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Polysorbate 80 Inhibition of *Pseudomonas aeruginosa* Biofilm Formation and Its Cleavage by the Secreted Lipase LipA▽

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Surface-associated bacterial communities known as biofilms are an important source of nosocomial infections. Microorganisms such as *Pseudomonas aeruginosa* can colonize the abiotic surfaces of medical implants, leading to chronic infections that are difficult to eradicate. Our study demonstrates that polysorbate 80 (PS80), a surfactant commonly added to food and medicines, is able to inhibit biofilm formation by *P. aeruginosa* on a variety of surfaces, including contact lenses. Many clinical isolates of *P. aeruginosa*, as well as gram-negative and gram-positive clinical isolates, were also inhibited in their ability to form biofilms in the presence of PS80. A *P. aeruginosa* mutant able to form biofilms in the presence of this surfactant was identified and characterized, and it was revealed that this mutant overexpresses a lipase, LipA. Surfactants such as PS80 can be cleaved by lipases, and we demonstrate that PS80 is cleaved by LipA at its ester bond. Finally, polyethoxylated(20) oleyl alcohol, a chemical with a structure that is similar to that of PS80 but that lacks the ester bond of PS80, can inhibit the biofilm formation of *P. aeruginosa* strains, including the mutant overexpressing LipA. Our results demonstrate that surfactants such as PS80 can inhibit bacterial biofilm formation on medically relevant materials at concentrations demonstrated to be safe in humans and suggest that the understanding of the mechanisms of bacterial resistance to such surfactants will be important in developing clinically effective derivatives.

Compounds that are tolerated by eukaryotic cells and that are able to inhibit bacterial biofilm formation would have widespread clinical value. Bacterial biofilms are surface-attached communities of microorganisms embedded in an extracellular matrix made of a mix of exopolysaccharides, DNA, and proteins. The bacteria growing in a biofilm are able to survive more successfully in hostile environments than non-surface-attached (planktonic) bacteria. Bacteria grown in a biofilm have increased resistance to antibiotics (17) and to the host immune system compared to that of their planktonic counterparts (11, 15). In the past decade, an increasing number of infections that involve bacterial biofilms have been identified (6, 9). The NIH has estimated that up to 80% of clinical infections are biofilm infections (8), with *Staphylococcus* spp. and *Pseudomonas aeruginosa* cited as frequently identified pathogens. Bacterial biofilms have been observed on medical implants such as catheters, joint prostheses, cardiac valves, and contact lenses (6, 9). Unlike most human tissues, these abiotic surfaces lack properties to prevent bacterial attachment or colonization and become a microbial reservoir that can lead to chronic infections. These infections lead to higher costs for patient care, since prostheses and implants must very often be removed and replaced to eliminate the infection (7).

Various approaches are being developed to reduce the attachment of microorganisms to abiotic surfaces with the goal of reducing the rate of infections related to biofilm formation. Several approaches relate to modifying the physical properties of the surface to render it antiadhesive, while others create a surface with antibacterial properties (for a recent review, see reference 25). To work toward achieving this goal, several studies have focused on coating abiotic surfaces with both synthetic surfactants and biosurfactants. Surfactants are amphiphilic molecules, as they contain both hydrophilic and hydrophobic moieties that allow them to be easily immobilized on a surface, and are commonly used to lower the surface tension of a material. Surfactants have been shown to inhibit the adhesion of gram-negative and gram-positive bacteria to solid surfaces (14, 18, 29, 33, 37). When the impact of surfactants on microbial life is considered, cationic surfactants (such as quaternary imidazolium compounds) are often the most toxic to microorganisms and are used as antimicrobials. Anionic surfactants like sodium dodecyl sulfate are usually less active and are mostly effective against gram-positive bacteria, whereas nonionic surfactants are considered to have no antibacterial properties (38).

Polysorbate 80 (PS80) is a nonionic surfactant commonly added to foods, cosmetics, and pharmaceutical preparations as an emulsifier and dispersing agent and is considered to be well tolerated when it is delivered to mucosal, intradermal, and intravenous sites (21). It is derived from polyoxylated sorbitol and oleic acid and is viscous but water soluble, which makes it an additive of choice for vaccines, oil-based medical preparations, as well as topical ophthalmic medications.

There have been reports on the effects of PS80 on bacteria for more than four decades. While it possesses little antimi-

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crobial activity alone, PS80 can increase bacterial cell permeability (4) and enhance the antimicrobial activity of a variety of antibiotics against *P. aeruginosa*, including chlorhexidine diacetate, benzalkonium chloride, and polymyxin B sulfate (2, 3, 5, 27, 28). On the basis of these findings and studies indicating that PS80 lyses spheroplasts (gram-negative bacteria in which the outer membrane has been removed), Brown and colleagues proposed that PS80 is primarily active against the inner membrane of *P. aeruginosa* (2). According to this view, its capacity to enhance the activity of antimicrobial agents that damage the outer membrane is thus a reflection of its ability to gain access to the inner membrane in the presence of these compounds.

In this report, we describe the PS80-mediated inhibition of biofilm formation by *P. aeruginosa* and other gram-negative and gram-positive bacterial species. We also present evidence that *P. aeruginosa* PA14 can resist the action of PS80 by cleavage of this surfactant by a secreted lipase, showing one possible resistance pathway for this organism.

MATERIALS AND METHODS

Bacterial strains and media. All *P. aeruginosa* strains used in this study were derived from *P. aeruginosa* PA14 (26), unless otherwise specified, and the mutants screened were obtained from the PA14 Transposon Insertion Library (16). All clinical isolates came from the laboratory strain collection. *Escherichia coli* JM109 *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] was used as the host strain for plasmid construction and propagation, whereas *E. coli* S17-1λ*pir recA thi hsdRM*$^+$ *RP4*::2-*Tc*::*Mu*::*Km* Tn*7* λ*pir* was used for conjugations with *P. aeruginosa*. *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen, Carlsbad, CA) was used for in vivo homologous recombination for plasmid construction, as described previously (31). Selections of the *S. cerevisiae* yeast strains were performed with a synthetic defined agar (0.67% yeast nitrogen base [Research Products International, Mt. Prospect, IL], 0.076% complete supplement mixture without uracil [Sunrise Science Products, San Diego, CA], 1.5% dextrose [Sigma, St. Louis, MO], 2% agar [Difco, Franklin Lakes, NJ]) without uracil.

All bacterial strains were grown in LB medium (30) at 37°C, unless otherwise noted, and their growth was monitored at an optical density of 600 nm (OD_{600}). All supernatants tested were obtained from overnight cultures centrifuged at 8,000 × *g* for 1 min and filtered through 0.2-μm-pore-size filters (Millipore, Billerica, MA). Ampicillin was used at 150 μg/ml for *E. coli*. Carbenicillin was used at 500 μg/ml for *P. aeruginosa*. Gentamicin was used at 10 μg/ml for *E. coli* and 50 to 100 μg/ml for *P. aeruginosa*.

PS80 (Spectrum Chemicals, Gardena, CA) was prepared as a 1% solution in double-distilled water (water is referred to as vehicle in the rest of the text) and was used at a concentration of 0.01%, unless otherwise noted. Oleic acid (Fluka/Sigma, St. Louis, MO) was prepared as a 10% solution in 95% ethanol. *Pseudomonas* species lipase (type XIII; Sigma) was prepared as a 10-mg/ml solution in Tris-EDTA buffer, and polyethoxylated(20) oleyl alcohol (Pragmatics Inc., Elkhart, IN) was used at a concentration of 0.01% from a 1% solution prepared in double-distilled water. For arabinose-inducible plasmid p*lipA*, 0.2% arabinose was added to the cultures.

Biofilm assays and microscopy experiments. Biofilm assays in microtiter dishes were performed as described previously (19, 23). All biofilms were incubated for 24 h at 37°C. The amount of biofilm present in each well was measured by solubilization of crystal violet with 50% acetic acid and quantification at an OD_{550}. All biofilm data presented come from one representative experiment, with four replicates per strain, of a minimum of three independent assays. Other similar experiments showed the same trend and were internally controlled, but because LB medium, which is a rich medium, was used, the data varied from one experiment to the other. Supernatants from overnight cultures were heated at 65°C for 20 min, when necessary.

Attachment to polyvinyl chloride (PVC) and contact lenses made of etafilcon A or vifilcon A (Surevue [Johnson & Johnson] and Focus [Ciba Vision], respectively) was visualized and measured by using a variation of the air-liquid interface coverslip assay (19). Tabs of PVC and contact lenses were cut with a sterilized pair of scissors. The PVC tabs were then sterilized by immersion in 70% ethanol. Each tab was added to 1 well of a 12-well plate. The wells were filled with 250 μl of M63 minimal medium (24) supplemented with $MgSO_4$ (1 mM), glucose (0.2%), and Casamino Acids (0.5%) containing a 1:100 dilution of the bacterial strains tested (from overnight cultures). The plates were incubated at 37°C for 24 h. The tabs were then removed, rinsed four times in sterile phosphate-buffered saline to remove the planktonic cells, and then placed individually in the wells of a 12-well plate containing enough phosphate-buffered saline to cover each tab. Phase-contrast microscopic observations were performed with a DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a cooled charge-coupled-device digital camera and a 63X PL Flotar objective lens. Digital images were captured and analyzed with OpenLab software (Improvision, Coventry, England).

CFU counts from biofilm cells. The direct enumeration of the bacteria present in biofilms grown in the wells of a PVC plate was performed by a previously published protocol (19). A minimum of four replicates was done per strain and condition.

Lipase activity plate assay. Tributyrin plates were prepared by a protocol modified from that of Smeltzer et al. (34). Briefly, a HEPES buffer (20 mM HEPES, 500 mM NaCl, pH 7.4) was prepared and was warmed to 50°C. One gram of agarose was added to 50 ml of HEPES buffer and melted in a microwave. Simultaneously, 500 μl of tributyrin (Sigma) was added to 50 ml of HEPES buffer. This mix was sonicated at 35% for 3 min to create a white emulsion that was then mixed with the agarose and poured into four petri plates. After solidification, 5 μl of filtered supernatant of each of the strains of interest was spotted on the plates. Five replicates were spotted per strain. The plates were incubated at room temperature, and the diameter (in millimeters) of the zone of clearing, which corresponded to the lipase/esterase activity of the sample, was measured from a minimum of three plates after 3 days of incubation. *Pseudomonas* species lipase (type XIII) was used as positive control.

Quantitative real-time PCR. Quantitative reverse transcriptase PCR (qRT-PCR) was done by the use of previously published protocols (12, 13) with the following modifications. The strains of interest were subcultured in LB medium at 37°C until they reached an OD_{600} close to 0.4. The cells were normalized to an OD_{600} of 0.4. After a brief centrifugation to pellet the cells, RNA was isolated and cDNA was prepared as published previously (13). In the qRT-PCR experiment, the expression of each gene was measured in triplicate. All gene expression profiles were normalized to that for the *rplU* housekeeping gene. Expression levels were quantified as the number of picograms of input cDNA by using a standard curve method for absolute quantification, and these values were normalized to the level of *rplU* expression. The primers used in the qRT-PCR assays were primers CT0140 (5′-CTCGCTGCCAGCCCTCTGATCC) and CT0141 (5′-GGTGGCGGAAGCGATCAGGTCG) for *lipA* and primers *rplU*1 (5′-CG CAGTGATTGTTACCGGTG) and *rplU*2 (5′-CAACCGCAATGGGCGCTAT TGC) for the *rplU* control.

Construction of *lipA*::pMQ87Ap. The single-crossover knockout vector pMQ87 (31) was first modified as follows. The gentamicin resistance cassette was replaced by the ampicillin resistance cassette by using a yeast-based cloning system (31). The ampicillin resistance cassette was amplified from pMQ70 (31) by PCR with primers p70for (5′-AGGAAGAGTATGAGTATTCAACCGAAT TGACATAAGCCTGTATGTATCCGCTCATGAGAC) and p70rev (5′-ACTC ACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGTCAGTGGAAC GAAAACTCAC). The 1,000-bp fragment was cloned in pMQ87 (which was linearized by digestion with BglII) by in vivo homologous recombination in yeast cells (31). Plasmids were recovered from the yeast as described previously (31) and transformed into *E. coli* S17-1λ*pir*, and plasmid candidates were screened on a plate (growth on ampicillin and not gentamicin) as well as by PCR with primers p70for and p70rev (see above). One candidate, pMQ87Ap, was selected for further use.

The single-crossover knockout mutant of *lipA* in the mutant 3A8 background was generated by using the newly obtained pMQ87Ap suicide vector. A 600-bp fragment (nucleotides 100 to 700) of *lipA* was amplified with primers CT0150 (5′-GGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCA CCCCATCGTGCTGGCCCACGGCATGC) and CT0151 (5′-GTGCCAAGCT TGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGAAGTTGGTCAG CGGCGAGGAACCGC). Both pMQ87Ap and the PCR fragment obtained were cut with the restriction enzymes BamHI and SacI. *E. coli* JM109 cells were transformed by heat shock with the ligation mix. The transformants obtained were checked by PCR with primers CT0142 (5′-CCAAGTACCCGCAGGGCA TCCC) and P729 (5′-CAGACCGCTTCTGCGTTCTG). The selected transformants were conjugated into *P. aeruginosa* 3A8 in order to obtain *lipA*::pMQ87Ap.

Construction of p*lipA*. Plasmid p*lipA* was constructed with the yeast-based cloning system (31). The *lipA* gene from PA14 was amplified by PCR with primers CT0144 (5′-CCATACCCGTTTTTTTGGGCTAGCGAATTCGAGCT

CGGTACCCATGAAGAAGAAGTCTCTGCTCCCCC) and CT0145 (5′-GCC AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCCTACAGGC TGGCGTTCTTCAGGCG). The 1,020-bp PCR fragment was cloned in the vector pMQ70 (ampicillin resistant; linearized by digestion with SmaI) by in vivo homologous recombination in yeast cells (31). Plasmids were recovered from the yeast as described previously (31) and transformed into *E. coli* JM109, and plasmid candidates were screened by PCR with primers CT0140 and CT0141 (see above) and sequenced with primers CT0140 and CT0143 (5′-CGTGCTGG CGGTAGACGCTGAC). Selected transformants were conjugated into *P. aeruginosa* PA14 for subsequent analysis.

Detection of oleic acid by HPLC. Bacterial cultures were grown overnight in LB medium supplemented with vehicle or PS80 (0.01 or 0.1%). In the case of the *Pseudomonas* species lipase sample, both the lipase (which was used at 0.1 mg/ml) and PS80 (which was used at 0.01%) were incubated in LB medium overnight. After filtration, 40 ml of the supernatants was acidified with 400 μl of 10% trifluoroacetic acid (TFA). SepPac cartridges (Waters Corporation, Milford, MA) were attached to 60-ml syringes and washed with 5 ml acetonitrile and then 10 ml of 0.1% TFA. The acidified supernatants were applied to the cartridges at a rate of about 2 ml per minute. The cartridges were washed with 5 ml of 0.1% TFA and then with 5 ml of a buffer containing 50% of 0.1% TFA and 50% acetonitrile. The cartridges were washed a third time with a buffer containing 5% of 0.1% TFA and 95% acetonitrile, and this fraction was dried under vacuum in a Speedvac apparatus. The samples were resuspended in 65% buffer B (0.13% TFA in acetonitrile)–35% buffer A (0.15% TFA in water) and analyzed by high-pressure liquid chromatography (HPLC) with a chromatograph equipped with a model G1316A diode array detector (Agilent Technologies Inc., Santa Clara, CA). The chromatographic separation was performed on a Waters symmetry C_{18} column (5 μm by 3.9 mm by 150 mm; Waters Corporation). A binary buffer system consisting of buffer A and buffer B was used. The column was equilibrated in 65% buffer B, and the gradient was developed over 30 min to 95% buffer B at a flow rate of 1.5 ml/min. The column was maintained at 35°C, and the column effluent was monitored at a wavelength of 210 nm with a bandwidth of 8 nm.

RESULTS

PS80 inhibits biofilm formation of *P. aeruginosa* PA14 without inhibiting planktonic growth. PS80 decreased the biofilm formation of *P. aeruginosa* PA14, as measured by crystal violet staining of microtiter wells (22), when it was used at a concentration of as low as 0.001% (Fig. 1A). The 0.01% concentration of PS80 was chosen for use in the remaining experiments for which results are presented, unless specified otherwise. We confirmed this biofilm result by counting the numbers of CFU of cells grown as biofilms on the wells of a 96-well PVC plate (Fig. 1B). Wild-type (WT) PA14 cells treated with 0.01% PS80 showed a 100-fold reduction in attachment to the wells of the PVC plate. The presence of PS80 did not alter the planktonic growth of PA14 under the conditions used in our assays, as measured by determination of the OD and the numbers of CFU (data not shown). We also obtained a similar result for biofilm formation when we tested a glass surface (data not shown).

The effect of PS80 on cell attachment was not specific to PVC but could be observed on several types of medically relevant abiotic surfaces (Fig. 1C). When *P. aeruginosa* PA14 cells were incubated with the vehicle, an average of 59 ± 10 cells per microscopy field was attached to the PVC tabs, an average of 50 ± 5 cells was attached to the etafilcon A contact lens, and an average of 42 ± 7 cells was attached to the vifilcon A lens. However, in the presence of PS80, the levels of attachment decreased more than 10-fold: 1 ± 1 cell was attached to the PVC tabs, 6 ± 2 cells were attached to the etafilcon A lens, and 3 ± 1 cells were attached to the vifilcon A lens.

Identification of a mutant resistant to the effect of PS80. To begin to elucidate the mechanism by which PS80 mediates

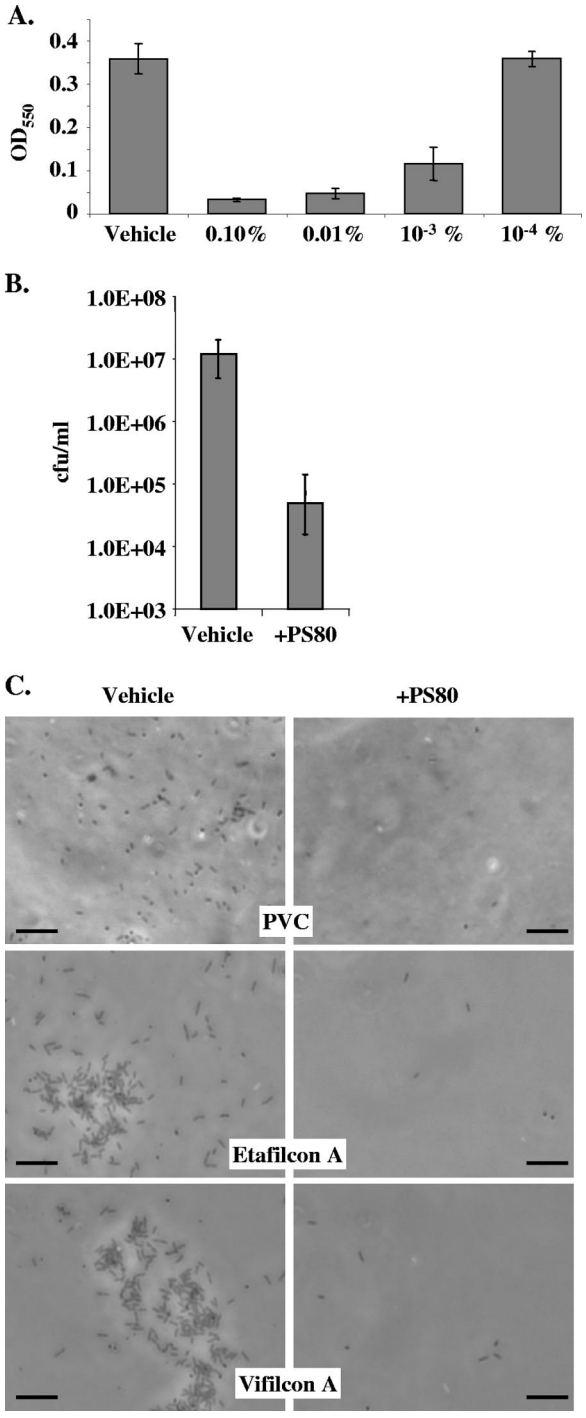

FIG. 1. PS80 inhibits biofilm formation of *P. aeruginosa* PA14. (A) Quantification of biofilms formed in PVC microtiter plates after a 24-h incubation at 37°C. The histograms represent the average of four replicates per condition tested, and the error bars denote the standard deviation. (B) CFU of biofilm cells attached to PVC wells of microtiter plates after a 24-h incubation at 37°C. (C) Top-down phase-contrast micrographs of biofilms formed on PVC tabs or two different contact lens polymers after a 24-h incubation at 37°C. Bars, 20 μm. Cells were grown in LB medium (A and B) or M63-glucose-Casamino Acids (C) supplemented with vehicle or PS80 (0.01%).

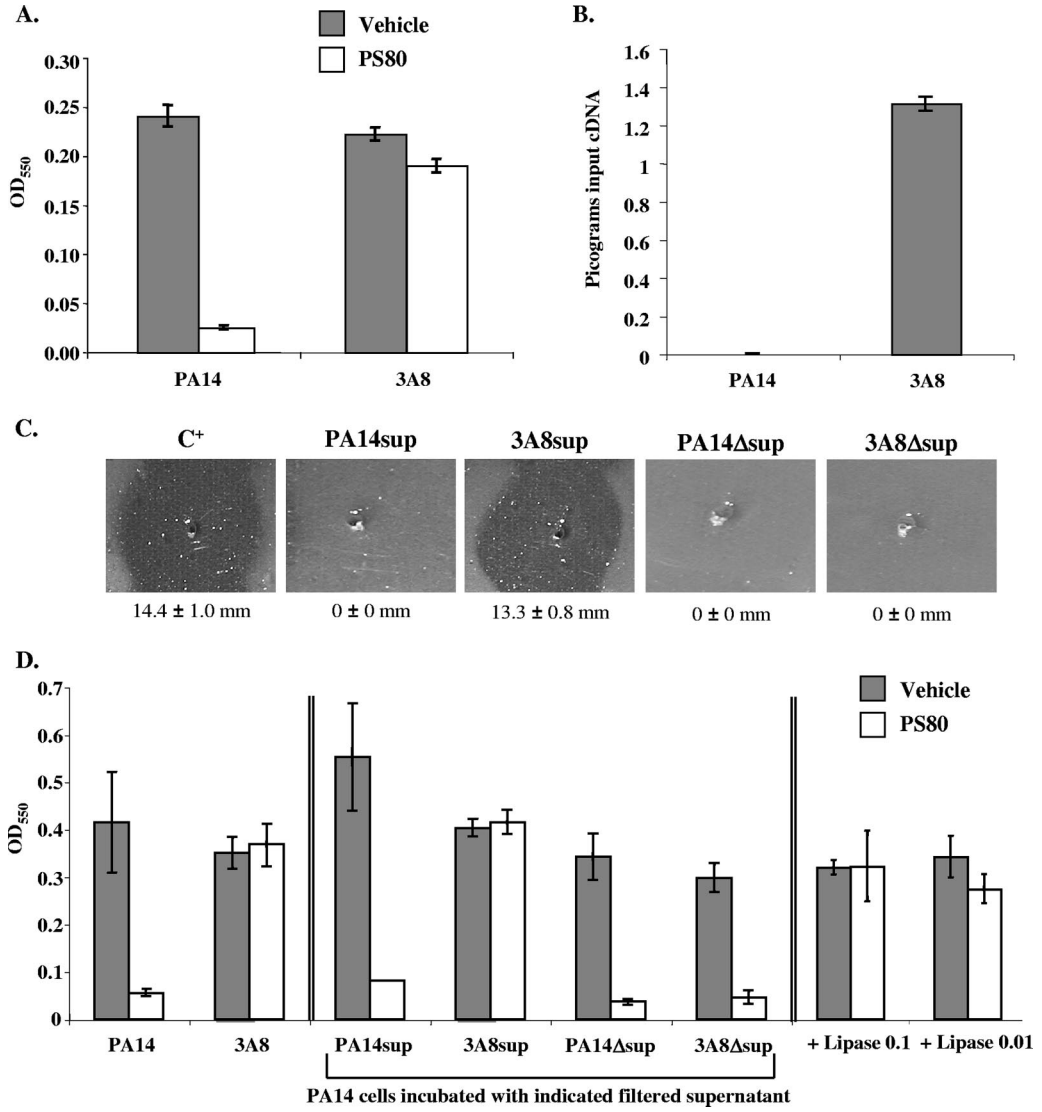

FIG. 2. Mutant 3A8 is resistant to the effect of PS80 and overexpresses the *lipA*-encoded lipase. (A) Quantification of biofilms formed by strain PA14 and mutant 3A8 in PVC microtiter plates after a 24-h incubation at 37°C. The graph presents the average and standard deviation of four replicates. (B) qRT-PCR measuring *lipA* gene expression (the results are averages of four independent experiments). Expression is plotted as the the number of picograms of input cDNA for each strain. (C) Lipase activity assay with a tributyrin emulsion plate (see Materials and Methods). C^+, purified *Pseudomonas* species lipase (type XIII, 0.1 mg/ml); sup, supernatant; Δsup, heat-inactivated supernatant. The average diameter of the zone of clearing is shown below each image. (D) The supernatants from overnight cultures of PA14 and 3A8 were filtered and added to the biofilm assays at a 0.1% concentration. A purified lipase (type XIII) at concentrations of 0.1 or 0.01 mg/ml was also added to a biofilm assay of PA14 cells. Gray bars, LB medium supplemented with vehicle; white bars, LB medium supplemented with PS80 (0.01%).

biofilm inhibition of strain PA14, we searched for mutants of PA14 with the capacity to form biofilms in the presence of 0.01% PS80. Toward that goal, we screened the transposon PA14 mutant library generated by Ausubel and colleagues (16). One mutant, named 3A8, showed strong and reproducible resistance to the effect of PS80 (Fig. 2A). The biofilm formation of 3A8 was comparable to that of the WT strain in the absence of PS80, but unlike the WT, its ability to form biofilms in the presence of PS80 was not affected.

The 3A8 mutant has a mariner transposon inserted at the 3′ end of *ligT* (PA14_27110), annotated as a 2′-5′ RNA ligase gene (http://v2.pseudomonas.com). The *ligT* gene is directly upstream of *lipA* (PA14_27100), which codes for an extracellular lipase. The lipases in bacteria have been extensively studied and are known to be able to cleave and degrade detergents such as PS80 (10).

We hypothesized that the known mariner transposon promoter might result in the increased expression of *lipA*. A qRT-PCR experiment demonstrated that the level of transcription of *lipA* was increased about 250-fold in mutant 3A8 (1.3129 ± 0.0034) compared to that in WT strain PA14 (0.0053 ± 0.0003) (Fig. 2B). This result suggested to us that the resistance to PS80 seen in 3A8 might be related to the overexpression of *lipA*.

The supernatant of 3A8 confers resistance to PS80. If the PS80 resistance of mutant 3A8 were due to higher levels of

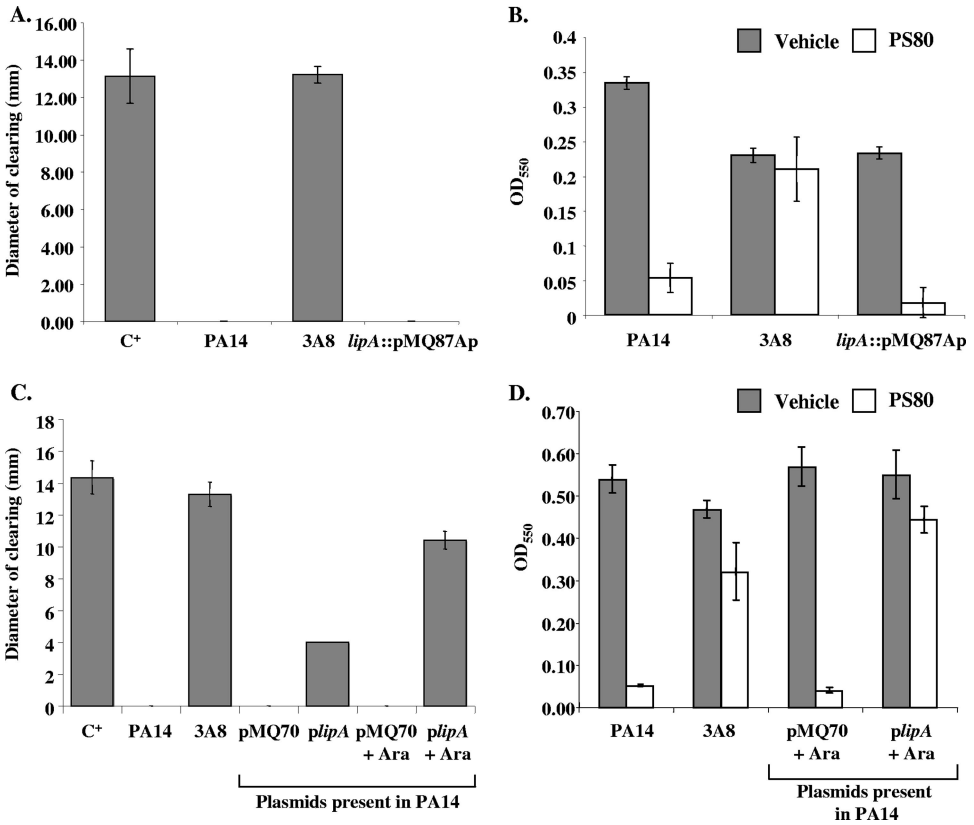

FIG. 3. The deletion and multicopy expression of *lipA* show that the overexpression of LipA is associated with resistance to PS80 in mutant 3A8. (A and C) Lipase activity assay on tributyrin elmulsion plates (see Materials and Methods). C^+, purified *Pseudomonas* species lipase (0.1 mg/ml); *lipA*::pMQ87Ap, single-crossover knockout mutant of *lipA* in the mutant 3A8 background; pMQ70, vector carried by WT strain PA14; p*lipA*, *lipA* gene cloned on pMQ70, carried by WT PA14. (B and D) Biofilm assay performed as described in the legend to Fig. 1. Gray bars, LB medium supplemented with vehicle; white bars, LB medium supplemented with PS80 (0.01%). Where indicated, strains carrying pMQ70 and p*lipA* were grown with 0.2% arabinose (+Ara).

expression and secretion of LipA, we would predict that filtered supernatants of 3A8 would contain a concentration of secreted LipA higher than that in the WT and would demonstrate a higher level of lipase activity. These cell-free supernatants were tested on a tributyrin emulsion plate, which is used to detect lipase activity through the clearing of the lipid emulsion. The WT PA14 supernatant had no detectable lipase activity even after 3 days of incubation (0 ± 0 mm of tributyrin clearing), whereas we could visualize strong lipase activity with the 3A8 supernatant (13.3 ± 0.8 mm of tributyrin clearing) by day 3 (Fig. 2C).

We tested the ability of WT strain PA14 to form biofilms in the presence of PS80 and a 1:10 dilution of filtered supernatant prepared from an overnight culture of either the WT strain or mutant 3A8. The WT cells incubated with the WT supernatant could not form biofilms in the presence of PS80, but the same cells incubated with the supernatant derived from the 3A8 mutant showed attachment to PVC in the presence of PS80 at the same level as in the presence of the vehicle alone (Fig. 2D).

To further show that the resistance of mutant 3A8 was due to the production of the LipA enzyme, we tested filtered supernatants after treatment at 65°C for 20 min to denature the proteins present in the sample. As expected, WT cells supplemented with heated 3A8 supernatant did not form a biofilm in the presence of PS80 (Fig. 2D). When the same supernatant was tested on a tributyrin plate, no lipase activity could be detected (Fig. 2C). Taken together these results suggest that the 3A8 mutant secretes a heat-labile lipase that is associated with resistance to PS80.

We also tested the effect of a purified, commercially available lipase (type XIII) purified from *Pseudomonas* species on the biofilm formation of WT PA14 cells. When the bacteria were grown in the presence of both PS80 and the purified lipase (at a concentration of either 0.1 or 0.01 mg/ml), they formed biofilms as well as they did when they were treated with the vehicle alone (Fig. 2D).

The overexpression of LipA is associated with the resistance to PS80 and biofilm formation. To provide additional evidence that the resistance phenotype observed in mutant 3A8 was due to the overproduction of LipA, we constructed a single-crossover knockout mutant of *lipA* (the *lipA*::pMQ87Ap mutant) in the 3A8 background. This mutant showed a drastic reduction in the level of *lipA* transcription by qRT-PCR (data not shown). On tributyrin emulsion plates, the filtered supernatant of a culture of *lipA*::pMQ87Ap showed no lipase activity, similar to the result for strain PA14 and in contrast to the result for its parental strain, 3A8 (Fig. 3A). In a biofilm assay, *lipA*::pMQ87Ap and 3A8 had similar levels of biofilm formation in

the absence of PS80, but when they were incubated with PS80, *lipA*::pMQ87Ap lost its ability to form biofilm compared to that of mutant 3A8 and showed a phenotype similar to that of PA14 (Fig. 3B).

We also cloned *lipA* under the control of the P_{BAD} inducible promoter and introduced this construct in WT strain PA14. On tributyrin emulsion plates, the filtered supernatant of a WT culture containing the p*lipA* construct showed some lipase activity without the addition of arabinose, whereas the control showed no lipase activity. However, when cells containing p*lipA* were grown in the presence of 0.2% arabinose, the lipase activity observed on these emulsion plates reached a level similar to what was measured for 3A8 (Fig. 3C). When WT cells transformed with p*lipA* were tested in a biofilm assay in the presence of PS80, they formed as much biofilm as they did when they were incubated without PS80, upon induction with 0.2% arabinose (Fig. 3D). Taken together, these results suggest that the overexpression of *lipA* in mutant 3A8 is directly associated with the resistance to PS80 and biofilm formation.

PS80 is degraded to oleic acid by the filtered supernatant of mutant 3A8. Some lipases are known to have an esterase activity that allows them to cleave detergents at their ester bond (10). We postulated that lipases, including LipA, are able to render PS80 inactive by cleaving it at its ester bond (Fig. 4A). This cleavage should lead to the production of an alcohol and oleic acid. Consistent with this premise, we noted a reduction in the pH of the 3A8 supernatant on modified lipase activity plates prepared with 0.01% PS80, in place of tributyrin, and amended with phenol red, a pH indicator (data not shown).

We next analyzed filtered supernatants of mutant 3A8 for their ability to degrade PS80. While we could not directly monitor the supernatants for PS80, we could assess the accumulation of oleic acid, one product of PS80 generated when this compound is cleaved at its ester bond. We incubated the supernatants of 3A8 with the vehicle or PS80 and analyzed the resulting supernatant by HPLC on a C_{18} HPLC column to detect the formation of oleic acid. Analysis of the supernatant of 3A8 incubated with PS80 (0.01%) led to the detection of a small peak at the retention time for oleic acid (~18.6 min) (Fig. 4B).

In a control experiment, no peak at the retention time for oleic acid could be detected in the supernatant of mutant 3A8 when it was incubated with vehicle alone (Fig. 4C). When 3A8 was incubated with 10-fold more PS80 (0.1%), we could detect a larger peak at the retention time corresponding to that for oleic acid (Fig. 4D). This result indicates that the production of oleic acid depends, at least in part, on the initial concentration of PS80 present in the solution.

To confirm our conclusion that the peak at 18.6 min corresponds to oleic acid, we spiked the mutant 3A8 supernatant containing 0.01% PS80 with commercially available purified oleic acid at a concentration of 0.01%. The peak detected in this spiked sample had a similar retention time of 18.6 min, suggesting that we had detected the production of oleic acid in our samples (Fig. 4E). No peak at the retention time of 18.6 min could be detected for samples purified from the WT strain incubated with vehicle alone (control experiment; Fig. 4F) or with PS80 (0.01%) (Fig. 4G), indicating that PS80 is not cleaved under the conditions tested. It should also be noted that, unlike PS80, commercially available oleic acid at a concentration of 0.01% did not lead to a reduction in the level of biofilm formation by WT strain PA14 (data not shown).

We also tested if the commercially available *Pseudomonas* species lipase (type XIII) was able to cleave PS80. When the mix of the lipase and PS80 was incubated overnight at 37°C, a peak at the retention time for oleic acid could be detected (data not shown). If a similar mix was tested by HPLC without incubation, no peak corresponding to oleic acid could be detected. Taken together, these data suggest that the cleavage of PS80 to oleic acid occurs in the presence of lipases and that a lipase capable of such activity is present in the supernatant of mutant 3A8.

A surfactant with an ether bond inhibits the biofilm formation of mutant 3A8. Given our evidence suggesting that PS80 is cleaved by LipA in the filtered supernatant of mutant 3A8, we postulated that a surfactant containing a lipase-resistant ether bond would inhibit the biofilm formation of the LipA-overproducing mutant. To address this question, we tested the surfactant polyethoxylated(20) oleyl alcohol, which has a chemical structure similar to that of PS80 (Fig. 4A). Importantly, polyethoxylated(20) oleyl alcohol contains an ether bond, which lipases cannot cleave, in place of the ester bond present in PS80.

Biofilm assays showed that both WT strain PA14 and mutant 3A8 were equally inhibited by polyethoxylated(20) oleyl alcohol (Fig. 5), and the presence of this compound in the medium did not lead to any alteration of the growth of the strains tested (data not shown).

PS80 inhibits biofilm formation of other clinical isolates of *P. aeruginosa* and other gram-negative and -positive bacteria. We were interested in whether the effect of PS80 on biofilm formation could be observed in *P. aeruginosa* clinical isolates as well as other bacterial species. We tested a collection of gram-negative and gram-positive clinical isolates and identified 255 strains that were able to form a biofilm when they were grown in LB medium supplemented with the vehicle. Seventy-five of the 255 isolates had reduced planktonic growth when they were incubated with PS80 and were not further tested. The remaining 180 isolates were tested for biofilm formation in the presence of 0.01% PS80 and were categorized as biofilm formation inhibited when at least a 50% decrease in the level of biofilm formation was observed in the presence of PS80 (Table 1).

Of the 180 remaining isolates, 101 were *P. aeruginosa*, and biofilm formation was inhibited by PS80 in 45% of these of 101 isolates. All *P. aeruginosa* clinical strains were also tested for lipase production on tributyrin emulsion plates. Of the isolates able to form biofilms in the presence of PS80, 25% showed increased lipase activity on the plates compared to that of WT strain PA14 (Table 1). Biofilm formation in the presence of polyethoxylated(20) oleyl alcohol was also tested; 80% of the isolates able to form biofilms in the presence of PS80 also formed biofilms in the presence of polyethoxylated(20) oleyl alcohol, but only 25% of these isolates had increased lipase activity (data not shown). The existence of PS80- and polyethoxylated(20) oleyl alcohol-resistant strains suggests that there are mechanisms for resisting the antibiofilm effects of surfactants that do not involve lipase-mediated degradation of the surfactant.

Incubation with PS80 inhibited the biofilm formation of all of

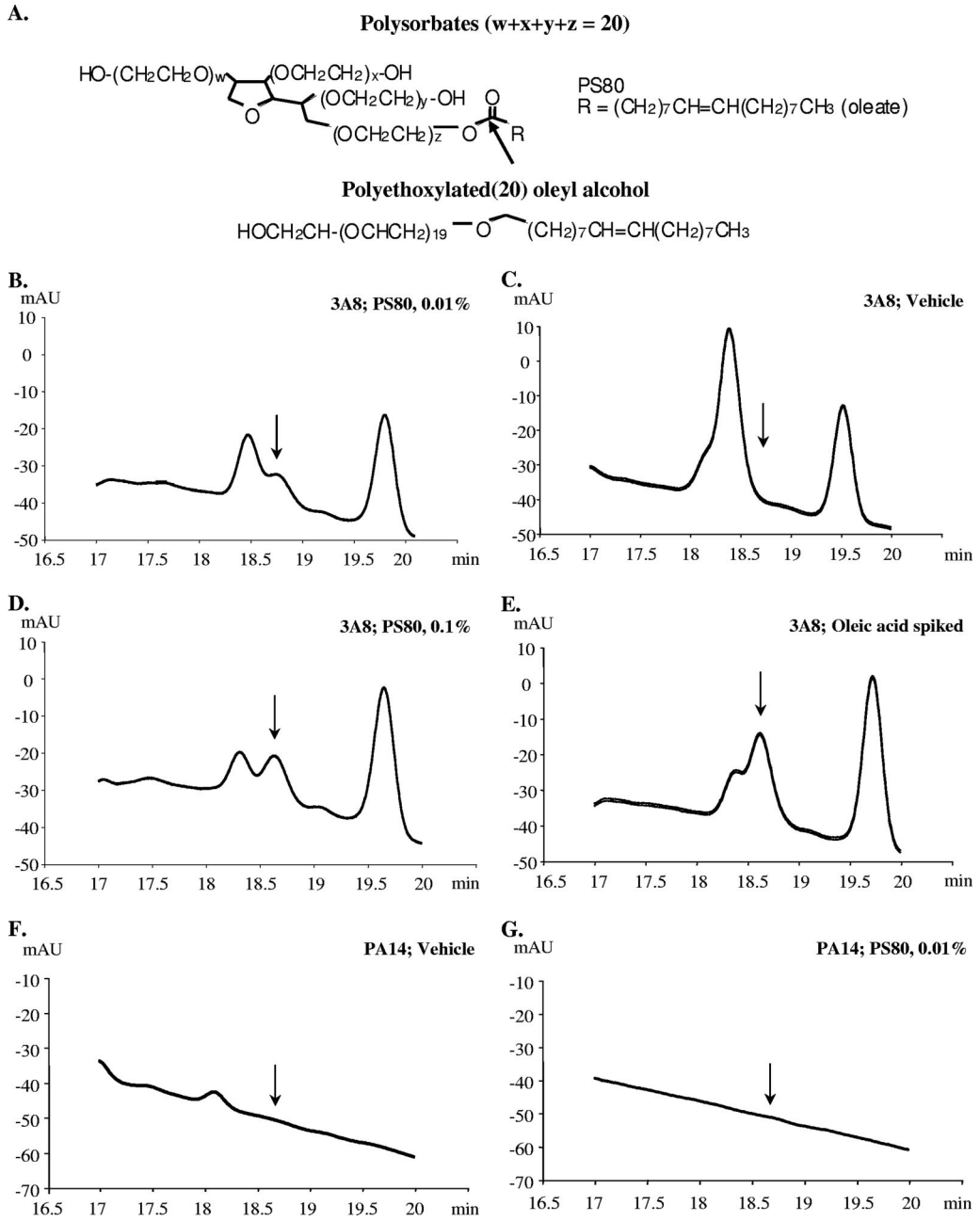

FIG. 4. Oleic acid is a product of LipA-mediated PS80 degradation. (A) Chemical structures of PS80 and polyethoxylated(20) oleyl alcohol (courtesy of Alex Pletnev, Department of Chemistry, Dartmouth College). The arrow shows the ester bond of PS80 expected to be cleaved by lipases. (B to G) HPLC traces of various supernatants. The arrows indicate the retention time for oleic acid. (B and D) Analysis of the supernatants of mutant 3A8 to which PS80 at 0.01% (B) and 0.1% (D) was added. The oleic acid peak is detected at the retention time of ~18.6 min and is dose dependent (compare the peaks in panels B and D). (C) Mutant 3A8 supernatant with no added PS80 used as a control. (E) Control in which the 3A8 supernatant was spiked with 0.01% oleic acid. (F) The WT PA14 supernatant with no added PS80 used as a control. (G) The WT supernatant with added PS80 (0.01%). mAU, milli-absorbance units.

the *Escherichia coli* and *Klebsiella* sp. isolates tested (Table 1). Biofilm formation was inhibited in most gram-positive bacterial species tested in the presence of PS80, including *Staphylococcus epidermidis* (100% of the tested isolates), *Staphylococcus saprophyticus* (100%), coagulase-negative *Staphylococcus* (97.5%), *Enterococcus* sp. (100%), and *Streptococcus mutans* (100%) (Table 1). In contrast, biofilm formation was inhibited in only 66.7% of *Staphylococcus aureus* isolates in the presence of PS80 (Table 1). Moreover, all *S. aureus* isolates able to form biofilms in the presence of PS80 showed high levels of lipase activity when it was measured on tributyrin emulsion plates, whereas none of the coagulase-negative staphylococci showed any lipase activity, reinforcing the idea that non-lipase-dependent mechanisms of resistance to PS80 exist (Table 1).

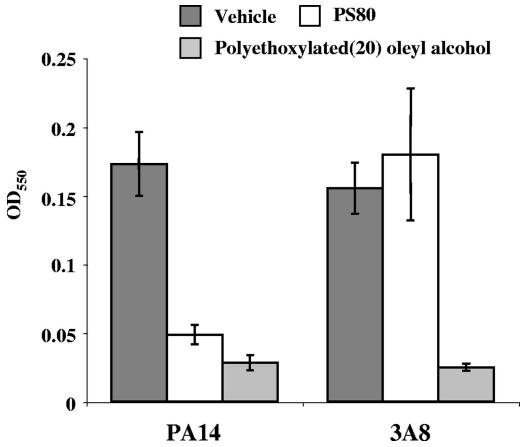

FIG. 5. Polyethoxylated(20) oleyl alcohol inhibits biofilm formation by WT strain PA14 and mutant 3A8. Biofilm assays were performed as described in Materials and Methods. LB medium was supplemented with either vehicle (dark gray bars), PS80 (0.01%; white bars), or polyethoxylated(20) oleyl alcohol (0.01%; light gray bars).

***P. aeruginosa* PAO1 is resistant to PS80 but does not overproduce LipA.** In addition to all the clinical isolates, we also tested the other commonly used *P. aeruginosa* laboratory strains, PAK and PAO1. Strain PAK was found to be inhibited in the presence of PS80 at concentrations similar to those that inhibited strain PA14 (data not shown), whereas *P. aeruginosa* PAO1 could still form a biofilm in the presence of 0.01% PS80 (Fig. 6A), as well as in the presence of polyethoxylated(20) oleyl alcohol. Strain PAO1 was able to form biofilms in the presence of PS80 at concentrations as high as 2% (data not shown). Unlike mutant 3A8, the filtered supernatant of strain PAO1 could not restore biofilm formation when it was added to a biofilm assay with PA14 cells in the presence of PS80 (Fig. 6A). Moreover, PAO1 did not show any lipase expression

TABLE 1. Effect of PS80 (0.01%) on ability to form biofilms by clinical isolates of gram-negative and gram-positive bacterial species

Species	No. of clinical isolates	% of clinical isolates with:	
		Inhibition of biofilm formation in presence of PS80[a]	Lipase activity[b] and ability to form biofilms in presence of PS80
Pseudomonas aeruginosa	101	45	25
Escherichia coli	6	100	NA[c]
Klebsiella spp.	7	100	NA
Staphylococcus aureus	12	66.7	100
Staphylococcus epidermidis	11	100	NA
Staphylococcus saprophyticus	1	100	NA
Coagulase-negative *Staphylococcus*	40	97.5	0
Enterococcus sp.	1	100	NA
Streptococcus mutans	1	100	NA

[a] None of the strains had an obvious growth defect in the presence of PS80 (data not shown).
[b] The lipase activity was measured on tributyrin emulsion plates (see Materials and Methods).
[c] NA, not applicable. None of the clinical isolates were able to form biofilms in the presence of PS80.

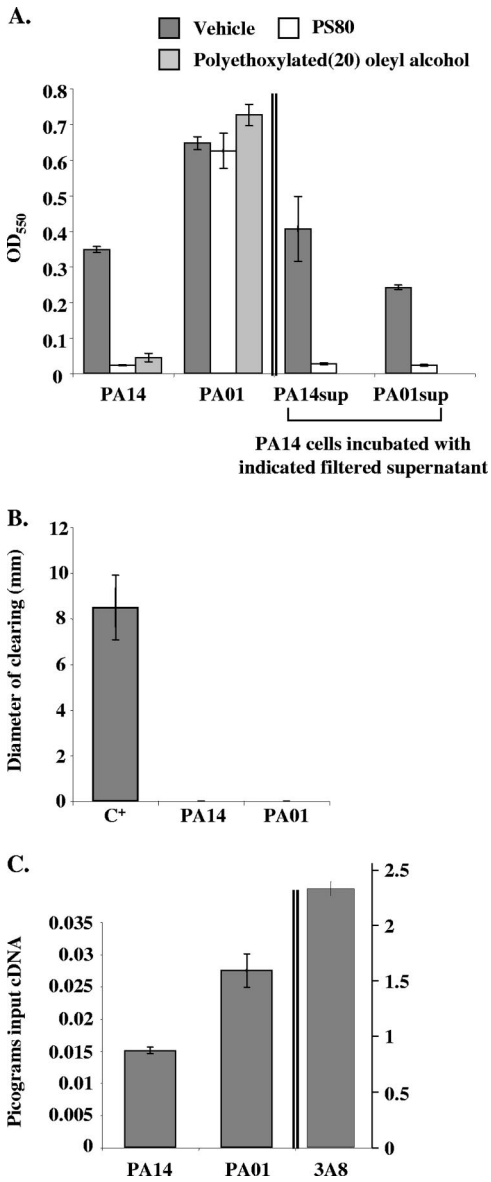

FIG. 6. Strain PAO1 is resistant to the effect of PS80, but the overexpression of a lipase is not involved. (A) Biofilm assays were performed as described in Materials and Methods. LB medium was supplemented with either vehicle (dark gray bars), PS80 (0.01%; white bars), or polyethoxylated(20) oleyl alcohol (0.01%; light gray bars). The supernatants from overnight cultures of strains PA14 and PAO1 were filtered and added to the biofilm assays with PA14 cells at a 0.1% concentration. (B) Lipase activity assay on tributyrin emulsion plates (see Materials and Methods). C^+, purified *Pseudomonas* species lipase (0.1 mg/ml). (C) qRT-PCR measuring *lipA* expression (the results are the averages of two independent experiments). Expression is plotted as the number of picograms of input cDNA for each strain. Data for the level of *lipA* expression in mutant 3A8 were added on the right of the graph to show the differences in scales between PA14, PAO1, and 3A8.

when it was measured on tributyrin emulsion plates (Fig. 6B), suggesting that the mechanism of resistance of this strain is different from that of mutant 3A8 and does not involve the overproduction of LipA. To verify this hypothesis further, we measured the level of expression of *lipA* by qRT-PCR and showed that PAO1 has a low level of transcription of *lipA*,

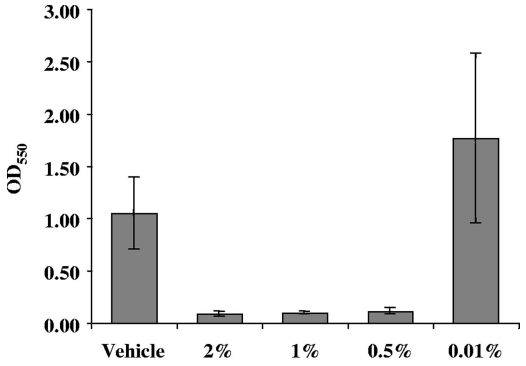

FIG. 7. Higher concentrations of PS80 lead to disrupted strain PA14 biofilms. The biofilm assay was performed as described in Materials and Methods. After a 24-h incubation, the LB medium was replaced with LB medium supplemented with either the vehicle or PS80 at a concentration of 2, 1, 0.5, or 0.01%. The biofilm assays were then incubated for another 24 h before they were quantified.

similar to that of PA14 and unlike that of 3A8, as shown in Fig. 6C. Taken together, these data suggest that PAO1 has a non-lipase-dependent pathway of PS80 resistance.

The addition of higher concentrations of PS80 can disrupt already formed biofilms. All the data presented so far show that PS80 does not allow WT strain PA14 cells to bind to the surface in order to develop a biofilm, but what about preexisting biofilms?

We tested the effect of PS80 on preformed biofilms and showed that the concentration of 0.01% used throughout this work was not able to disrupt an established biofilm formed by PA14 on PVC (Fig. 7). Nevertheless, when the concentration of PS80 was increased to 0.5, 1%, or 2%, no biofilm remained on the PVC wells (Fig. 7). This shows that bacterial biofilms could be disrupted in the presence of higher concentrations of PS80.

DISCUSSION

PS80 is a surfactant and emulsifier used by the cosmetic, food, and pharmaceutical industries at concentrations that range from 0.1% to over 1% (Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, http://www.cfsan.fda.gov/~dms/opa-appa.html). We have demonstrated that concentrations of PS80 at least 10 times lower than those commonly used can inhibit biofilm formation by *P. aeruginosa* PA14 as well as numerous other *P. aeruginosa* clinical isolates, including gram-negative and -positive isolates. The suppression of *P. aeruginosa* biofilm formation by PS80 was observed when it was tested with a variety of abiotic surfaces of medical relevance, including contact lenses. Additionally, at higher concentrations, such as 1%, PS80 was able to disrupt preformed biofilms of PA14. The detection of these effects at concentrations of PS80 known to be well tolerated by human tissues (21) suggests that PS80 or a related compound might have therapeutic value.

However, we found that many strains of *P. aeruginosa* are resistant to the effect of PS80. The identification of a transposon mutant (mutant 3A8) and studies with that mutant helped address the resistance of at least a subset of isolates. This mutated strain overexpresses the lipase LipA, which, when it is secreted, results in the cleavage of PS80 into an alcohol and oleic acid. Cell-free supernatants containing high levels of LipA alone or commercially available purified lipase conferred resistance to PS80 to WT cells. A similar effect was also observed when *lipA* was overexpressed on a plasmid in the WT cells. Taken together, these data lead to the conclusion that the PS80 resistance observed in mutant 3A8 is due to the presence of a higher concentration of LipA in the culture supernatant. Polyethoxylated(20) oleyl alcohol is structurally similar to PS80 but has an ether bond in place of the ester bond in PS80. Importantly, this surfactant was able to inhibit the biofilm formation of strain PA14 as well as that of mutant 3A8. We hypothesize that polyethoxylated(20) oleyl alcohol's activity against the 3A8 mutant is due to the presence of its lipase-resistant ether bond that links its hydrophilic and hydrophobic moieties, which renders it resistant to cleavage by the lipase LipA.

While some of the PS80-resistant clinical *P. aeruginosa* strains did show evidence of lipase secretion, approximately 75% did not. Moreover, 80% of all these PS80-resistant strains formed biofilms in the presence of polyethoxylated(20) oleyl alcohol, suggesting that additional mechanisms for resisting the antibiofilm effect of PS80 and related compounds exist. We reached a similar conclusion for the PAO1 strain of *P. aeruginosa*, which was resistant to the effect of PS80, even at high concentrations (up to 2%), as well as polyethoxylated(20) oleyl alcohol, but which did not show any involvement of a lipase in its resistance mechanism. More work is necessary to understand these non-lipase-dependent pathways to surfactant resistance.

Previous studies have addressed the ability of surfactants to alter biofilm formation and have obtained different results. Consistent with the idea that it can prevent bacterial attachment, PS80 (used at 0.01%) has been shown to play a role in the disruption of a microbe-peat association (1). While Mireles et al. (20) reported that surfactin as well as sodium dodecyl sulfate and PS80 (used at 0.25%) were effective at inhibiting biofilm formation by *Escherichia coli*, *Salmonella enterica*, and *Proteus mirabilis* on catheters, they did not observe any inhibition of biofilm formation by *P. aeruginosa*. As noted, we found that PS80's inhibitory effect applies to many but not all strains of *P. aeruginosa* tested, including the laboratory strain *P. aeruginosa* PAO1. The authors of that study did not specify the strain of *P. aeruginosa* used, so it is possible that they were testing *P. aeruginosa* PAO1, which we found to be resistant to PS80's inhibitory effect on biofilm formation. They go on to make the suggestion that there is an inverse relationship between the presence of surfactants and biofilm formation. Our data do not contradict this suggestion but suggest that some strains are resistant to these surfactant-mediated effects.

Potential clinical applications of the antibiofilm effect of PS80 or derivatives include the treatment of medical prosthetic devices, such as artificial joints and intraocular lenses, prior to implantation. For these types of implants, contamination typically occurs during implantation, but if the bacteria are cleared, reinoculation of the implant is unlikely. This application may be the most relevant, since our data indicate that PS80 prevents bacterial biofilm formation even when it is used at very low concentrations. In contrast, there are medical de-

vices such as catheters and contact lenses which experience bacterial reinoculation while in use. To be effective for these types of devices experiencing recontamination, it would be important to create a schedule of repeated applications of PS80 by the use of higher concentrations to disrupt biofilms that may form over time. Another strategy which might prevent bacterial attachment would be to permanently bind PS80 or a derivative to the surface of the device. Finally, clinical applications will require PS80 to work effectively in a polymicrobial environment. While our data showing that PS80 inhibits bacterial biofilm formation by a broad range of microbes are encouraging, additional work will be necessary to optimize these properties in a clinical setting.

Further work is also necessary to identify the mechanism by which PS80 inhibits biofilm formation. PS80 and related surfactants may act primarily by modifying the abiotic surface in such a way that it becomes resistant to bacterial attachment or by altering bacterial physiology to render the strains less fit to form biofilms. A better understanding of the mechanisms by which surfactants inhibit biofilm formation will help in the design of compounds of therapeutic value.

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