Electroporation of solar panel microbiome in the search of new biotechnological chassis

Final degree project – Degree in Biotechnology

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Summary

Abstract

Currently used synthetic biology chassis, most of them model organisms, are proved to be ineffective for some advanced and complex genetic engineering approaches. This leads to the need of new chassis that have genetic and molecular modules compatible with these new genetic techniques. In this work, we used a solar panel biofilm sample, the microbial community living on photovoltaic panel; in order to check its suitability as a source of new chassis, electric transformation was carried out, also know as electroporation. Eventually, we succeeded and an species (*Stenotrophomonas sp*) of this sample was electrotransformed. This work could open a new scenario in synthetic biology and biotechnology by focusing on environment microbiome as a source of novel organisms to be used as alternative chassis for bioengineering.

Introduction

Lightning-triggered origin and evolution of life

Life did not form spontaneously. This fact was proposed by Francesco Redi ("Omne vivum ex ovum") and by Lazzaro Spanllanzi, and was conclusively proved by Louis Pasteur[1] and by John Tyndall[2,3]. That is to say, there had to be a set of conditions which permitted the rise of it. Aleksandr Ivánovich Oparin, with his book "*Origin of life*" (1924)[4], stated the first ideas of our concept of origin of life by saying life had to be formed from organic compounds being present in primitive earth's oceans. He later proposed that the Earth had to have a reducing atmosphere in which the mentioned organic compounds would have been able to form and, for this, there needed to be whatsoever type of energy for primitive earth[5–8].

As Miller's 1959 work showed, inorganic compounds being part of primitive atmosphere could only absorb ultraviolet's wavelengths of less than 200 nm. Nevertheless, these compounds could also be influenced by electrical discharges. Indeed, it is known that these electric discharges are the main inductors of organic compounds synthesis[9]. Other scientist, like Eric T. Parker[10,11], R. D. Hill[5] and Alan W. Schwartz[12], proposed that lightning-caused synthesis of organic compounds could only be plausible in Archean volcanoes which have very reducing atmospheres and are a source of dust.

It is clear the essential role of lightning and electrical discharges the emergence of life, but it might also be vital in bacteria evolution. Bacteria division via fission creates very few genetic variations, leading to a relatively low degree of diversification over time. This cannot explain the biodiversity and adaptability found in bacteria. Horizontal Gene Transfer (HGT) (Figure 1) might have provided fast adaptation abilities to changing environments as well as harsh conditions[13], and it has been recognized as the main force that allowed bacteria to colonize most of the ecological niches on Earth[14].

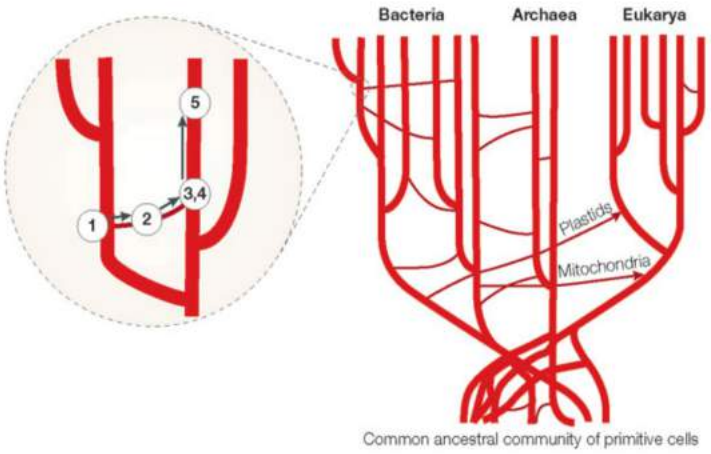


Figure 1: HGT between clades and the five steps leading to the stable inheritance of a transferred gene. Extracted from Smets *et al.* (2005)[140]

Specifically, HGT is present[15,16], despite intrinsic differences and evolutionary consequences, in genetic innovations or recombination (Fisher, 1930; and Muller, 1932): conjugation (plasmid-mediated), transduction (virus-mediated) and natural transformation (direct uptake of naked DNA)[17,18]. Natural transformation is the only HGT mechanism that may occurred without the active participation of two living cells, but a great proportion of natural bacteria are not intrinsically competent and may had difficulties on succeeding with this way of evolution[17]: approximately 1% of the described bacterial species

are known to be naturally transformable[19] (for example, *Escherichia coli* has not been proved to be at all[20,21]).

Natural competency[20], a process discovered by Frederick Griffith in 1928, is the physiological state in which prokaryotes have the ability of taking up environmentally-abundant genetic material, which can recombine with endogenous genetic material. This process requires expression of specific genes to develop a physiological step known as competence that promotes the uptake of DNA, and this is induced by specific signals and needs regulatory machinery not found in all bacteria[22].

Nature is wise and there is another way of increasing HGT frequency, even bacteria might intake genetic material naturally; a non-biochemical weather event does help on this transformation, lightnings[16]. That is bacteria electrotransformation or electroporation made possible by lightenings[17]. Electric current can permeabilize bacterial membranes, allowing the penetration of naked DNA. That is to say, electric current over a threshold value can permeabilize bacterial membranes (cell membrane resistance changes from 10^{-3} Siemens $\cdot$ cm^{-1} to 1 Siemens $\cdot$ cm^{-1})[23], producing a rapid and large increase of their electric conductivity and permeability. This forms metastable aqueous pores (according to the aqueous pore reference model) by penetration of water molecules into the lipid bilayer and by subsequent reorientation of the adjacent lipids with their polar headgroups towards this water[24], ; through these pores, electric current allows migration of exogenous DNA (Figure 2).

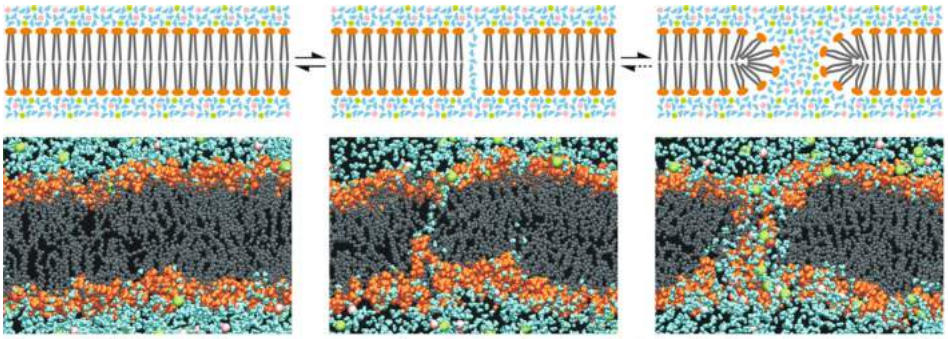


Figure 2: Metastable aqueous pore formation due to electric discharge's electric field. Extracted from Kotnik *et al.* (2013)[24]

The electrical parameters of this process have been extensively investigated and are now well characterized[25]. Lightning electrical currents penetrate the soil through an area of a few square centimeters with a current intensity varying from 10 to 200 kA[25]. Very close to the impact point of the lightning, the bacterial strains may be destroyed, but lightning creates an electrical field whose value decreases with the square of the radial distance[26]: there are some points in which current is compatible with electroporation (6 kV $\cdot$ cm^{-1} versus 12.5 kV $\cdot$ cm^{-1}) suggesting that lightning could naturally act as an *in situ* "electroporator" for mediating gene transfer among environmental bacteria (Figure 3)[27].

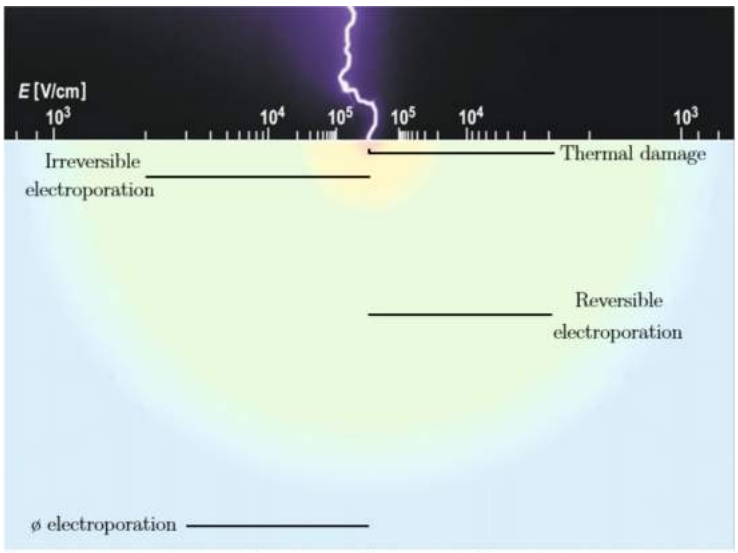


Figure 3: Relation between electric field-radial distance and lighting-triggered electroporation. Electric field decreases with the square of the radial distance[26] and this conditions the effect of lighting on the cells affected by discharge: as this physical magnitude lowers, cells suffered from thermal damage to absence of electroporation, in which there is no affection by lightning and only natural competent bacteria are transformed[24]. Modified from Kotnik *et al.* (2013)[24].

It has been proved that the transformation of added bacteria (DH10B *Escherichia coli*) to soil microcosm is possible and locally increased via lightning-mediated current injection; this laboratory-scale lightning had an electrical field gradient. (700 versus 600 $kV \cdot m^{-1}$) and current density (2.5 versus 12.6 $kA \cdot m^{-2}$) similar to those of full-scale lightning[27].

Cérémonie *et al.* (2004)[28] isolated two supposedly lightning-competent soil bacteria (*Pseudomonas sp.*) that show an electrotransformation frequency in soil several orders of magnitude higher than *Escherichia coli* (10^{-4} to 10^{-5} versus 10^{-5} to 10^{-8} electrotransformants per recipient cells, respectively) and this confirmed the potential significance of natural electrotransformation via lightning as a source of evolution, at least, for soil bacteria[27–29].

Electroporation as a genetic tool for transformation

Electroporation, developed in the 1960s by Frankenhaeuser *et al.* (1956)[30], is thus the application of an electric field leading to the electrical permeabilization of membranes[31]; following nature as an example (Figure 4). Electroporation is, thus, an alternative to calcium chloride-base, an older method for bacterial transformation[32]. Electroporation provides, not only a method for DNA intake[33], but also for DNA release[34].

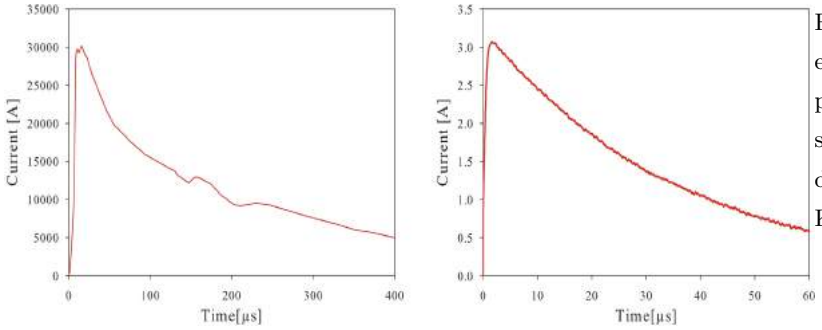


Figure 4: Comparison of time courses of the total electric current generated by lightning stroke and by parallel plate capacitor discharge. There is a similarity between the two processes, an exponential decay of electric current with time. Extracted from Kotnik *et al.* (2013b)[35].

Probably the first scientific description of a phenomenon suggestive of irreversible electroporation in tissue can be found in the 1754 Jean Antoine Nollet's book[36]. However, the first uses of high-voltage electric pulses focused on killing microorganism, and maybe the first report dates back to 1896, when the Louisville Water Company studied various methods for purifying river water[37]. Reversible electroporation, characterized by an special range of electrical field (Figure 6) and also known as electrotransformation or gene electrotransfer, was first achieved in mammalian cells in the early 1980s[33,38].

Electrotransformation is currently, therefore, a very well-known and efficient approach in various areas of biology, biotechnology, and medicine[24]. However, electroporation is far from being fully understood: its knowledge is phenomenological, based on measurements of electrical currents through planar bilayer membranes (BLM), under the influence of strong electric fields, and on molecular transportation of molecules into (or out of) the cells subjected to electric field pulses. That is to say, direct evidence of this physical process does not exist, but indirect evidence does (electron microscopy)[39].

When it comes to electroporation modulation, applied voltage and pulse amplitude are very important as they will induce pore formation. If prokaryotes are exposed to submillisecond electric pulses (this is the case of lightnings) and to pulse amplitudes (field strength) up to hundreds of V/cm, electroporation is undetectable. However, within a range spanning from hundreds of V/cm up to tens of kV/cm, electroporation is mostly reversible, and above this subrange it becomes mostly irreversible, and thermal damage starts to appear[24].

Cell death, due to electroporation, may be caused by two facts: one is electric damage and the other one is thermal damage, which can be independent from each other, The first one is based on the application of too high field strength (from 2 kV/cm) that create membrane pores so large that induce irreversible electroporation and necrosis; the second one consist on death due to Joule heating (temperatures above 42ºC may prevent biological repair mechanisms) caused by electrical pulse heat dissipation[40].

The main application of reversible electroporation is transformation, and efficiencies are something to be controlled and maximized. Molecular form of DNA being internalized plays a vital role in electrotransformation efficiency: generally, the efficiency is the highest for supercoiled circular double-stranded DNA (the indigenous form of plasmid and chromosomal DNA in many prokaryotes) and the lowest for linear double-stranded DNA with nonhomologous ends[24]. In contrast, for DNA concentrations in the range of pg/ml up to µg/ml, efficiency of gene electrotransfer in bacteria is roughly constant[24].

The optimal parameters of the successful gene transfer via electric pulse vary with bacterial species and even strains. In general, pulse amplitudes (electric fields) ranging from 2 to 30 kV/cm and pulse durations from milliseconds to tens of milliseconds[41–43] are desirable. What it has been proved is that applied field strength from 0.1 to 1 kV/cm and pulse duration from 0.4 to 1 s usually lead to survival in reversible electroporation[40].

Gene electrotransfer is effective both in Gram-negative and Gram-positive bacteria, although the latter generally require higher fields and/or yield lower efficiencies, measured as Colony-Forming Units per µg of DNA (CFUs/µg): when electrotransformed with plasmid DNA, typically up to 10^8 – 10^{10} CFU/µg for Gram-negative, and up to 10^6 – 10^7 CFU/µg for Gram-positive bacteria; this is associated with the thicker peptidoglycan layer of the Gram-positive wall[42,43].

Gene electrotransfer can in general occur in species of many archaeal and bacterial phyla[24]. It is compulsory to say that measuring genetic transformation sensitivity by this electrical shock-method can be only applied to species where a selectable genetic marker is reachable (such as an antibiotic-resistance allele). Other approach to quantify this competence, and hence, the capacity of transformation, is the study of this state by population genetics and by gene distributions genes that have been linked to natural competence[44].

Natural biodiversity exploit

Microorganism are the most extended form of life in terms of habitats: they live in almost every habitat one can think of, from 20 miles beneath the Earth's surface to 20 miles overhead[45], for example.

They adapt constantly to continuous disturbance of the environment, which has allowed them to colonize the most diverse ecosystems[46], thanks to the development of special traits that make life possible to them in those habitats. Examples of these habitats include soils[47] or air[48]; even extreme environments do contain microorganism, such as acid mine drainages[49] (for example, Mexico's Cueva Villa Luz[50]), deserts[51], under high temperature conditions[52] and hyper-saline environments[53].

Therefore, microorganism have constituted source of many applications and problem solutions. First use of microorganism may date back to 7000 years ago as there are findings of that-aged jars containing wine[54] and, what is more, Sumerian poem dated at 1700 B.C., "Hymn to Ninkasi", mentions beer brewing[55]. Many years later, Robert Hooke[56] and Antoni van Leeuwenhoek settled what is considered as the discovery of microorganism, and their exploitation peaked[57].

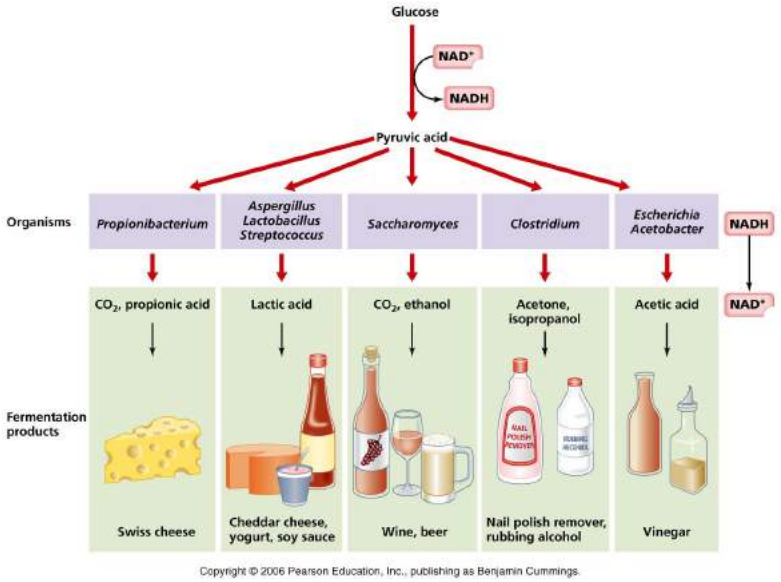


Figure 5: Microbial diverse use of piruvate for industrial applications.

Modern microbiology spreads to many fields, such as the development of pharmaceutical products, the use of quality-control methods in food and dairy product production, the control of disease-causing microorganisms in consumable waters and the industrial applications of microorganisms (vitamins, amino acids, enzymes, and growth supplements)[58,59]. Biotechnology uses microorganisms as living factories to fabricate products easily and with flexibility (Figure 5), such as human hormone insulin, industrial enzymes, third generation fuels, and biofertilizers; these are only a few applications, but others are worth

mentioned, like pollutant-resistant strains that are related to the biodegradation of recalcitrant molecules[60,61].

For biotechnological applications, even there are others examples, *Escherichia coli* and *Saccharomyces cerevisiae* have been the most spread Synthetic biology (SB) chassis[62], because of their adaptation to laboratory conditions, rapid growth, high heterologous protein production and availability of genetic and metabolic toolkits and resources. SB calls these kind of hosts "chassis", defined as organisms that serve as a foundation to physically contain genetic components, supporting them by providing resources to function[63].

However, as science develops and necessities increase, those chassis are getting rusty and insufficient, and its applicability is reducing: there has been a shift from simple gene mutations to complex synthetic circuits and creation of entirely synthetic genomes. At this point, *Escherichia coli* might not be any longer an adequate host for biotechnological and synthetic biologic genetic designs as their genetic and molecular modules might not be compatible with the inserted constructs[63].

Moving to new chassis bacteria is not as simple as porting the toolkits and circuits developed in *Escherichia coli* into another bacterial species since the genetic expression and regulation of synthetic circuits are highly host-specific (affinities of both DNA- and RNA-related enzymes and transcriptional factors, for example, are very different among species)[63].

New chassis need to be scaled-up from laboratory conditions to the level of industrial technologies to accomplish human needs. Also, genetic tools, such as electroporation, have to be applicable to or have to be developed in order to facilitate bioengineering[63]. Other requirements of the new chassis are that they should be able to grow under extreme conditions that could enhance expression of the inserted construct.

The development of new chassis is already under way, like Kushwaha *et al.* (2015)[64], who are making important progresses through the development of Universal Bacterial Expression Resource (UBER); or Chris Voigt and colleagues at the Massachusetts Institute of Technology[65] do: they are working to extend the bacterial programming language Cello beyond *Escherichia coli* to also work in other organisms, such as *Bacteroides sp.* and *Pseudomonas sp.*

Indeed, *Pseudomonas putida*[66] and *Bacillus subtilis* are becoming more and more spread SB chassis. Nevertheless, new chassis are in sight, such as *Geobacillus sp.*[67] (spore forming thermophile) and *Deinoccocus sp.*[68,69] (highly radiation and desiccation-resistant), being the former usable for applications at high temperatures and the latter, form stressful applications.

For novel chassis discovery, using bioprospecting of poorly studied microbial niches is required.

Solar panels microbiota and their applications

Extreme environments can host a very wide biodiversity as selective pressure has resulted in the development of efficient survival strategies (extremophiles).

An extreme habitat could be a high temperatures environment in which strategies like stability of cell components, saturation of lipids, repair methods of nucleic acids, acquisition of dormant forms, etc., are present. One interesting example is a black smoker hydrothermal vent at the Mid-Atlantic Ridge, where archaea *Pyrolobus fumarii* is able to grow under temperatures of 113 ºC and, thus, some autoclaving conditions can be found[52].

Another example of a termophilic environment are desserts, like Tataouine (south Tunisia), in which bacteria phyla like Actinobacteria, Firmicutes, Proteobacteria and Cytophaga-Flavobacterium-Bacteroides (CFB) group are frequent. Here, related to desiccation resistance, radiation resistance is often found[51].

Interestingly, solar panels are similar environments. Solar energy has the highest growing rate among all sustainable energies, covering around 4000 km[270], and it is thought to overtook the net growth of coal in 2015[71]. These data imply the fact that accessible extreme environment area from which science can take profit from is growing.

The microbiome of solar panels is starting to be studied[72–74] and they show great biodiversity even the extreme conditions they are subjected to. They contain more than 500 different species (Figure 6) per panel (Sphingobacteria and Deinococcus, among many others), most of which belong to drought-, heat- and radiation-adapted bacterial genera, and sun-irradiation adapted epiphytic fungi.

Most of the isolates showed resistance to very high salt concentrations (20–26% w/v NaCl) and short exposures to UV light, whereas only a few number of isolates proved resistant to a low pH or extreme heat[73]. These strategies permit their survival and derive from special physiological features (for example, diguanylate cyclase, bacterial L7/L12 and archaeal L7 proteins, membrane-bound proton-translocating pyrophosphatase mPP protein, GroEL, mPP and cold-shock protein and S-layer protein, lipoprotein 1)[73].

Species growing on solar panels are classified among mesophilic organism[73], but do face stressful dessert-like conditions of the surface of these panels (minimum water retention capacity and maximum sunlight exposure, all of which determine circadian and annual peaks of irradiation, desiccation and heat) and are adapted to them.

This outdoors human-made environment stands as a source of diverse microbial species which have developed strategies that may constitute new biotechnological applications.

Figure 6: Bacteria biodiversity of solar panels. This chart shows the bacteria biodiversity (in % of total bacteria) of a sample of a solar panel in Valencia, in 2013 summer solstice: the most abundant species are *Sphingomonas kaistensis* and *Novosphingobium tardaugens.*, both pertaining to the Sphingomonadaceae family. "Other" comprises species that individually represent a maximum of 1%. Modified from Dorado-Morales *et al.* (2016)[73].

One strategy that is also profitable for human is the production of carotenoids, which are know to confer radiation resistant due to its capacity to scavenge singlet molecular oxygen and peroxyl radicals generated during UV-derived photooxidation[75,76]. Therefore, solar panels microorganisms could be used to produce carotenoids for a wide range of applications[77,78], such as solar screens, food industry products or even paintings; these will only be achievable if science, and SB, develop new tools for the affordable use of these microorganisms, which may eventually constitute new SB and biotechnological chassis.

In this work, solar panels samples from Paterna (Valencia) were tested in terms of their electrotransformation capability by using antibiotic sensibility as electrotransformation negative control, in order to look for new SB chassis.

Materials and Methods

Microbial culture

Microbial culture was carried out on LB (Miller's Lysogeny Broth[79]) and R2A (Reasoner's 2A[80]) agar media (Table 1); the sterilization was carried out by autoclaving (30 minutes at 121ºC). Plating consisted of pouring approximately 20 mL of the media on 90 mm × 15 mm Petri dishes, which were inoculated with 50 µL of the corresponding sample.

Table 1: Growth media used. Microbial culture was carried out by inoculating 50 µL of the sample on 20 mL LB (I) or R2A (II) agar plates.

I.

Component	Final concentration (g/L)
NaCl	10
Triptone	10
Yeast extract	5
Agar	15

II.

Component	Final concentration (g/L)
Peptone	1
Glucose	0.5
Soluble starch	0.5
Yeast extract	0.5
K_2PO_4	0.3
Sodium pyruvate	0.3
$MgSO_4 \cdot 7\ H_2O$	0.05
Agar	15

Samples used and growth conditions

Three biological samples were used to perform the assays: solar panel's surface sample, DH5α *Escherichia coli*, NEB5 electrocompetent *Escherichia coli* and Ron Geller's (personal communication) homemade electrocompetent DH5α *Escherichia coli*.

Solar panel's surface sample (SP) was obtained after spraying 1.28 m^2 solar panels with a Sodium Phosphate Buffer (PBS) solution, so as to remove the superficial material with a sterilized scraper. This suspension was divided in 100 µL aliquots, rapidly frozen with solid CO_2 and stored at – 80°C until required. DH5-α *Escherichia coli* (DH5) was obtained from New England BioLabs NEB 5-α Electrocompetent *Escherichia coli*, C2989K, (NEB5); and electrocompetent DH10B *Escherichia coli* was kindly provided by Ron Geller, I^{2}SYSBIO's Viral Biology Group laboratory [81].

SP was cultured in the darkness and at room temperature (RT), whereas DH5, NEB5 and RG were cultured under darkness and at 37°C.

Growth was measured in terms of Colony-Formation Units per mL of sample (CFU/mL) or, in electroporation trials, in terms of Colony-Formation Units per µg of plasmid DNA used (CFU/µg); incubation time was 4 days for SP and 1 day for DH5, NEB5 and RG.

Microbial inhibition agents trial

SP sensitivity to growth inhibitors was assessed by using some antibiotics and antifungi (Table 2). This was performed to determine which resistance gen transfer could be used to assess SP electroporation. These growth inhibitors were added to the sterilized LB to the recommended final concentrations[82].

Table 2: Microbial growth inhibitors and concentrations used. Five microbial growth inhibitors were used, as samples, to test SP susceptibility to general growth inhibitors to perform following trails.

Susceptible microorganism	Growth inhibitor	Final concentration (mg/L)
Bacteria	Gentamicin	10
Bacteria	Kanamicin	50
Bacteria	Chloramphenicol	25
Bacteria	Tetracycline	10
Fungi	Nipagin A	100

Ten treatments (T0-9) were performed (Figure 27), using three technical replicates (dilutions) and two biological replicates (SP aliquots) in each of them: 6 CFUs/mL, per treatment, were analyzed.

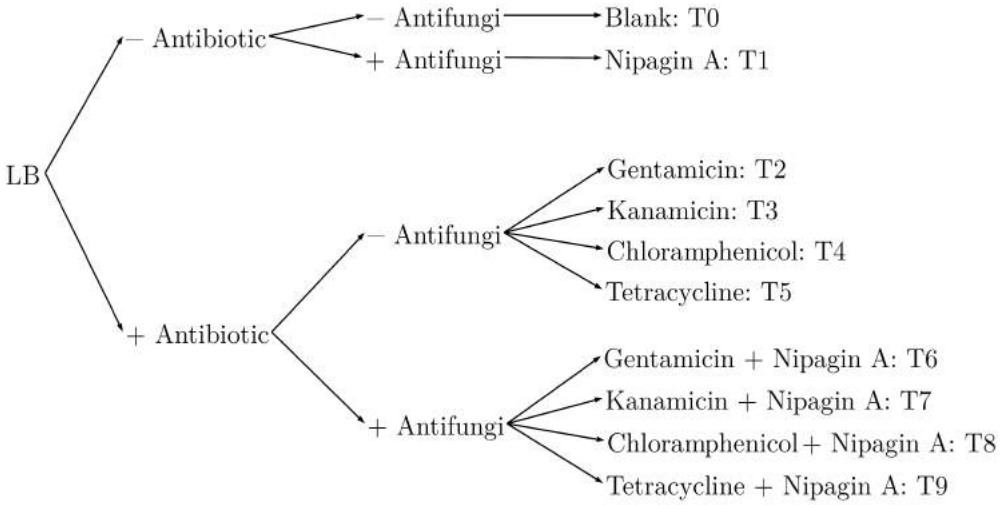
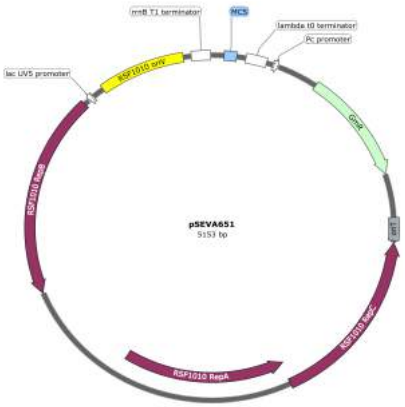


Figure 7: Treatments (T0-9) of solar panel samples for the growth inhibition assay. LB media were supplemented with antibiotics and/or antifungi and used to test SP susceptibility to these growth inhibitors in order to select the antibiotic resistance plasmid to be further used in electroporation.

Plasmid pSEVA651

Antibiotic resistance plasmid was the pSEVA651[83] (Figure 8); an antibiotic resistance gene Open Reading Frame (ORF) was pre-inserted in its Multiple Site of Cloning (MSC). Plasmid was obtained by mini-prep procedure, using Thermo Scientific GeneJET Plasmid Miniprep Kit (K0503)[84], and this plasmidic DNA solution was quantified in terms of its concentration and purity using the spectophotometric device Thermo Scientific Nanodrop 2000[85].

I.



II.

Characteristic	Value
Structure	Circular dsDNA
Length	5153 bp
Molecular weight	1.89 x 10^{11} mol/µg DNA

Figure 8: Antibiotic resistance plasmid pSEVA651. The vector chosen to carry the antibiotic resistance gene ORF was the plasmid pSEVA651 in whose MSC the mentioned ORF was previously inserted. Plasmid map (I) and information (II) generated by SnapGene software (from GSL Biotech; available at snapgene.com)[86].

Electroporation and culture recovery

Electroporation was assessed by using a BTX Gemini X^2 Harvard Electroporator (452006)[87] and with the predetermined program "*E. coli* #210 (Exponential Decay)" (Table 14).

Table 3: Electroporator programme for pSEVA651 electrotransfer, "E. coli #210 (Exponential Decay)".

Magnitude	Set value
Voltage	1800 V
Time constant	4.8 ms
Resistance	200 Ω
Capacitance	25 µF
Number of pulses	1
Gap width	1 mm

The process consisted of the electroporation of 20 µL of sample mixed with 2 µL of DNA solution, and processed in a pre-chilled (4°C) 1 mm gap-length electrocuvette; every item and sample was maintained at 4°C for optimal electroporation.

After electroporation, the recovery procedure was carried out. This consisted of the addition of 1 mL of the catabolite repression Super Optimal growth medium[88] (SOC; Table 4), pre-warmed at 37°C (for DH5, NEB5 and RG samples) or at 30°C (for SP sample); the resulting suspensions were incubated for 1h, at 37°C and 250 rpm (for DH5, NEB5 and RG samples) or at 30°C and 200 rpm (for SP sample), to induce expression of the pSEVA651 resistance gene.

In order to quantify the number of electrotransformants (CFUs/µg DNA), the recovered culture was inoculated in LB supplemented with the correspondent antibiotic. Negative control was also done by following the previous procedure, but without electroporation of the sample.

Viability change due to electroporation was also assessed by inoculating the electroporated sample and the negative control in LB.

Table 4: SOC as culture broth for electroporation recovery. After electroporation, cell walls holes have to close, which is induced by the cultivation of electroporated suspension in 1 mL of rich culture media, SOC.

Component	Final concentration (g/L)
Casein hydrolysate	20
Yeast extract	5
Glucose	3.6
$MgSO_4$	2.4
NaCl	0.
KCl	0.186

Solar panel sample influence on NEB5 electrotransformation

To test if components of SP samples affected electrotransformation efficiency, this trial consisted of electrotransfering pSEVA651 to NEB5 mixed with different volumes of SP, leading to various SP concentrations in NEB5 electroporation (Table 5); DNA concentration was constant (2 µL DNA in 22 µL of total volume).

In the same experiment, positive control was based on the electroporation of NEB5 by itself, in which high number of electrotransformants were expected (table 5).

Table 5: Solar panel sample inhibitory effect on NEB5 electrotransformation. In order to see how solar panel components may influence electroporation, NEB5 was electroporated with different concentrations of solar panel, constituting NEB5 and solar panel mixes; % SP is the % of the original SP sample (not diluted) referred to total volume.

Sample	µL NEB5	µL SP (dilution)	% SP (v/v)
NEB5	20	0	0
SP-D	0	20 (Direct)	100
SP-10^{-1}	0	20 (10^{-1})	1
SP-10^{-3}	0	20 (10^{-3})	0.1
SP-10^{-5}	0	20 (10^{-5})	0.001
Mix-D	10	10 (D)	50
Mix-10^{-1}	10	10 (10^{-1})	0.5
Mix-10^{-3}	10	10 (10^{-3})	0.05
Mix-10^{-5}	10	10 (10^{-5})	0.0005

Electrocompetence protocol

Electrocompetence was carried out following the protocol kindly provided by Ron Geller[81]; it was used for NEB5 and SP samples (Figure 9).

Firstly, 50 mL of the sample were pre-cultured in 50 mL LB:SOC (1:1, v:v) at 37°C and 250rpm (for NEB5 sample) or RT and 200 rpm (for SP sample), over night (o/n).

Fifty mL of the previous culture were transferred to fresh 450 mL LB:SOC (1:1), leading to a 1/10 dilution. This suspension was cultured at 37°C and 250rpm (for NEB5 sample) or 30°C and 200 rpm (for SP sample) so as to reach a OD_{600nm} between 0.3 and 0.4.

Growth was stopped by chilling the culture at 4°C and, then, cell suspension were transferred to pre-chilled 50 mL flasks; here, cells were harvested by centrifugation at 4ºC, 4000 x g for 20 minutes. Once carried out, cells were washed to remove traces of media and salts: three aqueous washings were done, consisting of centrifugation at 4ºC, 3500 x g for 15 minutes, decantation of supernatant and resuspension in 50 mL pre-chilled sterilized deionized water.

To finish, one last step of centrifugation was carried out, in which last centrigation's pellet was resuspended in 30 mL of pre-chilled aqueous glycerol 10% (v/v) and centrifuged at 4ºC, 3500 x g for 15 minutes. The final pellet was resuspended in 1 mL of pre-chilled aqueous glycerol 10% (v/v) and divided in 20 µL aliquots.

During the process, samples were taken to quantify growth by counting cells growing on LB (CFUs/mL) and by spectrophotometry (OD_{600nm}).

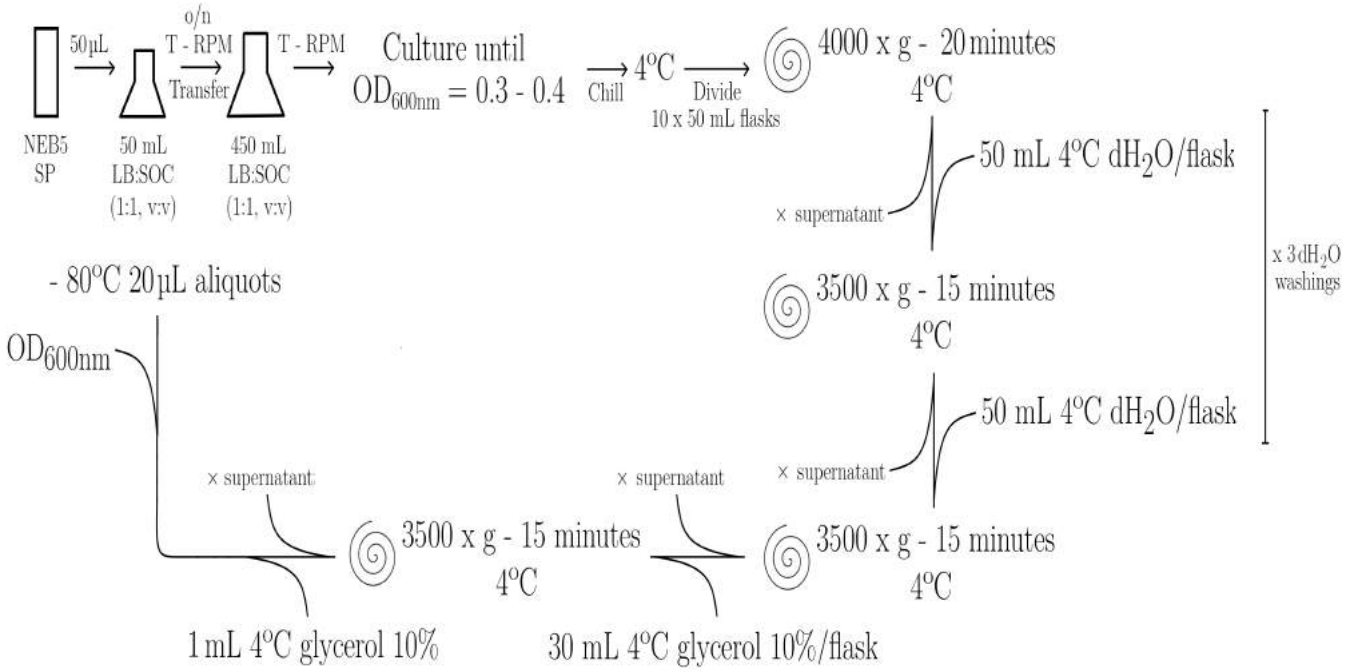


Figure 9: Electrocompetence protocol. It consisted of water and glycerol 10 % (v/v) to wash salts and other compounds, and purify the samples, maintaining ice-cold temperatures (4°C).

Verification of solar panel electroporation

Colonies of electroporated solar panel samples growing on LB supplemented with antibiotic were taken as positive colonies, colonies that might have been incorporated pSEVA651.

Confirmation of plasmid electrotransfer was carried out by plasmid extraction of the positive colonies, following the procedure mentioned above (Miniprep kit)[84]. Extraction result was quantified using spectrophotometric analysis, as mentioned above (Nanodrop[85]).

Afterwards, presence of the correct plasmid (pSEVA651) was checked by electrophoresis method. An agarose 0.8% gel (w/v, in Tris-Borate-EDTA (TBE) buffer) was prepared, in which fluorescent DNA intercalating agent 10000X GelRed (Biotium, #41003[89]) was added (2%, v/v); each lane was loaded with

5 µL of prepared sample, from a mix consisting of 5 µL extraction results + 1 µL 6X Loading buffer (TaKaRa, #SDO0503[90]). Electrophoresis was done by setting the voltage to 80 V, letting the DNA run for 2 hours; buffer used was TBE buffer.

Positive control (pSEVA651) was also used and DNA ladder used was DL2000 DNA Ladder (Mebep BioScience, #R-0401-01[91]), loading the same quantities than the samples.

Bands were checked using a transilluminator (VWR Imager2, #730-1458[92]) in which $A_{excitation}$ and $A_{emission}$ were those of DNA intercalating agent, 302 nm and 600 nm, respectively.

Confirmation of pSEVA651 plasmid electrotransfer

Once existence of plasmid was checked out, conclusive confirmation was done by a restriction enzyme trial.

Using SnapGene software[86], unique cutting restriction enzymes, which would produce linearizated forms of the vector, were searched on pSEVA561. HindIII, a type II restriction enzyme isolated from *Haemophilus influenzae* Rd[93], was chosen (New England BioLabs, Inc., #R0104[94])

The protocol was based on the incubation of the recommended mix (based on the ratio 10 enzyme units/µg DNA) for 1 hour, at the optimal temperature for enzyme activity (37ºC)[95]; the reaction was stopped by heat inactivation for 20 minutes, at the enzyme degradation temperature (80ºC)[96].

Enzyme restriction results were analyzed by electrophoresis, following the procedure mentioned above.

Identification of the electrotransformed colonies

Identification of colonies having introduced pSEVA651 consisted of the sequencing of 16S rRNA, which is known to provide unequivocal identification at genera level[97].

To do so, extraction of the total genomic DNA was carried out by following the procedure described by Latorre *et al.* (1986)[98], based on alkaline lysis. Resulting genomic DNA was measured in terms of quantity and quality by spectrophotometrical procedure (Nanodrop[85]).

Afterwards, PCR (PCR #1) of a 16S rRNA gene fragment was carried out in other to amplify this sequence. DNA electrophoresis was carried out in a 1.5% (w/v) agarose gel and voltage was set at 100V (running time was approximately 40 minutes).

I.

Component	Volume (µL)
10X Buffer[99]	4.5
dNTPs 2.5 mM[100]	1.5
Forward primer	1
Reverse primer	1
DNA-dependent DNA polimerase[99]	0.5
DNA	1
dH_2O	40.5
Total reaction volume: 50 µL	

II.

Stage	Composition
Initial denaturalization	96 °C – 5 minutes
Amplification cycles (x 30)	95 °C – 30 seconds
	54 °C – 30 seconds
	72 °C – 30 seconds
Additional extension	72 °C – 10 minutes
Hold	4 °C - ∞

III.

Forward primer[101]

5' - CTG GTT GAT CCT GCC AG - 3'

Reverse primer

5' - GTN TTA CNG CGG CKG CTG – 3'

Figure 10: PCR #1 characteristics. PCR of genomic 16S rRNA (PCR #1) was carried out and its characteristics are shown: I. refers to the reaction mix used, II. refers to the PCR programme and III. refers to the sequence of the pair of primers used.

To finish, sequencing of the amplified 16S rRNA was carried out: firstly, a PCR (PCR #2) of this DNA was done, using one single primer (reverse primer), to have 16S rRNA sequence copies whose nucleotides are fluorescently labeled; secondly, the resulting amplicons were sent to University of Valencia's SCSIE Genomic service[102] in order to sequence them by the Sanger method.

I.

Component	Volume (µL)
BigDye[103]	0.5
Buffer[103]	1.5
Primer	0.5
DNA	1
dH_2O	4.5

Total reaction volume: 8 µL

II.

Stage	Composition
Initial denaturalization	96 °C – 1 minutes
Amplification cycles (x 99)	96 °C – 10 minutes
	50 °C – 5 minutes
	60 °C – 4 minutes
Hold	4 °C - ∞

III.

Primer

5' - GTN TTA CNG CGG CKG CTG – 3'

Figure 11: PCR #2 characteristics. PCR of amplified 16S rRNA (PCR #2) was carried out and its characteristics are shown: I. refers to the reaction mix used, II. refers to the PCR programme and III..refers to the sequence of the primer used.

Statistical analysis and graphical representations

R programming language[104] was used to calculate means, and differences among replications and among samples. Differences among replications were based on standard deviation and differences among samples were calculated with a two-sided Student's t-test for two-sample comparison (normality was checked by using Shapiro Wilk test) and type III ANOVA test for multiple-sample comparison (spherical test confirmation was not possible due to low number of replicates per sample).

Graphs were constructed by using LibreOffice Calc (v6.0)[105] and by R programme language[104]. Images were edited using Kolourpaint[106] and GIMP[107].

Expressed values correspond, if repetitions were made, to the mean and error bars correspond to standard deviations.

Results and discussion

Solar sample characterization

SP sample was characterized in terms of growth (CFUs/mL), at day 4 of incubation; this was done using LB agar and R2A agar culture media.

Even growth quantification, in general, was done at the 4th day of incubation, these SP samples were left in incubation for 21 days in order to check growth and biodiversity differences (compared to 4 days of incubation, Figure 12).

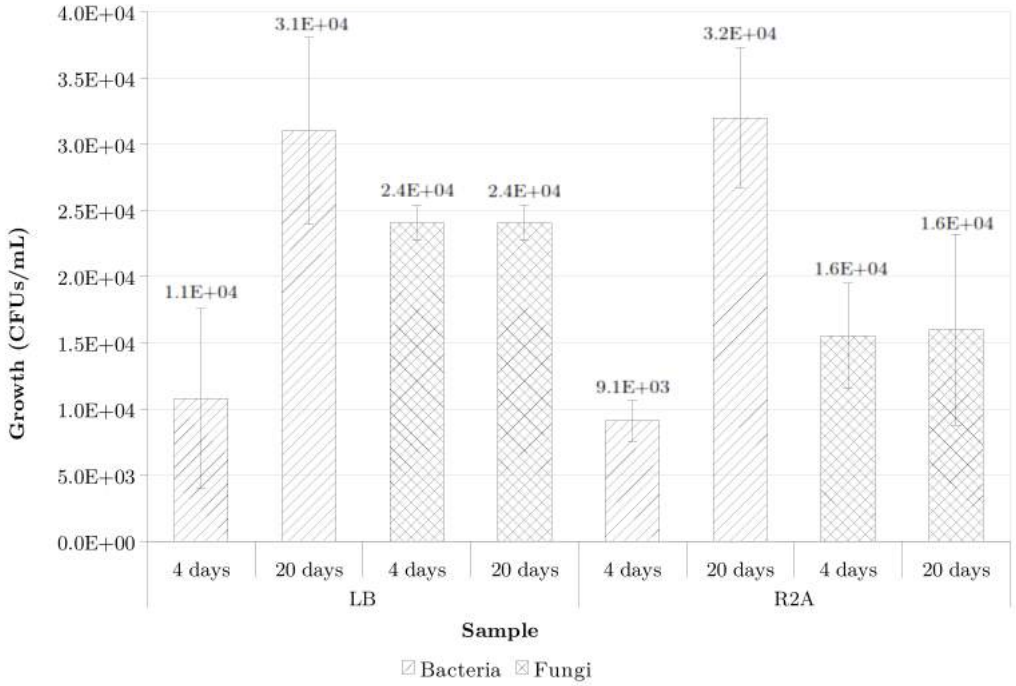


Figure 12: Solar panel growth characterization on solid media culture. Solar panel sample was characterized in terms of bacteria and fungi growth (CFUs/mL sample) in solid culture media, LB agar and R2A agar, on the 4th day and on the 20th day. Number of technical repetitions (n) = 3.

As seen in Figure 12, at the 4th day of incubation, from one hand, there is no significant bacteria growth difference between LB agar culture ((1.1 ± 0.68) x 10^4 CFUs/mL) and R2A agar culture ((9.1 ± 1.6) x 10^3 CFUs/mL). From the other hand, there is no significant fungi growth difference between LB agar culture ((2.4 ± 0.13) x 10^4 CFUs/mL) and R2A agar culture ((1.6 ± 0.40) x 10^4 CFUs/mL).

LB leads to a bacteria growth 1.21 times bigger that R2A does, while fungi growth is 1.50 times bigger in LB than R2A, this makes sense taking into account LB diverse and abundant nutrients, compared to R2A that is not that rich in nutrients (see Materials and Methods, Solar sample characterization). However, numerically, these differences are not statistically significant

Bibliography supports these growing values. LB is non-selective culture media, being rich thanks to its content of yeast extract; it is commonly used for genetically engineered *Escherichia coli*[108]. R2A is also a non-selective culture media but, in contrast, was formulated to isolate potable water bacteria, permitting slow-growing bacteria[80], bacteria that do not tolerate high concentrations of nutrients or stressed (sodium pyruvate increases the recovery of stressed cells), injured (soluble starch aids in the recovery of injured organisms by absorbing toxic metabolic by-products)[109] or chlorine-tolerant bacteria[110].

With these results, it was decided to only use LB agar culture in order to augment retrieved bacteria that could potentially be electrotransformed, even R2A is said to be used for cultivation of samples that derive from environment which are not rich in nutrients.

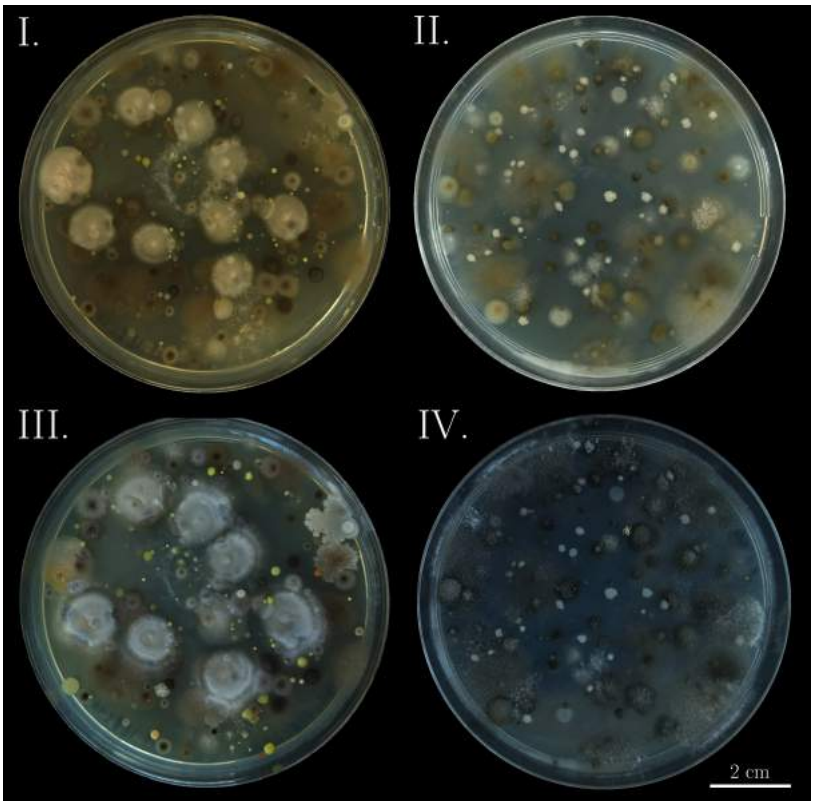


Figure 13: Solar panel sample growth on LB agar, 4th (I.) and 20th (III.) day, and on and R2A agar, 4th (II.) an 20th day (IV.).

Moreover, on day 20, bacteria growth was larger, whereas fungi stays constant visibly (Figure 12 and Figure 13): there was a significant bacteria CFUs/mL augment between day 4 and 20 in case of R2A culture (p-value = 0.02332); however, other growth differences were not significant. Even this, numerically and visibly, bacteria may need more time to growth, but fungi only needed a few days to grow.

Bioprospection states that, in order to try to get the highest biodiversity of a nature-derived sample, varied growth conditions should be used, such as source of carbon, nitrogen and phosphate, temperature and humidity, culture medium physical state, light conditions, day-night differences, micronutrients, O_2 conditions, etc. Taking into account this, SP should be grown under different growth conditions, but, to simplify procedures from now, only LB was used as mentioned above.

Gentamicin and nipagin A as solar panel sample growth inhibitors

To make electroporation results conclusive, reference of electroporation absence (negative control) was needed. This mentioned reference consisted of the SP antibiotic sensibility: gene electrotransfer would be based on a antibiotic resistance gene, contained in a plasmid (pSEVA651), which would give the sample the capacity of growth in a culture media supplemented with the correspondent antibiotic.

Therefore, a growth inhibition assay was assessed, using five growth inhibitors: four antibiotics (kanamicin, gentamicin, chloramphenicol and tetracycline) and one antifungi (nipagin A).

All 4 antibiotics are bacteriostatic that essentially inhibit protein synthesis[111], but their specific mechanism of action differs: for example, chloramphenicol inhibits peptide bond formation by binding to 25S rRNA and 50S subunit, inhibiting peptidyl transferase activity eventually[112]; and gentamicin provokes the misreading of mRNA by union to 30S-subunit proteins and 16S rRNA[113]. Nipagin A is an antifungi that inhibit fungi growth by suppressing their ability to grow or reproduce[114].

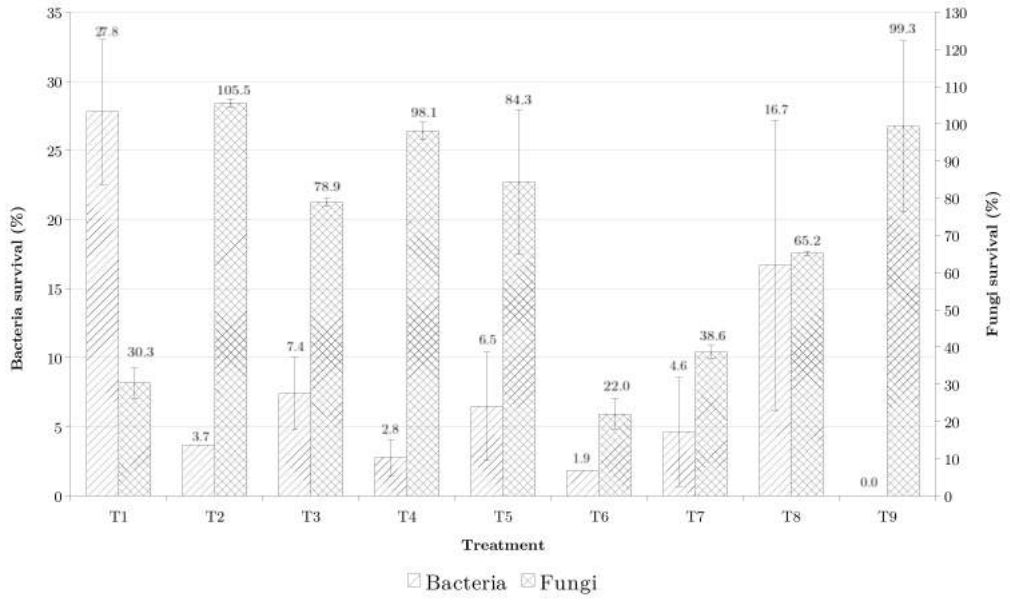


Figure 14: Solar panel growth on microbial inhibitors trial. Solar panel sample was tested in terms of its sensibility to a sample of growth inhibitors, four antibiotics and one antifungi: combination of them constituted nine treatments + one blank treatment; results are expressed in % of survival, relative to blank treatment (100 % survival). Number of technical replicates (n) = 2.

Growth inhibitors did affect SP growth in LB agar culture, as Figure 14 shows. In particular, the lowest bacteria survival was that of chloramphenicol, T4 (2.8 ± 1.3 %), or that of tetracycline + nipagin A, T9 (0 ± 0 %); whereas highest survival was seen in kanamicin, T3 (7.4 ± 2.6 %), or in chloramphenicol + nipagin A, T8 (16.4 ± 1.05%).

Nevertheless, fungi constitute a great deterrent for assays as they growth radially and impede proper bacteria count. Lowest fungi growth was achieved with the gentamicin + nipagin A treatment (T6, 22.0 ± 0 %) and bacteria growth on this treatment was similar to the treatment of lowest bacteria survival, T9 (1.8 ± 0 % vs. 0 ± 0 %, respectively).

Even the four antibiotics base their mechanism of action on the same molecular process, bacteria inhibition rates are statistically different (p-value = 0.04516). Fungi inhibition rates are also statistically different (p-value = 2.684 x 10^{-6}), even only one antifungi was used; this could suggest that the combination of antibiotics with the antifungi may affect fungi growth differently.

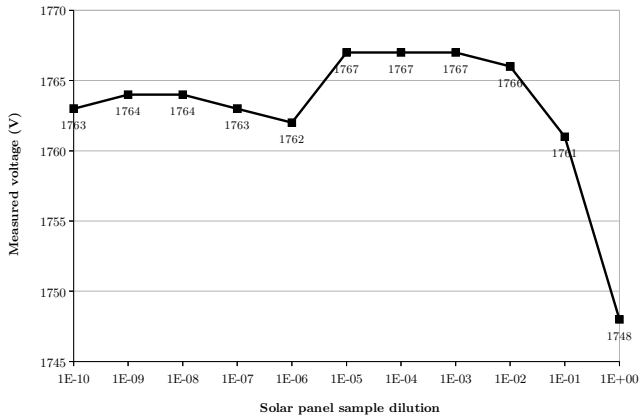
Despite what it has been said, to sum up, the treatment chosen was gentamicin + nipagin A: that is to say, transferred gene, contained in a plasmid, was one that would provide bacteria resistance to gentamicin; and culture would be done in LB supplemented with gentamicin and nipagin A.

Solar panel sample dilutions electroporation test

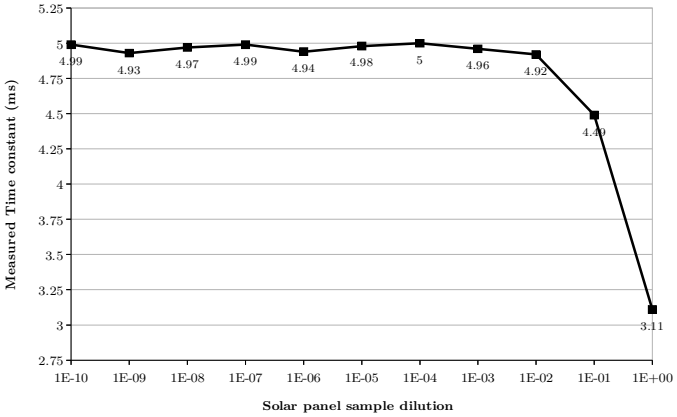
SP sample was taken from environment and, thus, its content is not controlled so substances like salts may challenge electroporation success.

To test how this SP content influence electroporation, SP sample was diluted with dH_2O and these resulting suspensions were electroporated, without adding DNA; differences in the output values of electroporation were expected, even electric arch in the most concentrated suspensions.

I.



II.



III.

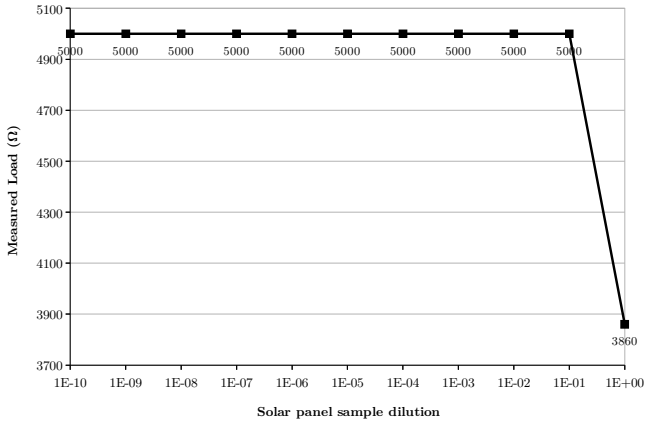


Figure 15: Electroporation of dilutions of solar panel sample. Solar panel sample may have a great quantity of deterrent substances for a procedure like electroporation, so electroporation of different SP aqueous dilutions was done to check influence of this substances. I. shows the measured voltage the sample receives (V), II. shows the measured time constant (ms) and III. shows the measured load (Ω).

Two output magnitudes were taken into account as these are the most important for electroporation as a physical process (Figure 15).

Firstly, the field strength is measured as the voltage delivered across an electrode gap and is expressed as kV/cm. However, taking into account electrode gap length (in the performed assay, 0.1 cm), it is expressed as voltage (V). This is related to the surpassing of the electrical potential of the cell membrane to allow pore formation[115].

Secondly, the pulse length is the duration of time the sample is exposed to electroporation pulse; in an exponential decay wave system (the electroporation system used in this work), this magnitude is called the "time constant", which is characterized by the rate at which the voltage is decayed to one-third the original set voltage[115,116]. It is said, for *Escherichia coli*, that time constants higher than 5.0 ms means that the pores were open too largely and the majority of cells probably died, whereas less than 4.0 ms means the pores were open too briefly to allow efficient uptake of DNA[115,116]

Thirdly, measured load is a synonym of measured resistance of the electroporated sample; this is known to be inversely proportional to salt media concentration[115]. Salt is known to be deterrent for electroporation procedure as it can induce electric arching and cell viability decrease; however, some can facility mobility of DNA thanks to ionic forces between ions and DNA[117].

In Figure 15, in every chart (I, II and III) one can see the constancy of the values throughout most diluted SP samples, but values start to drop as the concentration increases. In I and II charts, this fact can be explained with the fact that salts in the sample may be contributing to the electrical potential

of the cell membrane, so field strength is not able to surpass it and, thus, measured voltage and time constant decrease; in chart III, it can be accounted for the fact that the more salt the medium where cells are suspended has, the less resistance (load) is measured in it[115], so a low measure of this magnitude might be indicative of electric arching (related to high salt concentration on the medium).

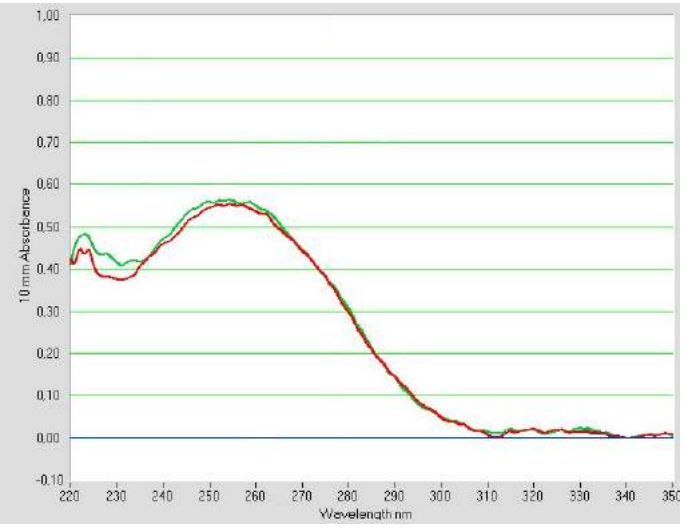
Nevertheless, no electric arch was obtained, not even with the most concentrated dilutions.

pSEVA651 plasmid extraction

Chosen antibiotic resistance gene was gentamicin, which was previously inserted in a plasmid (pSEVA651) in the form of gentamicin acetyltransferase (known to confer aminoglycoside resistance and, therefore, gentamicin's[118–120]).

This plasmid was inside a strain of *Escherichia coli*, so miniprep protocol was done with it to obtain the plasmid. The resulting solution containing the plasmid was analyzed spectrophotrometrically with Nanodrop (Figure 16).

I.



II.

A_{260} (au)	A_{280} (au)	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)	Yield (µg)
0.543 ± 0.0085	0.303 ± 0.0071	1.79 ± 0.0141	1.38 ± 0.078	27.16 ± 0.40	2.72 ± 0.04

Figure 16: pSEVA651 extraction by miniprep procedure. Plasmid containing gentamicin acetyltransferase was extracted from a strain of *Escherichia coli* by miniprep and analyzed by Nanodrop; I. shows the spectral patron of absorption and II. shows relevant measured magnitudes. Number of measures (n) = 2.

On one side, ratio A_{260}/A_{280} refers to contamination of DNA with proteins and other molecules, like phenols, and values equal or higher than 1.8 indicate pure DNA in this context: sample value in this ratio is 1.79 ± 0.01, near to 1.80, so DNA is pure in terms of proteins. On the other side, ratio A_{260}/A_{230} indicates the presence of organic compounds, chaotropic salts, urea, EDTA, carbohydrates and phenolate ions and it is usually higher than the previous ratio, so ratios higher than 2 are desirable: sample value in this is 1.36 ± 0.08, which is not good and indicates contamination with the above mentioned compounds.[121,122]

Concentration and, thus, yield obtained, are low (27.16 ± 0.40 ng/µL and 2.72 ± 0.04 µg, respectively) when compared to usual values miniprep offers: concentration is commonly above 50 ng/µL[123] and yield, between 50 µg and 100 µg[124]; however, it was proved to be sufficient for electroporation (see trials below: Electroporation of solar panel).

Solar panel inhibitory effect on NEB5 electrotransformation

It was previously known that SP samples would be difficult to electrotransformation due to the fact that this genetic tools had been designed and aimed at pure laboratory-made biological samples that accomplish certain chemistry characteristics, as the absence of phenols, salts or other compounds.

Due to that fact, firstly, it was desired to see how SP sample's components would affect electrotransformation. To test this, NEB5 was mixed with different aqueous dilutions of SP and assessed to plasmid electrotransfer; appropriate controls were made also (see Materials and Methods: G).

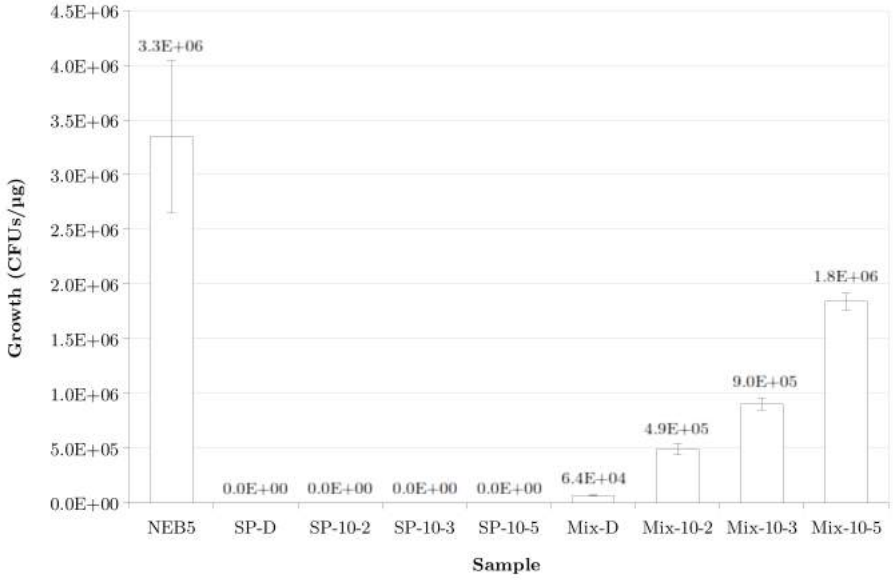


Figure 17: Solar panel inhibitory effect on NEB5 plasmid electrotransfer. Solar panel components were tested in terms of their deterrent effects on electroporations, in particular, NEB5 electrotransformation; for this purpose, NEB5 was electroporated with different final concentrations of solar panel sample. Number of technical replicates (n) = 2.

The fact that SP affects efficiency of NEB5 electrotransformation is clearly visible (Figure 17): there is a decrease in *Escherichia coli* electrotransformants (CFUs/µg) from NEB5 electroporation (3.3 x 10^6) to Mix-D electroporation (6.4 x 10^4), which may be caused by the presence of the different substances in SP sample that impede NEB5 electroporation, maybe by chelating DNA as some metal ions do[125,126].

Even that, comparing NEB5 sample's CFUs/µg to Mix samples' CFUs/µg (1:1), there are no significant differences. Nevertheless, when comparing all CFUs/µg together, differences are statistically different (p-value = 0.002258).

Moreover, if NEB5's CFUs/µg are depicted in reference to SP % (v/v), a meaningful graph is formed (Figure 18); in this, one can notice the direct numerical relationship between SP concentration and NEB5 electrotransformation efficiency.

The numeral relationship can be written as a logarithmic regression function (r^2 = 0.961), using every CFUs/µg - SP% pair of experimental values:

$$\text{NEB5's CFUs/µg} = -155278 \cdot \text{Ln (SP \% (v/v))} + 53784$$

(Eq. 1)

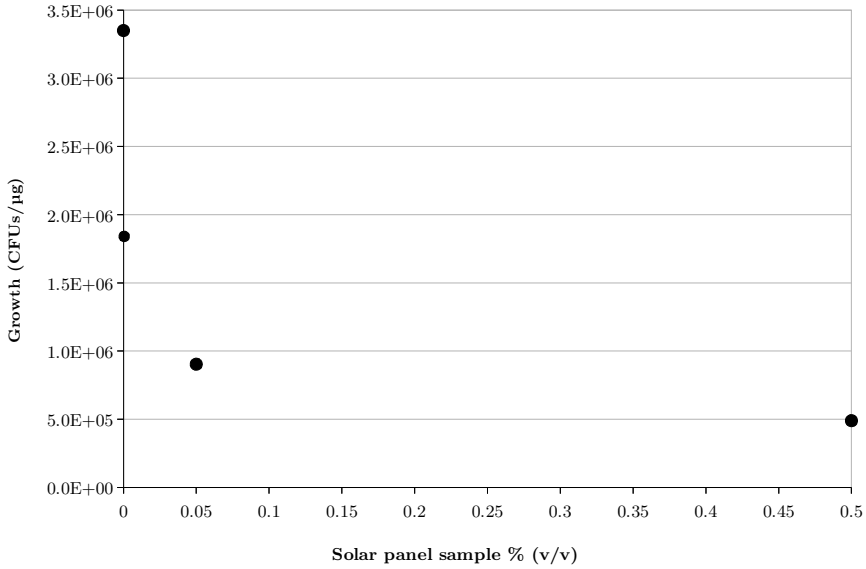


Figure 18: Graphical relationship between NEB5's electrotransformation efficiency and SP concentration; CFUs/µg correspondent to SP % = 50 was not included in the graph so as to avoid X axis dispersion.

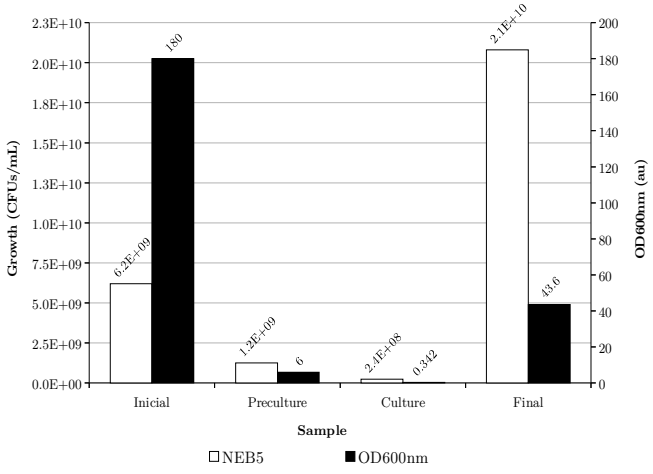
Electrocompetence procedure analysis

As there were not positive results on the electroporation of SP sample, that is to say, no colony grew on LB agar supplemented with gentamicin, an aliquot of RG sample was assessed to electroporation: positive results were found (data not shown), so electroporator failure and non integrity of extracted plasmid were discarded.

Consequently, the problem laid in SP sample, so electrocompetence protocol was applied to this sample to see if this epigenetic state could be achieved in the whole or part of the microbial community.

To test if electrocompetence protocol was applied well, this protocol was carried out with NEB5, which are electrocompetent: if it was applied correctly, NEB5 would keep on being electrocompetent (to test if electrocompetence stayed, results were compared to those of electroporation of NEB5 that had not been through electrocompetence protocol). Results (see Electroporation of solar panel) showed that NEB5 electrocompetence maintain, that is to say, electrotransformants of NEB5 and NEB5 gone through electrocompetence were not statistically different; thus, electrocompetence protocol was carried out properly.

I.



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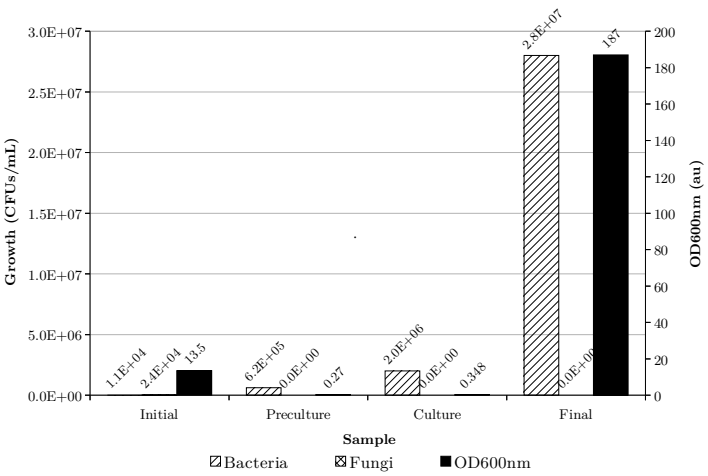

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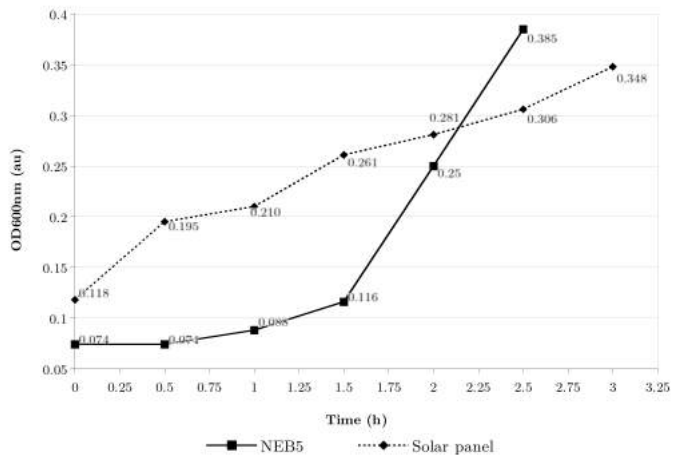


Figure 19: Electrocompetence protocol on NEB5 and solar panel. Solar panel was assessed with electrocompetence protocol and so was NEB5 as a positive control of this trial. On the one part, I. and II. show the characterization throughout the process on NEB5 and Solar panel, respectively, obtaining data from growth (CFUs/mL) and from density (OD_{600nm}); the stages of the process are as follow: "Initial" refers to the original sample, "Preculture" refers to the result of preculture stage, "Culture" refers to the result of the culture to OD_{600nm} = 0.4 au and "Final" refers to the result of the protocol. On the other part, III. shows the change of OD_{600nm} in the culture to OD_{600nm} = 0.4, in NEB5 and solar panel.

Growth curves at culture stage of electrocompetence protocol are exponential, noticeable in Figure 19 (I.), for NEB5; however, SP's (I., Figure 19) deviates graphically from it, which could be explained by the fact that SP is composed of different species and, thus, different growth rates, which would eventually mix and disturb the general growth curve.

In both samples, there was an increase of growth (CFUs/mL) due to stages of cultivation and of concentration (centrifugations). Moreover, there was a decrease in biodiversity of sample SP throughout the process, probably because of the predominant growth rate or capacity, in LB:SOC (1:1, v:v), of some species of SP, leading to a bias (Figure 20); a highlight is that fungi population vanished, so nipagin A would not be necessary in culture media.

This biodiversity decrease could be detrimental for the electroporation of this sample as there would be less number of different species, and thus chances, to potentially electrotransform; however, the protocol leads also to a decrease of the ratio bacteria vs. dust and solid microparticules, as well as fungi, which may be beneficial to electroporation as this new sample is purer.

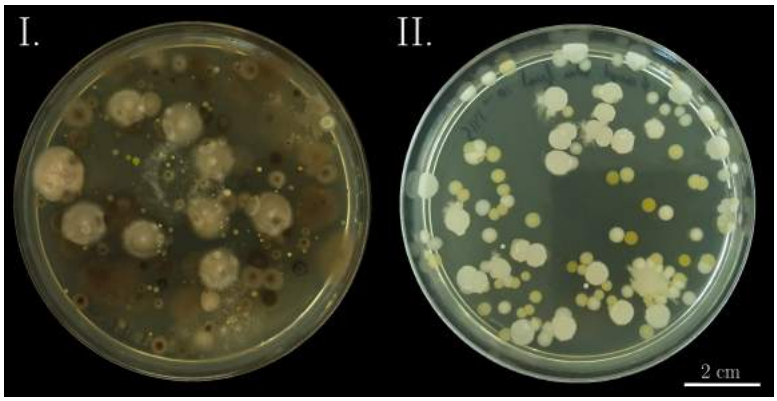


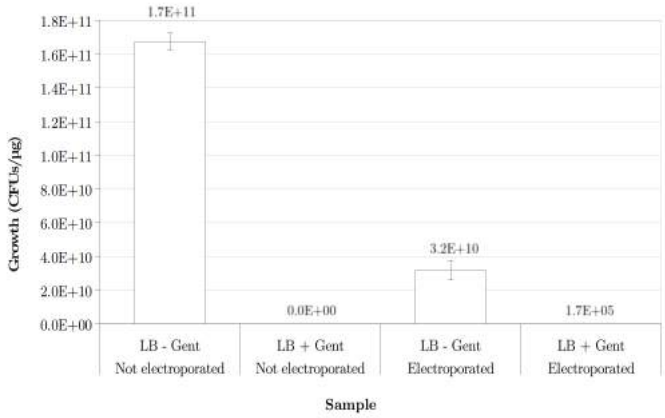
Figure 20: Solar panel biodiversity change due to electrocompetence protocol. Composition on species of solar panel visibly changed due to the culture and centrifugation stages the electrocompetence protocol is composed of: this can be seen in this figure, where I. shows solar panel (dilution 10^{-1}) culture on LB agar and II. shows culture on LB agar of the resulting sample of electrocompetence protocol of solar panel (dilution 10^{-4}); this last sample could not be culture at 10^{-1} dilution as this dilution created a microbial grass.

Electroporation of solar panel sample

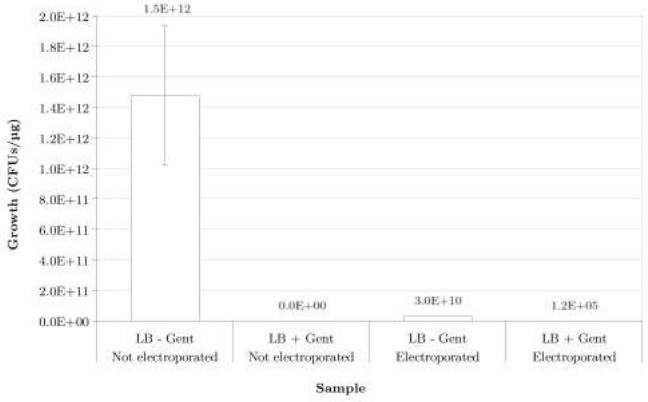
After carrying out the electrocompetence protocol on SP, this sample (electrocompetent SP) was electroporated. Also, NEB5 and NEB5 gone through the electrocompetent protocol (electrocompetent NEB5) was electroporated in order to see if electrocompetent protocol was carried out correctly (by comparing the results of NEB5 and electrocompetent NEB5) and as a positive control for the plasmid electrotransfer of electrocompetent SP (by comparing the results of electrocompetent SP and electrocompetent NEB5), as explained above.

Even previous trials showed no positive results for electroporation of SP, this sample was again electroporated to keep on seeking electrotransformants.

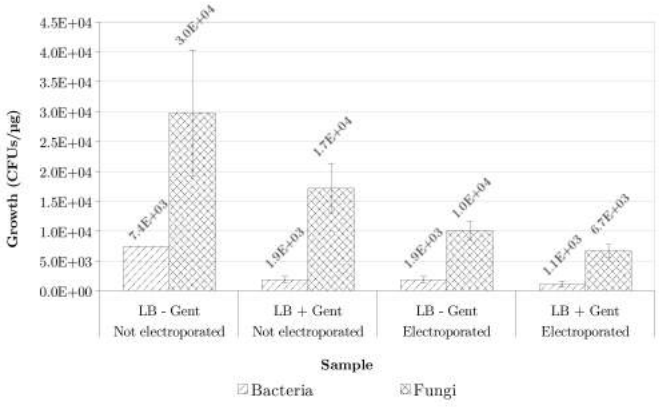
I.



II.



III.



IV.

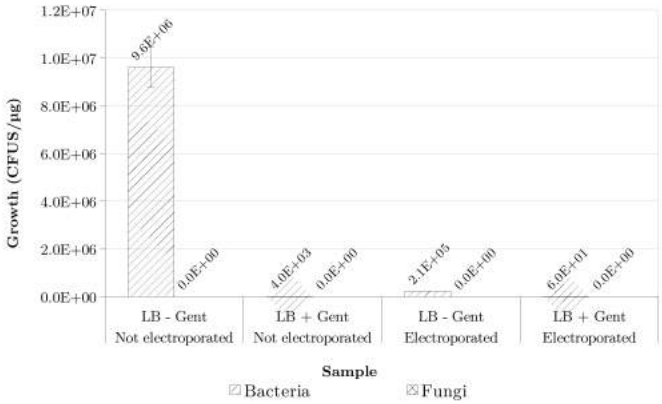


Figure 21: Electroporation of NEB5 (I.), Electrocompetent NEB5 (II.), Solar panel (III.) and Electrocompetent Solar panel (IV.). The four biological samples with which it has been working were electroporated and recovered by procedures described in Materials and Methods.

On one side, chart I and chart II (Figure 21) show there are no differences in terms of growth in the four treatments, supported by statistics (no significant differences, comparing each treatment, individually, in both samples). What is more, CFUs/µg in NEB5 and electroporated NEB5 are visible very similar as showed in Figure 22: correlation coefficient between these two samples is $r = 0.985402$, close to $r = 1$. These results indicate both samples act similarly in terms of electroporation and, thus, that electrocompetence protocol has not changed the original NEB5 sample electrocompetence.

On the other side, chart III and IV of Figure 21 show there is bacterial growth in LB agar supplemented with gentamicin, both before and after electroporation. Six morphologically different colonies (colonies 1-6: 1-3, from Electroporated electrocompetent SP; 4-6, from Electroporated SP) were found in total, but the fact that they could grow in the presence of gentamicin can be explained by natural resistance to gentamicin or by successful electrotransformation, so more trials were needed to confirm these results.

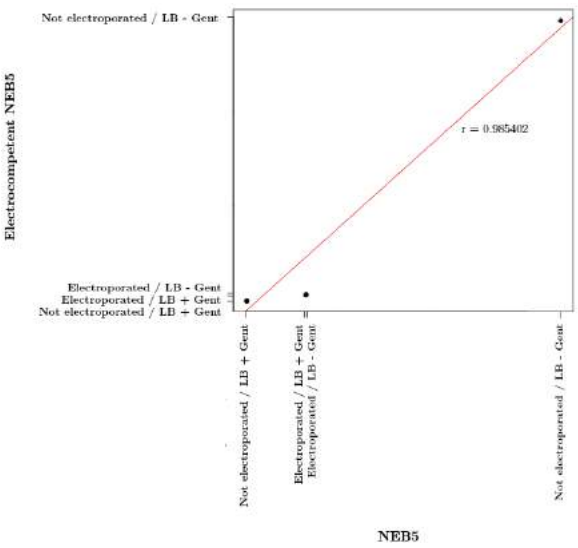


Figure 22: Scatter plot of growth of electroporated and not electroporated NEB5 and electrocompetent NEB5. This graph shows the correlation between the growth values of NEB5 and electrocompetent NEB5 in order to prove the similarity between them.

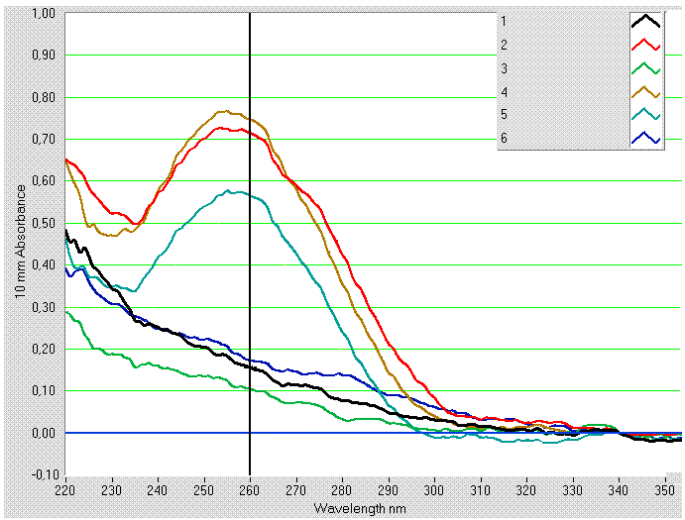
Interestingly, survival to electroporation was higher in the samples that had not been gone through electrocompetent protocol (NEB5, 19.03%; and SP, 25.00%) than those that had been gone through it (electrocompetent NEB5, 2.02%; and electrocompetent SP, 2.19%); this can be due to the disturbance that electrocompetence procedure induces to the samples.

Confirmation of solar panel sample electroporation

The mentioned six different colonies were purified by streaking on LB agar supplemented with gentamicin; two of them did not grow, which might mean they cannot be domesticated, as bibliography has previously reported. Even only four colonies were able to purify, verification was carried out for every colony (the six of them).

Firstly, plasmid extraction was carried out by miniprep[84] and plasmid samples were spectrophotometrically quantified by Nanodrop[85].

I.



II.

Colony	A_{260} (au)	A_{280} (au)	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)
1	0.154 ± 0.008	0.093 ± 0.0	1.70 ± 0.30	0.49 ± 0.02	7.68 ± 0.40
2	0.712 ± 0.006	0.408 ± 0.007	1.75 ± 0.04	1.47 ± 0.07	35.60 ± 0.29
3	0.117 ± 0.011	0.070 ± 0.020	1.77 ± 0.46	0.67 ± 0.04	5.85 ± 0.51
4	0.725 ± 0.008	0.348 ± 0.011	2.07 ± 0.06	1.78 ± 0.02	36.27 ± 0.38
5	0.541 ± 0.019	0.239 ± 0.024	2.27 ± 0.15	1.92 ± 0.05	27.06 ± 0.95
6	0.151 ± 0.021	0.084 ± 0.011	1.81 ± 0.06	0.55 ± 0.03	7.57 ± 1.04

Figure 23. Plasmid extraction of potentially electrotransformed colonies. Plasmid extraction of morphologically different electroporated colonies was carried out and plasmidic DNA was quantified with Nanodrop: I. shows the absorbance spectra of the plasmid samples and II. shows the quantification of the plasmid samples. Number of repetitions = 2.

Showed in Figure 23, three out of six colonies (two, four and five) yielded a significant quantity of plasmidic DNA, supported by their A_{260} peaks (Figure 23, I.). Nevertheless, all of them had a acceptable A_{260}/A_{280} ratio (> 1.8), but none of them got a decent A_{260}/A_{230} ratio (< 2.0).

Afterwards, plasmidic DNAs were analyzed by electrophoresis in a 0.8% (w/v) agarose gel.

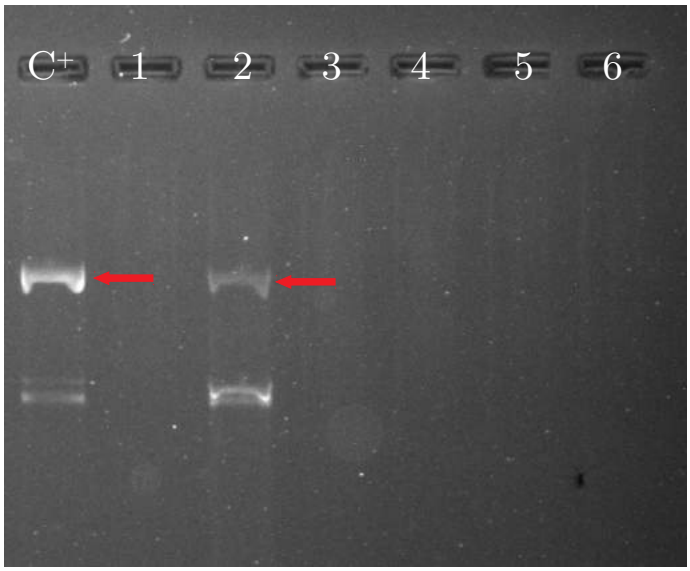


Figure 24: Agarose 0.8% electrophoresis of the plasmidic DNAs of positive colonies. Plasmidic DNA of the six positive colonies was loaded in a agarose 0.8% (w/v) electrophoresis; C+, positive control, which corresponds to pSEVA651; 1, 2, 3, 4, 5 and 6, plasmidic DNA of positives colonies 1-6.

As seen in Figure 24, only the plasmidic DNA of colony two eventually had DNA, but not colonies four and five; this could be due to the presence of denaturalizated or broke DNA in the samples of the fourth and the fifth colonies, but not plasmidic DNA so electrophoresis does not show any band in these lanes. As expected, colonies one, three and six did not provide any band. Colony two showed three bands which migrated the same as positive control; heaviest band might be pSEVA651 (red arrow) and the other two might be pieces of this plasmid.

To confirm that the highlighted band was, indeed, the plasmid used in the electroporation, an enzymatic restriction assay was carried out with plasmidic DNA of pSEVA651 (positive control) and of colony two: HindIII was selected as an example of enzyme that cuts the plasmid once, linearizating it.

Figure 25: Agarose 0.8% (w/v) electrophoresis of linearizated plasmidic DNA. pSEVA651 (C+, positive control) and colony two (2) were digested with HindIII as an unique cutter of the plasmid to obtain linearizated forms of it. DNA ladder was used, but biggest brand was insufficiently big for the molecular weight of linearizated plasmid, so it did not appear in the gel as it escaped from it.

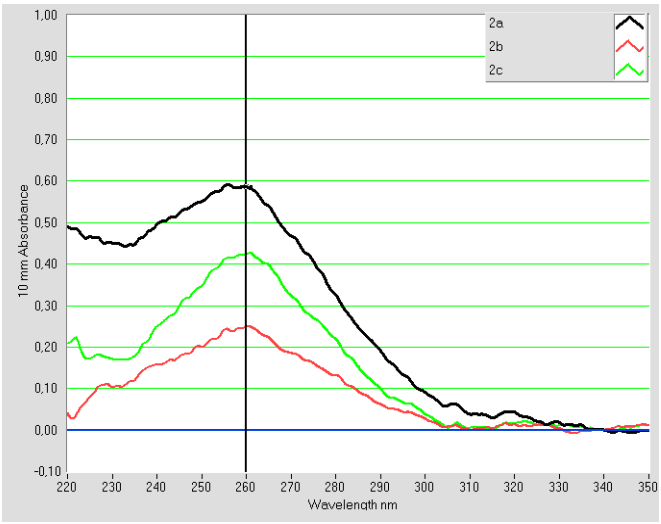
Colony two, derived from electroporated electrocompetent SP, was proved, as Figure 25 shows, that had been successfully transformed with pSEVA651 by electroporation: one out of six morphologically different electroporated colonies turned out to have incorporated pSEVA651.

Identification of electrotransformed colony

Sequencing of 16S rRNA was carried out with colony two (three procedures for the sample sample: 2A, 2B and 2C) in order to know what species it was.

Firstly, PCR (PCR #1) of extracted genomic DNA was carried out to amplify 16S rRNA; DNA was quantified by Nanodrop (Figure 26) and checked by agarose electrophoresis (Figure 28).

I.



II.

Rep.	A_{260} (au)	A_{280} (au)	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)
2A	0.487 ± 0.022	0.253 ± 0.012	1.93 ± 0.07	1.61 ± 0.06	24.37 ± 1.10
2B	0.227 ± 0.006	0.097 ± 0.014	2.39 ± 0.30	2.52 ± 0.57	11.34 ± 0.29
2C	0.481 ± 0.005	0.243 ± 0.014	1.98 ± 0.10	2.01 ± 0.03	24.04 ± 0.22

Figure 26: Genomic DNA extraction of the electrotransformed colony. Genomic DNA of electrotransformed colony two of solar panel sample was extracted following procedure described in Latorre *et al.* (1986)[98] in order to sequence it and identify the species; three procedures over colony two were done to ensure procedure and results; I. shows the average spectral patron of absorption and II. shows relevant measured magnitudes. Number of measures (n) = 3.

As seen in Figure 26, low concentrations[123] of genomic DNA were obtained; yields were not high either[124]. Nevertheless, A_{260}/A_{280} ratios were all good, informing of absence of protein contaminants; and A_{260}/A_{230} ratios were also good for 2B and 2B, informing of absence of other pollutants[121,122].

Amplification of genomic 16S rRNA by PCR (PCR #1) proved successful, proving as well the correct extraction of total genomic DNA, as seen in Figure 28. All three repetitions on the sample (colony two) had the same amplicon (red arrow): molecular weights, taking into account relative migration of DNA ladder's bands (Figure 28), are 548 bp, 591 bp and 528 bp (2A, 2B, 2C, respectively); mean ± standard deviation = 555 ± 32 bp.

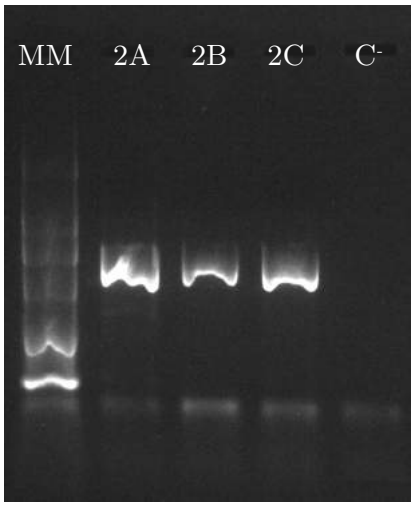


Figure 27: Agarose 1.5% (w/v) electrophoresis of amplified 16S rRNA of the electrotransformed colony. Extraction of total genomic DNA was carried out with electrotransformed colony two, three times (2A, 2B, 2C) and assessed to PCR to amplify 16S rRNA in order to know the species; "MM" corresponds to the DNA ladder, "2A", "2B", "2C" correspond to the samples 2A, B and C, and "C-" correspond to PCR negative control (absence of DNA).

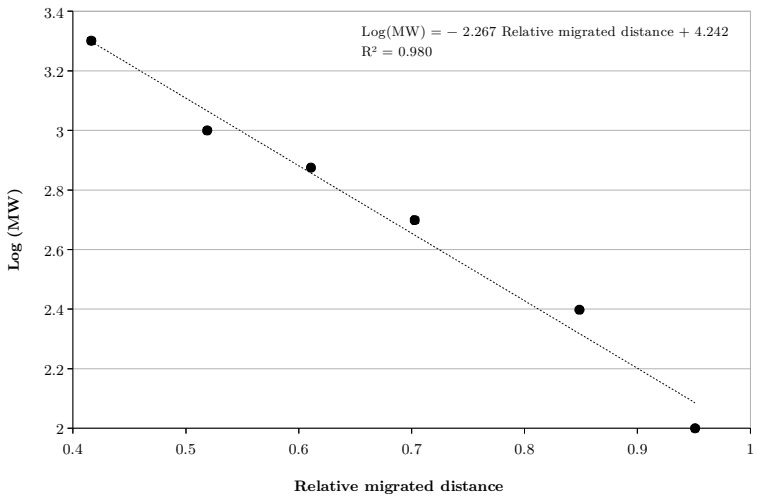


Figure 28: Lineal relation between decimal logarithm of molecular weight and relative migrated distance of DNA ladder's bands. Molecular weights of amplicons were calculated by interpolation on lineal regression curve of the relation between decimal logarithm of molecular weight and relative migrated distance (band migration/front migration) of DNA ladder's bands.

To finish, PCR (PCR #2) of the amplified 16S rRNA was done to obtain 16S rRNA sequences labeled with fluorescent nucleotides; these sequences were sequenced by Sanger sequencing at SCSIE[101]. With the obtained sequence, by BLASTn, colony two was identified as *Stenotrophomonas sp.* for the three replicates (% identity: 99%, E-value: 0; accession number: KT580638.1). Furthermore, obtained lengths of the sequences (2A, 2B and 2C) were 794 bp, 523 bp and 524 bp (614 ± 156 bp), respectively; these results, except that of 2A, are very similar to the calculated length of the amplicons of PCR #1.

This genera, firstly reported by Patronizer & Bradbury (1993)[127], is of great biotechnological relevance due to its varied applicability, mainly in environmental biotechnology and in introspection: heavy metal accumulation[128] like Cr(VI)[129], manganese[130] and chromato[131]; degradation of aromatic compounds such as dibenzothiophene[132] and chlorpyrifos[128], phosphorus pesticides like *DDT*[133,134], phenol[129] and p-nitrophenol[135], poly aromatic hidrocarbons[136],; and production of compounds of industrial interest like agarase[137].

Moreover, it is a Gram negative genera, which supports its electroporation as these bacterial group have a slimmer peptidoglycan layer than that of Gram-positive bacteria, augmenting frequency of potential electrotransformation[42,43].

As showed, this genera has metabolic and genetic flexibility, giving rise to lots of applications, and its proved transformation by electroporation may constitute an alternative of easy genetic modification, exploiting its practicability.

Conclusions

Wide-spread chassis, like *Escherichia coli*, are sometimes unappropriated for genetic engineering trails as they may have genetic characteristics, modules or constructs that are photogenically distant from the one artificially introduced; this reduces the potential of genetic modifications and applications.

The environment constitutes a source of varied and applicable microorganisms that may eventually emerge as new chassis as they offer new genetic resources that may accept modern genetic engineering, being more flexible with their applicability.

Here we showed electroporation, a 50-year-old technology, has been proved to be capable to transform a solar panel surface sample, a nature-derived biological sample, providing a new approach in the search of new and more diverse synthetic biology chassis. However, even possible, efficiency of

electrotransformation of this sample is still very low and this technique, as well as other supplementary or complementary, need to be developed so as to make this nature-derived sample usable. This improvement can involve sample modification, but this must avoid as much as possible in order to settle easy procedures and respect sample integrity and, thus, practicality. In this work, it was proved that carrying out an electrocompetence protocol may augment efficiency of electrotransfer, but this came with a decrease of biodiversity.

Despite the relevance of this work as an new approach in the development of synthetic biology, more research has to be carry out in order to set this scenario as a feasible alternative to commonly-used and sometimes insufficient chassis.

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