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Laboratory-Scale Evidence for Lightning-Mediated Gene Transfer in Soil

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Electrical fields and current can permeabilize bacterial membranes, allowing for the penetration of naked DNA. Given that the environment is subjected to regular thunderstorms and lightning discharges that induce enormous electrical perturbations, the possibility of natural electrotransformation of bacteria was investigated. We demonstrated with soil microcosm experiments that the transformation of added bacteria could be increased locally via lightning-mediated current injection. The incorporation of three genes coding for antibiotic resistance (plasmid pBR328) into the *Escherichia coli* strain DH10B recipient previously added to soil was observed only after the soil had been subjected to laboratory-scale lightning. Laboratory-scale lightning had an electrical field gradient (700 versus 600 kV m^{-1}) and current density (2.5 versus 12.6 kA m^{-2}) similar to those of full-scale lightning. Controls handled identically except for not being subjected to lightning produced no detectable antibiotic-resistant clones. In addition, simulated storm cloud electrical fields (in the absence of current) did not produce detectable clones (transformation detection limit, 10^{-9}). Natural electrotransformation might be a mechanism involved in bacterial evolution.

Ongoing sequencing and comparison of bacterial genomes are gradually modifying our understanding of how bacteria evolved. Although earlier ideas included a point mutation-based evolution occurring slowly and regularly, new theories propose a more erratic evolution in which drastic changes in the genome are due to irregular acquisition of new genetic information by horizontal gene transfers (HGT) (13). However, the frequency of HGT would have had to be so high during bacterial evolution that some evolutionists are questioning the accuracy of the phylogenetic analyses (5). Moreover, these analyses underestimate the actual number of transfers by missing the oldest events. Their efficiency is also limited to the transferred genes, which were successfully fixed in a microbial population by increasing the overall fitness. These sequence-based tools also miss the great majority of transfer events that conferred neutral or deleterious traits and therefore were subsequently deleted. In addition, numerous microcosm-based investigations devoted to studying these events, and specifically natural genetic transformation in environments, such as soil, sediment, and water, conclude that HGT would occur at extremely low frequencies (10, 15).

Natural transformation of bacteria in soil by extracellular DNA is a multistep process, precluding its occurrence at high frequency (15). When released into the soil at the death of organisms (or more actively for some bacteria), naked DNA has to avoid both enzymatic degradation and irreversible adsorption onto soil particles (4). Moreover, when taken up into the cell, transforming DNA still has to resist the numerous restriction and modification mechanisms and to be integrated into the host genome via homologous or illegitimate recombination (or to replicate autonomously). The intermediate step between these extra- and intracellular states corresponds to the transformation mechanism sensu stricto. When genetically encoded, this mechanism requires the bacterial cells to be in a competent state, which is another major limitation of transformation in situ (15).

The discrepancy between databases that indicate HGT has occurred frequently (13) in the past and the extremely low frequency measured in the environment (10, 15) has led to questions about whether experiments have targeted the appropriate bacteria, the important environmental habitats, and/or the correct processes. For instance, the soil bacterium *Ralstonia solanacearum,* a plant pathogen, exhibits a potential for HGT several orders of magnitude higher in plant tissues than in bulk soils (3). This is due to the rapid and extensive multiplication of the pathogen, leading to the development in situ of the physiological state of genetic competence. As a second example, some transformants of *Escherichia coli,* a bacterium which is not fitted with the appropriate molecular machinery to develop competence, were detected in freshwater, indicating that transformation can also be physically or chemically induced in situ (1). Therefore, our objective was to evaluate the possibility of other processes being involved in HGT.

Considerable data have already been collected concerning the mechanism and efficiency of gene exchange in laboratory devices using electrical fields and currents (6, 9, 11, 20). Thus, given that the environment is regularly subjected to thunderstorms and lightning discharges, our objective was to investigate whether the resulting electrical perturbations could lead to the "natural" transformation of bacteria. Thunderstorm development is a process which includes a progressive accumu-

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TABLE 1. Comparison of electrical parameters, averaged over the current injection period, between electroporation and artificial and natural lightning[a]

System	V_{crest} (kV)	E_{crest} (kV mm^{-1})	I_{crest} (A)	J_{crest} (A m^{-2})	$\int_{j(t)dt}$ (C m^{-2})
Electroporation	2.4	1.2	0.1	5,000	22.5
Simulated lightning	7.0	0.7	5.0	2,500	15
Natural lightning	I[a]	0.6[b]	26,000	12,000	4.15

[a] I, irrelevant.
[b] This value was calculated for an electrical resistivity of the soil tested of 50 $\Omega \cdot$ m.

lation of storm clouds followed by lightning discharges. The electrical parameters related to each of these steps have been investigated extensively and are now well characterized (2). An electrical potential difference builds up gradually with cloud growth. In temperate climates, clouds are positively charged in the upper zone and negatively charged at the bottom, resulting in a modification of the charge and of the electrical field distribution on the ground. The voltage difference between the cloud and the ground can reach up to 100 MV, corresponding to values around 16 kV m^{-1} (0.016 kV mm^{-1}) for flat ground surfaces, increasing considerably locally due to a tip effect (up to 0.7 kV mm^{-1}) (Table 1). Our experimental objectives were to determine whether these electrical fields related to cloud accumulation could be involved in the genetic transformation of bacteria, and if not alone, perhaps with the addition of electrical current in the form of lightning. Lightning discharges to the soil are characterized by an injection of current with the peak current intensity of the flashes varying from 10 to 200 kA (2), which is considered to flow through about 2 m^2 of soil surface. On average, this results in about 13 kA m^{-2} (Table 1). Our initial approach reported here was to test the possibility of this in situ electrotransformation by subjecting artificially seeded soil microcosms to electrical perturbations simulating both storm cloud electrical fields and lightning.

MATERIALS AND METHODS

The tests under simulated storm cloud electrical fields and lightning were performed in soil microcosms seeded with bacteria and plasmids. The laboratory-scale lightning system first simulated charge build up in clouds and then the lightning itself. Afterwards, selective medium was used in order to count those bacteria which had acquired the antibiotic resistance encoded by the plasmid. All of these components are described below. In addition, some comparison was made with electroporation in the presence and absence of soil.

Soil microcosms. Microcosms consisted of 50-mm-diameter petri dishes in which were placed 30 g (soil thickness, 10 mm) of air-dried and sieved (pore size, 2 mm) samples of a sandy loam soil (sand, 50%; silt, 41%; clay, 9%; organic matter, 40.6 g $\cdot$ kg of dry soil^{-1}; pH 6.8) collected at La Côte Saint André (Isère, France). We adapted the petri dishes to conduct electricity by sticking a steel wire through a hole in the bottom of the dish and then placing a round piece of aluminum paper inside the dish on the bottom. Thus, the wire went from the aluminum foil at the bottom of the petri dish, through the hole in the dish bottom and out to where it could be used as an electrical ground in the simulated-lightning experiments. Sterile soil conditions were obtained by gamma irradiating the microcosms with a 25-kGy dose from a ^{60}Co source (Conservatome, Dagneux, France). The moisture content for cloud and lightning simulation tests was 10% (wt/wt). The target moisture was achieved in part during the bacterial and plasmid inoculation (2.5 ml per 30 g of dried soil) and in part by adding sterile deionized water (0.5 ml per 30 g of soil). The soil pH after inoculation and immediately before being subjected to simulated lightning was maintained at 6.8. The soil samples were inoculated with 2.5 ml of the bacterial suspensions and mixed with a sterile pipette. Bacterial suspensions were made based on the required final bacterial concentrations in the soil microcosms, which were 10^7, 10^8, 10^9, and 10^{10} cells g of dry soil^{-1}. Each bacterial concentration was tested at least three times. The seeded soil samples were incubated at 28°C for 18 h before 100 μl of a 10-μg-ml^{-1} plasmid pBR328 (coding for resistance to ampicillin, chloramphenicol, and tetracycline) solution was added to the soil just prior to the lightning treatment. The controls were (i) soil with the added bacteria but without the plasmid, (ii) soil with both bacteria and plasmid added but not subjected to lightning, and (iii) soil with nothing added. Both sterile and non-sterile soils were tested.

Inoculum and plasmid preparation. The inoculum of *Escherichia coli* strain DH10B (Life Technologies, Cergy, France) was prepared by picking a colony maintained on agar and incubating it in 100 ml of liquid medium (Luria-Bertani [LB] medium) overnight, and then 2 ml of the culture was placed in 500 ml of LB medium and incubated for about 8 h. When the optical density (at 580 nm) of the liquid culture reached 1, the liquid was centrifuged for 15 min at 6,500 $\times$ *g* before resuspension in the appropriate volume of 0.8% NaCl in order to achieve the targeted final concentration in soil. Confirmation that *E. coli* strain DH10B could not be naturally transformed in vitro was done by mixing 40 μl of plasmid pBR328 DNA (Boehringer Mannheim, Meylan, France) and recipient cells on GTTP filters (Millipore, Bedford, Mass.) on solid LB medium. After incubation for 24 h at 37°C, the filters were resuspended in 2 ml of pure sterile water, and the suspensions were plated on selective medium (LB medium containing, per liter, ampicillin, 100 mg; chloramphenicol, 30 mg; and tetracycline, 12.5 mg). The plasmid pBR328 was isolated from *E. coli* by using the plasmid extraction kit from Qiagen Inc. (Chatsworth, Calif.).

Simulation of cloud-induced electrical field. When only the electrical field associated with clouds was tested without lightning, conditions corresponding to natural cloud-induced electrical fields were simulated by subjecting seeded soil samples to a static electrical field created through a large planar capacitor structure; the distance between the electrodes was 0.25 m. The apparatus was capable of delivering DC voltages ranging from 4 to 140 kV. In these experiments, when 140 kV (V_{crest}) was used over the 250 mm between electrodes, the gradient (E_{crest}) was about 0.56 kV mm^{-1}, which is similar to that of storm clouds (about 0.6 kV mm^{-1}) that produce lightning (Table 1). Soil samples were exposed to this field for different periods (1, 5, 10, and 60 min).

Lightning simulation. The experimental system was based on a high-voltage generator which could deliver impulses of up to 1 MV with 50 kJ of energy. This generator was constructed in order to test the performance of the high-voltage apparatus and the ability of different electronic equipment to survive lightning hits (A. Ben Rhouma, P. Auriol, and P. Dumas, Proc. 9th Int. Symp. High Voltage Eng., abstr. 7630, 1995). For the soil application, the current flowed from an electrode through the soil to the aluminum foil at the bottom of the petri dish, which was sitting directly on a solid bronze disk (0.5 m in diameter and 2 cm thick) which itself was sufficiently grounded for the applied current. The electrode was a cylindrical cell (bronze; 2.5 cm in diameter and 3.5 cm high) that was capable of applying voltage impulses up to 8.9 kV with a time constant value equal to 6 ms. Under these conditions, the current flowing through the soil can reach more than 6 A. For typical natural lightning, the duration of the current impulse of each return stroke can vary significantly from 0.1 to 1 ms (19).

Detection of transformants. Following the lightning discharge, a subsample of soil (approximately 2 g) was recovered and treated with DNase I (Boehringer, Mannheim, Germany) to degrade any persistent extracellular DNA (incubation for 2 h at 28°C with 1,000 U of DNase I). Detection protocols consisted of either (i) quantitative analysis by direct plating of part of the soil subsample (and dilutions of the subsample with the selective medium) on selective medium (LB medium containing, per liter, ampicillin, 100 mg; chloramphenicol, 30 mg; tetracycline, 12.5 mg; and, in order to inhibit fungal growth, amphotericin B, 1.25 mg) to enumerate transformants or (ii) qualitative analysis by incubating the whole 30 g of soil in the microcosm overnight at 28°C in 20 ml of selective medium before plating 100 μl five times on selective medium. Although this technique did not allow exact counting of transformants, it increased detection sensitivity, as any transformants could develop over the incubation period and therefore its theoretical detection limit was 1 transformant per 30 g of soil. Any apparent transformants were confirmed to contain the added plasmid by using a plasmid extraction kit (Qiagen Inc.) according to the manufacturer's instructions. For experiments with sterile soils, recipient bacteria (both those that had incorporated the plasmid and those that had not) were enumerated on LB medium.

Electroporation. Electrocompetent cells of *E. coli* strain DH10B were prepared as described by Drury (7). Electroporations were carried out with a gene pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) by using 40 μl of recipient cells mixed with 1 μl of plasmid pBR328 solution (100 mg liter^{-1}), incubated 1 min on ice, and electroporated in a 0.2-cm-diameter cuvette at 1.2 kV mm^{-1} (crest value) for 4 to 5 ms. LB medium (960 μl) was then immediately added, and the suspension was incubated for 1 h at 37°C before the dilutions

were plated on both LB medium alone and LB medium with antibiotics (as described above) to estimate survival and transformation frequencies, respectively. In addition, in order to try to incorporate the soil aspect in the electroporation tests, 75 mg of sterile soil was placed in the electroporation cuvette, 41 μl of the bacterium-plasmid suspension was added, and then an additional 75 mg of soil was added before the electroporation was performed.

To simulate our cloud experiments (where only an electrical field and no current is applied) with the electroporator, we used 0.45-cm-wide disposable Plastibrand cuvettes with electrodes added to the outside (hence not in contact with the solution); thus, with the application of voltage in the electroporator, no current occurred, but an electrical field was established. This was only tested on bacterial plasmid solutions in the absence of soil.

Comparison of electrical parameters for electroporation and lightning current propagation in soils. Three electrical parameters were used to compare the two laboratory-scale systems (electroporation and simulated lightning) and full-scale lightning (Table 1). The first parameter was the crest value of the electrical field (E_{crest}) which is obtained for natural conditions by dividing the maximum electrical tension value reached during the electrical impulse by the electrode distance (V_{crest}/d) or, for natural lightning, by multiplying the maximum value of the current density by the electrical resistivity of the soil tested ($J_{crest} \times \Omega$). The second parameter was the crest value of the current density (J_{crest}), which corresponds to the maximal current intensity divided by the cell cross section (I_{crest}/s), which provides data that can compare systems of different size scales. The third parameter was the amount of electrical charge flowing through the cell for a 1-m^2 surface ($\int_{j(t)dt}$). The laboratory conditions simulated very closely those occurring in soil during a lightning discharge. The crest values of the electrical field (E_{crest}) were nearly identical for natural and artificial lightning and differed by less than a factor of 2 for electroporation. The crest values of the current densities (J_{crest}) and the amounts of charge flowing through the soil were similar for the three different conditions in a range from 4 to 22 C m^{-2}, although the surface area and volume of soil for natural lightning could be determined only approximately. The variations in Table 1 are not significant with respect to our hypothesis when it is realized that, during the lightning event, considerable variation in these values occurs over the soil area hit by lightning (2, 19).

RESULTS AND DISCUSSION

Thunderstorm-related electrical parameters and potential biological effects. Simulated storm cloud electrical fields subjected soil seeded with recipient bacteria, *E. coli* strain DH10B, and donor DNA, plasmid pBR328, to static electrical fields as described above. These experiments failed to provide any detectable transformants under the various conditions tested, which included a range of recipient bacterium concentrations (from 10^7 to 10^{10} g of dry $soil^{-1}$), donor DNA concentrations (from 1 to 100 μg of plasmid pBR328 g of dry $soil^{-1}$), and amplitudes (from 0.16 to 0.6 kV mm^{-1}) and durations (1 to 60 min) of the generated electrical field. These results are consistent with the lack of transformants in vitro when the electroporation cuvette was modified to subject the bacteria only to electrical fields but without current. The efficiencies of transformation of *E. coli* strain DH10B (expressed as the number of transformants relative to that of recipient cells) by 0.5 μg of plasmid pBR328 DNA dropped from 1.3×10^{-5} with standard electroporation conditions to less than 7×10^{-9} (the detection limit) with modified conditions, indicating the requirement for electrical current in order to produce DNA transfer. These results imply that the accumulation of clouds alone (an electrical field without current) might not have any significant effect on HGT.

Artificial-lightning-related electrotransformation of bacteria in soil. Experiments were developed in order to subject soil samples seeded with recipient bacteria and donor DNA to laboratory-scale lightning delivered by a high-tension generator. The use of a non-naturally transformable bacterium such as *E. coli* DH10B and a plasmid harboring three antibiotic resistance marker genes (pBR328) allowed the easy detection of transformants.

Transformants were detected in all samples containing from 10^5 to 10^9 recoverable bacterial cells g of dry $soil^{-1}$ when the plasmid concentration was greater than 0.1 μg of DNA g of dry $soil^{-1}$ (Table 2). When the plasmid concentration was 0.1 μg of DNA g of dry $soil^{-1}$, transformants were detected qualitatively, but rarely in sufficient number to quantify the transformation frequency (Table 2). The qualitative method appeared to be quite sensitive to the presence of transformants, thus increasing the certainty that "no-lightning" controls did not produce any transformants (Table 2). Controls with lightning but without the plasmid also lacked any detectable transformants. When nonsterile soils were tested, *E. coli* DH10B was unable to maintain its population at elevated levels, and therefore, the actual target bacterial population was not identified and no clones were observed by the quantitative method, although the qualitative method did provide evidence of transformants.

Estimation of HGT levels in soils subjected to natural lightning. The experimental results indicate that bacteria in soils are affected by lightning-related electrical parameters, which, like those delivered by a standard electroporator, increase their permeability to extracellular DNA. The soils in our microcosms were homogenized in order to have the current delivered homogeneously throughout the soil sample. On the other hand, the current delivered by lightning discharges is going to flow along channels of reduced resistivity. These channels are characterized by very high current densities and a considerable increase in temperature, possibly subjecting bacteria to irreversible damage and death. Nevertheless, the magnitudes of the electrical parameters (current density and electrical field) decreases with distance from the axis of these conducting channels, creating electrical and thermic conditions compatible with bacterial survival and potentially with electrotransformation in a large volume of the soil surrounding these "overstressed" areas. Therefore, most lightning discharges would provide transformation-inducing electrical conditions at some places in the soil, independent of its structure, texture, and matrix characteristics, similar to that described here for the laboratory-scale lightning.

Another critical aspect of lightning-induced gene transfers in nature is the state of natural extracellular donor DNA. Significant amounts of indigenous extracellular DNA in a range from 5 to more than 35 μg g of dry $soil^{-1}$, depending upon the soil composition, can be routinely detected and even extracted (8). There are, however, few data on the transformation potential of these natural molecules. Even when a significant part of the DNA inoculated into nonsterile soils is rapidly degraded, another fraction could persist by adsorption onto soil particles and remain available to transformation mechanisms (4, 18). Moreover, the closed microhabitats due to soil aggregation (16) in which most indigenous soil bacteria develop, die, and release DNA might be much more favorable to natural electrotransformation than conditions outside the soil aggregate. The electrophoretic forces that are hypothesized to drive DNA into the transforming cells (11, 12) might be involved in the desorption of DNA molecules from soil particles (14), contributing to their increased availability. Also, intracellular DNA present in living, dying, and dead cells could be a major

TABLE 2. Laboratory-scale lightning-mediated transformation of *E. coli* strain DH10B by plasmid pBR328

System	Conditions			Results[e]
	Plasmid pBR328 concn[b]	*E. coli* DH10B concn[c]	No. of transformants after 24-h incubation[d]	
Laboratory-scale lightning (kV/A)[a]				
7.0/8.1	0.4	2.3×10^9	$>10^4$	1.1×10^{-8} (2.5×10^{-9})
7.0/8.1	0.1	2.3×10^9	$>10^3$	ND[f]
5.0/6.5	0.4	3.6×10^8	$>10^4$	1×10^{-8} (5.8×10^{-9})
5.0/6.5	0.1	3.6×10^8	$>10^2$	ND
6.0/5.2	0.4	1.2×10^8	$>10^3$	4×10^{-9} (1×10^{-9})
6.0/5.2	0.1	1.2×10^8	$>10^3$	ND
4.0/5.5	0.4	2.2×10^8	$>10^3$	ND
4.0/5.5	0.1	2.2×10^8	0	ND
5.0/4.3	0.2	3.3×10^8	$>10^4$	1.6×10^{-7} (1.3×10^{-7})
8.0/5.8	0.2	2.6×10^6	$>10^3$	ND
8.0/5.8	0.2	1.0×10^5	>10	ND
7.0/4.7	0.2	1.0×10^9[g]	$>10^2$	ND
	0.2	1.3×10^5	$>10^3$	2×10^{-5} (8×10^{-6})
	0.2	3.3×10^5	$>10^3$	ND
Controls without lightning[h]	0.4	4.0×10^8	0	ND
	0.1	4.0×10^8	0	ND
	0.4	2.3×10^8	0	ND
	0.1	2.3×10^8	0	ND
	0.2	3.3×10^5	0	ND
Controls with lightning	0	3.3×10^5	0	ND
	0	4.0×10^8	0	ND

[a] Electrical field potential (kV) and simulated lightning current (A) applied to soil microcosms.
[b] Concentrations are in micrograms of DNA gram of dry soil^{-1}.
[c] Concentrations are in CFU gram of dry soil^{-1}.
[d] Concentrations are in clones gram of dry soil^{-1}.
[e] Frequency based on number of clones per total *E. coli* strain DH10B cells counted in soil; standard deviations based on a minimum of three measurements are in parentheses.
[f] ND, frequency not determined due to lack of clones.
[g] This soil sample was not sterilized before the experiment. The quantity of recipient bacteria was not measured. The reported value is the initial inoculated concentration.
[h] Controls were identical in every way except for not experiencing laboratory-scale lightning.

source of donor DNA, in addition to extracellular DNA, based on evidence that electroporation was efficient in vitro in generating protoplast fusion (17) and even in transferring DNA between living bacterial cells (9). Finally, given that cells from nearly all bacterial taxa can be electroporated more or less efficiently and independently of their physiological state and with transforming DNA of prokaryotic or eukaryotic origin, the universality of this process needs to be considered.

Based on these varied experimental and theoretical data, the frequency of natural lightning-mediated gene transfers in soils might not be vastly different from a value of 10^{-9} resulting from our simulation experiments (Table 2). Natural lightning discharges are spread over 0.5 to 4 m^3 of soil containing more than 10^{12} to 10^{13} indigenous bacterial cells potentially affected by the electrical parameters, thus providing a theoretical number of transformants up to 10^4 at each discharge. The hundreds of lightning flashes subjecting the environment to thousands of coulombs each second on a world-wide scale could be participating in bacterial adaptation and evolution.

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