-2:Humidity and electrical parameters for each soil used

Soil	CSA	Roth 1	Roth 2
Date of sampling	November 2009	June 2010	March 2012
% of humidity	18.67 ± 0.29	17.92 ± 0.20	28.76 ± 0.11
% of humidity required for optimum PEF	9	21.5	16.5
Input Voltage (kV)	25	40-65	40-50
Output Voltage (kV)	9.4-9.6	4-9	7-10
Output intensity (A)	1.34-1.36	3-8	3-4

2. Monitoring of LinA-mediated lindane dechlorination:

In a pilot work (Lyon *et al.*, 2010), we verified that at least some of the soil bacteria were capable of taking up the pBLN plasmid by electrotransformation in a liquid medium and that these bacteria could express *linA*, as measured by lindane dechlorination evaluated *via* an increase in chloride concentration in the supernatant culture. Whereas no increase in chloride concentration could be detected in the negative controls and in the natural transformation assay, *in vitro* electrotransformation assays of both soil (CSA and MON) exhibited a significant increase in chloride concentration, indicating that lindane was being degraded in these cases.

The second step of our pilot work consisted of the addition of the pBLN plasmid into soil, applying a pulsed electric field (PEF) using a high-voltage generator, and monitoring lindane degradation, while also verifying the presence and expression of the degradation gene among soil bacteria. Lindane degradation was evaluated *via* an increase in chloride concentration in the supernatant of soil-water solutions. After three weeks in the CSA soil, the natural transformation sample (no shock), the electrotransformation sample and the positive controls for electrotransformation and lindane degradation (*Pseudomonas* sp. N3

electroporated with pBLN and *Sphingobium francense* sp+, respectively, added into soils with lindane) all showed significant increases in lindane degradation when compared with the negative control with lindane (soil with lindane). Overall, it appears that *in situ* electrotransformation increased the lindane-degrading capacity of the CSA soil, although not significantly more than natural transformation (*i.e.,* bacterial uptake of cell-free DNA from the environment) does; electrical discharge thus does not appear to improve the transformation efficiency. For the MON soil, the only significant increase in lindane degradation was in the positive control with *S. francense* sp+. Both the electrotransformation sample and electrotransformation positive control showed a slight (but not statistically significant) increase in chloride concentration. The lindane-degradation activity appears to have been more efficient in the CSA soil than in the MON soil. According to this result, the CSA soil was selected for a longer-term experiment and showed an increase in lindane degradation over time but not to the same extent as would be expected from the two-month result (especially for the electrotransformation sample). We therefore concluded that the majority of the degradation activity due to LinA had already occurred during the three weeks of incubation in presence of lindane after the electrotransformation of pBLN.

Lindane degradation monitoring over a two week period

The first part of our study aimed to better evaluate the time period needed for the dechlorination of soils by LinA and to improve the detection of lindane degradation and *linA* gene expression. In a new set of experiments, we evaluated lindane degradation after electrotransformation in the CSA soil over a two-week period based on gas chromatography mass spectrometry, a technique that allows the precise quantification of lindane and estimation of its degradation products (γ-PCCH, 1,4-TCDN and 1,2,4-TCB) (Figure III-3). This method was used in the previous study in 2010 to verify the absence of lindane in the soils prior to the experiments. Contrary to the methanol solvent previously used for lindane extraction (Lyon *et al.*, 2010), hexane was added to soil and soils agitated for 30 min, according to Rigas *et al.* (2007). The use of a sensitive analysis method such as GC-MS did not require lindane concentration as high as 10 mg lindane per gram of soil as used in 2010 – this is much higher than the concentrations routinely detected in contaminated sites - but this concentration was selected in order to facilitate lindane degradation detection and overcome any bioavailability constraints (Calvelo Pereira *et al.*, 2008). In our experiments a 100-fold reduced lindane concentration was added into soil samples.

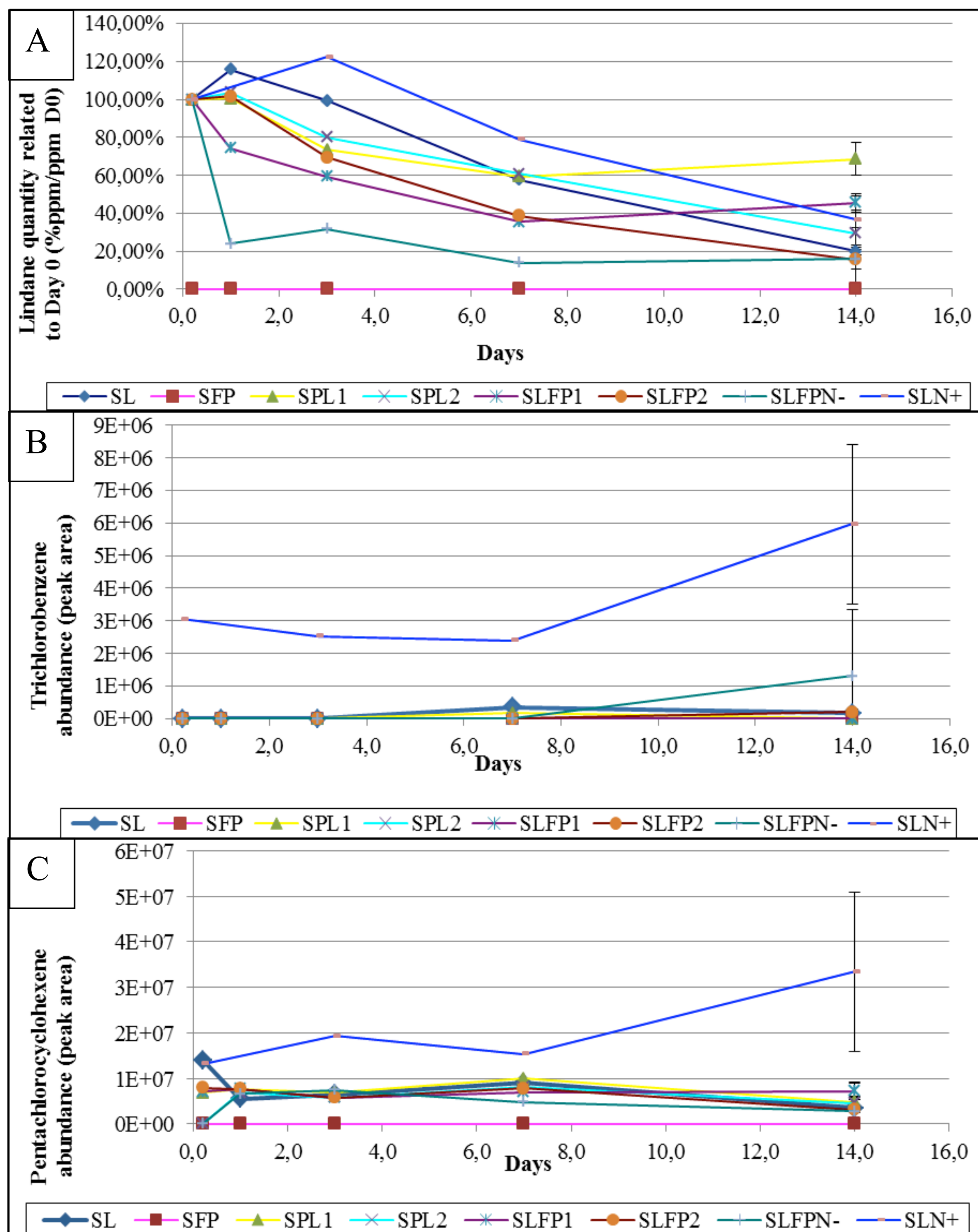


Figure III-3: Lindane quantities relative to Day 0 and relative abundance of TCB and γ-PCCH in peak area measured over time in the different samples of the CSA soil

SL: soil with lindane; SFP: Soil subjected to PEF with pBLN; SPL (x2): Soil with pBLN and lindane; SLFP (x2): Soil subjected to PEF with pBLN and lindane; SFPLN-: Soil subjected to PEF with pBLN, lindane and *P.* sp. N3; SLN+: Soil with lindane and *P.* sp. N+; D0: Day 0.

Although not statistically significant, lindane concentrations decreased in all samples overtime (Figure III-3A). This suggests adsorption of lindane on soil particles over time, as described by Kay and Elrick (1967) and limits the conclusions that can be proposed for lindane biodegradation.

γ-PCCH, resulting from the first lindane dechlorination by LinA, showed an increase in the positive control (bioaugmentation) (SLN+) after 14 days (Figure III-3C), although the figure was not statistically significant. This suggests that degradation of lindane might be promoted by inoculation of adapted bacteria. 1,4-TCDN produced during the second dechlorination by LinA was not detected, and this was certainly related to the spontaneous third dechlorination leading to TCB (Figure III-1) (Nagata *et al.*, 1999; Nagata *et al.*, 2007).

Only the positive controls for electrotransformation and lindane degradation, SFPLN- (Soil with lindane, *P.* sp. N3- and pBLN subjected to PEF) and SLN+ (Soil with lindane and *P.* sp. N3+) respectively, showed an increase in TCB abundance after 14 days - although again, this figure was not statistically significant - indicating a trend towards lindane degradation in these samples (Figure III-3B). The low levels of TCB may reflect poor electrotransformation and/or bioaugmentation success. Alternatively, this may have resulted from the subsequent degradation of TCB by indigenous bacteria (Brunsbach and Reineke, 1994; Wang *et al.*, 2007a) following its *P.* sp. N3 production by *linA* expression.

This new set of experiments showed that a two-week study timescale was too short, even though the electrotransformation and the majority of the degradation activity due to LinA had already occurred in the three weeks of our pilot work (Lyon *et al.*, 2010). The slight increase of degradation products (in spite of a lindane concentration decrease in all samples) does however hint promisingly towards the potential efficiency of bioaugmentation or *in situ* electrotransformation treatment involving inoculated bacteria.

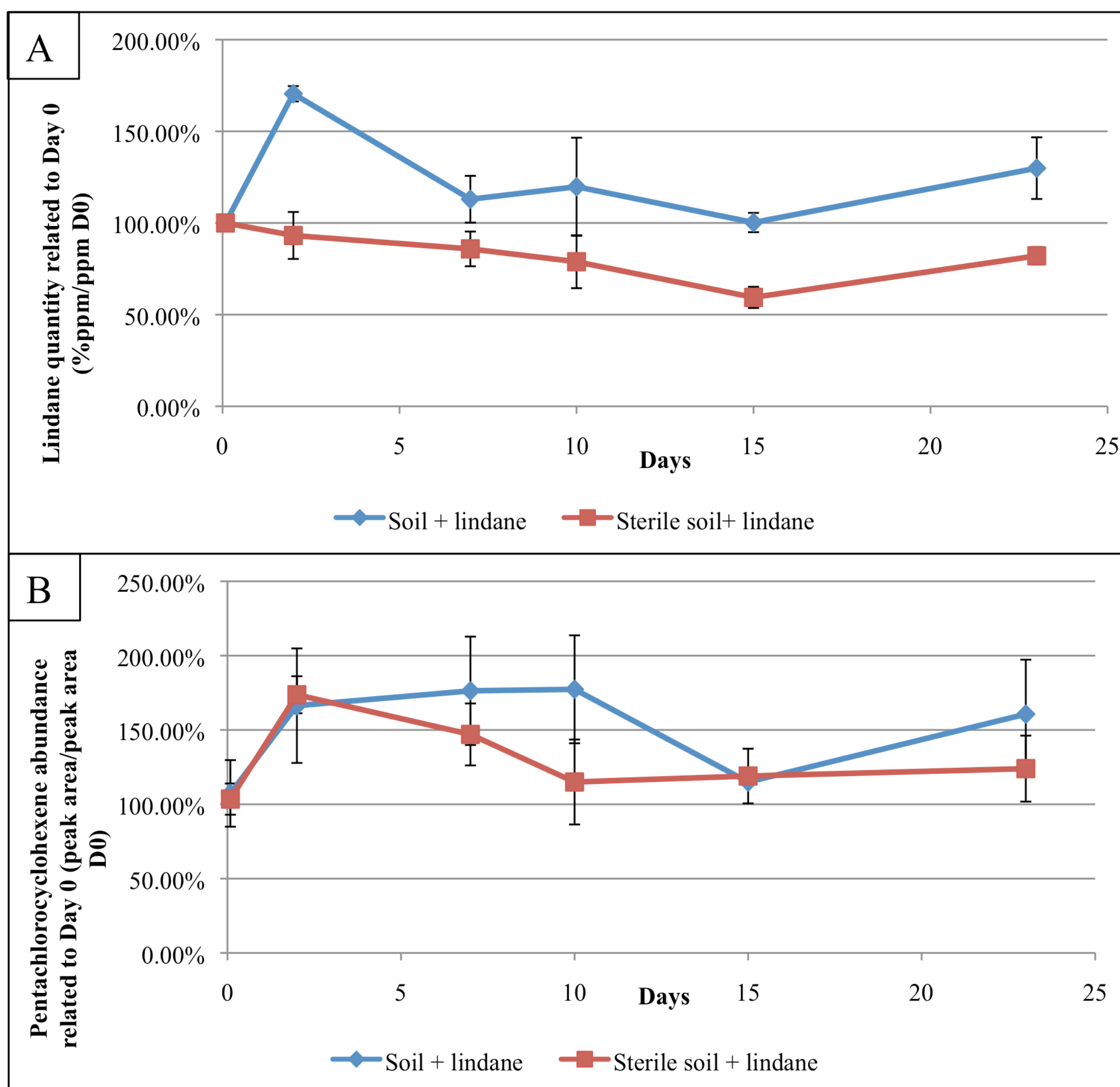


Figure III-4: Lindane quantities related to Day 0 and relative abundance of γ-PCCH in peak area related to Day 0 measured over 23 days in the Roth sterile or non-sterile soil with lindane

Evaluation of intrinsic bioremediation capacity of indigenous soil bacteria

GC-MS analyses were conducted on Rothamsted soil sampled in March 2012 (Roth 2) to search for the natural potential of indigenous bacteria to degrade lindane. Lindane and its degradation product (γ-PCCH) were quantified after incubation and extraction with hexane (after 1 h rather than 30 min) under agitation (to increase lindane desorption). Conditions included sterilized and non-sterile soil samples incubated for 23-days after confirmation (performed by Wessling company) of the absence of any trace of lindane.

The sterile soil supplemented with lindane showed a significant decrease in lindane concentrations compared to the sample containing non-sterile soil and lindane (Figure III-4A).

These results may be explained by an increased adsorption of lindane to soil particles after the soil bacteria were killed by sterilization and this left free space for lindane sorption. The increase of γ-PCCH relative abundance is not significantly different from that at Day 0, and no significant difference between the two samples (sterilized or not soil with lindane) was observed (Figure III-4B).

These results indicate that lindane would not be adsorbed on soil components in non-sterile samples and that γ-PCCH relative abundance was not significantly increased suggesting that indigenous bacteria were not able to perform natural bioremediation.

Monitoring of lindane degradation via electrotransformation over a two month period

The study was performed on Roth 2 and comprised (i) a direct *in situ* electrotransformation of bacteria in soil and (ii) an *in vitro* electrotransformation of bacteria extracted from soils (Nycodenz approach) before reinoculation into soil samples. Lindane degradation was monitored in soil microcosms incubated over a two-month period for various conditions (Table III-1). Lindane, TCB and γ-PCCH were detected (Figure III-5), but no other degradation products including 1,4-TCDN; 2,4,5 DNOL; 2,5 DDOL.

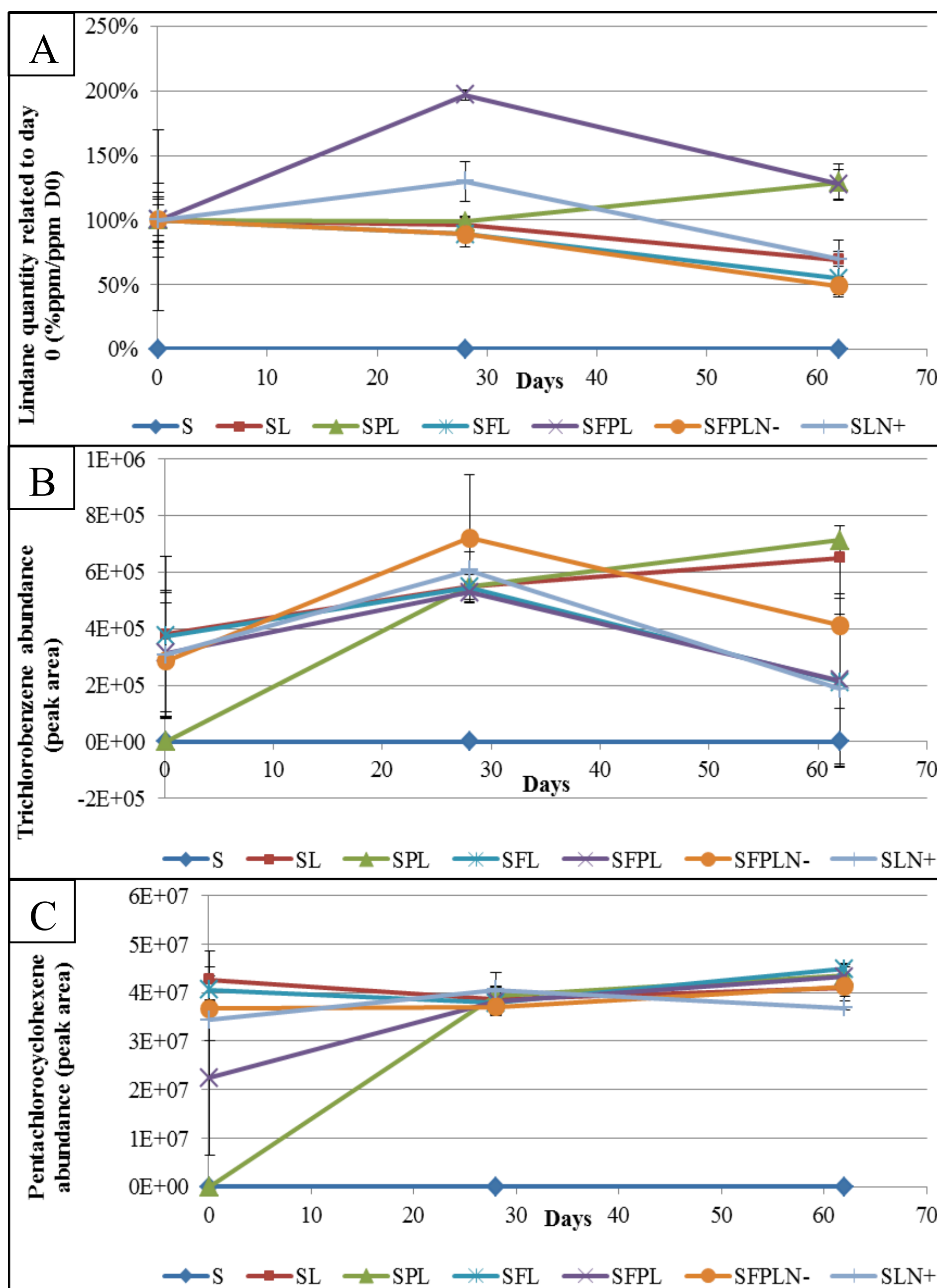


Figure III-5: Lindane quantity related to Day 0 and relative abundance of TCB and γ-PCCH in peak area measured over time in the different samples of the Roth soil

S: soil; SL: soil with lindane; SPL: Soil with pBLN and lindane; SFL: Soil subjected to PEF with lindane; SFPL: Soil subjected to PEF with pBLN and lindane; SFPLN-: Soil subjected to PEF with pBLN, lindane and *P.* sp. N3; SLN+: Soil with lindane and *P.* sp. N+; D0: Day 0.

After one month, the electrotransformation samples (SFPL) showed a significant increase in lindane concentration in comparison with Day 0 (Figure III-5A) and to the control in which the soil containing lindane was not subjected to electrotransformation (SL). This result was surprising, not least because this sample was expected to show a decrease in lindane concentration as a result of degradation following the integration of pBLN into indigenous bacteria and the subsequent expression of the *linA* gene. Lindane was present in equivalent quantities in all other samples, including SL.

One month later, all samples except the natural transformation assay (SPL) exhibited a decrease in lindane concentration, suggesting that adsorption of lindane on soil particles occurs when microcosms are incubated for a longer time (1 to 2 months *versus* the 23 days of the previous experiment). Contrary to expectations, the lindane decrease was significantly lower in the electrotransformation sample (SFPL) when compared with the control containing only soil and lindane (SL). This may be related to a possible imperfect homogeneity of lindane distribution throughout the soil microcosms.

After one month, as with lindane, the relative abundance of TCB did not significantly differ between samples (Figure III-5B). After two months, none of the samples showed a significant TCB increase when compared with the control containing soil and lindane (SL). Also, none of the samples showed a significant increase of γ-PCCH compared to the control containing soil and lindane (SL) after either one or two months (Figure III-5C).

Overall, this two-month experiment failed to confirm the weak lindane degradation observed during the CSA soil two-week study. As for CSA soil experiment, no other degradation product was detected, thus rejecting the hypothesis of further degradation of TCB. This lack of lindane bioremediation may in fact be due to the physicochemical properties and microbial community composition of soil, as showed by Baker *et al.* in 2010.

3. Monitoring of *linA* copy number and expression:

Both CSA and MON soil microbial extracts showed some basal presence of the *linA* gene in the *in vitro* test of our pilot study (Lyon *et al.*, 2010). Congruent with chloride measurements, no significant increase in *linA* copy number was noticed in the negative controls of either soil. In the assays of bacteria electroporated with pBLN and added to soils supplemented with lindane (electrotransformation assays), a significant increase in the *linA* copy number, indicative of the introduction of a significant amount of *linA* into the soil bacteria, was observed for the CSA sample only. This electrotransformation could therefore only augment bioremediation if the incorporated genes are functional. The comparison of the

linA copy numbers to the corresponding chloride concentrations clearly shows that CSA soil bacteria are capable of incorporating the vector pBLN carrying *linA* and of expressing the gene. In turn, for the MON sample, a chloride release was detected suggesting an increase of *linA* expression by bacteria despite the absence of increase in DNA copy numbers.

In the *in situ* test of our pilot study (Lyon *et al.*, 2010), after electrotransformation (day 0), the *linA* fragment targeted by qPCR was found in all MON and CSA samples. For the MON soil, the *linA* level was significantly increased in both natural and electrotransformation samples, which implies that both mechanisms were involved. The *linA* level, while lower in all samples after three weeks, was still significantly higher in the electrotransformed sample. However, only the positive control with *S. francense* sp+ showed a significant increase in chloride release. Therefore, electrotransformation-based gene bioaugmentation was not efficient in contrast to bioaugmentation using *S. francense* sp+ that contains all catabolic genes for lindane degradation. In the CSA samples, the initial *linA* levels were consistent in all samples, even though the electrotransformation was expected to introduce *linA* into some bacteria. This presence in all samples is peculiar because the *linA* gene is a mosaic and specific gene whose occurrence in environmental bacteria is extremely low, being restricted to highly lindane-contaminated soil bacteria (Boubakri *et al.*, 2006). After three weeks as well as after two months of incubation with lindane, the *linA* gene fragment was still detected in all tested samples in agreement with chloride measurements. However, the *linA* copy number continued to decrease through time, in opposition to chloride measurements that were significantly increased in the natural transformation sample (without shock), the electrotransformation sample and the positive controls for electrotransformation and lindane degradation (*Pseudomonas* sp. N3 electroporated with pBLN and *Sphingobium francense* sp+, respectively, added into soils with lindane). This is suggestive of an increase of *linA* expression by indigenous or added bacteria despite the absence of an increase in DNA copy numbers.

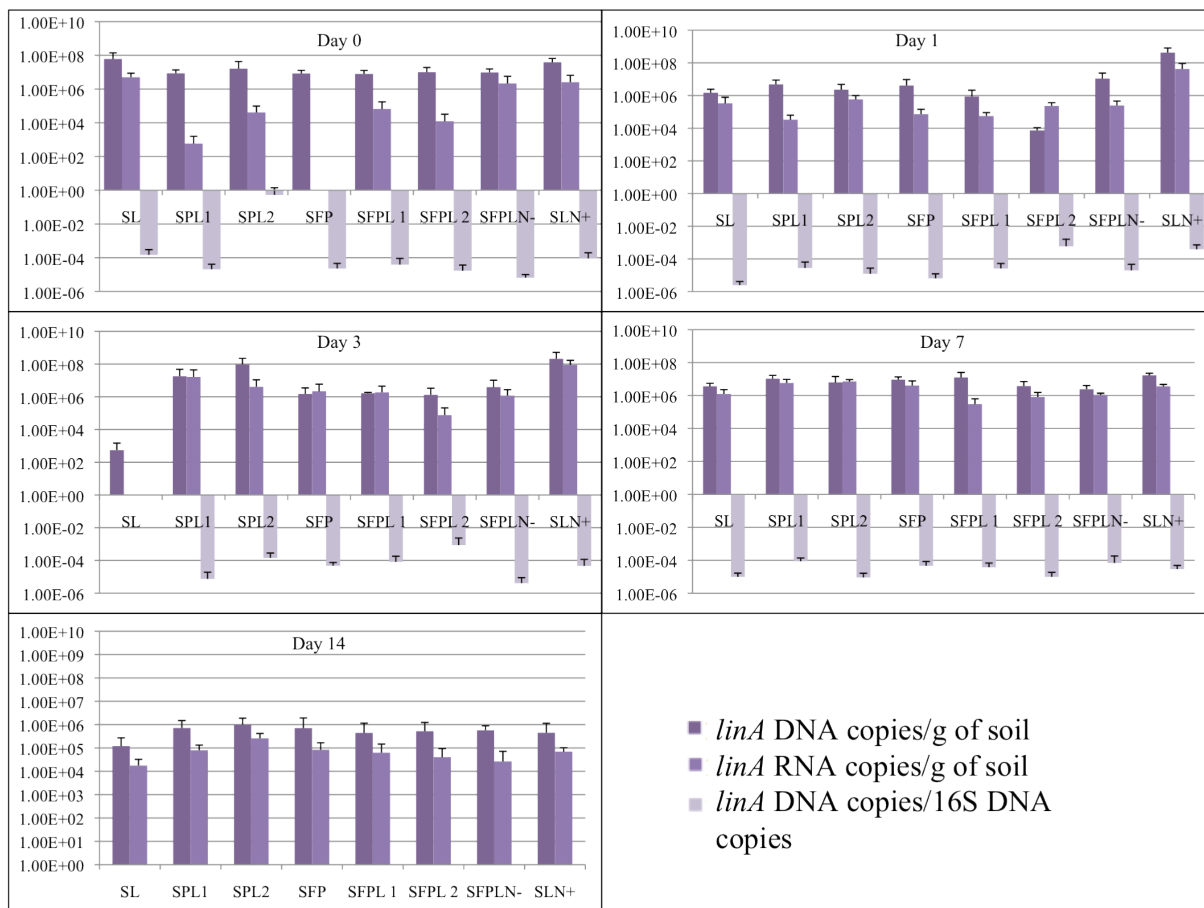


Figure III-6: *linA* presence and expression evaluated by *linA* DNA and mRNA copies.g^{-1} of soil quantified by qPCR and RT-qPCR as well as *linA*/SSU rDNA ratio over time for CSA soil (Day 0 to day 7)

SL: soil with lindane; SPL (x2): Soil with pBLN and lindane; SFP: Soil subjected to PEF with pBLN; SFPL (x2): Soil subjected to PEF with pBLN and lindane; SFPLN-: Soil subjected to PEF with pBLN, lindane and *P.* sp. N3; SLN+: Soil with lindane and *P.* sp.N+.

Based on this preliminary study, we tried to improve the electrotransformation-based bioremediation by indigenous bacteria. Using the CSA soil, we added four-fold higher number of plasmids, representing more than 300 pBLN molecules per soil bacterium, in order to optimize the proximity between bacterial cells and plasmids (10 µg pBLN/30 g of soil - Table III-1) and the probability of plasmid internalization by the bacteria. We first hypothesized that the *linA* DNA copy number would not vary between the samples as previously shown, while the *linA* expression might increase in electrotransformation samples and positive controls. After DNase treatment on CSA soil to remove any non-integrated plasmid molecules, DNA and RNA were extracted; *linA* DNA and mRNA copies were quantified over time by qPCR and RT-qPCR respectively (Figure III-6). SSU rDNAs were also quantified and used to normalize *linA* measurements, in addition to evaluating possible experimental biases.

Directly after electrotransformation (day 0), *lin*A was detected in all samples with no significant difference across samples (about 10^7 copies.g^{-1} of soil). The *linA*/SSU rDNA ratio was significantly higher than the negative control (SL) only in the second natural transformation assay (SPL2: Soil with pBLN and lindane) suggesting an increase of *linA* incorporation by soil bacteria after natural transformation. *linA* mRNA in this sample was significantly lower when compared with the negative control (SL). Therefore, indigenous or added bacteria did not appear to express *linA* and degrade lindane at time 0. After one day, the *linA*/SSU rDNA ratio of the electrotransformation sample SFPL2 was significantly higher than SL but the DNA and mRNA copy numbers of this sample were not. At Day 3, all samples contained copy numbers of *linA* DNA and *linA* mRNA and a *linA*/SSU ratio higher than the negative control SL. However, no copies of SSU rDNA were present in SL, suggesting a bias in the DNA extraction procedure and thus preventing us from either comparing this result with other samples or drawing conclusions for this time of sampling. After one and two weeks (Days 7 and 14), there was no significant difference among the samples in terms of *linA* DNA and mRNA, as well as for the *linA*/SSU rDNA ratio (SSU rDNA was not quantified at Day 14), suggesting a lack of *linA* expression from the soil bacteria in all samples at these times.

SLN+ was significantly higher than SL in *linA* DNA and mRNA copy number.g^{-1} soil and *linA*/SSU rDNA ratio at Day 1 only. Therefore, only the positive control achieving bioaugmentation did show a significant presence and expression of *linA,* suggesting its ability for degrading lindane.

In general, there was a decrease in all copy numbers over time in every sample, as previously shown by Lyon *et al.* (2010) after a three-week period, and furthermore after two months. We suggest that the plasmid was lost either because of the death of the electrotransformed bacteria or by the release of their plasmids (De Gelder *et al.*, 2007). This loss of plasmid in the system could be due to the lack of *in situ* selective benefits to the bacteria carrying and expressing the encoded genes and/or by the metabolic burden caused by pBLN on its host bacterium, resulting in the acidification of the local bacterial microenvironment (Cirpka *et al.*, 1999).

Several potential biases may be called upon to explain these results: (i) Bias in DNA extraction from bacterial cells may explain the observed lower number of SSU rDNA copies resulting in an increase of the *linA*/SSU rDNA ratio, while the *linA* copy number may be less affected. (ii) The DNase treatment prior to RNA and DNA extraction may lack efficiency because of the adsorption of DNase to soil particles (Demanèche *et al.*, 2001b). (iii) A possible DNA contamination of RNA with regard to the co-extraction of both nucleic acids using the RNA isolation kit may have occurred. Indeed, we relied on the manufacturer's claim and did not confirm the absence of DNA in RNA. The absence of DNA should thus be verified in further experiments by qPCR amplification on RNA samples. (iv) Although no pBLN was added as in Lyon *et al.* (2010), *linA* DNA and mRNA were also present in high copies in the negative control (SL). The addition of lindane was suggested to immediately select and increase the proportion of indigenous bacteria containing *linA* (Lyon *et al.*, 2010). Given the importance of the copy number and the lack of degradation shown in the GC-MS analyses, a contamination by pBLN is a more likely explanation. Further studies should involve the isolation and study of potential degrading soil indigenous bacteria.

Whereas Lyon *et al.* (2010) concluded that both the *in situ* electrotransformation and natural transformation increased the lindane-degrading capacity in the CSA soil, we observed a non-significant occurrence of *linA* DNA/mRNA in all samples and at all sample dates, which prevents us from drawing any conclusions. However, the statistical analyses used in the present study are more stringent than Lyon *et al.* (2010).

4. Assessment of repeated application of electric discharge:

We evaluated the impact of repeated shocks (three shocks twice weekly over three weeks) on the amount of incorporated plasmid in soil bacteria and on the bacterial diversity of the Roth 1 soil over a three-week period (Table III-1).

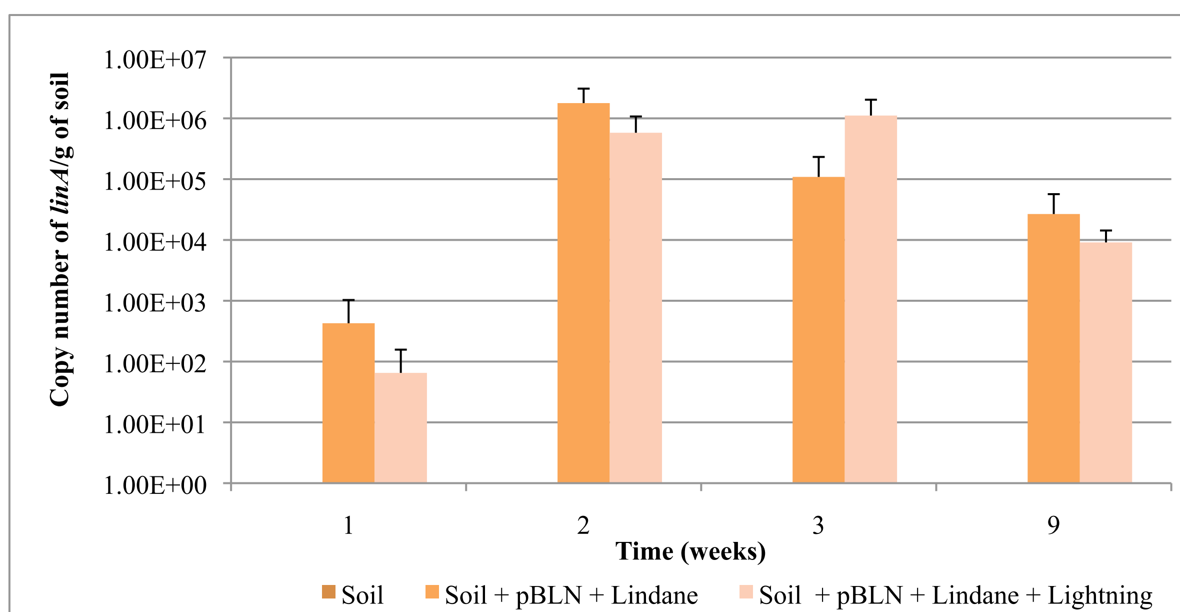


Figure III-7: *linA* DNA copies.g^{-1} of soil evaluated by quantification with qPCR over time for Roth 1 soil in three different samples

Indeed, *in vitro* experiments with mammalian cells have shown that multiple electroporations led to a higher efficiency of transformation (Brown *et al.*, 1992; Baron *et al.*, 2000; Song *et al.*, 2010). We again increased the quantity of added plasmids to reach more than 1000 pBLN per soil bacteria for electrotransformation optimization.

DNase treatment assessment:

Given the previous results suggesting the lack of efficiency of the DNase treatment prior to nucleic acid extraction, we performed several tests using several DNase quantities on 3 µg of pBLN added to 1 g of soil at different times of the procedure (Data not shown). We also tested several concentrations of EDTA to stop the DNase reaction (Data not shown). We concluded that 40 units of DNase were enough to degrade the non-incorporated pBLN added to soil and that 300 mM EDTA was necessary to stop the DNase reaction. After DNAse treatment of exogenous DNA, nucleic acids were extracted and quantified by fluorometry instead of spectrophotometry that was used in the previous study and may potentially overestimate the nucleic acid concentrations.

Monitoring of <u>linA</u> gene incorporation:

qPCR analyses were performed once a week over three weeks and then again six weeks later. Roth 1 soil (Figure III-7) and soil with just lindane (data not shown) did not contain any copies of *linA*.

After a week, the transformation and electrotransformation samples (respectively SPL, *i.e.*, soil with plasmid and lindane, and SFPL, *i.e.*, soil with plasmid and lindane subjected to lightning-simulated repeated PEF) contained equivalent number of *linA* copies (4.27×10^2 and 6.51×10^1 *linA*.g^{-1} of soil, respectively) (Figure III-7). The *linA* copy numbers of the electrotransformation sample increased over time (5.79×10^5 and 1.11×10^6 *linA*.g^{-1} of soil after two and three weeks, respectively) but was not significantly different from the transformation samples (respectively 1.78×10^6 and 1.09×10^5 *linA*.g^{-1} of soil after two and three weeks), suggesting, as for Lyon *et al.* (2010), that both natural and electro-transformation contributed to the incorporation of pBLN in indigenous soil bacteria. A six-week long additional incubation (nine weeks in total) without supplementary shocks showed a significant decrease of *linA* copies.g^{-1} of soil in the electrotransformation sample (2.67×10^4 and 9.11×10^3 *linA*.g^{-1} of soil for SPL and SFPL, respectively). This may be related to the loss of the plasmid because of a lack of selective advantage and the absence of electric shocks.

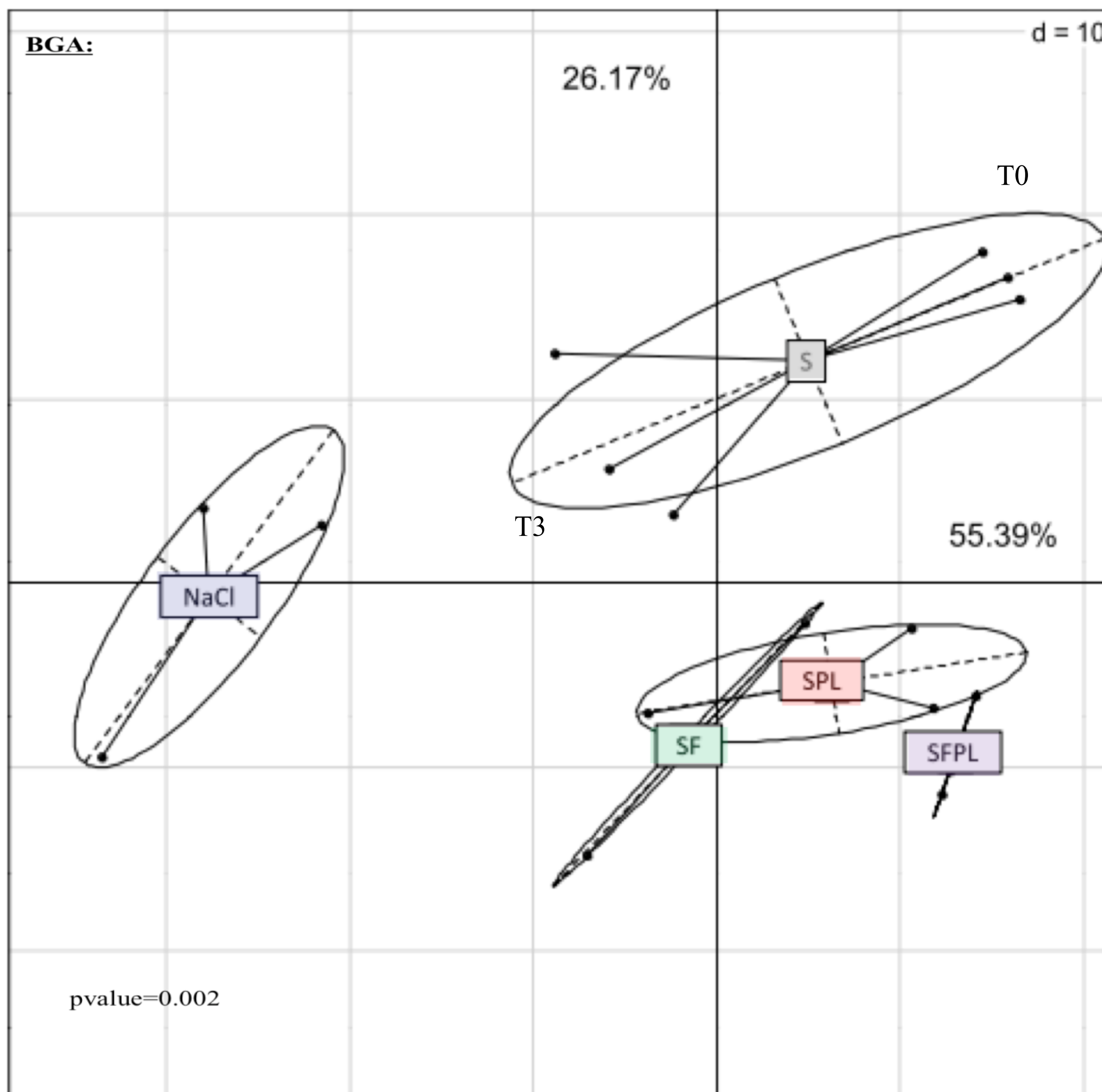


Figure III-8: Between Group Analyses of RISA from different samples (SF, SPL and SFPL) after three weeks of repeated shocks compared to the negative (S at 0 and 3 weeks) and positive controls (SNaCl)
S: soil; SF: Soil subjected to PEF; SPL: Soil with pBLN and lindane; SFPL: Soil subjected to PEF with pBLN and lindane; SNaCl: Soil with NaCl.

Impact on soil bacterial diversity:

In parallel with this study, we monitored the structure of the Roth 1 soil bacterial community to evaluate a possible effect of repeated shocks. After three weeks of repeated shocks, bacterial diversity changes were observed (Figure III-8) in comparison to the negative (Soil S at 0 and 3 weeks) and positive (SNaCl) controls. Indeed, salinity known to change electrical conductivity in soil significantly reduced the diversity of bacteria (Arshad and Farooq, 2003).

A major difference was observed for the positive control (with the SNaCl sample clearly separated from all other samples on the BGA first axis, explaining 55.39% of the RISA sample variability). The second most important difference (axis 2, explaining 26.17% of the variability) was between the negative control (S) and the treated samples (SF sample, *i.e.,* soil subjected to repeated PEF, SPL sample, *i.e.,* soil with pBLN and lindane, SFPL sample, *i.e.,* soil with plasmid and lindane subjected to repeated PEF). Additionally, the bacterial diversity changed over the three weeks of incubation (T0 samples *versus* T3 samples). Among the three week-incubated samples, SFPL was the sole one to be separated from the negative control (T3S) along axis 1, whereas SF and SPL samples overlapped with the latter. Both treatments altered the bacterial community structure, the largest change being due to electrotransformants. Bacteria able to resist repeated PEF, foremost when electrotransformed, may have risen within the bacterial community, as reported previously by Cérémonie *et al.* (2008) where PEF was shown to induce redistribution of soil compounds and increase of part of the soil bacteria.

After six weeks of additional incubation without supplementary electrical discharges (nine weeks in total), all samples exhibited similar overall bacterial community structure (data not shown). The only noticeable effect was related to the additional six weeks incubation period. Therefore, the modification of bacterial communities is not maintained in the absence of repeated electrical shocks.

Table III-3: Number of viable, culturable, antibiotic-resisting and lindane-degrading bacterial cells in soil after different treatment

	Viable cells.mL^{-1} with live/dead	CFU.mL^{-1} on 1/10 TSA-C	CFU.mL^{-1} on 1/10 TSA-CTK	CFU.mL^{-1} on 1/10 TSA-L	Presence of lindane-degradation zones	Survival of bacteria (%)
Soil bacteria (Control)	$2.06\times10^{8} \pm 5.57\times10^{1}$	$5.52\times10^{6} \pm 4.82\times10^{6}$	0	2.88×10^{6}	+	/
Soil bacteria + pBLN (Natural transformation control)	/	7.00×10^{5}	0	4.60×10^{5}	+	/
Soil bacteria electroporated with pBLN (Electro-transformation sample)	/	$3.01\times10^{5} \pm 2.55\times10^{4}$	$2.00\times10^{1} \pm 1.63\times10^{1}$	$2.36\times10^{5} \pm 2.83\times10^{4}$	++	43.00 ± 3.64%

Values with standard deviation are the mean of at least three experiment repetitions.

5. Bioremediation potential of gene-bioaugmented indigenous soil bacteria:

***In vitro* electrotransformation potential of indigenous soil bacteria:**

Electrotransformation potential of indigenous soil bacteria was ascertained *in vitro* by comparing the lindane degradation capacity and antibiotics resistance level of the electrotransformed isolates to those obtained after natural transformation of the plasmid, as well as to those observed in the absence of transformation.

Indigenous bacteria, which were extracted from Roth 2 soil amounted to approximately 2.10^8 viable cells per mL of sucrose, *i.e.,* 10^7 viable cells per experiment (50 µL), 2.68% of which were culturable on TSA supplemented with cycloheximide (TSA-C) (5.10^6 CFU of indigenous soil bacteria per mL of sucrose) (Table III-3). We established the level of natural resistance to the antibiotics used as markers in our experimental procedure and found no naturally tetracycline and kanamycin-resistant bacteria when grown on TSA-CTK medium. In turn, some clearance halos were detected on TSA supplemented with lindane (TSA-L) (Table III-3) suggesting that some of the Roth 2 soil bacteria did possess a natural lindane degradation potential.

The control sample for natural transformation also did not contain bacteria that were able to resist both antibiotics - but did contain some lindane degraders. Forty three percent of soil bacteria were able to survive the electrical shock, 20 $CFU.mL^{-1}$ (mean value) were able to resist both antibiotics when grown on TSA-CTK and these showed an increase in the size of the lindane-degradation zone on TSA-L, thus confirming the electrotransformation potential of the Roth 2 indigenous bacteria.

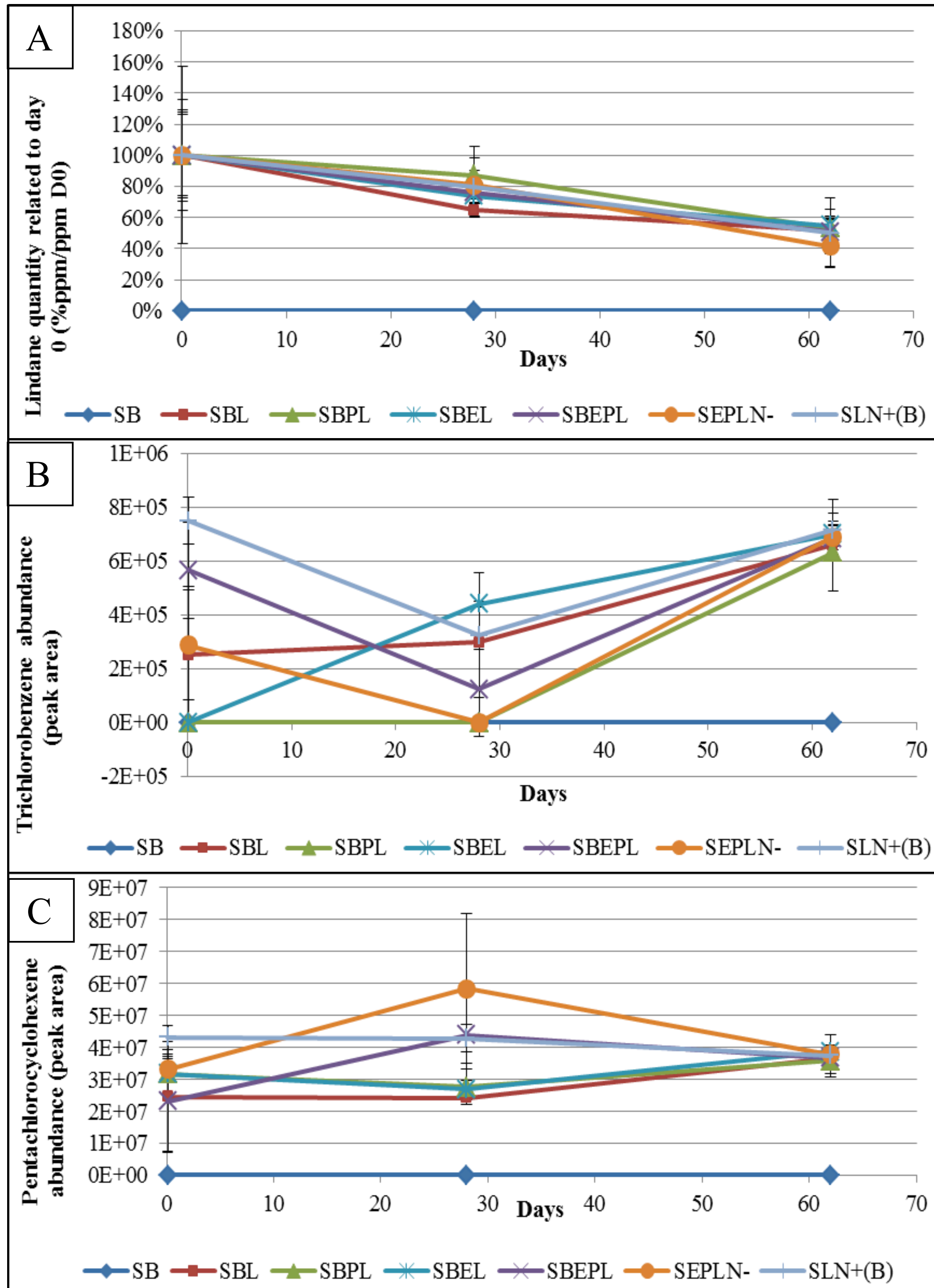


Figure III-9: Lindane quantity related to Day 0 and relative abundance of TCB and γ-PCCH in peak area measured over time in the different samples of the Roth soil

SB: Soil with indigenous bacteria; SBL: Soil with indigenous bacteria and lindane; SBPL: Soil with lindane and indigenous bacteria with pBLN; SBEL: Soil with lindane and electroporated indigenous bacteria; SBEPL: Soil with lindane and electroporated indigenous bacteria with pBLN; SFPLN-: Soil with lindane and electroporated *P.* sp. N3 with pBLN; SLN+: Soil with lindane and *P.* sp. N+; D0: Day 0.

***In situ* bioremediation potential of *in vitro* electrotransformed indigenous soil bacteria**

Roth 2 soil-extracted bacteria submitted *in vitro* to a single PEF were then replaced in soils to evaluate whether this process might translate into *in situ* bioremediation and/or be more efficient than the *in situ* soil experiment (Section 2.3 of Results and Discussion). As before, only lindane, TCB and γ-PCCH could be measured over time in the different samples (Figure III-9). No other degradation product was detected.

After one month, none of the samples showed any significant decrease in lindane compared to the control containing soil and lindane (SL) (Figure III-9A). After two months, all samples showed a similar decrease suggesting that adsorption to soil particles occurred, as in the *in situ* experiment on Roth 2 soil, again preventing us from drawing any conclusions regarding the lindane degradation. As for lindane, there were no significant differences in TCB relative abundance between samples after one month. We therefore sampled again one month later (Figure III-9B). After two months, none of the samples showed a significant increase in the lindane degradation product TCB compared to the control containing soil and lindane (SL). None of the samples showed a significant increase of γ-PCCH compared to the control containing soil and lindane (SL) after one or two months (Figure III-9C).

Thus, the *in vitro* electrotransformation experiments, although more successful, were not efficient when bacteria were reinocluated into soils, which is commonly reported in the literature due to the inherently complex nature of soils (Desaint *et al.*, 2003). Furthermore, the lack of an *in situ* selective pressure may explain why liquid electrotransformation experiments were more successful than the *in situ* soil experiments. The lindane dechlorination ability observed in our *in vitro* pilot study (Lyon *et al.*, 2010) may have result more from the selective pressure of both antibiotics (tetracycline and kanamycin) used for the bacterial incubation than from the presence of lindane. Therefore, for both *in situ* and *in vitro* experiments, we suggest that pBLN does not provide an *in situ* selective benefit to the bacteria carrying and expressing the encoded genes because of the lack of new carbon sources. Furthermore, lindane dechlorination represents a metabolic burden due to the acidification of the local environment and is likely to lead to plasmid loss.

CONCLUSION AND PROSPECTS

Our studies showed that *in situ* electrotransformation-mediated gene bioaugmentation could not serve as a bioremediation tool for lindane degradation in these conditions. Adsorption on soil particles appears to constitute a major logistical problem to both pBLN and lindane addition over time. Several enhancements could be applied: (i) Desorption of bounded-molecules to soil particles before lindane extraction and DNase treatment. Indeed, DNases (as well as nucleic acids) can be bound on soil particles (Demanèche *et al.*, 2001b) thereby reducing their bioavailability; (ii) Improvement of lindane extraction from soils by additional extraction steps with hexane as in Rigas *et al.* (2007) or other solvents as in Pereira *et al.* (2008) and Benimeli *et al.* (2008); (iii) Improved extraction of only incorporated plasmids by Nycodenz extraction of soil bacteria (Bertrand *et al.*, 2005) or (iv) use of liposomes or cells containing DNA for a better gene delivery (Sato *et al.*, 2005).

The system chosen in this research, lindane degradation *via* pBLN acquisition, aimed to demonstrate the feasibility of the general bioremediation method, but failed to provide a suitable model. In future attempts, the MGE chosen should confer a selective advantage to the bacteria carrying and expressing the encoded genes to enhance its *in situ* persistence in the bacterial community. In this study, pBLN contained the *linA* gene that encodes only the protein performing the first two-dechlorination steps of lindane, whereas pADP-1, for example (isolated from *Pseudomonas* sp. strain ADP) contains the complete catabolic sequences (Martinez *et al.*, 2001) for another widely used and dangerous pesticide, atrazine (Gammon *et al.*, 2005). This plasmid could confer a selective advantage to electrotransformed bacteria since they might use atrazine as a carbon source while suffering less from the resulting acidification of the local environment, as occurs during dechlorination of polychlorinated compounds such as lindane.

Although the stability and maintenance of MGEs has previously been shown to be difficult while attempting gene bioaugmentation (Desaint *et al.*, 2003), the stability of the degradation gene in the system can be improved by locating the gene in a transposon that could integrate into the chromosome of the host bacterium (Springael and Top, 2004; Shintani *et al.*, 2005). Degradation genes could also be engineered for higher efficiency and/or better control and then electrotransformed into indigenous bacteria. The addition of a PEF may be used to facilitate the uptake of these elements into bacteria that might not ordinarily participate in HGT.

Even if we overcame these difficulties with the changes proposed in experimental design and achieve an efficient bioremediation *via in situ* electrotransformation results, another hurdle would have to be faced - the actual application of this technology in the field. Two methods are proposed: firstly, *in situ* current generation or the application of current to smaller soil samples that are then replaced into the field. Technologies for *in situ* current generational ready exist, such as PEF used for disinfecting foods in food packaging plants (Wouters and Smelt, 1997; Wouters *et al.*, 1999). This technology would have to be developed and optimized for field situations. The second proposal, the use of microcosms to reinoculate the soil, would be more feasible with existing technologies. Soil bacteria could be extracted as performed for the Roth 2 soil or by Nycodenz gradient from the site to be remediated, then electrotransformed with the advantageous degradation genes of interest and then reinoculated into the site. This still presents an advantage over bioaugmentation, since indigenous bacteria are more adapted to the site than any foreign inocula. However, such an approach raises ethical and legal issues.

APPENDICES

Appendix III-1: Delina Y. Lyon, Jérémy Pivetal, Laurine Blanchard, and Timothy M. Vogel - 2010 - Bioremediation *via In situ* Electrotransformation - *Bioremediation Journal*, 14(2): 109–119, 2010

CONCLUSION AND FURTHER WORK

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Bacteria are the most abundant organisms in the biosphere. Their considerable genetic and functional diversity acquired during 3.5 billion years of evolution has allowed them to adapt to the continuous disturbance of the environment and to colonize all types of ecosystems (Whitman *et al.*, 1998). Bacterial activities are fundamental to many ecological and biogeochemical cycles (Evans, 1976; Falkowski *et al.*, 2008; Godfrey and Glass, 2011), and are essential to humans. Indeed, they contribute to human health (new medicine) (Ishige *et al.*, 2005), the health of the environment (treatment of chemical pollution of soils) (Vogel, 1996), and of food production (promotion of plant growth, protection against pathogens, cheese and yogurt production through fermentation) (Leroy and De Vuyst, 2004; Lucy *et al.*, 2004). Furthermore, humans and animals associate permanently with bacteria through beneficial relations (Thompson, 1978) even if their importance is overshadowed by the pathogenic character of a minority of them (Mansfield *et al.*, 2012; World Health Organization, 2013).

To diversify their genetic material and allow adaptation to environmental disturbances and colonization of new ecological niches (Wiedenbeck and Cohan, 2011), bacteria use various evolutionary processes including point mutations and endogenous sequence rearrangements (Matic *et al.*, 1997; Gordo *et al.*, 2011), as well as acquisition of new genetic information by horizontal genetic transfer (HGT) mechanisms including conjugation, transduction and transformation (Ochiai *et al.*, 1959; Syvanen, 1985; Jain *et al.*, 2002; Aminov, 2011; Popa and Dagan, 2011; Zhaxybayeva and Doolittle, 2011). Additionally, electro-transformation mediated by lightning-related electrical parameters has been proposed as a complementary gene transfer mechanism occurring in nature (Demanèche *et al.*, 2001c), long after the application of an electrical pulse started to be used in laboratories in the 1960s to introduce DNA into cells of most bacterial taxa, as well as in fungal, plant or animal cells (Maniatis *et al.*, 1982; Dower *et al.*, 1988; Chakraborty and Kapoor, 1990; Gilchrist and Smit, 1991; Drury, 1996; Weaver and Chizmadzhev, 1996; Lurquin, 1997; Newell, 2000; Villemejane and Mir, 2009).

Lightning creates electrical fields associated with currents in the same range of values as those developed by an electroporator. Preliminary experiments reporting the isolation of transformants from soil subjected to a simulation of lightning discharges confirmed that such meteorological phenomena might be implicated in the triggering of gene exchange among bacteria in a natural setting (Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006a). Considering the tremendous number of thunderstorms and lightning discharges worldwide, the electrical perturbations that they induce and the large volume of soil in which bacteria can

be affected by a "transforming" electrical activity, natural electrotransformation may contribute to the shaping of bacterial genomes. This could solve the discrepancy observed between the low frequency to which conjugation, transduction, natural transformation based HGT seem to occur under natural conditions and the high HGT frequencies deduced from *in silico* analyses of bacterial genomes (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994; Davison, 1999).

Beyond studies focusing on soil, other environmental bacteria may be subjected to lightning mediated electrotransformation, most notably bacteria living in clouds, which are the seat of electric fields and are subjected to many more lightning discharges than soil (Gary, 1999). The presence in clouds of bacteria exhibiting the ability to form ice nuclei (IN) that lead to precipitations (Morris *et al.*, 2004) and are involved in the triggering of lightning (Gonçalves *et al.*, 2012) associated with compatible electric field pulses (few $kV.cm^{-1}$) (Nucci *et al.*, 1988) led us to postulate that natural electrotransformation in clouds may affect bacteria in the same way as was observed in soils. Among IN bacteria, one of the most ice nucleate active bacteria is the global phytopathogen *Pseudomonas syringae* (Morris *et al.*, 2010; Mansfield *et al.*, 2012), assumed to spread worldwide through the water cycle (Morris *et al.*, 2008). This bacterium might take advantage of its ice-nucleate potential and induce the triggering of phenomena that would contribute to an increase in its adaptive potential, thereby improving its dissemination and plant pathogenic ability. This would require that these bacteria are able to survive lightning discharges and exhibit some electrotransformation abilities for using electric field pulses associated with lightning currents. Combined with a strong dissemination potential through precipitations from clouds, *P. syringae* therefore might be proposed as a model of a particularly adaptable microorganism for colonizing new niches.

We first determined if an ice nucleator bacterium could survive and acquire new genes in clouds based on lightning shock-simulating *in vitro* electroporation of *P. syringae* CC0094. In comparison with two other bacteria (*Pseudomonas* sp. N3 and *Escherichia coli* TOP10), *P. syringae* appears to be the best adapted for survival and for being genetically electro-transformed in clouds, which suggests that it would be able to survive and evolve while being transported in clouds. Furthermore, *P. syringae* may be involved in the triggering of lightning in clouds (Gonçalves *et al.*, 2012) that may help aerosolized bacteria to develop new competence such as phytopathogenicity, to improve their fitness and adapt to new niches and influence the indigenous microbial communities.

Secondly we evaluated the impact of lightning on survival, electro-transformation potential and diversity of rain bacteria. These resisted lightning better than laboratory strains,

possibly because they had been selected for when encountering the harsh conditions of an atmospheric journey. In turn, these maintenance conditions may have somehow "counter-selected" the laboratory strains. *P. rhizosphaerae* was the sole species to be retrieved from the electrotransformed samples only. *P. syringae* was relatively poorly represented among our rain isolates that encompassed different origins and life modes, including ice nucleators, cold-adapted bacteria, as well as mammal or plant pathogens, saprophytes or plant growth promoters and bacteria with biodegradation capacities, representatives of the variable sources of bacterial emissions from Earth. Although resistant to electrical discharges, *P. syringae* was not isolated either when naturally or electrically transformed, unlike our *in vitro* study of *P. syringae* CC0094 electrotransformation in simulated cloud conditions. This study, the first to address these questions to the best of our knowledge, suggests that bacteria aerosolized from diverse terrestrial ecosystems, can spread to new habitats through clouds. Unsurprisingly, most of the isolates we recovered from rain appeared to originate from vegetation, as reported by Šantl-Temkiv *et al.* (2013), who suggested that the storm cloud environment selected for plant bacteria because of the common stress factors shared between phyllosphere and atmosphere. Plant-associated bacteria appeared to be better adapted than soil ones to survival and growth in atmospheric conditions. In turn, organic matter is preferentially aerosolized from soils (Šantl-Temkiv *et al.*, 2013) and could support the growth and life of bacteria in clouds. Furthermore, soil dust is a major driver of ice nucleation in clouds leading to precipitations (Conen *et al.*, 2011). Soil organic matter could act synergistically with plant-associated bacteria to help these organisms to increase their adaptive potential through lightning formation within clouds and to disseminate through precipitations.

Both these studies suggest that while disseminating through clouds and inducing passively triggered lightning, ice nucleate active bacteria could acquire new genes in clouds *via* lightning electrotransformation, thereby possibly enhancing their genetic diversity. In addition to the harsh conditions that bacteria encounter in the atmosphere, protection against lightning as well as natural or electro-transformation may contribute to microbial evolution. Our results could explain why *Pseudomonas* are among the most ubiquitous and highly ice nucleation active bacteria found in nature, how they adapt to different habitats as well as cause economically important (plant) diseases. Furthermore, they may be causal agents of precipitation and their eradication may thus threaten a generative source of rain (Cohen, 2012).

Further work should include the testing of ice nucleation activities and pathogenicity of our isolates recovered from rain, most importantly *P. syringae* and of the electrotransformed

P. rhizosphaerae. For these organisms, additional investigations should aim at evaluating their ability for survival and genetic electro-transformation in the cloud-like conditions used in the first part of our study. Another avenue of research would be to confirm the effect of protection to lightning in icy conditions supposedly due to the IN activity by testing *ina*-mutants of the same *P. syringae* strain. Further corroboration of our results should be sought by performing similar studies in improved experimental conditions, as close as possible to the *in situ* cloud environment, e.g., the cloud chamber available at the AIDA (Aerosol Interaction and Dynamics in the Atmosphere) facility at Forschungszentrum Karlsruhe (Möhler *et al.*, 2008). Indeed, AIDA can be operated as a pumped expansion chamber to investigate aerosol-cloud processes at the simulated temperature and humidity conditions of atmospheric clouds.

Lightning-mediated natural electrotransformation contributing to the horizontal transfer of genes among soil bacteria (Gary, 1999; Demanèche *et al.*, 2001c; Cérémonie *et al.*, 2004, 2006a) led us to envisage that electrotransformation could be applied to the engineering of indigenous soil bacteria. The final part of our work thus consisted in evaluating how transferring cell-free plasmid DNA directly into bacteria at a field site using an electric exponential discharge application was able to affect a larger number and variety of bacteria. This study was performed in the context of developing a tool for bioremediation of lindane, a widely used pesticide prior to the 2006 ban on its agricultural use.

- A pilot experiment published in 2010 in Bioremediation Journal (Lyon *et al.*, 2010) demonstrated that *in situ* electrotransformation leading to gene bioaugmentation could serve as a bioremediation tool. However, the liquid electrotransformation experiments proved to be more successful than the *in situ* ones.
- Optimization of the *in situ* procedure involving careful control of the experimental conditions (adjustment of humidity and electrical parameters, improvement of the molecular biology and chemical processing steps, as well as testing of soils with different physical characteristics and monitoring over various periods of time) revealed that both natural and electro-transformation contributed to the incorporation by soil indigenous bacteria of a plasmid harboring a gene encoding the first lindane dechlorination steps. The bacterial diversity structure appeared to be affected by the different treatments, the largest change being for electrotransformants. Bacteria able to resist repeated PEF, foremost when electrotransformed, may have risen within the bacterial community.
- Finally, the testing of *in vitro* electrotransformation-based gene bioaugmentation of soil extracted bacteria prior to re-inoculation in their soil of origin, failed to show a significant

effect when they were returned to soils. This is suggestive of an absence of bioremediation potential *in situ* of electrotransformed indigenous bacteria despite their *in vitro* bioremediation ability.

Besides possible adsorption phenomena on soil particles over time (for the plasmid solution and/or pollutant that merit further testing), the poor electrotransformation-based bioremediation may result from the metabolic burden imposed upon bacteria that incorporate the plasmid, higher than the selective benefit that it was expected to provide. Further attempts should thus use mobile genetic elements that confer to bacteria new traits enhancing their perpetuation in the bacterial community. The best way for the application of this technology in the field should nevertheless be the *in vitro* electrotransformation of the soil-extracted bacteria prior to their reinoculation into the soil. Numerous modifications and adjustments are still needed to optimize the tool development for bioremediation purposes.

In conclusion, we showed that natural electrotransformation mediated by electrical discharges such as those occurring in clouds or reaching soils, can be involved in the horizontal gene transfer process among bacteria and, considering the importance of lightning worldwide, may play a role in the adaptation and evolution of these organisms. The next steps forward are to demonstrate that this is indeed occurring in nature and to investigate how important an evolutionary mechanism this may constitute.

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Abstract

To diversify their genetic material, allowing adaptation to environmental disturbances and colonization of new ecological niches, bacteria use various evolutionary processes, including the acquisition of new genetic material by horizontal transfer mechanisms such as conjugation, transduction and transformation. Electrotransformation mediated by lightning-related electrical phenomena may constitute an additional gene transfer mechanism occurring in nature. The presence in clouds of bacteria capable of forming ice nuclei that lead to precipitations and are involved in the triggering of lightning, such as the global phytopathogen *Pseudomonas syringae,* led us to postulate that natural electrotransformation in clouds may affect bacteria, by contributing to increase their adaptive potential. We first determined if the ice nucleator bacterium *P. syringae* could survive when in clouds and acquire exogenous genetic material through lightning shock-simulating *in vitro* electroporation. In comparison to two other bacteria, *P. syringae* appears to be best adapted for survival and for genetic electrotransformation under these conditions, which suggests that this bacterium would be able to survive and evolve whilst being transported in clouds. Secondly, we evaluated the impact of lightning shock-simulating *in vitro* electroporation on the survival, the electrotransformation potential and the diversity of bacteria collected from rain samples. These isolates better resisted lightning than the laboratory strains and some were able to electrotransform exogenous DNA. The rain bacteria we isolated were of different origins and were representative of life modes of the various sources of bacterial emissions on Earth. Our study suggests that bacteria aerosolized from diverse terrestrial ecosystems can spread to new habitats through clouds whilst also being able to acquire new genetic material *via* lightning-based electrotransformation, thereby potentially enhancing their genetic diversity. The final part of our work consisted of evaluating whether electrotransformation could be applied to the engineering of indigenous soil bacteria in order to develop a tool for the bioremediation of lindane, a once widely used pesticide. Optimized experiments revealed that both natural and electrotransformation contributed to the incorporation of a plasmid harboring a gene encoding the first lindane dechlorination steps by indigenous soil bacteria. In conclusion, we showed that natural electrotransformation mediated by electrical discharges such as those occurring in clouds or reaching soils can be involved in the horizontal gene transfer process among bacteria and, considering the importance of lightning worldwide, may play a role in the adaptation and evolution of these organisms.

Key words: lightning, electrotransformation, electroporation, HGT, cloud, ice nucleate active bacteria, rain, *Pseudomonas syringae*, survival, diversity, adaptation, lindane bioremediation.

Résumé

Pour diversifier leur matériel génétique, s'adapter aux perturbations environnementales et coloniser de nouvelles niches, les bactéries utilisent plusieurs processus évolutifs dont l'acquisition de matériel génétique par transfert horizontal de gènes comme la conjugaison, la transduction et la transformation. À ces trois mécanismes naturels s'ajoute l'électrotransformation due aux phénomènes électriques liés à la décharge de foudre. La présence dans les nuages de bactéries aérosolisées capables de former des noyaux de glace à l'origine des précipitations et impliquées dans le déclenchement de la foudre, telles que la bactérie phytopathogène à répartition mondiale *Pseudomonas syringae*, nous a conduit à proposer que l'électrotransformation naturelle dans les nuages pouvait affecter les bactéries, contribuant ainsi à augmenter leur potentiel adaptatif. Dans un premier temps, nous avons déterminé si la bactérie glaçogène *P. syringae* pouvait survivre à des électroporations simulant des décharges de foudre et acquérir du matériel génétique exogène dans les nuages. Comparée à deux autres bactéries, *P. syringae* se révèle être mieux adaptée pour la survie et l'électrotransformation génétique, ce qui suggère qu'elle serait capable de survivre et d'évoluer durant son transport dans les nuages. Nous avons ensuite évalué l'impact d'électroporations simulant les décharges de foudre sur la survie, le potentiel d'électrotransformation et la diversité de bactéries présentes dans des échantillons de pluie comme substitut des communautés bactériennes des nuages. Ces dernières étaient plus résistantes que les souches de laboratoire et certaines étaient capables d'acquérir de l'ADN exogène par électrotransformation. Les bactéries de la pluie isolées provenaient de différentes origines et présentaient différents modes de vie, représentatifs des sources probables d'émissions de bactéries terrestres. Cette étude montre que les bactéries aérosolisées de divers écosystèmes terrestres sont susceptibles de se disséminer dans de nouveaux habitats grâce aux nuages tout en étant capable d'acquérir de nouveaux gènes par éléctrotransformation, et d'augmenter ainsi potentiellement leur diversité génétique. La dernière partie de mon travail a évalué si l'électrotransformation appliquée aux bactéries indigènes du sol pouvait être employée pour dépolluer les sols contaminés par un pesticide largement utilisé autrefois, le lindane. L'optimisation des expériences met en évidence l'incorporation par les bactéries indigènes d'un plasmide contenant le gène codant les premières déchlorinations du lindane au travers d'une combinaison de transformation naturelle et d'électrotransformation. En conclusion, nous avons montré que l'électrotransformation naturelle liée aux décharges électriques, comme celles se produisant dans les nuages ou atteignant le sol, peut être impliquée dans le transfert horizontal de gènes chez les bactéries et, considérant l'importance de la foudre à travers le monde, pourrait jouer un rôle dans l'adaptation et l'évolution de ces organismes.

Mots-clés: foudre, électrotransformation, électroporation, THG, nuages, bactéries glaçogènes, pluie, *Pseudomonas syringae*, survie, diversité, adaptation, bioremédiation du lindane.