Biodegradation **7**: 415–423, 1997.

Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers

Pia A. Willumsen & Ulrich Karlson
Department of Marine Ecology and Microbiology, National Environmental Research Institute, P.O. Box 358, Frederiksborgvej 399, DK-4000 Roskilde, Denmark

Accepted 21 October 1996

Key words: biodegradation, bioemulsifier, biosurfactant, polyaromatic hydrocarbons, soil bacteria

Abstract

Fifty-seven bacterial strains were isolated from PAH-contaminated soils using PAH-amended minimal medium. The isolates were screened for their production of biosurfactants and bioemulsifiers when grown in liquid media containing selected PAHs. The results suggest that many, but not all, of the isolates are able to produce biosurfactants or bioemulsifiers under the experimental conditions. The majority of the strains isolated on phenanthrene, pyrene, and fluoranthene were better emulsifiers than surface tension reducers and the stability of the formed emulsions was in general high. The strains isolated on anthracene were in general better in lowering the surface tension than in forming emulsions. In all strains, reduction of surface tension and emulsion formation did not correlate. However, in the majority of strains the two factors were associated with the bacterial cell surfaces, rather than the culture supernatants. Nevertheless, supernatants from selected surfactant-producing anthracene isolates increased the aqueous solubility of anthracene. Although a significant potential for surfactant and emulsifier production in the microbiota of the PAH-contaminated soils was found in this study, the ability of individual strains to mineralize PAHs did not coincide with production of surface-active compounds.

Introduction

Bioremediation of PAH-contaminated soil is thought to be limited by the low bioavailability of sorbed and nonaqueous phase contaminants (Cerniglia 1992, Wilson & Jones 1993). The addition of chemically or biologically produced surfactants (surface-active agents) to PAH-contaminated soils has been suggested as a method of solubilization and of enhancing the availability of the hydrophobic aromatic compounds. Chemical surfactant solutions were shown to increase the aqueous solubility and the mobility of PAHs in soil (Edward et al. 1991; Liu et al. 1991; Vigon & Rubin 1989). Increased degradation of PAHs due to use of surfactants has been reported (Guerin & Jones 1988; Aronstein et al. 1991; Tiehm 1994). However, inhibition of degradation due to toxicity of the surfactants or reduced bioavailability of micelle solubilized PAHs has also been observed (Laha & Luthy 1991; Rock & Alexander, 1995; Volkering et al. 1995). The contradictory findings could stem from different surface properties of the bacterial degraders applied in those studies.

Surface properties of the bacterial cell, such as its ability to adhere to the surface of hydrophobic compounds, the hydrophobicity of the cell surface, and the biosurfactant or bioemulsifier-like chemistry of the cell envelope, are likely determinants of how resistant to added surfactants the cells are and of how well the cell walls are capable to interact with surfactant micelles. Furthermore, the excretion of biosurfactant may allow the organism to more intimately associate with surfactant micelles (surfactant solubilized PAHs).

Biosurfactants can be divided into two major categories. The low molecular weight surfactants (eg. glycolipids, sophorolipids, trehalose lipids, fatty acids and phospholipids) consist of a hydrophobic and a hydrophilic part. Such compounds have, when present in an aqueous medium, the capability to decrease the surface tension of the medium. The high mole-

cular weight polymers, the amphipathic polysaccharide bioemulsifiers, however, do not necessarily consist of hydrophobic/hydrophilic moieties. In an aqueous medium, bioemulsifiers affect the formation of an emulsion and its stability, but the presence of bioemulsifiers does not necessarily, like with biosurfactants, cause a reduction in the surface tension of the medium (Cooper 1986; Gutnick & Shabtai 1987; Van Dyke 1991).

An alternative to using surfactants in bioremediation may be the addition of biologically produced emulsifiers, or the stimulation of microbial production of biosurfactants and bioemulsifiers in the contaminated soil. A microorganism's ability to reduce the surface tension and/or to form an emulsion can either be due to excretion of extracelluar products or can be associated with characteristics of the cell surface (Zajic & Seffen 1984; Bosch et al. 1988, Van Dyke 1991). Either situation are of potential interest in a soil clean-up situation in order to enhance the mobilization and/or the solubilization of the hydrophobic PAHs.

Biosurfactants and bioemulsifiers are synthesized by bacteria, yeasts and fungi during growth on various carbon sources, in particular during growth on hydrophobic substrates (Gerson & Zajic 1979). Production of emulsifiers and surfactants by hydrocarbon-degrading bacterial strains has been reported. The environmental sources of these strains were diesel oil and hydrocarbon contaminated bottom sediment and surface water (Broderick & Cooney 1979); hydrocarbon contaminated subsurface soil (Mac Elwee et al. 1990; Francy et al. 1991) and unleaded gasoline contaminated soil (Allen et al. 1992). None of the surfactants or emulsifiers produced were directly related to growth on PAHs or coal tar. To our knowledge the bacterial production of such compounds in coal tar contaminated soil has not been investigated to date.

The purpose of this study was to determine whether bacterial strains isolated from PAH-contaminated soils are able to produce surfactants or emulsifiers when grown in the presence of selected PAHs. Fifty-seven isolates were screened for their production of surfactants or emulsifiers when grown in a liquid medium containing a selected PAH as the sole source of organic carbon. Furthermore, the strains' capacity to mineralize PAHs was determined.

Materials and methods

Chemicals and solutions

All chemicals were of analytical grade or better. Radiochemicals used were [4,5,9,10-^{14}C]pyrene, specific activity 56 mCi/mmol, >95% radiochemical purity (Amersham); [9-^{14}C]phenanthrene, specific activity 8.3 mCi/mmol, >98% radiochemical purity (Sigma); [sidering-U-^{14}C]anthracene, specific activity 11.2 mCi/mmol, >89% radiochemical purity (Amersham); and [3-^{14}C]fluoranthene, specific activity 45 mCi/mmol, >95% radiochemical purity (Sigma). Stock solutions of phenanthrene, anthracene, pyrene and fluoranthene were prepared in acetone.

Media

All media used in these studies were prepared in distilled water purified on a Milli-Q_{plus} apparatus (Millipore) to a resistivity > 18.2 MΩcm^{-1}.

For extraction and dilution of bacteria from soil a buffered Winogradsky-solution (W) (Holm & Jensen 1972) was used containing per litre: 0.25 g K_2HPO_4; 0.125 g $MgSO_4$; 0.125 g NaCl; 0.0025 g $Fe_2(SO_4)_3$; 0.0025 g $MnSO_4$. pH 6.5- 6.7. For the isolation of potentially PAH-degrading bacteria medium M9s (modified after Maniatis et al. 1982 and Heitkamp & Cerniglia 1988a) was used containing per litre: 6 g Na_2HPO_4; 3 g KH_2PO_4; 0.5 g NaCl; 1 g NH_4Cl; 250 µg yeast extract; 250 µg soluble starch; 250 µg peptone and 2.5 ml of the following solution of trace elements (Bauchop & Elsden, 1960) (per litre): 5.37 g MgO; 1 g $CaCO_3$; 2.25 g $FeSO_4 \cdot 7H_2O$; 0.887 g $ZnSO_4 \cdot 7H_2O$; 0.13 g $CuSO_4 \cdot H_2O$; 0.14 g $CoSO_4 \cdot 7H$- $_2O$; 0.01 g H_3BO_3; 0.42 g $MnSO_4 \cdot H_2O$; 2.5 g $FeSO_4 \cdot 7H_2O$; 61.62 g $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.4. For M9s-plates 7 g/l agarose (Sea Kem, GTG. FMC Bioproducts) was used. The minimal salt medium (MSu) used for screening of biosurfactant and bioemulsifiers production was identical to M9s, except for the N- source; 3 g/l $(NH_4)_2SO_4$. Liquid PAH-containing media were prepared by adding 20 mg/l of the respective PAH to sterile flasks. The solvent was allowed to evaporate before sterile M9s or MSu was added. The content was sonicated (Metason 200, Struers Denmark) for 5 min in order to increase the surface area of non-solubilized PAH-crystals.

The purity of the isolates was confirmed by plating on TSA which consisted of 3 g/l TSB (Tryptic Soy Broth, Difco) and 15 g/l Bacto agar (Difco).

PAH-coated M9s-plates were prepared by a method modified after Kiyohara et al. (1982). One ml of PAH-acetone stock solution (4 mg/ml) was transferred to M9s-agarose plates. The solvent was allowed to evaporate for 2 h.

Extraction and isolation of bacteria from soil

PAH-contaminated soil samples were obtained from a former gasworks site at Frederiksberg, Denmark. To extract bacteria from soil, an aliquot of soil was mixed with W-solution (1:9) in a blender (Waring, New Hartford, Connecticut) for 3 times 1 min with subsequent cooling on ice after each blending period. The slurry was allowed to rest for 10 min and serial dilutions in the W-solution were prepared from the supernatants.

Isolates were obtained by plating the serial dilutions on M9s-agarose plates coated with selected PAH-compounds. The plates were incubated in the dark at 15°C for 14–48 days. Colonies surrounded by a clearing zone in the PAH layer (PAH-degrading bacteria), as well as colonies not surrounded by a clearing zone (presumptive PAH-degrading bacteria), but representing different morphological types, were picked and purified by repeated transfer on TSA and M9s+PAH.

To identify and eliminate strains able to grow on residual acetone in the PAH surface layer and/or impurities in the agarose, the isolates were double-plated on M9s-plates without acetone and M9s-plates to which acetone had been transferred as for preparation of PAH-coated plates.

Selected strains were transferred to 60 ml glass tubes containing 10 ml M9s supplemented with 20 mg/l of one of the selected PAHs as the only carbon and energy source. The tubes were incubated in a tilted position on a shaker in the dark at 25 °C. The capability of the isolates to grow in the presence of the selected PAHs was determined by visual examination of turbidity in the medium. Isolates which failed to grow were excluded from further screening.

The known PAH-degraders, strains CRE7, CRE11 (Mueller et al. 1994); *Sphingomonas paucimobilis* EPA505 (Mueller et al. 1990) and *Mycobacterium* sp. (Heitkamp et al. 1988b and 1988c), were included in the experiments. All strains were maintained at -80 °C in glycerol (20% vol/vol).

Bacterial growth experiment

Inoculum for each experiment was pregrown in 25 ml MSu-medium containing 20 mg/l PAH as the sole carbon source. The flasks were incubated at 20 °C on a shaker table at 170 rpm. Growth was determined by visual examination of the turbidity. One ml of the pregrown culture was transferred into a 60 ml glass tube containing 25 ml MSu medium and 20 mg/l PAH as the sole source of carbon, resulting in an initial density of $OD_{450nm} \geq 0.05$. The tubes were incubated at 20 °C oblique on a shaker table at 170 rpm. Growth was followed in the tubes by measurement of optical density at 450 nm using a UV/VIS Spectrophotometer (Unicam 8625, Unicam, England). Growth was defined as an increase in the initial OD_{450nm} by ≥ 0.2). Each strain was inoculated in duplicate. MSu containing the respective PAH was used as a blank.

Screening for biosurfactant and bioemulsifier production

Reduction of surface tension (ST)

A good biosurfactant producer was defined as one being able to reduce the surface tension of the growth medium by $\geq$20 mN/m compared with distilled water. The definition of this threshold level was adapted from the literature where a culture is considered promising if it reduces the surface tension of a liquid medium to 40 mN/m or less (Cooper 1986). Subsamples of 6 ml were taken during the exponential and stationary growth phases of the growth experiment. The ST was measured using a model K10 tensiometer (Krüss, Hamburg, Germany). The ring was cleaned with 99% ethanol and heated to redness. All glass and plastic equipment used were acid washed (HCl, 0.1 M). Samples were allowed to equilibrate for 2 h before measurements were made. Corrections were made for the dial readings and the ring geometry in order to arrive at surface tension values. After measuring the surface tension of the culture samples, the cells were removed by centrifugation at 11300x g for 20 min at 4°C. Culture samples were measured within 4 hours after sampling. Culture supernatant samples were stored at 4 °C until measurement. Each experiment was done in duplicate. All measurements were performed at 25 °C. The ST of Milli-Q water at 25 °C was used as zero-control.

Emulsifying capacity

The emulsifying capacity was determined using a modification of a method described by Bosch et al. 1988. Subsamples of 6 ml were taken from the cultures when the growth entered stationary phase of the growth experiment. These subsamples of culture, as well as

culture supernatant (11300x g, 20 min, 4°C), were overlaid by 10% vol/vol diesel oil (Fuel 2, Ekofisk) and vortexed for 20 s followed by 20 s manual shaking to create an optimum emulsion. Each experiment was done in duplicate. All glass and plastic equipment used were acid washed (HCl, 0.1 M). An emulsification index, "EI", was calculated as the ratio of the height of the emulsified layer to the height of the total oil phase. By repeating the reading after 24 hours, an indication of the stability of the emulsions was obtained. EI=0 indicates no emulsification and EI=1, 100% emulsification. A good emulsifier-producing strain was designated as one having an EI $\geq$ 0.5 (equals 50% emulsification of the diesel oil layer) 2 h after shaking. The definition of this threshold level was adapted from the literature where a culture is considered promising if it creates emulsions higher than 40% (Bosch et al. 1988). An emulsion was defined as stable if the EI after 24 h was 50% or better of the emulsion at 2 h.

Mineralization studies

The potential of the isolated bacteria to mineralize selected PAHs was determined using ^{14}C-labeled phenanthrene, pyrene, anthracene and fluoranthene. Sterile duplicate 60 ml glass tubes containing 10 ml MSu and 20 mg/l PAH, labelled with 8.3 Bq/mg, were inoculated with 1 ml of the respective pregrown cultures. The tubes were sealed with sterile silicone stoppers and incubated at 20 °C for 4–8 weeks oblique on a shaker table at 170 rpm. Sterile uninoculated as well as autoclaved inoculated controls were included. The $^{14}CO_2$ produced through mineralisation of the PAHs was collected in 400 µl 1 M KOH contained in plastic vials suspended from the silicone stoppers by a steel hook. Growth was followed by measurement of optical density as for growth experiments. Traps were exchanged at daily to weekly intervals, depending on growth. The amount of $^{14}CO_2$ collected was quantified by mixing the KOH with 1.5 ml Ready Gel Scintillation cocktail (Beckman) and 1.0 ml H_2O. After resting for at least 4 h in the dark to eliminate chemiluminescence, the samples were counted using a model LS 1801 scintillation counter (Beckman). The detection limit for mineralization ($x \pm 2s$ for the abiotic background) was 6.6, 5.9, 7.1, and 4.7% for phenanthrene, anthracene, fluoranthene and pyrene, respectively.

Determination of the aqueous solubility of crystalline anthracene

Isolates were grown in 25 ml MSu-medium containing 20 mg/l anthracene or glucose as the sole carbon sources. The flasks were incubated as described for the growth experiments. When the culture entered stationary phase, the culture broths were centrifuged at 11300 x g for 20 min at 4 °C. Samples of 5 ml of culture supernatant were transferred to glass tubes containing anthracene crystals. The mixtures were incubated at 25 °C on a shaker for 24 h. Crystal free subsamples were transferred to quartz cuvettes using glass pipettes. Concentrations of dissolved anthracene were determined at 354 nm using a Beckman DU®-70 spectrophotometer. The standard curve for solubilized anthracene was prepared with methanol solubilized anthracene. The respective supernatants were used as blanks. The results were compared with the solubilization of anthracene in MSu alone and MSu containing glucose (controls).

Results

Microbial isolates

A total of 57 strains, isolated from plates with phenanthrene (20), anthracene (9), pyrene (19) and fluoranthene (9), were able to grow in PAH-minimal medium. Those isolates were used in the subsequent screening assay, using the same PAH-compound. Whether the strains were able to grow in the presence of more than one of the PAH compounds was not investigated.

Surface activity

The isolates were screened for production of biosurfactants and bioemulsifiers when growing in phenanthrene, anthracene, pyrene or fluoranthene-amended medium.

High variability in surface tension measurements, emulsification capability and growth performance between replicates was often observed. Therefore, only with few isolates the reduction in surface tension or the formation of an emulsion above the defined critical levels was significant. However, an isolate was scored positive as biosurfactant or bioemulsifier producer, when the average plus the standard deviation was above the defined threshold levels. Results based

Table 1. Ability of the isolates to reduce the surface tension of an aqueous medium

	Exponential growth phase						Stationary growth phase					
	culture*			culture sup.*			culture*			culture sup.*		
ST red	≤ 10	11–19	≥ 20	≤ 10	11–19	≥ 20	≤ 10	11–19	≥ 20	≤ 10	11–19	≥ 20
pyrene	69	15	15	77	15	8	68	32	0	74	26	0
fluoranthene	80	0	20	60	40	0	44	33	22	44	56	0
phenanthrene	67	22	11	78	17	6	85	5	10	60	30	10
anthracene	13	25	63	25	50	25	0	33	67	0	56	44

Culture sup.: culture supernatant; ST red: reduction in surface tension (mN/m) at 25 °C; *: percentage based on the number of strains tested in the specific experiment.

on only one sample (because of inconsistencies in growth between replicates, lack of sampling in exponential growth phase or loss of sample during sample preparation) are not shown and were not included in the evaluation, but do in general support the results based on the duplicate samples.

Results of surface tension measurements for the isolates are presented in Table 1. Less than 20% of the tested phenanthrene, fluoranthrene and pyrene isolates were able to reduce the surface tension by ≥ 20 mN/m. The strains grown in the presence of anthracene, however, were in general good biosurfactant producers. In the exponential and the stationary growth phase, respectively, 63 and 67% of the strains grown in the presence of anthracene were able to reduce the surface tension in culture samples by ≥ 20 mN/m (Table 1). It was investigated whether the growth of three of these strains in the presence of anthracene actually increased the aqueous solubility of crystalline anthracene. All supernatants of the selected cultures grown in the presence of anthracene were able to significantly increase the aqueous solubility of anthracene, in contrast to supernatants from cultures grown in the presence of glucose (Table 2).

The degree of emulsification and the stability of the emulsions formed by the strains is presented in Table 3. Sixty-seven percent of all isolates were, in the culture samples, able to produce detectable emulsions with diesel oil. Among the isolates grown in the presence of pyrene, fluoranthene and phenanthrene, 71%, 57% and 58%, respectively, produced good emulsions (EI≥0.5) in culture samples. The strains grown in the presence of anthracene were in general poor emulsifiers. The stability of the emulsions was overall quite high, and presence of the cells had a significant stabilizing effect on the emulsions (Table 3).

No correlation was found between the reduction of surface tension and the emulsification index (EI).

Table 2. Aqueous solubility of anthracene in stationary phase culture supernatant from selected surfactant producing strains after growth on two different carbon sources

Strains	mg Ant/l at 25°C		St red*
	MSu+Ant	MSu+glucose	
A3	0.40	0.05	27.6 ± 6.7
A7	1.19	0.12	12.4 ± 1.0
CRE 11	0.73	0.38	27.6 ± 5.3
control	0.08	0.10	0.0 ± 0.0

Ant: anthracene; ST red*: reduction in surface tension (mN/m) at 25 °C, measured on a stationary phase culture grown in MSu+Ant; MSu: minimal salt medium; control: respective medium, non-inoculated.

Location of surface activity

The ability to reduce surface tension by more than 20 mN/m was higher in culture samples than in culture supernatant samples (Table 1). The ability to create a good emulsion (EI≥0.5) was associated with the cells (Table 3). However, 11% of our strains showed good surfactant production activity in the culture supernatant samples (stationary growth phase) and 12% of the strains demonstrated good emulsions in culture supernatant samples, indicating excretion of extracellular surface-active compounds into the culture.

PAH-degrading potential

The bacterial strains isolated in this study were grown in a liquid minimal salts medium containing trace quantities of peptone, yeast extract and soluble starch (250 µg/l) and PAHs (20 mg/l) as the sole sources of carbon. All isolates were able to grow in this medium but lag phases between 100 and 500 hours were observed for both the start-up and the adapted cultures. The content of peptone, yeast and soluble starch as well

Table 3. Capacity of the isolates to emulsify diesel oil

PAH	EI > 0 after 2h*		EI ≥ 0.5 after 2h*		Stable emulsion**.	
	culture	culture sup.	culture	culture sup.	culture	culture sup.
pyrene	100	36	71	21	100	80
fluoranthene	71	17	57	17	100	0
phenanthrene	68	11	58	6	85	50
anthracene	75	40	40	25	67	67

EI: emulsification index; culture sup: culture supernatant; *: percentage of strains, based on the number of strains tested in the specific experiment; **: percentage of strains, whose EI after 24 h was ≥50% of the EI after 2h, based on the number of strains with EI>0 after 2h.

Table 4. Relationship between surface activity and the ability to degrade selected PAHs, in percent of strains, based on the number of strains (in parantheses) which scored positive in the degradation assays

PAH	EI ≥ 0.5 ST red < 20 mN/m	EI ≥ 0.5 ST red ≥ 20 mN/m	EI < 0.5 ST red ≥ 20 mN/m	EI < 0.5 ST red < 20 mN/m
A				
pyrene (12)	56	0	0	44
fluoranthene (7)	60	20	0	0
phenanthrene (17)	56	0	0	13
B				
pyrene (12)	33	0	0	33
fluoranthene (7)	20	0	0	0
phenanthrene (17)	0	0	7	0

EI: emulsification index; ST red: reduction in surface tension (mN/m) at 25 °C; A: culture samples; B: culture supernatant samples.

as small quantities of carbon present in water implies that growth in the presence of an added PAH does not prove growth at the expense of, i.e. degradation of this compound.

The capacity of the isolates to degrade selected PAHs was determined using mineralization of radiolabeled PAHs, formation of clearing zones on PAH-coated agarose plates (Kiyohara et al. 1982) and/or colour changes in the growth medium indicating accumulation of intermediates (Gibson et al. 1984; Harayama et al. 1989; Mueller et al. 1990). A number of 12, 7, 17, and none of the tested potential pyrene, fluoranthene, phenanthrene and anthracene degrading strains, respectively, were able to degrade the PAHs under the experimental conditions applied in the tests.

This study was based on a screening of unknown strains and the growth and mineralization conditions were not optimized for every strain tested. Lack of optimum growth conditions for the specific strain can explain the observed irregularities in growth performance, the relatively long lag phases as well as the fact that none of the strains isolated on anthracene, including strain CRE7 and CRE11, which are known to degrade 2- and 3-rings PAHs, were able to mineralize anthracene, to produce clearing zones or to colour the growth medium. Another explanation for the lack of anthracene mineralization can be that these strains are able to only co-metabolize anthracene and that the possibly produced co-metabolites were not detected with the three assays applied.

Based on the results obtained in the surface activity and degradation assays, the relationship between surface activity and the ability to degrade PAHs was investigated (Table 4). Approximately 60% of the pyrene, fluoranthene and phenanthrene degrading isolates had an EI ≥ 0.5 without being able to reduce the surface tension ≥ 20 mN/m. With exception of 20% of the fluoranthene degraders, none of the strains tested were both good emulsifiers and surface tension reducers.

Discussion

The intent of this study was to determine whether microorganisms isolated from PAH-contaminated soils

are producing surface-active compounds when grown in the presence of selected PAHs.

The type, quantity and quality of surfactants produced by bacteria is reported to be affected by the nature of the carbon substrate (Singer et al. 1983), the concentrations of N, P, Mg, Fe and Mn ions in the medium (Atlas 1981; Cooper et al. 1981; Guerra-Santos et al. 1984, 1986; Haferburg et al. 1986) and culture conditions such as pH, temperature and agitation (Margaritis et al. 1979, Guerra-Santos et al. 1984, 1986). Furthermore, as cells age, increased autolysis can release the intracellular pools of surfactants (Kosaric et al. 1983). The high variability observed in measurements of surface tension, emulsifying capabilities and growth performance in this screening may, thus, be related to cell age and the cell's sensitivity to variations in growth factors within the micro-environment.

One of the most effective biosurfactants reported is surfactin from *Bacillus subtilis* which can lower the surface tension of an aqueous medium from 74 mN/m to 27 mN/m (Cooper & Zajic 1980). Bosch et al. (1988) have isolated a strain whose surfactant product was able to reduce the surface tension of the medium to 26 mN/m. Not all of our fifty-seven isolates were able to produce surfactants when cultured at the growth conditions applied in this assay. Furthermore, none of our isolates produced a surfactant able to reduce the surface tension to 27 mN/m. However, three of the anthracene and one of the pyrene grown strains were able to lower the surface tension of the culture to 35–40 mN/m.

The isolates grown in the presence of anthracene were in general good biosurfactant producers compared with the isolates grown in the presence of phenanthrene, pyrene and fluoranthene (Table 1). This fact might be related to low bioavailability of anthracene. This compound has a very low aqueous solubility (0.07–0.08 mg/l at 25°C) compared to the three other PAHs (1.29 mg/l, 0.14 mg/l and 0.26 mg/l, for phenanthrene, pyrene and fluoranthene, respectively (Sims & Overcash 1983; Pearlman et al. 1984)). Our results suggest that the surfactants produced by the isolates grown in the presence of anthracene actually increase the aqueous solubility of anthracene (Table 2). The production of the surfactants might thus help the strains to adapt to the low aqueous solubility of the substrate.

The emulsification activity of hydrocarbon degrading bacteria has previously been reported. Broderick and Cooney (1979) have screened 129 hydrocarbon-degrading strains for their ability to form emulsions. They report that for all 129 isolates stable emulsions between kerosene and an aqueous medium containing the bacterial cells were formed. Francy et al. (1991) found that exposure to hydrocarbons and biostimulation (nutrients and oxygen addition) encouraged growth of emulsifiers. Allen et al. (1992) found that all microbial isolates from subsurface material contaminated with unleaded gasoline demonstrated emulsification when overlaid with unleaded gasoline, whereas emulsification by cultures overlaid with kerosene and diesel fuel was weaker.

We found that 67% of our isolates were able to form detectable emulsions with diesel oil. One might speculate, that this relatively low percentage of emulsifiers among isolates from soil contaminated with PAHs, as opposed to soil contaminated with aliphatic hydrocarbons, might indicate that growth on PAHs does not require emulsification to the same extent as growth on aliphatic hydrocarbons. Alternatively, some essential growth factors for emulsification may have been lacking in our study. Nevertheless, the ability to create a good emulsion appeared to be more pronounced among our PAH-degrading isolates than the ability to lower the surface tension of the culture samples. Similar to Broderick and Cooney (1979) and Bosch et al. (1988), we found that the stability of the emulsions formed was overall quite high. The stability of the emulsions has been reported to be of importance for both the performance and the effectiveness of the emulsifier (Zosim et al. 1982; Parkinson 1985). However, stable and thick emulsions might decrease the bioavailability of the PAHs, similar to the effect observed for substrates solubilized in chemical surfactant micelles (Volkering et al. 1995). Therefore, more research is needed to elucidate the effect of those stable emulsions on the hydrocarbon- and PAH-degradation.

For aliphatic hydrocarbon-grown strains, Allen et al. (1992) found that the capability to form an emulsion was due, at least in part, to the presence of intact whole cells. However, approximately 85% of their isolates did retain emulsification abilities after removal of cells. Similar results have been reported by Broderick and Cooney (1979). We found that the surface tension reducing activity and the emulsification capability for most of the strains were due to cell surface-associated products or to characteristics of the cell surfaces. However, 11 and 12% of the total number of isolates showed good surface and emulsifying activity, respectively, in the cell free broth samples.

The production of surfactant has often been reported to be associated with growth. Both biosurfactants

and bioemulsifiers are, therefore, expected to be produced and excreted during the exponential growth phase (Gerson & Zajic 1978; Cooper 1986; Bosch et al. 1988; Allen et al. 1992). For all our good surface tension reducers, we found that the reduction in surface tension was initiated in the exponential growth phase. This indicates for these strains a relationship between surfactant production and growth in the presence of the selected PAHs. However, for two out of 57 strains, the reduction of surface tension was only noted in the stationary growth phase. Surfactant production was probably not growth-associated in those two cases. With two other isolates the reduction in surface tension was significantly higher in the stationary than in the exponential growth phase. These observations could be due to:

- some physiological phenomena resulting from the states of growth, e.g., a higher number of surfactant producing cells in the stationary phase than in the exponential growth phase, or
- a result of cell lysis.

However, based on our observations it is not possible to distinguish between the two alternatives.

Biosurfactants and bioemulsifiers are chemically two different kinds of products (Cooper 1986). In our screening of bacterial isolates from PAH-contaminated soils, no correlation was found between the reduction in surface tension and the ability to form emulsions. E.g., among our isolates grown in the presence of anthracene, in spite of the good surface tension reducing abilities, only 40% were able to form good emulsions between the diesel oil and the aqueous medium. Similarly, no correlation between the reduction in surface tension and the ability to form emulsions has been found for hydrocarbon-grown cells (Broderick & Cooney 1979; Bosch et al. 1988 and Allen et al. 1992).

Although a significant potential for surfactant production in the microbiota of the PAH-contaminated soils was found in this study, degradation of the PAHs did not correlate with the production of surfactants and emulsifiers by our isolates (Table 4). These conclusions are comparable with observations reported for aliphatic hydrocarbon degrading microorganisms, where it has been reported that not all hydrocarbon degrading strains are dependent on production of emulsifiers or surfactants to be able to degrade hydrocarbons (Gerson & Zajic 1978; Rapp et al. 1979; Allen et al. 1992).

Acknowledgments

Financial support from the Strategic Environmental Research Program, Denmark, is gratefully acknowledged. The authors wish to thank Novo Nordisk, Bagsværd, Denmark for loaning the tensiometer, and Vibeke Mikkelsen, Bente Rose Hansen, Janne Skalshøj and Tina S. Boldt for skilled technical assistance.

References

Allen PG, Francy DS, Duston KL, Thomas JM & Ward CH (1992) Biosurfactant production and emulsification capacity of subsurface microorganisms. Soil Decontamination Using Biological Processes. Karlsruhe, DECHEMA, Fedeal Republic of Germany

Aronstein BN, Calvillo YM & Alexander m (1991) Effects of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soil. Enviro. Sci Technol. 25: 1728–1731

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 45: 180–209

Bauchop T & Elsden SR (1960) The growth of microorganisms in relation to their energy. J.Gen. Microbiol. 23: 457–469

Bosch MP, Robert M, Mercadé ME, Espuny MJ, Parra JL & Guinea J (1988) Surface active compounds on microbial cultures. Tenside Surfactants Detergents 25 (4): 208–211

Broderick LS & Cooney JJ (1979) Emulsification of hydrocarbons by bacteria from freshwater ecosystems. Dev. Ind. Microbiol. 24: 425–434

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation, 3: 351–368

Cooper DG & Zajic JE (1980) Surface active compounds from microorganisms. Appl. Microbiol. 26: 229–253

Cooper DG, MacDonald CR, Duff SJB & Kosaric N (1981) Enhanced production of Surfactin from *Bacillus subtilis* by continuous product removal and metal cation addition. Appl. Environ Microbiol. 42(3): 408–412

Cooper DG (1986) Biosurfactants. Microbiol. Sci. 3 (5): 145–149

Edward DA, Luthy RG & Liu Z (1991) Solubilization of polycyclic aromatic hydrocarbons in micellar nonionic surfactant solutions. Environ. Sci. Technol. 25 (1): 127–133

Francy DS, Thomas JM, Raymond RL & Ward CH (1991) Emulsification of hydrocarbons by subsurface bacteria. J. Ind. Microbiol. 8: 237–246

Gerson DF & Zajic JE (1978) Surfactant production from hydrocarbons by *Corynebacterium lepus* and *Pseudomonas asphaltericus*. Dev. Ind. Microbiol. 19: 577–599

Gerson DF & Zajic JE (1979) Microbial biosurfactants. Process Biochemistry 14(7): 20–29

Gibson DT & Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds, Vol 13 (pp 181–242)

Guerin WF & Jones GE (1988) Mineralization of phenanthrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. 54(4): 937–944

Guerra-Santos L, Käppeli O & Fiechter A (1984) *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Environ. Microbiol. 48(2): 301–305

Guerra-Santos LH, Käppeli O & Fiechter A (1986) Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant pro-

duction on nutritional and environmental factors. Appl. Microbiol. Biotechnol. 24: 443–448

Gutnick DL & Shabtai Y (1987) Exopolysaccharide biopolymers. In: Kosaric N et al. (Ed) Biosurfactants and Biotechnology, Vol 25 (pp 211–246). Marcel Dekker Inc, New York

Haferburg D, Hommel R, Claus R & Kleber H (1986) Extracelluar microbial lipids as biosurfactants. Adv. Biochem. Eng/Biotechnol. 33: 53–93

Harayama S & Timmis KN (1989) Catabolism of aromatic hydrocarbons by *Pseudomonas*. In: Hopwood DD & Chater CF (Ed) Genitics of Bacterial Diversity (pp 151–171). Academic Press, Inc. London

Heitkamp MA & Cerniglia CE (1988a) Mineralization of polyaromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl. Environ. Microbiol. 54(6): p 1612–1614

Heitkamp MA, Franklin W & Cerniglia CE (1988b) Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. Appl. Environ. Microbiol. 54(10): 2549–2555

Heitkamp MA, Freeman JP, Miller DW & Cerniglia CE (1988c) Pyrene degradation by a *Mycobacterium*sp.: identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. 54(10): 2556–2565

Holm E, Jensen V (1972) Aerobic chemoorganotrophic bacteria of a Danish beech forest. Oikos 23: 248–260

Kiyohara H, Nagao K & Yana K (1982) Rapid Screen for bacteria degrading water-insoluble hydrocarbons on agar plates. Appl. Environ. Microbiol. 43: 454–457

Kosaric N, Gray NCC & Cairns WL (1983) Microbial emulsifiers and de-emulsifiers. In: Rehm HJ & Reed G (Ed) Biotechnology, Vol 3 (pp 576–592). Verlag Chemie, Dearfield Beach, Fl

Laha S & Luthy RG (1991) Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. Environ. Sci. Technol. 25(11): 1921–1930

Liu Z, Laha S & Richard GL (1991) Surfactant solubilization of polyaromatic hydrocarbons in soil-water suspensions. Wat. Sci. Technol. 23: 475–485

MacElwee C, Lee H & Trevors JT (1990) Production of extracelluar emulsifying agent by *Pseudomonas aeruginosa* UG1. J. Ind. Microbiol. 5: 25–32

Maniatis T, Fritsh EF & Sambrook J (1982) Molecular Cloning: A Laboratory Manual (pp 440). Coldspring Harbour Press. New York

Margaritis A, Kennedy K, Zajic JE & Gerson DF (1979) Biosurfactant production by *Norcardia erythropolis*. Dev. Ind. Microbiol. 20: 623–630

Mueller JG, Chapman PJ, Blattmann BO & Pritchard P (1990) Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Appl.Environ. Microbiol. 56(4): 1079–1086

Mueller JG, Lantz SE, Devereux R, Berg JD and Pritchard PH (1994) Studies on the microbial ecology of PAH biodegradation. In: Hinchee RE, Leeson A, Semprini L, Ong SK (Eds) Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds (pp 218–238). Lewis Publishers, Boca Raton, FL

Parkinson M (1985) Bio-surfactants. Biotechnol. Adv. 3: 65–83

Pearlman RS, Yalkowsky SH & Banerjee S (1984) Water solubilities of polynuclear aromatic and heteroaromatic compounds. J. Phys. Chem. Ref. Data, 13(2): 555–562

Rapp P, Bock H, Wray V & Wagner F (1979) Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on n-alkanes. J. Gen. Microbiol. 115: 491–503

Rock F & Alexander M (1995) Biodegradation of hydrophobic compounds in the presence of surfactants. Environ. Toxicol.Chem. 14 (7): 1151–1158

Sims RC & Overcach MR (1983) Polynuclears in soil- plant systems. Residue Review, 88: 1–68

Singer MEV & Finnery WR (1990) Physiology of biosurfactant synthesis by *Rhodococcus* species H13-A. Can. J. Microbiol. 36: 741–745

Tiehm A (1994) Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. 60(1): 258–263

Van Dyke MI, Lee H & Trevors JT (1991) Applications of microbial surfactants. Biotech. Adv. 9: 241–252

Vigon BW & Rubin AJ (1989) Practical consideration in the surfactant-aided mobilization of contaminants in aquifers. J. Water Pollut. Control Fed. 61(7): 1233–1240

Volkering F, Breure AM, van Andel JG & Rulkens WH (1995) Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 61(5): 1699–1705

Wilson SC & Jones KJ (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. Environ. Pollut. 81: 229–249

Zajic JE & Seffens W (1984) Biosurfactants. Crit. Rev. Biotechnol. 1 (2): 87–107

Zosim Z, Gutnick D & Rosenberg E (1982) Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 Emulsan. Biotech. Bioeng. XXIV: 281–292