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Role of Adherence in Growth of *Acinetobacter calcoaceticus* RAG-1 on Hexadecane

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The high affinity of *Acinetobacter calcoaceticus* RAG-1 for liquid hydrocarbons permitted the isolation of a spontaneous nonadherent mutant, MR-481. Strain MR-481 exhibited no significant affinity for three test hydrocarbons, yet resembled the wild type in many properties, including production of the extracellular emulsifying agent emulsan. To study the role of adherence in growth on hydrocarbons, RAG-1 and MR-481 were compared for growth on hexadecane under conditions of limited agitation and at low initial cell densities. Adherent RAG-1 cells were able to grow rapidly under these conditions, whereas nonadherent MR-481 cells failed to grow for at least 54 h. However, the addition of emulsan either initially or at various times after inoculation enabled the nonadherent MR-481 cells to grow on hexadecane. Growth was not the result of reversion of MR-481 from nonadherent to adherent cells. The data demonstrate that adherence is a crucial factor in the growth of *A. calcoaceticus* RAG-1 on hexadecane in the absence of extracellular emulsification of the substrate.

The importance of cell adherence in the growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces (28). The initial step in the life cycle of *Bdellovibrio* cells is attachment to gram-negative bacteria (30, 33). Specific cell-to-cell interactions pervade the entire life cycle of myxobacteria (4, 29). Species-specific adhesion and tissue-specific adhesion are prerequisites for the successful colonization by microorganisms of animals (18) and plants (12). Microbial adhesion to surfaces has been the subject of several recent reviews (3, 11-13, 18).

In cases in which the carbon or energy source is a water-insoluble material such as cellulose or chitin, cell adhesion can facilitate growth (11, 29, 31), but cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, the growth of microorganisms on hydrocarbons presents a special problem, since not only are hydrocarbons immiscible in water, but also their breakdown cannot occur extracellularly. The first step in aromatic (6) or aliphatic (19) hydrocarbon degradation is the introduction of molecular oxygen into the molecules by cell-associated enzymes (5).

Two general types of hydrocarbon-cell interactions, depending upon the state and size of the oil droplets relative to the size of the microbial cells, have been postulated (9): the adherence of cells to large oil droplets and pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets. The relative contribution of each of these two types of interactions to the growth of bacteria on hydrocarbons has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon-aqueous interfacial area and growth rate can be used to support either hypothesis (16).

Hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1 has been shown both to adhere avidly to hydrocarbons and other surfaces possessing hydrophobic properties (23-26), and to produce a potent extracellular emulsifying agent (20). The purified emulsifying agent, referred to as emulsan, is an anionic heteropolysaccharide (37) containing fatty acid side chains (1), with an average molecular weight of 9.9×10^5 and an axial ratio of about 60. In this paper, we describe the isolation and characterization of a mutant of RAG-1 that is deficient in its ability to adhere to hydrocarbons. By using this mutant we have been able to demonstrate the importance of adherence in the growth of RAG-1 on hexadecane.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. calcoaceticus* RAG-1 (ATCC 31012) and a nonadhering derivative, MR-481, were used throughout this study. Cells were maintained on nutrient agar at 4°C and were subcultured every 2 months.

For measuring adherence to test hydrocarbons or for preparing inocula for growth experiments, 0.1 ml of bacteria from an overnight culture was inoculated into 50 ml of brain heart infusion broth (Difco Laboratories) and incubated in a New Brunswick model G-53 Gyrotory shaker at 150 rpm at 30°C. Cells were harvested in the stationary phase after 18 h of growth, washed twice, and suspended 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 $MgSO_4 \cdot 7H_2O$, distilled water to 1,000 ml [pH 7.1]).

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14-mm diameter) containing 4.2 ml of aqueous medium to give an initial turbidity of 5 Klett units. Acetate growth medium consisted of PUM buffer supplemented with 0.2% sodium acetate. Hexadecane growth medium consisted of 0.5 ml of hexadecane (olefin-free, 99% purity; Fluka AG, Buchs SG, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick Gyrotory water bath shaker (model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Isolation of nonadhering mutant MR-481. A spontaneous mutant strain of RAG-1 which is deficient in its ability to adhere to hydrocarbons was isolated by the following technique. RAG-1 cells were inoculated into 50 ml of nutrient broth (Difco) supplemented with 0.5% NaCl and incubated in a New Brunswick model G-53 Gyrotory shaker at 150 rpm at 30°C. Cells were harvested in the stationary phase after 18 h of growth, washed twice, and suspended in PUM buffer to an absorbance at 400 nm of ca. 1.5 (Gilford model 240 spectrophotometer; 1-cm light path). To 5 ml of the cell suspension was added 2 ml of *n*-octane, and the phases were mixed in a Vortex mixer for 5 min. After phase separation, the lower aqueous phase was transferred to a second test tube. Octane was again added and the phases were mixed as described above. This extraction process was continued until the cells in the lower aqueous phase were too few to be visible by microscopic examination. This final aqueous phase served as the inoculum for a second nutrient broth culture which was grown to the stationary phase as described above. Cells were again harvested, washed, and extracted with octane; the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even after several extraction attempts with octane. The final aqueous phase was plated on nutrient agar, and three individual colony types were isolated and tested for adherence to hydrocarbons. One strain, termed MR-481, exhibited no significant affinity towards the test hydrocarbons and was chosen for further study.

Adherence to hydrocarbons. The technique for measuring adherence of bacteria to hydrocarbons has been described previously (24). Cells were washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of ca. 1.5. Various volumes of the test hydrocarbons (*n*-hexadecane, *n*-octane, and *p*-xylene) were added to round-bottom acid-washed test tubes (10-mm diameter) containing 1.2 ml of washed-cell suspension, and the phases were mixed in a Vortex mixer uniformly for 120 s. After 15 min was allowed for the phases to separate, the absorbance of the lower aqueous phase of each tube was measured at 400 nm. Adherence was calculated as the percent loss in absorbance relative to that of the initial cell suspension.

Emulsan preparation. The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously (22). The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH), redissolving the precipitate in 0.5 M Na_2SO_4, and dialyzing it extensively against distilled water. The material was then deproteinized by the phenol method (37) and dialyzed. The final product had a reduced viscosity of 490 cm^3/g, an emulsifying activity of 150 U/mg, and an ester content of 0.5 μmol/mg and contained less than 0.5% protein. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and sterilized by autoclaving before it was added to RAG-1 and MR-481 in the growth experiments.

RESULTS

Isolation and characteristics of *A. calcoaceticus* MR-481. *A. calcoaceticus* RAG-1 adheres avidly to hydrocarbons (24–26). Adherent cells were observed exclusively on the surface, rather than in the interior, of the hydrocarbon droplets. A spontaneous mutant strain of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells which did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when it was grown under conditions for which RAG-1 adherence was optimal (Fig. 1). Upon being mixed, RAG-1 broke the hydrocarbon phase into small droplets that were covered by adherent cells. These droplets rose rapidly after the mixing procedure of the assay; a dramatic decrease in the absorbance of the lower aqueous phase, corresponding to removal of adhering cells, was observed. Mutant MR-481, however, was nonadherent, and the hydrocarbon phase rapidly coalesced after being mixed, leaving the cells in the aqueous phase.

Mutant MR-481 resembled the wild type in the following characteristics: colonial and cell morphology; sensitivity to two bacteriophages, AP-2 and AP-3 (O. Pines and D. L. Gutnick, Arch. Microbiol., in press), which are specific for *A. calcoaceticus* RAG-1 strains; production of extracellular emulsifying activity during growth on ethanol as the sole carbon and energy source (22); and growth rate on brain heart infusion broth (38 min at 30°C with aeration).

Growth of RAG-1 and MR-481 on hexa-

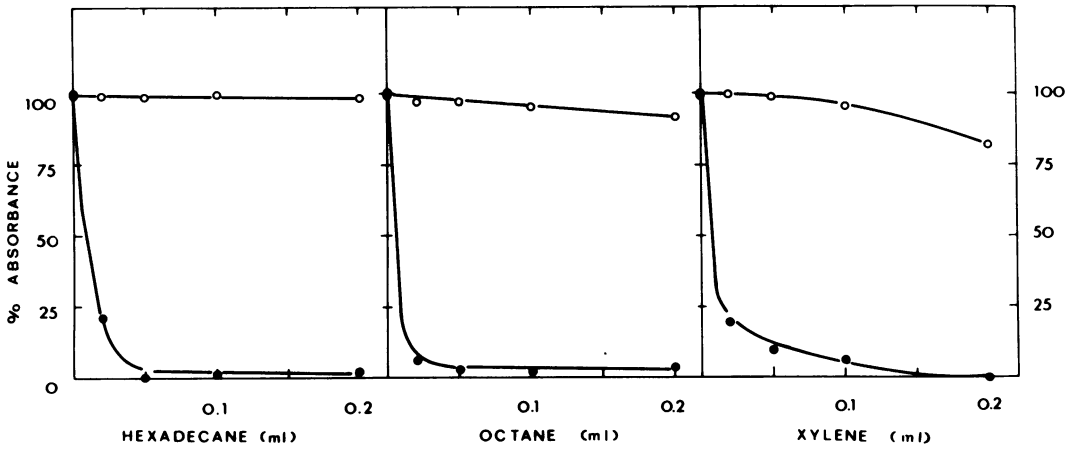

FIG. 1. *Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary-phase cells were harvested from brain heart infusion broth, washed twice, and suspended in PUM buffer to an absorbance at 400 nm of 1.45 to 1.50. To 1.2 ml of suspended RAG-1 (●) or MR-481 (○) cells were added various volumes of test hydrocarbon, and the mixtures were mixed in a Vortex mixer for 120 s. After phase separation, the turbidity of the lower aqueous phase was measured. The ordinate is the percentage of initial absorbance of the cell suspension.*

decane. It should be emphasized that in all growth experiments inocula were prepared in the same manner for measuring adherence as was described in the legend to Fig. 1. In defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Fig. 2). After a 2-h lag, both strains grew with a doubling time of 48 min.

With hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew with no appreciable lag, whereas the nonadherent mutant MR-481 failed to grow for at least 54 h (Fig. 3). The growth of RAG-1 was accompanied by breakage of the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered with patches of adhering cells. Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus, the observed turbidity of the lower aqueous phase was due to unbound cells. The values presented for the growth of RAG-1 on hexadecane should be considered minimal, since attached bacteria were not counted. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Fig. 4).

Effect of emulsan on growth of MR-481 on hexadecane. *A. calcoaceticus* RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan. It was of interest to examine the effect of a highly purified preparation of emulsan (see above) on the growth of

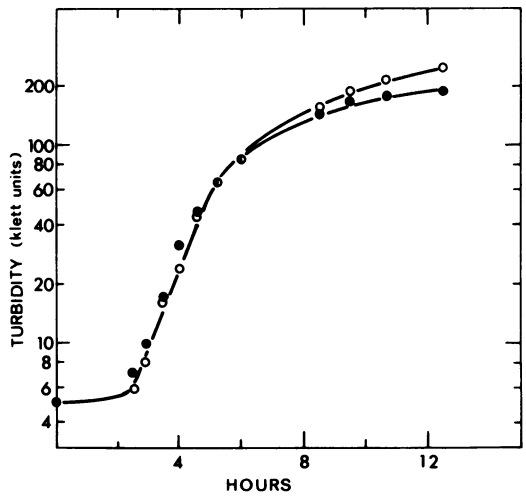

FIG. 2. *Growth of RAG-1 and MR-481 in acetate medium. Stationary-phase cells were harvested from brain heart infusion broth, washed twice, and suspended in PUM buffer supplemented with 0.2% sodium acetate. RAG-1 (●) and MR-481 (○) cells were inoculated into 4.2 ml of acetate medium in Klett tubes to an initial turbidity of 5 Klett units.*

RAG-1 and of the nonadherent mutant MR-481 on hexadecane (Fig. 5). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481. However, emulsan did permit the growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both emulsan and hexadecane began after about 6 h, whereas neither component alone supported growth for 54 h. At the weight

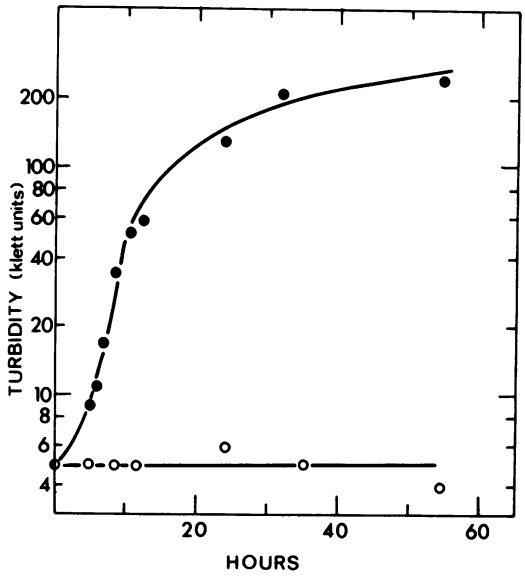

FIG. 3. *Growth of RAG-1 and MR-481 in hexadecane medium. Klett tubes containing 4.2 ml of PUM buffer were inoculated with either RAG-1 (●) or MR-481 (○) to an initial turbidity of 5 Klett units. The tubes were overlaid with 0.5 ml of hexadecane and incubated as described in the text.*

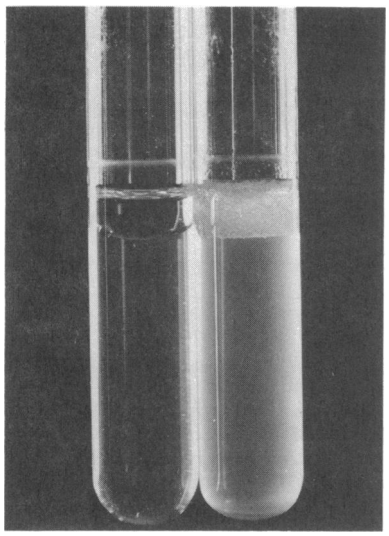

FIG. 4. *Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. The tubes were photographed after 48 h of incubation under the conditions described in the legend to Fig. 3.*

ratio of hexadecane to emulsan used, 1,925:1, the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements (Z. Zosim, unpublished data). Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several h of incubation (Fig. 6). In each case of emulsan addition, the resultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth after emulsan addition appeared to increase with the length of incubation before the addition of the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of a genotypic or phenotypic reversion of MR-481 from nonadherent to adherent cells. As shown in Table 1, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan as compared with that of the initial inoculum grown in rich medium (Fig. 1).

RAG-1 cells which were harvested and washed after growth on hexadecane both in the presence and in the absence of emulsan exhibited a high affinity for hexadecane (Table 1). Thus, the appearance of free RAG-1 cells in the

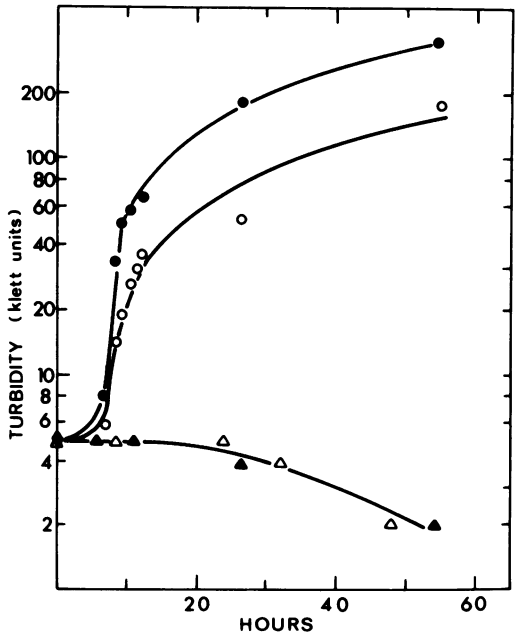

FIG. 5. *Effect of emulsan on the growth of MR-481 on hexadecane. The preparation of inocula and incubation conditions were as described in the legend to Fig. 3. RAG-1 (●) and MR-481 (○) were grown on hexadecane medium in the presence of 50 μg of emulsan per ml. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 μg of emulsan per ml in the absence of hexadecane.*

lower phase during growth on hexadecane did not result from a loss of their adherent properties.

DISCUSSION

Mudd and Mudd (15) reported over 50 years ago that certain microorganisms tend to concentrate at oil-water interfaces. Although this observation has been frequently corroborated during studies on the growth of microorganisms on liquid hydrocarbons (7, 9, 13, 14, 16, 17), there is no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons. Recent studies have shown that certain bacterial species can adhere to liquid hydrocarbons despite their inability to degrade paraffins (24) and that *Pseudomonas aeruginosa* cells exhibit a low affinity for hydrocarbons, even when they are grown on hexadecane (26). Certain investigators have argued that microbial growth on hydrocarbons is caused by emulsification of the water-insoluble substrate rather than by direct contact (2, 34, 35). *A. calcoaceticus* RAG-1 is an interesting microorganism in this regard because it both adheres avidly to hydrocarbons (24–26) and produces an extracellular emulsifying agent, emulsan, which has been extensively characterized (1, 20, 22, 37).

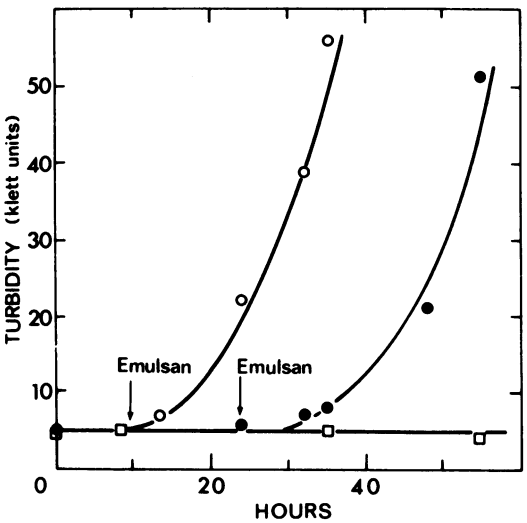

FIG. 6. *Induction of growth of MR-481 on hexadecane by emulsan addition. The preparation of inocula and incubation conditions were as described in the legend to Fig. 3. Emulsan was added to a final concentration of 50 μg/ml at 10* (○) *or 24 h* (●). *Tubes containing no emulsan served as controls* (□).

TABLE 1. *Adherence of RAG-1 and MR-481 after growth on hexadecane*

Strain	Turbidity[a]		Adherence (%)
	Initial	Final	
RAG-1[b]	1.42	0.09	94
RAG-1[c]	1.40	0.05	96
MR 481[c]	1.43	1.36	4.9

[a] Cells were washed twice and suspended in PUM buffer to initial turbidities (absorbance at 400 nm) as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension, and the mixture was mixed in a Vortex mixer for 120 s. After phase separation, the turbidity of the aqueous phase was measured.

[b] RAG-1 cells were harvested after 54 h of growth on hexadecane medium, washed twice, and suspended in PUM buffer.

[c] RAG-1 and MR-481 were harvested after 54 h of growth on hexadecane medium in the presence of 50 μg of emulsan per ml, washed twice, and suspended in PUM buffer.

The isolation of a mutant strain of *A. calcoaceticus* RAG-1 that is deficient in its ability to adhere to hydrocarbon permitted an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The nonadherent mutant strain MR-481 failed to grow on hexadecane under the moderate agitation conditions which supported growth of wild-type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane, insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system, were ruled out by the following data: (i) MR-481 and RAG-1 cells grew with similar kinetics on acetate medium under conditions identical to those employed for growth on hexadecane; (ii) MR-481 was able to grow on hexadecane medium after the addition of an emulsifier; and (iii) MR-481 cells were able to grow on solid medium supplemented with hexadecane vapors as the sole carbon and energy source. We conclude from these data that adherence is a prerequisite for the growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of the substrate.

Both RAG-1 and MR-481 produced a potent extracellular emulsifying agent which permitted growth of nonadhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell density-dependent phenomenon (10, 21). With the low cell density of the inoculum employed (5×10^7 cells per ml), the concentration of emulsan which the cells produced was too low to emulsify the hydrocarbon and permit growth.

These experiments suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and lim-

ited emulsification. Such conditions prevail in most natural environments. On the other hand, the presence of extracellular emulsifying agents and the provision of vigorous agitation permit the growth of nonadherent bacteria on hydrocarbons. However, such conditions, which are dependent on a diffusible substance, may be restricted to growth on hydrocarbon in closed systems such as laboratory flasks and fermentors.

The results presented here suggest that the choice of microbial strains and growth conditions largely dictates which of the two mechanisms (adherence or emulsification) will predominate in any given investigation. Similar conclusions were drawn by Nakahara et al. (16) based on the kinetics of growth of cells in fermentors. For example, models based on investigations which employed poorly adhering *Pseudomonas* strains and which were carried out in the presence of extraneous emulsifiers or with vigorous agitation (2, 8, 34, 35) tend to minimize the role of adherence in hydrocarbon degradation. In an investigation which employed conditions which prevented the emulsification of the substrate (14), direct contact was reported to be the more important factor.

An interesting phenomenon which has also been observed by other investigators during growth of *A. calcoaceticus* on hexadecane is the appearance of free cells in the aqueous phase. Neufeld et al. (17) reported that the appearance of unbound bacteria coincides with a decrease in the surface hydrophobicity of the cells. In the present study, no evidence was found that the cells which appeared in the aqueous phase during growth had lost their ability to adhere to hydrocarbon. RAG-1 cells which were harvested from the aqueous phase and washed after 54 h of growth still adhered avidly to hexadecane. The appearance of unbound RAG-1 cells in this study can best be explained by emulsifier production during growth on hexadecane (22). The presence of emulsifier in hydrocarbon fermentation is accompanied by a decrease in interfacial tension between the hydrocarbon and aqueous phases (14, 17) which may, in turn, affect the thermodynamic favorability of cell partitioning at the interface.

The importance of bacterial adherence in the successful colonization of a wide variety of surfaces has gained increasing recognition in recent years (11–13, 18, 27, 32, 36). In the present report, the role of adherence in the growth of RAG-1 on hexadecane has been demonstrated. Experiments are in progress to characterize the cell surface components responsible for the adherent properties exhibited by *A. calcoaceticus* RAG-1.

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