Biodegradation (2006) 17:545–557
DOI 10.1007/s10532-005-9025-7

A bioremediation approach using natural transformation in pure-culture and mixed-population biofilms

Sudeep Perumbakkam[1,2], Thomas F. Hess[3] & Ronald L. Crawford[2,*]
[1]*Department of Microbiology, Molecular Biology & Biochemistry, University of Idaho, Life Science 142, P.O. Box 443052, Moscow, ID, 83844-3052, USA;* [2]*Environmental Biotechnology Institute, University of Idaho, Food Research Center 204, P.O. Box 441052, Moscow, ID, 83844-1052, USA;* [3]*Department of Biological and Agricultural Engineering, University of Idaho, Engineering and Physics Building 401, P.O. Box 440904, Moscow, ID, 83844-0904, USA (*author for correspondence: e-mail: crawford@uidaho.edu)*

Accepted 27 September 2005

Key words: atrazine, biofilm, bioremediation, DNA transformation, xenobiotic compound

Abstract

Bacterial transformation by naked DNA is thought to contribute to gene transfer and microbial evolution within natural environments. In nature many microbial communities exist as complex assemblages known as biofilms where genetic exchange is facilitated. It may be possible to take advantage of natural transformation processes to modify the phenotypes of biofilm communities giving them specific and desirable functions. Work described here shows that biofilms composed of either pure cultures or mixed populations can be transformed with specific catabolic genes such that the communities acquire the ability to degrade a particular xenobiotic compound. Biofilms were transformed by plasmids bearing genes encoding green fluorescent protein (*mut2*) and/or atrazine chlorohydrolase (*atzA*). Confocal microscopy was used to quantify the number of transformants expressing *mut2* in the biofilms. Degradation of atrazine by expressed *atzA* was quantified by tandem mass spectrometry. PCR analysis was performed to confirm the presence of *atzA* in transformed biofilms. These results indicate that it should be possible to use natural transformation to enhance bioremediation processes performed by biofilms.

Abbreviations: CLSM – confocal laser scanning microscopy; EPS – exopolysaccharide; ES-MS/MS – electrospray tandem mass spectrometry; GFP – green fluorescent protein; M9 – minimal media; MSM – mineral salts media; PCR – polymerase chain reaction

Introduction

A large number of synthetic chemicals (xenobiotic compounds) have been released into the environment and are of concern because of their potential risk to human health and the environment. Most of these chemicals are associated with past or present agricultural, industrial, and military operations and often leach from soil into groundwater systems. Considering the large number of contaminating chemicals and their occurrence at thousands of sites, development of affordable groundwater remediation technologies is a major challenge. Bioremediation is often considered as a cost-effective technology and utilizes microorganisms for the mineralization or detoxification of pollutants (Tiirola et al. 2002; Timmis & Pieper 1999).

In environments such as groundwater most bacteria are thought to live in communities and form complex structures called biofilms (Costerton et al. 1987; Costerton et al. 1999; Davies et al. 1998; Mah & O' Toole 2001). The close physical associations of microbial community members in biofilms lead to interactions at the genetic level. Gene transfer occurs both at intra- and inter-species

levels and plays an important role in biofilm evolution and genetic diversity (Stewart & Costerton 2001). Transformation, conjugation, and transduction are facilitated within biofilm microbial populations. In particular, bacteria in contaminated environments appear to mutate and horizontally transfer genetic material, thereby acquiring improved abilities to degrade or detoxify hazardous substrates (Stoodley et al. 2002). Though it has been assumed that soil microbial communities are constantly evolving via the movement of genes within the community, gene transfer within biofilm communities has not been studied extensively *in vivo*.

Bacteria take up naked DNA through a process known as transformation. In a given population, not all bacteria are capable of natural transformation. Based on evidence collected from studies of pure cultures, DNA uptake is usually associated with specific competence factors. Bacterial competence is related to the phenomenon of quorum sensing, which indicates that a biofilm, where cell density is high, offers an environment conducive to transformation (Lorenz & Wackernagel 1994; O'Toole et al. 2002; Stewart & Carlson 1986). Bacteria may express competence factors during different stages in their growth cycles. For example, in the laboratory some strains of *Pseudomonas stutzeri* usually express competence factors during the stationary growth phase (Stewart & Carlson 1986; Stewart et al. 1983), whereas a strain like *Acinetobacter* sp. BD413 expresses these factors during the logarithmic phase (Hendrickx et al. 2003; Palmen & Hellingwerf 1997).

Because intrinsic processes of bioremediation are at times slow, actively engineered approaches are sometimes needed to speed the biological reactions required for pollutant breakdown. Hendrickx et al. (2003) suggested that it might be possible to use natural transformation to enhance biodegradation process in biofilms, though we are not aware that this approach has actually been tested with functional degradative genes. In work reported here, we evaluated the use of natural transformation for introducing exogenous genes into pure-culture biofilms of *Acinetobacter* sp. BD413 and into mixed-community soil biofilms using a gene (*mut2*) encoding green fluorescent protein (GFP). We similarly transformed these biofilm communities with the gene *atzA* (Mandelbaum et al. 1993) that encodes an enzyme (atrazine chlorohydrolase) that converts atrazine to hydroxyatrazine, the first product observed during bacterial degradation of the well-characterized herbicide (Martinez et al. 2001). The biofilms acquired and expressed these genes via natural transformation. We confirmed uptake and expression of *mut2* by observing fluorescence of transformed cells within biofilms and expression of *atzA* by exposing the biofilms to atrazine and then measuring atrazine removal by the biofilm using electrospray tandem mass spectrometry (ES-MS/MS).

Materials and methods

Bacterial strains and media

Table 1 lists the bacterial strains used in this study. *Acinetobacter* sp. strain BD413 was used for all pure-culture biofilm experiments. Soil collected on

Table 1. Bacteria, plasmids, and PCR primers used in this study

Bacterial strains and plasmids	Source or reference
Acinetobacter sp. BD413	ATCC 33305
Soil mixed culture	University of Idaho, Moscow campus
E. coli DH5α	University of Idaho collection
p519ngfp	Matthysse et al. (1996)
PMD4	Dubnau (1999)
PBBR1MCS5	Kuehn et al. (1998)
pSP1 *(mut2)*	This study
pSP2 *(mut2, atzA)*	This study
PCR primers for *atzA* gene	IDT Corp., Coralville, IA
5′ GTT AGC CAC CAT GTT CGC CC 3′	Based on NCBI accession number NC_004956
5′ TGC AAA CGC TCA GCA TCC AG 3′	Based on NCBI accession number NC_004956

the University of Idaho, Moscow, ID, USA, campus was used as an inoculum for producing soil-derived biofilms. Minimal media (M9) (Ausubel et al. 2002) and mineral salts media (MSM) (Mandelbaum et al. 1993) containing 0.1% glucose as a carbon source and 0.05% NH_4Cl as a nitrogen source were used for growing biofilms and for DNA transformation and atrazine breakdown experiments. An atrazine stock solution (250 mg ml^{-1}) was prepared by dissolving the herbicide in methanol and sonicating the solution in a water bath for 3 h (Mandelbaum et al. 1993). Appropriate aliquots of the stock solution were added to M9 or MSM to give a final atrazine concentration of 20 mg l^{-1} for all atrazine degradation experiments.

Gentamycin (10 μg ml^{-1}) was used as a selection agent for plasmid cloning and to enrich transformed bacteria within soil and pure-culture biofilms. Cells were cultivated in Terrific Broth (Ausubel et al. 2002) prior to their use for preparation of plasmid and chromosomal DNA.

Plasmids and cloning

Table 1 lists the plasmids used in the experiment. The broad host range plasmid pBBR1MCS5 (Kovach et al. 1994) was used for biofilm transformation experiments. The GFP gene used was obtained from plasmid p519ngfp (Matthysse et al. 1996). A fragment consisting of the *pnpt*2 promoter and the *mut2* gene was excised from p519ngfp as a *Hind III-EcoRI* fragment and then cloned into the *Hind III-EcoRI* digested pBBR1MCS5 to create pSP1. Colonies were selected by blue/white screening. The *atz*A gene from plasmid pMD4 (de Souza et al. 1995) was cleaved as an *avaI* fragment, blunt-ended using T4 polymerase, and then ligated to the *SmaI*-digested pSP1 to form pSP2. *Escherichia coli* DH5α was used for routine plasmid cloning. Standard microbiological techniques (plasmid extraction, DNA quantification, spectroscopy, bacterial quantification, and enzyme digestion and ligation) were performed as described in manufacturers' protocols or as detailed in Ausubel et al. (2002).

Biofilm growth and transformation experiments

A flow cell (model FC81, BioSurface Technologies Corporation, Bozeman, MT) was set up with a flow rate of 1.9 ml h^{-1} using a syringe pump (Model 600 910/920, Harvard Apparatus, Inc., Dover, MA). Glass slides used for growing biofilms were first washed with concentrated HCl and then thoroughly rinsed with distilled water. The setup was autoclaved along with the Teflon® tubing used to supply media. The flow cell was then inoculated with *Acinetobacter* sp. strain BD413 or a soil consortium and allowed to grow for 2 days at room temperature ($\sim$28 °C) in either M9 or MSM. After 2 days of growth, a total of 200 ng of plasmid DNA (pSP2; 0.5 μg μl^{-1}) was passed through the chamber over a period of 2 h. In order to enrich for the transformants in the biofilm population, the medium inflow was then switched to media containing gentamycin (10 μg ml^{-1}) and atrazine (20 mg l^{-1}). This feed was continued for an additional 3 days. The chamber effluent was collected using a fraction collector. Collected samples were stored at −80 °C until analysis.

Data reported here are from single experiments. To confirm that results were repeatable, three repetitions (data not shown) of each treatment for both pure-culture and soil-derived biofilms were performed as described above. To rule out the possibility that simple adsorption of atrazine to the biofilm matrix might be responsible for atrazine removal by transformed biofilms, pSP2 was replaced with pSP1, a *mut2* construct without *atzA*, and the experiments were repeated.

Confocal laser scanning microscopy (CLSM) analysis and estimating transformation efficiency

After a biofilm experiment was completed (5 days), the biofilm was stained with Syto 60 (Molecular Probes, Eugene, OR) according to the manufacturer's protocol by pumping the staining solution into the flow cell. The flow cell was left undisturbed for 1 h and then viewed using a BioRad MRC 1024 CLSM equipped with a krypton–argon laser on a Nikon Diaphot inverted microscope. Excitation lines were 488 nm for GFP and 647 nm for Syto 60. Data were acquired using Laser Sharp software (version 3.2; BioRad Labs, Hercules, CA). Emission spectra filters were 522 DF (506–538 nm) and 680 DF32 (664–696 nm) for GFP and Syto60, respectively. Objective lenses were a 40× Flour (0.85 N.A.) and a 60× oil Plan Apo (1.4 N.A.). The final resolutions of the images were of 400× and 600× magnitude. Confocal Assistant and Metamorph software packages

(Molecular Devices Corporation, Downingtown, PA) were used for further image processing.

Biofilm height was determined by using the *zx*-axis (two-dimensional analyses) of the confocal microscope. The resulting start and stop positions of the scan (measured in μm) provided a measure of biofilm height. The biofilm slide was divided arbitrarily into three equal sectors (1.25 cm long by 1 cm wide) based on distance from the medium inlet. These were sector 1 nearest the inlet, sector 2 in the middle of the slide, and sector 3 farthest from the inlet. Each sector was viewed at three different locations. Results were presented as the mean of these triplicate readings. A three-way factorial ANOVA was performed to test for significant differences in height among the three sectors.

Transformation efficiency was determined by counting the total number of cells (fluorescence of Syto 60) and transformed cells (emitting florescence due to the presence of GFP). Transformation efficiency was calculated as described previously by dividing the number of cells expressing GFP by the total number of cells appearing in the microscopic field (Hendrickx et al. 2003; Kuehn et al. 1998). A two-way factorial ANOVA was performed on the data obtained.

Electrospray tandem mass-spectroscopy (ES-MS/MS)

To confirm atrazine removal by *atzA*-transformed biofilms under various treatment conditions, we employed ES-MS/MS to examine chamber effluent samples for the amount of atrazine remaining in solution. Fractions collected from the chamber effluent were extracted with 30-mM ammonium formate buffer (pH 6.6) and analyzed using positive ion electrospray ionization (Quattro II, Micromass Ltd., UK). Samples were delivered into the source at a flow rate of 0.60 μl h^{-1} using a syringe pump (Harvard Apparatus, South Natick, MA). A potential of 3.5 kV was applied to the electrospray needle, and the sample cone was kept at an average of 15 V. Detector resolution was set at 15; source temperature was kept constant at 120 °C. The counter electrode, skimmer, and RF lens potential were tuned to maximize the ion beam. All spectra were collected as an average of 18 scans.

Quantitative data from ES-MS/MS were converted to the percentage of atrazine remaining in the chamber effluent samples as compared to atrazine concentrations observed in effluents from bacteria-free control chambers.

Polymerase chain reaction (PCR) analysis

PCR analyses were performed to confirm the presence of *atzA* in DNA extracted from transformed biofilm communities. After the experiments described above, the biofilm was stripped from the glass slide and inoculated into M9-glucose-NH_4Cl. After growth, cells were centrifuged and the pellet then resuspended in buffer containing Dnase I [10 mg ml^{-1} in $MgCl_2$ (4.2 mg l^{-1})]. After incubation for 20 min, the cells were washed twice with sterile saline (0.89% NaCl). PCR primers (Table 1) were designed based on the published sequence of *atzA* (atrazine chlorohydrolase: NCBI Gene Bank database accession number NC_004956) using Vector NT1 (Informax, Invitrogen, Inc., Carlsbad, CA). These primers amplify the complete 1400-bp sequence of *atzA*. The biofilm cell suspension was heated for 10 min at 95 °C in a PCR thermocycler (PTC100 M.J. Research, Inc., Watertown, MA), cooled, and centrifuged at 3000×*g* for 2 min; 1.0 μl of the supernatant was used as template for PCR analysis. The primers (100 pg; Table 1) were added to a 50-μl reaction volume as described by the manufacturer (Invitrogen, Inc., Carlsbad, CA). PCR was performed in the PTC100 Thermocycler under the following conditions: initial denaturation at 95 °C for 40 s, annealing at 55.3 °C for 30 s, and primer extension at 72 °C for 3 min (30 cycles), followed by a final extension time of 10 min at 72 °C.

Results

Transformation of Acinetobacter *sp. BD413 and soil-community biofilms*

To determine if there was any difference in transformation rates due to the use of two different replication origins, plasmids p519ngfp (Matthysse et al. 1996) and pSP1 were compared as transformation vectors. Plasmid DNA solution containing a total of 200 ng of pSP1 or p519ngfp at 0.5 μg μl^{-1} was injected into the biofilm chamber over a period of 2 h. Following an

additional three days of flow using media augmented with gentamycin to enrich for transformants, Syto 60 dye was used to counterstain the biofilm for observation of living cells under a confocal microscope. GFP fluorescence was also viewed to identify transformed cells expressing *mut2* (Figure 1).

Table 2 shows the number of transformants in the three biofilm sectors for both pure-culture and soil-derived biofilms. In the pure-culture biofilm of *Acinetobacter* sp. transformed with p519ngfp, the number of transformants was similar in both the first and second sectors. Sector 3, farthest from the chamber inlet, showed a 100-fold increase in number of transformants as compared to sectors 1 and 2. Sectors 1 and 3 contained statistically different numbers of transformants ($p \leq 0.0001$). When the pure-culture biofilm was transformed with pSP1, there were similar numbers of transformants in the first two sectors, while sector 3 showed a 10-fold increase in the number of transformants as compared to sectors 1 and 2. All sectors were statistically different ($p \leq 0.0001$). In the soil-based biofilm, the RSF1010-derived origin (plasmid p519ngfp) provided a 10-fold increase in number of transformants in the second and third biofilm sectors as compared to the first sector. Statistically significant differences were detected between sectors 1 and 2 and sectors 1 and 3 ($p \leq 0.0001$). A similar trend was observed when the pBBR1MCS5-based origin (pSP1) was used with significant differences detected among all sectors at $p \leq 0.0001$.

Height of Acinetobacter *sp. BD413 and soil biofilms*

After biofilms were grown for 5 days in the flow chamber, the three sectors were viewed under a confocal microscope to determine biofilm height. A two-dimensional *zx* analysis was performed to estimate the height of the biofilm. Three points in each sector were sampled and averaged to provide the values shown in Figure 2. In the pure culture, the sector 1 biofilm had an average height of 40 μm and a very similar height of 70 μm in the remaining two sectors. Pure-culture biofilm heights were significantly different in

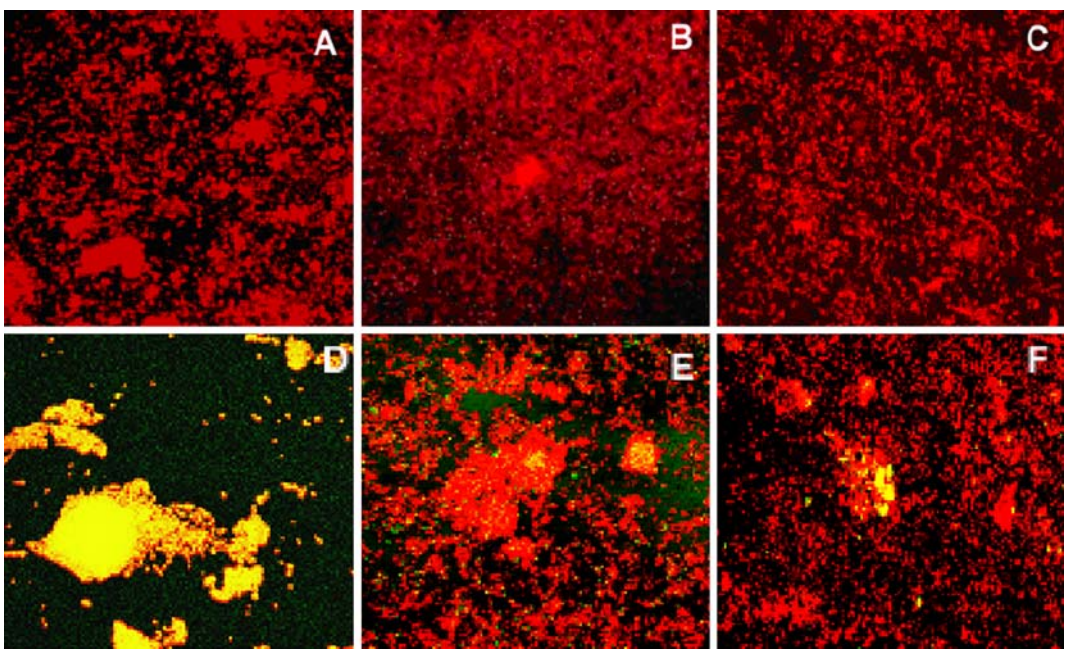

Figure 1. CLSM analyses of *Acinetobacter* sp. BD413 and soil mixed culture biofilms. A (40×): untransformed *Acinetobacter* sp. BD413 biofilm; B (40×): untransformed soil biofilm; C (60×): untransformed soil biofilm; D (40×): pSP2 treated biofilm of *Acinetobacter* sp. BD413 (the yellowish-green color identifies transformants that give this color due to superimposition of red Syto 60 and green GFP fluorescence); E (40×): soil biofilm transformed with *atzA* (pSP2; the yellow color represents the transformants and the red color the untransformed population); F (60×): closer view of a soil biofilm with a pSP2 transformed population (the red color is the untransformed live population stained by Syto 60).

Table 2. Transformation frequencies (±standard deviations) of *Acinetobacter* sp. strain BD413 and soil mixed culture biofilms with pSP1 (pBBR1MCS5-based) and p519ngfp (RSF1010-based) plasmids obtained by confocal scanning laser microscopy. A and C; G and H; G and I; D, E, and F; J, K, and L were significantly different in transformation frequency ($p \leq 0.0001$)

	Sector 1	Sector 2	Sector 3
Acinetobacter sp. transformants			
p519ngfp	$1.3\times10^{-4} \pm 0.120\times10^{-4}$ (A)	$4.7\times10^{-4} \pm 1.200\times10^{-4}$ (B)	$6.3\times10^{-2} \pm 0.460\times10^{-2}$ (C)
pSP1	$1.2\times10^{-4} \pm 0.562\times10^{-4}$ (D)	$3.2\times10^{-4} \pm 0.654\times10^{-4}$ (E)	$7.9\times10^{-3} \pm 1.030\times10^{-3}$ (F)
Soil transformants			
p519ngfp	$2.4\times10^{-4} \pm 0.281\times10^{-4}$ (G)	$6.3\times10^{-3} \pm 0.534\times10^{-3}$ (H)	$8.3\times10^{-3} \pm 0.372\times10^{-3}$ (I)
PSP1	$2.9\times10^{-3} \pm 0.830\times10^{-3}$ (J)	$5.8\times10^{-2} \pm 1.200\times10^{-2}$ (K)	$9.0\times10^{-2} \pm 0.264\times10^{-2}$ (L)

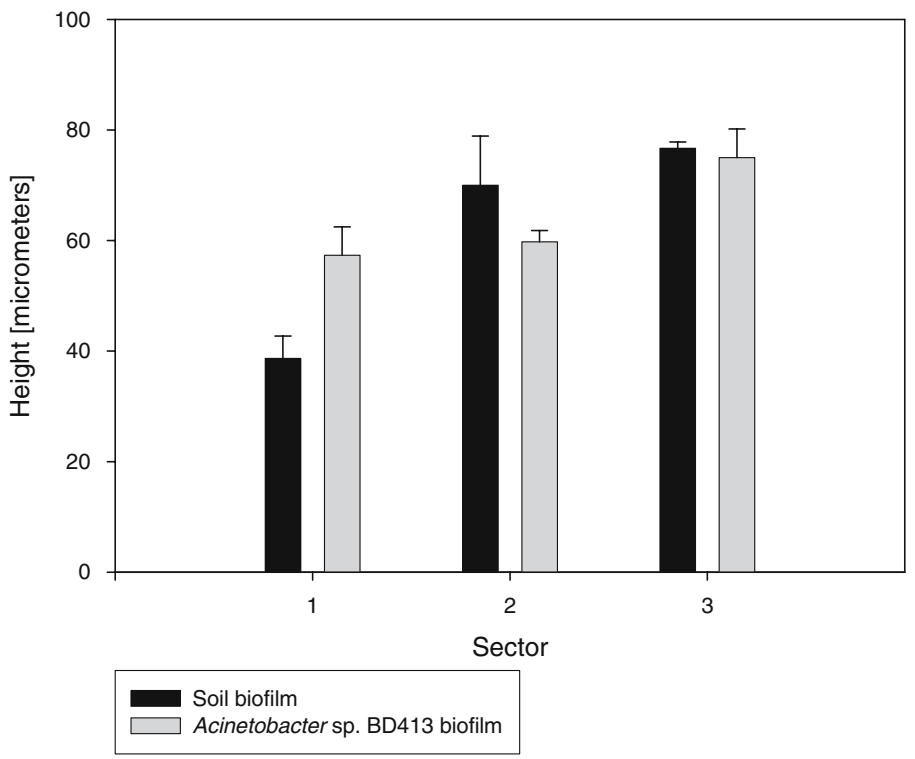

Figure 2. Average biofilm heights of *Acinetobacter* sp. BD413 and soil mixed culture biofilms. Error bars represent ±1 standard deviation of three replicate experiments. Biofilm heights of the pure-culture *Acinetobacter* and soil-based biofilms were significantly different ($p \leq 0.05$) only in sector 1 of the biofilm chamber.

sectors 1 and 3 ($p \leq 0.0008$). Soil-derived biofilm height varied between 65 μm and 75 μm (observed in sector 3). For the soil-based biofilm, significant differences existed between sectors 1 and 2 and between sectors 1 and 3 ($p \leq 0.0001$). When heights of pure-culture and soil-based biofilms were compared, sector 1 was the only sector found to be statistically different ($p \leq 0.0001$).

Atrazine transformation by Acinetobacter *sp. BD413 and soil consortia biofilms in M9 medium*

After biofilm growth from either *Acinetobacter* sp. BD413 or a soil-derived consortia, pSP2 containing the *atzA* was used to transform the mature biofilms. Figures 3 and 4 show atrazine removal percentages from atrazine-supplemented M9 passed over transformed biofilms. The *Acinetobacter*

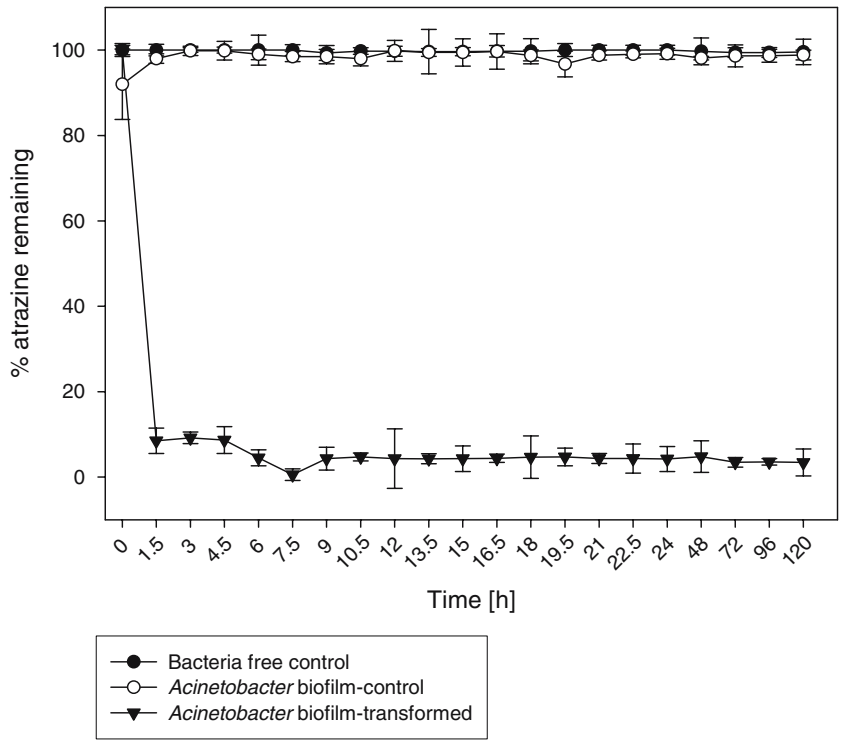

Figure 3. Atrazine removal in *atzA*-transformed *Acinetobacter sp*. BD413 biofilms grown on M9. Shown are the percentages of atrazine remaining in the chamber outflow from a bacteria-free control (●), an untransformed *Acinetobacter* control (○), and an *Acinetobacter* pSP2-transformed biofilm (▼). The initial concentration of atrazine was 20 mg l^{-1}. Chamber effluent samples were collected at 90-minute intervals for the first day and thereafter once for the remaining experimental time period (4 days). Values indicate the percentage of the input atrazine concentration remaining in the chamber effluent at the various time points. Error bars represent ±1 standard deviation of three replicate analyses.

sp. BD413 biofilm clearly expressed genes borne on pSP2 as shown by its degradation of atrazine (Figure 3). The percentage of atrazine degradation remained stable throughout the three-day period of the experiment. An untransformed biofilm (*Acinetobacter* control) did not remove atrazine over a similar incubation period.

A similar trend of atrazine removal was observed in a pSP2-transformed soil biofilm; however, the native population of the untransformed soil control also removed some atrazine (Figure 4). This loss of atrazine in the control biofilm accounted for a maximum of only 20% of the total available atrazine after 5 days. A bacteria-free control (no biofilm) showed no atrazine removal. Thus, in both the soil consortium and pure-culture *atzA*-transformed biofilms, there was a stable loss of 80–90% of the available atrazine over the 5-day experimental period (only 21 hourly time points are shown in the figures). This loss of atrazine can be attributed to uptake and expression of *atzA* by biofilm cells.

To determine if these experiments were repeatable, all transformation experiments were replicated three times for both soil and *Acinetobacter* sp. BD413 biofilms. Similar results were observed in all cases (data not shown).

Atrazine degradation by Acinetobacter *sp. BD413 and soil consortia biofilms in MSM medium*

To determine if media type would affect atrazine degradation percentages, MSM media was substituted for M9 in another series of experiments. Stable trends in atrazine removal similar to those observed using M9 were also observed when MSM was used (Figures 5 and 6). The untransformed soil biofilm control once again showed some background atrazine removal similar to that

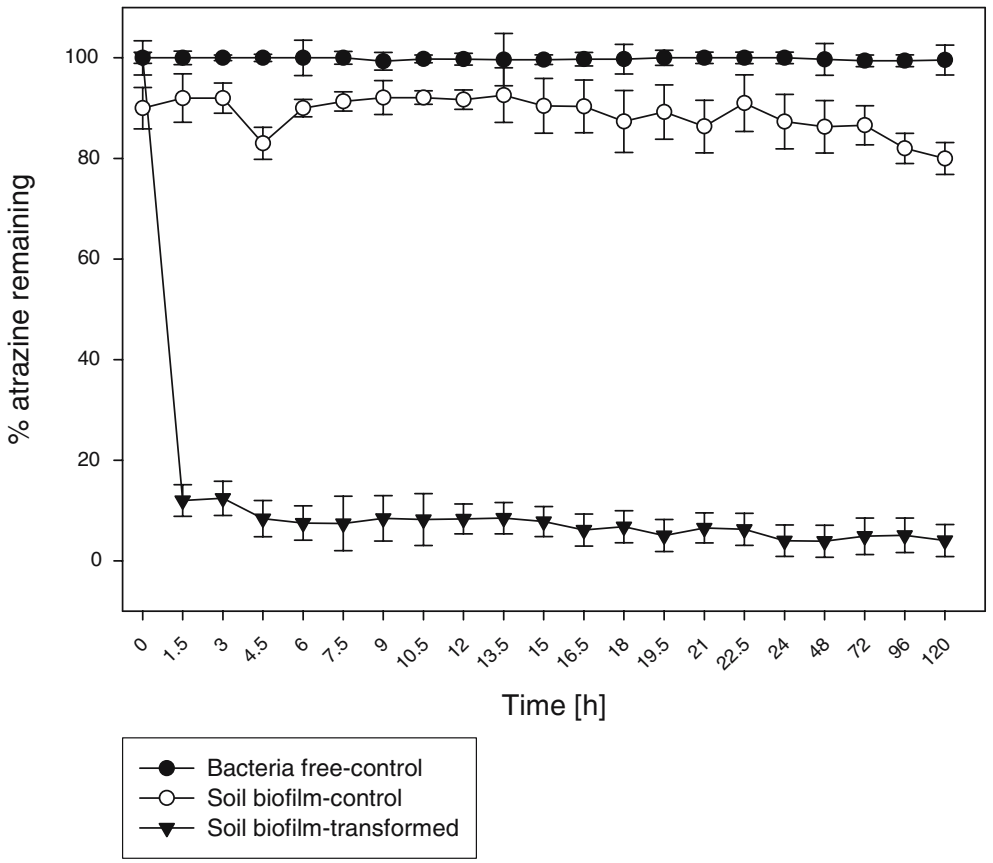

Figure 4. Atrazine removal in soil-derived biofilms grown on M9. Shown are the percentages of atrazine remaining in the chamber outflow from a bacteria-free control (●), an untransformed soil biofilm control (○), and a soil biofilm transformed with pSP2 (▼). Values indicate the percentage of the input atrazine concentration remaining in the chamber effluent at the various time points. Error bars represent ±1 standard deviation of three replicate analyses.

seen using M9. Thus, removal of atrazine was similar in both soil and pure-culture biofilms and remained stable for the three-day period after the biofilm was transformed with pSP2, regardless of the medium used.

To confirm that atrazine removal by the transformed biofilms was not the result of surface adsorption but was indeed caused by the introduced *atzA* gene, a *mut2*-containing plasmid that did not contain *atzA* was used to transform control biofilms, atrazine-bearing media were passed through the transformed biofilms, and chamber effluent samples were collected and analyzed for residual atrazine. Such controls did not remove atrazine, ruling out simple adsorption as a removal mechanism.

Biofilms that were grown from *Acinetobacter* sp. BD413 or the soil consortium and then transformed with pSP1 lacking *atzA* genes did not degrade or adsorb atrazine (Figure 7).

PCR analysis

Figure 8 shows results obtained by PCR-based analyses of biofilms transformed with pSP2. The primer set employed was designed to amplify the complete *atzA* open reading frame. Results showed the presence of the expected 1400-bp product amplified from the *atzA*-transformed *Acinetobacter* biofilm (lane 4) but no product from the untransformed control *Acinetobacter* biofilm (lane 3). Lanes 5 and 6 show similar expected results for the untransformed and transformed soil biofilms, respectively, where *atzA* was amplified from the transformed biofilm but not the untransformed control. Positive and negative

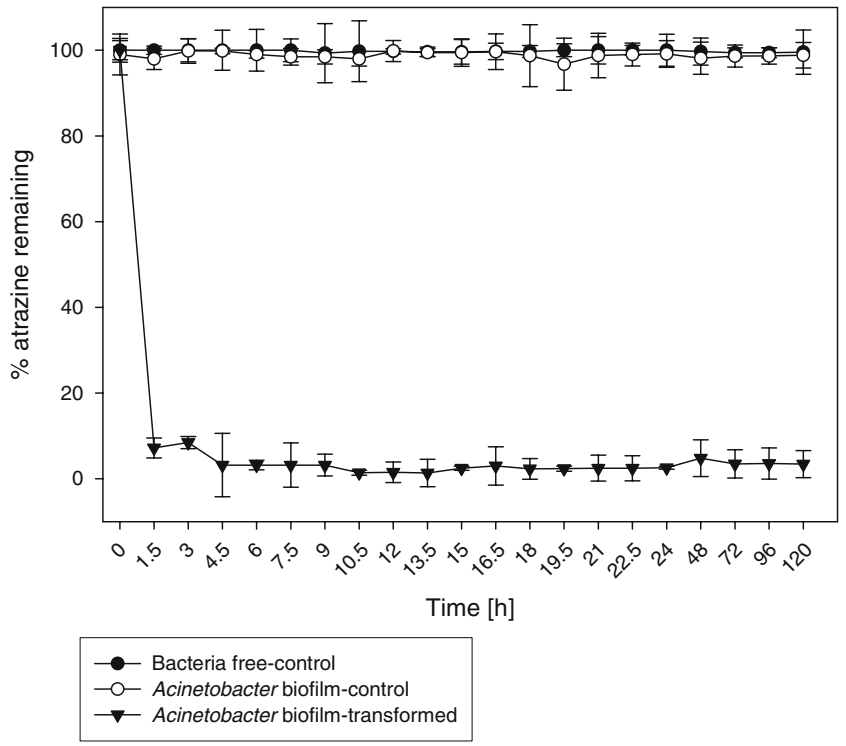

Figure 5. Atrazine removal in *Acinetobacter sp*. BD413 biofilms grown on MSM. Shown are the percentages of atrazine remaining in the chamber outflow from a bacteria-free control (●), an untransformed *Acinetobacter* biofilm control (○), and an *Acinetobacter* biofilm transformed with pSP2 (▼). Error bars represent ±1 standard deviation of three replicate analyses.

controls (lanes 1, 2, 3, and 8) all showed expected results. Specifically, plasmids pMD4 and pBBRIMCS5 carrying the *atzA* gene (lanes 1 and 2) showed the 1400-bp amplicon. The untransformed *Acinetobacter* (*atzA*; lane 3) showed no amplicon; likewise for a no-template control (lane 8). The soil-derived biofilm control (lane 5) showed some non-specific amplification by these primers but no product at 1400 bp.

Discussion

Transformation is thought to be one of the principal mechanisms by which genes are exchanged between bacterial cells in nature. Gene transfer via transformation may occur at both the inter- and intra-species levels. Here we investigated the potential for using transformation to introduce genes of value for bioremediation into natural biofilms. In this work we employed a special flow cell designed to mimic the growth and typical structural architecture of natural biofilms. We grew biofilms from both pure cultures and soil microbial consortia within the flow cells and then used the biofilms we had developed for *in vivo* transformation experiments.

The condition of "competence" for DNA uptake by bacterial cells is widely acknowledged as a crucial phenomenon that allows natural transformation to proceed at significant rates (Stewart & Carlson, 1986). Our model biofilm pure culture *Acinetobacter* sp. BD413 is an example of a highly competent bacterial species, so a large number of cells in its population are competent for DNA uptake at any point in time (Palmen & Hellingwerf 1997). Thus, a biofilm comprised of *Acinetobacter* sp. BD413 cells should be a good system to examine the hypothesis that natural transformation can be a useful approach to modify biofilm microbial communities with genes of value for bioremediation. Our results confirmed the value of this model system. We observed that *Acinetobacter* biofilms were readily transformed by plasmids bearing both the *mut2* reporter gene and the *atzA* gene, which is involved in atrazine biodegradation.

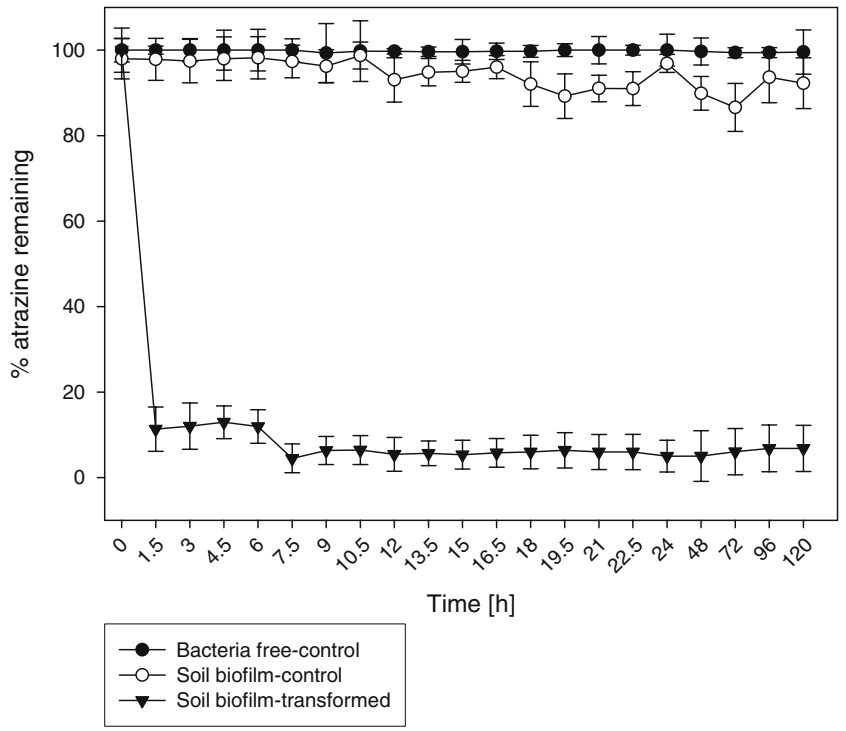

Figure 6. Atrazine removal in soil biofilms grown on MSM. Shown are the percentages of atrazine remaining in the chamber outflow from a bacteria-free control (●), an untransformed soil biofilm control (○), and a soil biofilm transformed with pSP2 (▼). Error bars represent ±1 standard deviation of three replicate analyses.

Both genes were expressed within the biofilm matrix.

A more challenging test of our hypothesis involved our attempts to transform a mixed-community biofilm with plasmids bearing *mut2* and *atzA*. Such biofilms harbor a diversity of bacteria. It is likely that a smaller component of such a mixed population would be competent for DNA uptake at any one time as compared to a pure-culture-based biofilm. Our results showed, however, that transformation efficiencies for the pure-culture-based and soil-derived biofilms were similar (Table 2). This is a highly encouraging result for eventual application of this technology in the field (Strong et al. 2000).

Acinetobacter sp. BD413 (Juni & Janik 1969) is an exopolysaccharide negative mutant; however, despite this mutation the strain formed a reasonably thick biofilm, 40–70 μm in height (Figure 2). Its biofilm was thicker in sector 3 of the biofilm chamber than in sectors 1 and 2 suggesting that sector location plays a role in determining biofilm height and transformation frequency. This may reflect its higher susceptibility to shear forces near the medium inlet that are likely responsible for some biofilm dissociation and dispersion in those sectors (Stoodley et al. 1999). In comparison, the height in the soil community-derived biofilm remained constant throughout the chamber. This mixed community clearly formed a stronger biofilm than the pure culture *Acinetobacter*, probably by way of its production of a stronger polysaccharide matrix. Despite the differences in biofilm architecture, both biofilms were effectively transformed by the employed plasmids.

Confocal analyses confirmed the presence of genes carried by pSP2 in the transformed biofilms as indicated by synthesis of fluorescent GFP. In order for us to observe GFP fluorescence, *mut2* not only had to enter the cells within the biofilm but it also had to be expressed. PCR analyses and atrazine degradation confirmed the presence and expression of *atzA* in the transformed biofilms. Prior to the PCR analyses, the biofilm material was treated with DNase to remove any adsorbed DNA that might have been bound to the biofilm

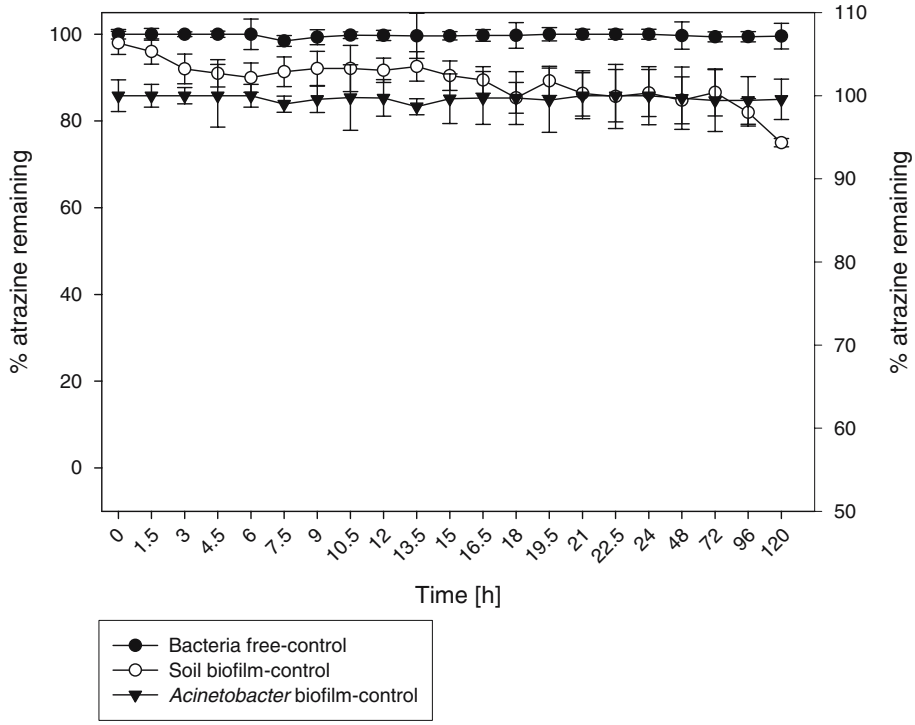

Figure 7. Lack of adsorption of atrazine to *Acinetobacter* sp. BD413 and soil biofilms lacking *atzA*. Symbols are ●, bacteria-free control (scale on left); ○, transformed *Acinetobacter* biofilm control (scale on left); ▼, transformed soil biofilm (scale on right). Error bars represent ±1 standard deviation of three replicate analyses.

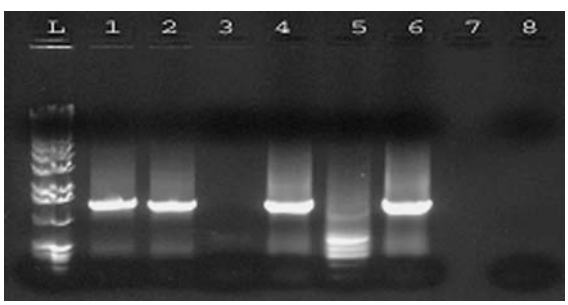

Figure 8. Biofilm PCR analysis. Lane L: 1-kb ladder; Lane 1: PMD4 carrying *atzA*; Lane 2: pBBR1MCS5 carrying *atzA*; Lane 3: *Acinetobacter* DNA control (untransformed – no *atzA* gene); Lane 4: *Acinetobacter* biofilm transformed; Lane 5: soil control (untransformed); Lane 6: soil biofilm transformed; Lane 7: no sample; Lane 8: PCR control (no template).

matrix. This treatment was employed to address the unlikely possibility that if the biofilms had adsorbed some of the added DNA, it may not have been degraded over the period of the experiment (5 days) by biofilm community nucleases.

Although the soil biofilm community was not characterized during this study, other techniques such as 16S rDNA fingerprinting could be used to identify members of the soil biofilm community. It would also be of interest to isolate representative transformants from the post-transformation biofilms and determine if the introduced genes are maintained as plasmids or have been integrated into the microbial chromosomes, as plasmid retention and homologous or illegitimate recombination are all possible mechanisms of gene incorporation.

Soil biofilms, unlike the *Acinetobacter* biofilms, showed some background activity for atrazine removal. This activity was minimal considering the diversity of bacteria present and the potential number of non-specific enzymes that could be present in these mixed soil populations. Such biofilms may contain an organism(s) capable of atrazine degradation via a related or completely different mechanism (Herzberg et al. 2004; Lawrence et al. 2001). However, we did not observe removal of more than 20% of the influent atrazine from non-transformed control soil biofilms. There did appear to be some enrichment of atrazine degraders over the course of the experiment since the percentage removal increased from an initial

level of 5–10% to about 20% after 5 days (Figure 4).

Atrazine was passed through the *atzA*-transformed biofilm chambers at a fairly high concentration of 20 mg l^{-1} and at an environmentally realistic flow rate. Under these conditions bacteria within the biofilms had sufficient time in contact with atrazine to degrade the herbicide. Atrazine was effectively removed by both the *Acinetobacter* biofilm and the soil-derived biofilm; 80–85% of the initial concentration of atrazine was removed in both systems. Changing the medium composition flowing through the reactors from M9 to MSM resulted in no difference in removal percentages of atrazine, indicating that simple mineral-based media were fully capable of supporting atrazine degradation by transformed biofilms. Most groundwater systems are also minimal, oligotrophic environments. This indicates that eventual application of this transformation technology to real-world groundwater sites is a promising idea.

Additional questions that need to be addressed prior to conducting field-based experiments include the following. Will this approach also work for genes encoding degradation of pollutants other than atrazine? What is the minimal number of genes in a pathway that will be required to accomplish acceptable detoxification of a contaminant such as atrazine? For example, is the conversion of atrazine to hydroxyatrazine sufficient to allow its ultimate complete mineralization or will additional atrazine pathway genes also be required? We detected hydroxyatrazine in chamber effluents from pure-culture biofilms, but no hydroxyatrazine remained in effluent from mixed soil biofilms. Therefore it appears that the *atzA* gene alone was insufficient for complete atrazine degradation in our pure-culture model system but did result in complete degradation when added into the mixed population biofilm. Another logical question is how much flow might be needed to adequately expose a transformed biofilm to contaminated water to allow for enough contact time for detoxification? There may be insufficient flow in some aquifers. Finally, will supplementation with an alternative carbon source such as glucose be required to support degradation of some contaminants if only one or a few new genes are introduced into the community?

In summary, data reported here demonstrate that it is possible to modify microbial biofilms through natural transformation to improve their biodegradative capabilities. Ultimately the technique could be applied to natural community biofilms such as are found in aquifers or bioreactors. This would demonstrate the utility of this technique for bioremediation enhancement. Such enhancement would involve genetic manipulation of the environment but would not require the highly regulated and controversial addition of genetically engineered microorganisms to the environment.

Acknowledgments

We would like to thank Drs. Andrzej Paszczynski and Allan Caplan for their help with mass spectroscopy and molecular biology techniques, respectively. We thank Ann Norton at the CLSM Center at the University of Idaho for assisting with confocal microscopy and Cornelia Sawatzky for editorial assistance. This research was funded by the U.S. Department of Defense via grant #DAAD 19-00-1-0078.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K (2002) Current Protocols in Molecular Biology. John Wiley & Sons, Englewood Cliffs, NJ

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC & Dasgupta & Marrie M TJ (1987) Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. 41: 435–464

Costerton JW, Stewart PS & Greenberg EP (1999) Bacterial biofilms: A common cause of persistence infections. Science 284: 1318–1322

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW & Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280: 295–298

de Souza ML, Wackett LP, Boundy-Mills KL, Mandelbaum RT & Sadowsky MJ (1995) Cloning, characterization, and expression of a gene region from *Pseudomonas* sp. strain ADP involved in the dechlorination of atrazine. Appl. Environ. Microbiol. 61: 3373–3378

Dubnau D (1999) DNA uptake in bacteria. Ann. Rev. Microbiol. 53: 217–244

Hendrickx L, Hausner M & Wuertz S (2003) Natural genetic transformation in monoculture *Acinetobacter* sp. strain BD413 biofilms. Appl. Environ. Microbiol. 69: 1721–1727

Herzberg M, Dosoretz CG, Tarre S, Beliavski M & Green M (2004) Biological granulated activated carbon fluidized bed reactor for atrazine remediation. Water Sci. Technol. 49: 215–222

Juni E & Janik A (1969) Transformation of *Acinetobacter alcoaceticaus* (*Bacterium antitratum*). J. Bacteriol. 98: 281–288

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop II RM & Peterson KM (1994) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175–176

Kuehn M, Hausner M, Bungartz H-J, Wagner M, Wilderer PA & Wuertz S (1998) Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms. Appl. Environ. Microbiol. 64: 4115–4127

Lawrence JR, Kopf G, Headley JV & Neu TR (2001) Sorption and metabolism of selected herbicides in river biofilm communities. Can. J. Microbiol. 47: 634–641

Lorenz MG & Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev. 58: 563–602

Mah TF & O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9: 34–39

Mandelbaum RT, Wackett LP & Allan DL (1993) Mineralization of the s-triazine ring of atrazine by stable bacterial mixed cultures. Appl. Environ. Microbiol. 59: 1695–1701

Martinez B, Tomkins J, Wackett LP, Wing R & Sadowsky MJ (2001) Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. J. Bacteriol. 183: 5684–5697

Matthysse AG, Stretton S, Dandie C, McClure NC & Goodman AE (1996) Construction of GFP vectors for use in gram-negative bacteria other than *Escherichia coli*. FEMS Microbiol. Lett. 145: 87–94

O'Toole G, Kaplan HB & Kolter R (2002) Biofilm formation as microbial development. Ann. Rev. Microbiol. 54: 49–79

Palmen R & Hellingwerf KJ (1997) Uptake and processing of DNA by *Acinetobacter calcoaceticus*- a review. Gene 192: 179–190

Stewart GJ & Carlson CA (1986) The biology of natural transformation. Ann. Rev. Microbiol. 40: 211–235

Stewart GJ, Carlson CA & Ingraham JL (1983) Evidence for an active role of donor cells in natural transformation of *Pseudomonas stutzeri*. J. Bacteriol. 156: 30–35

Stewart PS & Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358: 135–138

Stoodley P, Lewandowski Z, Boyle JD & Lappin-Scott HM (1999) Structural deformation of bacterial biofilms caused by short-term fluctuations in flow velocity: an *in situ* demonstration of biofilm viscoelasticity. Biotech. Bioeng. 65: 83–92

Stoodley P, Sauer K, Davis DG & Costerton JW (2002) Biofilms as complex differentiated communities. Ann. Rev. Microbiol. 56: 187–209

Strong LC, McTavish H, Sadowsky MJ & Wackett LP (2000) Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. Environ. Microbiol. 2: 91–98

Tiirola MA, Wang H, Paulin L & Kulomaa MS (2002) Evidence for natural horizontal transfer of the *pcpB* gene in the evolution of polychlorophenol-degrading *Sphingomonads*. Appl. Environ. Microbiol. 68: 4495–4501

Timmis KN & Pieper DH (1999) Bacteria designed for bioremediation. Trends Biotechnol. 17: 200–204