Appl Microbiol Biotechnol (2010) 85:1131–1139
DOI 10.1007/s00253-009-2216-0

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Effect of two types of biosurfactants on phenanthrene availability to the bacterial bioreporter *Burkholderia sartisoli* strain RP037

Robin Tecon · Jan Roelof van der Meer

Received: 19 June 2009 / Revised: 19 August 2009 / Accepted: 19 August 2009 / Published online: 3 September 2009
© Springer-Verlag 2009

Abstract Biosurfactants are tensio-active agents that have often been proposed as a means to enhance the aqueous solubility of hydrophobic organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs). Biosurfactant-producing bacteria such as those belonging to the genus *Pseudomonas* might therefore enhance PAH availability to PAH-degrading bacteria. We tested the effects of two types of biosurfactants produced by *Pseudomonas* sp., cyclic lipopeptides and rhamnolipids, on phenanthrene bioavailability. Bioavailability was judged from growth rates on phenanthrene and from specific induction of a phenanthrene-responsive GFP-reporter in *Burkholderia sartisoli* strain RP037. Co-culturing of strain RP037 with the lipopeptide-producing bacterium *Pseudomonas putida* strain PCL1445 enhanced GFP expression compared to a single culture, but this effect was not significantly different when strain RP037 was co-cultivated with a non-lipopeptide-producing mutant of *P. putida*. The addition of partially purified supernatant extracts from the *P. putida* lipopeptide producer equally did not unequivocally enhance phenanthrene bioavailability to strain RP037 compared to controls. In contrast, a 0.1% rhamnolipid solution strongly augmented RP037 growth rates on phenanthrene and led to a significantly larger proportion of cells in culture with high GFP expression. Our data therefore suggest that biosurfactant effects may be strongly dependent on the strain and type of biosurfactant.

Keywords Bioreporter · GFP · Rhamnolipid · Lipopeptide · Polycyclic aromatic hydrocarbons (PAH)

The bioreporter strain *B. sartisoli* RP037 is available on request to Prof. Jan Roelof van der Meer, Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne, Switzerland. JanRoelof.Vandermeer@unil.ch.

R. Tecon (✉) · J. R. van der Meer
Department of Fundamental Microbiology,
University of Lausanne,
Lausanne CH-1015, Switzerland
e-mail: Robin.Tecon@unil.ch

J. R. van der Meer
e-mail: JanRoelof.Vandermeer@unil.ch

Present Address:
R. Tecon
Centre for Terrestrial Ecology,
Netherlands Institute of Ecology (NIOO-KNAW),
Heteren 6666 GA, The Netherlands
e-mail: r.tecon@nioo.knaw.nl

Introduction

Biosurfactants, i.e. surface-active molecules that are produced by living organisms, are frequently presented in the scientific literature as a means to increase the apparent solubility and/or bioavailability of hydrophobic organic contaminants (HOCs) (Van Dyke et al. 1991; Zhang and Miller 1992; Bertrand et al. 1994; Desai and Banat 1997; Garcia-Junco et al. 2001; Mulligan 2005; Urum et al. 2006). They are produced by a variety of microbes and can be of various chemical types, such as glycolipids, lipopeptides, phospholipids, and fatty acids (Desai and Banat 1997; Mulligan 2005). Rhamnolipids, a major class of glycolipids produced by certain *Pseudomonas* species, represent common biosurfactants which are commercially exploited as emulsifiers and dispersants (Mulligan 2005; Soberón-Chávez et al. 2005). Common features of biosurfactants are their amphiphilic properties, their ability to reduce the surface tension of water, and their enhancement of hydrocarbon emulsification. Compared to synthetic surfactants, biosurfactants are in general non-toxic and biodegradable. Most of them

increase the apparent solubility of HOCs by dissolving them into micelles, and for this reason their potential for augmenting the biodegradation rate of HOCs was extensively investigated. Biosurfactant production was shown a key characteristic of alkane-degrading bacteria, for which this serves to augment alkane bioavailability and thus degradation rate (Zhang and Miller 1992; Yakimov et al. 1998; Noordman and Janssen 2002; Olivera et al. 2009). A similar effect has been proposed for the bacterial degradation of polycyclic aromatic hydrocarbons (PAHs), but it appears that biosurfactant production is not an essential trait of PAH degraders (Johnsen et al. 2005). The external addition of biosurfactants, however, was shown to increase the solubilization of PAHs from non-aqueous phase liquids and solid particles (Garcia-Junco et al. 2001, 2003). Yet, an augmentation of PAH solubilization is not necessarily associated with an equivalent increase of its bioavailability to microorganisms (Shin et al. 2004), and the nature of biosurfactants effects on PAH degradation rate are complex and is debated for more than a decade (Volkering et al. 1997). In fact, it might be possible that molecules dissolved in micelles are actually less available to certain bacteria than freely dissolved molecules, when these are incapable of releasing the molecules from the micelles. Also, biosurfactants themselves might be used as a preferential substrate by microorganisms, which would lead to a reduction of the degradation rate of the HOC (Volkering et al. 1997).

Pseudomonas putida strain PCL1445 is a rhizosphere inhabitant that produces two different lipopeptides (referred to as putisolvin I and II), consisting of a 12 amino acid cyclic peptide with a hexanoic acid chain at the N terminus (Kuiper et al. 2004). Putisolvin I and II inhibited biofilm formation of *Pseudomonas* on hydrophobic surfaces, and both compounds lowered the surface tension of culture medium while increasing its emulsifying activity (Kuiper et al. 2004). The biosynthesis of lipopeptides in strain PCL1445 was shown to be influenced by nutritional and environmental conditions, and is controlled by quorum sensing and the two-component regulatory system GacA/GacS (Dubern et al. 2005, 2006; Dubern and Bloemberg 2006).

Here we tested the effect of lipopeptides on the bioavailability of phenanthrene (PHE) to *Burkholderia sartisoli* strain RP037, a model degrader for low molecular weight PAHs. In addition, *B. sartisoli* RP037 is equipped with a phenanthrene-inducible green fluorescent protein (GFP) reporter. Two types of assays were performed in this study. In the first, semi-purified lipopeptide extract from PCL1445 was added directly to RP037 cultures growing on PHE to examine the effects on PHE growth rate and on GFP induction, as measure for PHE availability. In the second, we tested co-culturing of RP037 and the lipopeptide-producing strain PCL1445 or one of the mutants impaired in lipopetide biosynthesis (PCL1436). Although a large number of publications addressed the effects of pure biosurfactants, very few investigated co-inoculation of a biosurfactant-producing and a HOC-degrading bacterium (Dean et al. 2001; Straube et al. 2003). The in situ use of a bacterium-producing surfactants may be advantageous for certain bioremediation strategies. Finally, we compared the results obtained with lipopeptides with pure rhamnolipids.

Material and methods

Chemicals and biosurfactants

Phenanthrene (97% purity) was purchased from Fluka (Buchs, Switzerland). PHE crystals were of various sizes with an approximate average diameter of 0.10 mm. The rhamnolipid solution JBR215 was obtained from Jeneil Biosurfactant Company (Saukville, USA), and was provided as a 15% aqueous solution. It was produced from sterilized and centrifuged fermentation broth. The two major components in the solution, a mono- and a di-rhamnolipid, have molecular mass of 504 and 650 D, respectively, and are present in an approximate ratio of 1:1. The critical micellar concentration (CMC) of JBR215 is 37 μM, or ~20 mg/L (Gu and Chang 2001). In our experiments, we used the rhamnolipid solution at a concentration of 0.1%, which corresponded to 150 mg/L, or ~270 μM, i.e. ~7 times above the CMC.

Strains and growth conditions

The wild-type strain *B. sartisoli* RP007 was described by Laurie and Lloyd-Jones (1999). The derived strain *B. sartisoli* RP037 is a GFP-based biosensor described previously (Tecon et al. 2006). RP037 was routinely grown at 30°C on Tryptone Yeast (TY, contains 3 g/L of yeast extract and 5 g/L of Bacto tryptone) agar plates or liquid cultures with 50 mM NaCl, supplemented with 50 μg/ml of kanamycin to select for the reporter plasmid pJAMA37 (Tecon et al. 2006). Type 21C minimal medium (Gerhardt et al. 1981) with 10 mM of sodium acetate and 50 μg/ml of kanamycin was used as a defined medium for RP037 growth. *P. putida* PCL1445 is the wild-type strain that produces the lipopeptides putisolvin I and II with biosurfactant activity (Kuiper et al. 2004). *P. putida* PCL1436 is a transposon mutant of PCL1445, which has lost the ability to synthesize the two lipopeptides (Kuiper et al. 2004). *P. putida* PCL1438 is another transposon mutant, which still produces biosurfactants but not lipopolysaccharides (LPS). All *P. putida* strains were routinely grown in Luria-Bertani medium or in type 21C minimal medium with 2% (*w*/*v*) glycerol at 30°C without antibiotics.

Co-cultivation of *B. sartisoli* and *P. putida*

RP037 and *P. putida* strains were pre-cultured separately overnight in TY and LB medium, respectively. The cells were centrifuged at 1,200×*g* for 10 min and the supernatant was removed. The cells were then resuspended in minimal medium in order to obtain a culture turbidity at 600 nm of ~0.4. Twenty milliliters of minimal medium containing 10 mM glycerol but no antibiotics were inoculated with 2% of each preculture (RP037 plus one of the *P. putida* strains). The cultures were incubated at 30°C with shaking at 180 rpm in the presence or absence of PHE crystals (at 0.25 mg/ml of medium). The number of colony forming units in culture was determined from serially diluted samples plated on TY with 50 mM NaCl agar plates for RP037, or LB agar for *Pseudomonas*. GFP fluorescence intensities of individual RP037 cells were measured using flow cytometry or epifluorescence microscopy, as described previously (Tecon et al. 2006). In brief, fluorescence intensities of individual cells were expressed as 'average gray values' derived from the digitalization of images or flow diagrams. Cellular AGVs were then plotted in cumulative ranking from lowest to highest to visualize and calculate population-derived descriptors of fluorescence, such as the 75th percentile. Confidence intervals on such percentile descriptors were calculated by bootstrapping, as explained elsewhere (Tecon et al. 2006).

Lipopeptide extraction from *P. putida* cultures

P. putida strains were grown for 2 days in minimal medium with 2% (*w*/*v*) glycerol. Cells were removed by centrifugation and the supernatant was further filtered through 0.45 µm. In a glass beaker, 50 ml of supernatant were mixed with 50 ml of ethyl acetate and agitated at room temperature for 1 h with a magnetic stirrer. The organic phase was recovered and evaporated using a Rotavap system. Subsequently the extract was resuspended in 5 ml of ethyl acetate, transferred to a glass vial, and evaporated using a stream of N_2. Finally, the dried extract was resuspended in 500 µl of dimethylsulfoxide (DMSO).

Droplet-collapsing assay

Twenty-five microliters of bacterial culture or culture supernatant to which lipopeptide or rhamnolipid was added were pipetted on a hydrophobic surface (parafilm). After 5-min incubation at room temperature, the diameter of the droplet was measured using graduated paper.

Flow cytometry analysis

One milliliter of cell culture was centrifuged at 1,200×*g* for 15 min. The supernatant was discarded and the cells were resuspended in 50 µl of phosphate-buffered saline (PBS). Two microliters of this cell suspension was further diluted in 500 µl of PBS in a 5-ml polystyrene tube (Becton Dickinson, USA), then analyzed with a FACS Calibur system (Becton Dickinson). Specific settings were optimized for the detection of RP037 cells based on the forward and side scattered light channels. A gate was defined to properly quantify RP037 cells and exclude background events from the analysis. In each sample we measured the green fluorescence intensity on the FL1-H channel for 10,000 cells. The 488-nm laser intensity was set as such that the fluorescence of uninduced RP037 cells (i.e., grown on acetate alone) was below 10 arbitrary units.

Results

Co-cultivation of *B. sartisoli* strain RP037 with *P. putida* strain PCL1445 or PCL1436

First we tested whether strains *P. putida* PCL1445 and its derivative PCL1436, carrying a Tn*5*-insertion in the genes for lipopeptide biosynthesis (Kuiper et al. 2004), were able to grow and produce lipopeptide in the minimal medium used to cultivate *B. sartisoli* with acetate or glycerol as sole carbon source. Growth on glycerol promoted higher production of lipopeptides than on acetate, which was evident from a lower surface tension in the culture medium measured by a drop-collapsing assay (data not shown). Single cultures of PCL1445 and PCL1436 grew equally well on minimal medium with glycerol (Fig. 1a). *P. putida* and *Burkholderia* were then co-cultured in minimal medium with glycerol, in the presence or absence of PHE crystals. Precultures of RP037 and PCL1445 or PCL1436 were washed and mixed in an inoculum so as to have approximately ten times more colony forming units (CFU) per milliliter for *Burkholderia* than for *Pseudomonas*. Co-cultures of RP037 and PCL1445 grew similarly than a co-culture of RP037 plus PCL1436 on glycerol and PHE or glycerol alone ($p>0.05$ with a bilateral Student *t* test; Fig. 1b). Growth of RP037 alone on PHE was obvious after 62 h, but the strain essentially did not grow with glycerol only, which suggested that in the co-cultures most of the glycerol was used by *P. putida*. After 44 and 68 h of incubation, we sampled the cultures in order (1) to determine the GFP production in the bacterial biosensor RP037 and (2) to estimate the proportion of *Burkholderia* and *Pseudomonas* cells by plating (Fig. 1c, d). *P. putida* strains were not fluorescent and thus were automatically excluded from epifluorescence microscope GFP measurements on individual cells. After 44 h, the GFP intensities in the population of RP037—expressed here as the 75th percentile of individual cellular fluorescence intensities in

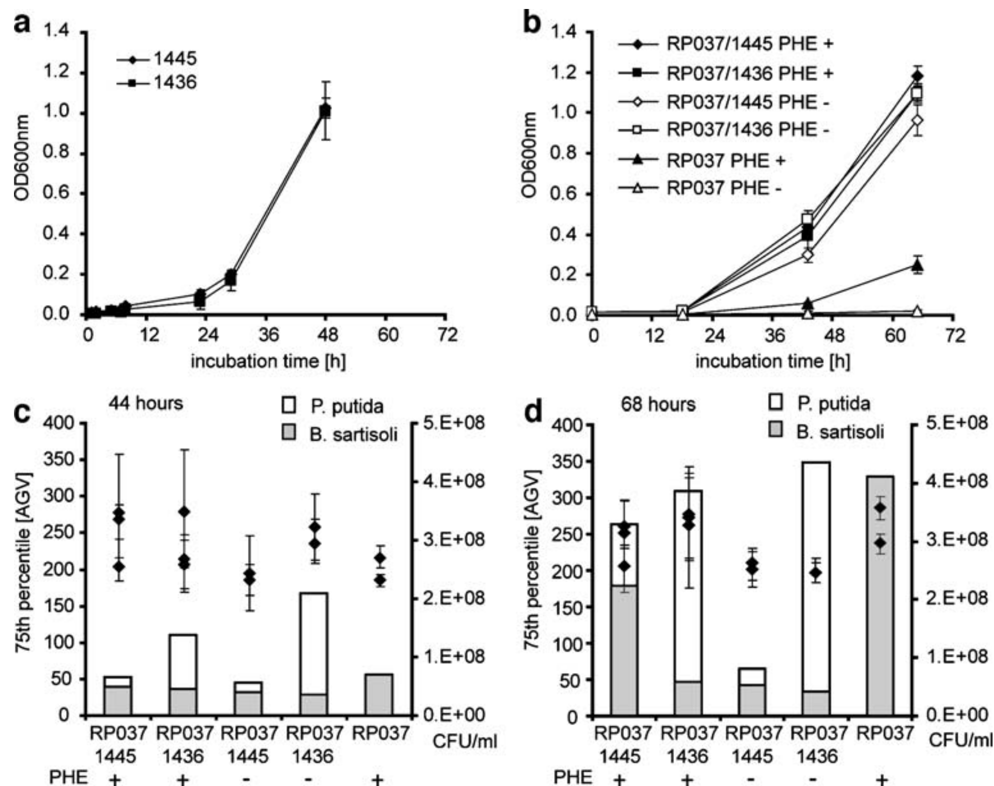


Fig. 1 Co-cultivation of *B. sartisoli* strain RP037 with *P. putida* strain PCL1445 or PCL1436, compared to RP037 alone. Development of culture turbidity in minimal medium with 10 mM glycerol, for *P. putida* single cultures (**a**) or *P. putida*/*B. sartisoli* co-cultures and *B. sartisoli* single cultures (**b**), without (PHE−) or with phenanthrene (PHE+, 0.25 mg/ml), measured at 600 nm (OD600nm). *Error bars* indicate standard deviations from triplicate (co-cultures) or duplicate (single cultures) measurements. **c** GFP fluorescence intensity of RP037 cells after 44 h. **d** Similar after 66 h of cultivation. *Black diamonds* represent the 75th percentile of the individual cellular fluorescence intensities in a bacterial sample, expressed as average gray value produced by the microscope and imaging program (AGV). *Error bars* indicate the 95% confidence interval for the percentile, calculated by bootstrapping in the program R (Tecon et al. 2006). *Histogram bars* show the number of CFU per milliliter of culture for *B. sartisoli* and *P. putida*

the bacterial culture (Tecon et al. 2006)—were slightly higher in certain replicate co-cultures with PCL1445 in the presence of PHE. GFP expression was also higher in co-cultures with PCL1436 irrespective of the presence of PHE. But because of triplicate variability none of the differences were significant for the treatment. The single cultures of strain RP037 exposed to glycerol alone without PHE did not grow sufficiently well for a proper GFP analysis. The number of CFU of RP037 in co-cultures at the end of the cultivation was approximately similar, but that of *Pseudomonas* varied more importantly. In particular strain PCL1445 produced far fewer CFU per milliliters than strain PCL1436, and much less than was expected from the culture turbidity (Fig. 1c). Microscopic observations of independent cultures revealed that *P. putida* had the tendency to form aggregates of 10 to 100 cells, and that this tendency was much more pronounced for PCL1445 than for PCL1436. After 68 h of incubation, the intensity of GFP fluorescence was higher in RP037 cells exposed to PHE compared to glycerol only, irrespective of the presence or nature of the co-inoculum. The number of CFU per milliliter of strain RP037 was highest in single culture, about twice as low in co-culture with PCL1445 and four times as low with PCL1436, suggesting partial inhibition.

Cultivation of *B. sartisoli* strain RP037 in presence of culture extracts from *P. putida*

We employed a simple procedure to extract and concentrate the lipopeptides from the supernatant of PCL1445 cultures grown on minimal medium with glycerol. The addition of the concentrated extract from a culture of PCL1445 to water lowered its surface tension, as indicated by the flattening of a small droplet on parafilm. Such effect was not observed with an extract from a culture of mutant PCL1436, impaired for the synthesis of lipopeptides. We cultivated RP037 in minimal medium with acetate and with 1% (*v*/*v*) of lipopeptide extract from either PCL1445 or PCL1436 cultures, in presence or absence of PHE crystals in the medium.

Cultures grew similarly on acetate and reached an optical density at 600 nm of ~0.45 after 24 h (Fig. 2a). Once all

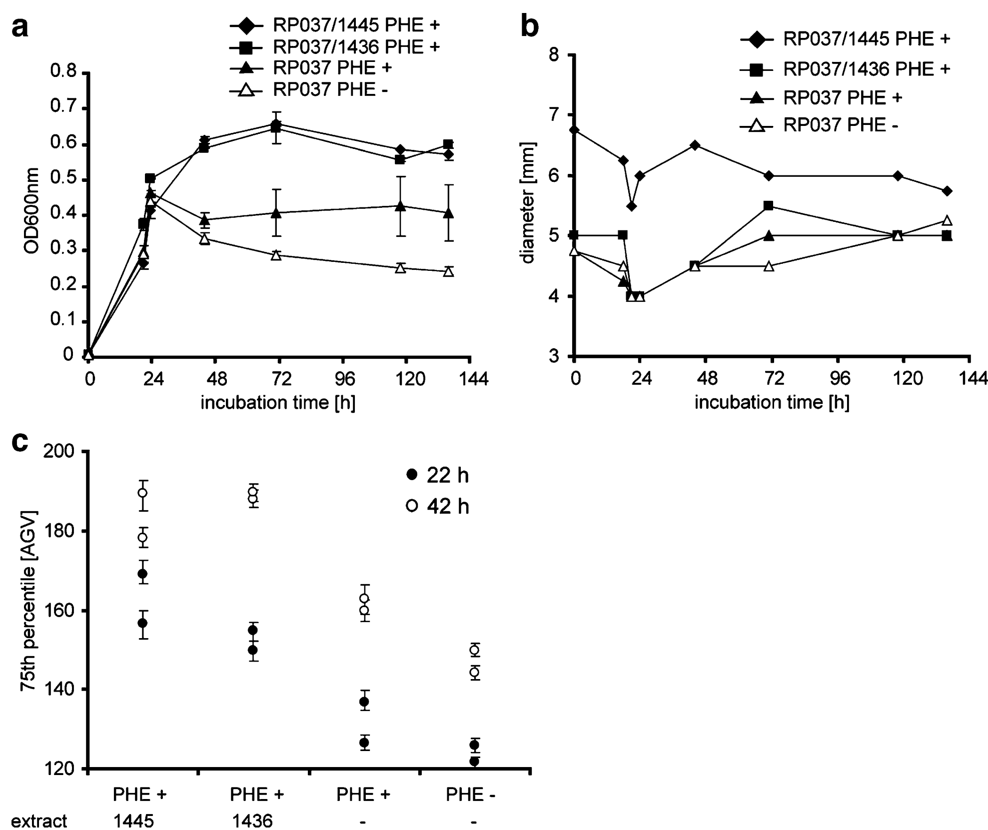


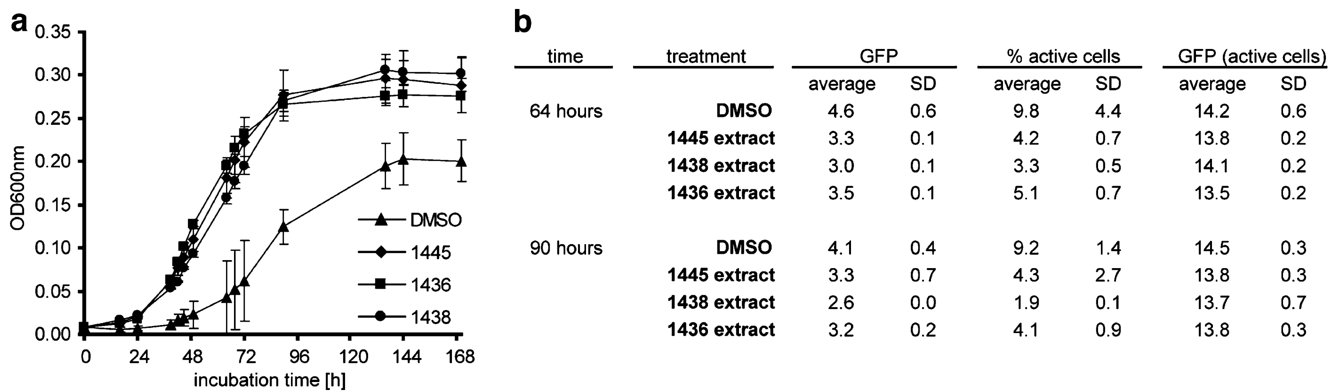
Fig. 2 Cultivation of *B. sartisoli* strain RP037 in the presence of supernatant extract from *P. putida* strain PCL1445 or PCL1436. **a** Culture turbidity at 600 nm of RP037 in minimal medium with 10 mM acetate, 1% supernatant extract in DMSO (1445 or 1436), and without (PHE−) or with phenanthrene crystals (PHE+, 0.10 mg/ml). *Error bars* indicate standard deviations from duplicate measurements. **b** Culture droplet (25 µl) diameters on parafilm. An increase of the droplet diameter indicates a decrease of surface tension. Measurement accuracy is ±0.5 mm. **c** GFP fluorescence intensity in RP037 cells after 22 and 42 h of incubation. *Circles* represent the 75th percentile of the individual cellular fluorescence intensities in the culture sample. *Error bars* indicate the 95% confidence intervals for the percentile, calculated by bootstrapping

acetate was used, the cells cultivated in absence of extract stopped to grow, whereas the presence of extract supported some extra growth of strain RP037. PHE addition prevented the decrease of turbidity that was observed with control cells, sustaining a flux of carbon sufficient to maintain the cells but not to allow extra growth. At selected time intervals, the cultures were sampled and their surface tension was assessed using a simple drop-collapsing assay (Fig. 2b). The cultures supplemented with an extract from a PCL1445 culture but not with one from a PCL1436 culture showed a reduction of the surface tension of the medium, which was attributed to the presence of lipopeptides. This difference persisted at least for 5 days of cultivation, although it appeared to fade with time (Fig. 2b). After 22 and 42 h of cultivation, the RP037 population was sampled and the GFP fluorescence intensity in individual cells was measured by epifluorescence microscopy. Figure 2c indicates the 75th percentiles of GFP fluorescence intensities in independent RP037 cultures. After 22 h of incubation, cultures without extract and PHE expressed the lowest percentile values, followed by the cultures without extract, but with PHE. Interestingly, RP037 cultures supplemented with *P. putida* PCL1445 extract showed the highest GFP expression. After 42 h, however, RP037 cells incubated with PHE and with *P. putida* extract, irrespective of it originating from PCL1445 or PCL1436 displayed higher GFP expression than cultures without extract.

In a similar experiment, we cultivated RP037 in minimal medium in presence or absence of *P. putida* extract, with PHE but without acetate. In addition to PCL1445 and PCL1436 extracts, we tested the influence of an extract from a culture of *P. putida* strain PCL1438, another transposon mutant that was impaired in the production of LPS but still capable to produce lipopeptide (I. Kuiper, personal communication). All extracts promoted faster bacterial growth as well as a higher final culture density than the culture with PHE only (Fig. 3a). After 64 and 90 h of incubation, cultures were sampled and the GFP fluorescence intensity in individual RP037 cells was measured by flow cytometry (Fig. 3b). Surprisingly, after 64 h of incubation the mean GFP fluorescence intensity of the whole population was significantly higher in the cells that were not exposed to *Pseudomonas* extract ($p<0.05$ with a unilateral Student t test). After 90 h of incubation, the percentage of active cells (i.e., those above a predefined threshold gate) was also significantly higher ($p<0.05$). Among the different supernatant extracts, the PCL1438 extract produced the lowest values for GFP expression.

Cultivation of *B. sartisoli* strain RP037 in presence of the biosurfactant JBR215

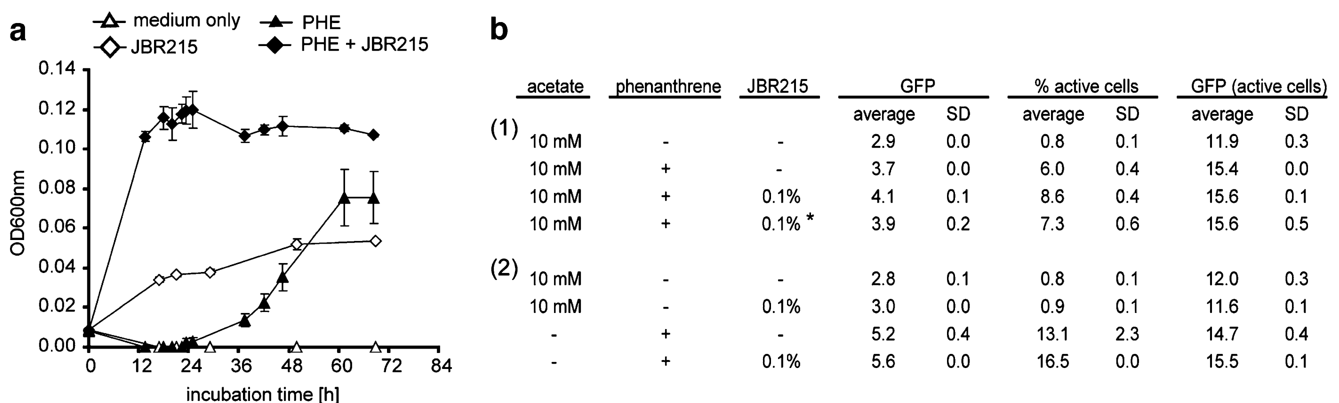
Finally, we tested whether a different biosurfactant would be more favorable in increasing the availability of PHE to



time	treatment	GFP average	GFP SD	% active cells average	% active cells SD	GFP (active cells) average	GFP (active cells) SD
64 hours	**DMSO**	4.6	0.6	9.8	4.4	14.2	0.6
	1445 extract	3.3	0.1	4.2	0.7	13.8	0.2
	1438 extract	3.0	0.1	3.3	0.5	14.1	0.2
	1436 extract	3.5	0.1	5.1	0.7	13.5	0.2
90 hours	**DMSO**	4.1	0.4	9.2	1.4	14.5	0.3
	1445 extract	3.3	0.7	4.3	2.7	13.8	0.3
	1438 extract	2.6	0.0	1.9	0.1	13.7	0.7
	1436 extract	3.2	0.2	4.1	0.9	13.8	0.3

Fig. 3 Effect of supernatant extract from *P. putida* strains PCL1445, PCL1438, or PCL1436 on growth of RP037 with PHE. **a** Culture turbidity development at 600 nm of RP037 in minimal medium with PHE (0.15 mg/ml), plus 0.75% (*v/v*) of culture extract of strain 1445, 1436, or 1438 in DMSO, or DMSO alone as a control. *Error bars* indicate standard deviations from triplicate measurements. **b** GFP fluorescence intensity of RP037 cells after 64 h measured by flow cytometry. Three indicators are shown: the mean individual cellular fluorescence intensity in the sample (*GFP*), the percentage of cells with higher fluorescence intensity than a defined fluorescence background (*% active cells*), and the mean individual cellular fluorescence intensity in this cell subpopulation (*GFP (active cells)*). Data show averages and standard deviations calculated from three independent cultures

RP037. Here we used the commercial biosurfactant JBR215, which consists of a mixture of mono- and di-rhamnolipids from *Pseudomonas aeruginosa*. RP037 cells were cultivated in minimal medium with a thin layer of solid PHE on the surface of the glass flask in absence or presence of biosurfactant solution at a concentration of 0.1%. At this concentration, the surface tension of the medium was strongly reduced, as indicated by a droplet-collapsing assay, and this effect persisted at least for 2 days of incubation (data not shown). The bacteria cultivated in presence of JBR215 and exposed to PHE grew very rapidly until they stabilized at a certain cell density, whereas those exposed to PHE only showed a long lag phase followed by growth, which stopped as the dissolution rate of PHE became limiting (Fig. 4a). Bacteria exposed to biosurfactant solution only showed limited but significant growth, indicating that strain RP037 was able to degrade some of the rhamnolipids, or that the biosurfactant solution contained other available carbon sources. This supplementary growth corresponded approximately to the differences in final culture densities between PHE only, biosurfactant only, and PHE plus biosurfactant. Growth on the biosurfactant product was not sufficient to explain the more rapid growth rate observed in cultures on PHE plus



	acetate	phenanthrene	JBR215	GFP average	GFP SD	% active cells average	% active cells SD	GFP (active cells) average	GFP (active cells) SD
(1)	10 mM	-	-	2.9	0.0	0.8	0.1	11.9	0.3
	10 mM	+	-	3.7	0.0	6.0	0.4	15.4	0.0
	10 mM	+	0.1%	4.1	0.1	8.6	0.4	15.6	0.1
	10 mM	+	0.1% *	3.9	0.2	7.3	0.6	15.6	0.5
(2)	10 mM	-	-	2.8	0.1	0.8	0.1	12.0	0.3
	10 mM	-	0.1%	3.0	0.0	0.9	0.1	11.6	0.1
	-	+	-	5.2	0.4	13.1	2.3	14.7	0.4
	-	+	0.1%	5.6	0.0	16.5	0.0	15.5	0.1

Fig. 4 Effect of biosurfactant JBR215 on growth and GFP expression in *B. sartisoli* strain RP037 on PHE. **a** Culture turbidity development at 600 nm of RP037 in minimal medium without acetate but with 0.1% of JBR215 in the presence or absence of a PHE crystalline layer (0.05 mg/ml). *Error bars* indicate standard deviations from triplicate (PHE+) or duplicate (PHE−) measurements. The graph results from the combination of two separate experiments (*black and white symbols*). **b** GFP expression in RP037 cultures with or without 10 mM acetate, absence or presence of JBR215, and with or without crystalline PHE (0.05 mg/ml). JBR215 was added directly at time 0 or after 24 h of incubation (*). GFP expression was recorded by flow cytometry after 44 h (*1*) or 38 h (*2*), and is represented by the mean individual cellular fluorescence intensity in the sample (*GFP*), the percentage of cells that showed higher fluorescence intensity than a defined fluorescence background (*% active cells*) and the mean individual cellular fluorescence intensity in this cell subpopulation (*GFP (active cells)*). Averages and standard deviations were calculated from three independent cultures

biosurfactant (Fig. 4a). We observed that the addition of the JBR215 product partly disrupted the PHE layer, producing many small and insoluble PHE crystals. In addition to the effect of surfactant micelles on the solubilization of PHE, this may have modified PHE mass transfer rates to the bacteria by increasing the contact surface between PHE and the aqueous phase. GFP expression in RP037 was then assessed by flow cytometry (Fig. 4b). RP037 cells grown with acetate, PHE, and JBR215, or with PHE plus JBR215, or with PHE alone, all expressed GFP higher than in the absence of PHE ($p<0.01$; Fig. 4b). In addition, cultures with PHE to which biosurfactant was added expressed significantly more GFP than in the absence of biosurfactants ($p<0.05$). In particular, biosurfactant addition seemed to increase the proportion of 'active' cells in culture (i.e., those expressing GFP, Fig. 4b). A stepwise addition of JBR215 to the culture during growth produced intermediate results. Results with rhamnolipids thus suggest that a temporary increase of the number of active cells may allow faster growth of RP037 on PHE.

Discussion

We used the bacterial biosensor *B. sartisoli* strain RP037 as a model bacterium to test the hypothesis as to whether biosurfactants can enhance the bioavailability of PHE to cells in liquid culture. Two assays were deployed to test our hypothesis. One in which RP037 was co-cultured with a biosurfactant producer. The other in which purified biosurfactant was added from the beginning of cultivation on PHE. Two biosurfactant types were tested, lipopeptides and rhamnolipids. In general, *Pseudomonas* lipopeptides did not seem to enhance PHE bioavailability to RP037. This was concluded from the absence of more rapid growth of RP037 on PHE in co-cultures with a lipopeptide-producing strain (PCL1445) compared to a non-lipopeptide isogenic strain (PCL1436), from the absence of more rapid growth on PHE with purified lipopeptide extract from a producer culture compared to a non-producer culture, and from the absence of augmented GFP induction in RP037 cells compared to non-supplemented cultures of RP037 on PHE. Some effects were seen, which will be discussed in more detail below, but which could not be attributed to lipopeptides. In contrast, the rhamnolipid JBR215 induced both more rapid growth of RP037 on PHE and a higher proportion of GFP-producing cells compared to a non-amended RP037 culture on PHE. Although we ignore the exact mechanism of this effect, as discussed below, we can conclude that rhamnolipids can enhance PHE bioavailability to RP037.

We realized that co-cultivation of two bacterial species, although in principle the simplest bacterial 'community' that can be produced in the laboratory, show a much more complex and unpredictable behavior than pure cultures (An et al. 2006). Two bacterial species can interact in a variety of direct (e.g., contact, aggregation) and indirect ways (e.g., competition for nutrients, use of non-self metabolites, production of bacteriocides). Whereas our initial hypothesis was that lipopeptide production by PCL1445 could enhance the utilization rate of PHE by RP037, our results demonstrate that possibly metabolites other than lipopeptides can (slightly) enhance RP037 growth on PHE. The optimized growth media conditions (i.e., glycerol as carbon source) we employed for co-culturing *B. sartisoli* and *P. putida* essentially sustained growth of *P. putida* only while allowing lipopeptide production. Growth of RP037 in co-culture thus had to come from PHE utilization exclusively, and any enhancement of PHE utilization rates might have originated in lipopeptide production or other secondary metabolites excreted by *P. putida*. Acetate, in contrast, could be utilized by both bacteria, but proved to be less favorable for biosurfactant production.

Culture droplet-collapsing measurements indicated that PCL1445 indeed lowered the surface tension of the medium, but this was more pronounced at the end of the growth phase (≈3 days). Since the production of lipopeptides by PCL1445 is known to take place at late exponential phase (Kuiper et al. 2004), it might thus be that the lipopeptide production was not high enough during the most critical time of PHE utilization by RP037. In contrast, GFP production in RP037 was slightly stimulated in co-cultures with both PCL1445 and PCL1436 at early growth stage (44 h), suggesting that some compounds other than lipopeptides influenced the biosensor activity of RP037. This tendency disappeared after 3 days (68 h) of incubation.

The proportion of the two bacterial types in the community is known as another important variable for its behavior (Mellefont et al. 2008). We ensured RP037 sustenance by using an inoculum approximately ten times higher than that of *P. putida*. Our results indicated that *P. putida* rapidly colonized the suspended community at the expensive of its exclusive carbon source glycerol, which RP037 was unable to productively utilize. Interestingly, we also observed that the RP037 population doubled twice or thrice even in the absence of PHE in the culture medium, which suggested that RP037 utilized some carbon source released by *P. putida* during growth on glycerol. Compared to the control on PHE only, RP037 after 3 days had multiplied less rapidly in the presence of *P. putida*, with the non-lipopeptide producer having a stronger effect than PCL1445. It is unclear if this inhibition was due to competition for nutrients or other factors.

To rule out the possibility that lipopeptide production by PCL1445 had not occurred at the right moment for RP037 to enhance its utilization rate of PHE, we employed

partially purified lipopeptides and demonstrated that a 1% extract from strain PCL1445 indeed lowered the surface tension of the medium (Fig. 2b). Furthermore, we ensured that surfactant effects would act only on the apparent aqueous solubility of PHE by using PHE crystals, and not on dispersion of PHE dissolved in non-aqueous phase liquid such as heptamethylnonane employed before (Tecon et al. 2006). However, also in this case we could not demonstrate a direct effect of lipopeptides on PHE availability. Interestingly, *P. putida* culture extracts, both from PCL1445 or PCL1436 increased RP037 growth rates (Figs. 2a and 3a), which reinforces the conclusion drawn from co-culture experiments that some other compound(s) produced by *P. putida* serve as a carbon source for RP037. Both extracts were even able to increase GFP expression in RP037 cells compared to non-amended cultures with PHE, which may be due to an overall higher metabolic activity of the cells rather than to increased PHE bioavailability. This stimulatory result on GFP expression could not be confirmed when the experiment was repeated in cultures with PHE (with or without extract) but without acetate (Fig. 3b).

Contrary to the lipopeptides, a 0.1% commercial solution of rhamnolipids, produced by *P. aeruginosa* species, had a spectacular effect on RP037 growth on PHE. Rhamnolipids has before been shown to enhance PAH biodegradation rates by a *Pseudomonas* species (Shin et al. 2004; Hickey et al. 2007). Addition of rhamnolipids also increased the proportion of active GFP expressing RP037 cells in culture, which suggests that the PHE availability in solution increased importantly compared to a non-amended control. Despite this strong effect, we cannot firmly conclude the mechanism of rhamnolipid action on PHE availability. We observed that a 0.1% concentration of rhamnolipids 'disrupted' the layer of crystalline PHE deposited at the bottom of the culture flask. Instead of increasing the apparent PHE solubility in the aqueous phase, the rhamnolipids may have caused a breakup of the confluent PHE layer into numerous very small crystals. This may have produced an enormous increase in the contact surface between PHE and aqueous phase, and, therefore, a higher dissolution flux. The higher PHE flux could then have sustained a larger proportion of cells degrading PHE.

Obviously, the utilization of biosurfactants holds great promise for bioremediation technologies, because it might increase the bioavailability of HOCs for bacterial degradation. HOC biodegradation rates, which otherwise and often are limited in the environment by mass transfer processes, may thus be enhanced by biosurfactant application. Despite its promise and successful applications (Straube et al. 2003), there is no general rule for the most optimal type of biosurfactant or for the best application method. For example, Mulligan et al., considered in situ biosurfactant production easier and more cost efficient than external addition (Mulligan 2005), but we show here that in situ production may lead to numerous secondary effects that have nothing to do with increasing HOC availability. For this reason, there is an interest in better understanding the interactions between surfactant producers and pollutant degraders, in particular because it was demonstrated that the observed effects can be strain-dependent (Dean et al. 2001). This was corroborated by our results here, which showed no effects for lipopeptides but strong effects for rhamnolipids on PHE availability by RP037. Given that numerous secondary effects can play a role, a detailed understanding of biosurfactant mechanisms on HOC bioavailability is necessary. For instance, biosurfactants have been proposed not only to increase the apparent aqueous phase solubility of a HOC, but also to modify the bacterial cell membrane. This could render the membrane more hydrophobic and lead to increased HOC concentrations in the cell (Soberón-Chávez et al. 2005). Increased cell wall hydrophobicity could also promote better attachment to HOC phases or particles, which can reduce mass transport distances and thereby increase HOC degradation rates. We think that simple pathway induction assays like the ones with bioreporters deployed here, can provide an effective means for screening other biosurfactants and perhaps, obtain more generalized knowledge on the different bioavailability mechanisms.

Acknowledgments The authors are very grateful to Mrs. Fatimatou Baldé for her help in performing the experiments with lipopeptides. We also thank Terry J. McGenity from the University of Essex (UK) for the gift of the commercial rhamnolipid solution JBR215.

References

An D, Danhorn T, Fuqua C, Parsek MR (2006) Quorum sensing and motility mediate interactions between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* in biofilm cocultures. Proc Natl Acad Sci U S A 103:3828–3833

Bertrand J-C, Bonin P, Goutx M, Gauthier M, Mille G (1994) The potential application of biosurfactants in combatting hydrocarbon pollution in marine environments. Res Microbiol 145:53–56

Dean SM, Jin Y, Cha DK, Wilson SV, Radosevich M (2001) Phenanthrene degradation in soils co-inoculated with phenanthrene-degrading and biosurfactant-producing bacteria. J Environ Qual 30:1126–1133

Desai J, Banat I (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64

Dubern J-F, Bloemberg GV (2006) Influence of environmental conditions on putisolvins I and II production in *Pseudomonas putida* strain PCL1445. FEMS Microbiol Lett 263:169–175

Dubern J-F, Lagendijk EL, Lugtenberg BJJ, Bloemberg GV (2005) The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445. J Bacteriol 187:5967–5976

Dubern J-F, Lugtenberg BJJ, Bloemberg GV (2006) The *ppuI-rsaL-ppuR* quorum-sensing system regulates biofilm formation of

Pseudomonas putida PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II. J Bacteriol 188:2898–2906

Garcia-Junco M, De Olmedo E, Ortega-Calvo J-J (2001) Bioavailability of solid and non-aqueous phase liquid (NAPL)-dissolved phenanthrene to the biosurfactant-producing bacterium *Pseudomonas aeruginosa* 19SJ. Environ Microbiol 3:561–569

Garcia-Junco M, Gomez-Lahoz C, Niqui-Arroyo J-L, Ortega-Calvo J-J (2003) Biosurfactant- and biodegradation-enhanced partitioning of polycyclic aromatic hydrocarbons from nonaqueous-phase liquids. Environ Sci Technol 37:2988–2996

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Briggs Philipps G (1981) Manual of methods for general bacteriology. American society for microbiology, Washington, D. C

Gu MB, Chang ST (2001) Soil biosensor for the detection of PAH toxicity using an immobilized recombinant bacterium and a biosurfactant. Biosens Bioelectron 16:667–674

Hickey A, Gordon L, Dobson A, Kelly C, Doyle E (2007) Effect of surfactants on fluoranthene degradation by *Pseudomonas alcaligenes* PA-10. Appl Microbiol Biotechnol 74:851–856

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71–84

Kuiper I, Lagendijk EL, Pickford R, Derrick JP, Lamers GEM, Thomas-Oates JE, Lugtenberg BJJ, Bloemberg GV (2004) Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Mol Microbiol 51:97–113

Laurie AD, Lloyd-Jones G (1999) The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. J Bacteriol 181:531–540

Mellefont LA, McMeekin TA, Ross T (2008) Effect of relative inoculum concentration on *Listeria monocytogenes* growth in coculture. Int J Food Microbiol 121:157–168

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133:183–198

Noordman WH, Janssen DB (2002) Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. Appl Environ Microbiol 68:4502–4508

Olivera NL, Nievas ML, Lozada M, del Prado G, Dionisi HM, Siñeriz F (2009) Isolation and characterization of biosurfactant-producing *Alcanivorax* strains: hydrocarbon accession strategies and alkane hydroxylase gene analysis. Res Microbiol 160:19–26

Shin K-H, Kim K-W, Seagren EA (2004) Combined effects of pH and biosurfactant addition on solubilization and biodegradation of phenanthrene. Appl Microbiol Biotechnol 65:336–343

Soberón-Chávez G, Lépine F, Déziel E (2005) Production of rhamnolipids by *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 68:718–725

Straube WL, Nestler CC, Hansen LD, Ringleberg D, Pritchard PH, Jones-Meehan J (2003) Remediation of polyaromatic hydrocarbons (PAHs) through landfarming with biostimulation and bioaugmentation. Acta Biotechnol 23:179–196

Tecon R, Wells M, van der Meer JR (2006) A new green fluorescent protein-based bacterial biosensor for analysing phenanthrene fluxes. Environ Microbiol 8:697–708

Urum K, Grigson S, Pekdemir T, McMenamy S (2006) A comparison of the efficiency of different surfactants for removal of crude oil from contaminated soils. Chemosphere 62:1403–1410

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

Volkering F, Breure AM, Rulkens WH (1997) Microbiological aspects of surfactant use for biological soil remediation. Biodegradation 8:401–417

Yakimov M, Golyshin P, Lang S, Moore E, Abraham W, Lunsdorf H, Timmis K (1998) *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int J Syst Bacteriol 48:339–348

Zhang Y, Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl Environ Microbiol 58:3276–3282