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Emulsan Production by *Acinetobacter calcoaceticus* in the Presence of Chloramphenicol

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When exponentially growing cultures of *Acinetobacter calcoaceticus* RAG-1 or RAG-92 were either treated with inhibitors of protein synthesis or starved for a required amino acid, there was a stimulation in the production of emulsan, an extracellular polyanionic emulsifier. Emulsan synthesis in the presence of chloramphenicol was dependent on utilizable sources of carbon and nitrogen and was inhibited by cyanide or azide or anaerobic conditions. Radioactive tracer experiments indicated that the enhanced production of emulsan after the addition of chloramphenicol was due to both the release of material synthesized before the addition of the antibiotic (40%) and de novo synthesis of the polymer (60%). Chemical analysis of RAG-1 cells demonstrated large amounts of polymeric amino sugars; it was estimated that cell-associated emulsan comprised about 15% of the dry weight of growing cells. The data are consistent with the hypothesis that a polymeric precursor of emulsan accumulates on the cell surface during the exponential growth phase; in the stationary phase or during inhibition of protein synthesis, the polymer is released as a potent emulsifier.

Bioemulsifiers have received increasing attention in recent years from the point of view of the growth physiology of the microorganisms which produce them (4, 6–8, 21, 22) and because of their potential commercial exploitation (2, 5, 23). We have shown previously that hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1 produces a potent extracellular emulsifier (14, 18), referred to as emulsan, which is a highly asymmetric anionic polymer with a molecular weight average of 9.9×10^5 (24). Although the chemical structure of emulsan has not yet been elucidated, it appears to consist of a D-galactosamine-containing polysaccharide backbone with fatty acid side chains (1, 24). Protein (approximately 15%) associated with the polysaccharide can be removed by hot phenol treatment without destruction of activity. Emulsan stabilizes a wide variety of hydrocarbon-in-water emulsions by forming a strong film on the interface (23a). Although the physiological function(s) of emulsan is unknown, evidence has been presented for the role of emulsan as a bacteriophage receptor (13) and a stimulator of the growth of a nonadherent mutant of *A. calcoaceticus* RAG-1 on hydrocarbons (16).

As is the case with many other extracellular polysaccharides (20), emulsan is produced largely after the cells reach the stationary phase (18). In this paper, we report studies on factors which affect the synthesis and release of emulsan. Evidence is presented for a cell-associated form of the bioemulsifier.

MATERIALS AND METHODS

Organisms and culture conditions. The organisms used in these studies were *A. calcoaceticus* RAG-1, RAG-92, and AG-1. Strain RAG-92 is a lysine auxotroph of *A. calcoaceticus* RAG-1 (ATCC 31012). The growth characteristics and emulsan production of strain RAG-92 are similar to those of strain RAG-1 (13). Strain AG-1 is an emulsan-defective mutant of strain RAG-1 which was isolated by selecting for fast growth on minimal medium containing a low (0.25% [vol/vol]) concentration of ethanol. Strain RAG-92 was cultivated routinely in EL medium containing, per liter, 14.8 g of $K_2HPO_4 \cdot 3H_2O$, 4.84 g of KH_2PO_4, 1.8 g of urea, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of L-lysine, and 25 ml of absolute ethanol. The same medium without lysine was used for strains RAG-1 and AG-1. Growth experiments were performed in Erlenmeyer flasks filled to 10% of their capacity; incubation was at 30°C with rotary shaking at 400 rpm (New Brunswick model G-25). Growth was determined by measuring the turbidity of the cultures in a Klett-Summerson photoelectric colorimeter (model 800-3) with a green filter. Appropriate dilutions were prepared to perform measurements in the linear range of the colorimeter (30 to 150 Klett units). An exponentially growing culture of strain RAG-92 on EL medium with a turbidity of 100 Klett units corresponded to a cell dry weight of 0.43 g/liter.

Emulsan production in the presence of CM. Exponential-phase RAG-92 cells (200 to 400 Klett units)

were harvested at 10,000 × *g* at 4°C for 15 min, washed twice with cold sterile EL medium (without ethanol and lysine), and then suspended in EL medium containing 50 μg of chloramphenicol (CM) per ml to a turbidity of about 300 Klett units. Incubation conditions were identical to the growth conditions described above. At timed intervals, samples were removed and centrifuged at 10,000 × *g* for 15 min, and the supernatant fluids were assayed for emulsifying activity as previously described (18) with hexadecane:2-methylnaphthalene as the hydrocarbon mixture. The values reported for emulsan are the averages of at least three determinations. One unit of emulsan per ml yields 100 Klett units in the standard assay. Purified emulsan preparations have specific activities of 250 to 350 U/mg (dry weight).

Radioactive experiments. L-[*U*-^{14}C]lysine (specific activity, 342 mCi/mol) and [1-^{14}C]ethanol (specific activity, 56 mCi/mol) were products of the Radiochemical Centre, Amersham, England. The radioactive ethanol was mixed with sterile water, divided into several parts, and stored at −18°C. Incorporation of label into cells was measured by applying, in duplicate, 0.1-ml culture samples onto Whatman 3 MM filter disks (3.5-cm diameter), precipitating macromolecules by immediately submerging the filters into cold trichloroacetic acid, and washing the filters sequentially with ethanol, ethanol-ether (1:1 [vol/vol]), and ether. The radioactivity remaining on the air-dried disks was determined in a Packard liquid scintillation counter (model B.P.L.) with Packard toluene scintillation fluid. Determination of radioactivity in aqueous samples (e.g., emulsan solutions) was performed in Hydroluma scintillation fluid (Lumac Systems AG, Basel).

Column chromatography. A mixture of radioactive emulsan produced in the presence of CM and highly purified carrier emulsan (24) was applied to a Sepharose 4B column (90 by 2 cm; Pharmacia Fine Chemicals, Uppsala) which had been equilibrated with TM buffer (0.2 M Tris buffer [pH 7.5] containing 10 mM $MgSO_4$) at 4°C. Elution was performed with TM buffer at 14 ml/h; fractions (2.3 ml each) were collected and assayed for emulsifying activity and radioactivity.

General analytical methods. Protein concentrations were determined by the method of Lowry et al. (11) with bovine serum albumin as the standard. *O*-Acyl was estimated by a modification of the Hestrin method (19) with acetohydroxamate as the standard. Hexosamines were determined by the indole-nitrite method (3) with galactosamine as the standard. Cell dry weight was determined after drying a sample of washed cells overnight at 80°C. Viscosity was measured on 1.0-ml samples in an Ostwald-Fenske microviscometer at 30°C as previously described (24). Values reported for protein, ester, and hexosamine content; cell dry weight; and viscosity are the averages of three determinations. Thin-layer chromatography was performed on 0.1-mm thick cellulose F plates (E. Merck, Darmstadt) by using pyridine-ethyl acetate-water-acetic acid (5:5:3:1 [vol/vol]) for development. Multiple chromatograms were stained for sugars and amino sugars by (i) alkaline silver nitrate and (ii) 0.2% ninhydrin in acetone, followed by heating at 105°C for 2 to 3 min.

TABLE 1. Effect of antibiotics on emulsan production[a]

Antibiotic	Final concn (mg/ml)	Turbidity (Klett units)	Emulsan (U/ml)
None		875	38
Nalidixic acid	0.03	400	30
Bacitracin	1.76	355	12
Ampicillin	1.00	625	27
Tetracycline	0.05	435	91
Streptomycin	0.13	365	52
CM	0.05	400	97

[a] An exponentially growing culture of RAG-92 cells (300 Klett units) was harvested, washed, and suspended in fresh EL medium to a turbidity of 300 Klett units. The suspension was then divided into flasks containing different antibiotics. After 7 h of incubation, culture turbidity and extracellular emulsifying activity were measured.

RESULTS

Earlier reports from this laboratory indicated that emulsan was produced by *A. calcoaceticus* growing either on hydrocarbon (14) or on ethanol (18) media primarily after the cells reached the stationary growth phase. In an attempt to uncouple growth from emulsan production on ethanol, the effect of several antibiotics was investigated with RAG-92 cells (Table 1). Nalidixic acid inhibited growth, measured by increase in culture turbidity by over 80%, but lowered emulsan production by only 19% compared to the control. Typical of other *Acinetobacter* strains, RAG-92 was quite resistant to β-lactam antibiotics (9); a concentration of 1 mg of ampicillin per ml caused only a partial inhibition of growth and emulsan formation. Bacitracin inhibited both growth and production. The most interesting results were obtained with protein synthesis inhibitors such as tetracycline, streptomycin, and CM; increase in culture turbidity was inhibited by 76 to 89%, but emulsan production actually increased by 37 to 155%. Since CM was the antibiotic that caused maximum emulsan production, it was selected for further investigation. None of the antibiotics tested had any effect on the emulsification assay itself.

Table 2 summarizes a series of experiments defining the requirements for emulsan production in the presence of CM. Emulsan formation was totally dependent on a utilizable source of carbon and energy. Acetate could partially replace the ethanol requirement. A variety of sugars and amino sugars, including glucose, galactose, glucosamine, and galactosamine, did not even partially replace the ethanol requirement for either growth or emulsan production. In the presence of respiratory inhibitors, such as cyanide or azide, or under anaerobic conditions, emulsan production was completely blocked. A utilizable nitrogen source, such as urea or am-

TABLE 2. Requirements for the production of emulsan in the presence of CM

Treatment[a]	Turbidity (Klett units)	Emulsan (U/ml)
Control (−CM)	920	57
Complete system	470	71
−Ethanol	380	4
−Urea	470	7
$-Mg^{2+}$	470	26
−Lysine	480	63
−Phosphate[b]	380	47
−Lysine, CM	430	63
+1 mM KCN	390	4

[a] Experiments were performed as described in footnote *a* of Table 1, except that the initial turbidity was 375 Klett units. The complete system (EL medium) contained 2.5% (vol/vol) ethanol, 30 mM urea, 0.02% $MgSO_4 \cdot 7H_2O$, 100 mM phosphate buffer (pH 7.0), 0.2 mg of lysine per ml, and 50 μg of CM per ml.

[b] In the absence of phosphate, pH was controlled by 100 mM Tris buffer (pH 7.0).

monium sulfate, was also required for the production of emulsan.

Since strain RAG-92 is a lysine auxotroph, it was of interest to measure emulsan production during lysine starvation in both the absence and presence of CM. The data in Table 2 indicated that emulsan was made during lysine starvation regardless of whether the antibiotic was present. Thus, emulsan was produced when protein synthesis was inhibited either by specific antibiotics or by starvation for a required amino acid.

To compare the properties of emulsan prepared by CM treatment with those of emulsan purified from stationary-phase cultures (24), 1 liter of exponentially growing RAG-92 cells (220 Klett units) was harvested, washed, and suspended in the same volume of fresh EL medium. After 7 h of incubation in the presence of 50 μg of CM per ml, the cells were removed by centrifugation, and the supernatant fluid was dialyzed extensively against distilled water and then lyophilized. Emulsan prepared in this manner (yield, 1.14 g) exhibited the following properties: specific activity, 260 U/mg; protein, 11.2%; hexoasmine content, 25.3%; *O*-acyl content, 0.5 μmol/mg; reduced viscosity, 245 cm^3/g. These values are similar to those obtained from standard preparations of emulsan (24).

The kinetics of growth and emulsan formation in the presence and absence of CM are shown in Fig. 1. Although growth was strongly inhibited, cells treated with antibiotic began to produce emulsan within 1 h and reached a maximum level at 5 h. In contrast, the parallel culture without the antibiotic grew normally, but produced little emulsan during the initial 3 h. The upper panels of Fig. 1 present specific production values. At 5 h, the specific production of the CM-treated cells was more than seven times the values of the control cells. The specific production level obtained with the antibiotic in 5 h was similar to the maximum values reached with stationary-phase cultures under optimal conditions.

The specific production of emulsan in batch cultures varied greatly with ethanol concentration (Fig. 2). Although the growth yield was proportional to ethanol concentration over the range of 0.01 to 0.5% (vol/vol) ethanol, very little emulsan was produced under these conditions of substrate limitation. With between 0.5 and 2.0% (vol/vol) ethanol, the ratio of emulsifying activity to growth yield increased dramatically. The failure of cells to produce emulsan at low concentrations of ethanol was most likely due to the requirement for a carbon and energy source for emulsan production (Table 2); when cells reached the stationary phase because of substrate limitation (Fig. 2), they were unable to produce emulsan. In support of this concept was the fact that cells growing even on low concentrations of ethanol produced emulsan when growth was terminated by the addition of CM or the limitation of a required amino acid. For example, RAG-92 cells grown to stationary

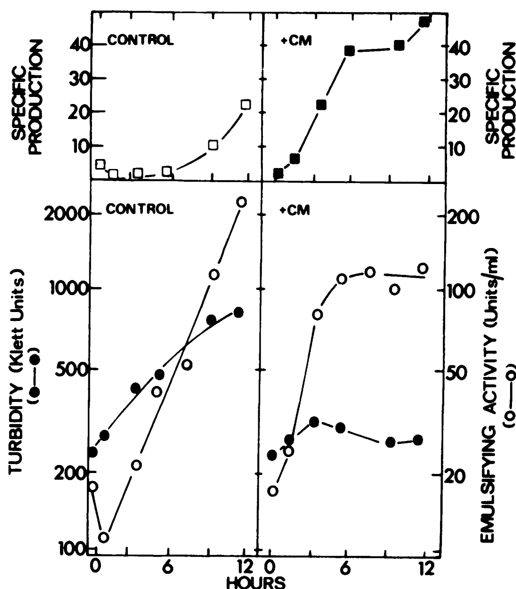

FIG. 1. Kinetics of growth and emulsan production in the presence of cm. Experiments were performed as described in footnote *a* of Table 1 with an initial turbidity of 240 Klett units. The upper panels show the specific production of emulsan at each time point. Specific emulsan production is defined as [emulsan (units per milliliter)/culture turbidity (Klett units)] × 100.

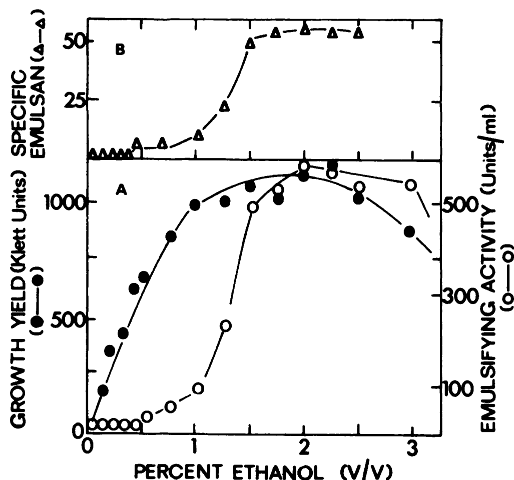

FIG. 2. Growth yields and emulsifying activity as a function of ethanol concentration. Washed cells were inoculated into EL medium containing various concentrations of ethanol. After 72 h of incubation, turbidities and emulsifying activities were measured. Specific emulsan production is defined in the legend to Fig. 1.

phase on 0.5% ethanol medium containing 50 μg of lysine per ml reached a culture turbidity of 520 Klett units and produced 170 U of emulsan per ml; a parallel culture grown on 0.5% ethanol medium containing 100 μg of lysine per ml reached a culture turbidity of 740 Klett units but produced only 16 U of emulsan per ml.

To determine whether emulsan production in the presence of CM was a result of de novo synthesis of the polymer(s) or release of preformed emulsan, radioactive tracer experiments were performed. Two radioactive compounds were used: [^{14}C]lysine to label protein and [^{14}C]ethanol as a general carbon label. The isotopes were added either several generations before CM treatment (prelabel) or during exposure to the antibiotic (postlabel). Table 3 shows that CM inhibited the incorporation of lysine into both cells and emulsan by over 98%. Thus, there is no evidence that synthesis of the protein fraction associated with emulsan is more resistant to CM inhibition. It follows that the protein fraction associated with emulsan (approximately 10% by weight) was synthesized during the growth phase and was released into the medium after CM addition. No detectable radioactivity was found in the polysaccharide fraction of emulsan (phenol treated) when [^{14}C]lysine was used as the label. With [^{14}C]ethanol as the tracer (Table 3), about 40% (2,900 cpm/ml) of the radioactivity in the emulsan fraction was incorporated before CM addition, and 60% (4,400 cpm/ml) was incorporated during the antibiotic treatment. When prelabeled log-phase cells were incubated for 7 h with the antibiotic and unlabeled ethanol, 56% of the radioactivity remained associated with the cells and 30% was released as emulsan (the remaining 14% presumably was metabolized). During the postlabeling period, 59% of the incorporated radioactivity went into cell-free emulsan, and only 41% was cell associated.

The fact that the radioactivity in the emulsan fractions was due to emulsan, rather than some other extracellular, non-dialyzable impurity, was established by chromatography with authentic emulsan on a Sepharose 4B column (Fig. 3). Over 90% of the radioactivity from prelabeled and postlabeled emulsan fractions cochromatographed with highly purified emulsan. The specific radioactivities of column-purified prelabeled and postlabeled emulsans were 3,250 and 4,980 cpm/mg, respectively.

Emulsan contains fatty acids (0.5 μmol/mg) joined to the polysaccharide through ester linkages (1). To determine whether the polymer was esterified with fatty acid before or after CM addition, the prelabeled and postlabeled emulsans were hydrolyzed in alkali and fractionated (Table 4). The alkaline conditions were sufficiently strong to hydrolyze all of the esters (as measured by the hydroxylamine reaction) without destroying the polymeric backbone. Fatty acids were extracted into ether after acidification, and their radioactivity was determined. The ratio of labeled fatty acids to deesterified polymers was the same for pre- and postlabeled

TABLE 3. Incorporation of labeled precursors into cells and emulsan (label added during growth phase or CM treatment)[a]

Precursor	Fraction	Incorporation (cpm/ml)	
		Prelabel	Postlabel
[^{14}C]lysine	CM-treated cells	25,300	460
	Emulsan	6,000	90
[^{14}C]ethanol	Log-phase cells	9,600	
	CM-treated cells	5,400	3,000
	Emulsan	2,900	4,400

[a] Prelabeled RAG-92 cultures were grown in EL medium supplemented with either [*U*-^{14}C]lysine (0.1 μCi/ml) or [1-^{14}C]ethanol (0.5 μCi/ml) to 300 Klett units, harvested, washed extensively, and then suspended in the same volume of fresh unlabeled EL medium containing 50 μg of CM per ml. Postlabeled cells were treated in the same manner except that the label was added only during the CM treatment. In both cases, the cultures were harvested after 7 h of incubation with the antibiotic. Emulsan was obtained from the supernatant fluid after extensive dialysis. CM-treated cells were the final washed pellet fraction.

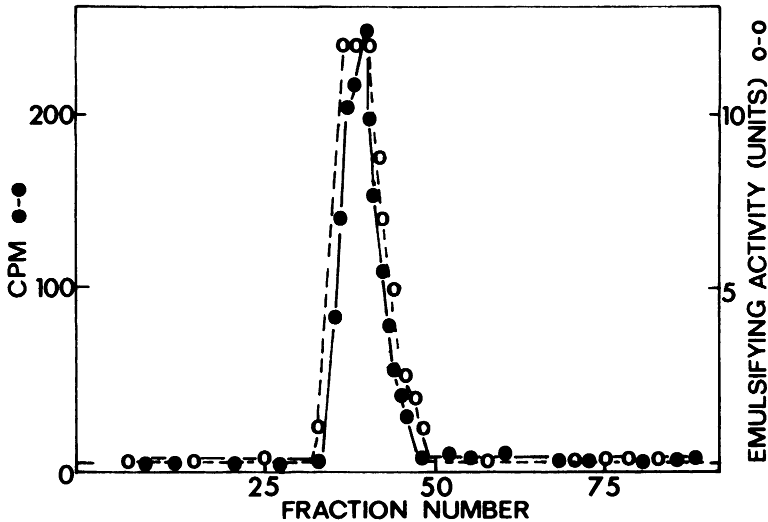

FIG. 3. Sepharose 4B chromatography of a mixture of purified unlabeled emulsan and ^{14}C-labeled emulsan produced in the presence of CM. A mixture of highly purified emulsan and ^{14}C-labeled emulsan obtained from a CM-treated culture (postlabeled) was applied to a Sepharose 4B column (2-cm diameter and 90-cm length). The column was equilibrated and run with TM buffer at 4°C. The sample, 0.6 ml of emulsan solution (1.2 mg/ml) contained 111 emulsifying units and 1,980 cpm. The column was eluted at 14 ml/h. Fractions (2.3 ml each) were collected and assayed for radioactivity and emulsifying activity.

emulsan. Thus, it appears that esterification of the polymer occurred both before and after CM treatment.

Further evidence for a cell-associated form of emulsan came from chemical analyses of washed cells (Table 5). Exponentially growing RAG-1 cells contained 48.2 μg of hexosamine per mg of cells (dry weight). After CM treatment, the hexosamine content of the cells decreased to 6.9 μg per mg of cells. Similarly, stationary-phase RAG-1 cells and log-phase cells of an emulsan-negative mutant had relatively low amounts of hexosamine: 13.9 and 6.1 μg per mg of cells (dry weight), respectively.

The difference in the hexosamine content of log-phase RAG-1 cells and CM-treated stationary-phase and emulsan-negative cells, 34 to 42 μg/mg of cells, is most likely due to the amino sugars in emulsan. However, it was also possible that cell wall and other amino sugar-containing materials contributed to this difference.

TABLE 4. Distribution of radioactivity in alkaline-hydrolyzed prelabeled and postlabeled emulsan[a]

Fraction[b]	Prelabeled emulsan		Postlabeled emulsan	
	cpm	%	cpm	%
Ether phase	2,900	13	3,900	15
Aqueous phase	19,800	86	22,400	84
Interphase	300	1	300	1

[a] [^{14}C]ethanol-labeled emulsan preparations were obtained from the experiment described in footnote *a* of Table 3.

[b] ^{14}C-labeled emulsan was hydrolyzed in 0.1 N NaOH for 5 min at 100°C. The hydrolysates were acidified to pH 2 with HCl and then extracted with an equal volume of diethyl ether. The ether phase was washed with an equal volume of water; the aqueous phases were combined.

TABLE 5. Hexosamine content of hydrolyzed cells[a]

Cell fraction	Dry wt (mg)	Hexosamine	
		mg	%
Log-phase RAG-1	8.3	0.40	4.82
Log-phase RAG-1, CM-treated[b]	7.5	0.052	0.69
Stationary-phase RAG-1	7.2	0.10	1.39
Log-phase AG-1 (emulsan negative)	7.9	0.048	0.61

[a] Exponentially growing RAG-1 and AG-1 cells, as well as stationary-phase RAG-1 cells, were harvested, washed once in cold water, and then suspended in water to about 2,000 Klett units. Samples (1 ml each) were used for the determination of cell dry weight and hexosamine content. Hexosamines were determined after hydrolysis in 5 N HCl at 100°C for 20 min and neutralization with NaOH.

[b] CM treatment was as described in footnote *a* of Table 1.

Thus, washed exponential- and stationary-phase RAG-1 cells were extracted with 0.1 N NaOH at 4°C for 1 h (according to a procedure developed by Y. Shabtai; unpublished data) to solubilize the cell-bound emulsan. After centrifugation at 12,000 × *g* for 20 min, portions of the supernatant fluids and resuspended pellets were hydrolyzed as described in Table 5 and assayed for hexosamine content. The supernatant fluids from stationary- and exponential-phase cells contained 4.0 and 33.5 μg of hexosamine per mg of cells (initial dry weight), respectively, whereas both pellet fractions contained about 9 μg of hexosamine per mg.

The clarified alkaline extract of exponential-phase RAG-1 cells was neutralized with dilute HCl, dialyzed to remove salts, and then hydrolyzed with 5 N HCl at 100° for 30 min. After removing the HCl in vacuo, sugars were examined qualitatively by thin-layer chromatography. The chromatographic pattern was identical to that obtained from hydrolyzed emulsan: galactosamine (major component) and an aminouronic acid (24). No glucosamine was detected.

DISCUSSION

Although bioemulsifier production by microorganisms is generally associated with growth on hydrocarbons (6, 8, 10, 14), *A. calcoaceticus* RAG-1 produced at least as much emulsan when grown on ethanol as on hexadecane medium (18). Many of the technical difficulties associated with studying microbial growth on hydrocarbons, such as sampling and measurement of growth parameters, were avoided by using the water-soluble substrate ethanol for studying the relationship between growth and emulsan production.

In previous reports, the term emulsan referred only to the extracellular polymeric emulsifying agent produced by strain RAG-1. The following data provide strong evidence for a cell-associated form of emulsan which, for the purposes of this discussion, is referred to as proemulsan. (i) Thirty percent of the carbon atoms in washed, uniformly labeled, exponential-phase cells were excreted as labeled emulsan into the medium after CM treatment in non-radioactive medium (Table 3). The most direct explanation for this finding is that proemulsan exists in exponentially growing cells and is released in an energy-dependent reaction (Table 2) during CM treatment. The alternative explanation, de novo synthesis from monomers produced by massive breakdown of prelabeled protein or other polymers, is unlikely because (a) over 90% of the [^{14}C]lysine prelabeled protein was conserved (as cell-bound plus emulsan-associated protein) after CM treatment, (b) no radioactivity from the prelabeled protein was subsequently found in the emulsan polysaccharide, and (c) the distribution of label between polysaccharide and fatty acids was the same for pre- and postlabeled emulsan. (ii) Chemical analysis of exponential-phase RAG-1 cells demonstrated high quantities of polymeric amino sugars; the polymer was solubilized by mild alkaline treatment and shown qualitatively to contain the same monosaccharides as emulsan. By comparison with stationary-phase cells or with an emulsan-deficient mutant, it was possible to estimate that growing RAG-1 cells contained an excess of about 38 μg of hexosamine per mg of cells (dry weight). Since acid-hydrolyzed emulsan yielded about 25% amino sugar by this test, the estimated amount of proemulsan is 15% by weight of the dry cells. (iii) By using an enzyme-linked immunosorbent assay with an emulsan-specific antibody preparation, it was shown that exponential-phase RAG-1 cells bore high amounts of cell-associated emulsan which decreased rapidly as cells reached the stationary phase (S. Goldman, Y. Shabtai, C. Rubinowitz, E. Rosenberg, and D. L. Gutnick, submitted for publication).

What is the mechanism for release of proemulsan from growing cells when protein synthesis is inhibited? One possibility is that a minor component of emulsan (protein or fatty acids) which is absent in proemulsan during the growth phase is needed for the release process. Data from the radioactive experiments argue against this possibility because (i) all the emulsan protein that appeared in the extracellular polymer was synthesized before the addition of CM and (ii) the fatty acid ester content of prelabeled and postlabeled emulsan was similar. Thus, there is no evidence that the lack of one of these components prevented release during the exponential growth phase.

The existence of relatively large amounts of proemulsan associated with RAG-1 cells during the growth phase should be taken into account when considering possible natural functions for emulsan. Evidence has already been presented for the role of cell-bound emulsan as a receptor for bacteriophage ap3 (13). In general, if one considers proemulsan as a major component of the minicapsule of RAG-1, then proemulsan could play a role in many of the functions attributed to bacterial capsules. More specifically, the relative importance of emulsan as an extracellular emulsifier to its role on the cell surface during growth on hydrocarbons is currently under investigation.

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