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Hydrocarbon Assimilation and Biosurfactant Production in *Pseudomonas aeruginosa* Mutants

ANDREAS K. KOCH,[1] OTHMAR KÄPPELI,[2] ARMIN FIECHTER,[1] AND JAKOB REISER[1]*

Institute for Biotechnology, ETH-Hönggerberg, CH-8093 Zürich,[1] and BIDECO AG, CH-8600 Dübendorf,[2] Switzerland

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We isolated transposon Tn5-GM-induced mutants of *Pseudomonas aeruginosa* PG201 that were unable to grow in minimal media containing hexadecane as a carbon source. Some of these mutants lacked extracellular rhamnolipids, as shown by measuring the surface and interfacial tensions of the cell culture supernatants. Furthermore, the concentrated culture media of the mutant strains were tested for the presence of rhamnolipids by thin-layer chromatography and for rhamnolipid activities, including hemolysis and growth inhibition of *Bacillus subtilis*. Mutant 65E12 was unable to produce extracellular rhamnolipids under any of the conditions tested, lacked the capacity to take up ^{14}C-labeled hexadecane, and did not grow in media containing individual alkanes with chain lengths ranging from C_{12} to C_{19}. However, growth on these alkanes and uptake of [^{14}C]hexadecane were restored when small amounts of purified rhamnolipids were added to the cultures. Mutant 59C7 was unable to grow in media containing hexadecane, nor was it able to take up [^{14}C]hexadecane. The addition of small amounts of rhamnolipids restored growth on alkanes and [^{14}C]hexadecane uptake. In glucose-containing media, however, mutant 59C7 produced rhamnolipids at levels about twice as high as those of the wild-type strain. These results show that rhamnolipids play a major role in hexadecane uptake and utilization by *P. aeruginosa*.

Many procaryotic and eucaryotic microorganisms satisfy their carbon and energy requirements by using compounds, such as hydrocarbons, that are poorly soluble in aqueous media. The growth on hydrocarbons is often associated with the production of surface-active compounds. Surface-active molecules contain hydrophilic and hydrophobic components, a property that enables such molecules to concentrate at interfaces and to reduce the surface tensions of aqueous media. Several different microbial products that exhibit surface-active properties have been identified in the past. These so-called biosurfactants are produced by certain bacteria and by a number of yeasts and filamentous fungi. They include low-molecular-weight glycolipids, lipopeptides, and high-molecular-weight lipid-containing polymers such as lipoproteins, lipopolysaccharide-protein complexes, and polysaccharide-protein-fatty acid complexes. The common hydrophobic (lipophilic) moiety in biosurfactants is the hydrocarbon chain of a fatty acid, whereas the polar or hydrophilic group is derived from the ester or alcohol functional groups of neutral lipids, from the carboxylate group of fatty acids or amino acids, or, in the case of glycolipids, from carbohydrates. When the surfactants are extracellular, they cause the emulsification of the hydrocarbon. When they are cell wall associated, they facilitate the penetration of hydrocarbons to the periplasmic space. Many of the biosurfactants known today have been investigated with a view toward possible technical applications. Because biosurfactants are readily biodegradable and can be produced in large amounts by microorganisms and thus are not dependent on petroleum-derived products, they might well be able to replace, in some instances, the traditional synthetic surfactants. The structures, properties, and production of biosurfactants have been reviewed extensively; the overview of Reiser et al. (34) is the most recent.

* Corresponding author.

The rhamnose-containing glycolipids produced by *Pseudomonas* spp. (20, 22, 38) are among the biosurfactants to be studied most intensively. The rhamnolipids from *Pseudomonas aeruginosa* were first described in 1949 (24), and studies on the biosynthesis of these compounds were carried out in vivo by Hauser and Karnofsky (14–16), who showed that these glycolipids were secreted into the medium during the stationary phase of growth. They also defined the optimal conditions for rhamnolipid production by this organism from various radioactive precursors, such as acetate, glycerol, glucose, and fructose. Burger et al. (6, 7) described the complete enzymatic synthesis of a rhamnolipid by extracts of *P. aeruginosa*. They were able to show that the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyl transferase, and that TDP-rhamnose is an efficient rhamnosyl donor in the synthesis of the rhamnolipid according to the following reactions: TDP-L-rhamnose + β-hydroxydecanoyl-β-hydroxydecanoate $\xrightarrow{\text{transferase 1}}$ TDP + L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and TDP-L-rhamnose + L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate $\xrightarrow{\text{transferase 2}}$ 2 TDP + L-rhamnosyl-L-rhamnosyl-β-hydroxydecan-oyl-β-hydroxydecanoate. L-Rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate have been designated as rhamnolipids 1 and 2, respectively. Whereas rhamnolipids 1 and 2 are the principal rhamnolipids produced in liquid cultures, rhamnolipids 3 and 4, containing two sugar moieties and one fatty acid moiety and one sugar and one fatty acid moiety, respectively, appear to be produced exclusively by resting cells (38). The production of rhamnolipids is regulated, and rhamnolipid formation by *P. aeruginosa* in a mineral salt medium with 2% alkanes as a C source is increased after NO_3^- limitation during the stationary growth phase (39). A direct relationship between increased glutamine synthetase activity and enhanced biosurfactant production was recently found in *P. aeruginosa* cells

grown in nitrate: increased ammonium and glutamine concentrations repressed both phenomena (28). Evidently, the control of rhamnolipid production is linked in some way to the control of nitrogen metabolism.

Hisatsuka et al. (17–19) isolated a "protein-like activator for *n*-alkane oxidation" from *P. aeruginosa*. It had a molecular weight of about 14,300 and 147 amino acid residues. This activator was able to emulsify hexadecane and water and stimulated the growth of *P. aeruginosa* on hexadecane. *P. aeruginosa* rhamnolipids and the proteinlike activator interacted with each other during emulsification of hexadecane (18). Other pseudomonads such as *Pseudomonas fluorescens* have also been reported to produce rhamnolipids, particularly when glycerol is used as a carbon source (39), and *Pseudomonas* strains with the capacity to produce biosurfactants other than rhamnolipids when growing on sucrose were isolated from the environment by a simple screening procedure (31). One of these isolates, *P. fluorescens* 378, was shown to produce a novel surface-active compound of high molecular weight consisting mainly of carbohydrates and a protein with a molecular weight of 10^6 and an isoelectric point of 9.1 (32).

The exact physiological role(s) of rhamnolipids is unclear at this point. They appear to be involved during growth of *P. aeruginosa* on water-insoluble substrates such as paraffin (23), and they may serve as virulence factors during colonization of the lung tissue (21). Our long-term goal is to arrive at a better understanding of the synthesis and roles of rhamnolipids in pseudomonads by using molecular genetic and biochemical tools; as a first step toward this goal, we report here on the isolation and analysis of mutants that are affected in rhamnolipid production.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* DSM 2659 (our strain PG201 [11]) was used throughout this study, and *Bacillus subtilis* PG203 (a gift from L. Guerra-Santos) was used in the growth inhibition assay. Plasmid pCHR84 containing a derivative of Tn*5* was obtained from C. Sasakawa (37).

Growth media. Luria broth (LB) was used to propagate *Escherichia coli*, *B. subtilis*, and *P. aeruginosa*. A minimal medium (11) containing 2% glucose or 0.5% hexadecane was used in some of the experiments. Antibiotics were applied at the following concentrations: 50 μg of kanamycin sulfate per ml, 20 μg of gentamicin sulfate per ml, 10 μg of trimethoprim per ml, and 20 μg of tetracycline hydrochloride per ml for *E. coli* and 100 μg of gentamicin sulfate per ml or 150 μg of tetracycline hydrochloride per ml for *P. aeruginosa*.

Transposon Tn*5*-GM mutagenesis of *P. aeruginosa*. *E. coli* MC1061 cells harboring pCHR84 were grown in LB containing trimethoprim and gentamicin at 30°C for 12 h, and *P. aeruginosa* was cultivated in LB at 43°C for 24 h. The cultures were washed twice with sterile 10 mM Tris buffer (pH 7.5) and resuspended in 5 ml of the same buffer. A membrane filtration technique (40) was applied for the conjugation step. Serial dilutions of the mating mixture were plated on LB agar plates containing gentamicin and incubated at 30°C. The colonies appearing after 24 h were collected and transferred to fresh LB containing gentamicin. After shaking at 30°C overnight, serial dilutions of the cultures were plated onto LB agar containing gentamicin and incubated at 43°C for 48 h.

Screening for mutants defective in rhamnolipid synthesis. Single colonies obtained after the mating procedure were inoculated into microtiter plate wells (Dynatech) containing LB with gentamicin, and the plates were incubated at 30°C overnight. Part of the culture liquid was then transferred with the help of a spiked cover fitting to a microtiter plate containing 2% hexadecane minimal medium with gentamicin. The microtiter plates were incubated at 30°C for 60 h, and the wells were then checked for growth of the bacteria.

Detection, quantification, and isolation of rhamnolipids. For the detection of rhamnolipids, four independent tests were carried out. These included the lowering of the interfacial tension (IFT) by rhamnolipids (10), the detection of rhamnolipids by thin-layer chromatography (26), the hemolysis of erythrocytes by rhamnolipids (25), and the growth inhibition of *B. subtilis* exerted by rhamnolipids (22). Culture supernatants were concentrated as follows. The pH of 10 ml of the culture supernatant was adjusted to 6.5, and $ZnCl_2$ was added to a final concentration of 75 mM (12). The precipitated material was dissolved in 10 ml of 0.1 M sodium phosphate buffer (pH 6.5) and extracted twice with an equal volume of diethyl ether. The pooled organic phases were evaporated to dryness, and the pellets were dissolved in 100 μl of methanol. Concentrated culture supernatants were spotted onto paper filter discs (6.0-mm Whatman AA discs), which were then put onto a layer of LB soft agar containing freshly grown *B. subtilis* cells (approximately 10^9/ml) or onto agar plates containing 5% sheep blood (Becton Dickinson). Typically, 10 ml of a culture supernatant was concentrated down to 100 μl, and 10-μl aliquots were applied to the paper discs. The *B. subtilis* plates were put at 37°C overnight, and the blood agar plates were incubated at room temperature for 2 days; the zones of inhibition and hemolysis were then measured. As an indirect method for the quantification of biosurfactants, the cell-free culture liquid was diluted in 0.1 M sodium phosphate buffer (pH 6.5), and the IFT was measured with an autotensiomate against a mixture of the following aliphatic hydrocarbons: 0.4% *n*-dodecane, 3.8% *n*-tridecane, 24.3% *n*-tetradecane, 32.6% *n*-pentadecane, 26.6% *n*-hexadecane, 12.0% *n*-heptadecane, and 0.3% *n*-octadecane (10).

The orcinol assay (9) was used to directly assess the amount of glycolipids in the sample: 333 μl of the culture supernatant was extracted twice with 1 ml of diethyl ether. The ether fractions were pooled and evaporated to dryness, and 0.5 ml of H_2O was added. To 100 μl of each sample 900 μl of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added; after heating for 30 min at 80°C, the samples were cooled for 15 min at room temperature and the A_{421} was measured. The concentrations of rhamnolipids were calculated by comparing the data with those of rhamnose standards between 0 and 50 μg/ml.

For the growth stimulation experiments rhamnolipids were purified by the procedure of Reiling et al. (33).

[^{14}C]hexadecane uptake studies. Studies on the incorporation of ^{14}C-labeled hexadecane into alkane-induced cells were performed in a way similar to the protocol described by Bassel and Mortimer for the yeast *Yarrowia lipolytica* (2). Single colonies grown on LB agar were inoculated into LB and cultivated at 37°C at 180 rpm for 16 h; 0.3-ml samples were inoculated into 50 ml of a 2% glucose minimal medium and shaken in baffled 500-ml flasks at 190 rpm and 30°C for 22 h. The cells were washed twice in 50 ml of H_2O and then resuspended in minimal medium containing 0.3% hexadecane and rhamnolipids (50 μg/ml) and incubated for 16 h. The cells were washed twice with water and finally resuspended and diluted into minimal medium to give a final optical density at 600 nm (OD_{660}) of 0.04. A 10-ml sample of this

suspension corresponding to 0.4 mg (dry mass) of cells was transferred to a 50-ml Erlenmeyer flask, and then 5 μl of an undiluted *n*-[1-^{14}C]hexadecane stock solution (100 μCi/ml, 61 mCi/mmol, 268 mCi/g; Amersham) was added. Control experiments contained rhamnolipids (50 μg/ml, final concentration) in addition. The cultures were shaken at 200 rpm at room temperature, and 1-ml aliquots were withdrawn at various times and filtered through glass fiber filters (0.7-μm pore size, 2.4-cm diameter; GF/F Whatman). The cells were then washed twice with 5 ml of ice-cold ethanol, the filters were placed into scintillation vials, 20 ml of Aqualuma (Lumac) was added, and the samples were counted.

RESULTS

Screening for mutant strains of *P. aeruginosa* affected in hydrocarbon assimilation. It has been shown that the addition of low concentrations of detergents such as Triton X-100 and Brij 35 to the culture medium stimulates the growth of *P. aeruginosa* cells on water-insoluble substrates such as hexadecane (4). Moreover, we and others (20, 23) have found that purified rhamnolipids from *P. aeruginosa* at concentrations of around 10 μg/ml significantly accelerate the growth of *P. aeruginosa* on hexadecane-containing media. These observations, which are in line with there being a need for amphiphilic compounds capable of solubilizing the water-insoluble substrate, formed the basis for our design of a screening procedure for mutants affected in the production of extracellular rhamnolipids. This procedure makes use of the fact that the growth of cells lacking the ability to synthesize rhamnolipids is retarded in media containing hexadecane as a carbon source. We used transposon Tn*5*-GM (37), which carries a gentamicin resistance gene, to mutagenize *P. aeruginosa* PG201 (10). This strain was found to be resistant to kanamycin at concentrations up to 200 μg/ml, therefore precluding the use of transposon Tn*5* carrying a kanamycin resistance gene. Individual gentamicin-resistant colonies (10,000) were picked into the wells of microtiter plates containing LB medium, and aliquots of the grown cultures were then transferred into wells containing hexadecane-minimal medium. The growth of the cultures in 138 cases (1.4%) was either retarded or absent even after 240 h of incubation, indicating that these strains may have defects in the biosynthesis of amino acids, nucleic acid precursors, vitamins, etc., or that they are affected in the assimilation of hydrocarbons.

Identification of mutants affected in alkane uptake and utilization. Since a number of different biochemical reactions are involved in alkane utilization in bacteria, including the uptake of alkanes, their terminal hydroxylation, and the β-oxidation reactions (for a recent review, see reference 41), a strategy was designed to unequivocally identify mutants affected in rhamnolipid production. To differentiate between mutants affected in the production of rhamnolipids and mutants deficient in other functions involved in hexadecane assimilation, the 138 mutant strains that were affected in their growth in hexadecane-minimal medium were inoculated into a minimal medium containing 2% glucose and incubated at 37°C for 48 h. As expected, many of the mutants turned out to be affected in their growth in minimal media containing glucose, indicating that they are affected in steps other than hexadecane uptake and/or utilization. Twenty different strains that grew well in glucose-minimal media were analyzed further for their growth behavior in media containing hexadecane and in media containing hexadecane supplemented with rhamnolipids. In shake flask experiments, the growth of all 20 of the strains tested could be stimulated by the addition of purified rhamnolipids, although the growth behaviors of the individual strains were quite different. Two strains, 59C7 and 65E12, were analyzed in more detail in minimal media containing either glucose (Fig. 1A) or hexadecane (Fig. 1B) as a carbon source. It is evident from Fig. 1B that the addition of purified rhamnolipids at a concentration of 0.01% permitted the growth of the two mutant strains, whereas in the absence of rhamnolipids growth was not possible within 45 h of incubation.

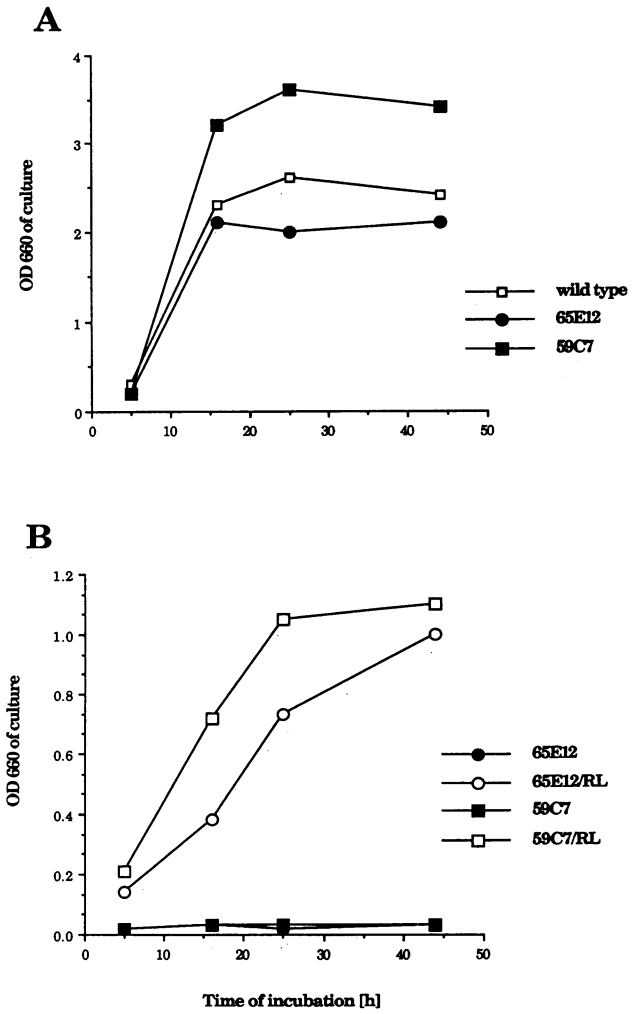

FIG. 1. Growth of *P. aeruginosa* PG201 and mutants 65E12 and 59C7 in minimal media containing glucose or hexadecane. (A) Samples of 0.2 ml of overnight cultures in LB medium were inoculated into 20 ml of minimal medium containing 2% glucose. Cultivation took place at 37°C and 180 rpm. The OD_{660} values of 10-fold-diluted culture supernatants were determined at various times. (B) Samples of 0.4 ml of an overnight culture in LB medium were inoculated into 20 ml of minimal medium containing 1% hexadecane and incubated at 37°C. To the samples indicated by "RL," rhamnolipid was added at a concentration of 0.01%.

To see whether the growth behavior of the two mutant strains in minimal media containing hydrocarbons other than hexadecane was also affected, a series of alkanes ranging from dodecane to nonadecane was tested (Fig. 2). *P. aeruginosa* PG201 was able to use efficiently alkanes ranging from C_{12} to C_{19} as carbon sources (Fig. 2A). Moreover, both

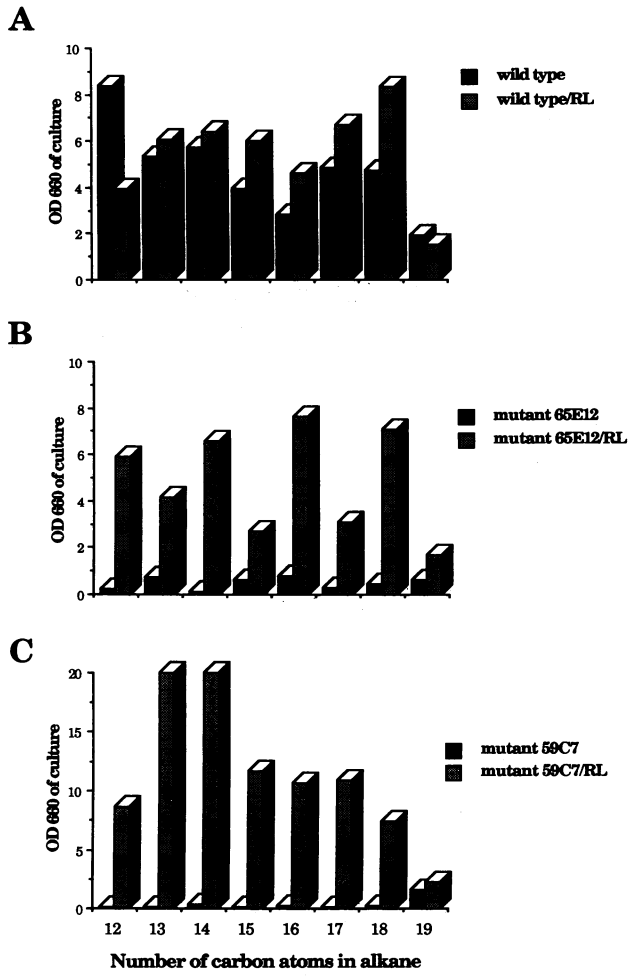

FIG. 2. Growth of *P. aeruginosa* PG201 (A) and mutant 65E12 (B) and 59C7 (C) strains on aliphatic C_{12} to C_{19} hydrocarbons. The growth of PG201 and its transposon-carrying derivatives in a 0.5% alkane minimal medium was monitored for 140 h. At the end of the incubation period the OD_{660} of the culture was measured. To the samples indicated by "RL," rhamnolipid was added at a final concentration of 0.005%.

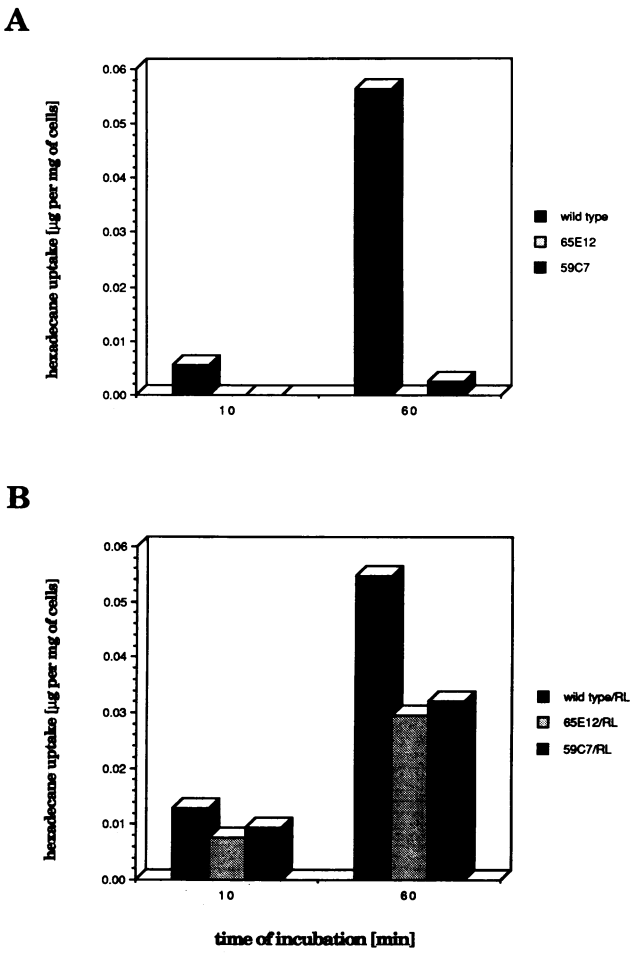

FIG. 3. Hexadecane uptake by *P. aeruginosa* PG201 and mutant strains 65E12 and 59C7. Glucose-grown cells were inoculated in a minimal medium containing 0.3% hexadecane and rhamnolipids at concentrations of 50 µg/ml and incubated for 16 h at 200 rpm. After three washing steps, cell aliquots corresponding to an OD_{660} of 0.04 were inoculated into 10 ml of a minimal salt medium. To the cultures shown in panel B, purified rhamnolipids at a concentration of 50 µg/ml were added. Then 5 µl of *n*-[1-^{14}C]hexadecane (100 µCi/ml, 61 mCi/mmol) was added. Incubation took place at room temperature. Samples of 1 ml were withdrawn at the times indicated; the cells were filtered, washed twice with ethanol, and counted.

mutant strains appear to be generally affected in their assimilation of alkanes; growth on alkanes ranging from C_{12} to C_{19} was not possible without the addition of purified rhamnolipids (Fig. 2B and C). Surprisingly, mutant 59C7 reached much higher OD values than did the wild type when growing on C_{13} and C_{14} in the presence of low amounts of rhamnolipids (Fig. 2C).

To gain more information concerning the phenotypes of the two mutant strains, a hexadecane uptake experiment was conducted (Fig. 3). Alkane-induced cells of *P. aeruginosa* PG201 and the 65E12 and 59C7 mutant strains were analyzed for their capacity to take up radioactively labeled hexadecane within 60 min. Since the two mutant strains do not grow in hexadecane-containing media, the induction of the cells had to be carried out by adding rhamnolipids to the preculture medium. After careful washing, the cells were added to a minimal medium containing ^{14}C-labeled hexadecane; samples were withdrawn after 10 and 60 min, filtered, and washed twice with ethanol to remove any extracellular hexadecane. A significant accumulation of hexadecane occured under these conditions in wild-type *P. aeruginosa* cells but not in the two mutant strains (Fig. 3A). However, the accumulation of hexadecane by the mutant strains could be restored upon adding purified rhamnolipids to the cultures (Fig. 3B). Our measurements have indicated that in the order of 1.4% of the added hexadecane was taken up by wild-type cells in 1 h, corresponding to roughly 0.6 µg of hexadecane per mg (dry weight) of cells.

Alkane-nonutilizing mutants affected in rhamnolipid production. The results described above are consistent with the view that the mutant strain 65E12 lacks the capacity to produce extracellular rhamnolipids and that this lack causes the cells to be unable to take up and utilize the hydrocarbons in the growth medium. To directly detect and quantify extracellular rhamnolipids in cultures of the wild type and the two mutant strains, four independent tests that have previously been used to detect rhamolipids from *P. aerugi-*

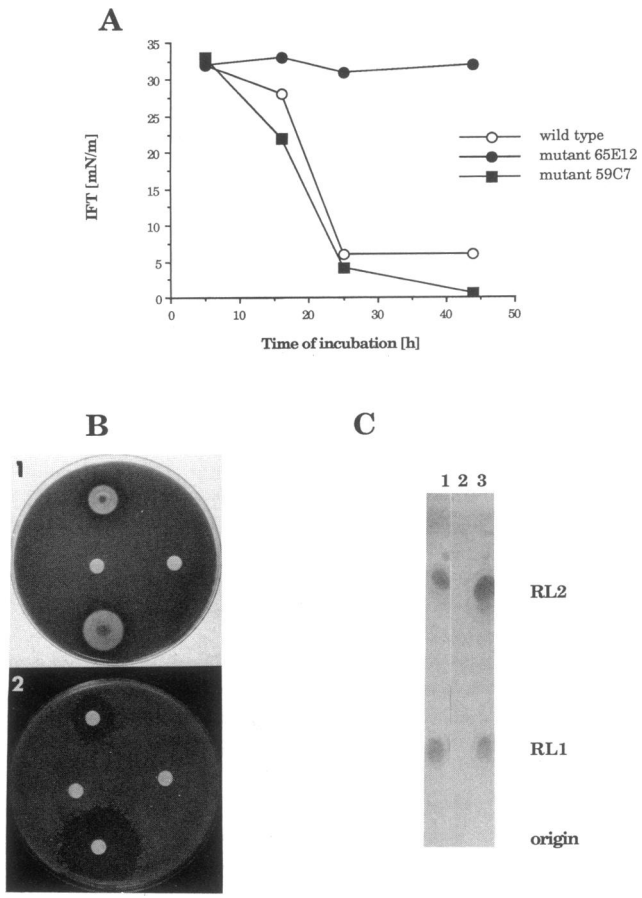

FIG. 4. Quantification of rhamnolipids produced by *P. aeruginosa* PG201 and mutants 65E12 and 59C7. (A) Determination of IFT. Culture aliquots were withdrawn and centrifuged at the times indicated. The supernatant was diluted 10 times in 0.1 M phosphate buffer (pH 6.5), and the IFT was measured against those of a mixture of aliphatic hydrocarbons. (B) Hemolytic activity of culture supernatants (plate 1) and inhibition of *B. subtilis* by concentrated culture supernatants (plate 2). Glucose-grown cultures of *P. aeruginosa* PG201 and mutant strains 65E12 and 59C7 were cultivated until the stationary growth phase was reached, and the culture supernatants were concentrated. Then 10 μl of 100-fold-concentrated supernatants were loaded either onto filter paper discs on top of agar plates containing 5% sheep blood or onto a soft agar overlay containing *B. subtilis* cells. The blood agar plates were left at room temperature for 48 h, and the plates of *B. subtilis* cells were incubated at 37°C overnight. Methanol (10 μl) was spotted on the disc shown on the right. The top discs contained the concentrated supernatant of strain PG201, the discs in the center contained the concentrated supernatant from mutant 65E12, and the bottom discs contained the concentrated supernatant from mutant 59C7. (C) Thin-layer chromatographic analysis of culture supernatants obtained from glucose-grown cells. The concentrated rhamnolipids corresponding to 1 ml of culture supernatant were spotted onto silica gel plates and developed as described previously (10). Lanes: 1, concentrated supernatant of strain PG201; 2, concentrated supernatant of mutant 65E12; 3, concentrated supernatant of mutant 59C7.

nosa were carried out. These included lowering of the IFT by rhamnolipids (10), detection of rhamnolipids in thin-layer chromatograms (26), hemolysis of erythrocytes by rhamnolipids (25), and growth inhibition of *B. subtilis* by rhamnolipids (22). Figure 4A summarizes the changes in IFT of the culture supernatants of wild-type and mutant cells grown in a glucose-containing minimal medium. It was obvious from this experiment that there was no change in the IFT of the culture medium of mutant 65E12, probably because of the lack of extracellular rhamnolipids. Surprisingly, however, the culture supernatant from mutant 59C7 showed a clear drop in the IFT that was similar to the IFT drop in the culture supernatant of wild-type cells. Thus, mutant 59C7 appears to produce rhamnolipids in glucose-containing minimal media. These findings could be confirmed by making use of the fact that rhamnolipids have hemolytic properties. For this purpose the concentrated culture supernatants from glucose-grown wild-type, 59C7, and 65E12 cells were spotted onto filter paper discs on top of an agar plate containing 5% sheep blood. The culture supernatants from wild-type and mutant 59C7 cells contain abundant amounts of a hemolysin that is lacking in the culture supernatant of strain 65E12 (Fig. 4B, culture 1). The same overall pattern was seen in the *Bacillus* inhibition test shown in Fig. 4B, culture 2. In this case the concentrated culture supernatants had been applied to filter paper discs located on an agar plate containing a soft agar overlay with *B. subtilis* cells. In the thin-layer analysis shown in Fig. 4C, the concentrated culture supernatants had been applied to a silica gel thin-layer plate. In contrast to wild-type and mutant 59C7 supernatants from glucose-grown cells, the mutant 65E12 culture supernatant did not reveal the two typical glycolipid-containing spots after orcinol-sulfuric acid staining of the thin-layer plate. The following analytical steps resulted in a quantitative measure of the sugar lipids produced by the two mutant strains. The glycolipids of glucose-grown culture supernatants were extracted with diethyl ether, concentrated by evaporation, and dissolved in water. Sulfuric acid and the sugar-specific orcinol reagent (9) were added, and the intensity of the appearing color was measured. The orcinol reaction revealed that *P. aeruginosa* wild-type cells produced in the order of 0.5 mg of glycolipids per ml of culture supernatant and that mutant 59C7 supernatants showed roughly double this amount (on the order of 1.1 mg/ml; Table 1). Mutant 65E12, on the other hand, did not give rise to any color reaction in the orcinol assay, indicating that no extracellular glycolipids were produced. The IFT was quantified by determining the dilution factor required to give IFT values between 0 and 2 mN/m, thus yielding the F_{cmc} values shown in Table 1. (Increasing F_{cmc} values indicate increasing concentrations of the surface-active compounds.) Ac-

TABLE 1. Quantification of rhamnolipids produced by *P. aeruginosa* PG201 and mutant derivatives 65E12 and 59C7

Strain	F_{cmc}[a]	Glycolipids[b] (μg/ml)	Diameter (mm) of zones indicating[c]:	
			Hemolytic activity	Inhibition of *B. subtilis*
PG201	5	525	11.5	23
65E12	0	0	0	0
59C7	10	1,125	15.5	35

[a] The IFTs of different dilutions of the culture supernatant were measured against those of a mixture of aliphatic hydrocarbons. The dilution factors still giving rise to values between 0 and 2 mN/m are expressed as F_{cmc}.

[b] The ether-soluble fraction of the culture supernatant was acidified and treated with an orcinol solution as described in Materials and Methods. The data represent the concentration of glycolipids in the undiluted culture supernatants.

[c] Diameters of the hemolytic and growth inhibition zones presented in Fig. 4B.

cording to this analysis, mutant 59C7 produced twice as much surfactant as did the wild-type strain, yielding F_{cmc} values of 10 and 5, respectively. Mutant 65E12 supernatants on the other hand, did not lower the IFT at any of the dilutions tested. Table 1 also indicates the sizes of the hemolytic and growth inhibition zones shown in Fig. 4B. Taken together, our results indicate that mutant 65E12 is unable to produce rhamnolipids in glucose- and hexadecane-containing minimal media supplemented with low concentrations of rhamnolipids (data not shown). Mutant 59C7 appears to overproduce rhamnolipids in glucose-containing minimal media at levels about twice as high as those of wild-type cells. As shown in Fig. 1B, low concentrations of rhamnolipids stimulated the growth of mutant 59C7 in hexadecane-containing media. Concentrated culture supernatants from such cells contained rhamnolipids at concentrations comparable to those of the wild-type. Evidently, this mutant is able to produce rhamnolipids, provided that triggering amounts of exogenous rhamnolipids are added.

To verify the presence of transposon Tn*5*-GM in the two mutant strains, DNA was isolated and analyzed by Southern blotting. In both cases a single transposon insertion was found (data not shown), thus indicating that the change in a single chromosomal locus in each strain is responsible for the observed mutant phenotypes.

DISCUSSION

Biosurfactants are produced by certain bacteria, yeasts, and filamentous fungi during cultivation on various carbon sources, in particular during growth on hydrophobic substances such as hydrocarbons. These observations led to the assumption early on that biosurfactants serve to emulsify the hydrocarbons in the growth medium thus facilitating their uptake. A correlation between surfactant production and growth on water-insoluble substrates could indeed be shown by Itoh and Suzuki (23). They demonstrated the stimulating effect of rhamnolipids on the growth of a mutant strain of *P. aeruginosa* on *n*-paraffin. This strain, designated PU-1, had been identified as an *n*-paraffin nonutilizer after nitrosoguanidine mutagenesis. Since this strain was still capable of producing rhamnolipids, albeit at substantially reduced levels compared with those in the wild-type strain, the growth-stimulatory role(s) of the exogenously added rhamnolipids could not be explained unequivocally. Rhamnolipids are also produced by *P. aeruginosa* in media containing glucose or glycerol as a carbon source, particularly when the cells become limited for nitrogen (11, 28, 39), indicating that they may serve other roles besides being involved in solubilizing hydrophobic substrates. The mutants that we describe here were isolated with a view toward investigating the physiological role(s) of rhamnolipids and getting access to the genes responsible for their production.

In the literature there are a number of approaches to the identification of mutant bacterial cells that are impaired in the synthesis or secretion of extracellular and cell-bound emulsifiers. Matsuyama et al. (27) used direct colony thin-layer chromatography to isolate *Serratia marcescens* mutants that were defective in the production of cyclodepsipeptides, and the capacity of bacterial colonies to attach to hydrophobic surfaces was explored to develop a screening system to isolate mutants that were affected in the synthesis of wetting agents. Bar-Ness et al. (1) presented evidence for an increased cell surface hydrophobicity in the *S. marcescens* NS38-9 mutant strain compared with that in the wild type. This change in cell surface hydrophobicity was caused by the lack of serratamolide production. Adhesion to polystyrene was shown by Rosenberg (35) to be a powerful tool to distinguish mutants of the *Acinetobacter calcoaceticus* RAG-1 strain that were impaired in the hydrophobicity of their cell surfaces from the corresponding wild-type cells. To isolate, out of a pool of cells, the spontaneous nonadhering mutant derivative MR-481 of the *A. calcoaceticus* RAG-1 strain, Rosenberg and Rosenberg (36) used a modification of the procedure described above. Surface-active strains of *Pseudomonas putida* and *P. fluorescens* were identified by Bunster et al. (5), who used polystyrene as a hydrophobic surface for the screening of individual culture liquid droplets, which, in the presence of surfactants, spread over the surface. The extracellular aminolipid surfactin of *B. subtilis* is known to be a powerful hemolysin. Nakano et al. (29) isolated Tn*917* insertion mutants that were defective in surfactin production by screening individual colonies on blood agar.

We have tried, without success, to adapt the direct colony thin-layer procedure to detect rhamnolipids in *P. aeruginosa* PG201. Our lack of success was possibly due to the facts that the amounts of rhamnolipids produced by a single colony were below the level of detection and that rhamnolipids are probably not cell associated as they are in *S. marcescens*. Hemolytic zones on blood agar plates are readily apparent with *P. aeruginosa* colonies. However, since *P. aeruginosa* is known to produce two kinds of hemolytic activities (due to the heat-labile hemolysin, which is a potent phospholipase [3], and to the heat-stable hemolysins, which are the rhamnolipids [25]), hemolysis at the colony level cannot be taken as a direct measure for rhamnolipid production. The strategy that we have chosen for isolating rhamnolipid nonproducers is an indirect one, selecting for mutants that are affected in rhamnolipid production and in alkane uptake. The question therefore remains as to whether rhamnolipid-negative mutants that would be screened for with a more direct approach would always be affected in hexadecane uptake as well. In other words, it remains unclear whether rhamnolipids are the sole key factor in facilitating the uptake of water-insoluble substrates in vivo or whether additional factors such as the proteinlike activator for *n*-alkane oxidation (17–19) also play a crucial role in this.

At this point we can only speculate about the biochemical lesions in the *P. aeruginosa* 65E12 and 59C7 mutant strains. For rhamnolipid production, the biosynthesis of TDP-rhamnose and hydroxy fatty acids is needed to provide the building blocks that are eventually coupled by transferases (6, 7, 15). Thus, mutations in any of the above reactions would result in rhamnolipid nonproducers, provided that the mutation is not lethal for the cell. Viable mutants of *E. coli* Y10 that are impaired in the biosynthesis of TDP-rhamnose have been described (30), and it is likely that mutant strains of *P. aeruginosa* affected in the first of the two rhamnosyl transferases involved in the synthesis of rhamnolipids would be viable as well. Although strain 65E12 did not appear to produce rhamnolipids under any of the conditions tested and thus is likely to carry a mutation affecting any of the reactions discussed above, the behavior of mutant 59C7 was more complex. This strain, in glucose-containing minimal media, produced about double the amount of rhamnolipids compared with the wild type. On hexadecane-containing minimal medium, however, growth was not possible without the addition of purified rhamnolipids, but in hexadecane-containing minimal media supplemented with low concentrations of rhamnolipids substantial amounts of rhamnolipids were produced. It is thus possible that mutant 59C7 carries a

mutation affecting a gene whose product is involved in the control of rhamnolipid biosynthesis. A mutant of *P. aeruginosa* that is capable of overproducing an emulsifier-type compound with chicken fat as a carbon source has been described by Chakrabarty (8). Since the nature of the affected emulsifying agent(s) has not been documented, the relationship to rhamnolipid synthesis is not clear.

One of our long-term interests is to produce rhamnolipids in strains other than the opportunistic human pathogen *P. aeruginosa*. The isolation of DNA sequences that are capable of complementing the defect in rhamnolipid biosynthesis and the transfer of such sequences to other gram-negative organisms might bring us a step closer to this goal. Further investigations on surfactant production and secretion, emulsion formation and stabilization, and uptake of emulsified hydrocarbons are needed to elucidate the complex behavior of growth on water-insoluble compounds. The first results of remediation work in the enhanced removal of spilled oil from Alaskan gravel show some of the promising applications of *P. aeruginosa* surfactants (13).

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REFERENCES

1. **Bar-Ness, R., N. Avrahamy, T. Matsuyama, and M. Rosenberg.** 1988. Increased cell surface hydrophobicity of a *Serratia marcescens* NS38 mutant lacking wetting activity. J. Bacteriol. **170**:4361–4364.
2. **Bassel, J. B., and R. K. Mortimer.** 1985. Identification of mutations preventing n-hexadecane uptake among 26 n-alkane non-utilizing mutants of *Yarrowia* (*Saccharomycopsis*) *lipolytica*. Curr. Genet. **9**:579–586.
3. **Berka, R. M., G. L. Gray, and M. L. Vasil.** 1981. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. Infect. Immun. **34**:1071–1074.
4. **Breuil, C., and D. J. Kushner.** 1980. Effects of lipids, fatty acids, and other detergents on bacterial utilization of hexadecane. Can. J. Microbiol. **26**:223–231.
5. **Bunster, L., N. Fokkema, and B. Schippers.** 1989. Effect of surface-active *Pseudomonas* spp. on leaf wettability. Appl. Environ. Microbiol. **55**:1340–1345.
6. **Burger, M. M., L. Glaser, and R. M. Burton.** 1963. The enzymatic synthesis of a rhamnose-containing glycolipid by extracts of *Pseudomonas aeruginosa*. J. Biol. Chem. **238**:2595–2602.
7. **Burger, M. M., L. Glaser, and R. M. Burton.** 1966. Formation of rhamnolipids of *Pseudomonas aeruginosa*. Methods Enzymol. **8**:441–445.
8. **Chakrabarty, A. M.** 1985. Genetically-manipulated microorganisms and their products in the oil service industries. Trends Biotechnol. **3**:32–38.
9. **Chandrasekaran, E. V., and J. N. Bemiller.** 1980. Constituent analyses of glycosaminoglycans, p. 89–96. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry. Academic Press, Inc., New York.
10. **Guerra-Santos, L., O. Käppeli, and A. Fiechter.** 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. **48**:301–305.
11. **Guerra-Santos, L., O. Käppeli, and A. Fiechter.** 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotechnol. **24**:443–448.
12. **Guerra-Santos, L. H.** 1985. Doctoral dissertation. Swiss Federal Institute of Technology, Zürich.
13. **Harvey, S., I. Elashvili, J. J. Valdes, D. Kamely, and A. M. Chakrabarty.** 1990. Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by microbial surfactant. Bio/Technology **8**:228–230.
14. **Hauser, G., and M. L. Karnovsky.** 1954. Studies on the production of glycolipide by *Pseudomonas aeruginosa*. J. Bacteriol. **68**:645–654.
15. **Hauser, G., and M. L. Karnovsky.** 1957. Rhamnose and rhamnolipide biosynthesis by *Pseudomonas aeruginosa*. J. Biol. Chem. **224**:91–105.
16. **Hauser, G., and M. L. Karnovsky.** 1958. Studies on the biosynthesis of L-rhamnose. J. Biol. Chem. **233**:287–291.
17. **Hisatsuka, K., T. Nakahara, Y. Minoda, and K. Yamada.** 1975. Capacity to oxidize *n*-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S_7B_1. Agric. Biol. Chem. **39**:999–1005.
18. **Hisatsuka, K., T. Nakahara, Y. Minoda, and K. Yamada.** 1977. Formation of protein-like activator for *n*-alkane oxidation and its properties. Agric. Biol. Chem. **41**:445–450.
19. **Hisatsuka, K., T. Nakahara, and K. Yamada.** 1972. Protein-like activator for *n*-alkane oxidation by *Pseudomonas aeruginosa*. Agric. Biol. Chem. **36**:1361–1369.
20. **Hisatsuka, K. I., T. Nakahara, N. Sano, and K. Yamada.** 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. Agric. Biol. Chem. **35**: 686–692.
21. **Iglewski, B.** 1989. Probing *Pseudomonas aeruginosa*, an opportunistic pathogen. ASM News **55**:303–307.
22. **Itoh, S., H. Honda, F. Tomita, and T. Suzuki.** 1971. Rhamnolipid produced by *Pseudomonas aeruginosa* grown on *n*-paraffin. J. Antibiot. **24**:855–859.
23. **Itoh, S., and T. Suzuki.** 1972. Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in *n*-paraffin-utilizing ability. Agric. Biol. Chem. **36**:1233–1235.
24. **Jarvis, F. G., and M. J. Johnson.** 1949. A glyco-lipide produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. **71**:4124–4126.
25. **Johnson, M. K., and D. Boese-Marrazzo.** 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect. Immun. **29**:1028–1033.
26. **Koch, A. K., J. Reiser, O. Käppeli, and A. Fiechter.** 1988. Genetic construction of lactose-utilizing strains of *Pseudomonas aeruginosa* and their application in biosurfactant production. Bio/Technology **6**:1335–1339.
27. **Matsuyama, T., M. Sogawa, and I. Yano.** 1987. Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. Appl. Environ. Microbiol. **53**:1186–1188.
28. **Mulligan, C. N., and B. F. Gibbs.** 1989. Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **55**:3016–3019.
29. **Nakano, M. M., M. A. Marahiel, and P. Zuber.** 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. J. Bacteriol. **170**:5662–5668.
30. **Okazaki, R., T. Okazaki, J. L. Strominger, and A. M. Michelson.** 1962. Thymidine diphosphate 4-keto-6-deoxy-D-glucose, an intermediate in thymidine diphosphate L-rhamnose synthesis in *Escherichia coli* strains. J. Biol. Chem. **237**:3014–3026.
31. **Persson, A., and G. Molin.** 1987. Capacity for biosurfactant production of environmental *Pseudomonas* and *Vibrionaceae* growing on carbohydrates. Appl. Microbiol. Biotechnol. **26**: 439–442.
32. **Persson, A., E. Oesterberg, and M. Dostalek.** 1988. Biosurfactant production by *Pseudomonas fluorescens* 378: growth and product characteristics. Appl. Microbiol. Biotechnol. **29**:1–4.
33. **Reiling, H. E., U. Thanei-Wyss, L.-H. Guerra-Santos, R. Hirt, O. Käppeli, and A. Fiechter.** 1986. Pilot plant production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **51**:985–989.
34. **Reiser, J., A. K. Koch, K. Jenny, and O. Käppeli.** 1989. Structure, properties, and production of biosurfactants, p. 85–97. *In* J. W. Obringer and H. S. Tillinghast (ed.), Advances in biotechnology for aerospace applications. The Portfolio Publishing Co., The Woodlands, Tex.

35. **Rosenberg, M.** 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. **42**:375–377.
36. **Rosenberg, M., and E. Rosenberg.** 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. **148**:51–57.
37. **Sasakawa, C., and M. Yoshikawa.** 1987. A series of Tn5 variants with various drug-resistance markers and suicide vector for transposon mutagenesis. Gene **56**:283–288.
38. **Syldatk, C., S. Lang, and F. Wagner.** 1985. Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas spec.* DSM 2874 grown on *n*-alkanes. Z. Naturforsch. Teil C Biochem. Biophys. Biol. Virol. **40**:51–60.
39. **Wagner, F., H. Bock, A. Kretschmer, S. Lang, and C. Syldatk.** 1983. Production and chemical characterization of surfactants from *Rhodococcus erythropolis* and *Pseudomonas sp.* MUB grown on hydrocarbons, p. 55–60. *In* J. E. Zajic, D. G. Cooper, T. R. Jack, and N. Kosaric (ed.), Microbial enhanced oil recovery. PennWell Publishing, Tucson, Ariz.
40. **Walter, M. V., A. Porteous, and R. J. Seidler.** 1987. Measuring genetic stability in bacteria of potential use in genetic engineering. Appl. Environ. Microbiol. **53**:105–109.
41. **Witholt, B., M.-J. de Smet, J. Kingma, J. B. van Beilen, M. Kok, R. G. Lageveen, and G. Eggink.** 1990. Bioconversion of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors: background and economic potential. Trends Biotechnol. **8**:46–52.