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Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons

Abstract Polycyclic aromatic hydrocarbon (PAHs)-degrading bacteria may enhance the bioavailability of PAHs by excreting biosurfactants, by production of extracellular polymeric substances, or by forming biofilms. We tested these hypotheses in pure cultures of PAHs-degrading bacterial strains. Most of the strains did not substantially reduce the surface tension when grown on PAHs in liquid shaken cultures. Thus, pseudo-solubilization of PAHs in biosurfactant micelles seems not to be a general strategy for these isolates to enhance PAHs-bioavailability. Three semi-colloid *Sphingomonas* polysaccharides all increased the solubility of PAHs (Gellan 1.3- to 5.4-fold, Welan 1.8- to 6.0-fold and Rhamsan 2.4- to 9.0-fold). The increases were most pronounced for the more hydrophobic PAHs. The polysaccharide-sorbed PAHs were bioavailable. Mineralization rates of 9-[^{14}C]-phenanthrene and 3-[^{14}C]-fluoranthene by *Sphingobium* EPA505, were similar with and without sphingans, indicating that mass-transfer rates from PAHs crystals to the bulk liquid were unaffected by the polysaccharides. Biofilm formation on PAHs crystals may favor the diffusive mass transfer of PAHs from crystals to the bacterial cells. A majority of the PAHs-degraders tested formed biofilms in microtiter wells coated with PAHs crystals. For strains capable of growing on different PAHs; the more soluble the PAHs, the lower the percentage of cells attached. Biofilm formation on PAHs-sources was the predominant mechanism among the tested bacteria to overcome mass transfer limitations when growing on poorly soluble PAHs.

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Introduction

Microbial degradation of polycyclic aromatic hydrocarbons (PAHs) in soil and sediments is thought to be limited by the mass transfer of PAHs to the water phase; sorbed, crystalline and "non-aqueous phase liquid"-solubilized PAHs being unavailable to the PAHs-degrading bacteria (Volkering et al. 1992, Harms and Bosma 1997, Bosma et al. 1997). Generalizations regarding the bioavailability of PAHs may be inappropriate as there are important organism-specific differences in gaining access to PAHs (Guerin and Boyd 1992).

It is well known that bacteria growing on alkanes may produce biosurfactants to increase the alkane bioavailability (Itoh and Suzuki 1972; Oberbremer and Müller-Hurtig 1989). A similar strategy has been suggested for PAHs-degrading bacteria. Biosurfactants produced by a *Pseudomonas aeruginosa* growing on phenanthrene and naphthalene were shown to increase PAHs solubility, suggesting that the microorganism was promoting the bioavailability of its substrate (Déziel et al. 1996). Willumsen and Karlson (1997) on the other hand, screened 57 PAHs-degrading isolates for production of biosurfactants, but found no correlation between biosurfactant production and PAHs mineralization.

PAHs-degrading bacteria isolated from soil often belong to the sphingomonads (sensu lato) and the mycobacteria (Kästner et al. 1994; Mueller et al. 1997; Bastiaens et al. 2000; Ho et al. 2000; Johnsen et al. 2002b). Sphingomonads have an outer gluco-sphingolipid cell membrane, consisting of a hydrophilic mono- or tetra-saccharide and a lipophilic dihydrosphingosine residue (Kawahara et al. 1991; Kawasaki et al. 1994). The structure of the gluco-sphingolipids resembles the structure of surfactants. In this study, we tested the hypothesis that sphingomonads growing on PAHs enhance the bioavailability of their substrates by excretion of surfactants (gluco-sphingolipids).

Sphingomonads are also known to excrete anionic heteropolysaccharides (sphingans) (Pollock 1993) with a common repeating unit (Chandrasekaran and Radha

1995). It may be speculated that these sphingans are involved in PAHs-degradation by accumulating PAHs in biofilms in non-steady-state environments like soil, where water flowing through soil pores after rainfall may supply pulses of water-solubilized PAHs. Furthermore, the semicolloid sphingans may increase the aqueous solubility of the PAHs by adsorption of the PAHs. Therefore, we investigated the effect of commercially available sphingans on the solubility of PAHs, and on the mineralization of fluoranthene by a sphingomonad.

A third mechanism for increased mass transfer from crystalline PAHs to bacterial cells has been demonstrated by Wick et al. (2001, 2002). Cells of *Mycobacterium frederiksbergense* LB501T were shown to form biofilm on anthracene crystals, the nearest cells being less than 1 μm away from the crystal surface. According to Fick's first law of diffusion, the small distance between the solid PAHs and the biofilm cells, combined with a high PAHs affinity, strongly favors the diffusive mass transfer of PAHs to the cells by steepening the aqueous concentration gradient. This will increase the mass transfer as long as the distance between the cells and the crystals is smaller than the thickness of the diffusive boundary layer surrounding the PAHs-source, all other factors being equal and assuming uniform mixing by mass flow outside the diffusive boundary layer. It has been suggested that the characteristically hydrophobic cell surfaces of mycobacteria aid in the attachment of the cells to PAHs crystals (Bastiaens et al. 2000). We screened a number of PAHs-degraders for formation of biofilms on PAHs crystals, the rationale being that hydrophobic bacteria (i.e., *Mycobacterium* and *Nocardia*) may overcome low aqueous phase substrate concentrations by efficient substrate-to-cell contact mechanisms. For the more hydrophilic strains (i.e., sphingomonads), biofilm formation would not be expected and the cells would be dependent on the concentration of PAHs in the bulk liquid.

Materials and methods

Bacterial strains and culture media

The genus *Sphingomonas* has recently been divided into four genera: *Sphingomonas* (sensu strictu), *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi et al. 2001). These are collectively referred to as the sphingomonads. The bacterial strains (Table 1) were stored in 40% (v/v) glycerol at −80°C. Bacterial cells were grown as shaken batch cultures (room temperature) in a phosphate minimal medium (PMM) (Johnsen et al. 2000) supplemented with glucose and glycerol (inocula), or PAHs crystals.

Screening strains for biosurfactant production

All glassware, silicone stoppers and pipette tips were acid-washed (HCl, 0.1 M), and rinsed once with tap water and three times with demineralized water. All experiments were performed in triplicate. PAHs (2.6 mg) in acetone solution was added to sterile glass tubes (60 ml, Ø=2.5 cm i.d.). The tubes were capped with aluminium Cap-O-Test caps and the acetone was evaporated overnight. PMM (12 ml) was added to each tube and the tubes were sonicated for 15 min to increase the surface area of the PAHs crystals. Inoculum

was washed twice in PMM and diluted to OD_{540} =0.5. The tubes were inoculated with 0.5 ml inoculum, sealed with silicone stoppers and incubated in a tilted position on a shaker-table (170 rpm, room temperature). The air-phase of the tubes was exchanged with fresh, sterile air every 7 days to prevent depletion of oxygen and accumulation of carbon dioxide. The increase in OD_{540} was followed by placing the tubes in a spectrophotometer three times a week. The reduction of the surface tension was measured when the cultures reached the pseudo-stationary growth-phase, i.e., when the mass-flow of PAHs from the PAHs crystals equaled the cells' basic maintenance energy requirements, leading to a constant biomass (Wick et al. 2001). The cultures were centrifuged (8,000 g, 10 min) to remove cells and PAHs crystals. The surface tension of the supernatant was measured using a model K10 ring-tensiometer (Krüss, Hamburg, Germany). The ring was cleaned with 96% ethanol and heated to redness. The samples were equilibrated for 1 h at 25°C before measurements were made. The surface tension of Milli-Q water was used as a zero control, PMM was used as a negative control and PMM containing 0.2% (w/v) sodium dodecyl sulfate (SDS) as a positive control. The PAHs-degrading, biosurfactant-producing strain *P. aeruginosa* CRE11 was also used as a control.

Adsorption of PAHs to sphingans

The method was adapted from that of Barkay et al. (1999). Three *Sphingomonas* exopolysaccharides (sphingans), Gellan, Welan and Rhamsan, produced by Kelco (San Diego, Calif.), were obtained as follows. Clarified Gellan was purchased from Sigma-Aldrich (Copenhagen, Denmark) under the trade name Phytagel. Industrial grade Welan and Rhamsan were a gift from Mikael Grathwohl (Ringsted og Semler A/S, Copenhagen, Denmark). Alasan was obtained from E. Rosenberg (Barkay et al. 1999). Sphingans and Alasan were hydrated in Tris buffer (500 μg ml^{-1}, 20 mM Tris-HCl, 10 mM CaCl$_2$, pH 7.0) for 1 h, centrifuged 30 min at 10,000 g and autoclaved 20 min at 121°C. Pyrex test tubes (10 ml) with screw-caps were washed three times in Milli-Q water and autoclaved. Flourene, phenanthrene, fluoranthene and pyrene were dissolved in acetone (10 mg ml^{-1}) and pipetted into the glass tubes to give 200 μg PAHs per tube. The acetone was evaporated in a sterile flow hood and the air-phase of the tubes was exchanged with sterile air to remove acetone fumes. A 6 ml aliquot of polysaccharide solution was added to each tube followed by incubation in a horizontal position on a tilting table for 18–22 h at approximately 17°C. Buffer and polysaccharide solutions incubated without PAHs were used as negative controls. The Alasan solution was used as a positive control. All treatments were performed in triplicate. After incubation, the content of each tube was checked for sterility by streaking 1 μl on 10% Tryptone Soy Broth (TSB) plates. The contents of the tubes were filtered through glass wool to remove remaining PAHs crystals; 2.5 ml filtrate was mixed with 2.5 ml hexane and shaken heavily for 3 min to extract the PAHs, followed by centrifugation (20 min, 3,000 g) to separate the phases. The PAHs concentration of the hexane extracts were measured spectrophotometrically at 250 nm (phenanthrene), 261 nm (fluorene), 236 nm (fluoranthene) and 273 nm (pyrene), read against hexane. The absorbance of controls without PAHs was subtracted from all PAHs readings. Calibration curves were made by dissolving the PAHs in hexane. Limits of detection by spectrophotometry were (rejecting absorbance readings below 0.010): phenanthrene 0.02 μg ml^{-1}, fluorene 0.06 μg ml^{-1}, fluoranthene 0.03 μg ml^{-1} and pyrene 0.04 μg ml^{-1}.

Purification of commercial sphingans

For PAHs-mineralization experiments, clarified Gellan was used as purchased. Industrial grade Welan and Rhamsan were purified to avoid increased mineralization of the PAHs due to bacterial growth on cell fragments, proteins, vitamins etc. possibly present. Welan and Rhamsan were dissolved in Milli-Q water (1.00 mg ml^{-1}) by stirring for 1 h, the solutions were centrifuged (30 min, 10,000 g) to spin down cell fragments and the supernatants were decanted.

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Table 1 Polycyclic aromatic hydrocarbon (PAHs)-degrading bacterial isolates used in this study and their production of biosurfactants measured as reduction of the surface tension at 25°C compared to Milli-Q water. *PMM* Phosphate minimal medium, *Flu* fluorene, *phe* phenanthrene, *ant* anthracene, *fla* flouranthene, *pyr* pyrene

Strain	Identification	Reference	Reduction in surface tension (mN m^{-1})	PAHs[a]
LB501T	*Mycobacterium frederiksbergense*	(Bastiaens et al. 2000)	12.2±1.7	ant
Fan9	*Mycobacterium frederiksbergense* (DSM44346)	(Willumsen et al. 2001)	22.6±1.8	ant
PYR-1	*Mycobacterium vanbaalenii* (DSM7251)	(Kelley et al. 1993)	2.6±0.6	phe
GJ-3p	*Mycobacterium gilvum* (DSM44382)	G. Zaitsev, pers. comm.	2.6±1.8	ant
LB307T	*Mycobacterium gilvum*	(Bastiaens et al. 2000)	6.2±0.5	phe
LB208	*Mycobacterium gilvum*	(Bastiaens et al. 2000)	3.4±1.1	phe
VF1	*Mycobacterium* sp. (DSM11096)	(Kästner et al. 1994)	2.0±0.1	fla
VM503	*Mycobacterium* sp.	D. Springael[b]	1.0±0.7	phe
VM466	*Mycobacterium* sp.	D. Springael[b]	3.2±0.4	ant
VM505	*Mycobacterium* sp.	D. Springael[b]	2.4±0.8	ant
VM572	*Mycobacterium* sp.	D. Springael[b]	2.3±0.6	pyr
VM582	*Mycobacterium* sp.	D. Springael[b]	2.3±0.4	pyr
VM451	*Nocardia asteroides*	D. Springael[b]	18.8±1.6	ant
BP9	*Gordona* sp. (DSM11095)	(Kästner et al. 1994, 1998)	6.7±1.5	pyr
LH162	*Novosphingobium* sp.	D. Springael[b]	0.3±0.4	phe
LH128	*Novosphingobium subarctica*	(Bastiaens et al. 2000)	1.5±1.3	phe
10–1	*Sphingomonas* sp. (DSM12247)	(Ho et al. 2000)	3.4±0.0	fla
CO6	*Sphingomonas* sp.	(Ho et al. 2000)	0.5±0.3	fla
LB126	*Sphingobium chungbukensis*	(Bastiaens et al. 2000)	1.7±1.3	flu
EPA505	*Sphingobium* sp. (DSM7526)	(Mueller et al. 1990)	2.8±0.7	phe
JAJ137	*Pseudomonas fluorescens*	(Johnsen et al. 1996)	3.4±1.2	phe
CRE11	*Pseudomonas aeruginosa*	(Mueller et al. 1989, 1997)	20.8±1.0	phe
	PMM	Negative control	1.0±0.7	–
	0.2% SDS	Positive control	38.3±0.1	–

[a] Carbon source used in the biosurfactant assay
[b] Environmental Technology, Flemish Institute for Environmental Research (VITO), Mol, Belgium

Dissolved Welan in the supernatant was precipitated on ice with two volumes, and Rhamsan with three volumes, of cold (5°C) acetone. The precipitates were "fished" out with a spatula and dried overnight in a fume hood.

Effect of sphingans on the mineralization of PAHs

The sphingans (700 mg l^{-1}) were hydrated in Milli-Q-water for 1 h and autoclaved (121°C, 20 min). The sphingan solutions were mixed with double strength PMM in a 1:1 ratio (sphingan-PMM). Mineralization of 9-[^{14}C]-phenanthrene (Sigma-Aldrich, purity >98%) and 3-[^{14}C]-fluoranthene (Sigma-Aldrich, purity >95%) was tested in 250 ml sterile Erlenmeyer flasks. PAHs (2 mg; specific activity: phenanthrene, 205 Bq mg^{-1}; fluoranthene, 470 Bq mg^{-1}) in acetone solution were added to sterile Erlenmeyer flasks. The acetone was evaporated and 50 ml sphingan-PMM was added to the flasks to give a final PAHs concentration of 40 mg l^{-1}. The flasks were sonicated for 10 min to increase the area of the PAHs crystals, and shaken for 24 h to give saturated PAHs-solutions. *Sphingobium* sp. strain EPA505 inoculum was grown to late exponential phase, washed once and resuspended in PMM. Inoculum (0.5 ml) was added to each flask. The flasks were sealed with sterile silicone stoppers and incubated on a shaker table (200 rpm, room temperature) for 10 days. Flasks without bacteria and flasks without sphingans were used as controls. The $^{14}CO_2$ produced during the mineralization of the PAHs was collected in 400 μl KOH, contained in glass vials suspended from the silicone stoppers by steel hooks. The amount of $^{14}CO_2$ was quantified by mixing the KOH with 1 ml water and 1.5 ml Ready Gel Scintillation cocktail (Beckman), and counted on a LS1801 Beckman scintillation counter. The negative controls (no bacteria) never counted above background level.

Bacterial growth on nutrients originating from the sphingans was tested: 60-ml glass tubes containing 10 ml sphingan-PMM were inoculated with EPA505 and incubated for 5 days (200 rpm, room temperature). The OD$_{540}$ was measured at the beginning and at the end of the incubation.

Attachment of bacterial cells to PAHs-coated microplate wells

Inocula were grown in PMM with glucose and glycerol, harvested in the exponential phase, washed once in PMM and diluted in PMM to OD$_{540}$ =0.3. Attachment to PAHs crystals was determined using a modification of a WST-1 microplate method for determination of growth on crystalline PAHs (Johnsen et al. 2002a). In brief, after coating the walls of microplate wells (sterile, 96-well, flat-bottom microtiter plates; Nalge Nunc International, Roskilde, Denmark, product no. 269787) with phenanthrene, fluoranthene or pyrene, 200 μl PMM and 10 μl diluted inoculum were added to each well. Inoculum (1 μl) was streaked on TSB-agar plates to check for purity. The microplates were wrapped in plastic bags and incubated at room temperature on a shaker table at 300 rpm. All treatments were carried out in quadruplicate. After 5 days, the liquid contents of the wells were transferred to new microplates, to assay the potential metabolic activity of the planktonic cells. The original plates were filled with fresh medium to assay the potential

metabolic activity of the attached cells; 50 μl e-donor solution (glucose, pyruvate and succinate,16.6 mM each; Tris buffer, 40 mM; final pH: 6.5) was added to the microplate wells together with 10 μl WST-1 reagent. The plates were incubated on a shaker table (300 rpm) at room temperature for 5 h. Absorbance was measured at 450 nm with a reference wavelength at 630 nm on a microplate spectrophotometer at regular intervals. The absorbance at time zero was subtracted from the subsequent readings. The change in absorbance was plotted against time and the potential respiratory activity of each well was calculated by linear regression. Attached respiration was expressed as percentage of the sum of attached and planktonic respiration.

Results

Biosurfactants

Most of the PAHs-degraders tested in our study did not substantially reduce the surface tension (Table 1). In particular, none of the tested sphingomonads produced biosurfactants. Minor surface tension reductions were obtained for *Mycobacterium* strains LB307T, LB501T and Fan9, *Nocardia* sp. VM451, *Gordona* sp. BP9.

Sorption of PAHs to sphingans

The three sphingans Gellan, Welan and Rhamsan all increased PAHs solubility (Table 2), Gellan showing the

Table 2 Effect of Gellan, Welan and Rhamsan on the aqueous solubility of four PAHs. Buffer was used as a negative control, the PAHs-binding polysaccharide Alasan was used as a positive control. The effect is given as apparent aqueous solubility and as the ratio of the PAHs concentrations in the presence ($C_{polysacc}$) and the absence (C_{buffer}) of polysaccharides

|	PAHs concentration (μg ml^{-1})	$C_{polysacc}/C_{buffer}$
Fluorene		
Buffer	0.47±0.08	1.0
Gellan	0.60±0.12	1.3
Welan	0.82±0.05	1.8
Rhamsan	1.11±0.09	2.4
Alasan	2.35±0.11	5.0
Phenanthrene		
Buffer	0.38±0.05	1.0
Gellan	0.56±0.09	1.5
Welan	0.71±0.15	1.9
Rhamsan	1.23±0.04	3.2
Alasan	4.70±0.80	12
Fluoranthene		
Buffer	0.05±0.01	1.0
Gellan	0.14±0.03	2.9
Welan	0.31±0.05	6.0
Rhamsan	0.46±0.19	9.0
Alasan	1.51±0.25	30
Pyrene		
Buffer	0.06±0.02	1.0
Gellan	0.31±0.04	5.4
Welan	0.22±0.03	3.9
Rhamsan	0.34±0.04	6.1
Alasan	1.50±0.23	27
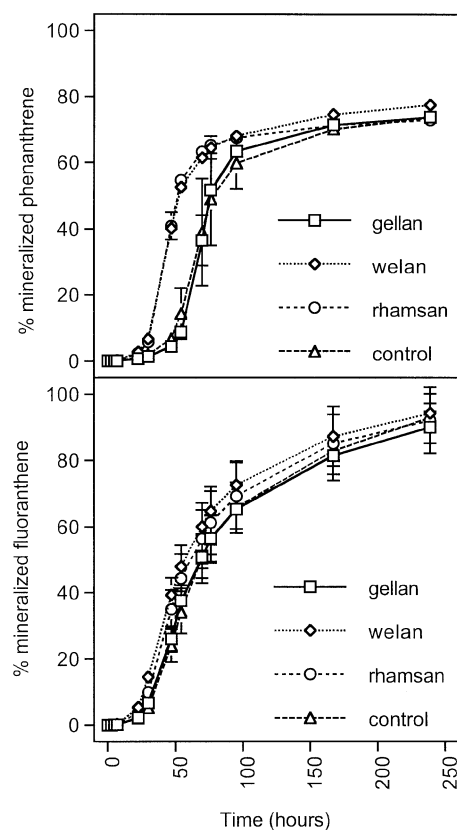
Fig. 1 Effect of the three polycyclic aromatic hydrocarbon (PAHs)-sorbing *Sphingomonas* exopolysaccharides Gellan, Welan and Rhamsan on the mineralization of ^{14}C-phenanthrene and ^{14}C-fluoranthene by *Sphingobium* sp. EPA505

least increase (1.3- to 5.4-fold), Welan being intermediate (1.8- to 6.0-fold) and Rhamsan giving the highest increase (2.4- to 9.0-fold). Though increasing the PAHs solubility, the effects of the sphingans are not nearly as great as the effect of the positive control, Alasan (5.0- to 30-fold).

Mineralization of PAHs as affected by sphingans

The initial growth phases (initial non-linear ^{14}CO$_2$ evolution) were shortened in the presence of Welan and Rhamsan. This effect was most pronounced for phenanthrene, time to onset of linear release of ^{14}CO$_2$ being reduced from 59 h to 30 h. At the onset of the phenanthrene experiment, roughly 4–7% of the total phenanthrene was dissolved in the combined aqueous and Welan/Rhamsan sorbed pools, calculated on the basis of the data in Table 2, whereas only 2–3% was dissolved in the Gellan and the control experiments. This suggests that the sphingan-sorbed phenanthrene was readily bioavailable and accounted for the faster initial growth. Welan and Rhamsan increased the initially dissolved fluoranthene fraction 6- to 9-fold, but this did not produce measurably faster initial growth (Fig. 1), as the initially dissolved fluoranthene amounts were much smaller than

Table 3 Attachment to PAHs-coated microtiter plate wells during growth on various crystalline PAHs. Attachment is expressed as the attached respiration in percent of the total respiration ±1 SD. *Sph* Sphingomonad, *Noc Nocardia, Myc Mycobacterium*

	Sph 10-1	Sph EPA505	Sph LH128	Sph CO6	Noc VM451	Myc LB501T	Myc Fan9	Myc VM572	Myc VM582	Myc LB307T
Phenanthrene (1.15)[a]	1±7	5±5	84±3	99±1	41±3	15±3	8±3	55±4	65±9	33±6
Fluoranthene (0.26)[a]	4±1	11±7	–	–	12±3	59±31	51±42	–	–	99±1
Pyrene (0.14)[a]	–	–	–	–	57±19	72±4	23±6	95±2	99±8	87±5
Anthracene (0.043)[a]	–	–	–	–	–	78±4	28±6	–	97±2	–

[a] Aqueous solubility (mg l^{-1}, SRC PhysProp database)

for phenanthrene, i.e., the initially dissolved fluoranthene pool was too small to significantly contribute to cell growth. Control experiments showed no increase in OD$_{540}$ when the sphingan-media were inoculated with EPA505. Thus, the higher growth rates in the presence of Welan and Rhamsan were not caused by contamination of the sphingans with sugars, proteins, nucleic acids, etc.

Attachment

For each strain, we investigated attachment to PAHs degraded by that specific strain. Results were omitted when the total respiration was lower than the total respiration of the control wells (without PAHs), which was probably due to the accumulation of toxic metabolites. The respiration associated with the PAHs-coated well surfaces (as a percentage of total respiration) varied considerably between different strains and between the different PAHs for strains capable of growing on several PAHs (Table 3). According to their attachment to PAHs-coated wells, the strains could be divided into four groups.

The first group consisted of *Novosphingobium subartica* LH128 and sphingomonad CO6. The respiring cells attached to the phenanthrene-coated surfaces, as there was only a small amount of respiration in the suspended phase.

The second group consisted of the *Mycobacterium* spp. VM572, VM582 and LB307T. They also attached to the PAHs-coated surfaces, but there was a tendency that the higher the solubility of the PAHs, the lower the percentage of respiration attached, suggesting that attachment was correlated with low aqueous solubility of the substrate.

The third group consisted of *Sphingobium* sp. EPA505 and sphingomonad sp. 10-1, which did not attach well to the PAHs-coated surfaces. These strains seemed to depend on PAHs solubilized in the bulk liquid.

The fourth group contained *Mycobacterium* spp. LB501T and Fan9, and *Nocardia* sp. VM541. Attachment of respiring cells of these strains was highly variable, not only when comparing attachment to different PAHs, but also between replicates grown on the same PAHs.

Discussion

Biosurfactants

The method for screening PAHs-degraders for biosurfactant production was adapted from earlier work (Willumsen and Karlson 1997). The PAHs concentration was increased from 20 mg l^{-1} to 200 mg l^{-1} because the critical micelle concentration (CMC) of biosurfactants often lies in the range 10–30 mg l^{-1} (Horowitz et al. 1990; Van Dyke et al. 1993; Yakimov et al. 1995). The biosurfactant concentration must exceed the CMC to give a significant increase (in this context) in the aqueous PAHs concentration. Furthermore, surfactant molecules

may influence the dissolution process by attaching to the PAHs-water interface. Here, they form hemi-micelles that may accelerate PAHs-dissolution (Volkering et al. 1998).

A bacterium is generally considered a good biosurfactant producer if it reduces the surface tension to ≤ 40 mN m^{-1} at 25°C (Cooper and Zaijic 1980) corresponding to a reduction of 32 mN m^{-1} compared to water. None of the tested strains were convincing biosurfactant producers. Thus, for the strains tested, increased solubility by pseudo-solubilization of PAHs in biosurfactant micelles does not seem to be a strategy to increase PAHs bioavailability. It has previously been questioned, whether surfactant-solubilized PAHs are bioavailable at all (Volkering et al. 1995; Zhang et al. 1997; Willumsen et al. 1998; Willumsen and Arvin 1999). It cannot be ruled out, however, that the limited biosurfactant release by some of the actinomycetes (Table 1) may lead to formation of hemi-micelles at the PAHs-water interface and accelerated PAHs dissolution. On the other hand, microorganisms produce biosurfactants for many other purposes than increasing the bioavailability of hydrophobic substrates (Ron and Rosenberg 2001).

Sorption to sphingans

Sorption of PAHs to sphingomonad exopolysaccharides (sphingans) might be an alternative strategy for sphingomonads to increase the bioavailability of PAHs. The increases in apparent solubility were most pronounced for the four-ring PAHs (pyrene and fluoranthene), probably because these compounds are more hydrophobic than the three-ring PAHs.

Sorption of aromatic compounds to extracellular polymeric substances (EPS) has been reported previously. The monoaromatics benzene, toluene and xylene sorbed to EPS in biofilms (Späth and Wuertz 1998). In another study, 24 out of 28 microbial polymers acted as phenanthrene sorbents and carriers, facilitating transport of phenanthrene in sand columns (Dohse and Lion 1994). Diclofop methyl (methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]-propanoate), a two-ring chlorinated herbicide, accumulated in biofilms by sorption to microbial exopolymers (Wolfaardt et al. 1994) and the accumulated dichlofop was metabolized by the biofilm community during starvation (Wolfaardt 1995). The biofilm regions responsible for diclofop accumulation were shown to be hydrophobic by binding of the hydrophobic fluorescent dye Nile Red (Wolfaardt and Lawrence 1998).

In the presence of high concentrations of monovalent cations (e.g., K^+) or low concentrations of divalent cations (e.g., Ca^{++}), sphingans form tightly packed semi-colloid structures. Pairs of sphingan double helices are aligned in an antiparallel fashion and are connected by strong carboxylate$\cdots Ca^{2+} \cdots$carboxylate or carboxylate$\cdots K^+ \cdots$water$\cdots K^+ \cdots$carboxylate interactions (Chandrasekaran and Radha 1995). Hydrophobic attractions between one hydrophobic and one hydrophilic moiety in water is quite common (Van Oss 1995). Furthermore, the monomeric sugar moieties of polysaccharides can be hydrophobic or hydrophilic, dependent solely on their three-dimensional conformation. The hydrophilicity is closely linked to the maximum extent of freedom of movement of the constituent monomer moieties of the polysaccharides (Van Oss 1995). It may be speculated that sphingans contain hydrophobic regions due to the very rigid molecular architecture when divalent cations are present, and these hydrophobic regions may be responsible for the observed increases in the aqueous PAHs solubility of the sphingan solutions (Table 2).

We have previously demonstrated that sphingomonads with PAHs-degrading capabilities excrete exopolysaccharides when grown as biofilm in flow-channels (Johnsen et al. 2000), but attempts to isolate sphingans from liquid cultures of three PAHs-degrading sphingomonads have been all negative (data not shown).

Sphingobium sp. strain EPA505 was grown on fluoranthene in the presence of Gellan, Welan and Rhamsan. EPA505 was chosen because it does not attach well to fluoranthene crystals (Table 3) and therefore depends mostly on fluoranthene dissolved in the bulk liquid. Increased fluoranthene pseudo-solubility in the presence of sphingans would lead to an increased mass transfer of fluoranthene from the crystals to the bulk liquid, due to a steeper fluoranthene concentration gradient within the diffusive boundary layer surrounding the crystals. This would in turn lead to increased mineralization rates. Increased dissolution rates in the presence of Alasan have been documented recently (Barkay et al. 1999). Our results clearly demonstrate that the mineralization rates, i.e., the slopes of the linear $^{14}CO_2$-evolution, were the same with and without sphingans (Fig. 1), indicating that mass-transfer rates from crystals to the bulk liquid were unaffected by the sphingans. The reason might be either that the sorption and desorption rates are much smaller than the crystal to bulk liquid dissolution rates or that the diffusion of the PAHs-sphingan complexes within the boundary layer may be slow compared to monodispersed PAHs molecules, thereby diminishing the effect of the increased solubility.

Although the binding of PAHs did not affect PAHs transfer rates, it may be speculated that sphingan-producing organisms that accumulate PAHs, may show increased fitness in periods of famine. It is, however, unclear whether sorption of PAHs to EPS materials serves to increase PAHs bioavailability, rather than being a non-specific side effect of EPS production for other purposes.

Attachment

For the actinomycetes, there was a tendency that the more soluble the PAHs, the higher the percentage of respiration of cells in suspension. Such a split between attached and planktonic populations was not found for the sphingomonads, in which either the majority of respiring cells were associated with the crystals, indicating biofilm formation, or the respiring cells were suspended. Thus, it

seems that biofilm formation was a common mechanism among the isolates to overcome low PAHs bioavailability in the presence of crystalline substrates. *Mycobacterium frederiksbergense* strain LB501T has previously been reported to form very luxuriant biofilms on the surface of anthracene crystals in shaken liquid cultures (Wick et al. 2001, 2002).

Mycolic acids constitute one of the major types of biosurfactants (Desai and Banat 1997); therefore, some surface tension reduction by *Mycobacteriun*, *Nocardia* and *Gordona* was not surprising as mycolic acids are a major component of the cell wall of these genera (Goodfellow 1992). Production of cell-bound biosurfactants may change hydrophobic cell surfaces to charged and hydrophilic, and excretion of biosurfactants may change a substratum from hydrophobic to charged and hydrophilic (Neu 1996). It has been suggested that these hydrophobicity/hydrophilicity shifts are a mechanism for cells to regulate attachment to, and detachment from, substrata (Neu 1996). It may be speculated that the observed biosurfactant excretion is a means of *Mycobacterium* and *Nocardia* to release the cells from a hydrophobic nutrient source when cells at the bottom of the biofilm become oxygen-limited or when the nutrient source on which they are growing becomes depleted. It has previously been demonstrated that the non-toxic chemical surfactants Triton X-100 and Dowfax 8390 inhibited adhesion of anthracene-utilizing *Mycobacterium* and *Pseudomonas* at concentrations equal to one-half of the CMC (Stelmark et al. 1999). Growth on solid anthracene was depressed in the presence of the two surfactants, indicating that mass transfer of anthracene to the cells was optimized by attachment to the anthracene crystals (Stelmark et al. 1999).

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