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Inhibition of Bacterial Adherence to Hydrocarbons and Epithelial Cells by Emulsan

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Acinetobacter calcoaceticus RAG-1 and BD413, as well as *Streptococcus pyogenes* M-5, adhered to octane. Adherence was inhibited by emulsan (100 μg/ml), the polymeric emulsifying agent produced by *A. calcoaceticus* RAG-1. Emulsan also inhibited adherence of *S. pyogenes* and RAG-1 to buccal epithelial cells. The mean values of bound *S. pyogenes* per epithelial cell were 57.2 and 20.7 for the control and emulsan-containing suspensions, respectively; mean values of bound RAG-1 per epithelial cell were 221 for the control and 40 for the suspension containing 100 μg of emulsan per ml. Desorption of previously bound RAG-1 from epithelial cells by emulsan was concentration dependent: a maximum of 80% desorption was obtained with 200 μg of emulsan per ml. The data showing that emulsan desorbed 70% of the indigenous bacterial flora from buccal epithelial cells suggest that hydrophobic interactions mediate not only the in vitro adherence of laboratory strains to epithelial cells, but actually govern the adherence of the majority of the bacteria that colonize this surface. The advantages of using emulsan as an antiadherence agent include its chemical purity, stability, and polymeric nature.

In many cases, adherence of microorganisms to tissues within the host is a prerequisite for subsequent colonization (5) and thus an essential factor in the disease process. Several different mechanisms have been shown to be involved in adhesion of bacteria to mammalian cells. One such mechanism, hydrophobic interactions, has been reported to be involved in the adherence of enteropathogenic *Escherichia coli* (3, 15, 18), rough strains of *Salmonella typhimurium* (7), and streptococci (1, 6, 11, 16) to mammalian cells. Hydrophobic interactions may also play a role in the adherence of certain oral species to the tooth surface (18a). Cell surface hydrophobicity has also been reported to be an important factor in phagocytosis (17).

Bacterial adherence to liquid hydrocarbons is a general method for measuring cell surface hydrophobicity (9–14). Recently, this technique was employed to demonstrate correlations between the ability of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 and the oral pathogen *Streptococcus pyogenes* M-5 to adhere to buccal epithelial cells and hydrocarbons (11). *A. calcoaceticus* RAG-1 mutant MR-481, selected for its inability to adhere to hydrocarbon (12), also failed to adhere to epithelial cells.

A. calcoaceticus RAG-1 produces a potent extracellular bioemulsifier referred to as emulsan (8). Emulsan is a polyanionic heteropolysaccharide with a molecular weight average of 9.9×10^5 (20). Emulsan consists of a D-galactosamine-containing polysaccharide backbone with covalently linked fatty acid side chains (2, 20). Protein (approximately 15%) associated with emulsan can be removed by hot phenol treatment or calcium nitrate precipitation without destroying its emulsifying activity. Emulsan stabilizes a wide variety of hydrocarbon-in-water emulsions by forming a strong film at the interface (19).

The high affinity of emulsan for hydrocarbon-water interfaces (19) suggested that it might be useful for studying the role of hydrophobic interactions in bacterial adherence phenomena. In this report, data are presented which demonstrate that emulsan prevents adherence of *A. calcoaceticus* and *S. pyogenes* to hydrocarbon and buccal epithelial cells. Emulsan was also shown to be highly effective in removing bound indigenous bacteria from epithelial cells.

MATERIALS AND METHODS

Bacterial growth conditions. *A. calcoaceticus* RAG-1 (ATCC 31012) was grown in nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl and harvested in stationary phase after overnight incubation at 30°C with shaking. For adherence to octane, the cells were then diluted 1:100 into fresh

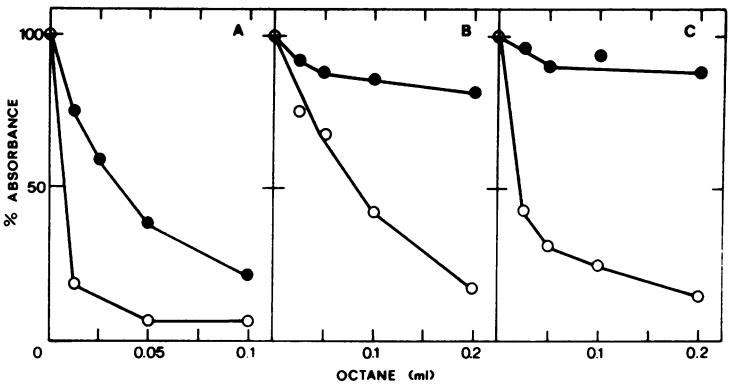

FIG. 1. Adherence of *A. calcoaceticus* RAG-1 (A), *A. calcoaceticus* BD413 (B), and *S. pyogenes* M-5 (C) to octane. Washed cells were suspended in PUM buffer, and adherence to octane was carried out as described previously (9–14) in the presence of 100 μg of emulsan per ml (○) or in the absence of the biopolymer (●). Results are expressed as percentage of the initial absorbance at 400 nm of the aqueous phase (after mixing) as a function of hydrocarbon volume added.

medium and allowed to grow for 6 h before harvesting. *A. calcoaceticus* BD413 (4) was grown overnight in the same medium. *S. pyogenes* M-5 (H type 5) was grown at 37°C in brain heart infusion broth (Difco) under static conditions and harvested after 48 h.

Epithelial cells. Epithelial cells were obtained as described previously (11) by scraping the buccal mucosa of several healthy donors with wooden sticks. Samples were taken in the morning before the donors had put anything into their mouths. The cells were washed twice and suspended in phosphate-buffered saline to a concentration of 10^6 cells per ml. Viability of epithelial cells, as determined by trypan blue exclusion staining, was 40 to 60% in the presence or absence of emulsan.

Emulsan. Emulsan, the polyanionic emulsifying agent of *A. calcoaceticus* RAG-1, was purified from the cell-free supernatant fluid obtained from an ethanol-grown culture (8). After dialysis and lyophilization, the powder was washed in warm ethanol to remove colored impurities and then extracted into 80% ethanol; emulsan was then precipitated by the addition of a saturated solution of $Ca(NO_3)_2$ in 90% ethanol. The extraction of emulsan into 80% ethanol and precipitation with $Ca(NO_3)_2$ was repeated. The product was then dissolved in water, dialyzed extensively against distilled water, and lyophilized. Stock solutions of 2 mg of emulsan per ml were used routinely. The emulsan used in these studies had an emulsifying activity of 150 U/mg (8), an O-ester content of 0.5 μmol/mg, a reduced viscosity of 350 cm^3/g, and a residual protein content of less than 5%.

RESULTS

As previously demonstrated (10), *A. calcoaceticus* RAG-1 and BD413, as well as *S. pyogenes* M-5, adhered to octane (Fig. 1). Under the conditions employed, *A. calcoaceticus* RAG-1 cells were more adherent than *S. pyogenes*, which was slightly more adherent than *A. calcoaceticus* BD413. The adherence of all these strains to octane was inhibited by 100 μg of emulsan per ml. With the more hydrophobic RAG-1 cells, emulsan was more efficient at lower octane volumes, whereas with the other two strains, emulsan was effective over the entire range of octane concentrations examined. Higher concentrations of emulsion could not be used to prevent the bacteria from adhering to the hydrocarbon-water interface because of the formation of stable emulsions, which interfered with turbidity measurements. Similarly, the use of octane as a test hydrocarbon in these experiments prevented the formation of stable emulsions, as this hydrocarbon is poorly emulsified by emulsan (19).

The emulsan-mediated inhibition of RAG-1 adherence to octane was not due to a measurable change in cell viability nor to an irreversible binding of emulsan to the bacterial cell surface. Pretreatment of RAG-1 cells with 200 μg of emulsan per ml for 30 min had no effect on cell viability. Furthermore, when RAG-1 cells were harvested by centrifugation after emulsan treatment and then resuspended in PUM buffer (9), the cells adhered to hydrocarbons with the same efficiency as untreated controls.

As seen in Fig. 2, emulsan prevented adherence of RAG-1 to buccal epithelial cells. In the absence of emulsan, 70% of the epithelial cells contained more than 150 adherent RAG-1 cells, whereas in the presence of 100 μg of emulsan per ml, 67% of the epithelial cells had 50 or less adhering RAG-1 cells. The mean values of RAG-1 cells per epithelial cell were 221 and 40 for the control and emulsan-containing suspensions, respectively.

Emulsan was also effective in preventing adherence of *S. pyogenes* M-5 to epithelial cells (Fig. 3). In the absence of emulsan, 94% of the epithelial cells contained more than 20 adherent *S. pyogenes* cells, whereas in the presence of

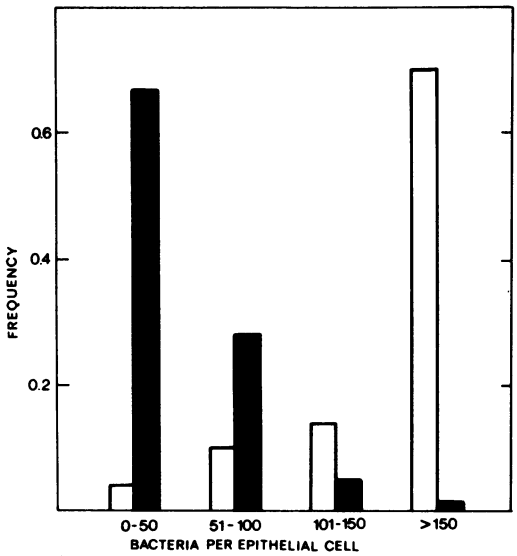

FIG. 2. Adherence of *A. calcoaceticus* RAG-1 to buccal epithelial cells. Equal volumes of washed *A. calcoaceticus* RAG-1 (1.0×10^9 cells per ml) and epithelial cells (1.1×10^6 cells per ml) were incubated together for 15 min in the presence of 100 μg of emulsan per ml (black bars) or in the absence of the biopolymer (open bars), and adherence was measured after filtration, as described in the text. The data were pooled from three independent experiments in which a total of 600 epithelial cells were scored microscopically. The background values of adherent indigenous bacteria were subtracted in all cases.

200 μg of emulsan per ml, 61% of the epithelial cells had 20 or less adhering *S. pyogenes* cells. The mean values of *S. pyogenes* cells per epithelial cell were 57.2 and 20.7 for the control and emulsan-containing suspensions, respectively.

Emulsan, in addition to its ability to inhibit bacterial adherence to epithelial cells, was also found to be effective in desorbing previously bound RAG-1 cells (Fig. 4). Desorption of RAG-1 from the epithelial cells was dependent on the emulsan concentration. A maximum of 80% desorption was obtained at 200 μg of emulsan per ml.

While examining the effects of emulsan on adherence of *A. calcoaceticus* RAG-1 and *S. pyogenes* M-5 to epithelial cells, it became evident that a significant fraction of the bacterial population indigenous to the buccal epithelial cell surface was removed by the emulsan treatment. Thus, a study of emulsan-mediated desorption of the indigenous bacteria (with no bacteria added) was carried out (Fig. 5). The 500 control epithelial cells scored (no emulsan) showed a wide range in the number of bacteria per epithelial cell; the mean and median values of bacteria per epithelial cell were 61 and 39, respectively. Epithelial cells treated with 200 μg of emulsan per ml had mean and median values of bacteria per epithelial cell of 18 and 8, respectively. Thus, 70% of the bound indigenous bacteria were removed by emulsan.

Treatment of epithelial cells with 200 μg of emulsan per ml for 30 min had no significant effect on the viability of the epithelial cells as determined by trypan blue exclusion staining. However, when emulsan-treated epithelial cells were centrifuged, washed twice and resuspended in phosphate-buffered saline, adsorption of RAG-1 cells to them was inhibited by 82%, suggesting that the epithelial cell surface was either modified by emulsan or that emulsan bound to the epithelial cells with high affinity.

DISCUSSION

In a previous report, we observed that adherent *A. calcoaceticus* RAG-1 cells were desorbed from hydrocarbon droplets during growth on hexadecane (12). The possibility that this was due to the production of emulsan by the growing cells (9) led us to examine the efficacy of emulsan in inhibiting bacterial adherence to hydrocarbon and other hydrophobic surfaces.

The results presented here clearly demonstrate that emulsan inhibits RAG-1 adherence to hydrocarbon. Emulsan-mediated interference of

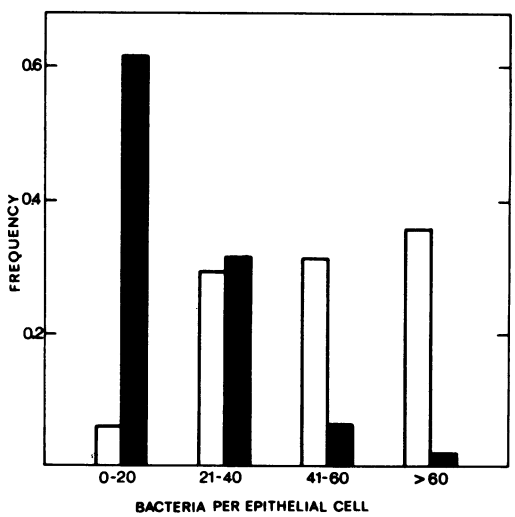

FIG. 3. Adherence of *S. pyogenes* M-5 to buccal epithelial cells. Equal volumes of washed *S. pyogenes* M-5 (1.0×10^9 cells per ml) and epithelial cells (1.1×10^6 cells per ml) were incubated together for 15 min in the presence of 200 μg of emulsan per ml (black bars) or in the absence of the biopolymer (open bars). Nonadherent bacteria were removed by centrifugation at $100 \times g$ for 5 min; epithelial cells were then suspended in phosphate-buffered saline, and adhering bacteria were measured as described in the text. Background values of adherent indigenous bacteria were subtracted.

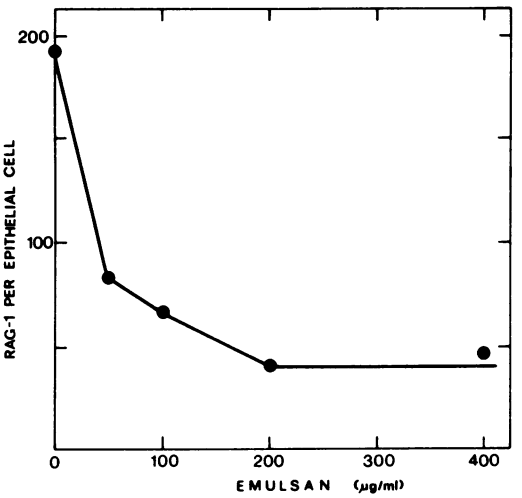

FIG. 4. Desorption of *A. calcoaceticus* RAG-1 from buccal epithelial cells by emulsan. After incubation of *A. calcoaceticus* RAG-1 and epithelial cells for 15 min as described in the legend to Fig. 2, the cell suspension was divided into five tubes, each containing different concentrations of emulsan. The mixtures were then incubated for an additional 15 min before filtration and determination of adhering bacteria. For each concentration of emulsan, the average number of bacteria per 100 epithelial cells was determined. Background values of adherent indigenous bacteria were subtracted.

adherence was neither limited to a particular bacterium nor specific for a particular hydrophobic surface. Adherence to hydrocarbons of another *A. calcoaceticus* strain, BD413, which produces a rhamnose-glucose exopolysaccharide (N. Kaplan and E. Rosenberg, submitted for publication) rather than emulsan, was also strongly inhibited by emulsan. Results similar to those obtained with *Acinetobacter* strains were also observed for *S. pyogenes* M-5; adherence of *S. pyogenes* to epithelial cells has been reported to be due to hydrophobic interactions resulting from the exposure of the lipid moiety of lipoteichoic acid (6).

In a previous study (11), correlations were found between the adherence of RAG-1 and *S. pyogenes* M-5 to hydrocarbons and adherence of these strains to buccal epithelial cells. It was thus of interest to determine whether emulsan would similarly inhibit adherence of these strains to buccal epithelial cells. Low emulsan concentrations (0.01 to 0.02%) proved highly effective in preventing adherence of both RAG-1 and *S. pyogenes* M-5 to buccal epithelial cells. In addition to its ability to prevent adherence, emulsan was able to remove 80% of RAG-1 cells which were previously bound to epithelial cells.

The amphipathic nature of the emulsan polymer (2, 20) enables it to coat hydrocarbon droplets with a thin, stable film which lowers the interfacial tension (8) and accounts for the excellent emulsifying properties of emulsan (8, 19). The data presented here demonstrate the ability of emulsan to act as a soluble antiadherence factor, inhibiting bacterial adherence to hydrocarbon and epithelial cells and removing bacteria bound to these surfaces. We propose that a common mechanism accounts for the potent emulsifying and antiadherence properties of emulsan: formation of a surface film which renders hydrophobic surfaces hydrophilic and prevents attachment of bacteria through hydrophobic interactions. It was previously suggested that hydrophobic interactions mediate adherence of RAG-1 and *S. pyogenes* M-5 to hydrocarbons and buccal epithelial cells (11). The ability of emulsan to inhibit adherence of these strains to both types of surfaces strengthens this contention. The reduced bacterial-binding ability of emulsan-pretreated epithelial cells suggests that emulsan either forms a film on epithelial cells in much the same manner as it coats hydrocarbon droplets or, alternatively, interferes with a bacterial receptor on the epithelial cell surface.

Of particular interest in this context is the effect of emulsan in removing adherent indigenous microbial flora from the buccal epithelial cell surface. Among 500 epithelial cells scored, 70% of the adherent autochthonous bacterial flora were removed by emulsan. These results suggest that hydrophobic interactions mediate not only the in vitro adherence of laboratory

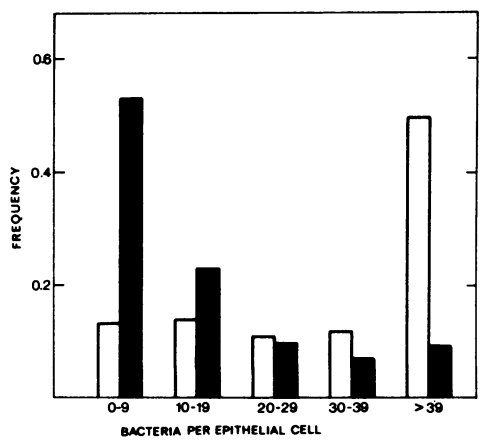

FIG. 5. Desorption of indigenous bacteria from buccal epithelial cells by emulsan. Washed human buccal epithelial cells were incubated for 15 min in the presence of 200 μg of emulsan per ml (black bars) or in the absence of the biopolymer (open bars), and the remaining adherent bacteria were determined. The data were pooled from five independent experiments in which a total of 500 emulsan-treated epithelial cells and 500 control epithelial cells were examined.

strains to buccal epithelial cells, but may actually govern the adherence of the majority of the indigenous bacteria that normally populate this surface. Our preliminary finding that over 95% of the cultivable bacteria released from buccal epithelial cells partitioned to the hydrocarbon-water interface provides additional support for this concept. Thus, whereas surface hydrophobicity of pathogenic streptococcal strains such as M-5 may promote their adherence to and subsequent colonization of the buccal mucosa (1, 5, 6, 11, 16), similar mechanisms may enable the establishment of the natural flora on the epithelial cell surface.

The use of emulsan as an antiadherence agent has several advantages: (i) emulsan has been extensively purified and characterized, both physically and chemically (2, 8, 19, 20); (ii) it is stable over a wide pH range and retains activity after autoclaving; and (iii) as opposed to low-molecular-weight molecules, the polymeric nature of emulsan reduces the possibility of its penetration into the bacterial cell or mammalian tissue. Thus, it should be possible to use emulsan as a molecular probe in desorbing, isolating, and further characterizing bacteria which adhere with high affinity to hydrophobic surfaces.

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