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MITIGATION OF MEMBRANE BIOFOULING BY HARNESSING BACTERIAL CANNIBALISM

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Frank Huang
Snezna Rogelj

NEW MEXICO WATER RESOURCES RESEARCH INSTITUTE
New Mexico State University
MSC 3167
Box 30001
Las Cruces, New Mexico 88003-0001
Telephone (505) 646-4337 FAX (505) 646-6418
email: nmwrri@wrri.nmsu.edu

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By

Frank Huang, Associate Professor
Department of Civil & Environmental Engineering
New Mexico Tech

Snezna Rogelj, Professor
Department of Biology
New Mexico Tech

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New Mexico Tech

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ABSTRACT

In 2002, about 113 million barrels (bbls) of produced water were generated in New Mexico during crude oil and natural gas production. The majority of the produced water is re-injected back into the same geological formation to enhance recovery of fuel reserves while the remainder is disposed of via deep injection wells. The amount of water disposed of through the injection wells is vast. Even partial desalination of this water for use in industry, agriculture, and recreation would dramatically decrease pressures on freshwater aquifers and provide more water for beneficial needs. Membrane desalination, being a well-established and effective separation process, is used routinely to reclaim small quantities of produced water. Large-scale implementation of the membrane processes for desalinating produced water, however, is hampered by the recurring biofouling of the membranes and the associated high operating costs. Acid and alkaline/detergent cleaning of biofouling are generally found to be ineffective.

In this research, the applicability of bacterial cannibalism on biofouling control was studied. *Bacilli* and *Bdellovibrio bacteriovorus* have been shown to degrade biofilms and thus were chosen as the candidates to induce cannibalism or predation. Evaluation of the protease and DNase activities showed that the 36-hour conditioned media (CM) by *B. subtilis* and *Bdellovibrio bacteriovorus* exhibited a significant proteolytic and DNA-degrading activity, respectively. Consequently, the 36-hour CM could potentially be used to control biofouling. In order to assess the degree of degradation on pre-formed biofilms, *Pseudomonas fluorescens* biofilms were cultivated at the air/growth media interface and characterized using Scanning Electron Microscopy with a special sample preparation process. The pre-formed *Pseudomonas fluorescens* biofilms were then immersed either in control saline, or *B. subtilis* conditioned media either in the absence or the presence of the *living B. subtilis* cells, or *E. coli-Bdellovibrio* conditioned medium. The effectiveness of biofilm removal was gauged by staining the remaining biomass after treatment with crystal violet. From the results, the treatment of the pre-formed *P. fluorescens* biofilm with the *B. subtilis* or *Bdellovibrio* conditioned medium appeared to have reduced the *P. fluorescens* biofilm accumulation over time.

Keywords: biofouling, produced water, bacterial cannibalism, membrane desalination

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INTRODUCTION

In 1993, about 25 billion barrels (bbls) of produced water from crude oil production and approximately 300 million bbls from natural gas production were generated in the U.S.. A large portion of the produced water (65%) is re-injected into the same geological formation to enhance recovery of fuel reserves with the remaining water disposed of via deep injection wells.[1] The amount of water disposed through the injection wells is vast. Lea County, New Mexico, for example, disposes of roughly 70,000 acre-feet of oilfield-produced water (brine)/year, enough to sustain a city of 300,000 for a year, *if* it could be purified. Water produced from oil and gas operations is therefore increasingly seen as a potential asset, as opposed to an environmental liability. Even partial desalination of water for use in industry, agriculture, and recreation would dramatically decrease pressures on freshwater aquifers and provide more water for beneficial uses.

Membrane desalination, being a well-established and effective separation process, is used routinely to reclaim small quantities of produced water. Large-scale implementation of the membrane processes for desalinating produced water, however, is hampered by the recurring biofouling of the membranes and the associated high operating costs. Acid and alkaline/detergent cleaning of biofouling are generally found to be ineffective.[2] In this research, the applicability of bacterial cannibalism (used loosely to also designate inter-species predation) on biofouling control was studied. *Bacillus subtilis* and *Bdellovibrio bacteriovorus* were chosen as the potential bacteria to induce cannibalism or predation. *Bacillus subtilis*, an ubiquitous bacterium, has been shown to engage in cannibalism of surrounding bacteria when subjected to environmental stress in order to delay the spore formation.[3] Given that *Bacillus subtilis* also possesses the enzymes to degrade some of the most recalcitrant compounds found in nature, such as lignin, terpenes, and hemicellulose, it may be an ideal candidate for membrane biofouling control. *Bdellovibrio bacteriovorus*, a predatory bacterium, also exhibits the ability to control biofilm. [4] In this studes we extend these findings to investigate whether *Bdellovibrio bacteriovorus* and *B. subtilis* can exhibit the ability to control biofilm accumulation as elaborated by other bacterial species.

Specific aims of this research are to:

1) Establish bacterial release of degrading enzymes into conditioned media
2) Establish growth conditions for *Bdellovibrio bacteriovorus* in *Escherichia coli*

3) Establish *Pseudomonas fluorescens* biofilms
4) Measure the effect of bacterially conditioned media on *Pseudomonas fluorescens* pre-formed biofilm

RESEARCH RATIONALE

General Information

It is known that in the natural world, most bacteria (~99.5%) aggregate in biofilms where they behave very differently from their planktonic form.[5-10] In membrane purification processes, organics and other impurities are retained on the membrane surface by design, and thus this continuously available food source frequently leads to biofouling of the membrane. The formation of biofilm on surfaces can be modeled so as to proceed in the following stages (1) reversible surface attachment of planktonic bacteria, (2) irreversible attachment and microcolony formation, and (3) biofilm maturation.[11] Environmental factors, such as nutrient levels, pH, temperature, shear, and surfaces, can greatly influence numerous aspects of the biofilm formation.[12, 13] A mature biofilm normally consists of microbial cells embedded in a mass of extracellular polymeric substances (EPS).[14] EPS provide a highly hydrated gel matrix in which microbial cells can establish stable synergistic consortia.[15] Generally EPS are composed of polysaccharides, but are likely to also contain proteins, nucleic acids, and other polymeric lipophilic compounds.[16, 17]

Acid and alkaline/detergent cleaning have often been found to be ineffective in removing membrane biofouling. In studying the biofouling of a pilot nanofiltration system fed with conventionally treated Ohio River water, Speth and others observed only a short-term flux recovery after acid and alkaline-detergent treatments.[2] Enzyme treatment to mitigate biofouling has been explored in a limited number of studies with mixed success.[18] One of the major obstacles of using degradative enzymes for biofouling control is the lack of broad-spectrum enzymes for the diverse biofilms encountered in nature. If bacterial cannibalism can be induced with controlled environmental conditions, the process may offer a plausible solution to membrane biofouling by having living machines (*Bacilli* or *Bdellovibrio*) determine how the biofilms should be degraded.

Reasoning for Aim #1

Several bacterial species, including biofilm-forming and sporulating types, are known to elaborate and discharge into their surrounding media enzymes, which are capable of degrading biological materials. The multifold purposes of these processes are likely to include the release of individual bacteria from captivity in a biofilm or breakdown of biological macromolecules into fragments that can subsequently serve as a resource of nutrients. While the biochemical nature of the released degrading enzymes is generally complex and may vary from species to species and is subject to great changes determined by the specific environmental circumstances, it is expected that potent, non-specific proteases and DNase are a critical component of these released enzymes. To assess the possibility that the released enzymes would be able to degrade biofilms (may be composed of protein and DNA-containing extracellular polymeric substances), we needed to identify the bacterial strains as well as the culture conditions that trigger the protease and the DNase release into the conditioned media.

Reasoning for Aim #2

Bdellovibrio bacteriovorus are Gram-negative, motile uniflagellated bacteria that have an obligatory parasitic life cycle. *Bdellovibirios* attack other (exclusively) Gram-negative cells, penetrate into and multiply in their periplasm, and eventually burst out of the host cells that are killed in this process. These aerobic bacteria, which are found in wet soil and brackish or marine water, have recently been shown to attack bacteria within Gram-negative biofilms and decrease their biomass.[4] We therefore postulated that we might be able to use their native parasitic properties to mitigate biofilms accumulated on the filtration membranes.

Reasoning for Aim #3

Using a drip-flow system, we were able to grow thick *Pseudomonas fluorescens* (a Gram-negative bacterium) biofilms. Unfortunately, this process is complicated and very time consuming, yielding only a few biofilm samples per week. We therefore needed to develop a simpler, model-biofilm system that would yield many replica biofilm samples that could then be exposed to a variety of treatments and tracked as biomass through time. For that reason, we developed a method that permits for accumulation of a *Pseudomonas fluorescens* biofilm ring on the walls of a 96-well plastic plate. This biofilm ring accumulates at the air-water interface and can be used as a substrate for the potentially degradative treatments.

Reasoning for Aim #4

To measure the effect of bacterially conditioned media (which would contain the elaborated degradative enzymes), we grew biofilms in the 96-well format for two days and then exposed them to the putative biofilm-degrading conditions for varied time periods. At the end of the treatment period, the media and the planktonic bacteria within the well were removed, and the residual biofilm ring was stained with crystal violet, washed with water, and then the dyed biofilm was dissolved in ethanol, whose optical density, measured by the plate reader, was proportional to the of the residual mass biofilm. A decrease in mass of the *Pseudomonas fluorescens* biofilm would thus be seen as a decrease in the crystal violet stain in the well.

Materials and Methods

Bacterial strains: The bacterial strains used in this study were obtained either from ATCC [*Bdellovibrio bacteriovorus* 114 (ATCC 25631), *Bacillus licheniformis* (ATCC 14580 and 10716), *Staphylococcus epidermidis* (ATCC 35984), and *Escherichia coli* (ATCC 25922)] or from Wards [*Bacillus subtilis* (85W 0228)].

Bacterial culture: All cells were cultured in LB broth at 37 °C with vigorous shaking. *P. fluorescens* biofilms grow either as a ring at the media-plastic interface and adhere to the plastic or as a floating biofilm when supported by a floating surface. The floating biofilm (for SEM analysis) was prepared by floating ethanol-sterilized chips (pre-cut to 5x5mm) of Permanox cell culture slides (Nunc) on the surface of the LB media inoculated with *P. fluorescens* (starting OD_{600} ~0.05) and incubating overnight in the absence of movement at 37 °C. Thicker biofilms can be obtained by longer incubation.

Preparation of conditioned media: Freshly inoculated bacteria (starting OD_{600} ~0.05) were grown in LB for the indicated periods of time, the cells removed from the media by a 5-minute centrifugation at 12,000G, and the supernatant (conditiond media) collected for the subsequent analysis for the presence of DNase and protease activity.

Determination of the protease activity: 10µls of the BioRad Rad (Bio-Rad Laboratories, Hercules, CA) pre-stained protein molecular weight marker were mixed with 10µl of the

conditioned media and incubated at 37 °C for the indicated periods of time. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiled for 5 minutes. The samples were analyzed for the presence of the remaining protein bands on 12% SDS-PAGE gels. Since the protein substrate was already pre-labeled, there was no need to stain and destain the gels.

Determination of the DNase activity: One µl of the Bio-Rad (Bio-Rad Laboratories, Hercules, CA) KB DNA ladder was incubated in the presence of the conditioned media for the indicated periods of time. After addition of the DNA sample dye and EDTA (2mM final concentration), the samples were electrophoresed on 1% TBE agarose gels containing 0.1µg/ml ethidium bromide.

Crystal violet staining: Total mass of a biofilm in a well was determined by colorimetric assay using crystal violet stain. The biofilms in wells were washed twice with PBS and then stained with 0.1% crystal violet (Sigma, St. Louis, MO). The wells were washed trice with PBS, and then the stained biomass dissolved in an appropriate volume of 95% ethanol and were read on a plate reader at 495 nM after a 15-minute dye-extraction period.

SEM sample preparation: Biofilm samples were fixed in 1% Paraformaldehyde, 1% Gluteraldehyde in 1x PBS (phosphate-buffered saline) for 30 minutes, washed three times with 1x PBS for 10 minutes each, and then treated with 1% OsO4 (Electron Microscopy Sciences, Hatfield, PA) in 1x PBS for 30 minutes. After washing three times with nanowater for 10 minutes each, the samples were stained with 3.5% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) in nanowater for 15 minutes, then washed twice in nanowater for 5 minutes each. Samples were then dried in ethanol using increasing steps of 70%, 80%, 90%, 95%, and 100% ethanol for 5 minutes each. The samples were then treated with hexamethyldisilizane (HMDS; Electron Microscopy Sciences, Hatfield, PA) by submersing the samples in a 1:1 mixture of 100% Ethanol:HMDS for 5 minutes, and then 100% HMDS for 5 minutes. HDTMS was removed, and the biofilm samples were allowed to dry slowly. The samples were subsequently gold sputter coated and SEM viewed using SEM JEOL 6100.

RESULTS AND DISCUSSION

Protease Activity

Several strains of bacteria, *Bdellovibrio bacteriovorus, Bacillus subtilis*, *Bacillus licheniformis, Escherichia coli, Pseudomonas fluorescens* and *Staphylococcus epidermidis*, were grown in LB medium, which was then tested, at different times after inoculation and growth saturation, for elaboration of non-specific protease and DNase activities in their conditioned medium. We reasoned long-term active bacterial growth would result in depletion of the nutrients, which would then lead to cannibalistic behavior that is characterized by the release of degradative enzymes into the nutrition-depleted medium. Indeed, as expected and as shown in Figure 1, we have found protease activity in the 36-hour conditioned medium which by that time would have been depleted of nutrients. Interestingly and also as expected, the protease was only found in the cultures of *B. subtilis*, and *Bacillus licheniformis* (Figure 1, Lanes 2, 5 and 8) , the two microbial strains known to indulge in cannibalistic behavior prior to their sporulation.

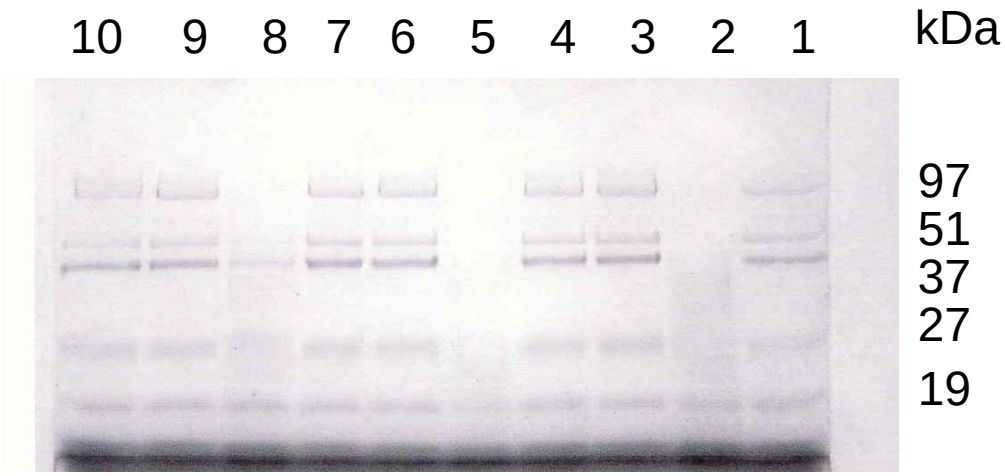


FIGURE 1. *Expression of protease activity in medium conditioned for 36 hours by a variety of different strains of bacteria*. Lane 1: LB only; Lane 2: *B. licheniformis* (ATCC 14580); Lane 3: *B. licheniformis* (ATCC 10716); Lane 4: *B. licheniformis* (ATCC 10716), duplicate of Lane 3; Lane 5: *B. licheniformis* (ATCC 14580), same as in Lane 2; Lane 6: *S. epidermidis*; Lane 7: *E. coli*; Lane 8: *B. subtilis*; Lane 9: *P. fluorescens*; Lane 10: *B. bacteriovorus*. Pre-stained molecular weight markers were subjected to 30-minute incubation with conditioned media (CM) and electrophoresed on the SDSP-PAGE gel. Sizes of the protein molecular weight markers are shown on the right. The disappearance of the bands indicates the presence of protein-degrading activity.

Expression of this proteolytic activity was tracked as a function of time. As shown in Figure 2, the protease activity, undetectable at 14 hours, begins to be accumulated by *Bacillus licheniformis* at 22 hours and then increases thru 36 hours of incubation.

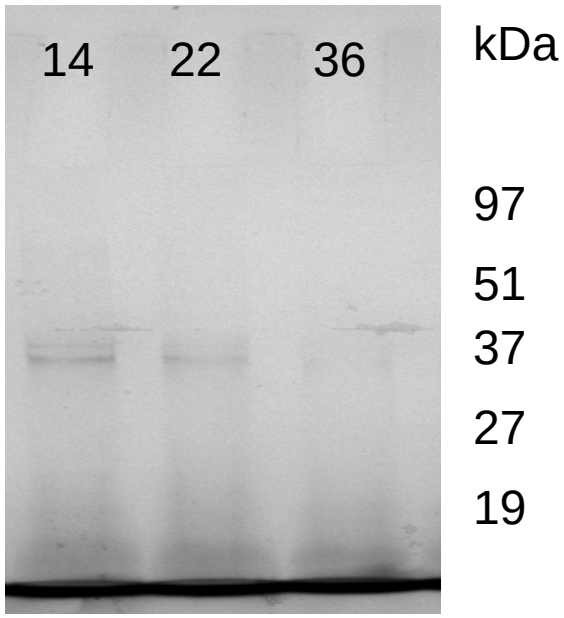


FIGURE 2. *Expression of protease activity in* Bacillus licheniformis *as a function of time*. Pre-stained molecular weight markers were subjected to 30-minute incubation with CM collected at the indicated times after inoculation and electrophoresed on the SDS-PAGE gel. The disappearance of the bands (especially evident at 22 and 36 hours) indicates the presence of protein-degrading activity. Molecular weights of the pre-stained protein markers used as a substrate are indicated on the right.

DNase Activity

We tested for the presence of released DNase in the conditioned media of the above listed bacterial strains. As shown in Figure 3, none of the tested strains released significant amounts of DNase after 14 hours of active growth.

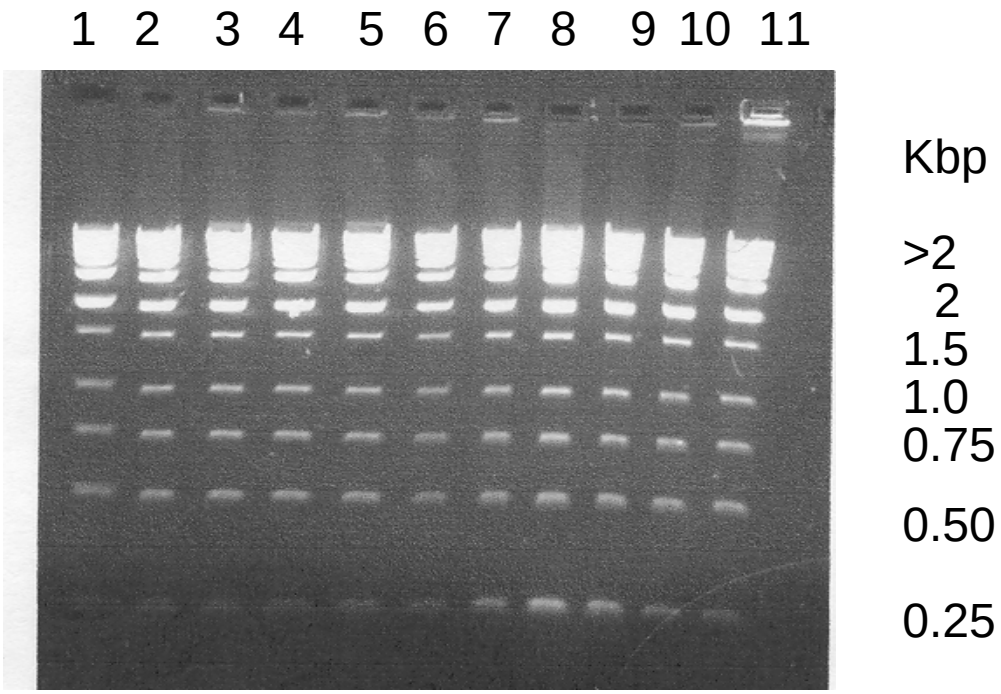


FIGURE 3. *DNase activity is absent in bacterially conditioned medium at 14 hours.* The bands represent DNA molecular weight markers that were used as a substrate to detect DNase activity. Lane 1: Substrate DNA only; Lane 2: DNA plus LB control (no bacteria); Lane 3: DNA plus CM from *B. licheniformis* (ATCC 14580); Lane 4: DNA plus CM from *B. licheniformis* (ATCC 10716); Lane 5: DNA plus CM from *B. licheniformis* (ATCC 10716), same as in Lane 4; Lane 6: DNA plus CM from *B. licheniformis* (ATCC 14580), same as Lane 3; Lane 7: DNA plus CM from *S. epidermidis*; Lane 8: DNA plus CM from *E. coli*; Lane 9: DNA plus CM from *B. subtilis*; Lane 10: DNA plus CM from *P. fluorescens*; Lane 11: DNA plus CM from *B. bacteriovorus*.

With one exception, these bacterial strains do not produce any DNase even at later times (data not shown). However, *Bdellovibrio bacteriovorus*, grown in the *E. coli* host, produced significant amounts of potent DNase by 36 hours (Fig. 4).

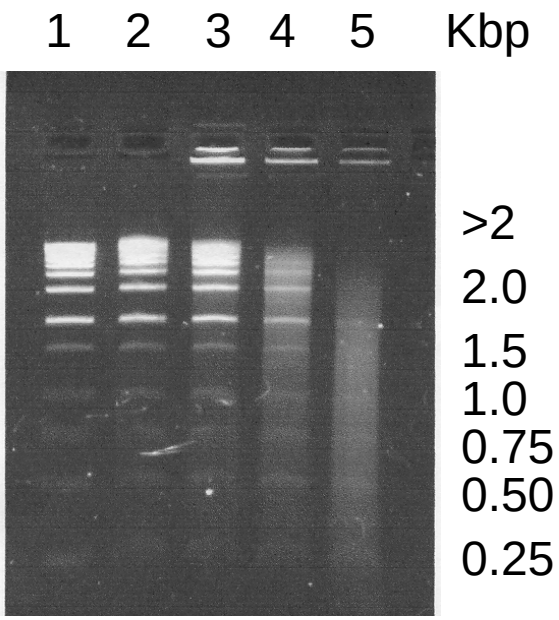


FIGURE 4. *Time course: DNase activity is elaborated by* Bdellovibrio bacteriovorus*, grown in* E. coli *host and released into conditioned medium as a function of time.* The time course of DNase expression of these bacteria shows that accumulation already begins at 22 hours and increases thereafter. Lane 1: Substrate DNA only; Lane 2: DNA plus LB control (no bacteria); Lane 3: DNA plus CM from *B. licheniformis* (ATCC 14580) grown in *E. coli* at 14 hours; Lane 4: DNA plus CM from *B. licheniformis* (ATCC 14580) grown in *E. coli* at 22 hours; Lane 5: DNA plus CM from *B. licheniformis* (ATCC 14580) grown in *E. coli* at 36 hours. The size of the molecular weight markers (in kilo base-pairs) is shown on the right.

We therefore concluded that it is the 36-hour conditioned media (CM) by *B. subtilis* and *Bdellovibrio bacteriovorus* that might be able to degrade the *Pseudomonas fluorescens* pre-formed biofilm.

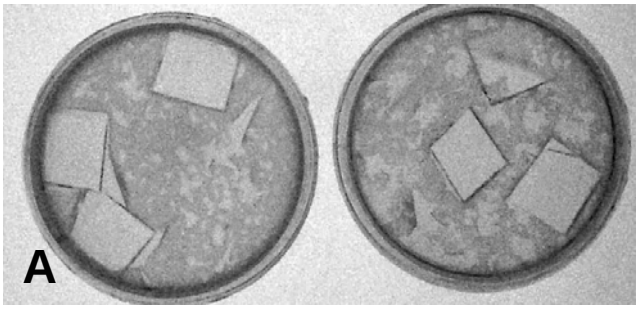
Bdellovibrio bacteriovorus

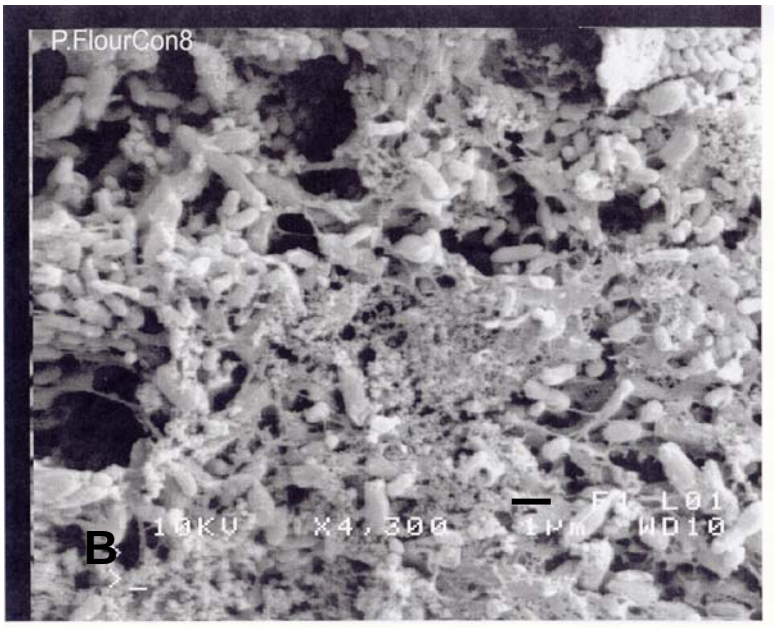
Bdellovibrio bacteriovorus is an obligatory parasite, thus the growth conditions for this bacterium in its *E. coli* host had to be established first. Rapidly grown *E. coli* were infected with *Bdellovibrio* at different ratios and co-cultured at 37 ºC. Unfortunately, there is no straightforward biochemical manner with which the growth of the two bacteria can individually be determined, so the success of the *Bdellovibrio* growth rate was assessed visually under a microscope by their differential morphology. *Bdellovibrio* is a rapidly motile rod, while *E. coli*

is larger and less rapidly moving. The increase in the extracellular *Bdellovibrio* population showed a healthy, rapidly expanding co-culture.

Pseudomonas fluorescens

To develop a rapidly forming *Pseudomonas fluorescens* biofilm that would serve as the cell and EPS-containing target for degrading activity, we grew these bacteria planktonically in a culture plate. Placement of floating plastic initiated formation of a biofilm at the air-media interface. Figure 5A is a picture of the biofilm as it floats on the air-media interface (grey material between the floating plastic disks). Figures 5B and C show this healthy biofilm with many resident bacteria, attached to each other with abundant EPS (B), and forming a series of water-filled channels (C). Careful sample preparation techniques were used to preserve the original microbial morphology.





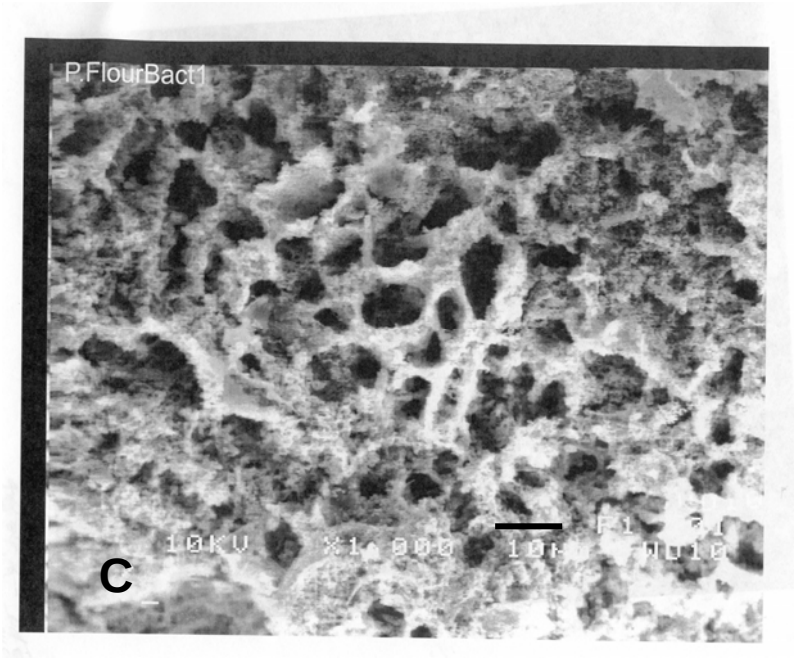


FIGURE 5. P. fluorescens *biofilm*. A) Growth of the biofilm in petri dishes at the air-liquid interface as initiated and supported by floating Permanox plastic squares. B) Individual bacteria, as well as abundant EPS, are readily visible on this SEM micrograph. The bar represents 1 μm. C) Large numbers of bacteria aggregate and form channels that permit nutrient transfer. The bar represents 10 μm on this SEM micrograph.

Analogous biofilm, a ring around the wells at the air-media interface, forms when bacteria are grown in 96-well plates. Because this biofilm adheres firmly to the walls of the well, it is difficult to be removed for the preparation of SEM samples. However, this 96-well format is ideal for quantifying the accumulated or retained biofilm mass as it lends itself to selective removal of the planktonic bacteria through washing and the dissolution of the retained dye in ethanol. The optical density of the dissolved crystal violet is proportional to the mass of the biofilm and permits for the straightforward quantification of the biofilm in the well[19]. In addition, the large number of the wells allows for inclusion of many replicas into an experiment.

Biofilm Degradation

Having developed a satisfactory model substrate biofilm and defined conditions under which the bacteria release degradative enzymes, we exposed the biofilms, accumulated on the sides of the wells in a 96-well dish (each containing only 100 μl of fluid), to a variety of potential biofilm-degrading conditions. The grown biofilm ring was immersed in one of the following:

control saline, *B. subtilis* conditioned media either in the absence or the presence of the living *B. subtilis* cells, or *E. coli-Bdellovibrio* conditioned medium that still contains living *E. coli and Bdellovibrio* cells. Figure 6 shows that the three experimental conditions, on average, decreased the growth rate of the biofilm—or resulted in biofilm degradation—as compared to the saline control. We believe, in retrospect, that the difficulties in obtaining good replicas were due to uneven evaporation of our samples and the subsequent failure of the biofilm ring to be submersed under the enzymes in the replaced (conditioned) media.

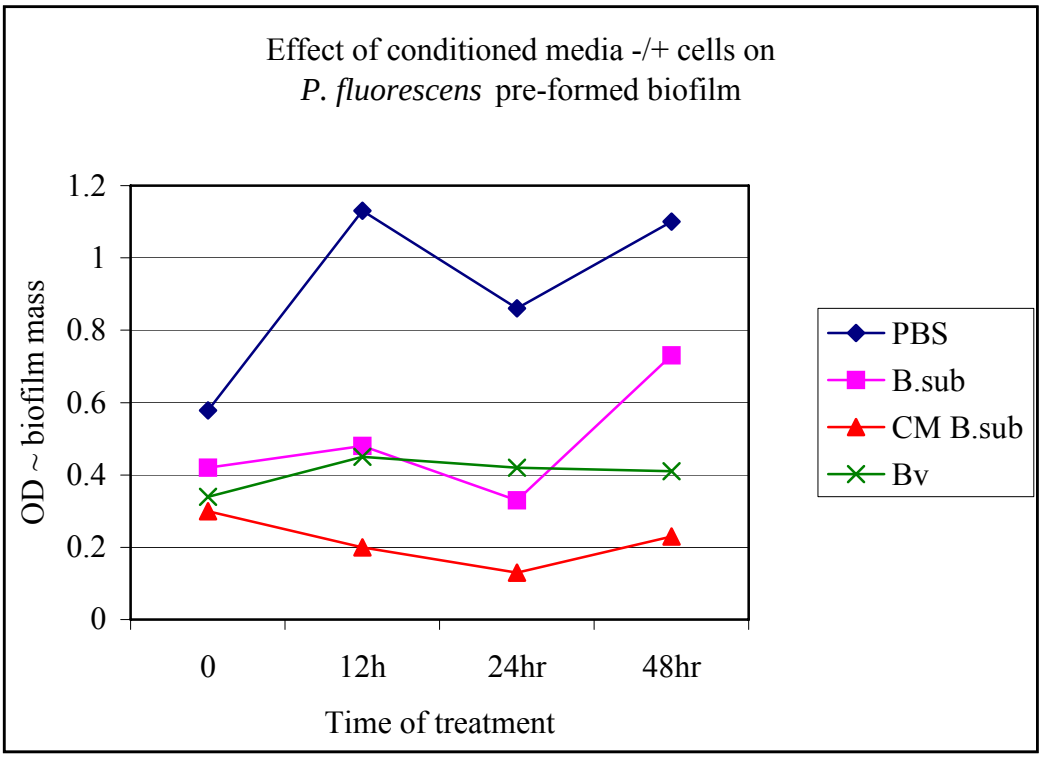


FIGURE 6. *Effect of bacterially conditioned media on* P. fluorescens *biofilm growth as a function of time.* PBS = phosphate buffered saline; B. sub = *B. subtilis* conditioned media with the bacteria still present; CM B. sub = *B. subtilis* conditioned media devoid of bacteria, and Bv is media that was conditioned – and still contains – *E. coli* and *Bdellovibiro*. Averages of three experimental points are shown.

Essentially analogous results were obtained when media, in which sporulation was induced by limitation of nutrients, were added to the actively growing *P. fluorescens* biofilm. In this experiment, 20% of the fluid in the wells with a pre-grown biofilm was replaced by either the control media, or *B. subtilis* conditioned media before sporulation (including living cells) or after sporulation (cells included), or cultures of *E. coli* with *Bdellovibrio* conditioned

medium. Figure 7 shows that these experimental conditions did indeed, on average, decrease the growth rate of the biofilm as compared to the control. Unfortunately, the replicates were again rather poor, possibly due to insufficient liquid coverage of the pre-formed biofilm with the new media.

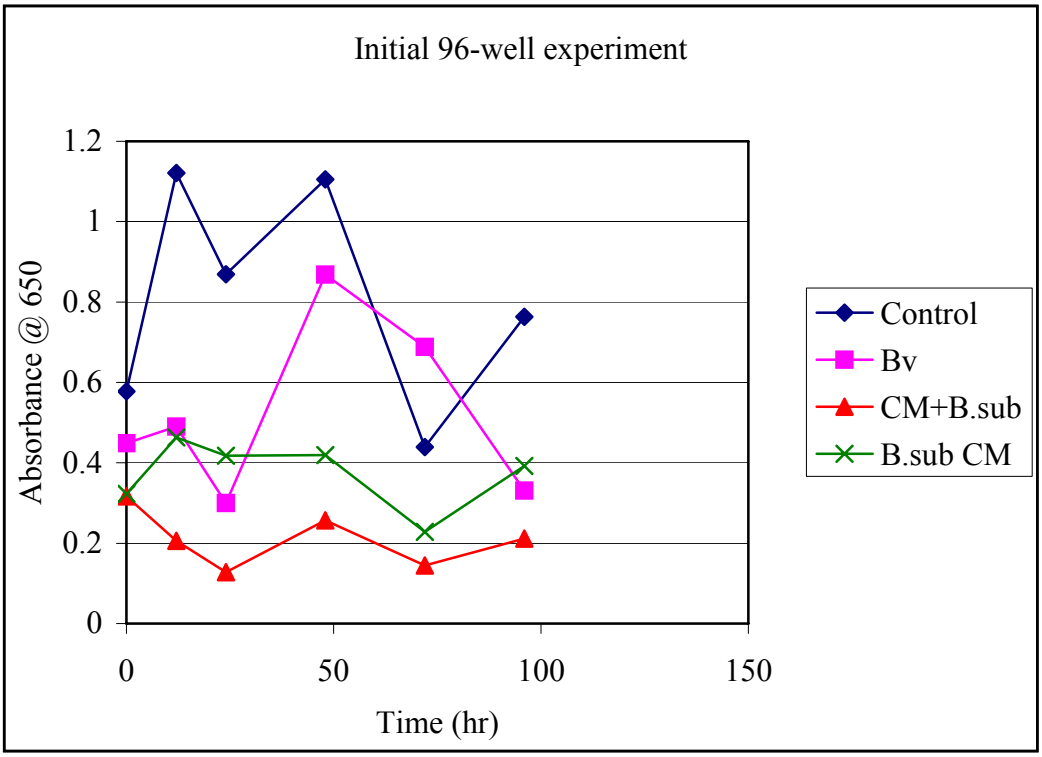


FIGURE 7. *Effect of bacterially conditioned media on* P. fluorescens *biofilm growth as a function of time.* PBS = phosphate buffered saline; Pre-sporBsCM = pre-sporulated *B. subtillis* conditioned media with the bacteria still present; Post-sporBsCM = post-sporulated *B. subtillis* conditioned media with the bacteria still present; and Bv = media that was conditioned – and still contains – *E. coli* and *Bdellovibiro*. Averages of three experimental points are shown.

CONCLUSION

In summary, we have found that the treatment of the pre-formed *P. fluorescens* biofilm with the *B. subtilis* or *Bdellovibrio* conditioned medium leads to either a decrease in *P. fluorescens* biofilm accumulation, degradation of the pre-formed biofilm, or a combination of these two processes.

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