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Adherence of *Acinetobacter calcoaceticus* RAG-1 to Human Epithelial Cells and to Hexadecane

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The ability of *Acinetobacter calcoaceticus* RAG-1 to adhere to human epithelial cells was investigated and compared with its ability to adhere to a test hydrocarbon (hexadecane). RAG-1, a microorganism originally isolated for growth on hydrocarbon, adhered to epithelial cells when grown under conditions which promote its adherence to hexadecane; similarly, RAG-1 cells adhered poorly to epithelial cells when grown under conditions which cause the cells to possess low affinity towards hexadecane. A mutant derived from RAG-1, MR-481, deficient in its ability to adhere to hydrocarbon, was similarly unable to adhere to epithelial cells. RAG-1 adherence to epithelial cells was not blocked by a number of sugars tested. *Streptococcus pyogenes*, whose adherence to epithelial cells has been previously attributed to hydrophobic interactions, was also able to adhere to hexadecane. Results suggest that hydrophobic interactions mediate adherence of the strains studied to both epithelial cells and hydrocarbon.

There is increased interest in the investigation of adherence of various microorganisms in their natural environment and the mechanisms involved (2). In such studies. bacteria are routinely assayed under laboratory conditions for adherence to those surfaces which they normally colonize in nature. Bacteria, however, may also adhere to surfaces which they do not normally encounter. Marine bacteria, for example, can adhere to and colonize plastic surfaces (4, 6, 7). Similarly, a recently developed assay revealed that adherence to hydrocarbon droplets is not restricted to hydrocarbon-degrading microorganisms (15). Preliminary observations indicated that *Streptococcus pyogenes* M-5, a strict colonizer of epithelial cells, also adhered to hydrocarbon. It was therefore of interest to see whether a hydrocarbon-degrading *Acinetobacter* strain (14, 16), whose adherence to hydrocarbon has been studied previously (15), would adhere to epithelial cells that are normally colonized by pathogenic bacteria but rarely, if ever, inhabited by *Acinetobacter* species.

MATERIALS AND METHODS

Bacterial growth conditions. *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) was grown in either nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% NaCl or brain heart infusion broth (Difco); cells were grown with shaking at 30°C and harvested as indicated. *S. pyogenes* M-5 (H type 5) was grown at 37°C in brain heart infusion broth under static conditions and harvested at 48 h.

Epithelial cells. Epithelial cells were obtained as described previously (12) by scraping the buccal mucosa of several healthy donors with wooden sticks. The cells were washed twice and suspended in phosphate-buffered saline to a concentration of 10^5 cells per ml.

Adherence to hexadecane. A rapid and simple assay for bacterial adherence to hexadecane has recently been reported (15) and is described here in brief. Harvested bacteria were washed twice and resuspended in PUM buffer (22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, and distilled water to 1,000 ml), pH 7.1. To 1.2 ml of a turbid bacterial suspension (absorbance at 400 nm, 1.4 to 1.6) in acid-washed test tubes, 0.2 ml of hexadecane was added. After 10 min of preincubation at 30°C, the two phases were mixed under controlled conditions for 120 s. After the phases were allowed to separate, the aqueous phase was carefully removed, and its light absorbance was measured by using a Gilford model 240 spectrophotometer. The fraction of adherent cells was taken as the percent decrease in absorbance of the aqueous phase after mixing and phase separation as compared with that of the original suspension.

Bacterial adherence to epithelial cells. An epithelial cell suspension (0.5 ml) was mixed with an equal volume of bacterial suspension (2×10^{10} cells per ml), conditions which enable optimal adherence. The mixtures were rotated end over end for 30 min at

37°C (12). The epithelial cells were separated from the nonadherent bacteria by filtration through polycarbonate filters (12-μm pore size) (Nuclepore Corp., Pleasanton, Calif.), washed extensively with phosphate-buffered saline and smeared onto glass microscope slides. The preparations were stained following drying with Hemacolor rapid stain (Merck & Co., Inc., Rahway, N.J.) and examined with a bright-field microscope. Adherence was recorded as the percentage of the epithelial cells bearing 100 or more attached bacteria. Clumping of bacteria was not observed in either adherence assay.

Addition of sugars (of the highest purity obtainable) to the adherence assay was performed as described previously (12).

Isolation of a nonadhering mutant of *A. calcoaceticus* RAG-1. Strain MR-481, a mutant deficient in its ability to adhere to hydrocarbon. was isolated by repeated transfers of RAG-1 cells which remained in the aqueous phase after mixing with *n*-octane. This mutant resembled the wild type in phage sensitivity, agglutination by specific antibodies, and production of emulsifying activity during growth on ethanol as sole carbon source (16).

RESULTS

S. pyogenes M-5 is a pathogen with high affinity for epithelial cells (1, 11). In routine tests, over 30% of the epithelial cells scored were covered by 100 or more bacteria. Strain M-5 was found to adhere to three test hydrocarbons examined (hexadecane, octane, and *p*-xylene). Adherence of *S. pyogenes* M-5 to hexadecane is shown in Fig. 1.

A. calcoaceticus RAG-1, a hydrocarbon-degrading bacterium, has previously been found to adhere avidly to hydrocarbons (15) and to other hydrophobic surfaces, such as polystyrene (M. Rosenberg, unpublished data). The adherence of RAG-1 to hexadecane increased greatly with culture age (Fig. 2). Early-log-phase cells grown on nutrient broth exhibited very low affinity towards hexadecane, whereas late-log-phase cells adhered to the hydrocarbon. It was of interest to see whether a similar dependence on culture age could be observed in RAG-1 adherence to epithelial cells. Adherences of early- and late-log-phase cells of RAG-1 to buccal epithelial cells and hexadecane were compared (Fig. 3). RAG-1 cells harvested during late exponential phase adhered with high affinity to both epithelial cells and hexadecane droplets, whereas early-exponential-phase cells exhibited low affinity towards both surfaces, even at the high ratio of bacterial to epithelial cells employed.

Attempts were made to inhibit RAG-1 adherence to epithelial cells by addition of monosaccharides (12). None of the sugars tested (D-mannose, D-glucose, methyl-β-D-glucoside, D-glucosamine, *N*-acetyl-D-glucosamine, D-galactose, methyl-α-D-galactoside, maltose, and L-arabinose) blocked RAG-1 adherence to epithelial cells at concentrations as high as 25 mg/ml, suggesting that RAG-1 adherence to epithelial

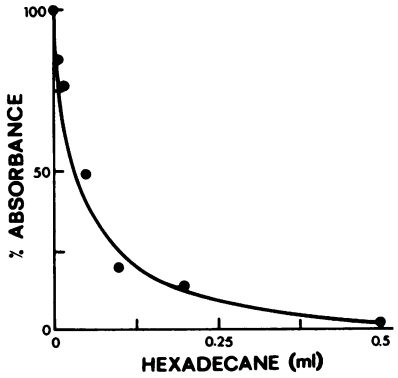

FIG. 1. *Adherence of S. pyogenes M-5 to hexadecane. Cells were grown on brain heart infusion broth to stationary phase (48 h), washed twice, and suspended in PUM buffer. Adherence to hexadecane was carried out as described in the text. Results are expressed as the percentage of the initial absorbance at 400 nm of the aqueous suspension (after mixing) as a function of hydrocarbon volume added.*

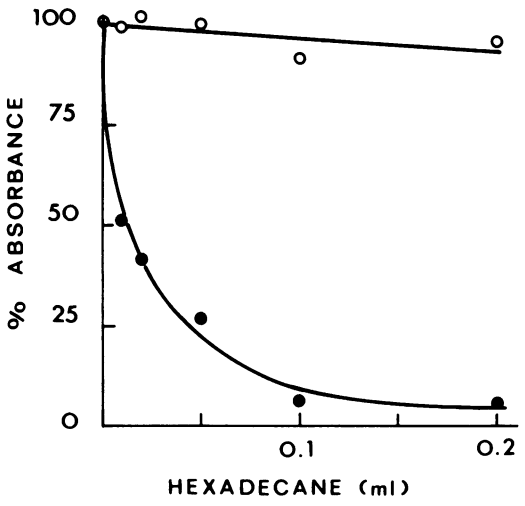

FIG. 2. *Adherence of early- (○) and late- (●) log-phase A. calcoaceticus RAG-1 cells to hexadecane. RAG-1 cells were grown in nutrient broth containing 0.5% NaCl and harvested from the same culture at 15 Klett units (early logarithmic phase) and at 65 Klett units (late logarithmic phase) as measured in a Klett-Summerson colorimeter fitted with a green filter. Cells were washed and assayed for adherence to hexadecane as described in the text. Results are expressed as the percentage of the initial absorbance at 400 nm of the aqueous suspension (after mixing) as a function of hydrocarbon volume added.*

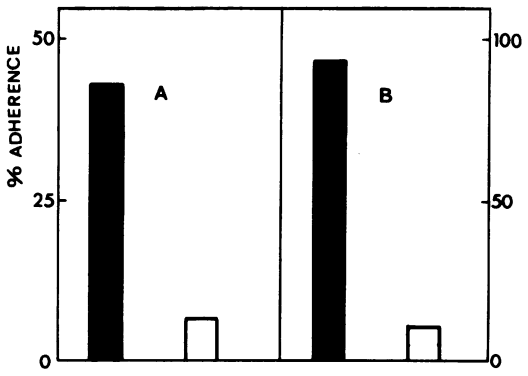

FIG. 3. *Adherence of A. calcoaceticus RAG-1 to hexadecane and epithelial cells. RAG-1 cells, grown in nutrient broth containing 0.5% NaCl, were harvested from the same culture at 20 Klett units (early logarithmic phase; open bars) and 80 Klett units (late logarithmic phase; black bars). Cells were washed and assayed for adherence to both hexadecane and epithelial cells as described in the text. Adherence to epithelial cells is shown as the percentage of epithelial cells with 100 or more attached bacteria (A). Adherence to hexadecane is shown as the percentage of bacteria removed from the aqueous phase (after mixing) in the presence of 0.2 ml of hexadecane (B).*

cells is not mediated by recognition of monosaccharide residues.

Isolation of a mutant of RAG-1 deficient in its ability to adhere to hydrocarbon enabled further studies to be carried out on the above correlation between RAG-1 adherence to epithelial cells and to hexadecane. This mutant, referred to as MR-481, adhered neither to epithelial cells nor to hexadecane, even when grown under conditions which enhanced adherence of wild-type RAG-1 cells to both surfaces. In a representative experiment, RAG-1 and mutant MR-481 cells were grown to stationary phase in brain heart infusion broth, harvested, washed in buffer, and tested for adherence (Fig. 4). Although 100% of the wild-type cells adhered to hexadecane under these conditions, only 2.6% of the mutant cells adhered to the test hydrocarbon. Similarly, RAG-1 cells adhered with an extremely high affinity to epithelial cells (66% of the epithelial cells scored were covered by 100 or more bacteria), whereas only 3% of the epithelial cells incubated with MR-481 cells were covered by 100 or more bacteria.

DISCUSSION

In a previous report (15), we described a simple, quantitative method for measuring bacterial adherence to liquid hydrocarbons. Although most strains examined showed little or no affinity for the test hydrocarbons (hexadecane, octane, and xylene), others were highly adherent and were thus increasingly removed from the lower aqueous phase upon rapid mixing with increasing volumes of hydrocarbon. Adherent cells were observed microscopically on the surface of the hydrocarbon droplets. Addition of surfactants, such as isopropanol, resulted in the release of adherent cells back into the aqueous phase and recovery of most of the initial turbidity, demonstrating that lysis did not occur in the assay procedure. The adherence to hydrocarbon found for various strains (*Serratia marcescens*, *Staphylococcus aureus*, and *Escherichia coli* J-5) correlated with previous reports of hydrophobic interactions involving these and similar bacterial strains (5, 9, 10) and suggested that adherence of bacteria to hydrocarbon provides a simple method for measuring cell surface hydrophobicity.

The present study relates to the ability of bacteria to adhere to surfaces which they do not colonize under natural conditions. Certain pathogenic microorganisms, such as streptococci and staphylococci (15), can adhere to hydrocarbons, although they almost exclusively colonize epithelial cells. Conversely, an oil-degrading *Acinetobacter* strain, which was originally isolated for ability to degrade petroleum (14), was shown in the present report to adhere avidly to epithelial cells. *Acinetobacter* species, although sometimes isolated in clinical studies, are probably not pathogenic, and rarely, if ever, colonize buccal epithelial cells (8).

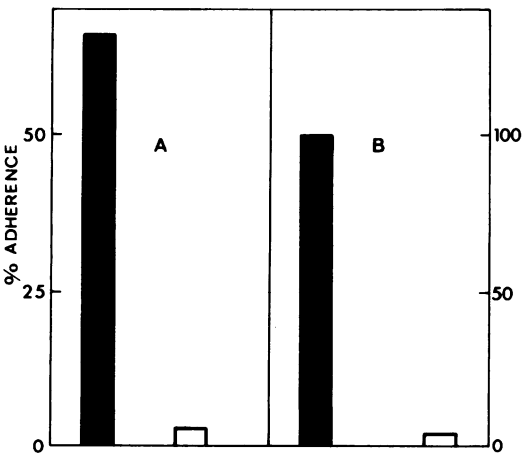

FIG. 4. *Adherence of A. calcoaceticus RAG-1 and mutant MR-481 to hexadecane and epithelial cells. Cells of RAG-1 and mutant MR-481 were inoculated 1:1,000 and harvested at stationary phase after 18 h. Determination of adherence of RAG-1 (black bars) and MR-481 (open bars) to epithelial cells (A) and hexadecane (B) was as described in the legend to Fig. 3.*

Why then, should a given species of bacteria develop characteristics which enable it to adhere to surfaces with which it rarely comes into contact? The simplest explanation is that the target surfaces studied here share common characteristics, thus enabling adherence of bacteria by a common mechanism. Results presented here show that a correlation exists between *A. calcoaceticus* RAG-1 adherence to epithelial cells and adherence to hexadecane. This correlation was strengthened by the inability of a mutant deficient in adherence to hexadecane to bind to epithelial cells.

If a common mechanism is indeed involved, then hydrophobic interactions between the bacteria and the surfaces studied here may play an important role. Hydrophobic interactions contribute to the adherence of pathogenic bacteria to various host cells (9, 13, 17) and the adherence of bacteria to hydrophobic plastic surfaces (4, 6, 7). Bacterial hydrophobicity has also been shown to be an important factor in phagocytic attachment (19). In a previous report (15), it was suggested that adherence of RAG-1 to hydrocarbon is mediated by a general hydrophobic interaction, rather than specific recognition of the hydrocarbon substrate; RAG-1 adherence to epithelial cells may involve similar interactions between hydrophobic sites on the bacterial and epithelial cell surfaces.

A similar case may be made for streptococcal adherence. The lipid moiety of lipoteichoic acid on the streptococcal surface was found to mediate adherence of these organisms to epithelial cells by a hydrophobic interaction mechanism (3, 11). Tylewska et al. reached similar conclusions, using phenyl-Sepharose columns (18). Adherence of streptococci to hexadecane, as reported here, may involve these same covalently bound lipids.

In the present report, we have shown correlations between the ability of bacterial cells to adhere to epithelial cells and to hydrocarbon. Similar correlations between the ability of bacteria to bind to animal cells and surface hydrophobicity have been reported, using other techniques (9, 13, 17-19). Nevertheless, further studies with other bacterial strains are required to determine whether the participation of hydrophobic interactions in bacterial adherence to epithelial cells is a general phenomenon. The use of bacterial adherence to hydrocarbon as an indication of bacterial hydrophobicity has certain advantages over other methods: (i) the surface to which the bacteria adhere (i.e., liquid hydrocarbons) is well defined, (ii) intricate equipment is not required, and (iii) quantitative results are rapidly and easily obtained.

Results presented here suggest that the mere potential of bacteria to adhere to epithelial cells may not be sufficient to initiate colonization and subsequent infection of mucosal surfaces. Conversely, the ability of a strict pathogen (i.e., *S. pyogenes*) to adhere to hydrocarbon is not sufficient to enable it to colonize such surfaces in nature. Factors such as nutritional requirements and resistance to deleterious physical and chemical pressures probably play a crucial role in bacterial colonization. Adherence to a given surface, although insufficient, is certainly a prerequisite to colonization. Thus, the characterization of the bacterial surface components responsible for bacterial adherence to hydrocarbon may help clarify some of the mechanisms through which microorganisms recognize and are recognized by the surfaces with which they interact.

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