Isolation of Lightning-Competent Soil Bacteria

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Artificial transformation is typically performed in the laboratory by using either a chemical (CaCl$_2$) or an electrical (electroporation) method. However, laboratory-scale lightning has been shown recently to electrotransform *Escherichia coli* strain DH10B in soil. In this paper, we report on the isolation of two "lightning-competent" soil bacteria after direct electroporation of the Nycodenz bacterial ring extracted from prairie soil in the presence of the pBHCRec plasmid (Tc^r, Sp^r, Sm^r). The electrotransformability of the isolated bacteria was measured both in vitro (by electroporation cuvette) and in situ (by lightning in soil microcosm) and then compared to those of *E. coli* DH10B and *Pseudomonas fluorescens* C7R12. The electrotransformation frequencies measured reached 10^{-3} to 10^{-4} by electroporation and 10^{-4} to 10^{-5} by simulated lightning, while no transformation was observed in the absence of electrical current. Two of the isolated lightning-competent soil bacteria were identified as *Pseudomonas* sp. strains.

Bacterial survival can be considered a successful adaptation to changing environmental conditions. Antibiotic resistance and xenobiotic degradation are common examples of the adaptability of the bacterial community (7, 17). Horizontal gene transfer among bacteria has influenced both rapid environmental adaptation and slow species evolution processes (7, 27). Genetic transformation, described as the stable acquisition of exogenous DNA, has been commonly divided into natural and artificial transformations. Natural transformation (5, 18) is an active process of DNA uptake requiring specific genes and a competence state in bacteria. Artificial transformation is considered a passive process requiring chemical (e.g., salt) or electrical (e.g., electroporation) methods in order to affect the cellular membrane and allow DNA introduction. Electroporation is commonly used in laboratories to introduce DNA into bacterial, fungal, plant, or animal cells by subjecting cells to an electric field (10, 20, 22, 26). Nonpermanent pores are formed in the lipid bilayer and other components of the cell membrane (19). While electrotransformation can be used on a large range of cells, the electrotransformation of bacteria often differs among species and generally requires some cell-specific preparation.

While horizontal gene transfer has been shown to occur in situ (9, 14, 18), the distinction between natural and artificial transformation has weakened due to observations of the influence of natural salt or electrical discharges on bacterial transformations in situ. The possible salt-dependent transformation of *Escherichia coli* strains was described when an *E. coli* strain, which was incompetent in the absence of salt, underwent chemical transformation in calcareous freshwater (3). In soil, salt concentrations are described as compatible with the induc-

tion of a competent phase (18). In addition, Demanèche et al. (8) showed that the electrical parameters occurring during electroporation (in vitro) were similar to those in soil microcosms subjected to lightning-mediated current injection (in situ). Moreover, these authors provided evidence for lightning-mediated gene transfer (electrotransformation) of an *E. coli* strain in sterile soil. However, the possibility of soil bacteria capable of lightning-induced electroporation was not investigated.

The aim of this work was to isolate lightning-transformable soil bacteria. Thus, we used the electroporation technique on the Nycodenz-extracted bacterial community with a selective plasmid to isolate electrotransformable soil bacteria, which were subsequently tested for their ability to incorporate free DNA when exposed to laboratory-scale lightning in soil microcosms.

Isolation of electrocompetent soil bacteria. Improvement of cell extraction methodology for soil genomic approaches has focused on maintaining the greatest bacterial diversity possible. The dispersion of the soil particles and the separation of the cells from soil particles by centrifugation constitute the two crucial steps for direct cell extraction (15, 25). Among the different methodologies tested, the high-speed centrifugation method based on Nycodenz density gradient centrifugation provided the best results (1, 16). Soil-derived cell suspensions obtained by the Nycodenz gradient method have been shown to be representative of the original community (6, 12, 24) with minimal effects on cell integrity and physiology (13, 16, 21). The Nycodenz-mediated extraction of bacteria from the soil matrix was performed as previously described (6). Soil from Montrond constituted the source of the bacterial soil community since it provided a high density of bacteria compared to soil from Côte Saint-André, for which the Nycodenz ring was difficult to obtain. Nycodenz-extracted cells (40 μl) were electroporated in the presence of the pBHCRec plasmid (400 ng) in a 0.2-cm-gap electroporation cuvette (Equibio, Ashford, United Kingdom) with one pulse (Gene Pulser II; Bio-Rad,

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TABLE 1. Characteristics of plasmids used in this study

Plasmid	Characteristic(s)	Resistance	Reference
pLEPO1	7 kb, plastid (*accD-rbcL*) sequences flanking the *aadA* gene		14
pBBR1MCS-3	5 kb, broad host range	Tc[r]	13
pBAB1	pUC18 containing *accD-rbcL* genes	Ap[r]	14
pBHC	8.1 kb, pBBR1MC-S3 derivative containing *accD-rbcL* genes	Tc[r]	This study
pBHCRec	9.7 kb, pBBR1MC-S3 derivative containing *accD-rbcL* genes	Tc[r], Sp[r], Sm[r]	This study
pGEMTeasy	Cloning vector	Ap[r]	Promega

Hercules, Calif.) at 12.5 kV/cm, 200 Ω, and 25 μF. The pBHCRec plasmid (Table 1) was constructed by introducing the PvuII DNA fragment (4,493 bp) from plasmid pLEPO1 into the SmaI site of the broad-host-range plasmid pBBR1MCS-3 (Table 1). The pBHCRec transformants were screened at 28°C on Luria-Bertani medium (0.5% NaCl) or 1/10 Trypticase soy broth based on these plasmid markers: streptomycin, 100 μg/ml; spectinomycin (Sigma-Aldrich, Saint-Quentin Fallavier, France), 75 μg/ml; and tetracycline (Boehringer Mannheim France SA, Meylan, France), 20 μg/ml. Two fungicidal drugs (nystatin [50 μg/ml] and cycloheximide [200 μg/ml]; ICN Biomedicals, Inc., Aurora, Ohio) were also required to avoid growth of the vegetative forms of spores present in the Nycodenz cell suspension. Two colonies (Ee2.1 and Ee2.2) were isolated after 2 days during the first experiment; one colony (N3) was isolated after 4 days during the second experiment. Cell-DNA mixtures without electrical discharge (natural transformation) and cells alone with and without electrical discharge (spontaneous mutation and native resistant) were used for control experiments. While all of the controls were negative after 2 days, 20 morphologically similar colonies were detected after 4 days. These colonies did not contain the plasmid. The N3 strain was selected based on its morphological difference with the spontaneous antibiotic-resistant colonies. The high number of resistant colonies on 1/10 Trypticase soy broth medium prevented the isolation of any transformants. Other more appropriate plasmids for better discrimination between natural resistance bacteria and electrotransformants are under investigation, with the goal of evaluating the size and diversity of the electrotransformable soil community.

Plasmid presence in the three isolated soil bacteria was confirmed by PCR with two sets of primers (415-416 and Chloro1-416). The presence of nonbacterial (plastid) sequences confirmed that the transformants were not members of native naturally antibiotic-resistant soil bacteria. The thermal cycling parameters used were 3 min at 94°C; 30 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 55°C, and elongation for 60 s (70 s for Chloro1-Chloro2) at 72°C; and a final extension of 7 min at 72°C. The Chloro1-416 primer set was used to amplify part of the plastid and *aadA* genes (14). The 415-416 primer set was used to amplify the *aadA* gene.

Electrotransformation versus natural transformation. Natural transformation requires a specific state of competence (18). To determine whether the isolated transformants were specifically obtained after electrotransformation instead of natural transformation, the isolates were tested for their natural transformability. The strains were first cured of the pBHCRec plasmid by subculturing without any selective pres-

sure. The absence of the plasmid was confirmed by PCR (415-416 primer set). Ee2.1 could not be cured of the plasmid, so only strains Ee2.2 and N3 were used for all of the following transformation experiments. The pBHCRec-cured isolates were then harvested (40 μl) at different growth states, washed, and put in contact with the pBHC plasmid (570 ng) on a filter or on liquid medium for 1 night or 2 h of incubation, respectively. The pBHC plasmid (2,896-bp PvuII fragments from plasmid pBAB1 inserted into the SmaI site of plasmid pBBR1MCS-3) (Table 1) was used for the following electrotransformation experiments in order to be certain that the electrotransformants we were able to obtain did not correspond to siblings of uncured pBHCRec strains. No transformants were detected in the absence of electrical discharges (detection threshold of 10^{-9}) at any of the tested growth phases (Fig. 1). The growth curves were also used to define the correlations between the bacterial densities and the optical densities; an optical density at 600 nm of 1 corresponded to 3.1 $\times 10^8$ and 2.6 $\times 10^8$ CFU/ml for strains N3 and Ee2.2, respectively.

Strain identification. The electrotransformable isolates, Ee2.2 and N3, were then identified based on the 16S ribosomal DNA sequence. The *rrn* gene was amplified from total genomic DNA (DNeasy tissue kit; QIAGEN, Courtaboeuf, France) with PA-PH primers (4) and cloned into the pGEMTeasy plasmid (cloning kit; Promega, Madison, Wis.). Plasmids were isolated from *E. coli* DH5α with a Miniprep extraction kit (QIAGEN) and sent to Genome Express (Meylan, France) for sequencing. Database comparisons (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) of the *rrn* genes of Ee2.2 (1,450 bp) and N3 (1,460 bp indicated 99% identity with the *rrn* gene of *Pseudomonas jessenii* strain PS06 and "*Pseudomonas reactans*," respectively. "*Pseudomonas reactans*" is an unclassified bacterium considered to be saprophytic and mushroom associated (21a). Both strains belonged to the *Pseudomonas* genus but differed morphologically and genotypically (*rrn* genes had 97% identity). As *Pseudomonas* are considered fast growing, dominant soil bacteria and are often transformable (2), the experimental condition used might have favored them. However, *Pseudomonas* spp. are not necessarily the only electrotransformable bacteria present in soil, nor the most efficiently electrotransformed.

Electroporation frequencies. The electrotransformation frequencies of *Pseudomonas* sp. strains N3 and Ee2.2 were compared to those of *E. coli* strain DH10B and *P. fluorescens* strain C7R12 (11). Before electroporation, cells (optical density at 600 nm of 0.6 to 0.7) were washed either once with sterile distilled water at room temperature or three times with 0.5 M sucrose at 4°C and then concentrated 100-fold. The sucrose
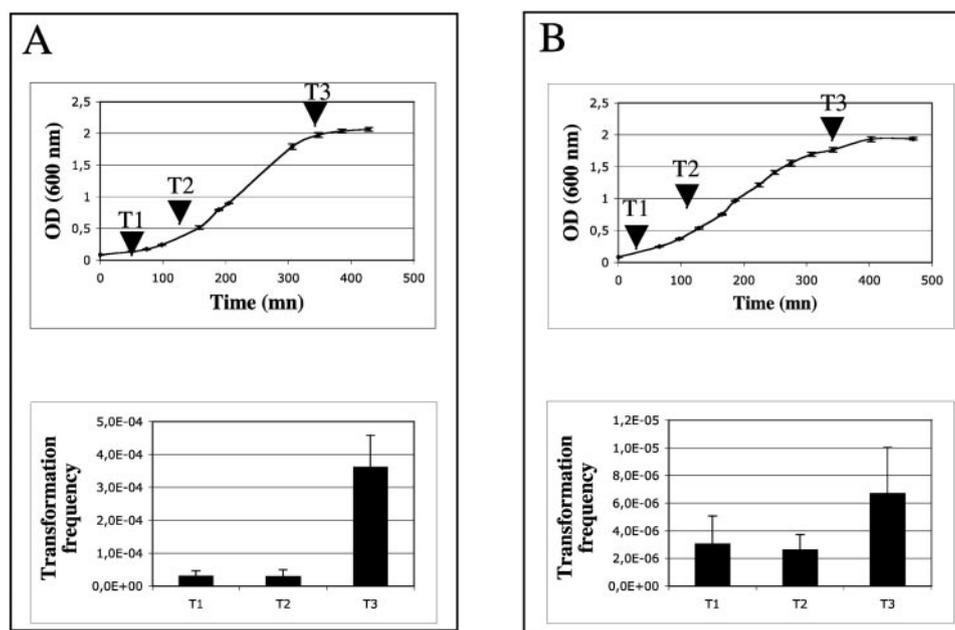
FIG. 1. Effect of growth phase on electroporation-induced transformation frequencies of *Pseudomonas* sp. strains N3 (A) and Ee2.2 (B). The error bars represent the standard deviations. The electrotransformation frequencies are indicated for the sampling times (T1, T2, and T3).

method is considered the typical preparation method for electrocompetent cells (2). The pure bacterial suspension (40 μl) and the pBHC plasmid (570 ng) were introduced into chilled or nonchilled 0.2-cm-gap electroporation cuvettes (Equibio) and exposed to one pulse (Gene Pulser II; Bio-Rad) at 12.5 kV/cm, 200 Ω, and 25 μF. The frequencies of electrotransformation were determined as the number of tetracycline-resistant colonies divided by the number of recipient cells after incubation for 2 h at 28 or 37°C. All of the transformation frequencies reported in this article are the means of results from three independent experiments. Both of the isolated strains, Ee2.2 and N3, had electroporation frequencies on the order of magnitude of 10^{-4}. Published transformation efficiencies for other *Pseudomonas* spp. generally resulted from experiments using optimal conditions. Results obtained with the isolated strains were consistent with those described in the literature (2), even though conditions were not optimized. The electrotransformation frequencies ranged from 10^{-2} to 10^{-3} when cells were treated with sucrose solution and a cold temperature before electrical discharge (Table 2). The laboratory strain, *P. fluorescens* C7R12, did not demonstrate significant electrotransformation frequencies (Table 2). This *P. fluore-*

scens strain could be transformed only if $MgCl_2$ (10 mM) was introduced into the culture during the growth phase. The *E. coli* strain DH10B, washed with water, had an electroporation frequency comparable to those of isolated electrocompetent bacteria (Table 2). The electroporation efficiencies for the two isolated strains, Ee2.2 and N3, ranged between 10^3 and 10^5 CFU μg of DNA^{-1}, depending on the electroporation conditions (Table 3).

Lightning-induced transformation frequencies in sterile soil. Pure cultures of strains Ee2.2, N3, DH10B, and C7R12 were added to sterile soil and subjected to laboratory-scale lightning. *E. coli* strain DH10B was used as the positive control (8), and *P. fluorescens* C7R12 was used as the *Pseudomonas* reference strain. The soil experimental system used (soil from Côte Saint-André, France) was the same as that previously described (8). The soil was dried before γ-sterilization (25-kGy; IONISOS, Dagneux, France). The day before the lightning current injection, the soil (35 g) was hydrated with distilled water to between 13 and 15% (wt/wt) humidity. The water-holding capacity of this soil is around 28%. The working humidity was dependent on the electrical parameters required. A drier soil led to lightning breakdown, and a more humid soil

TABLE 2. Electroporation and lightning-induced transformation frequencies of bacterial isolates and controls with the plasmid pBHC[a]

Strain	Electrotransformation frequency		Lightning-induced transformation frequency[b]	
	Without sucrose (22°C)	With 0.5 M sucrose (4°C)	Experiment 1 (20°C)	Experiment 2 (20°C)
Pseudomonas sp. strain Ee2.2	4.9×10^{-4} (1.7×10^{-4})	2.7×10^{-3} (1.5×10^{-3})	1.2×10^{-5}	5.3×10^{-7} (2.8×10^{-7})
Pseudomonas sp. strain N3	2.7×10^{-4} (2.2×10^{-4})	1.9×10^{-2} (1.1×10^{-3})	5.3×10^{-4} (2.8×10^{-4})	7.2×10^{-6}
E. coli DH10B	3.6×10^{-4} (2.7×10^{-4})	1.4×10^{-6} (8.5×10^{-7})	$<10^{-9}$	ND
P. fluorescens C7R12	$<10^{-8}$	10^{-7c}	$<10^{-9}$	ND

[a] Standard deviations are shown in parentheses. ND, not determined.
[b] There were 5 months between experiments 1 and 2.
[c] $MgCl_2$ (10 mM) was required.

TABLE 3. Transformation efficiencies of bacterial strains subjected to electroporation[a]

Strain	Transformation efficiency (CFU/μg of plasmid DNA)	
	Without sucrose (22°C)	With 0.5 M sucrose (4°C)
Pseudomonas sp. strain Ee2.2	4.9×10^3 (1.9×10^3)	4.1×10^4 (2.3×10^4)
Pseudomonas sp. strain N3	1.3×10^4 (1.4×10^3)	5×10^5 (1.1×10^5)
E. coli DH10B	2.6×10^4	4×10^3
P. fluorescens C7R12	ND	ND

[a] Standard deviations are shown in parentheses. ND, not determined.

led to a decrease in electrical tension. A 50-mm-diameter petri dish containing a round disk of aluminum foil at the bottom (electrode) was filled with the soil. The cell preparation and plasmid quantities were identical to those used for electroporation. The bacteria-DNA mixture was then deposited onto the surface of the soil. Approximately 10^8 cells harvested at the exponential phase were mixed with the pBHC plasmid. The second electrode was then placed on the surface of the soil to permit current injection. The microcosms were subject to electrical tensions ranging from 5 to 5.6 kV/cm. In nature, the lightning flash and thunder are due to the flow of high electrical currents into an ionized channel which connects the ground to the cloud. At ground level, these currents penetrate the soil through a small surface (a few square centimeters) and spread more or less uniformly, depending on the soil homogeneity. The flow of current in the soil creates an electric field which is applied to the living material. Very near the impact point of the lightning, the strains may be destroyed, but as the current density decreases with the distance from this point, there is a wide area where the electric field is in a good range to favor electrotransformation (a few kilovolts per centimeter) (8).

Around 2 g of inoculated soil was then placed into 100 ml of Luria-Bertani medium and incubated for 2 h at 28 or 37°C. Negative controls corresponded to the cell-DNA mixture without electrical discharge. The first objective was to determine if the isolated soil bacterial strains would be transformed when subjected to lightning. Transformation of all of the strains by the pBHC plasmid was detected after overnight incubation and confirmed by PCR (Chloro1-Chloro2 primer set). The lightning transformation frequencies of all of the strains after 2 h of incubation were then compared to the electroporation frequencies (Table 2). The N3 and Ee2.2 lightning electrotransformants were detected at 10^{-4} and 10^{-5} frequencies, respectively (Table 2, experiment 1). The electroporation and lightning transformation frequencies with the pBHC plasmid differed by about 1 log for strains N3 and Ee2.2. The strong correlation between electroporation and lightning frequencies observed for the isolated strains suggests that the electroporation transformation frequencies observed in vitro have some ecological significance. The lightning-induced transformation frequencies, which ranged from 10^{-4} to 10^{-5} with an 8-kb plasmid in soil, could be extrapolated to transform at least 10 and up to 100 *Pseudomonas* spp. bacteria per gram of soil per lightning strike. Natural transformation frequencies ranged from 10^{-8} to 10^{-9} with *P. fluorescens* and *Agrobacterium tumefaciens* strains under equivalent conditions in terms of plasmid size, DNA concentration, and cell density (9). Moreover, many frequencies of natural transformation are measured by using complex high-nutrition growth media, supplemented soil

extract, or nutriment-amended soil (18). Electrotransformation frequencies reported here were measured directly in soil. The isolation of "lightning-competent" bacterial strains, their lightning-induced transformation frequencies, and the accessibility of diverse sources (extracellular, living, and dying) of DNA in soil (18, 23) could increase our understanding of the importance of lightning-induced gene transfer for bacterial adaptation and evolution. The influence of electrical, DNA, and soil parameters on the ecological significance of lightning transformation is under investigation. The control bacteria, *E. coli* strain DH10B and *P. fluorescens* C7R12, did not exhibit a measurable lightning-induced transformation frequency ($<10^{-9}$) with the incubation period of 2 h. Lightning-induced transformation can probably be extended to a number of bacteria. However, the frequencies described in this study do not seem to be a universal characteristic of *Pseudomonas* since *P. fluorescens* strain C7R12 behaved differently from *Pseudomonas* strains Ee2.2 and N3. In experiment 2, 3.8×10^3 and 5.9×10^4 transformants μg of DNA^{-1} were counted in lightning-hit soil for strains Ee2.2 and N3, respectively.

Effect of growth phase on electrotransformability. Frequencies of electrotransformation in the presence of the pBHC plasmid were tested at different growth phases (lag, exponential, and stationary). *Pseudomonas* sp. strain N3 had the highest electrotransformation frequency in the stationary phase (Fig. 1A), while the physiological state for strain Ee2.2 had no apparent effect on its electrotransformability (Fig. 1B). The important difference in transformation requirements between lightning transformation and natural transformation resides in the bacterial state of transformability. While a specific state of competence is required for naturally competent bacteria (18), the lightning-competent isolates have been transformed during all of their growth phases both in vitro and in situ (data not shown).

The approach described in the present study has shown that the bacterial community directly isolated from soil by Nycodenz extraction constituted an effective pool of bacteria for finding examples of those that were lightning competent. Direct Nycodenz extraction might have avoided significant modifications of microbial diversity and cell physiology linked to a cultivation step (6). While Nycodenz extraction of soil bacteria has been used extensively for genomic and diversity studies (25), only recently have the Nycodenz-extracted bacteria been considered a pool of physiologically active soil bacteria (24, 28).

Nucleotide sequence accession numbers. Sequences of the *rrn* genes of Ee2.2 and N3 have been deposited under accession numbers AY625608 and AY625609, respectively.

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