APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1986, p. 146–151
0099-2240/86/070146-06$02.00/0

Vol. 52, No. 1

Enhanced Emulsan Production in Mutants of *Acinetobacter calcoaceticus* RAG-1 Selected for Resistance to Cetyltrimethylammonium Bromide

YOSEF SHABTAI† AND DAVID L. GUTNICK*

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

Received 26 November 1985/Accepted 1 April 1986

Mutants of *Acinetobacter calcoaceticus* RAG-1 that produced elevated levels of the polymeric bioemulsifier emulsan were isolated on the basis of their resistance to the cationic surfactant cetyltrimethylammonium bromide (CTAB). Such mutants showed maximum enhancement in both overall yield and specific productivity of some two- to threefold over that of the wild type. In addition, the effect was also observed in a resting cell system in the presence of chloramphenicol, indicating that the mutation is not simply the result of faster growth. When CTAB-tolerant mutants were subjected together with the sensitive parent to the detergent under growing conditions, only the mutants were found to grow. The results suggest that the mutation for CTAB resistance leads to enhanced capsule production. This was confirmed quantitatively by a specific enzyme-linked immunosorbent assay for the cell-bound emulsan minicapsule.

Much attention has been focused in recent years on microbial surfactants and emulsifiers as potential products of industrial fermentation (6, 7, 27, 28). One such product currently being produced on a commercial scale is the polymeric emulsion stabilizer emulsan. Emulsan is an extracellular lipoheteropolysaccharide polyanionic bioemulsifier produced by the oil-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 (8, 16–18, 29). The polysaccharide backbone of the polymer consists of three amino sugars, D-galactosamine, D-galactosamine uronic acid (pK 3.01), and a third unidentified hexoseamine (30). The amphipathic properties of the polymer are due in part to the presence of fatty acids linked to the polysaccharide backbone via ester and amide linkages (4).

A cell-associated form of emulsan has been shown to constitute a minicapsular layer on the surface of exponential RAG-1 cells which is released into the medium as the cells approach stationary phase (8). The release process recently has been shown to involve the activity of an exocellular esterase (23). The cell-associated form of emulsan serves as a receptor for the specific RAG-1 phage ap3 (13–15). Moreover, we have recently shown that the presence of the emulsan capsule on the cell surface enhances the tolerance of RAG-1 cells to the cationic detergent cetyltrimethylammonium bromide (CTAB) (22). Protection from CTAB was also observed when the medium was supplemented with the purified bioemulsifier. Because CTAB is used to precipitate emulsan during the purification of the polymer (8, 30), the neutralization of the detergent is most likely due to a direct interaction with the emulsifier. Therefore, at least one class of RAG-1 mutant capable of growing in the presence of high concentrations of CTAB might be expected to produce elevated levels of emulsan. This approach was used in this study to isolate such emulsan overproducing mutants. In addition to producing more emulsan, these mutants produced emulsan earlier in the growth cycle.

* Corresponding author.
† Present address: Center for Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel.

MATERIALS AND METHODS

Organisms and culture conditions. *A. calcoaceticus* RAG-1 (ATCC 31012) and RAG-92, a lysine-requiring auxotroph of RAG-1 (19), were grown in a minimal salt (ETMS) medium containing the following, per liter: 22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 4.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 25 ml of absolute ethanol. ETMS medium was supplemented with L-lysine (final concentration, 0.2 g/liter) when required. Semisolid medium contained the same constituents, with the addition of agar 2% (wt/vol). CTAB-containing media were prepared by diluting a sterile stock CTAB solution (2%) into fresh minimal media to the required concentration. Growth experiments were performed in Erlenmeyer flasks filled to 20% of their capacity. The cultures were shaken at 250 rpm at 30°C in a gyratory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Growth was determined by turbidity measurements in a Klett-Summerson colorimeter with a green filter. Samples were first diluted to the linear range of the measurement (100 to 150 Klett units). Culture turbidity of 100 Klett units corresponds to 0.36 g of cell dry weight (CDW) per liter. Cells were washed twice (10,000 × *g*, 15 min) and suspended in phosphate buffer (0.15 M, pH 7.0). CDW was determined after drying 10-ml fractions at 80°C to constant weight.

Emulsan preparation and assay. Emulsan was prepared from culture supernatant as described previously (8, 18, 30) and was purified by the CTAB preparation method (8, 22). To release the CTAB bound to the cell-free emulsan produced in cultures growing in the presence of the detergent, sodium sulfate was added to the supernatant to a final concentration of 50 mM, and the detergent was precipitated as the iodide salt with the addition of KI to a final concentration of 0.3 mg/ml. The precipitate was separated by centrifugation at 10,000 × *g* for 30 min before the assay. Emulsan activity was measured by a standard emulsifying assay (17, 18) based on emulsification of a mixture of hexadecane–2-methylnapththalene in Tris buffer (10 mM, pH 7.2, containing 20 mM $MgSO_4$). One unit corresponds to about 6.5 μg of the purified emulsifier (155 U/mg [dry weight]). In addition, emulsan was also estimated by the

immunochemical method based on an enzyme-linked immunosorbent assay (ELISA) as described previously (8).

CTAB tolerance assay. The CTAB-tolerance assay has been described previously (22) and is based on exposure of cells to various CTAB concentrations in defined minimal media with series of culture tubes containing 1.9 ml of ETMS medium and increasing CTAB concentrations up to 20 μg/ml at intervals of 0.5 μg/ml. The cultures were shaken at 30°C for 24 h. At the end of the incubation the cultures were examined for growth. CTAB tolerance (expressed in micrograms of CTAB per milliliter) was determined as the highest concentration of the detergent in the presence of which growth occurred.

Direct selection of CTAB-tolerant mutants on agar plates. The technique for isolation of CTAB-tolerant mutants was that of Ames and Whitefield (2). Culture fractions (0.1 ml), containing 10^5 RAG-1 cells per ml previously grown overnight in standard ETMS medium, were spread on ETMS agar plates supplemented with CTAB at a final concentration of 10 μg/ml. A crystal of *N*-methyl *N*-nitro-*N*-nitrosoguanidine (NTG; Sigma Chemical Co., St. Louis, Mo.) was deposited at the center of each plate, and the plates were incubated for 30 h at 30°C. Colonies which appeared on the selection plates were transferred to fresh CTAB-containing (10 μg/ml) plates. Mutants that were found to grow on the CTAB plates were isolated, grown on ETMS medium; and examined for growth and emulsan production under standard conditions. A specific production unit (units per milligram of CDW) was defined as the ratio of emulsan concentration (in units per milliliter) to cell mass (in milligrams CDW per milliliter) at the end of incubation for 72 h.

Isolation of CTAB-tolerant mutants after selection and enrichment in liquid medium. The procedure for isolation of CTAB-tolerant mutants was a modification of the technique of Adelberg et al. (1). Mutant 92 was grown to 100 Klett units (1.3×10^9 cells per ml) in standard ETMS medium containing lysine. The cells were collected by centrifugation and washed once with phosphate buffer and twice with 20 mM citrate buffer (pH 6.0). The cells were concentrated 10-fold (to 2 ml of 9.5×10^9 cells per ml), and the suspension was then diluted 10-fold with citrate buffer. NTG was added to the suspension to a final concentration of 250 μg/ml. The cell suspension was incubated for 1 h at 30°C with slow gyratory shaking. At the end of the incubation the viable count of the culture was measured. The suspension was washed twice with phosphate buffer and diluted 10-fold into ETMS medium containing 0.2 mg of lysine per ml to allow for phenotypic expression. After 15 min of incubation at 30°C, samples were inoculated into fresh ETMS medium containing lysine and CTAB at a final concentration of 50 μg/ml. These cultures were incubated at 30°C for 6 h. At the end of the selection cells were collected, washed, suspended in fresh medium, and incubated for 8 h. A second selection was then performed by incubating the cells for 6 h in fresh medium containing 50 μg of CTAB per ml. After the second enrichment, fractions of these cultures were spread on agar plates containing CTAB at final concentrations of 50, 75, 100, and 200 μg/ml. Resistant colonies were isolated and restreaked onto agar plates containing CTAB. Mutants which appeared to be tolerant to the detergent were characterized according to (i) CTAB tolerance, (ii) colony size at 48 h, and (iii) colony appearance.

Emulsan production in a resting cell system in the presence of chloramphenicol. A washed cell suspension of exponential-phase cells (1.5 g of CDW per liter) was incubated with shaking at 30°C in the presence of chloramphenicol (50 μg/ml), as described previously (19). The suspensions were shaken for 8 h, during which time samples were removed at regular intervals and assayed for cell mass and extracellular emulsan activity.

TABLE 1. Production of emulsan by CTAB-tolerant mutants

Strain[a]	Cell mass (mg of CDW/ml)	Emulsan (U/ml)	Specific production (U/mg of CDW)
RAG-1	3.9	240	62
RAG-92	4.3	185	43
CTR			
10-3	3.6	290	80
10-7	3.9	320	82
10-12	4.4	360	82
10-17	4.2	370	88
10-19	3.8	290	76
10-27	4.2	500	119
10-34	4.3	460	106
10-38	4.0	460	115
10-49	4.3	770	179
10-51	4.4	580	132
10-72	4.2	370	88
10-80	4.4	380	86
10-90	4.0	330	82
10-113	4.2	340	80
CTRL			
50-1	4.2	310	74
50-2	4.0	420	105
75-1	4.6	350	76
75-2	3.7	440	119
100-1	4.1	520	127
100-2	3.9	300	77
100-3	3.3	180	54

[a] Mutants marked CTR were derived from RAG-1 by direct isolation from plates containing 10 μg of CTAB per ml. Mutants marked CTRL were isolated after enrichment in liquid medium containing 50 μg of CTAB per ml and were derived from RAG-92.

Emulsan production under lysine starvation conditions. The method to determine emulsan production under lysine starvation conditions was essentially the same as that described above for determination in the presence of chloramphenicol, except that protein synthesis was arrested by starvation of RAG-92 and its derivatives for lysine (19). The release of emulsan was achieved by washing and suspending exponential-phase cells in complete minimal medium in the absence of the required amino acid. The cultures were then incubated and assayed as described above.

Measurement of cell-bound emulsan. The relative amount of emulsan minicapsule was assayed by the specific ELISA with an emulsan-specific antibody cross-linked to alkaline phosphatase, as described previously (8). The results are expressed as A_{405} (arising from the phosphatase assay) per 10^6 cells.

RESULTS

Isolation of CTAB-tolerant mutants. Previous results from this laboratory have shown that the parental RAG-1 cells are tolerant to CTAB at a maximum concentration of 2 μg/ml on ETMS agar plates and 0.6 μg/ml in liquid ETMS medium (22). A total of 114 CTAB-tolerant mutants were isolated on ETMS agar plates containing 10 μg of CTAB per ml, and 7 isolates were obtained following mutagenesis and enrichment in liquid medium containing 50 μg of CTAB per ml, as described above. The CTAB-tolerant isolates were screened

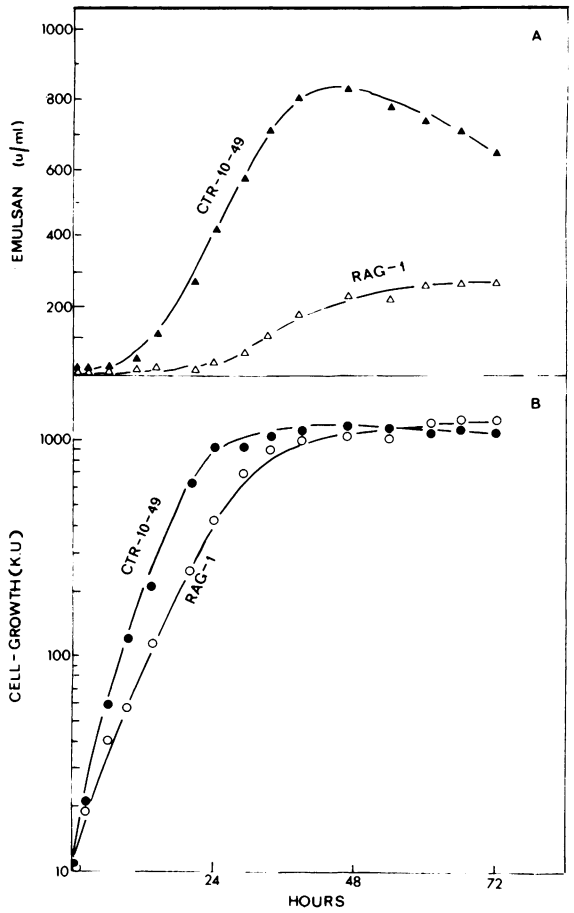

FIG. 1. Cell growth and emulsan production in CTR-10-49 and RAG-1. K.U., Klett units.

for emulsan yields and productivity. Twenty of the CTAB-tolerant mutants showed enhanced production of emulsan over that of the wild type (Table 1). The majority of these mutants exhibited enhancement of between two- and three-fold in both rate of production (5 to 7 U of emulsan per ml h) and polymer yield (90 to 170 U of emulsan per mg of CDW). Moreover, none of the 121 mutants isolated showed a lower productivity than the parental strains. Two mutants, CTR-10-49 and CTRL-100-1, were chosen for further study on the basis of their higher productivity. Mutant CTR-10-49, derived from RAG-1 on semisolid media, was sensitive to 10 μg of CTAB per ml on plates and 2 μg of CTAB per ml in liquid media. The mutant CTRL-100-1 was derived from the lysine auxotroph RAG-92 by enrichment in liquid media, in which it showed a tolerance to 18 μg of CTAB per ml. This mutant was found to be tolerant to 100 μg of CTAB per ml on plates.

Emulsan production by CTAB-tolerant mutants. The two mutants each produced about 600 U of emulsan per ml and about 4 mg of CDW per ml during a 72-h growth period. In sharp contrast, the parental strains RAG-1 and RAG-92 produced only 240 and 220, U of emulsan per ml, respectively. It was of interest, therefore, to examine the kinetics of emulsan production throughout the growth cycle.

A number of differences were observed when the growth and emulsan production of strain CTR-10-49 was compared with that of RAG-1 (Fig. 1). In the case of the parent RAG-1, extracellular emulsan began to accumulate only after about 20 h (at the end of exponential growth). This production extended into the stationary phase and reached a maximum at about 48 h. In contrast, when the mutant CTR-10-49 was grown on ethanol under the same conditions, the accumulation of emulsan began earlier, at about 12 h, and continued for the next 20 h. Between 12 and 32 h the rate of emulsan production by CTR-10-49 was about 12 U/mg of CDW per h, while during the same period the rate of emulsan production with the parent RAG-1 was only 5 U/mg of CDW per h. A maximum emulsan yield of 800 U/ml was observed in the case of the mutant, while the parent RAG-1 gave a yield of about 300 U/ml. The final emulsan-to-cell ratio at 72 h was 190 U/mg of CDW for CTR-10-49 and 60 U/mg of CDW for RAG-1. It should be noted that the emulsan activity which accumulated in the cell-free broth of the parent remained constant during the 24-h period between 48 and 72 h. In contrast, the mutant activity decreased some 25% during this period. Finally, mutant CTR-10-49 exhibited a faster initial growth rate (growth rate, 0.63 h^{-1}) than RAG-1 (growth rate, 0.36 h^{-1}). It was of interest to determine whether the enhanced production rate in the mutant was related to its faster growth rate on ethanol, or whether the rate (and extent) of emulsan production itself was directly affected by the mutation.

Emulsan production in resting cells. To distinguish the possibility of emulsan production in resting cells, emulsan production was examined with a resting cell system in which growth and protein synthesis were arrested in the presence of chloramphenicol (8, 19). As described previously (8, 19), emulsan production by RAG-1 cells in the exponential phase was accelerated under these conditions (Fig. 2C). Moreover, mutant CTR-10-49 released emulsan at a rate of 30 U/mg of cell per h, which was almost twice the parental rate in this resting cell system (17 U/mg of cell per h). The final yield of emulsan was about 220 U/ml in the case of the mutant, compared with 110 U/ml for the parent.

Similar results were obtained when the CTAB-resistant mutant CTRL-100-1 was compared with RAG-92 in a resting cell system in which growth was arrested by starvation for

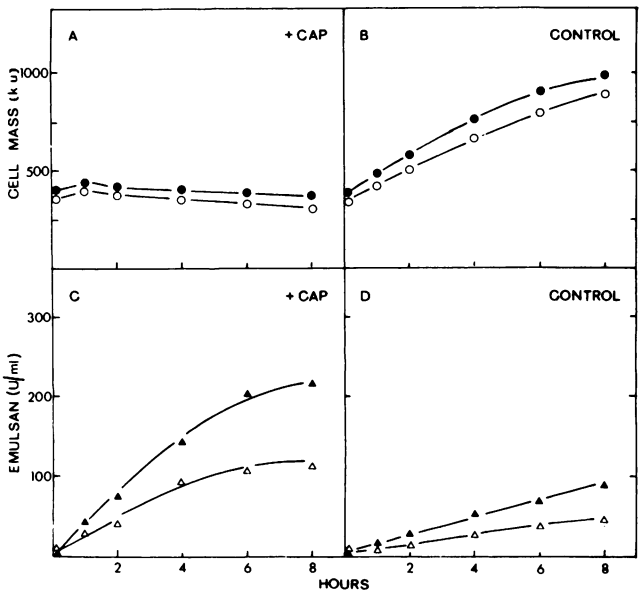

FIG. 2. Emulsan production in the presence of chloramphenicol (CAP). k.u., Klett units. Symbols: open symbols, RAG-1; closed symbols, CTR-10-49.

lysine (Fig. 3). The overproducing lysine auxotroph CTRL-100-1 released emulsan at a rate of 70 U/mg per h under conditions of lysine starvation, while the parent RAG-92 released emulsan at a rate that was about threefold lower (20 U/mg per h).

Emulsan mediated protection against CTAB toxicity. A previous report from this laboratory showed that CTAB tolerance of RAG-1 cells could be correlated with the production of extracellular emulsan (20). Similar results were obtained with mutant CTR-10-49 (Fig. 4). It is interesting that emulsan produced by both mutant and parent protected the cells against CTAB toxicity to the same extent (about 1.6 U/μg of CTAB [22]). When emulsan was removed from the growth medium in the whole culture and washed cells were examined for CTAB tolerance, it was found that cells of the mutant showed a fourfold increase in CTAB tolerance over that of the parent RAG-1 (2 μg/ml compared with 0.5 μg/ml, respectively). Furthermore, when the washed cells were analyzed for the presence of the emulsan minicapsule by an emulsan-specific ELISA (8), cells of mutant CTR-10-49 exhibited two- to fourfold more emulsan capsule than did the parental cells (A_{405} of 0.5 to 0.7 per 10^6 cells of the mutant and A_{405} of 0.2 per 10^6 cells of the parent; data not shown).

Although extracellular emulsan was shown to protect the cells against the toxic effects of CTAB (22) (Fig. 4), the tolerance to the very high concentrations of CTAB in the medium during the selection of resistant mutants could not be explained solely on the basis of elevated levels of cell-free emulsan. The possibility that enhanced tolerance was due, in part, to larger quantities of tightly bound cell-associated emulsan was indicated in the experiments with washed cells shown in Fig. 4. In addition, when cells of CTRL-100-1 and the parent RAG-92 were inoculated together in liquid culture and CTAB (200 μg/ml) was added after 24 h of incubation, growth of the parent ceased immediately, while the tolerant mutant was barely affected by the toxic surfactant (Fig. 5B). The concentration of emulsan at the time of exposure to CTAB was about 100 U/ml and continued to rise to about 600 U/ml during the subsequent 30 h. The presence of cell-free emulsan at the time of CTAB addition did not affect the sensitivity of the parental cells (compare Fig 5B and D). In the mixed culture without CTAB, slightly higher levels of emulsan were obtained than in the presence of CTAB, owing to the additional production by the parental cells (Fig. 5C).

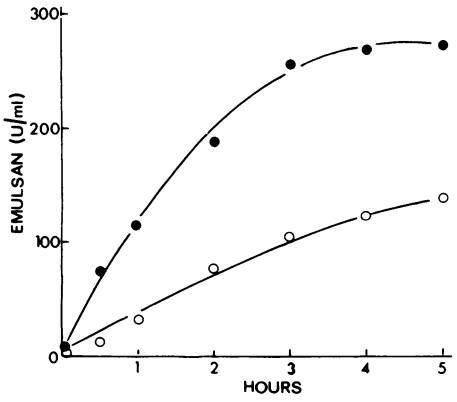

FIG. 3. Emulsan release during lysine starvation. Symbols: ●, mutant CTRL-100-1; ○, parent RAG-92.

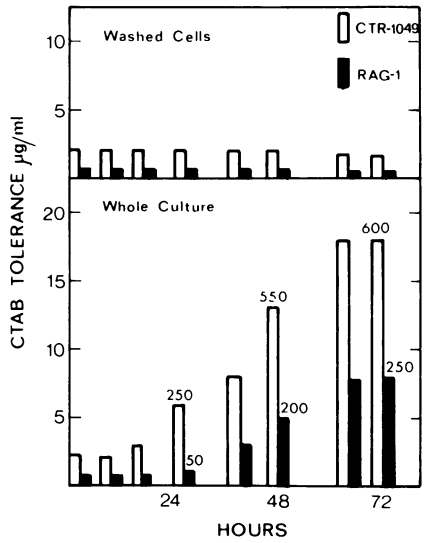

FIG. 4. CTAB tolerance of washed cells and whole cultures. The numbers above the shaded columns represent the concentration of emulsan (in units per milliliter) in the growing cultures at the time of inoculation into the CTAB medium.

DISCUSSION

Previous results from this laboratory have indicated that the extracellular bioemulsifier emulsan can protect the cell from the toxic effects of the amphipathic cation CTAB. This finding formed the basis for this study, in which a system is described to selectively enrich for emulsan overproducers. About 6% of all mutants which were selected directly on CTAB-containing plates showed enhanced emulsan production; none of the mutants produced less emulsan than the parent. The fact that fewer numbers of mutants were isolated from CTAB-containing liquid medium than from plates may be due to the presence of higher concentrations of CTAB in the liquid medium. This material in turn would be expected to be more toxic to cells which had lost their productive emulsan shield (22).

The overproducing mutants showed about a threefold enhancement of volumetric and specific productivity over that of the corresponding parents. Whereas emulsan appeared earlier in the growth cycle, enhanced production was also observed in a resting cell system. It thus appears that the genetic modification involved an alteration in an emulsan-specific step which was not directly linked to the growth of the mutant. Moreover, the mutations did not appear to involve a weakening of the binding of the emulsan minicapsule to the external surface of the cell, because washed cells of the mutant were at least fourfold more resistant to CTAB than were the parents. In addition, the mixed culture experiments showed no effect of extracellular emulsan produced by the mutant in sparing the CTAB-mediated inhibition of parental growth. The results of such competition experiments demonstrate a role for cell-bound emulsan in protecting parental strains (but not emulsan-deficient mutants) against CTAB toxicity (5, 10, 20, 21, 26). It is interesting that mutations to CTAB resistance in strains of *Klebsiella* and *Corynebacteria* (3, 11) showed a weaker association of the capsule with the cell. Moreover, mucus production by wild-type strains of *Pseudomonas aeruginosa* was found to be elevated in the presence of CTAB (9).

Mutant CTRL-100-1 was tolerant to 100 μg of CTAB per

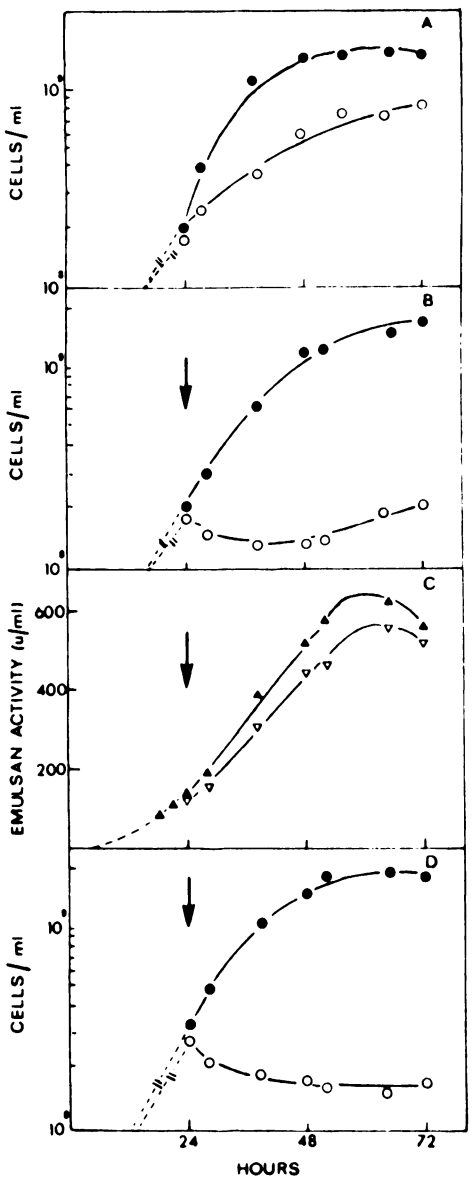

FIG. 5. Effect of CTAB on growth and emulsan production of CTRL-100-1 and RAG-92 in mixed cultures. (A) Mixed culture of RAG-92 (○) and CTRL-100-1 (●) without CTAB. (B) Mixed culture of CTRL-100-1 and RAG-92 with CTAB. (C) Emulsan activity in cell-free supernatants of mixed culture without (▲) and with (▽) CTAB. (D) Growth of CTRL-100-1 (●) and RAG-92 (○) in separate independent cultures in the presence of CTAB. Arrows in panels B through D indicate the time of exposure to CTAB.

ml, while CTR-10-49 was 10-fold less tolerant, even though both mutants produced about the same amount of emulsan per cell. In addition, mutant CTRL-100-1 produced emulsan at a significantly higher rate than CTR-10-49. The correlation between rapid production rate and CTAB tolerance may be explained by the rapid encapsulation of the mutant cells, which renders them more resistant to the higher concentration of CTAB. As a capsule, emulsan may bind more of the positively charged CTAB than the cell-free polymer. Alternatively, a second CTAB-binding factor may be made and transported to the outer surface of the cell in such mutants. Strain improvement leading to enhanced production of economically important polysaccharides is likely to be of major significance (7, 12, 25). The rate-limiting step in extracellular polysaccharide production involves the same amount of lipid carrier in the membrane which is available for transport of water-soluble precursors to the outside. Sutherland and Ellwood (24, 25) have suggested that selection for bacitracin resistance could be used to select mutants with elevated levels of polyprenol phosphate. The selection procedure described in this report is not necessarily restricted to emulsan overproducers. Its application in strain improvement for producers of a variety of polyanionic polysaccharides is currently in progress.

ACKNOWLEDGMENTS

This study was supported in part by a grant from Petroleum Fermentations, N.V.

We thank Rina Avigad for excellent technical assistance.

LITERATURE CITED

1. **Adelberg, F. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for a mutagenesis by N-methyl-N-Nitro-N-Nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Ames, B. N., and H. J. Whitefield.** 1966. Frameshift mutagenesis in *Salmonella*. Cold Spring Harbor Symp. Quant. Biol. **31**:221–225.
3. **Andreeson, M., and H. G. Schlegel.** 1974. A new coryneform hydrogen bacterium: *Corynebacterium autotrophicum* strain 7C. II. Isolation of slime-free mutant. Arch. Microbiol. **100**:351–361.
4. **Belsky, I., D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: determination of emusifier bound fatty acids. FEBS Lett. **101**:75–178.
5. **Chaplin, C. F.** 1951. Observation of quaternary ammonium disinfectants. Can. J. Bot. **29**:373–382.
6. **Cooper, D. G., and J. E. Zajic.** 1980. Surface-active compounds from microorganisms. Adv. Appl. Microbiol. **26**:229–253.
7. **Gabriel, A.** 1979. Economic value of biopolymers and their use in enhanced ol recovery, p. 191–204. *In* R. C. W. Berkeley, G. W. Gooday, and D. C. Ellwood (ed.), *In* Microbial polysaccharides and polysaccharases. Academic Press, Inc., London.
8. **Goldman, S., Y. Shabtai, C. Rubinovitz, E. Rosenberg, and D. L. Gutnick.** 1982. Emulsan production in *Acinetobacter calcoaceticus* RAG-1: distribution of cell-free and cell-associated cross-reacting material. Appl. Environ. Microbiol. **44**:165–170.
9. **Govan, J. R. W.** 1975. Mucoid strains of *Pseudomonas aeruginosa*: the influence of culture medium on the stability of mucus production. J. Gen. Microbiol. **8**:513–522.
10. **Hotchkiss, R. D.** 1946. The nature of the bactericidal action of surface active agent. Ann. N.Y. Acad. Sci. **46**:479–493.
11. **Norval, M., and I. W. Sutherland.** 1969. A group of *Klebsiella* mutants showing temperature dependent polysaccharide synthesis. J. Gen. Microbiol. **57**:369–377.
12. **Pace, G. W.** 1980. Production of extracellular microbial polysaccharides. Adv. Biochem. Eng. **15**:41–70.
13. **Pines, O., E. A. Bayer, and D. L. Gutnick.** 1983. Localization of emulsan-like polymers associated with the cell surface of *Acinetobacter calcoaceticus*. J. Bacteriol. **154**:893–905.
14. **Pines, O., and D. L. Gutnick.** 1981. Relationship between phage resistance and emulsan production, interaction of phages with the cell-surface of *Acinetobacter calcoaceticus* RAG-1. Arch. Microbiol. **130**:129–133.
15. **Pines, O., and D. L. Gutnick.** 1984. Specific binding of a bacteriophage at a hydrocarbon-water interface. J. Bacteriol. **157**:179–183.
16. **Reisfield, A., E. Rosenberg, and D. Gutnick.** 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. **24**:363–368.
17. **Rosenberg, E., A. Perry, D. T. Gibson, and D. L. Gutnick.** 1979. Emulsifier of Archrobacter RAG-1: specificity of hydrocarbon

substrate. Appl. Environ. Microbiol. **37**:409–413.
18. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: Isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402–408.
19. **Rubinovitz, C., D. L. Gutnick, and E. Rosenberg.** 1982. Emulsan production by *Acinetobacter calcoaceticus* in the presence of chloramphenicol. J. Bacteriol. **152**:126–132.
20. **Salton, R. J.** 1961. The adsorption of cetyltrimethylammonium bromide by bacteria: its action in releasing cellular constituents and its bactericidal effects. J. Gen. Microbiol. **5**:391–404.
21. **Salton, R. J., R. W. Horne, and V. E. Cosslett.** 1951. Electron microscopy of bacteria treated with cetyltrimethylammonium bromide. J. Gen. Microbiol. **5**:405–407.
22. **Shabtai, Y., and D. L. Gutnick.** 1985. Tolerance of *Acinetobacter calcoaceticus* RAG-1 to the cationic surfactant cetyltrimethylammonium bromide (CTAB)—role of the bioemulsifier, emulsan. Appl. Environ. Microbiol. **49**:192–197.
23. **Shabtai, Y., and D. L. Gutnick.** 1985. Exocellular esterase and emulsan release from the cell surface of *Acinetobacter calcoaceticus* RAG-1. J. Bacteriol. **161**:1176–1181.
24. **Sutherland, I. W.** 1979. Microbial exopolysaccharides: control of synthesis and acylation, p. 1–34. *In* R. C. W. Berkeley, G. W. Gooday, and D. C. Ellwood (ed.), Microbial polysaccharides and polysaccharases. Academic Press, Inc., London.
25. **Sutherland, I. W., and D. C. Ellwood.** 1979. Microbial exopolysaccharides—industrial polymers of current and future potential, p. 107–150. *In* A. C. Brill, E. C. Ellwood, and C. Ratledge (ed.), Microbial technology current state, future projects, Symposium 29. Cambridge University Press, Cambridge.
26. **Ulitzur, S., and M. Shilo.** 1970. Effect of *Prymnesium pavum* toxin, cetyltrimethylammonium bromide and sodium dodecyl sulfate on bacteria. J. Gen. Microbiol. **62**:363–370.
27. **Zajic, J. E., and C. J. Panchal.** 1976. Bioemulsifiers. Crit. Rev. Microbiol. **5**:36–66.
28. **Zajic, J. E., and W. Seffans.** 1984. Biosurfactants. Crit. Rev. Biotechnol. **1**:87–107.
29. **Zosim, Z., D. Gutnick, and E. Rosenberg.** 1982. Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter calcoaceticus* RAG-1 emulsan. Biotechnol. Bioeng. **24**:281–292.
30. **Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. **37**:414–420.