Effect of Pyocyanin on a Crude-Oil-Degrading Microbial Community

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Pseudomonas aeruginosa is an n-alkane degrader that is frequently isolated from petroleum-contaminated sites and produces factors that enhance its competitiveness and survival in many environments. In this study, one such factor, pyocyanin, has been detected in an oil-degrading culture containing P. aeruginosa and is a redox-active compound capable of inhibiting microbial growth. To examine the effects of pyocyanin further, an oil-degrading culture was grown with and without 9.5 μM pyocyanin and microbial community structure and oil degradation were monitored for 50 days. Denaturing gradient gel electrophoresis (DGGE) analysis of cultures revealed a decrease in the microbial community diversity in the pyocyanin-amended cultures compared to that of the unamended cultures. Two members of the microbial community in pure culture exhibited intermediate and high sensitivities to pyocyanin corresponding to intermediate and low levels of activity for the antioxidant enzymes catalase and superoxide dismutase, respectively. Another member of the community that remained constant in the DGGE gels over the 50-day culture incubation period exhibited no sensitivity to pyocyanin, corresponding to a high level of catalase and superoxide dismutase when examined in pure culture. Pyocyanin also affected the overall degradation of the crude oil. At 50 days, the culture without pyocyanin had decreased polycyclic aromatic hydrocarbons compared to the pyocyanin-amended culture, with a specific reduction in the degradation of dibenzothiophenes, naphthalenes, and C29 and C30 hopanes. This study demonstrated that pyocyanin influenced the diversity of the microbial community and suggests the importance of understanding how interspecies interactions influence the degradation capability of a microbial community.

Degradation of crude oil in the environment by autochthonous microbial communities has been well documented, and individual microorganisms capable of metabolizing components of crude oil have been isolated from a variety of ecosystems (2, 28, 32). Individual microorganisms are capable of degrading only a limited number of crude oil components; thus, more extensive degradation of oil depends on the presence of metabolically diverse microbial communities (2, 8, 18, 21, 29, 39). However, microbial communities are not static, and factors affecting the composition of the community will alter their degradation potential. Alterations in community degradation potential due to physicochemical changes (i.e., temperature, nutrient availability) have been characterized (4, 6, 14, 37). However, little is known about the connection between crude oil and microbial interspecies interactions and how this influences the overall degradation capacity of the community.

Microbial community profiles shift as a result of environmental exposure to crude oil because of the growth and loss of microorganisms capable and incapable of metabolizing crude oil components (19, 41). However, within populations of crude oil-degrading microorganisms, interactions resulting from each microorganism’s metabolic capability can be synergistic or detrimental to further oil degradation (3, 13). For example, the production of rhamnolipid biosurfactants by Pseudomonas aeruginosa has been shown to enhance the rates of octadecane (46) and hexadecane (1) uptake and degradation. However, it has also been demonstrated that the presence of P. aeruginosa isolates in crude oil-degrading enrichment cultures decreases microbial diversity and aromatic degradation (21). In the later study, two oil-degrading microbial communities were initiated with soil from a polycyclic aromatic hydrocarbon (PAH)-contaminated site (20, 21). One community was enriched in the laboratory on oil containing n-alkanes, while the second was enriched on oil lacking n-alkanes. The culture developed in the presence of n-alkanes was characterized by reduced microbial diversity, the presence of P. aeruginosa, and substantially reduced PAH degradation, while the culture developed in the absence of n-alkanes contained a more diverse community, lacked the presence of P. aeruginosa, and was capable of aromatic degradation. Thus, the presence of P. aeruginosa in crude oil-degrading cultures may influence microbial community diversity, hence influencing the efficiency of crude oil degradation.

P. aeruginosa is frequently isolated from petroleum-contaminated sites and is capable of producing metabolites (i.e., alginate, rhamnolipid, pyocyanin) that enhance its competitiveness and survival (24, 46). For example, the water-soluble secondary metabolite pyocyanin (1-hydroxy-5-methylphenazine) has demonstrated antimicrobial activity against a variety of microorganisms (5, 24, 33). While the direct mechanism of pyocyanin toxicity remains unclear, the wide range of biological activity is thought to be due to its ability to catalyze the formation of toxic radicals such as superoxide (O2·−) and hydrogen peroxide (H2O2) (24). It has been proposed that microorganisms expressing high levels of the antioxidant enzymes catalase and superoxide dismutase (SOD) could tolerate pyocyanin more effectively than microorganisms not expressing or expressing low levels of the enzymes (5, 24, 25). Further, it is thought that pyocyanin production may be coupled to intracellular ATP...
levels, resulting in increased pyocyanin production under carbon- or nutrient-limited conditions (43). Thus, under limiting conditions, the growth of P. aeruginosa and the subsequent production of pyocyanin may alter the microbial community structure by inhibiting the growth of microorganisms sensitive to pyocyanin.

Little is known about how microbial interspecies interactions influence the degradation of crude oil. In this study, an antimicrobial compound, pyocyanin, was isolated and identified from a crude oil-degrading culture containing two strains of P. aeruginosa (20, 31). The remaining oil was extracted three times with dichloromethane, dried over sodium sulphate, and analyzed by gas chromatography (GC) with flame ionization detection (FID). The resulting degraded BLC (DBLC) oil is similar to the parent oil, with the exception that it lacks most of the n-alkanes (see Fig. 5a).

**MATERIALS AND METHODS**

**Oil composition.** To generate the oil used in this study, n-alkane-containing oil (Bonny Light Crude [BLC]; 56% saturates, 31% aromatics, 11% polars, 2% asphaltenes, 35.3° API gravity) was predegraded for 30 days with a coculture of P. aeruginosa (20, 31). The remaining oil was extracted three times with dichloromethane, dried over sodium sulphate, and analyzed by gas chromatography (GC) with flame ionization detection (FID). The resulting degraded BLC oil was observed to degrade culture containing two strains of P. aeruginosa and the functional diversity of the culture was examined over a 50-day period. Addition of pyocyanin to crude oil-degrading cultures in the laboratory decreased the diversity of the microbial community and resulted in reduced oil degradation.

**Experimental design.** To examine the effects of pyocyanin on a crude oil-degrading microbial community, 1% transfers of the original DLC enrichment culture described above were grown in 10 ml of BMTM (23) supplemented with 125 ml Erlenmeyer flasks with Telfon-lined screw caps for 30 days at 200 rpm and 30°C and have been transferred monthly (4% inoculum) for approximately 80 months (20, 21). The LC enrichment culture was developed on BLC oil, while the DLC enrichment culture was developed on DBLC oil (20). The LC culture is characterized by reduced microbial diversity, the presence of two distinct strains of P. aeruginosa (31), and degradation of n-alkanes and more complex saturates [i.e., C28 17α(H)21β(H)-bopane] found in crude oil but reduced aromatic and heterocyclic degradation (9, 20, 21). The DLC enrichment culture was observed to have a more diverse microbial community, lacked P. aeruginosa, and has been shown to degrade n-alkanes, hopanes, aromatics, and heterocycles. Spectrophotometric analysis (15) of LC and DLC culture supernatants after 30 days of growth on BLC or DBLC oil revealed an average of 9.5 μM pyocyanin in triplicate LC but not DLC culture supernatants. The concentration of pyocyanin observed in our cultures is similar to that observed by others and was the foundation of the studies described below.

**Microbial community structure.** At each time point, 1-ml aliquots were removed from triplicate cultures and total community DNA was obtained by cell lysis, phenol-chloroform extraction, and ethanol precipitation by the CTAB method, followed by PCR amplification with primers 5570F and 9206GCR (17). Amplification was done with a Techne Geneclamp universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) (30). Briefly, samples were run on an 8% polyacrylamide gel in 1× TAE containing a 40 to 60% denaturing gradient (7 M urea and 40% formamide). Electrophoresis was carried out for 18 h at 50 V and 60°C. The gels were stained for 1 h with SYBER Green I (Molecular Probes, Eugene, Ore.) and analyzed with a Bio-Rad model 1000 VersaDoc imaging system (Bio-Rad Laboratories).

**Phylogenetic analyses.** Sequences were confirmed and hand aligned with the BioEdit sequence alignment editor (22). Sequences were submitted to the advanced BLAST search program (National Center for Biotechnology Informa-
RESULTS

Pyocyanin identification. To determine if pyocyanin was present in crude oil-degrading cultures, chloroform-acid extracts of supernatant from the LC and DLC enrichment cultures were analyzed by HPLC fractionation and mass spectrometry. Analysis of culture extracts after 30 days of growth on oil by reverse-phase HPLC yielded a single UV-absorbing species in the LC but not in the DLC culture extracts (Fig. 1). Further analysis of this peak by mass spectromscopy demonstrated a protonated molecular ion cluster at *m/z* 211/212, thus identifying the peak as pyocyanin (Fig. 2), similar to previous research (42). Pyocyanin isolated from the LC culture demonstrated a mass spectrum identical to that of the pyocyanin standard (data not shown). In addition, throughout the time course experiment, HPLC analysis of the pyocyanin-amended DLC cultures demonstrated that pyocyanin remained stable at an average of 9.5 μM in triplicate cultures over the 50-day incubation period.

Microbial community structure. DGGE profiles were used to examine the overall effect of pyocyanin on microbial community structure. At day 3, the DGGE profile of the DLC enrichment culture without pyocyanin showed six observable bands (B1 to B6; Fig. 3a). The profile of the DLC culture
amended with pyocyanin showed four bands that are similar to those of the unamended culture (B2, B3, B4, and B6), while two bands (B1 and B5) were not resolved. The band between B2 and B3 is a result of nonspecific amplification of B4 and is observed in both pyocyanin-amended and unamended cultures. After 14 days, the banding profile of the unamended culture remained similar to that seen on day 3, with the exception of the appearance of a new band (B7), which was not apparent in the pyocyanin-amended culture. Also, at day 14, the faint appearance of B1 was observed in the pyocyanin-amended cultures. At 50 days, the unamended DLC culture remained stable, demonstrating seven distinct bands (B1 to B7). After 50 days, all bands, with the exception of B5, were observed in the pyocyanin-amended cultures. Serial dilution

FIG. 4. Phylogenetic tree, constructed by the neighbor-joining method, showing the relationship of bands excised from DGGE gels to 16S rRNA gene sequences of previously described bacteria. Numbers represent confidence estimates determined by bootstrap analysis with 1,000 replicates. Thermotoga subterranea was used as the outgroup.
isolates (DLC21, -22, -23, and -25) were compared to com-
bers of the microbial community. DNAs extracted from these
and plating of the DLC cultures with and without pyocyanin
amended with pyocyanin. All traces are representative of triplicate
DBLC remaining after 50 days of incubation with the DLC culture
amended with pyocyanin. (d) DBLC remaining without pyocyanin. (c) DBLC remaining after 14 days of incubation with the DLC culture
amended with pyocyanin. (b) Uninoculated DBLC control at 14 days. (a) Unamended DLC cultures.

FIG. 5. GC-FID traces of DBLC oil extracted after 14 and 50 days
of incubation with the DLC enrichment culture with and without
pyocyanin-amended DLC cultures.

Concentrations of PAHs were normalized to oleanane as a measure of PAH degradation.

TABLE 1. Ratios of PAHs to oleanane and concentrations of DBTs, NPHs, and hopanes remaining after 14 and 50 days of incubation
with pyocyanin-amended or unamended DLC enrichment cultures

<table>
<thead>
<tr>
<th>Incubation time and culture</th>
<th>PAH/oleanane ratio</th>
<th>Concn (ng ml of oil$^{-1}$) ± SEM$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DBT</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBLC$^{b}$</td>
<td>9.5 ± 0.7</td>
<td>233 ± 15</td>
</tr>
<tr>
<td>D$^{d}$</td>
<td>7.5 ± 0.9</td>
<td>167 ± 34</td>
</tr>
<tr>
<td>D/P$^{d}$</td>
<td>9.7 ± 0.7</td>
<td>229 ± 5</td>
</tr>
<tr>
<td>50 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBLC$^{b}$</td>
<td>8.1 ± 0.8</td>
<td>264 ± 2</td>
</tr>
<tr>
<td>D$^{d}$</td>
<td>6.3 ± 1.1</td>
<td>181 ± 6</td>
</tr>
<tr>
<td>D/P$^{d}$</td>
<td>8.6 ± 0.6</td>
<td>157 ± 5</td>
</tr>
</tbody>
</table>

$^{a}$ Concentrations of PAHs were normalized to oleanane as a measure of PAH degradation.

$^{b}$ The data are for triplicate samples. C1 to C4 represent carbon numbers of alkyl groups in alkylated homologues.

$^{c}$ Uninoculated control.

$^{d}$ Unamended DLC cultures.

Pyocyanin-amended DLC cultures.
the control. However, after 50 days, both the DLC cultures with and without pyocyanin had decreased concentrations of DBTs compared to the control (29 and 22% reductions, respectively). The second class of compounds affected by the presence of pyocyanin was the NPHs. At days 14 and 50, GC-MS analysis demonstrated decreased concentrations of NPHs remaining in the DLC cultures without pyocyanin (61 and 36%, respectively) while the concentrations in the pyocyanin-amended cultures remained similar to those in the controls. After 14 days, no substantial degradation of either C29 or C30 hopane was demonstrated by the DLC cultures with and without pyocyanin compared to that of the control. However, after 50 days, the DLC culture without pyocyanin contained decreased concentrations of both C29 and C30 hopanes (55 and 48%, respectively) while the DLC culture amended with pyocyanin remained similar to the control. The individual isolates from the DLC culture (DLC21, -22, -23, and -25) demonstrated no growth on either DBT or NPH.

Catalase and SOD activities. When they were grown on TSB and amended with 9.5 μM pyocyanin at the onset of log phase, the growth of isolates DLC21, -22, and -23 was affected to various degrees compared to that of unamended cultures. DLC25 was unable to grow on TSB, so it was not tested further. The final cell densities of DLC21 and DLC22 were significantly (P < 0.01, analysis of variance) reduced, by 15 and 10%, respectively, when they were amended with pyocyanin, while the cell density of DLC23 was not affected (Fig. 6). Furthermore, protein extracts from the three isolates demonstrated different catalase and SOD activity levels (Table 2). Isolate DLC21 demonstrated the lowest activity levels for both catalase and SOD, while isolate DLC23 demonstrated the highest levels of activity in both assays. Compared to DLC21 and -23, DLC22 demonstrated intermediate catalase and SOD enzyme activity levels. In this study, individual isolates demonstrated no growth on crude oil, thus requiring pyocyanin inhibition to be measured under nutrient-rich conditions. While isolates DLC21 and -22 demonstrated reduced growth in the presence of pyocyanin under these conditions, it is possible that pyocyanin would have an increased effect on these members of the microbial community grown on crude oil.

**DISCUSSION**

Biodegradation of crude oil is a complex process requiring a metabolically diverse microbial community. Throughout this process, the microbial community profile often shifts corresponding to the degradation of each oil fraction (2). For example, during the degradation of n-alkanes, which are among the first compounds attacked, bacteria within the oil-degrading community possessing group 1 alkane hydroxylase genes are first selected, providing for the degradation of short-chain alkanes (34). This selection is often followed by the growth of bacteria possessing group 2 and 3 alkane hydroxylase genes and capable of degrading long-chain alkanes. This suggests that upon initial exposure to crude oil, the microbial community profile would shift toward microorganisms, such as *P. aeruginosa*, capable of degrading the n-alkane component of crude oil. This population shift may establish either beneficial or antagonistic interspecies interactions that ultimately result in altered overall community degradation potential. For example, we have previously demonstrated that the presence of *P. aeruginosa* in crude oil-degrading enrichment cultures decreases microbial diversity and aromatic degradation (20).

In this study, the antibacterial compound pyocyanin was identified by liquid chromatography-MS from the supernatant of a crude oil-degrading enrichment culture containing two isolates.
distinct strains of _P. aeruginosa_ (31), and it was further demonstrated that pyocyanin reached a concentration of 9.5 µM in the culture supernatants. While the direct mechanism of pyocyanin-induced bactericidal activity remains unclear, previous research has demonstrated bacterial toxicity to micromolar concentrations of pyocyanin (5, 24). For instance, when the medium was amended at mid-exponential phase with pyocyanin at the same concentration used in our studies (9.5 µM), the growth and viability of the gram-positive bacterium _Micrococcus luteus_ were significantly reduced (5). Also, Hassan and Fridovich demonstrated that the generation time of _Escherichia coli_ cells growing on glucose-minimal medium increased from 43 to 145 min when they were supplemented with 10 µM pyocyanin (24). Therefore, the concentration of pyocyanin found in our crude oil-degrading enrichment culture could influence pyocyanin-sensitive members of the microbial community.

Analysis of the DLC enrichment culture with and without pyocyanin amendment over a 50-day incubation period demonstrated that the presence of pyocyanin altered the composition of the community. DGGE analysis and single-isolate studies revealed that individual members of the community exhibited various degrees of sensitivity to pyocyanin. For example, the growth of isolates DLC21, -22, and -25 was suppressed by the presence of pyocyanin while the growth of DLC23 was not affected. Various levels of susceptibility to pyocyanin have been reported for individual microorganisms, and susceptibility to pyocyanin is thought to depend on the rate of pyocyanin uptake and the level of antioxidant enzyme (SOD and catalase) activity (5, 24, 25, 27, 33, 40). For example, pyocyanin toxicity has been shown to decrease when _E. coli_ cells are grown on nutrient-rich medium that supports higher catalase and SOD activity levels. Also, _P. aeruginosa_ isolates are known to increase antioxidant activity when grown under conditions stimulating pyocyanin production (low nutrient), resulting in cellular protection (25). Similarly, in this study, DLC23 contained the highest catalase and SOD activity levels of the three bacteria isolated from our crude oil-degrading cultures and was not sensitive to pyocyanin. While it is currently assumed that differential expression of catalase and SOD activities is the principal means of pyocyanin resistance, it is also possible that other general antibiotic resistance mechanisms play a role in pyocyanin resistance (5).

The presence of pyocyanin not only affected the composition of the community but also altered the degradation capability of the culture. In pyocyanin-amended cultures, the degradation of DBTs was decreased at day 14 but not at day 50 compared to that in unamended cultures. While it is unable to grow on DBT alone, it is interesting that the growth of DLC22 was suppressed early during the time course and corresponded to reduced degradation of DBTs. However, after 50 days, DLC22 was observed in the pyocyanin-amended cultures, which may correspond to the increased degradation of DBTs. The ability of DLC22 to overcome complete inhibition by pyocyanin could be related to an increase in the catalase and SOD activity levels. Furthermore, DLC21 contains the lowest catalase and SOD activities of the isolates tested and is not observed in DGGE profiles of pyocyanin-amended cultures throughout the time course. Also, while catalase and SOD activities were not available for DLC25, DGGE analysis indicates that this isolate appears to be moderately sensitive to pyocyanin, with growth suppressed only early in the time course. It is possible that these pyocyanin-sensitive bacteria are responsible for the degradation or partial transformation of the aromatic and complex saturates examined in this study.

The microbial interactions occurring during crude oil degradation are not well understood and most likely include numerous positive and negative interactions. For example, toxicity of PAHs, such as NPH, to certain bacteria has been observed (10). Furthermore, degradation of PAHs by individual isolates often does not mirror PAH degradation in complex mixtures such as crude oil (12, 35). In these complex contaminant mixtures, partial transformation of PAHs, often occurring through what has been described as a cometabolic process (7, 36), is more likely to occur (13). Thus, while the degradation of DBTs, NPHs, and hopanes has not yet been linked to the isolates examined in this study, the decreased degradation of these compounds in the presence of 9.5 µM pyocyanin suggests that pyocyanin-sensitive bacteria may be involved in their partial or complete degradation.

While much is known about the effects of _P. aeruginosa_-produced factors in clinical settings, much less is known about how they influence microbial community interactions within environmental settings. In this study, pyocyanin was demonstrated to influence the functional diversity of a crude oil-degrading enrichment culture. While pyocyanin is one of many factors that could influence community diversity and degradation potential, the presence of this factor in our cultures resulted in reduced microbial diversity, as well as reduced overall community degradation capability. This represents one of the first studies to examine how pyocyanin production by an individual member of a crude oil-degrading microbial community can influence the overall composition and function of that community. Lastly, the data suggest the importance of understanding how environmental contaminants impact interspecies interactions within a microbial community during a bioremediation process.

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