Establishment and characterization of dual-species biofilms formed from a 3,5-dinitrobenzoic-degrading strain and bacteria with high biofilm-forming capabilities

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Received 18 April 2007; accepted 6 August 2007.
First published online December 2007.
DOI:10.1111/j.1574-6968.2007.00913.x

Editor: Gilbert Shama

Keywords
biodegradation; dual-species biofilms; nitrobenzoic acid; immobilization; bioaugmentation.

Abstract
In this study, the possibility of establishing a dual-species biofilm from a bacterium with a high biofilm-forming capability and a 3,5-dinitrobenzoic acid (3,5-DNBA)-degrading bacterium, Comamonas testosteroni A3, was investigated. Our results showed that the combinations of strain A3 with each of five strains with a high biofilm-forming capability (Pseudomonas sp. M8, Pseudomonas putida M9, Bacillus cereus M19, Pseudomonas plecoglossicida M21 and Aeromonas hydrophila M22) presented different levels of enhancement regarding biofilm-forming capability. Among these culture combinations, the 24-h dual-species biofilms established by C. testosteroni A3 with P. putida M9 and A. hydrophila M22 showed the strongest resistance to 3,5-DNBA shock loading, as demonstrated by six successive replacements with DMM2 synthetic wastewater. The degradation rates of 3,5-DNBA by these two culture combinations reached 63.3–91.6% and 70.7–89.4%, respectively, within 6 h of every replacement. Using the gfp-tagged strain M22 and confocal laser scanning microscopy, the immobilization of A3 cells in the dual-species biofilm was confirmed. We thus demonstrated that, during wastewater treatment processes, it is possible to immobilize degrader bacteria with bacteria with a high biofilm-forming capability and to enable them to develop into the mixed microbial flora. This may be a simple and economical method that represents a novel strategy for effective bioaugmentation.

Introduction
Nitrated aromatic compounds are considered to be toxic and recalcitrant chemicals. There are very few microorganisms capable of degrading these pollutants in the natural environment. Bioaugmentation with degrading microorganisms in the wastewater treatment system has been shown to enhance the degradation and removal of these pollutants (Boon et al., 2000; Yu & Mohn, 2001; Farrell & Quilty, 2002; Said & Spiros, 2005). However, this bioaugmentation process does not always work well (Bokhamy et al., 1997). There are a number of reasons for this, including the fact that the added bacteria may be washed out from the system or grazed by protozoa. Thus, the persistence of the added bacterium in the system for a long time is a prerequisite for maintaining its degrading capability. The immobilization of microorganisms has been suggested as a strategy for maintaining efficient degradation in bioaugmentation systems, and many ways of immobilizing degrading bacteria have been developed. For example, Quan et al. (2003) reported that 2,4-dichlorophenol-degrading mixed culture could be immobilized in polyvinyl alcohol jel beads and added to sequencing batch reactors to treat wastewater containing 2,4-dichlorophenol (2,4-DCP). Mohan et al. (2005) studied an application of a bioaugmentation strategy in an anaerobic sequencing batch biofilm reactor that involved inoculation with enriched sulphate-reducing bacteria in an alginate-immobilized matrix for the enhanced treatment of sulphate-bearing chemical wastewater. Gentili et al. (2006) examined the potential of chitin and chitosan flakes obtained from shrimp waste as a carrier material for a hydrocarbon-degrading bacterial strain and tested the potential of an immobilized hydrocarbon-degrading bacterial strain for crude-oil-polluted seawater bioremediation in...
seawater microcosms. However, these methods may be complex or expensive.

Biofilm reactors are especially well suited to the treatment of industrial wastewater containing slowly biodegradable or toxic compounds owing to their low sensitivity to toxicity and environmental stresses (Armenante, 1998; Morton et al., 1998). Some degrading bacteria were once immobilized or incorporated into the existing biofilm and used to augment the system containing persistent compounds containing wastewater (Coughlin et al., 2003). However, many degrading bacterial strains are often weak in terms of biofilm-forming capacity on the surface of vectors.

Bacteria with strong biofilm-forming capabilities have attracted much attention in medical and industrial enterprises (David et al., 1998; O’Toole et al., 2000). It has been found that some of these bacteria coaggregate with other microorganisms to develop biofilms, and that these biofilms are capable of holding other microorganisms (EI-Azizi et al., 2004; Alex et al., 2002; Rickard et al., 2004). However, little is known about the relationships between the bacteria with high biofilm-forming capabilities and specific degrading bacteria. The objective of this study is to investigate whether a mixture inoculation of bacteria with high biofilm-forming capabilities and degrading bacteria can result in the formation of biofilms and promote the immobilization of degrading bacteria in the biofilms. In this study, the co-inoculation of a 3,5-dinitrobenzoic acid-degrading strain and each of five strains with high biofilm-forming capabilities was applied, and the dual-species biofilms that developed were subjected to comparison in terms of biofilm-forming capacity on the surface of vectors.

Materials and methods

Bacterial strains and media

Comamonas testosteroni A3 (Li et al., 2007b) was isolated from a laboratory-scale nitrobenzene-compound wastewater treatment system and found to be able to mineralize 3,5-dinitrobenzoic acid (3,5-DNBA). The mineralization of 3,5-DNBA by the A3 strain was demonstrated in batch culture by measuring the cell growth yields and total organic carbon (TOC) in a medium with 3,5-DNBA as the only carbon and energy source. Pseudomonas sp. M8, Pseudomonas putida M9, Bacillus cereus M19, Pseudomonas plecoglossicida M21 and Aeromonas hydrophila M22 (Li et al., 2007a) were isolated and screened from the biofilms of the above wastewater treatment system and small carpolites in soil, and found to have higher biofilm-forming capacities than strain A3 and 12 other isolates from the same sample. They are thus referred to as biofilm-forming strains. The biofilm-forming strains cannot degrade 3,5-DNBA.

3,5-dinitrobenzoic acid mineral medium (DCMM) consists of 3,5-DNBA, 200 mg L⁻¹; NH₄NO₃, 400 mg L⁻¹; KH₂PO₄, 100 mg L⁻¹; K₂HPO₄, 30 mg L⁻¹; MgSO₄, 200 mg L⁻¹; NaCl 30 g L⁻¹. DCMM supplemented with 2% (w/v) Luria-Bertani (LB) broth was named DM2M.

Construction of a gfp-tagged strain of A. hydrophila M22

Aeromonas hydrophila M22 was selected to accept a GFP plasmid using a method described in Kerry et al. (2004). The GFP expression plasmid pTRGFP (Amp⁴, Tc⁴) is a broad host vector plasmid and was constructed in our laboratory (Qiu et al., 2004). pTRGFP in Escherichia coli DH5α was introduced into M22 (spe¹) by triparental mating, in which the plasmid transfer function was provided in trans by E. coli HB101 (Km², triparental mating helper strain containing pRK2013). The resultant mating cells were screened on LB agar containing spectinomycin (100 mg L⁻¹) to exclude E. coli, and ampicillin (100 mg L⁻¹) and tetracycline (20 mg L⁻¹) to select for the pTRGFP-harbouring cells of M22. The obtained isolate was referred to as A. hydrophila M22/GFP.

Growth-interaction assay

The growth interaction between strain A3 and five biofilm-forming strains was examined using a method described in Al-Bakri et al. (2004).

In order to determine the growth-inhibition effect of 3,5-DNBA on the biofilm-forming strains, M8, M9, M19, M21 and M22, grown individually on LB agar plates at 30 °C for 24 h, were harvested and then resuspended in sterile phosphate-buffered saline (PBS) to an optical density of 1.0 at 600 nm (OD₆₀₀nm). Then, 500-µL volumes of these suspensions were inoculated into 50 mL of DCMM2 containing 0 mg L⁻¹, 200 mg L⁻¹ or 400 mg L⁻¹ of 3,5-DNBA, and cultured at 30 °C for 24 h. Bacterial growth was monitored by reading the absorbance at a wavelength of 600 nm.

Biofilm formation assay

Biofilm culture was performed as described previously by Bechet & Blondeau (2003) and Tait & Sutherland (2002). The culture system comprised new 150-mL Erlenmeyer flasks containing 50 mL of DCMM2 synthetic wastewater, two pieces of microscope glass slides (24.5 × 76.2 × 0.8 mm), and five pieces of polyvinylchloride (PVC) carrier (100 × 12 × 0.1 mm). Flasks were inoculated in triplicate to obtain c. 10⁷ cells mL⁻¹, consisting of one species or a 1:1 mixture of two species. The cultures were then incubated at 30 °C with shaking at 100 r.p.m.
The biofilm biomass was determined through a method modified from Zhu & Mekalanos (2003). Briefly, after 24 h of growth, the DCMM2 synthetic wastewater containing suspended cells was removed and the flakes rinsed twice with distilled water, and the remaining attached biomasses were stained for 30 min with 50 mL 0.1 % (w/v) crystal violet in water. The flakes were washed thoroughly with water and dried overnight. The retained crystal violet was dissolved in 10 mL of ethanol-acetone (80:20 v/v), and absorbance (OD_{570 nm}) was measured at 570 nm.

**Biofilm shock assay**

Biofilms, after being established for 24 h, were shocked by replacement of the medium with the fresh DCMM2 synthetic wastewater medium every 6 h, and then the ability of the biofilm to degrade 3,5-DNBA in the presence of continuously changing DCMM2 synthetic wastewater was determined.

**3,5-Nitrobenzoic acid determination**

The determination of 3,5-dinitrobenzoic acid content was conducted by first centrifuging the culturing solution for 2 min at 5500 g and then obtaining the absorbance of the supernatant with a UV/VIS spectrophotometer with a spectrophotometer (UV-2201, SHIMADZU, Japan) at 241 nm. The concentration of 3,5-nitrobenzoic acid was finally calculated based on a standard curve.

**Viable cell counts for the biofilm**

Viable counts of the cells attached to the glass slides were estimated as follows. After removal of the glass slide from the flask and gentle washing in three changes of sterile PBS, the glass slide was placed in a sterile flask containing 10 mL of sterile PBS and vortexed for 10 min to remove the adhered cells. The serial dilution suspension was plated onto DCMM agar for A3 CFU and onto LB agar for total bacterial CFU.

**Visualization of biofilm structure**

In order to survey the distributions of both biofilm-forming and degrading strains in the biofilm, the dual-species biofilm of strain A3 with strain M22/GFP on the glass slide was developed as described above.

The biofilm stain method is based on the work of Nancharaiah et al. (2005) and Lucy et al. (1998). The dual-species biofilms were stained with 100 μL of 15 μM red fluorescent nucleic acid stain SYTO 60 (Sigma) for 15 min. The biofilms were then rinsed with PBS for 30 min to remove unbound stains in order to decrease background fluorescence.

The structure of the dual-species biofilms was observed using the method described in Thurnheer et al. (2004). Biofilms were examined using a DM IRB E inverted microscope (Leica Mikroskopie, Wetzlar, Germany). Filters were set to 450–500 nm for the detection of cells expressing GFP, and to 610–660 nm for cells stained with SYTO 60. Confocal images were obtained using 100 × (numeric aperture 1.4) oil immersion objectives. Each biofilm was scanned at randomly selected positions. Z-series were generated by vertical optical sectioning at every position with the thickness of the slices set to 0.5 μm.

**Results**

Preliminary investigations into the interactions between A3 and each of the strains M8, M9, M19, M21 and M22 demonstrated no growth-inhibition effects (data not shown). Hence, these five A3-pairing strains with high biofilm-forming capability were selected for further study. The five biofilm-forming strains were cultured in DCMM2 synthetic wastewater containing 0, 200 or 400 mg L\(^{-1}\) of 3,5-DNBA at 30 °C for 24 h, and the absorbance (OD\(_{600\text{nm}}\)) of all uncentrifuged media was in the range 1.18–1.31. No differences were observed in the biomass production of each of the five strains at different concentrations of 3,5-DNBA synthetic wastewater (P > 0.05), indicating that 200 mg L\(^{-1}\) and even 400 mg L\(^{-1}\) of 3,5-DNBA had no inhibitory effect on the growth of these five biofilm-forming strains.

**Biofilm-forming capability**

The biofilm biomass of A3 and its five pairing biofilm-forming bacteria, as well as the co-cultures of A3 and each of the biofilm-forming strains were investigated after culturing in glass flasks for 24 h at a shaking speed of 100 r.p.m. The results showed that the biofilm biomass formed by A3 or each of the five biofilm-forming strains was much higher than that of each of the single cultures (Fig. 1). The

**Fig. 1.** Biofilm formation in single and mixed cultures of the five biofilm-forming bacteria and degrading strain A3. Biofilms were assayed after incubation for 24 h at 30 °C in DMM2 synthetic wastewater. The bars represent ± SD of the assay performed in triplicate.
results also showed that the biofilm-forming strains presented different levels of enhancement in biofilm-forming capability in mixed cultures. Among all combinations, the mixed culture of A3 with M9 or M22 formed visible and rough biofilms at the air—liquid interface of the flask wall, their biofilm biomasses were highest, and the biofilm biomasses obtained from these two culture combinations were 5.95 and 4.86 times higher, respectively, than that formed by the single A3 culture.

**Resistance of the biofilm to shock loading and fixation of strain A3**

The results of the experiment showed that the 3,5-DNBA degradation rates of the mixed cultures and of the A3 single culture were similar in 6 h (46.7–55.1%) and 12 h (85.5–94.4%) at 30 °C. After the DCMM2 synthetic wastewater had been refreshed, the planktonic population was eliminated. The degradation capabilities of biofilms established for 24 h are shown in Fig. 2. The dual-species biofilms, A3 with M9 and A3 with M22, had a strong resistance to 3,5-DNBA shock loading during successive replacements of DCMM2 synthetic wastewater, and the degradation rate reached 63.3–91.6% and 70.7–89.4% within 6 h of each replacement. By contrast, the levels of resistance against 3,5-DNBA shock loading of the A3 single-species biofilm and the A3/M19 and A3/M21 dual-species biofilms were relatively low. The 3,5-DNBA degradation percentages of the A3 single-species biofilm and the A3/M19 and A3/M21 dual-species biofilms were 11.0 to 65.4% (Fig. 2a), 10.3 to 50.7% (Fig. 2d), and 11.9% to 58.5% (Fig. 2e), respectively, under a shock loading by successively replacing the DCMM2 synthetic wastewater.
In order to estimate the number of A3 cells fixed in the biofilm, the CFUs of total and A3 cells in the dual-species biofilm established on a glass slide at 24 h and 60 h were counted (Fig. 3a–f). Before the successive shock loading, it was found that the total CFUs and A3 CFUs of biofilms formed by A3 with M22 or M9 were significantly higher than those of the other three dual-species biofilms. In the A3–M9 biofilm, after being shocked six times, the A3 CFU and the total CFU were 3.7 × 10^7 and 1.4 × 10^8 CFU per slide, respectively. In the A3–M22 biofilm, the same figures reached 5.9 × 10^6 and 9.2 × 10^7 CFU per slide. This result demonstrates that both the biofilms adhered firmly to the flask wall but still retained quite high numbers of cells. The relatively high quantity of degrading bacteria in the biofilms also shows that strain A3 can be well maintained in these two dual-species biofilms over time.

It could be seen with the naked eye that the A3–M19 dual-species biofilm developed easily and could be detached from the glass surface in large pieces. The total CFU was reduced from 2.4 × 10^5 to 3.6 × 10^4 CFU per slide with the replacement of the synthetic wastewater (Fig. 2d). Before and after successive shock loadings, the strain A3 CFUs in the A3–M21 dual-species biofilm were 2.9 × 10^3 and 4.0 × 10^4 CFU per slide, respectively (Fig. 2e). The number of A3 cells in the dual-species biofilm was not significantly different from that in the single-species biofilm of A3 (7.7 × 10^4 and 3.4 × 10^4 CFU per slide) (Fig. 2a). Biofilms A3–M19 and A3–M21 resisted shock loading only weakly, as demonstrated in the biofilm detachment assay and the relatively small number of A3 cells in these biofilms. Consequently, they had the reduced 3,5-DNBA degradation rates in the synthetic wastewater.

**Dual-species biofilm structure of A3 with M22/GFP**

The dual-species biofilms of A3 with M22/GFP were coloured with red fluorescence stain, SYTO60. The structure of the dual-species biofilms established for 24 h was observed under confocal laser scanning microscopy (CLSM) (Fig. 3). GFP was not masked by the presence of SYTO60 in the cell, and therefore A3 cells appeared red and M22/GFP cells appeared green or yellow after overlay of red with green images. Observation of the three-dimensional biofilm structure showed that A3 was embedded in the biofilm, which further demonstrates that strain A3 can be well fixed in dual-species biofilms of A3 with M22/GFP.

**Discussion**

The strains *Pseudomonas* sp. M8, *P. putida* M9, *B. cereus* M19, *P. plecoglossicida* M21 and *A. hydrophila* M22 used in this study were screened from 18 bacterial strains isolated from a biofilm of a wastewater treatment system of nitrated aromatic compounds and from small carpolites in soil. They have relatively high biofilm-forming capabilities compared with other isolates (data not shown). *Pseudomonas putida* and *A. hydrophila* have been studied previously in their capacity as bacteria with high biofilm-forming capabilities (Rosalina et al., 2003; Martín et al., 2002). The fact that these two bacterial species were also included in our group of high-biofilm-forming isolates from the wastewater treatment system suggests that they are probably very common in natural biofilms.

The mixed culture of the five biofilm-forming strains with the degrading strain A3 resulted in the formation of biofilms with varying quality in terms of 3,5-DNBA degradation. For instance, although the mixed culture of A3 with M19 could form a large amount of biofilm, this biofilm was easily detached, making the 3,5-DNBA degradation less effective when the synthetic wastewater was often replaced. The biofilm biomass of the mixed culture of A3 with M21 was low, and the number of A3 cells in this dual-species biofilm was not significantly higher than that in a single culture of A3. Thus, strains M19 and M21 did not enhance A3 fixed in biofilm. By contrast, mixed cultures of M9 or M22 with A3 most effectively promoted large amounts of biofilm production and enhanced the establishment of A3 in the biofilm. It was also demonstrated that the A3–M9 and A3–M22 dual-species biofilms were the most capable of coping with the shock loading produced by
3,5-DNBA. The presence of M9 and M22 played a crucial role in the formation of the biofilm, and markedly increased the number of A3 cells in the dual-species biofilms. It therefore seems feasible to use the M9 and M22 strains as viable options for bioaugmentation to enhance the establishment of 3,5-DNBA-degrading bacteria in biofilms in an engineering setting.

From the shocking experiment, it was found that the ability of the biofilm to degrade 3,5-DNBA was enhanced after the shock-loading treatment. However, we did not see a significant increase in the A3 CFU in the biofilm after the shock loading, suggesting that the enhanced ability to degrade was not a result of increased A3 cell numbers. From the experiment we found that the successive propagation of strain A3 in DCMM or DCMM2 synthetic wastewater led to an increase in the degrading rate. Even a very small inoculation can degrade almost 90% of the 3,5-DNBA within 2–6 h. Thus it seems that the increased ability to resist the shock loading made a contribution to the increased ability of A3 to degrade.

A previous study found that, after adhering to the solid surface, the bacteria first formed a micro-colony and then developed into a mature biofilm (Mikkel et al., 2003). The dual-species biofilm image of A3 with M22/GFP by CLSM clearly showed the inner structure of the biofilm. It can be seen from the three-dimensional image of the A3-M22/GFP biofilm that the degrading strain A3 integrated well with the biofilm-forming strain M22/GFP and was embedded in the biofilm (Fig. 3). The degrading bacteria were distributed homogenously both on the surface and inside the biofilm.

At this time, we do not understand the process and mechanism underpinning how M9 and M22 promote the immobilization of A3. Nevertheless, our study has demonstrated that the use of bacteria with strong biofilm-forming capabilities to promote the immobilization of specific degrading bacteria in wastewater treatment systems may be a novel bioaugmentation strategy to be used in maintaining the degrading bacteria for purposes of pollutant degradation.

Acknowledgements

We thank Professor Meng Xiangxun at Soochow University and Dr Gao Weimin at Oak Ridge National Laboratory for helpful discussions and help with the English. This work was supported by grant 30600016 from the Chinese National Science Foundation and grants 2005DKA21201-2 of platform project of the resource of natural science and technology from the Ministry of Science and Technology.

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