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***Appl. Environ. Microbiol.* 1996, 62(6):1908.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1996, p. 1908–1912 Vol. 62, No. 6
0099-2240/96/$04.00+0

Biosurfactant Production by a Soil *Pseudomonas* Strain Growing on Polycyclic Aromatic Hydrocarbons

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Received 9 October 1995/Accepted 18 March 1996

The capacity of polycyclic aromatic hydrocarbon (PAH)-utilizing bacteria to produce biosurfactants was investigated. Twenty-three bacteria isolated from a soil contaminated with petroleum wastes were able to form clearing zones on mineral salt agar plates sprayed with solutions of PAHs. Naphthalene and phenanthrene were utilized as sole substrates. Biosurfactant production was detected by surface tension lowering and emulsifying activities from 10 of these strains grown in an iron-limited salt medium supplemented with high concentrations of dextrose or mannitol, as well as with naphthalene or phenanthrene. Glycolipid determinations showed that in cultures of *Pseudomonas aeruginosa* 19SJ on naphthalene, the maximal productivity of biosurfactants was delayed compared with that in cultures grown on mannitol. However, when small amounts of biosurfactants and naphthalene degradation intermediates were present at the onset of the cultivation, the delay was markedly shortened. Production of biosurfactants was accompanied by an increase in the aqueous concentration of naphthalene, indicating that the microorganism was promoting the solubility of its substrate. Detectable amounts of glycolipids were also produced on phenanthrene. This is the first report of biosurfactant production resulting from PAH metabolism.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants occurring mostly as a result of fossil fuel combustion and as by-products of industrial activities. Since many are known or suspected carcinogens and mutagens, exposure to PAHs may represent a significant health risk to human populations (43), and therefore their fate in nature is of great environmental concern. Microorganisms with the ability to degrade many PAHs have been described, and their mechanisms of action have been studied (7).

The bioremediation of soils contaminated with PAHs is limited by the poor availability of these hydrophobic contaminants to microorganisms (24). Surfactants can help, by solubilization or emulsification, to release hydrocarbons sorbed to soil organic matter and increase the aqueous concentrations of hydrophobic compounds, resulting in higher mass transfer rates (1). Contradictory results are found in the literature about the effects of addition of synthetic and biologically produced surfactants on PAH biodegradation (33). However, recent studies indicate that they can enhance hydrocarbon biodegradation by increasing microbial accessibility to insoluble substrates (38, 45). Several researchers have investigated the addition of biosurfactants to enhance the biodegradation of hydrocarbons (15, 17, 28, 45).

The composition, structure, and properties of a variety of surfactants produced by bacteria, yeasts, and fungi have been described (2, 11). Among the best-studied biosurfactants are the rhamnolipids of *Pseudomonas aeruginosa* (15). Most extracellular surface-active compounds are synthesized by microorganisms growing on poorly soluble substrates, principally *n*-alkanes (16). Biosurfactant production is often associated with the capacity of microorganisms to utilize hydrocarbons as substrates (18, 27). Koch et al. (22) have isolated a mutant of *P. aeruginosa* PG201 which had lost its capacity to grow on hexadecane because it failed to secrete its extracellular rhamnolipids. It has been suggested that biosurfactant growth-stimulating activity is more specific for the hydrophobic substrate used to produce it (3, 10) and is restricted mainly to the producing organism itself (15, 18, 26).

More knowledge about the specific mechanisms used by microorganisms to get access to PAHs may provide useful information to improve PAH biodegradation applications. Since microbial growth on water-immiscible aliphatic hydrocarbons has been associated with the production of surfactants, we were interested in finding out if PAH-metabolizing bacteria might be able to secrete surface-active agents.

Our objective is to determine if production of surfactants by PAH-metabolizing microorganisms is part of their strategy for growing on such poorly available substrates. As a first step toward this goal, we report here the isolation of bacterial strains producing surfactants while growing on naphthalene or phenanthrene. A detailed study of one of the isolates, *P. aeruginosa* 19SJ, was conducted.

MATERIALS AND METHODS

Source of microorganisms. Bacteria were isolated from a sandpit that received important quantities of oil refinery wastes during the 1960s. Biosurfactant-producing reference strains *P. aeruginosa* UG2 and PG201 were obtained from H. Lee and J. T. Trevors (University of Guelph, Guelph, Ontario, Canada) and J. Reiser (Institute for Biotechnology, Zurich, Switzerland), respectively.

Maintenance and identification of bacteria. Microorganisms were stored at −70°C in glycerol and subcultured on tryptic soy agar (Difco) plates before use as inoculum. Selected bacterial isolates were characterized by using API 20E strips (bioMérieux Vitek, Inc., Hazelwood, Mo.) and several physiological and biochemical tests (23).

Isolation of PAH-degrading microorganisms. Isolates were plated on Bushnell-Haas agar (BH) (Difco) and sprayed (20, 37) with a 2% PAH stock solution in acetone. Presumptive PAH users were distinguished by formation of a clearing zone or a coloration around the colonies. Sprayed-plate experiments were performed in duplicate. Naphthalene dioxygenase activity was detected by the formation of blue-indigo colonies when indole (1 mM) was added to the agar (9).

The ability to grow on naphthalene and phenanthrene (Aldrich Chemical Co., Milwaukee, Wis.) was confirmed by using 250-ml Erlenmeyer flasks containing

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25 ml of BH and incubated in the dark at 29°C and 150 rpm. PAH utilization was initially assessed visually by disappearance of PAH crystals, color changes, and increase in optical density of the medium. For the determination of residual PAHs, whole cultures were adjusted to pH 12.0 with 1 N NaOH to prevent potential biosurfactant interferences such as formation of emulsions, extracted with diethyl ether, and analyzed with a gas chromatograph (Hewlett-Packard model 5890A) equipped with a flame ionization detector.

Screening for biosurfactant activity. PAH-utilizing bacteria were first screened for the ability to produce surfactants by cultivation in liquid BH supplemented with 2% dextrose. Many *Pseudomonas* strains are known to produce biosurfactants on dextrose, glycerol, or mannitol (31, 39). The drop-collapsing test was used for rapid determination of surface tension lowering (19). The emulsifying activity of culture supernatants was estimated by adding 0.5 ml of sample fluid and 0.5 ml of hexadecane to 4.0 ml of distilled water. The tube was vortexed for 10 s, held stationary for 1 min, and then visually examined for turbidity of a stable emulsion.

Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg/ml; Sigma Chemical Co., St. Louis, Mo.) and methylene blue (5 μg/ml) (34) were used to detect extracellular glycolipid production with 2% dextrose or mannitol as a substrate. Biosurfactants were observed by the formation of dark blue halos around the colonies.

Biosurfactant production and quantification. The mineral salt medium utilized for the blue agar plates (34) was supplemented with 10 mg of $FeSO_4 \cdot 7H_2O$ liter^{-1} for use as an optimized medium (designated SWF medium) for biosurfactant production in liquid cultures (pH 6.7). Growth conditions favorable for the production of surfactants require an elevated C-to-N ratio and limiting iron concentrations (14). Therefore, a high concentration (2%) of mannitol, naphthalene, or phenanthrene was used as a substrate. For biosurfactant production on PAHs, the strains were first grown on naphthalene in order to induce their naphthalene catabolic genes. Growth was monitored by measuring the A_{600}. Surface tension was measured by the ring method with a du Nouy tensiometer (Fisher Scientific, Toronto, Ontario, Canada).

Assuming that the surfactants secreted by our *P. aeruginosa* strain were rhamnolipids, the concentration of extracellular glycolipids was evaluated in triplicate by measuring the concentration of rhamnose with a modified orcinol method (8, 22). A volume of 200 μl of the acidified culture supernatant was extracted three times with 1 ml of diethyl ether, and then the fractions were pooled, dried, and resuspended in 1 ml of 0.05 M sodium bicarbonate. A 200-μl sample was treated with 1.8 ml of a solution of 100 mg of orcinol in 53% H_2SO_4 and boiled for 20 min. After cooling at room temperature for 15 min, the A_{421} was measured. Rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose and expressed as rhamnose equivalents (in milligrams per milliliter).

Determination of naphthalene apparent solubility. In order to estimate the solubilizing effect of the surfactants produced when the bacteria were grown on naphthalene, samples of culture (1.3 ml) were centrifuged at 500 × *g* for 30 min and the concentration of pseudosolubilized naphthalene was quantified from 1 ml of supernatant.

Cell surface hydrophobicity test. A modified assay of microbial adhesion to hydrocarbons (32) was utilized to determine changes in cell surface hydrophobicity during growth in SWF liquid medium with naphthalene as a substrate. Hexadecane (0.5 ml) and washed cells (2 ml) were vortexed in a test tube. After equilibration, the loss in absorbance of the aqueous phase relative to that of the initial cell suspension was measured, and hydrophobicity was estimated by calculating the percentage of cells adhering to hexadecane. Reported results are means of duplicate measurements.

Analysis of naphthalene polar intermediates. The concentration of hydroxylated aromatic metabolites from naphthalene degradation was determined by an adaptation (13, 21) of the method outlined by Box (6), which uses the Folin-Ciocalteu reagent. A standard curve was prepared with sodium salicylate, and the concentration of metabolic intermediates was estimated as salicylate equivalents in milligrams per milliliter.

RESULTS

PAH utilization. Twenty-three bacteria able to form clearing zones on BH agar plates coated with naphthalene or phenanthrene (designated PAH$^+$ strains) were isolated. Experiments with BH liquid cultures confirmed their capacity to grow on these PAHs as sole substrates. All PAH$^+$ bacteria formed blue-indigo colonies on indole-containing plates, indicating the presence of naphthalene dioxygenase activity (10, 23). Biosurfactant-producing reference strains UG2 and PG201 did not clear PAH-sprayed plates or convert indole to indigo. All PAH$^+$ strains were fluorescent pseudomonads.

Screening for the ability to produce biosurfactants. When grown in BH with 2% dextrose, 10 of the PAH$^+$ strains decreased the culture medium surface tension below 35 mN/m and stabilized hexadecane-water emulsions. They formed halos on blue agar plates with dextrose and mannitol.

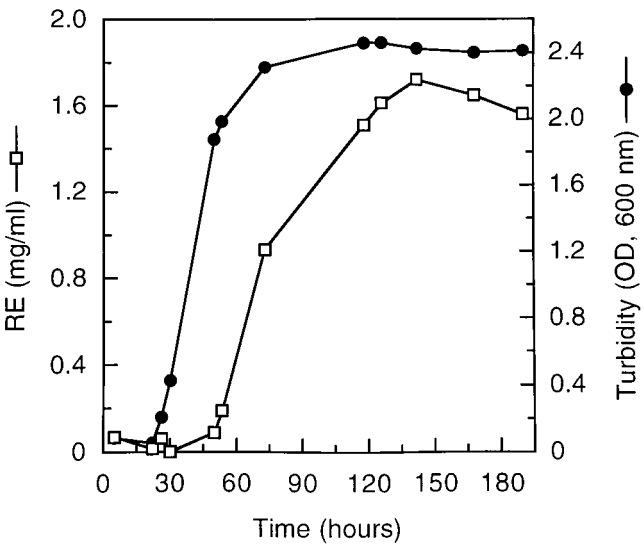

FIG. 1. Production of biosurfactants (glycolipids) by *P. aeruginosa* 19SJ in SWF mineral salt medium with 2% mannitol. Incubation was done at 25°C with shaking at 200 rpm. OD, optical density. Biosurfactant levels are expressed as rhamnose equivalents (RE).

These 10 strains also synthesized biosurfactants with naphthalene or phenanthrene as the sole substrate. When cultured in SWF medium with 2% naphthalene, indications of biosurfactant production were seen within 3 days of incubation. Hexadecane-emulsifying activity was observed and the surface tension of the culture supernatants was lowered to between 30 and 35 mN/m after 4 to 6 days, depending on the strain. The amounts of biosurfactants produced were therefore sufficient to reach the lowest possible surface tension, the critical micelle concentration.

The strain which displayed the highest biosurfactant productivity was selected for a more detailed analysis. It was identified as *P. aeruginosa* 19SJ. Figure 1 shows the profile of biosurfactant production obtained when this strain was cultured in SWF medium with mannitol. As expected, since biosurfactants are secondary metabolites, maximal glycolipid production started near the end of the exponential growth phase.

Biosurfactant production during growth on PAHs. In the study of biosurfactant production on PAHs with strain 19SJ, starting the cultures with washed cells compared with whole culture broth affected the kinetics of biosurfactant production, cell behavior, and naphthalene degradation. Glycolipid accumulation (expressed as rhamnose equivalents) was delayed when washed cells were used as inoculum (Fig. 2a) instead of whole culture broth (Fig. 2b). However, enough glycolipids were secreted at the end of the exponential growth phase to cause an important drop in surface tension (Fig. 2a). Accumulation of naphthalene degradation products in the supernatant was accompanied by a decrease in pH. Actually, the major metabolite was salicylic acid, as determined by gas chromatography-mass spectrometry. As expected, an increase in the apparent aqueous solubility of naphthalene was observed when concentrations of glycolipids exceeded the critical micelle concentration (which is indicated by the lowered surface tension). Because of its volatility, the actual concentration of solubilized naphthalene was probably slightly higher than indicated by the data obtained. Bacterial degradation of naphthalene was not

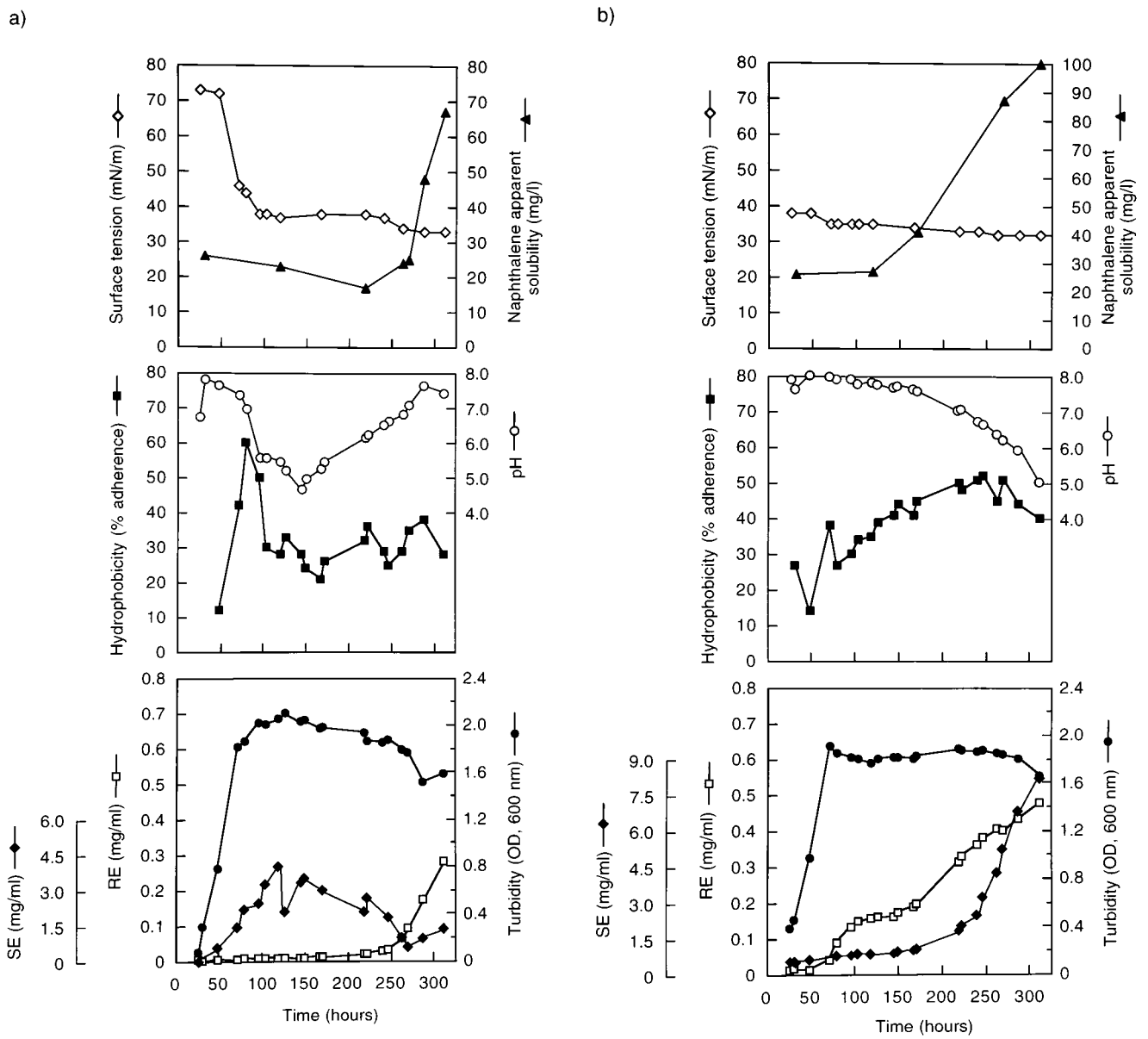

FIG. 2. Production of biosurfactants (glycolipids) by strain 19SJ grown in 150 ml of SWF medium with 2% naphthalene as a substrate. Results shown are from one experiment of duplicate experiments. (a) Inoculation with washed cells from 5 ml of a 15-day-old culture. (b) Inoculation with 5 ml from a 15-day-old culture. Incubation was done at 27°C with shaking at 200 rpm. OD, optical density. Levels of naphthalene degradation metabolites are expressed as salicylate equivalents (SE); levels of biosurfactants are expressed as rhamnose equivalents (RE).

directly quantified because of its volatility and utilization of very high concentrations (20 g/liter). Degradation of naphthalene is therefore indirectly shown by the accumulation of glycolipids and metabolic intermediates.

Washed cells of strain 19SJ were cultivated in SWF medium with 2% phenanthrene under the same conditions used previously for naphthalene. After 10 days of incubation, the drop-collapsing test was positive and an emulsion of supernatant and hexadecane was stable. However, concentrations of biosurfactants obtained were low (about 40 μg of rhamnose equivalents per ml; minimal surface tension reached around 45 mN/m, after 450 h of incubation) compared with those obtained with naphthalene.

DISCUSSION

Observation of both tensio-active and emulsifying activities indicated that biosurfactants were produced by 10 of 23 PAH-utilizing bacterial strains. This production was observed when cells were grown on soluble substrates, such as mannitol or dextrose, or on poorly soluble PAHs, such as naphthalene or phenanthrene. Our method of strain selection probably ensured resistance to high concentrations of naphthalene, which has been reported to be toxic for many PAH-metabolizing bacteria (5, 25, 42).

The biosurfactants synthesized by *P. aeruginosa* 19SJ are most probably a mixture of rhamnolipids, the amphiphilic surface-active glycolipids usually secreted by *P. aeruginosa* (15,

36). Accordingly, this strain formed halos on blue agar plates, which detect the production of extracellular anionic glycolipids by *Pseudomonas* spp. (34). Overproduction of biosurfactants on mannitol occurred as the cells ceased to grow. This was expected, since growth-limiting conditions are required for production of rhamnolipids by *P. aeruginosa* (14, 39).

The pattern of biosurfactant production from naphthalene in cultures inoculated with washed 19SJ cells was very different from that of cultures inoculated with the whole broth. Biosurfactant production, like cell growth, depends on the availability of the substrate. Naphthalene solubility (31 mg/liter) was high enough to allow unrestricted growth of both washed and unwashed cells, as illustrated by their similar increase in turbidity (Fig. 2). However, at high cell density, the availability of poorly soluble substrates becomes limiting (40) because PAHs are utilized only in the dissolved state (44). The fact that glycolipid accumulation started more rapidly when the cultures were inoculated with the whole broth may be partially explained by the concomitant addition of biosurfactants that could increase naphthalene availability and hence bacterial utilization. The inoculum culture fluid may also have contained some amounts of the diffusible autoinducers known to regulate rhamnolipids synthesis in *P. aeruginosa* (29). On the other hand, washed cells required an adaptation before reaching the stage of maximal surfactant secretion. An important increase in surface hydrophobicity was observed at the beginning of the stationary phase of growth, coinciding with the first stage of biosurfactant production. This may facilitate cell adhesion and access to naphthalene, as suggested by the subsequent accumulation of degradation intermediates.

Smaller amounts of glycolipids were produced from phenanthrene than naphthalene. Since it is much less water soluble (1.3 mg/liter) than naphthalene, it is probable that cells required more time to adapt themselves to the utilization of this poorly accessible PAH.

The rationale behind biosurfactant production on PAHs is that it should stimulate itself by enhancing the substrate availability. An accumulation of naphthalene degradation metabolites was observed when concentrations of glycolipids in the medium started to increase abruptly. This can be explained by the increasing amount of available naphthalene due to the solubilizing effect of biosurfactants (as indicated by the concentration of pseudosolubilized naphthalene). Similar observations in the presence of synthetic surfactants have been reported (12, 25, 38).

The exact reason why some microorganisms produce surfactants is unclear. However, biosurfactant-producing bacteria are found in higher concentrations in hydrocarbon-contaminated soils (30). Accordingly, our results also suggest that the capacity to secrete surface-active agents is a common feature among PAH-degrading soil bacteria.

Until now, the few researchers who have looked for surfactant production by PAH-degrading bacteria have been unsuccessful (35, 41). UG2 was reported to display some emulsifying activity when grown on naphthalene (4), but under our experimental conditions it could not utilize naphthalene or express any naphthalene dioxygenase activity. To our knowledge, this is the first published report of microorganisms producing surface-active compounds while growing on PAHs. Experiments are in progress to characterize the optimal conditions for biosurfactant production on PAHs and to determine the potential role biosurfactants may play in PAH metabolism by the producing organism.

ACKNOWLEDGMENT

We thank Zeina Yared for excellent technical assistance in bacterial identification and screening for PAH utilization.

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