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Role for Emulsan in Growth of *Acinetobacter calcoaceticus* RAG-1 on Crude Oil

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When *Acinetobacter calcoaceticus* RAG-1 was grown together with an emulsan-deficient mutant on crude oil, only the emulsan-producing RAG-1 was found to grow, regardless of whether the medium was supplemented with emulsan. The results suggested that the cell-associated form of the bioemulsifier is the biologically active species required for growth on crude oil. A revertant of an emulsan-deficient strain was isolated which simultaneously regained the ability to produce both cell-associated and cell-free emulsan as well as the ability to grow on crude oil.

The petroleum-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012) has been shown to produce an extracellular emulsifying agent termed emulsan (7, 11). Purified emulsan is an anionic heteropolysaccharide with an average molecular weight of 9.9×10^5 and an axial ratio of about 60:1 (14). The polymer consists of D-galactosamine, D-galactosamine uronic acid (pK_a, 3.01), and an unidentified hexosamine. The amphipathic properties of emulsan are due in part to the presence of fatty acids linked to the polysaccharide backbone in both ester and amide linkages (1, 14).

A cell-associated form of emulsan constitutes a minicapsular layer on the surface of exponential-phase RAG-1 cells which is released into the medium as the cells approach stationary phase (3, 4). Upon its release from the cell surface, the polymer acquires emulsifying activity. In addition, the released cell-free emulsan loses its ability to function as a receptor for the specific RAG-1 bacteriophage ap3 (5, 6). Mutants resistant to phage ap3 are deficient in extracellular emulsan activity and have been shown to lack both the emulsan minicapsular layer and the extracellular emulsan antigenic cross-reacting material (4, 6). Results from this laboratory have recently demonstrated a role for an exocellular cell surface esterase in emulsan release (13).

The ready availability of emulsan-deficient mutants provides a useful tool for studying the possible biological advantages of an emulsan-producing strain.

A series of emulsan-deficient mutants derived from either RAG-1 or a lysine auxotrophic derivative of RAG-1, RAG-92, were selected on the basis of resistance to the emulsan-specific phage ap3 (4, 5). Cells were grown on filtered Mediterranean seawater supplemented with ammonium sulfate (final concentration, 0.2%) and one of the following carbon sources: ethanol (0.5%), sodium acetate (0.2%), hexadecane (2% [vol/vol]), or Agha Jari Iranian crude oil (2% [vol/vol]; obtained from the Haifa Refineries, Haifa, Israel). The crude oil was allowed to stand in an open bottle at 50°C for 72 h and then was autoclaved before use. Cells were grown in 4.5 ml of medium in standard Klett tubes (inner diameter, 14 mm) starting with an initial titer of 3×10^7 to 8×10^7 CFU /ml. When cultures were grown on water-insoluble substrates, samples of the aqueous phase for viable counts were removed, after they had settled with a syringe fitted with a long needle which was passed through the oil layer. Total CFU were determined by adding emulsan to a final concentration of 500 μg/ml and vigorously mixing with a vortex mixer (Tuttenauer Inc., Jerusalem) for 2 min (since emulsan has been shown to remove adherent cells from oil droplets) (8–10). After a settling period of 20 min, cells were removed from the lower aqueous phase. The recovery of input-adhering CFU by this method was between 65 and 80%. Emulsan production (3), adherence to hexadecane (9), and cell-bound hexosamine (2, 12) were determined as previously described. Parental cells and emulsan-deficient mutants were distinguished from one another on the basis of phage resistance (6) and colony morphology (emulsan-deficient mutants form translucent rather than white mucoid colonies).

When crude oil was added to seawater medium under conditions of limited mixing, emulsan-producing strains RAG-1 and RAG-215 achieved turbidities of 55 and 75 Klett units (KU), respectively, after 70 h of growth. In contrast, emulsan-deficient mutants barely grew within 70 h on crude oil (between 3 and 26 KU) under the same conditions. It should be noted that the starter cultures of all strains from broth medium adhered avidly to hexadecane (93 to 98% adherence in the standard adherence assay). In addition, both parents and mutant adhered equally well to crude oil (92 and 96%, respectively).

When grown on crude oil, RAG-1 exhibited a 20-h lag and grew to about 4×10^8 CFU/ml within 50 h. At 20 h about 80% of the RAG-1 cells were associated with the oil phase. By 50 h, however, only 30% of the population had adhered to the oil. Late in stationary phase, nearly all of the cells were recovered from the aqueous phase. In contrast to RAG-1, an emulsan-deficient mutant strain, TR3, exhibited a lag period of 48 h and achieved the same growth yield only after 80 to 100 h. When RAG-1 and TR3 were grown with ethanol as the sole source of carbon and energy, both strains exhibited similar growth characteristics, suggesting that emulsan deficiency affects growth on crude oil specifically.

Since emulsan is both a cell-associated capsule and a cell-free product, it was of interest to determine whether an effect of the mutation to emulsan deficiency could be observed during growth of mixed cultures of the parent (RAG-1) and a mutant (TR3) on crude oil. A mixed culture of RAG-1 and TR3 inoculated with an equal number of each of the two strains was grown for 120 h (Fig. 1). Although the

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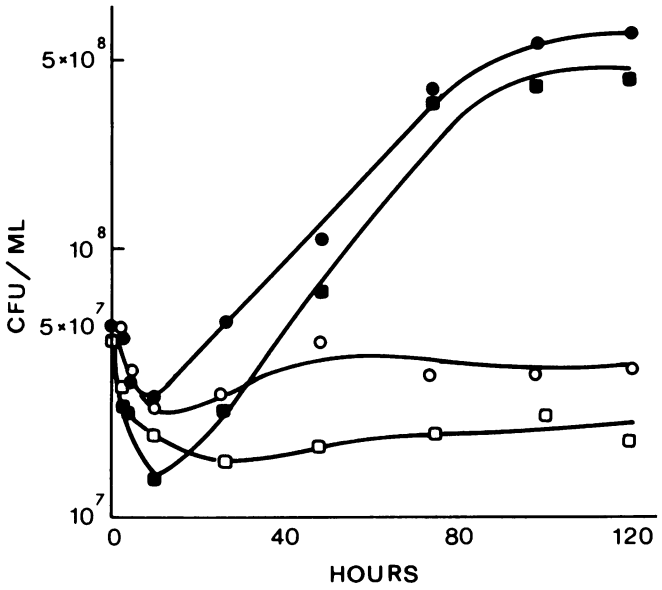

FIG. 1. Growth of a mixed culture of *A. calcoaceticus* RAG-1 and TR3 in seawater medium supplemented with 2% (vol/vol) crude oil. Unbound RAG-1 CFU/ml (■), total RAG-1 CFU/ml (●), unbound TR3 CFU/ml (□), and total TR3 CFU/ml (○).

medium was inoculated with an equal number of parent and mutant cells, the TR3 CFU did not increase above the initial titer during the entire course of the experiment. In sharp contrast, the parent RAG-1 grew to a final cell concentration of 6×10^8 CFU/ml, which represents a 25-fold enrichment over the mutant. Moreover, the addition of emulsan to the growth medium at a concentration sufficient to bring about emulsification of the crude oil did not enhance the growth of the mutant.

To test the hypothesis that cell-associated emulsan is involved in the growth of RAG-1 on crude oil, the growth pattern of emulsan-producing revertants of TR3 was examined. It was expected that growth on crude oil under the specific conditions described above might provide sufficient selective pressure to stimulate the growth of emulsan-producing revertants within an emulsan-negative population. To rule out the possibility of contamination, the wild type was replaced with a double auxotroph, RA15, which requires both tryptophan and lysine for growth. The emulsan-negative derivative of strain RA15, TRA15, was then selected by its resistance to phage ap3 (6).

A culture of TRA15 was grown on crude oil, transferred to fresh crude oil-containing medium, and grown as described above. After 48 h of growth, wild-type colonies appeared in the transferred culture at a frequency of 8×10^{-5} times that of the total bacterial population in the aqueous phase. Table 1 summarizes the relevant characteristics of strain RA15, its emulsan-deficient derivative TRA15, and REV15 (a revertant of TRA15). Although stationary-phase cultures of mutant TRA15 contained only about 25% of the emulsan activity and 17% of the cell-free hexosamines of the parental strain, the revertant strain, REV15, produced 68% of the emulsan activity and 81% of the cell-free hexosamines of the original parent strain RA15. In addition, washed exponential-phase cells of the original strain, RA15, and of the revertant, REV15, contained about 40 μg of hexosamine per mg (dry weight) of cells. In contrast, the emulsan-deficient mutant TRA15 had only about 16 μg of hexosamines per mg (dry weight). Moreover, the change in cell-bound emulsan associated with the reversion of the emulsan deficiency was correlated with an acquired sensitivity to phage ap3. Finally, the revertant strain REV15 regained the capacity to grow on crude oil (64 KU for the revertant and 10 KU for the mutant).

TABLE 1. Reversion of the emulsan-deficient phenotype

Strain	Stationary-phase supernatants			Exponential-phase cells	
	Growth (KU) at 72 h[a]	Emulsan activity (U/ml)	Hexosamine (mg/ml)	Dry wt (mg/ml)	Hexosamine (mg/ml)
RA15	1,300	203	0.153	3.0	0.110
TRA15	1,210	51	0.026	3.2	0.051
REV15	1,255	139	0.124	3.2	0.136

[a] Cultures were grown for 72 h in minimal medium supplemented with 2% (vol/vol) ethanol.

In this report we show that the emulsan minicapsule provides a distinct advantage to the wild-type cell growing on crude oil under conditions of poor mixing. This conclusion is based on the following facts: (i) emulsan-deficient mutants of RAG-1 grew very poorly on crude oil when compared with the parent strain, regardless of whether emulsan was present in the medium; (ii) growth of the two strains on water-soluble substrates was similar; and (iii) both emulsan deficiency and inability to grow on crude oil appeared to be simultaneously eliminated in a single revertant.

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