Cell surface properties result from the unique chemical structure of the cell surface. In the case of a gram-negative bacterium such as Pseudomonas aeruginosa, this structure is the outer membrane, the outer leaflet of which is primarily composed of lipopolysaccharide (LPS). The outer leaflet contains LPS molecules which are composed of three components (25). The first is the lipid A tail which is anchored into the hydrophobic region of the outer membrane. The second is the core oligosaccharide, which contains a unique eight-carbon sugar called 2-keto-3-deoxyoctonic acid (KDO). The core oligosaccharide is connected to the lipid A tail through its reducing end and is positioned at the surface of the membrane in a manner analogous to the glycerol-phosphate head group of a phospholipid. The core oligosaccharide is negatively charged, and the association of adjacent LPS molecules is stabilized by Mg\(^{2+}\) ions at the membrane surface. The third component of LPS is the O antigen which consists of 15 to 20 repeating monomers of a three- to five-sugar subunit. The O antigen is attached to the nonreducing end of the oligosaccharide and extends out from the cell surface into the environment (28). The presence of smooth LPS results in a relatively hydrophilic cell surface that is permeable to small hydrophilic molecules (molecular weight <600) but excludes hydrophobic molecules (16).

Several studies have shown that it is possible to modify the outer membrane of gram-negative bacteria by mutation or by the addition of chemical agents, such as EDTA. This results in overall changes in cell surface properties. For example, LPS mutants which have lost the O antigen component have increased affinity for hydrophobic probes (14, 23, 25, 29, 31, 32). EDTA treatment of cells has been shown to cause partial release of LPS (4, 5, 15, 30), resulting in cells that are more susceptible to the action of hydrophobic antibiotics (15, 16). The mechanism of EDTA-induced LPS loss is through chelation of divalent cations, namely Mg\(^{2+}\), which results in a weakening of LPS-LPS interaction and release of LPS to the growth medium (25). The loss of LPS from the outer leaflet of the outer membrane may cause a temporary exposure of the hydrophobic phospholipid fatty acid tails associated with the inner leaflet of the outer membrane. Alternatively, loss of LPS may decrease the compaction of the outer leaflet of the outer membrane, allowing increased passage of large hydrophobic compounds. Nikiado and Nakae (24) further suggest that lost LPS may be replaced with phospholipids, resulting in a cell surface with increased hydrophobic character.

The ability of Pseudomonas spp. to utilize hydrocarbons as a source of energy is well known (22). In most cases, however, the rate of utilization is slow compared to readily soluble compounds like sugars. Studies in pure culture have shown that biosurfactants, in particular rhamnolipid produced by P. aeruginosa, enhance hydrocarbon biodegradation rates (3, 9, 12, 13, 33, 35–37). It appears from this work that there are two mechanisms by which biosurfactants enhance the biodegradation of slightly soluble organic compounds. First, biosurfactants can solubilize hydrophobic compounds within micelle structures, effectively increasing the apparent aqueous solubility of the organic compound and its availability for uptake by a
cell. Second, biosurfactants can cause the cell surface to become more hydrophobic, thereby increasing the direct physical contact between the cell and the slightly soluble substrate (20, 33). For example, Zhang and Miller (36) found that rhamnolipid not only increased apparent hydrocarbon solubility but also modified the cell surface, resulting in increased hydrophobicity. Further, Herman et al. (8) showed that the addition of rhamnolipid at concentrations less than the critical micelle concentration (CMC) induced formation of multicellular aggregates, implying that the cells forming these aggregates are hydrophobic in nature. Taken together, these results indicate that the effect of rhamnolipid on the intrinsic ability of a cell to interact with hydrocarbons is not simply a function of increased solubility, but also results from changes in cell surface properties that are similar to those that have been described for LPS mutants or EDTA-treated cells.

The objective of this study was to investigate the rhamnolipid-induced chemical and structural changes that cause increased the cell surface hydrophobicity of P. aeruginosa. Two P. aeruginosa strains, P. aeruginosa ATCC 27853 and P. aeruginosa ATCC 9027, were used in this study. These organisms both degrade aliphatic hydrocarbons but can be characterized as having either fast (ATCC 27853) or slow (ATCC 9027) rates of growth on these substrates. Both the fatty acid and LPS content of these cells were measured during growth on a soluble substrate (glucose) and a slightly soluble substrate (hexadecane). To determine these results in detail, the effect of rhamnolipid on the intrinsic ability of a cell to interact with hydrocarbons is not simply a function of increased solubility, but also results from changes in cell surface properties that are similar to those that have been described for LPS mutants or EDTA-treated cells.

Materials and Methods

Bacterial cultures. Two P. aeruginosa strains were used in this study: P. aeruginosa ATCC 27853 and P. aeruginosa ATCC 9027. These two strains were selected because of their variable growth patterns on hydrocarbons; ATCC 27853 has a relatively fast intrinsic rate of growth while ATCC 9027 has a slower rate of growth on hydrocarbons (36). Both isolates were maintained from stocks at 70% of growth on hydrocarbons (36). Both isolates were maintained from stocks preserved in glycerol at -80°C by using a standard curve prepared with bovine serum albumin. The limit of detection was found to be 1 mg of protein per liter. Cultures supplied with glucose were sampled every 4 h, and cultures containing hexadecane were sampled every 1 to 4 days until growth reached the stationary phase.

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SEM. Cell samples (30 ml) were centrifuged at 10,000 \times g and were resuspended in 30 ml of 4% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) until samples were processed further. Between each of the following steps, samples were centrifuged at 10,000 \times g, and the supernatant was siphoned off with a vacuum. Samples were resuspended in 0.1 M phosphate buffer (pH 7.4) for 30 s and then rinsed twice for 5 min. Next, half of the samples were postfixed in 1% osmium tetroxide in nanopure \( \text{dH}_2\text{O} \), and the other half were fixed in 1% rhenium tetroxide in nanopure \( \text{dH}_2\text{O} \) for 30 min each. The samples were then washed once for 30 s and then twice in dd\( \text{H}_2\text{O} \) for 5 min. Samples were then run through an ethanol dehydration series of 30, 50, 70, 95, and three changes of 100% ethanol for 10 min each. Finally, samples were critical-point dried (Polaron; Energy Beam Sciences, Agawam, Maine). The dried specimens were mounted on aluminum mounts with Avery Spot o’ Glue and 3/8-in. polyester silver tape (3M, St. Paul, Minn.). Lastly, the samples were coated with a 10- to 20-nm layer of platinum in a magnetron sputtering device (Hummer 6; Anatech Ltd., Springfield, Va.). The material was examined by using a Hitachi S-4500 scanning electron microscope (Hitachi Scientific Instruments, Mountain View, Calif.). The samples were viewed at 0° tilt at 10 kV.

**RESULTS**

**Growth and cell surface hydrophobicity.** Rhamnolipid had a slight negative effect on the growth of ATCC 27853 on glucose as determined by protein analysis. This effect was manifested as a minor reduction in cell yield (Fig. 1). In terms of cell surface hydrophobicity, as measured by the BATH test, ATCC 27853 grown on glucose in the absence of rhamnolipid exhibited a relatively low cell surface hydrophobicity at all stages of growth. In contrast, rhamnolipid addition caused cell surface hydrophobicity to slowly increase throughout the exponential phase to reach 30% adherence in the late exponential phase and then rapidly increased to 79% adherence by the end of stationary phase.

The effect of rhamnolipid on \( P. \) aeruginosa ATCC 9027 during growth on glucose was quite different (Fig. 2). Rhamnolipid addition did not affect growth but caused a rapid change in cell surface hydrophobicity, reaching 78% adherence by the end of exponential phase. Following the onset of stationary phase, adherence declined sharply until reaching the basal level of approximately 16%. In the absence of rhamnolipid, cell surface hydrophobicity did not exceed 27% adherence.

A similar set of experiments were performed with hexadecane as a substrate. Growth of ATCC 27853 on hexadecane was characterized by a faster rate of growth (see slope of growth curves) upon addition of rhamnolipid (Fig. 3). The relative cell surface hydrophobicity of cells grown on hexadecane alone showed a slow, steady increase to 33% adherence over a period of 400 h. The addition of rhamnolipid to the growth medium caused a rapid increase in cell surface hydrophobicity during the exponential phase of growth, reaching 78% adherence to hexadecane in 100 h, after which adherence dropped to background levels.

Figure 4 shows the effect of rhamnolipid addition on ATCC 9027 grown on hexadecane. While ATCC 9027 naturally grows more slowly on hexadecane than ATCC 27853, rhamnolipid had a stimulatory effect on growth similar to that observed for ATCC 27853. Also similar to ATCC 27853, ATCC 9027 had a slow and steady increase in relative cell surface hydrophobicity to 40% adherence during growth on hexadecane alone (Fig. 4). The cultures grown on hexadecane in the presence of rhamnolipid showed a more rapid increase in relative cell surface hydrophobicity during exponential phase to 75% adherence, after which the adherence declined to less than 20% by the end of sampling at 500 h.

**Total cell lipids.** Analysis of total cell lipids showed no difference in the types of fatty acids produced by cells in the presence and absence of rhamnolipid. The major fatty acids produced were similar to those reported by others (6, 11): \( \text{C}_{16:0} (-40\%) \), \( \text{C}_{18:1} (-40\%) \), and \( \text{C}_{16:1} (-10\%) \). However, the amount of fatty acids produced was much lower in cells that were exposed to rhamnolipid. For example, there was a 6% loss of the \( \text{C}_{16:0} \) fatty acid, a 53% loss of the \( \text{C}_{18:1} \) fatty acid, and a 75% loss of the \( \text{C}_{16:1} \) fatty acid for ATCC 9027 in the presence of rhamnolipid.

**Total cellular and supernatant LPS content.** The lower fatty acid content in rhamnolipid-treated cells led us to hypothesize that fatty acid loss was due to the release of LPS from the outer membrane. Therefore, total cellular LPS content as well as LPS release to the supernatant was measured for strains by using KDO analysis. For total cellular LPS, cells of both strains were exposed to glucose to the late exponential phase in the absence of rhamnolipid. Results showed that both strains contain similar amounts of LPS: 7.4 \( \mu\)g of LPS per ml of ATCC 27853 culture and 8.1 \( \mu\)g of LPS per ml of ATCC 9027 culture. In this case, 1 ml of culture was centrifuged, the supernatant was discarded, and the LPS content of the pelleted cells was determined.

The amount of LPS released from these strains to the culture supernatant during growth was then measured to determine the effect of added rhamnolipid on the release of LPS.
from the cell surface. As shown in Fig. 1 and 2, for cells grown on glucose and not exposed to rhamnolipid, there was a slow release of LPS over the course of the experiment. This LPS release totaled 0.39 mg per ml of ATCC 27853 and 0.1 mg per ml of ATCC 9027. The amount of LPS released represents a small proportion of the total cellular LPS: 7% for ATCC 27853 and 1% for ATCC 9027. These values appear to represent a background level of LPS release that is normal under these experimental conditions. LPS release was significantly higher for both strains when exposed to rhamnolipid, reaching 2.0 mg/ml for ATCC 27853 and 0.27 mg/ml for ATCC 9027. These values represent a 25% release of total cellular LPS for ATCC 27853 and a 33% release of LPS for ATCC 9027. Both values represent a significant increase over controls that were not treated with rhamnolipid. Results in Fig. 3 and 4 show a similar pattern of LPS release for cells grown on hexadecane. Rhamnolipid caused an increase from 8 to 38% LPS release for ATCC 27853 and from 3 to 14% for ATCC 9027 in the presence of hexadecane.

The dependence of LPS release on rhamnolipid concentration is further examined in Fig. 5 and 6. For ATCC 27853, LPS release was significant at rhamnolipid concentrations as low as 0.04 mM (20 mg/liter). At higher rhamnolipid concentrations, the amount of LPS released exceeded 2.7 μg per ml of cell suspension, almost 36% of the total LPS content of the cell. For ATCC 9027, LPS was released at similarly low rhamnolipid concentrations, but the total LPS release was much less, reaching only 0.32 μg per ml of cell suspension (4% of the total LPS content of the cell). While the amount of LPS release was low, it seems that this release was enough for the bacterium to acquire a relatively hydrophobic surface, as seen in Fig. 2. The dependence of LPS release on rhamnolipid concentration was also different for ATCC 9027. While release of LPS from ATCC 27853 could be described by a second-order polynomial curve (r² = 0.977), release of LPS from ATCC 9027 proceeded in two phases: the first phase was characterized by a high, linear (r² = 0.9995) rate of increase in LPS loss from 0 to 0.1 mM rhamnolipid and a subsequent lower linear (r² = 0.990) rate of increase from 0.1 to 10 mM rhamnolipid.

The BATH assay results can be compared to LPS release. As shown in Fig. 1 to 4, the increase in cell surface hydrophobicity coincided with LPS release to the medium. However, once reaching a maximum value of 75 to 89%, cell surface hydrophobicity decreased sharply in all cases except for that of ATCC 27853 grown on glucose. Even though cell surface hydrophobicity declined, LPS remained high in the culture su-
permatants. This suggests that these cells regenerated LPS to become more hydrophilic rather than taking up the released LPS from solution.

**SDS-PAGE of LPS.** SDS-PAGE analysis of LPS was performed on concentrated supernatants taken from ATCC 27853 cultures incubated in the presence and absence of rhamnolipid for 8 or 24 h. As shown in Fig. 7, rhamnolipid (lane 2) is not visualized by the silver stain procedure. Protein (lane 3) is visualized but does not produce a characteristic LPS banding pattern. However, characteristic LPS banding patterns were clearly observed in culture supernatant samples (lanes 4 to 7) and *P. aeruginosa* serotype 10 LPS standards (lanes 8 to 10). The LPS banding patterns seen in supernatants of cells not treated with rhamnolipid (lanes 4 and 6) represent a background level of LPS release similar to that seen in the KDO analysis (Fig. 5). In contrast, the LPS banding patterns seen in cell supernatants treated with rhamnolipid for 8 or 24 h (Fig. 7, lanes 5 and 7) were much more intense. These results clearly demonstrate that rhamnolipid does not interfere with the staining procedure (lane 2) and that the more-intensive banding pattern is caused by rhamnolipid-induced LPS release. The LPS banding patterns observed in supernatants from cell suspensions treated with 6 mM rhamnolipid for only 2 h were also more intense than in those in suspension supernatants not treated with rhamnolipid (data not shown), indicating that rhamnolipid induces LPS release within 2 h.

**Electron microscopy.** The ATCC 27853 cell surface was examined using SEM in the absence and presence of rhamnolipid. Cells shown in Fig. 8A (no rhamnolipid) and B (with rhamnolipid) were prepared from the same samples that were used for the SDS-PAGE analysis shown in Fig. 7, lanes 4 and 5. The rhamnolipid-treated and untreated cells were fixed identically and show clear differences. The untreated cells shown in Fig. 8A have a rougher texture than the rhamnolipid-treated cells (Fig. 8B). The rough appearance of the untreated cell surface may be due to the LPS-protein complexes on the cell surface. Similar results were seen for *P. aeruginosa* ATCC 9027 (data not shown).

**DISCUSSION**

This study was initiated to explain the structural changes in *Pseudomonas* cell surfaces that result in increased cell surface hydrophobicity upon exposure to the biosurfactant rhamnolipid. The results of this research demonstrate that rhamnolipid, at very low concentrations, causes release of LPS from the outer membrane. As a result, the cell surface becomes more hydrophobic. The quantity and type of LPS found on the cell surface has a profound effect on the nature of interactions between the cell and its environment (18). In wild-type gram-negative bacteria, the predominant LPS is smooth-form LPS which contains the O antigen (polysaccharide side chain). This gives the bacterium a relatively hydrophilic surface suitable for normal environmental settings. Bacteria with smooth-form LPS are resistant to the action of hydrophobic antibiotics because such compounds are unable to penetrate the highly hy-
drophilic zone formed by the polysaccharide chains of the dense LPS layer (25). In contrast, bacteria that have rough-form LPS (no or short O antigen), mutant LPS, or no LPS are all more susceptible to the action of hydrophobic antibiotics. Similarly, the strains in this study that were treated with rhamnolipid showed loss of LPS, and this resulted in increased cell surface hydrophobicity. Concomitant with LPS loss and increased hydrophobicity was an increase in the rate of growth on the hydrophobic substrate hexadecane. This can be explained by the ability of hydrophobic bacteria to more easily make direct physical contact with hexadecane, a vital preliminary step for substrate uptake and biodegradation.

Rhamnolipid may interact with LPS in two ways to cause removal from the outer membrane. The first is that rhamnolipid causes the direct removal of LPS by solubilization of LPS. Clearly, rhamnolipid has detergency properties, as demonstrated by its ability to enhance hydrocarbon solubility (35). The second is that rhamnolipid causes the indirect removal of LPS through complexation of Mg$^{2+}$ in the outer membrane. Magnesium is a metal that is crucial for maintaining strong LPS-LPS interactions in the outer membrane. Its removal results in the destabilization of the LPS-LPS interaction and loss of LPS from the membrane (as described for EDTA [25]). This mechanism is supported by the fact that rhamnolipid has been shown to effectively complex divalent metal cations, such as magnesium (7, 34). The conditional stability constant (logK) describing the strength of the rhamnolipid-magnesium complex is 2.66 (26). To put this in context, this value is similar to the reported stability constants for other naturally occurring materials with magnesium: 1.27 for acetic acid, 3.43 for oxalic acid, 3.37 for citric acid, and 2.2 for fulvic acid (26). It is 6 orders of magnitude lower than the reported stability constant for EDTA and magnesium of 8.79 (19).

The effect of rhamnolipid on LPS release was different for the two strains used in this study. While ATCC 27853 released a larger proportion of its LPS (25 to 38%) in the presence of rhamnolipid, ATCC 9027 released only 3.3 to 14%. However, cell surface hydrophobicity increased similarly for both strains in the presence of rhamnolipid. Thus, even a minimal LPS release can lead to development of high adherence to hydrocarbons. This resulted in enhanced biodegradation of the hy-

FIG. 6. Effect of rhamnolipid on LPS released from cell suspensions of *P. aeruginosa* ATCC 9027. Cell suspensions were adjusted to an OD of 1.0 and were incubated with the respective concentration of rhamnolipid for 8 h, and supernatant samples were assayed for LPS. Each point represents the average and standard deviation of three replicate samples.

FIG. 7. A silver-stained SDS-PAGE of concentrated (10×) supernatants of suspensions of *P. aeruginosa* ATCC 27853. Lane 1, buffer; lane 2, 300 µg of rhamnolipid; lane 3, 5 µg of bovine serum albumin; lane 4, supernatant of *P. aeruginosa* ATCC 27853 treated only with MSM for 8 h; lane 5, supernatant of *P. aeruginosa* ATCC 27853 treated with 6 mM rhamnolipid for 8 h; lane 6, supernatant of *P. aeruginosa* ATCC 27853 treated only with MSM for 24 h; lane 7, supernatant of *P. aeruginosa* ATCC 27853 treated with 6 mM rhamnolipid for 24 h; lane 8, 5 µg of *P. aeruginosa* serotype 10 LPS; lane 9, 50 µg of *P. aeruginosa* serotype 10 LPS; lane 10, 1 µg of *P. aeruginosa* serotype 10 LPS.

FIG. 8. Scanning electron micrograph of *P. aeruginosa* ATCC 27853 grown on glucose in the absence of rhamnolipid (A) and presence of 6 mM rhamnolipid (B) for 8 h. The scanning electron micrographs shown are of cells fixed with 1% rhenium tetroxide. Rhenium tetroxide was considered superior to osmium tetroxide because of its ability to preserve anything larger than a simple hexose structure. Thus, this fixative better preserves external cell morphology and prevents artifacts due to condensation of supernatant materials onto the cell surface.
drophobic substrate hexadecane. Following hexadecane bio-
degradation, cells entered stationary phase and the cell surface hydrophobicity of both strains returned to normal levels, sug-
gest that LPS was regenerated (Fig. 3 and 4). In contrast, glucose experiments showed that addition of rhamnolipid did not have much impact on growth of either strain even though cell surface hydrophobicity was increased. In stationary phase, ATCC 9027 recovered in terms of cell surface hydrophobicity, but ATCC 27853 did not. The results presented show that while relative cell surface hydrophobicity is changed similarly for both strains in the presence of rhamnolipid, the temporal nature of the change is different and is a function of the bacterial strain tested, as well as its growth stage, and the type of growth substrate present.

In conclusion, this study demonstrates that rhamnolipid, even at concentrations much less than the CMC (0.1 mM), causes release of LPS which results in an increase in cell surface hydrophobicity. This allows increased association of cells with hydrophobic substrates resulting in increased degra-
dation rates. These results help explain why biosurfactants enhance biodegradation rates even at sub-CMC concentra-
tions where solubilization of the hydrocarbon is not a factor (8). In fact, similar unexplained observations have been made for some, but not all, synthetic surfactants (1). Understanding how biosurfactants impact biodegradation rates at low concen-
tration is important because, in practical terms, addition of low levels of surfactants, either biological or synthetic, will be more cost-effective for remediation than addition of the high levels of surfactant that are required to achieve solubilization effects.

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