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Genetic Manipulation of an Electroactive Marine Microbial Community

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14. ABSTRACT

The objective of this project was to develop the tools to transform and genetically manipulate mixed microbial biofilms on conductive surfaces. Development of this capability would allow us to bypass the isolation of environmental microorganisms, and to engineer systems for microbial electrosynthesis and control over ennoblement in Naval contexts. I have made progress toward this on several fronts: further characterization of the electroactive Biocathode MCL biofilm, development of electrochemical flow cells, and testing and design of genetic elements in a mixed biofilm.

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NRL Memorandum report

Genetic manipulation of an electroactive marine microbial community

Lina Bird

NRL CBMSE, Code 6920

Abstract:

The difficulty in isolating many environmental bacteria makes it difficult to study a number of environmentally relevant metabolisms, as an isolated strain is typically a prerequisite to investigations using molecular genetic techniques. One way to circumvent this difficulty is to find ways of using tools for genetic manipulation in mixed communities rather than on single isolates. Such a capability also has applications in synthetic biology, where a mixed community can be engineered for a specific purpose, such as electrosynthesis or chemical sensing. This study move these goals forward by exploring tools to transform and genetically manipulate mixed microbial biofilms on conductive surfaces. Development of this capability would not only allow us to better understand environmental microorganisms and communities but potentially to engineer systems for control over ennoblement in Naval contexts, among other future applications. In the work described here, the model community for this study, Biocathode MCL, was further characterized to obtain baseline information for genetic manipulation, and testing and design of genetic elements in the mixed biofilm was completed. Finally, contributions were made toward testing biofilms in electrochemical flow cells, an important technology for screening genetically engineered electromicrobial communities.

Introduction:

Although microbiologists are able to cultivate a wide range of microorganisms in the laboratory, many environmentally relevant microorganisms remain uncultivable, possibly due to unique growth requirements or because they are adapted to live in communities. These recalcitrant organisms are often of relevance to the Navy, with applications in energy generation, corrosion, bioremediation, and microbial electronics. Techniques to genetically manipulate bacteria within a mixed microbial would allow us to bypass the isolation of environmental microorganisms, and to engineer systems for microbial electrosynthesis and control over ennoblement in Naval contexts. Once such mixed microbial biofilm is Biocathode MCL, a marine bacterial community with the ability to produce cathodic current when grown on an electrode. Previous work by Leonard Tender, Sarah Glaven, and others at NRL have characterized this community, showing that it consists of about 20 bacterial species, with 3 dominant groups: *Marinobacter, Chromatiaceae,* and *Labrenzia*, from which it gets the name MCL. The member of the *Chromatiaceae* family *Candidatus* "Tenderia electrophaga", is an electroautotroph thought to be the bulk of the current produced[1-3].

The Biocathode MCL, in addition to being of interest for electrosynthesis and bioelectronics, is an excellent model community for developing tools to work with biofilms. It has several advantages: 1) Stability, with the community composition changing very little over the years. 2) An existing biofilm can be used to inoculate a fresh reactor. 3) It grows and begins producing current relatively quickly. 4) It is will characterized, with the keystone organism

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known and sequenced. 5) Current production sensitive to several antibiotics, allowing for selection of genetic transformants of the keystone organism.

In the work described here, the antibiotic sensitivities are determined for ampicillin and kanamycin, a method for transforming members of the community with foreign DNA is described, and a design for a targeted construct is presented.

Methods:

Strains and plasmids

Strains and plasmids are listed in Table 1. *E. coli* donor strains were grown on LB broth (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) with di-aminopimelic acid at 10 mM to supply the DAP auxotrophy of the donor strains. Conjugation was performed on BB broth plates (17.5 g/L marine broth, 5 g/L NaCl, 2.5 g/L yeast extract, 5 g/L tryptone, 15 g/L bacto-agar).

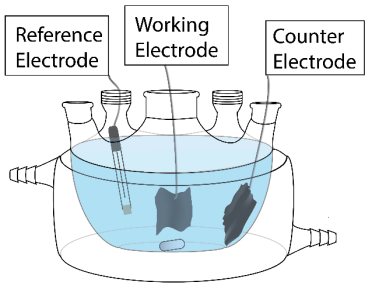

Figure 1: Electrochemical setup for growing Biocathode MCL. Diagram courtesy of Elizabeth Onderko.

Table 1	
Strains	
WM3064	*E. coli* strain with donor conjugation machinery and DAP auxotrophy for counter selection
Wang donor strain	*E. coli* strain with donor conjugation machinery, DAP auxotrophy for counter selection, and constitutive mCherry expression from a chromosomal insertion.
Biocathode MCL	A stable marine microbial community able to grow using a cathode as the sole added energy source. Contains a mix of organisms, with one autotroph, *Ca.* "Tenderia electrophaga"
Plasmids	
pBBr1-mcs2::GFP	A broad range vector with constitutive GFP and kanamycin resistance
pGT-Ah1,2,3,4	Plasmids from Ronda et al. with transposon carrying *gfp* and *amp* resistance.
pGT-B1, B2	Broad range vector (pBBR1 origin of replication) carrying constitutive *gfp* and *amp* resistance
pGT-S1, S2	Broad range vector (RSF1010 origin of replication) carrying constitutive *gfp* and *amp* resistance

Electrochemical reactor setup

Electrochemical reactors were set up as shown in Figure 1. This setup is known as the 3-electrode configuration, with a working electrode a silver-silver chloride reference electrode, and a counter electrode all in a single chamber. Both working and counter electrodes were made of graphite flags attached to titanium wires using titanium nuts and screws. The temperature is controlled via the water jacket surrounding the reactor, and the reactors is placed on a magnetic stirmplate to maintain a constant rate of stirring. Electrodes were attached to a potentiostat (Biologic) to maintain a constant potential while monitoring current for the duration of each experiment.

Construct design

Constructs were designed and visualized using the molecular biology suite of tools by Benchling.

Results:

Biofilm characterization

Initial work on this project related to further characterizing the electroactive biofilms targeted for transformation. To that end, I have revised and resubmitted a paper that included data on antibiotic sensitivity of the cathodic biofilm, which is vital information for genetic manipulation (Figure 1). This work showed that Biocathode MCL is sensitive to ampicillin and kanamycin, both commonly used as selective markers. Knowledge of antibiotic sensitivity allowed us to design experiments to transform genetic material into the biofilm using antibiotic markers.

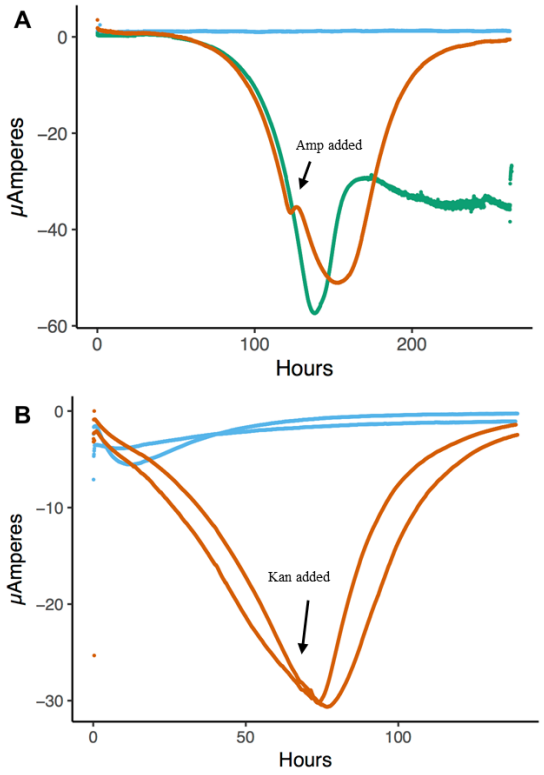

Figure 2: Effect of ampicillin (A, Amp) and kanamycin (B, Kan) on current production by Biocathode MCL. Blue, antibiotics added at inoculation; orange, antibiotics added at time point as marked; green, no antibiotics.

Plasmid and transposon integration in Biocathode MCL

Based on this knowledge of antibiotic resistance, the initial attempts to transform the biofilm were made with a plasmid carrying kanamycin resistance. The plasmid, pBBr1-mcs2::*gfp*, is known as a broad range vector able to replicate in a wide variety of bacteria, and carries both a GFP insert and the kanamycin resistance gene under constitutive promoters. These initial attempts were unsuccessful (Figure 3), with current dropping and not recovering when kanamycin was added post transformation. In Biocathode MCL, one organism, "*Candidatus* Tenderia electrophaga" is

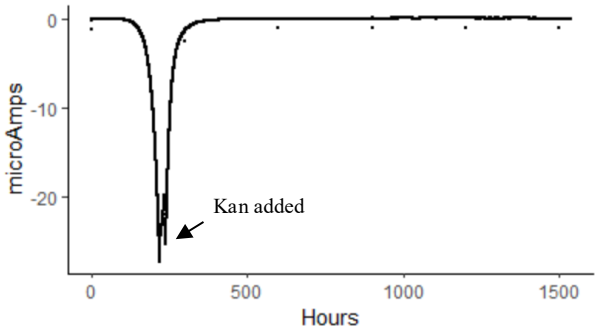

Figure 3: Conjugation of plasmid with kanamycin resistance, and addition of kanamycin post-conjugation.

thought to be responsible for the bulk of the current produced, so a failure to transform this one organism would lead to a lack kanamycin resistance as measured by current.

Following the unsuccessful use of the pbbr1-mcs2::*gfp* plasmid, I tested a set of plasmids and transposons from collaborators at Columbia University with a variety of promoters and origins of replication for successful transformation into Biocathode MCL. The mix contained plasmids in two categories: broad range vectors (the B and S series) expected to replicate and maintain themselves in the organisms within the biofilm, and transposons expected to insert into the genome of the target organisms (the Ah series)[4]. All of the plasmids carried ampicillin resistance, and were transferred to the target organisms via conjugation using an *E. coli* donor strain expressing mCherry fluorescent protein, in order to distinguish it from recipient organisms. Unlike kanamycin resistance, which requires each cell to have the resistance gene in order to grow, the ampicillin resistance enzyme degrades ampicillin in the medium. The experimental set up and initial results are shown in Figure 4. In this experiment, we saw indications that some members of the biofilm were becoming ampicillin resistant and protecting the cells around them. The fact that, post transfer, the biofilm was able to produce current in the presence of ampicillin showed that ampicillin was being cleared from the medium. The transformation was checked by microscopy; the plasmids, in addition to carrying ampicillin resistance, also expressed green fluorescent protein (GFP). The fluorescence allowed us to confirm the successful uptake of the plasmid by members of Biocathode MCL (Figure 4).

Imaging of the biofilm showed that that despite the salty environment and the genetic auxotrophy of the *E. coli* donor strain, many *E coli* cells remained attached to the MCL biofilm and metabolically active, as cells expressing both GFP and mCherry (which only the *E. coli* donor strain would do) were observed. However, after a single transfer, the only cells producing GFP were observed by microscopy, indicating that successful transformation of members of Biocathode MCL.

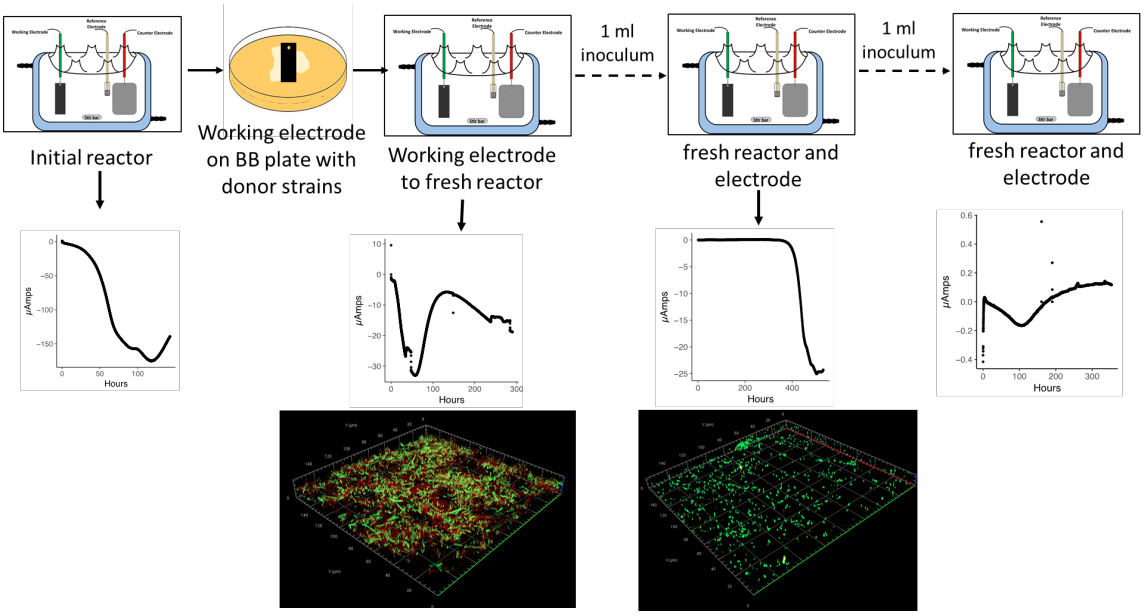

Figure 4: Conjugation of plasmid mixture with ampicillin resistance. Top: experimental flow. Middle: electrochemical traces showing current production of each transfer. Bottom: imaging of the electrochemical biofilm from select reactors.

Finally, the two categories of plasmids (wide range and transposon based) were tested separately. The initial results were similar for both the wide range vector and transposon groups (Figure 5). However, fluorescence microscopy imaging showed cells expressing GFP but not mCherry only in the transformation with the broad range vector group, not in the transposon group. Additionally, when transferred, only the culture transformed with the broad range plasmid was able to grow in the presence of ampicillin (Figure 6). Although we do not yet know which members of the community were successfully transformed, we have shown that conjugation with *E. coli* is a viable option for transforming members of an intact biofilm.

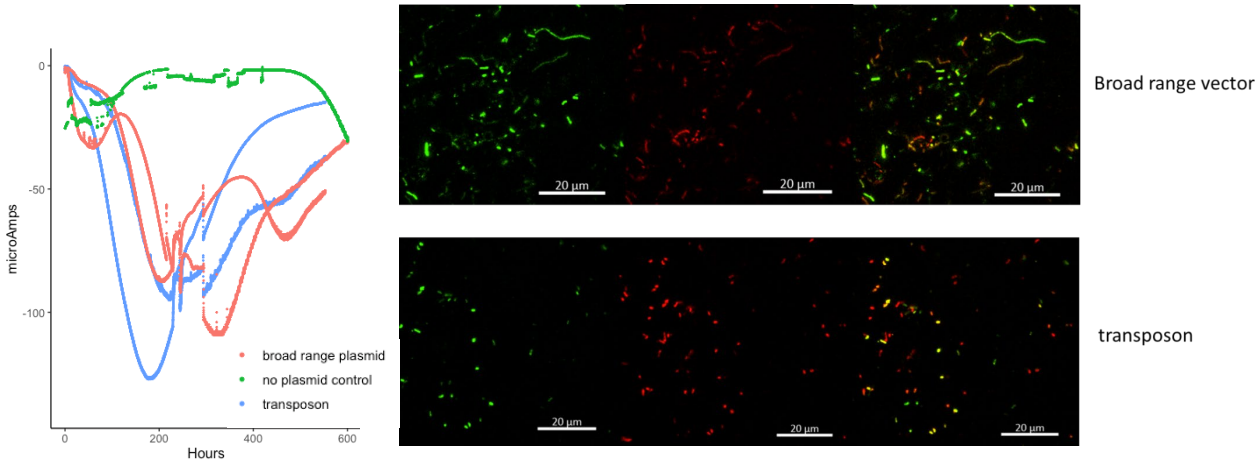

Figure 5: Left: current production of Biocathode MCL cultures grown in the presence of ampicillin after conjugation with a mix of broad range plasmids (red), transposing carrying plasmids (blue) and a no-plasmid control (green). Right: Fluorescence microscopy images of post conjugation cultures with the broad range plasmid (tope) and transposon containing plasmid (bottom). Red/yellow cells are the E. coli donor strain, while green cells are members of Biocathode MCL that have been successfully transformed.

The reason why the transposon based transformation was unsuccessful has not been fully determined; however, it may have been because the promoters used in the transposons were from a segmented filamentous bacteria, a bacterial group distantly related to the organisms in Biocathode MCL. It is possible that these promoters were not recognized in the transformed bacteria, leading to a failure of the transformation.

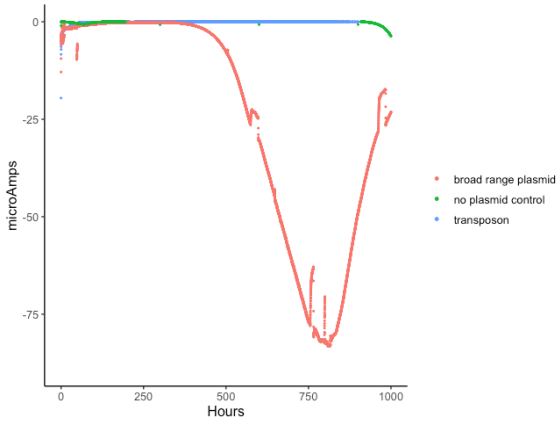

Figure 6: Current production of transformed Biocathode MCL cultures in the presence of ampicillin after transfer.

Design of targeted construct

With the successful transformation of members of the Biocathode MCL community, the next goal is to target that transformation to specific members of the community. I therefore designed a plasmid specifically for targeting *Ca* "Tenderia electrophaga". The construct, shown in Figure 7, uses homologous recombination, a technique for genome insertion that has been used in a wide variety of bacteria. The technique works through the transformation of a plasmid unable to replicate in a target organism, with a construct that contains sequences homologous to the genome of the target organism. The homology allow for recombination into the genome of the target organism, and only the target organism. In this way, a specific organism can be genetically manipulated, even if it is in a mixed community.

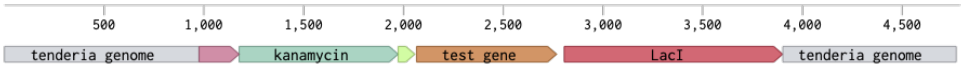

Figure 7: Construct design for insertion into the Ca "Tenderia electrophaga" genome. The kanamycin gene is under control of the rpoB promoter, the 100 bp region upstream of the rpoB gene, while the test gene (GFP initially) is under control of the inducible lac promoter.

Electrochemical flow cells for improved screening

A major component of building genetic tools for mixed biofilms is the screening of genetic modifications. To that end, I assisted colleagues in testing genetically modified biofilm forming strains in a commercially available electrochemical flow cell which reduced the volume needed to test strains, and can test 8 conditions in parallel. The results of this work was published in Bioelectrochemistry, as listed below.

Conclusions and future directions:

With the support of the Karle fellowship, I was able to make progress in engineering whole mixed community biofilms, currently an extremely challenging area of research. The Biocathode MCL has proven to be an excellent model for this type of work, due to its community makeup: the ability of *Ca.* "Tenderia electrophaga" to survive the presence of ampicillin so that other members of the community can clear it have made it possible to complete successful transformations of some members of the Biocathode MCL community, even if not all of them are successfully transformed. Its sensitivity to a non-clearable antibiotic (kanamycin) also allows assessment of whether the keystone organism (*Ca.* "Tenderia electrophaga")

In order to continue this work, we have secured a grant from ONR to use CRISPR tools for more directed engineering of mixed biofilms. This work is currently underway, and will replace the homologous recombination approach with a higher efficiency technique: CRISPR-guided insertion of genetic constructs. The higher efficiency with both increase the rate at which constructs can be created, and will increase the likelihood of success, as the efficiency is likely to be a large part of successful genome insertion.

Published Papers:

Yates, M.D., **Bird, L.J**., Eddie, B.J, Onderko, E.L., Voigt, C.A., and S.M. Glaven 2020. Nanoliter scale microbial electrochemistry towards high throughput characterization of engineered living electronic materials. *Bioelectrochemistry, Volume 137* https://doi.org/10.1016/j.bioelechem.2020.107644

Bird, L.J., Tender, L., Phillips, D., and Glaven, S. 2021. Survival and growth of a cathodic biofilm on unpoised electrodes. *Environmental Microbiology*. **In review**.

Presentations:

Bird, L. J., Phillips D., Tender L., and Glaven, S. Sustained Ennoblement by Electrotrophic Biofilms. *Tri-service Biocorrosion workshop, March 4, 2021*

Systems and synthetic biology for in situ biofilm engineering
Glaven S.M, **Bird, L.J**., Systems and synthetic biology for in situ biofilm engineering. *Tri-Service Biofilm workshop, March 26 2021*

Patents:

Lina J. Bird, applicant; Leonard M. Tender, applicant; Sarah M. Glaven "Use of conductive materials to drive bacterial carbon dioxide fixation through a passive, non-photosynthetic process to synthesize molecules without the addition of external energy"; Navy Case #111475, Provisional filed December 2019, IEB approved on May 22, 2020

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3. Eddie, B.J., et al., *'Candidatus Tenderia electrophaga', an uncultivated electroautotroph from a biocathode enrichment.* Int J Syst Evol Microbiol, 2016. **66**(6): p. 2178-2185.
4. Ronda, C., et al., *Metagenomic engineering of the mammalian gut microbiome in situ.* Nature Methods, 2019. **16**(2): p. 167-+.