Lightning-Triggered Electroporation as a Mechanism for Horizontal Gene Transfer

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Abstract

Horizontal gene transfer (HGT) plays a prominent role in evolution and genetic variability of life. Five biotic mechanisms of HGT among prokaryotes have so far been extensively characterized: conjugation, competence, transduction, gene transfer agent particles, and transitory fusion with recombination; but it seems questionable whether they can account for all ongoing HGT, and it is even less clear how HGT could have proceeded before any of these mechanisms – themselves products of evolution – had developed. An alternative and perhaps more general path to HGT is offered by non-biochemical, yet natural mechanisms of destabilization of the membranes enveloping the genetic material: freeze-thaw cycles, abrasive action of gravel and sand, and electroporation triggered by lightning strokes. This chapter focuses on the latter mechanism of gene transfer – DNA uptake and heritable expression based on reversible electroporation (electrotransformation), which is by far the most efficient technique of artificial HGT, reported to date for bacteria from at least 13 of their 29 currently recognized taxonomic phyla, archaea from at least two of their five phyla, microalgae from at least three of their six phyla, and yeasts from both their phyla. As a complement, irreversible electroporation is a mechanism of DNA release (electroextraction), although less efficient in the laboratory than chemical extraction. It is shown that conditions for electroporation-based DNA release, uptake, and transformation are present in many natural habitats exposed to lightning strokes, with quantitative estimates that the number of microorganisms subjected to conditions for lightning-triggered HGT, particularly in freshwater habitats, may well exceed 10^{17} per year.

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D. Miklavčič (ed.), *Handbook of Electroporation*,
DOI 10.1007/978-3-319-32886-7_25

Keywords

Horizontal gene transfer • Evolution • Electroporation • Electroextraction • Electrotransformation • Lightning strokes • Lightning-triggered transformation

Contents

Introduction

DNA sequencing has revolutionized our understanding of evolutionary relationships between organisms, yet it also revealed that evolution does not only proceed solely by gradual divergence of species due to random mutations and their natural selection but also by interchange of genetic material between species (horizontal gene transfer – HGT). This started to emerge in the early 1990s from comparison between genomes, which revealed bacterial genes present in some eukaryotes yet absent from any archaea, though eukaryotes are phylogenetically closer to archaea than to bacteria, and it was corroborated by comparison between nucleotide sequences in individual essential genes, from which it emerged that phylogenetic trees inferred from different such genes can differ drastically.

Horizontal gene transfer is now widely recognized as a major contributor to genetic variability of prokaryotes, with at least five natural mechanisms extensively documented:

- Competence: uptake by an organism of DNA from its surroundings
- Conjugation: transfer of DNA between two organisms in direct contact
- Transduction: transfer of DNA from one organism into another via an infection by a virus (phage)
- GTA-mediated transfer: transfer of DNA by gene transfer agents (GTAs), viruslike particles synthesized by some bacteria
- Transitory fusion with recombination: pairwise fusion of some archaea into a hybrid, followed by homologous recombination of their DNA and the hybrid's fission back into two archaea

As all these mechanisms are biotic, i.e., based on biomolecules synthesized and utilized by the organisms involved in HGT, it follows that these evolution-accelerating mechanisms are themselves products of evolution. Thus, though it is now widely accepted that HGT has been ongoing since the earliest stages of evolution, it is unclear whether – and how – HGT could have proceeded before any of its biotic mechanisms had developed.

It also seems questionable whether each occurrence of HGT identified to date can be explained by the five biotic mechanisms outlined above. Namely, although competence, conjugation, and transduction are found in both prokaryotic kingdoms, i.e., both in archaea and in bacteria, it is questionable whether at least one of these mechanisms can act in every archaeal and bacterial species. Competence is thus estimated to occur naturally in only ~1% of all bacterial species, and likely in an even lower fraction of archaeal species, and hindering the efficiency of competence further is rapid degradation of free DNA in natural habitats. For conjugation, efficiency quickly decreases with increasing genetic distance, as the proteins utilized in conjugative coupling are highly adjusted to a particular organism's envelope. Regarding transduction, most viruses performing this mechanism of HGT (phages) infect selectively, only transferring genes among genetically very close organisms – often even only within a single strain of a single species. Moreover, evidence is rapidly accumulating for HGT in eukaryotic organisms, in particular microalgae and yeasts, although neither conjugation nor competence exist in eukaryotes, and they are also in general not infectable by bacterial or archaeal phages.

As so often in biology, at least some of these limitations may have exceptions. The bacterium *Acinetobacter baylyi* can take up and express even highly fragmented and degraded DNA (Overballe-Petersen et al. 2013), and this ability may be present in other naturally competent bacteria. A mechanism somewhat resembling conjugation is used by the bacterium *Agrobacterium tumefaciens* to transfer its genes into cells of some flowering plants, inducing tumorigenesis (Zupan et al. 2000). Also, several viruses with a broad host range and/or adaptive host specificity are known both in bacteria (Koskella and Meaden 2013) and eukaryotes (Bandin and Dopazo 2011). As a consequence, it is highly questionable whether there is a case of HGT for which it could be inferred that none of the known biotic HGT mechanisms can explain it. While our knowledge and understanding of HGT are rapidly increasing, we are thus still far from a reliable assessment of the relative importance of each of the known biotic HGT mechanisms and farther still from a conclusion whether there are no additional such mechanisms of relevance.

An alternative and perhaps more general path to HGT, in particular among unicellular organisms – both prokaryotes and eukaryotes – is offered by abiotic, i.e., non-biochemical, yet natural mechanisms of destabilization of the membranes enveloping the genetic material: freeze-thaw cycles, abrasive action of gravel and sand, and electroporation triggered by lightning strokes (Kotnik and Weaver 2016). This chapter discusses the latter mechanism – lightning-triggered electroporation of microorganisms' envelopes, causing DNA release, uptake, and transformation, thus acting as a natural abiotic mechanism of HGT among them. Following this introductory section, section "Reversible Electroporation as a Mechanism of DNA

Uptake and Transformation in Microorganisms" discusses reversible electroporation as a mechanism of DNA uptake and transformation in microorganisms (electrotransformation), and section "Irreversible Electroporation as a Mechanism of DNA Release" treats irreversible electroporation as a mechanism of DNA release (electroextraction). Section "Lightning-Triggered Electrotransformation as a Contributor to HGT in Microorganisms" outlines the emergence of the hypothesis of lightning-triggered HGT, several experiments providing indirect empirical support to its feasibility, and theoretical considerations further corroborating that conditions for both electroextraction and electrotransformation are present in natural habitats subjected to lightning strokes. Finally, section "Assessing the Feasibility and Importance of Lightning-Triggered HGT" outlines some guidelines for improved assessment of the feasibility and importance of lightning-triggered HGT.

Reversible Electroporation as a Mechanism of DNA Uptake and Transformation in Microorganisms

Artificial electroporation-induced uptake of DNA with subsequent expression, termed gene electrotransfer, is, if properly designed and optimized, achievable in most biological cells; in those of multicellular eukaryotes, however, such transfer is in most cases not heritable (electrotransfection), while in prokaryotes, as well as many unicellular eukaryotes, it is generally possible to achieve both expression and heritability (electrotransformation). Electrotransfection was first achieved in mammalian cells in the early 1980s (Wong and Neumann 1982), while in bacteria the first studies suggested any form of gene electrotransfer to be achievable only after a complete removal of the cell wall. This was, however, largely due to insufficient available electric field amplitudes, and subsequent development of more powerful pulse generators quickly led to successful electrotransformation of a broad range of microorganisms with an intact wall, including not only bacteria and archaea but also unicellular eukaryotes – both microalgae and yeasts. To date, successful electrotransformation has thus been reported for bacteria from at least 13 of the 29 currently recognized taxonomic phyla, for archaea from at least 2 of their 5 phyla, for microalgae from at least 3 of their 6 phyla, and for yeasts from both their phyla (Table 1).

Electrotransformation is effective both in Gram-negative and Gram-positive bacteria, although the latter generally require higher fields and/or yield lower transformation efficiencies, which is attributed to the thicker peptidoglycan layer of the cell wall in Gram-positive bacteria; thus, with plasmid DNA, the optimized efficiencies are typically up to 10^8–10^{10} transformants per μg DNA for Gram-negative bacteria and up to 10^6–10^7 transformants per μg DNA for Gram-positive bacteria. In the same vein, bacteria possessing in addition an outer polysaccharide capsule are generally electrotransformed with even lower efficiencies, but these can still exceed 10^4 transformants per μg DNA (again with plasmid DNA) for bacteria in the exponential growth phase, in which the capsular synthesis rate decreases.

Table 1 A sample of successfully electrotransformed microorganisms (Reproduced from Kotnik et al. (2015) with permission. References to most reports summarized in this table are given in Kotnik 2013a)

Phylum	Species
Archaea	
Crenarchaeota	*Metallosphaera sedula*, *Sulfolobus acidocaldarius*, *Sulfolobus islandicus*, *Sulfolobus solfataricus*
Euryarchaeota	*Methanococcus voltae*, *Pyrococcus furiosus*
Bacteria	
Actinobacteria	*Brevibacterium lactofermentum*, *Corynebacterium diphtheriae*, *Mycobacterium smegmatis*
Bacteroidetes	*Bacteroides fragilis*, *Bacteroides uniformis*, *Prevotella ruminicola*
Chlamydiae	*Chlamydia psittaci*, *Chlamydia trachomatis*
Chlorobi	*Chlorobium vibrioforme*
Cyanobacteria	*Arthrospira platensis*, *Fremyella diplosiphon*, *Synechococcus elongatus*
Deinococcus-Thermus	*Deinococcus geothermalis*, *Thermus thermophilus*
Firmicutes	*Bacillus cereus*, *Clostridium perfringens*, *Enterococcus faecalis*, *Lactobacillus casei*, *Streptococcus pyogenes*
Fusobacteria	*Fusobacterium nucleatum*
Planctomycetes	*Planctomyces limnophilus*
Proteobacteria	*Campylobacter jejuni*, *Escherichia coli*, *Salmonella enterica*, *Sinorhizobium meliloti*, *Yersinia pestis*
Spirochaetes	*Borrelia burgdorferi*, *Serpulina hyodysenteriae*
Tenericutes	*Mycoplasma pneumoniae*
Thermotogae	*Thermotoga maritima*
Unicellular algae (microalgae)	
Chlorophyta	*Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, *Chlorella vulgaris*, *Dunaliella salina*, *Scenedesmus obliquus*
Heterokontophyta	*Nannochloropsis sp. W2J3B*, *Phaeodactylum tricornutum*
Rhodophyta	*Cyanidioschyzon merolae*
Unicellular fungi (yeasts)	
Ascomycota	*Candida maltosa*, *Ogataea polymorpha*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*
Basidiomycota	*Cryptococcus neoformans*, *Pseudozyma antarctica*, *Pseudozyma flocculosa*

Efficiency of electrotransformation in bacteria also depends strongly on the molecular form of DNA being transferred. Generally, the efficiency is the highest for supercoiled circular double-stranded DNA (the indigenous form of plasmid and chromosomal DNA in many prokaryotes), somewhat lower for relaxed circular double-stranded DNA, much lower for circular single-stranded DNA (indigenous to most ssDNA viruses) and linear double-stranded DNA with homologous ends (indigenous to eukaryotes), and lower still for linear double-stranded DNA with non-homologous ends (Kimoto and Taketo 1996).

For DNA concentrations spanning from pg/ml up to μg/ml, efficiency of electrotransformation in bacteria is roughly constant, implying that within this range and under fixed experimental conditions, for each bacterium the probability of being transformed increases roughly proportionally to the concentration of DNA surrounding it. The optimal parameters of the electric pulses used to achieve electrotransformation vary with bacterial species and even strain, but generally, pulse amplitudes (electric fields) range from 2 to 30 kV/cm and pulse durations from milliseconds to tens of milliseconds.

In eukaryotic cells, the efficiency of gene electrotransfer, particularly at low DNA concentrations, is improved if the electroporating pulse is followed by a contiguous, longer but much weaker pulse (tens or hundreds of milliseconds, tens of V/cm) that exerts an electrophoretic drag on the DNA molecules, and while this effect does not seem to have yet been investigated in bacteria or archaea, it is worth noting that with the now prevailing rectangular-pulse generators, the electrophoretic "tail" of the pulse has to be formed by appending a second pulse of lower voltage, while with the simpler exponential-decay-pulse generators, it was inherent to the pulse shape, which is also the case for natural discharges, including lightning strokes.

Efficiency of electrotransformation in bacteria can also be improved by hyperosmolarity of the medium, typically achieved by dissolving sorbitol or mannitol at 0.5–1.5 M concentrations; in nature, hyperosmolarity of aqueous media is generally a consequence of high concentrations of salts, but the efficiency of electrotransformation in high-salinity media does not seem to have yet been studied, likely due to the fact that for lab transformation protocols, similarity to natural conditions is less important than efficiency and practical feasibility. With respect to the latter, unlike sorbitol or mannitol, added salts increase the electric conductivity of the medium considerably; for a fixed electric field, as delivered by the prevalent generators of pulses with a fixed voltage, this increases the electric current and the heating of the medium, while for a fixed electric current, this reduces the electric field induced by this current.

As described above, the highest efficiencies of electrotransformation in bacteria are generally achieved with plasmid DNA. Among strains of the same species, transfer can be efficient also with unaltered indigenous plasmids, but between distant species, and particularly between phyla, the highest efficiency is usually obtained with artificially engineered chimeric plasmids (shuttle vectors). The principal aim of implementing gene transfer in bacteria is generally to achieve the most efficient transformation possible; furthermore, if the transferred gene originates from a eukaryote, using the most efficient form of DNA for transformation – plasmids – is only possible if this gene is inserted into a plasmid. As a consequence, in most reports on electrotransformation in prokaryotes, the result is obtained with pre-engineered plasmid DNA, and there has not been much motive for a systematic investigation of feasibility of interspecies electrotransfer of unaltered natural – either plasmid or chromosomal – DNA. Still, as Table 1 testifies, electrotransformation can in general occur in species of many archaeal and bacterial phyla.

Irreversible Electroporation as a Mechanism of DNA Release

Perhaps the earliest reported use of high-voltage electric pulses for killing of microorganisms dates back to 1896, when the Louisville Water Company studied various methods of purifying river water (Fuller 1896). The first scientific study of destruction of bacteria by irreversible membrane electroporation was published in 1967, showing that the lethal effect is nonthermal and results from extensive membrane disruption and leakage of intracellular contents, including DNA (Hamilton and Sale 1967). Since then, irreversible electroporation has become a well-known method for nonthermal inactivation of microorganisms, as well as for extraction of biomolecules, termed electroextraction (Kotnik et al. 2015).

Electroextraction of DNA has been demonstrated from both prokaryotes and eukaryotes (particularly from microalgae and yeasts), but as a laboratory technique, it was long considered inferior in efficiency to the standard DNA extraction method of alkaline lysis with purification in CsCl-ethidium bromide density gradients. As such, it was mostly used in those applications where low yields were acceptable, yet the extraction had to be fast and with limited contamination by debris (DNA can also be extracted rapidly by intense vortexing with glass microbeads or by intense ultrasonication, but these techniques disintegrate the exposed microorganisms, so the extract is a highly heterogeneous mix of very diverse biomolecules and multimolecular fragments of the cell's components). Still, the fact that irreversible electroporation results in release of DNA – both chromosomal and plasmid – is undisputable, and furthermore, even regarding the yields, it was recently shown that with electroextraction, they can, at least for plasmid DNA and sufficient optimization, be comparable or even superior to alkaline lysis (Haberl et al. 2013).

Lightning-Triggered Electrotransformation as a Contributor to HGT in Microorganisms

Emergence of the Hypothesis and Tentative Empirical Support

Perhaps the first mention in scientific literature of the possibility that lightning strokes could cause both DNA electroextraction (from microorganisms electroporated irreversibly) and subsequent electrotransformation (of nearby microorganisms electroporated reversibly), thus resulting in HGT, occurred in 1990. In July of that year, James Pfau and Philip Youderian from the University of Southern California published a brief report showing that the standard approach to laboratory electrotransformation, where alkaline lysis is used for DNA extraction and then electroporation for transformation, can be simplified by mixing the DNA-donor and DNA-recipient microorganisms and then applying electric pulses both for extraction and transformation. They showed that a yield of transformants, albeit highly suboptimal, is obtained even when a single electric pulse is applied to such a mixture, and in the closing sentence of their report, they referred to the possibility of natural gene transfer triggered by lightning strokes as their "speculation" (Pfau

and Youderian 1990). Five years later, a review of molecular evolution in bacteria referred to lightning-driven HGT – again only in passing – as an "interesting concept" (Trevors 1995). Six further years then passed until this topic was investigated again by a group of researchers from the University of Lyon I in France (Demanèche et al. 2001); by delivering pulses not through a discharge arc but through electrodes in direct contact with the bacteria-containing sample, this study did not differ much methodologically from earlier investigations and applications of electrotransformation, but it was the first in which its authors declared as their goal to investigate experimentally the feasibility of lightning-triggered HGT.

Still, at least four experimental studies published in 1990–1992, including that by Pfau and Youderian already discussed, and one predating it by 4 months, can be viewed as providing empirical support to the feasibility of lightning-triggered HGT – at least to the extent achieved a decade later in the work of Demanèche and coworkers cited in the preceding paragraph, i.e., with a single pulse applied to a mix of DNA-donors and DNA-recipients, although with delivery of the pulse without a discharge arc.

In the first such study submitted for publication in March 1990, David Summers and Helen Withers from the University of Cambridge reported exposing a mix of two *Escherichia coli* strains to a single pulse of 12 kV/cm and 4.6 ms, obtaining transfer of plasmid DNA in about $\sim 1/10^6$ exposed bacteria (Summers and Withers 1990).

Pfau and Youderian, in their abovementioned report written in July 1990, exposed a mix of *E. coli* and *Salmonella typhimurium* – species belonging to the same family (Enterobacteriaceae), but different genus (*Escherichia*, *Salmonella*) – to a single pulse of 20 kV/cm and 5 ms, getting transfer of plasmid DNA in both directions: at $\sim$1/1500 from *S.t.* to *E.c.* and $\sim$1/500,000 from *E.c.* to *S.t.* (Pfau and Youderian 1990).

In June 1991, John Kilbane and Barbara Bielaga from the Institute of Gas Technology in Chicago reported exposing a mix of *E. coli* and *Pseudomonas aeruginosa* – same class (Gammaproteobacteria), different order/family (Enterobacteriales/Enterobacteriaceae, Pseudomonadales/Pseudomonadaceae) to a single pulse of 12.5 kV/cm, obtaining detectable transfer of both plasmid and chromosomal DNA in both directions (Kilbane and Bielaga 1991).

Finally, in May 1992 a group of researchers from the Pasteur Institute in France reported exposing a mix of *E. coli* and *Mycobacterium smegmatis* – same kingdom (Bacteria), different phylum/class (Proteobacteria/Gammaproteobacteria, Actinobacteria/Actinobacteridae) to a single pulse of 12.5 kV/cm, getting transfer of plasmid DNA in $\sim 1/10^6$ exposed bacteria (Baulard et al. 1992).

The main aim of these studies was to assess the possibility of simplifying the apparatus and streamlining the protocols for electrotransformation, and the single-pulse approach was largely dismissed as too inefficient for practical applications; namely, efficiencies achievable in single-pulse exposures were by at least 2–3 orders of magnitude lower than if the DNA-donors were electroporated and the supernatant transferred to the DNA-recipients that were then electroporated separately and by a further order of magnitude inferior to the standard procedure of DNA extraction by alkaline lysis and pulses only delivered to electroporate DNA-recipients. Still, these

studies can be viewed as providing tentative support for feasibility of lightning-triggered HGT among prokaryotes, largely unaffected as the genetic distance between them increases.

Theoretical Analysis

With cloud-to-ground lightning strokes, even after the air ionization is completed and the electric current's path through the air is fully established, the electrical resistance of this path (typically several km long) dominates over the resistance of the ground through which the electric current's propagation then continues; as a result, the magnitude of the lightning stroke's electric current is largely independent of the local composition of the ground it enters (be it highly resistive dry soil or sand, moderately resistive freshwater, or highly conductive seawater). Limiting the analysis to aquatic habitats in which DNA diffuses the most easily, it is also reasonable to assume the stroke's electric current (I) spreads out roughly radially outward and downward from its point of entry, so that the resulting electric current density (J) and the electric field strength it induces (E) decrease roughly inversely proportionally to the square of the distance (r) from this point (Kotnik 2013a):

$$J = \frac{I}{2\pi r^2}, E = \frac{J}{\sigma} = \frac{I}{2\pi\sigma r^2}, \tag{1}$$

where σ denotes the electrical conductivity of the medium through which the current is propagating. Denoting by $I_{\max}$ the peak current, we thus have

$$J_{\max} = \frac{I_{\max}}{2\pi r^2}, E_{\max} = \frac{J_{\max}}{\sigma} = \frac{I_{\max}}{2\pi\sigma r^2}. \tag{2}$$

The electric field thus decreases radially in a continuous and monotonic manner, so there is generally an innermost region where this field is sufficient for irreversible membrane electroporation, causing DNA release, and adjacent to it outward is a region where the field is insufficient for irreversible yet sufficient for reversible electroporation, allowing for DNA uptake, expression, and heritability (Fig. 1).

Assuming for the lightning stroke the statistically determined median of its peak current, $I_{\max} \approx 30$ kA, and for the medium the average electrical conductivity of seawater, $\sigma \approx 40$ mS/cm, an electric field exceeding 9 kV/cm (sufficient for reversible electroporation of most microorganisms) is induced at radial distances up to ~3.6 cm; hence, in seawater hit by a typical lightning stroke, electroporation can occur in a hemispherical volume of at least ~100 cm^3. Similarly, an electric field exceeding 30 kV/cm (sufficient for irreversible electroporation of most microorganisms) is induced at radial distances up to ~2 cm, so that within the ~100 cm^3 where electroporation can occur, in the inner ~20 cm^3 it is predominantly irreversible and in the rest of the volume largely reversible. With ~3×10^9 cloud-to-ground strokes per year, and with ~1% of them striking the seas, this corresponds to ~2400 m^3 of seawater per year subjected to conditions for reversible electroporation, which at the

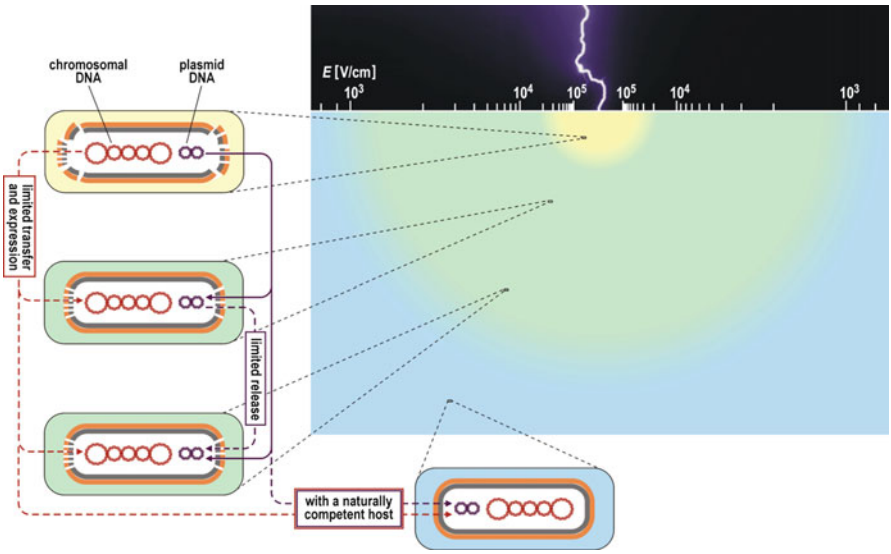

Fig. 1 Lightning-triggered HGT. Molecules of DNA – both chromosomal (*red*) and plasmid (*violet*) – are released from organisms in the region of irreversible electroporation (*yellow*) and plasmid DNA to a more limited extent also in the region of reversible electroporation (*green*). For host organisms without natural competence, transformation is restricted to the region of reversible electroporation, while naturally competent hosts can also be transformed by the released DNA in the region without electroporation (*blue*). For both electroextraction and electrotransformation, DNA has to traverse all layers of the organism's envelope, pictured here as consisting of a plasma membrane (*gray*) and a wall (*orange*), but in some organisms additionally comprising an outer membrane and/or a capsule (Reprinted from Kotnik (2013a) with permission)

microorganisms' concentrations typically exceeding 10^{11} per m^3 of seawater (see Table 1 in Whitman et al. 1998) implies that at least $\sim 10^{14}$ microorganisms per year are subjected in seawater to conditions under which electrotransformation can occur.

In freshwater lakes, due to their much lower electrical conductivity, the volumes subjected to irreversible and reversible electroporation at the same peak electric current are about three orders of magnitude larger: assuming the average electrical conductivity for freshwater lakes, $\sigma \approx 2$ mS/cm, an electric field of 9 kV/cm is exceeded in a volume of ~ 9000 cm^3 and 30 kV/cm in ~ 1500 cm^3. As $\sim 3 \times 10^9$ cloud-to-ground strokes ($\sim 99\%$ of all such strokes) strike continents and islands, and freshwater lakes cover more than 0.5% of the total continents' surface area, assuming that lightning strokes are roughly uniformly distributed over the land as the lakes, at least $\sim 1.5 \times 10^7$ cloud-to-ground strokes hit the lakes, with a volume of $\sim 10^6$ m^3 of freshwater per year subjected to conditions for reversible electroporation, and with microorganisms' concentrations typically exceeding 10^{11} per m^3 also in freshwater (see Table 1 in Whitman et al. 1998) implies that at least $\sim 10^{17}$

microorganisms per year are subjected in freshwater to conditions under which electrotransformation can occur.

To summarize, these rough estimates suggest that at least $\sim 10^{17}$ microorganisms per year are subjected to conditions suitable for lightning-triggered electrotransformation, the majority of them in freshwater habitats (Kotnik and Weaver 2016).

Assessing the Feasibility and Importance of Lightning-Triggered HGT

The quantitative estimates outlined above suggest that sufficiently close to the surface of natural aquatic habitats, lightning-triggered HGT may well be feasible. A comprehensive theoretical analysis of the dependence between the abundance of microorganisms in those habitats (expressed, e.g., as their number per unit volume) and the probability of DNA released from one of them by electroporation to come into contact with another capable of its uptake and transformation due to either reversible electroporation or natural competence would contribute significantly to the quantitative understanding of this issue. Still, from the fact that transformation by means of natural competence occurs in many aquatic bacteria it follows that at least in some such habitats, bacteria are sufficiently abundant for DNA released from one bacterium to come into contact with another bacterium in a manner allowing this DNA to be taken up by this bacterium and transform it. Lightning-triggered electroporation is one such mechanism of DNA release (irreversible electroporation for both chromosomal and plasmid DNA, reversible electroporation for plasmid DNA and to a more limited extent), and in regions with frequent thunderstorms, it is perhaps also not insignificant compared to other natural causes of bacterial death.

In addition, and perhaps of particular importance from the aspect of the early evolution, a recent study has provided some convincing arguments why early life is likely to have evolved in shallow ponds (Mulkidjanian et al. 2012), which, unlike the deep-sea hydrothermal vents that are also considered a possible habitat of the earliest organisms, are not accessible to lightning strokes.

The experimental studies employing a single electric pulse for both electroextraction of donor microorganisms and electrotransformation of recipient microorganisms, discussed in section "Emergence of the Hypothesis and Tentative Empirical Support," can also be viewed as providing tentative support for feasibility of lightning-triggered HGT among microorganisms, and the general applicability of electrotransformation to a very broad range of microorganisms is apparent from Table 1. Still, a rigorous and critical assessment leads to some clear misgivings against treating these results as a proof-of-principle for feasibility of lightning-triggered natural HGT, and based on these misgivings, the guidelines for a proper (re)assessment of such feasibility can be formulated, as outlined in the following subsections.

Delivery and Propagation of Electric Current

All currently commercially available electric pulse generators developed for electrotransformation of microorganisms are designed as to deliver electric pulses through electrodes in direct contact with the sample; this provides a well-controlled and thus easily reproducible and optimizable exposure, but compared to the lightning strokes and arc discharges in general, such an exposure lacks the acoustic shockwave and the ultraviolet light burst that accompany arc discharges. As the results presented in Table 1 and section "Emergence of the Hypothesis and Tentative Empirical Support" were all obtained with commercial generators, none featured an arc discharge, but these accompanying phenomena can influence both the microorganisms' viability and their ability for DNA uptake. Thus, it was recently reported that compared to electric pulses delivered in direct contact with the biological sample, arc discharges result in a more efficient DNA uptake and expression (Broderick et al. 2011) and also cause a more extensive release of intracellular molecules (Boussetta et al. 2013), although it should be noted that both these studies were performed on eukaryotic cells.

For a reliable assessment of lightning-triggered HGT, the electric pulses should be delivered as an arc discharge, with the current entering the sample from above, traversing an air gap separating the sample from the current-emitting electrode. To achieve also radial propagation of the electric current from its point of entry, thus emulating what occurs with lightning stroke's current upon its entering the ground (be it solid or liquid), the receiving electrode can be designed as a ring (for two-dimensional radial propagation) or a hemispherical shell (for three-dimensional radial propagation) surrounding the sample. A recently designed experimental system for controlled delivery of arc discharges to biological samples is depicted in Fig. 2.

Electric Pulse Waveform

The electric current of lightning strokes is characterized by a rapid rise to the peak value (median zero-to-peak time of ∼5 μs) and subsequent exponential decrease (median time constant of ∼100 μs or, equivalently, peak-to-half time of ∼70 μs). Incidentally, the waveforms of pulses used in commercially available electric pulse generators for electrotransformation are also exponentially decreasing, partly because such pulses are the simplest to form, requiring only a discharge of a precharged capacitor and perhaps also due to the inherent "tail" of such pulses that exerts an electrophoretic drag on DNA, thus possibly improving the efficiency of DNA uptake (see section "Reversible Electroporation as a Mechanism of DNA Uptake and Transformation in Microorganisms"). Still, the generators used for electrotransformation typically deliver electric pulses with a time constant of exponential decrease in the range of 1–5 ms, while those of typical lightning strokes are, as mentioned in the preceding paragraph, an order of magnitude shorter. As the results presented in Table 1 and section "Emergence of the Hypothesis and Tentative

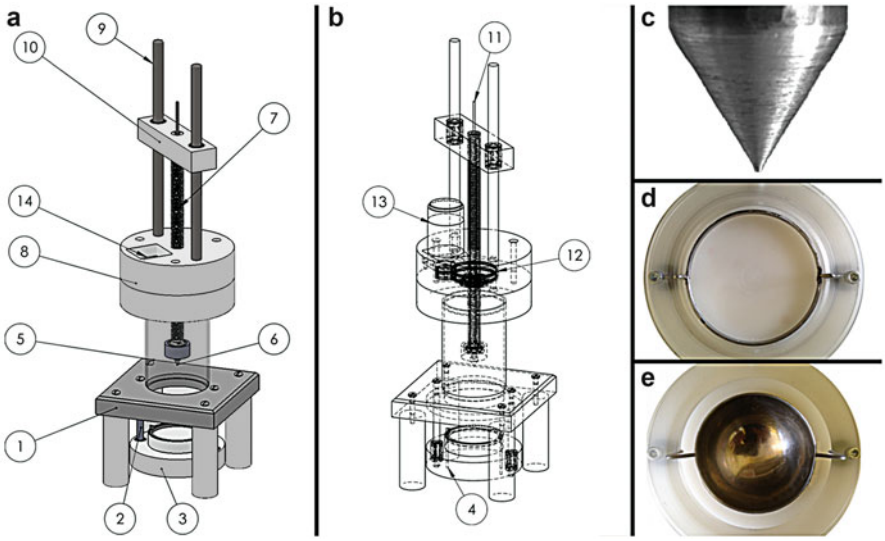

Fig. 2 An exposure system for emulating exposures of biological samples to lightning strokes. (**a**, **b**) Major components in solid (**a**) and wireframe (**b**) representation: (*1*) base, (*2*) dock guide, (*3*) sample loading dock, (*4*) receiving electrode connector, (*5*) transparent tube container, (*6*) emitting electrode tip, (*7*) emitting electrode encasement, (*8*) core, (*9*) emitting electrode guide, (*10*) upper stabilizer, (*11*) emitting electrode connector, (*12*) central cogwheel, (*13*) stepper motor with its cogwheel, (*14*) stepper motor slot. (**c**) A conical emitting electrode. (**d**) A ring-shaped receiving electrode (for two-dimensional radial propagation of the electric current). (**e**) A hemispherical-bucket-shaped receiving electrode (for three-dimensional radial propagation of the electric current) (Reprinted with permission from Marjanovič and Kotnik (2013); see this reference for further images, including photographs of the whole actual system and its use in arc delivery into various biological samples)

Empirical Support" were all obtained with commercial generators used for electrotransformation, none delivered exponentially decreasing pulses with a time constant below 1 ms, and this cannot be viewed as a proper empirical support for the feasibility of lightning-triggered HGT. At least one study did report attaining electrotransformants, in non-negligible yields, with a pulse of ∼100 μs duration, but of a rectangular waveform (Grenier et al. 2008).

For a reliable assessment of lightning-triggered HGT, the electric pulse used for electrotransformation should thus, in addition to its delivery as an arc discharge (see section "Delivery and Propagation of Electric Current"), also resemble the time course of the electric current – both its waveform and overall duration – of a typical lightning stroke. As none of the contemporary electroporation-based technologies and treatments utilizes such pulses, such an assessment calls for development of custom-designed generators. While downscaling of the amplitude of the electric current is almost unavoidable, it causes no essential loss of emulation consistency; as the lightning stroke's current always dissipates away from its point of entry into the ground, a downscaling of its amplitude in exposure systems merely reduces the size

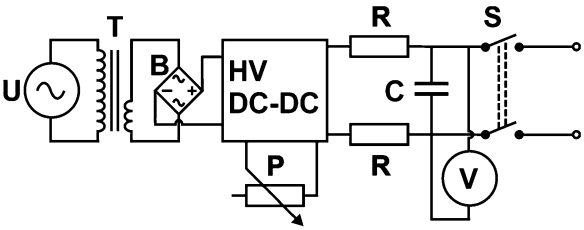

Fig. 3 Schematic diagram of the pulse generator for lightning emulation. U, grid power supply. T, isolation transformer. B, bridge rectifier. HV DC–DC, high-voltage DC–DC converter. P, linear potentiometer. R, resistors (200 kΩ each). C, capacitor (1 μF, 5 kV). S, high-voltage relay. V, digital voltmeter. The 1 μF capacitance C was chosen for use with the system depicted in Fig. 2, for which the impedance (including the biological sample in a 90-mm Petri dish) was measured at ∼100 Ω (Reprinted with permission from Reberšek et al. (2015); see this reference for further images, including photographs of the arc delivery into various biological samples and its effects as well as of the short-circuiting artifact mentioned in the body text and the elimination of this artifact)

of concentric areas subjected to various ranges of current density and electric field strength it induces. Certainly, the very highest current densities of lightning strokes are absent in downscaled exposure systems, but those are lethal to all living organisms and thus of no interest to investigation of HGT, while the electric field sufficient for both irreversible and reversible electroporation – and thus for both DNA electroextraction and electrotransformation – is consistently reached even with downscaling of the lightning stroke's current by a factor of 1000 (Kotnik 2013b).

A schematic diagram of a simple generator satisfying both the requirement of electric current waveform resembling that of a lightning stroke (∼5 μs zero-to-peak time, ∼100 μs time constant of exponential decrease) and the requirement of delivery as an arc discharge is outlined in Fig. 3. A capacitor discharge circuit generates an output current with a waveform very similar to those of lightning strokes, while the output of the generator is isolated from the line voltage by an isolation transformer both for safety reasons and to minimize the leakage current. A high-voltage direct current converter supplies the output stage of the generator, while the output voltage of the converter is set by a potentiometer and monitored by a voltmeter. The two resistors separate the converter from the output, while the capacitor stores the energy for the pulse during the charging phase, and the high-voltage relay releases this energy into the load during the discharging phase. The capacitance of the capacitor is chosen so that together with the impedance of the exposure system to which the pulse generator is connected (including the biological sample), the discharge generates an arc current with a time constant of exponential decrease of ∼100 μs, similar to those of lightning strokes (e.g., if the impedance of the exposure system is measured at 100 Ω, a capacitor of 1 μF should be used in the generator).

A possible alternative to the relay used in this design would be an insulated-gate bipolar transistor (IGBT), but these are generally more limited in their peak current and its risetime and much more prone to irreparable damage. With exposure systems such as the one depicted in Fig. 2, a problem encountered empirically is the gradual

lateral drift of the initially vertical arc discharge from the emitting electrode into the sample, which for discharges exceeding ~10 μs often results in short-circuiting between the emitting and the receiving electrode, but this artifact can be eliminated by incorporating an insulating cylinder concentrically between the emitting electrode and the receiving electrode, so that it forms a tight contact with the surface of the sample and thus precludes the discharge from evading the sample from above and short-circuiting the electrodes (Reberšek et al. 2015).

Microorganisms, DNA, and Exposure Conditions

For a reliable assessment of lightning-triggered HGT, the microorganisms of primary interest are those for which natural habitats are accessible to lightning strokes. Similarly, the DNA used in such assessment, be it plasmid or chromosomal, should be natural and devoid of artificial modifications often introduced in the laboratory to improve transfer and/or expression or to prevent DNA degradation by the host; successful lightning-triggered transformation with a shuttle vector or another type of engineered or altered DNA molecule does not necessarily imply that natural plasmid or chromosomal DNA can be transferred in the same manner.

The medium in which the microorganisms are exposed to electric pulses should resemble their natural habitat, free of conditions and substances used to adjust osmolarity, conductivity, microorganisms' permeability and/or viability, as well as DNA stability and/or transferrability. And in the same vein, also other experimental conditions absent from natural environments, such as centrifugation and filtration, should be avoided throughout all the relevant stages of the experiment – at least from the start of the exposure to electric pulses to the evaluation of the resulting transformation and expression. The experimental studies in which the adequate choice of microorganisms, DNA molecules (if not electroextracted), as well as the nature-emulating medium and experimental conditions will all be respected are currently in their initial stages, and most of the work still lies ahead.

Conclusions

The above considerations suggest that under contemporary conditions, per year, at least 10^{17} microorganisms are subjected to lightning strokes that generate conditions suitable for electrotransformation. To estimate the actual number of lightning-induced electrotransformants per year in natural habitats, this number, or an improved estimate thereof, would have to be multiplied by the transformation efficiency at naturally occurring concentrations of environmental DNA suitable for transformation, and to complicate matters further, these concentrations are combinations of DNA released by the mechanism under consideration and DNA released due to other causes of cell death. Clearly, uncertainties in such estimates are large, perhaps spanning many orders of magnitude, and reliable answers will certainly require both extensive and elaborate measurements and experiments.

Furthermore, such an assessment, if sufficiently perfected, may elucidate importance (or unimportance) of lightning-triggered HGT for evolution of current microorganisms under contemporary conditions, but it will not say much about the potential role of this mechanism during early evolution, when the biotic mechanisms had not yet evolved. For example, primordial microorganisms' envelopes may have differed considerably from modern ones in their intact permeability to nucleotides and their polymers, and the lightning stroke rates were likely much higher during periods with intense volcanic activity.

An even harder question to answer is that of the relative importance of lightning-triggered HGT compared to biotic HGT; even the assertions on the relative importance of each biotic HGT mechanism still vary wildly, with some studies claiming that competence, conjugation, and transduction explain virtually all-known HGT in prokaryotes, and others positing that GTAs are the predominant mechanism of HGT in oceans. Thus, perhaps the only aspect that the existing evidence suggests rather clearly is that in the sense of attainable phylogenetic distance between the DNA-donor and DNA-recipient microorganisms, electrotransformation in general acts more broadly than any of the biotic mechanisms. Other than this, to assess what fractions of the natural HGT are due to each individual mechanism, and particularly which HGT mechanism dominated during the early evolution, will likely require substantial progress in our knowledge of HGT – both biotic and abiotic.

Acknowledgment This work was supported by the Slovenian Research Agency (Grant P2-0249) and conducted in the scope of the European Laboratory of Pulsed Electric Fields Applications (LEA EBAM) and within networking efforts of the COST Action TD1104 – European Network for Development of Electroporation-Based Technologies and Treatments (EP4Bio2Med).

Cross-References

► Gene Delivery by Electroporation in Vitro: Mechanisms

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