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# Localization of Emulsan-Like Polymers Associated with the Cell Surface of *Acinetobacter calcoaceticus*

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Various immunochemical techniques were employed to probe the relationship between the extracellular emulsifying agent (emulsan) and the cell-associated form of the polymer in *Acinetobacter calcoaceticus* RAG-1. Using an emulsan-specific antibody preparation, immunocytochemical labeling revealed that an emulsan-like antigen is a major component of the 125-nm minicapsule which envelopes the exponential-phase cell of the parent strain. The marked reduction of this capsule in stationary-phase cells was correlated with the production of extracellular emulsifying activity. Crossed immunoelectrophoresis techniques demonstrated that the major antigenic component (S1) of the culture supernatant fluid is immunochemically identical to purified emulsan, yet electrophoretically distinct. The characteristics of the parent strain were compared with those of two phage-resistant mutant strains which are defective in extracellular emulsan production. One of these mutants, termed TR3, lacked both the emulsan-like capsule on the cell surface and the extracellular S1 component. A second phage-resistant emulsan-defective mutant (TL4) was characterized by an antigenically altered and inactive form of extracellular emulsan. A relatively small amount of emulsan-like capsular material was consistently demonstrated on the cell surface of this mutant. The correlation between phage sensitivity and extracellular emulsan production was strengthened by the fact that emulsan-specific antibodies inhibited both emulsification activity and phage adsorption onto cells of the parent strain.

Emulsan is a polyanionic D-galactosamine-containing extracellular bioemulsifier produced by the hydrocarbon-degrading microoganism *Acinetobacter calcoaceticus* RAG-1 (18, 27). In addition to the heteropolysaccharide backbone, the polymer contains fatty acid esters and amides, as well as noncovalently associated protein. The protein can be removed by phenol extraction to yield a water-soluble polymer, termed apoemulsan, which retains the emulsifying activity. In addition to its high molecular weight ($9.9 \times 10^5$), emulsan differs from other bioemulsifiers (26) in that its production does not require prior growth of RAG-1 on hydrocarbon substrates.

Although emulsan was initially identified as an extracellular emulsifier, recent reports from this laboratory suggest that an emulsan-like polymer may also be associated with the cell surface of RAG-1. For example, mutants defective in the production of extracellular emulsan were found to be incapable of adsorbing a bacteriophage specific for the wild-type strain, indicating an altered cell surface (15). Moreover, antibodies prepared against purified emulsan were shown to block the adsorption of such phages to the wild-type cells (15). An enzyme-linked immunosorbent assay using these antibodies has been used to demonstrate a difference in the amount of cell-associated cross-reacting material between exponential and stationary-phase cells (7, 8).

In the present report, these antibodies were used to probe the presence and disposition of an emulsan-like polymer on the cell surface of RAG-1. The characterization included immunoadsorption to produce an emulsan-specific immunoglobulin preparation which was used for crossed immunoelectrophoresis (CIE) analysis and specific immunocytochemical labeling of both parent and emulsan-defective mutants.

## MATERIALS AND METHODS

**Bacterial and phage strains and growth conditions.** *A. calcoaceticus* RAG-1 (ATCC 31012) was isolated as previously described (16). *A. calcoaceticus* RAG-TR3 and RAG-TL4 were selected on the basis of resistance

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to phage ap3 from the wild-type RAG-1 and from the lysine auxotroph RAG-92, respectively. Unless otherwise stated, the bacteria were cultivated in a minimal medium containing 22.2 g of $K_2HPO_4$, 7.26 g of $KH_2PO_4$, 4.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$ in 1,000 ml of distilled water. Unless otherwise stated, ethanol (2% [vol/vol]) was added as a carbon source, and lysine (100 μg/ml) was added to the culture medium of the auxotroph (TL4). Starter cultures were prepared by inoculating single colonies into 2 ml of minimal medium and incubating them overnight on a New Brunswick Gyrotory incubator shaker (model G25) at 30°C at 350 rpm. The overnight starter was diluted 1:100 into fresh minimal medium (20 ml in 100-ml flasks) and grown under the same conditions. Exponential-phase cells were harvested at a turbidity of 40 to 70 Klett units (K.U.) measured in a Klett-Summerson colorimeter with a green filter. Stationary-phase cells were usually harvested after 2 days of growth unless otherwise stated. Standard techniques for the isolation, characterization, propagation, and titration of phage ap3 have been described previously (15).

**Preparation of culture supernatants.** Cells were grown for 72 h in minimal medium supplemented with 2% (vol/vol) ethanol. The cultures were centrifuged at 12,000 × *g* for 10 min in a Sorvall SS-34 rotor, and the supernatant fluids were used for further analyses.

**Apoemulsan preparation and emulsification assay.** Emulsan was prepared by ammonium sulfate fractionation (18). Apoemulsan, which was kindly provided by Z. Zosim, was prepared by hot phenol extraction of emulsan (27). The resulting purified protein-free preparation used in this study had an emulsifying activity of 150 U/mg and a reduced viscosity of 490 $cm^3/g$. Emulsification activity and viscosity measurements were performed as reported earlier (18).

**Inhibition of phage adsorption.** A sample (1 ml) of exponentially growing RAG-1 cells ($10^7$ cells per ml, 5 K.U.) was incubated with 0.2 ml of the appropriate antibody preparation for 30 min at 30°C before the addition of phage. These cultures were then infected with phage ap3 to a final titer of $3.5 \times 10^7$ PFU/ml. After 10 min of incubation at 30°C, the cells were removed by centrifugation (12,000 × *g* for 10 min in a Sorval SS-34 rotor), and the supernatant fluid was titrated for residual PFU (15).

**Preparation of antibodies.** The antibody preparation used in this study was described previously (7, 8, 15). Antiserum was raised in rabbits by S. Goldman against a purified preparation of emulsan (containing 15% residual protein). The immunoglobulin G (IgG) fraction was purified from the antiserum by ammonium sulfate precipitation and subsequent ion-exchange chromatography on DEAE-cellulose (12).

**Adsorption of antibodies onto cells.** Cultures (100 ml) of the appropriate *A. calcoaceticus* strain were grown for 24 h and centrifuged (12,000 × *g* for 15 min in a Sorvall SS-34 rotor), and the pellet was washed three times in 150 mM potassium phosphate buffer, pH 7.0. The washed pellet was suspended in 2 ml of the IgG preparation, and the suspension was shaken gently for 5 h at 4°C. The cells were then centrifuged as described above, and the supernatant fluid was used to suspend a fresh washed cell pellet. The procedure was repeated, and the resulting supernatant fraction comprised the appropriate adsorbed antibody preparation.

**CIE.** CIE was performed essentially by the method of Weeke (23). Agarose (1% Pharmacia agarose A) was dissolved in 0.02 M barbital buffer (pH 8.6), which was also used as the running buffer in both the first and second dimensions. Electrophoresis was performed on glass plates (76 by 51 mm; Chance Bros. Ltd., Smethwick, United Kingdom) coated with the agarose gel (2.0 mm thick). Samples (7 μl) of the appropriate culture supernatant fluid (undiluted) or purified emulsan (20 μg) were run in the first dimension at 200 V for approximately 1.5 h (bromphenol blue was used as a marker) in a Shandon electrophoresis chamber. In the second dimension, a 15-mm intermediate gel strip was included to separate the antigen- and antibody-containing gels. To the antibody-containing gel, 10 μl of the undiluted immunoglobulin was added per ml of agarose (total volume, 5 ml). For crossed-line immunoelectrophoresis, supernatant fluids of either RAG-1. TL4 (10 μl), TR3 (25 μl), or 20 μg of purified apoemulsan were added to the intermediate gel strip. For tandem CIE, a second antigenic well (containing 20 μg of purified apoemulsan) was punched into the gel strip 4.0 mm to the anodal side of the reference antigen (appropriate supernatant fraction). Second-dimensional electrophoresis was performed at 45 V for 16 h.

**Immunocytochemical labeling procedure.** A suspension of the desired *A. calcoaceticus* RAG-1 strain containing approximately $10^9$ cells was washed twice by centrifugation (8,000 × *g*, 10 min) with 0.1 M cacodylate buffer (pH 6.5). The sedimented cells were suspended in the same buffer containing the TR3 cell-adsorbed antibody preparation (to a final concentration of 0.5 mg/ml). The resultant suspension was incubated for 15 min at room temperature and for another 45 min at 4°C. The cells were then centrifuged, and the pellet was washed gently with cacodylate buffer. The cell pellet was prefixed for 1 h with a solution of cacodylate buffer containing 1% glutaraldehyde and 0.002% ruthenium red. Before interaction with the cells, the prefixation solution was passed through a HA 0.2-μm filter (Millipore Corp., Boston, Mass.) and centrifuged subsequently at 100,000 × *g* for 3 h at 4°C in a Beckman fixed-angle type 50 rotor. After prefixation with ruthenium red, the cells were washed three times with cacodylate buffer, enrobed in 4% agar, and fixed in Karnovsky fixative, pH 6.5 (2, 10). The samples were washed three times with cacodylate buffer, postfixed with 1% $OsO_4$ for 1 h, washed with distilled water, and dehydrated in graded solutions of ethanol. The dehydrated cells were embedded in Epon (13), and sections (gray interference color) approximately 60 nm thick were obtained with an LKB-III ultramicrotome. The sections were mounted on Formvar-coated 200-mesh copper grids and coated with carbon. The sectioned samples were observed in a JEOL 100B electron microscope at an accelerating voltage of 80 kV.

**General methods.** Hexosamines were determined by the indole nitrite method (4) with galactosamine as the standard. The hexosamine content of cells was determined for cultures washed and suspended in water (1,000 K.U.), hydrolyzed in 5 N HCl for 20 min, and neutralized with NaOH. The cell dry weight was determined after the drying of a portion of washed cells overnight at 80°C. The viscosity was measured on 1.0-ml samples in an Ostwald Fenske microviscometer at 30°C as previously described (27).

## RESULTS

**Analysis of cell-free supernatant fluid by CIE.** When the cell-free culture fluid of RAG-1 was subjected to CIE with antibodies raised against emulsan, three distinct components, designated CIE peaks S1, S2, and S3 (Fig. 1a), were consistently found. Similar CIE patterns were obtained with less specific anti-whole cell (1; E. A. Bayer et al., J. Gen. Microbiol., in press) and antisupernatant antibodies. Using these less specific antibodies in the same CIE analysis, we observed that S1-like peaks were immunochemically identical to emulsan (data not shown). Peak S1 (Fig. 1a) apparently contained the major quantity of cross-reacting antigenic material which interacted with the antibody preparation.

Three distinct CIE peaks were also consistently observed in the supernatant fluids produced by the majority of phage ap3-resistant mutant strains. However, the mutants always exhibited an alteration in the major peak, S1. The most typical CIE pattern of ap3-resistant mutants was obtained with RAG-TR3. The CIE pattern of this mutant type was characterized by the presence of peak S1a (Fig. 1b). Peak S1a migrated slightly faster than S1 of the wild type (RAG-1) and exhibited a completely different precipitate morphology. In contrast, peaks S2 and S3 of RAG-TR3 appeared identical to those of RAG-1.

RAG-TL4 is an example of another type of phage ap3-resistant mutant, which was isolated at a much lower frequency. The phenotypic characteristics of this emulsan-defective mutant strain were very similar to those of all of the other phage ap3-resistant mutants; however, the CIE pattern (Fig. 1c) of supernatant fluids from RAG-TL4 revealed an alteration in the major component. This altered peak (designated S1b) was characterized by a diffuse, but heavy, precipitate morphology and exhibited an electrophoretic mobility faster than that of the wild-type S1. As in the case of mutant TR3, components S2 and S3 were similar to those of RAG-1.

**Comparison of purified apoemulsan with cell-free supernatant components.** Apoemulsan, purified from the supernatant fluid of RAG-1 as described above, had a residual protein content of less than 1%. This preparation gave a characteristic double peak in CIE gels, suggesting two closely related conformations or constituents (Fig. 2). To determine which component(s) of the RAG-1 supernatant fluid corresponded to purified apoemulsan, we performed both tandem CIE and crossed-line immunoelectrophoresis. Crossed-line CIE of purified apoemulsan in which RAG-1 supernatant fluid was included in the intermediate gel strip demonstrated complete identity between S1 and apoemulsan (Fig.

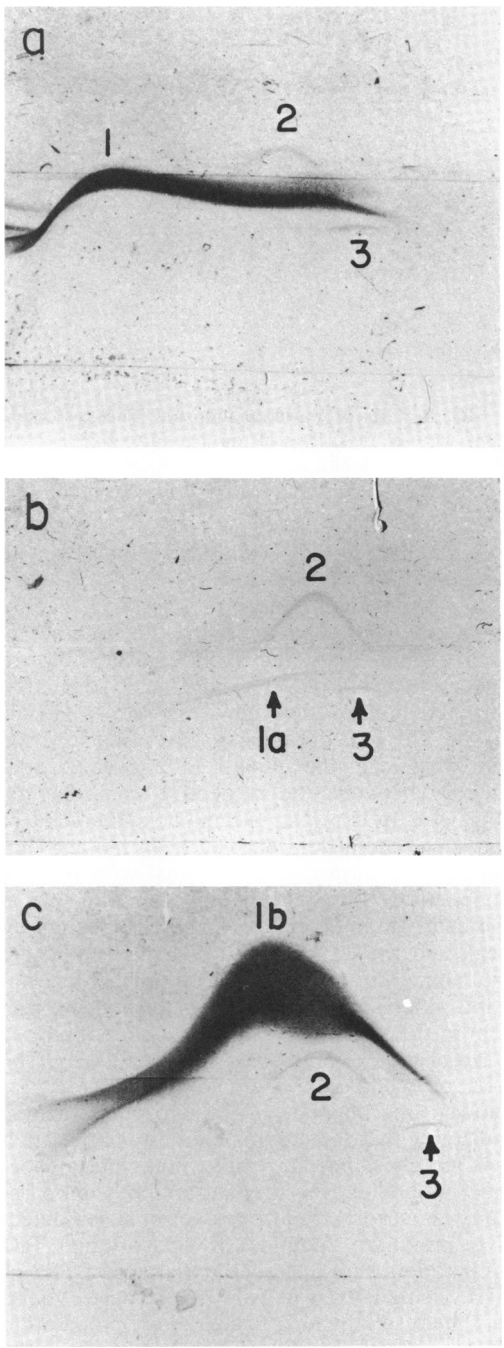


FIG. 1. CIE patterns of supernatant fluids derived from (a) RAG-1, (b) TR3, and (c) TL4. The origin is located at the bottom left of all gels; the anode is on the right in the first dimension and at the top in the second. The agarose in the second dimension contained unadsorbed antibodies. No antibodies were added to the intermediate gel. The precipitin peaks were enumerated as shown.

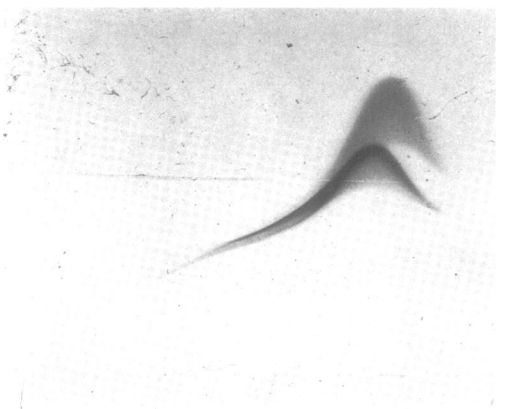

FIG. 2. CIE of purified emulsan. The gel conditions were as described in the legend to Fig. 1. Note the double peak and cathodic trail.

3a). Similar results were also obtained with tandem CIE (data not shown).

It was of further interest to determine whether the components characterizing the supernatant fluids of mutants defective in emulsan (Fig. 1b and c and Table 1) were antigenically related to the apoemulsan derived from the wild-type strain. The CIE of apoemulsan versus supernatant fluid from the mutant TR3 revealed nonidentity between the respective component(s) (Fig. 3b). In contrast, the major component of the supernatant fluid derived from mutant TL4 showed partial antigenic identity with purified apoemulsan (Fig. 3c). The presence of a spur at the cathodic intercept between the apoemulsan peak and the TL4 antigen (S1b) line indicated that some, but not all, of the antigenic determinants associated with the apoemulsan were present in the supernatant fluid of mutant TL4.

**Adsorption of antibodies by mutant cells.** The CIE of wild-type supernatant fluid (Fig. 1a) showed one major (S1) and two minor bands, indicating that the original antibody preparation was multispecific. To remove non-emulsan-specific antibodies, the original antibody preparation was adsorbed onto cells of emulsan-defective mutants. Samples of the original IgG preparation were adsorbed onto cells of TR3 or TL4. Using RAG-1 cells as a control, we found that the resultant adsorbed antibody preparation was completely inactive, i.e., no antigenic peaks were visible by CIE (data not shown). This indicated that the three antigenic components shown in Fig. 1a originated intact on the cell surface of the parent strain.

The adsorption of the IgG preparation onto mutant TR3 cells yielded an antibody preparation termed Ab-3. When this latter preparation was used, the CIE patterns of RAG-1 and TL4

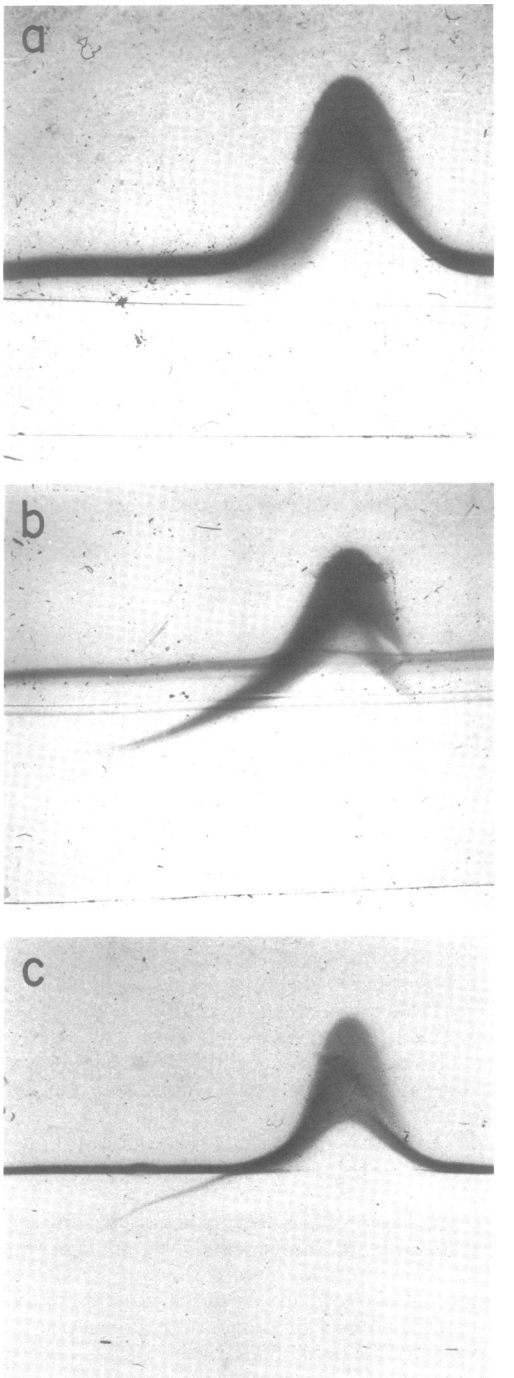


FIG. 3. Identity tests between purified emulsan and components contained in supernatant fluids. The crossed-line immunoelectrophoresis of purified emulsan (first dimension) was performed against (a) RAG-1, (b) TR3, and (c) TL4 supernatant fluids included in the respective intermediate gels. Note the complete identity of all emulsan determinants with the major precipitation line (S1) of the RAG-1 supernatant. A

TABLE 1. Characteristics of *A. calcoaceticus* RAG-1 strains used in this study

| Strain | Growth at 72 h (K.U.) | Sensitivity to phage ap3 | Emulsan yield[a] | |
|---|---|---|---|---|
| | | | (U/ml) | $\eta_{sp}$ |
| RAG-1 | 1,030 | Sensitive | 323 | 0.32 |
| TR3 | 940 | Resistant | 22 | 0.07 |
| TL4 | 1,100 | Resistant | 40 | 0.17 |

[a] The emulsan yield is expressed both in terms of emulsan activity and specific viscosity ($\eta_{sp}$) of the cell-free culture medium after 72 h of growth.

TABLE 2. Inhibition of emulsan activity by various antibody preparations[a]

| Antibody | Emulsan activity (U/ml) | % Activity |
|---|---|---|
| None | 83.5 | 100 |
| Unadsorbed | 24.0 | 29 |
| Ab-3 | 28.5 | 34 |
| Ab-4 | 84.0 | 101 |
| RAG-1 adsorbed | 81.0 | 97 |
| Preimmune serum | 91.0 | 109 |

[a] The appropriate antibody preparation (0.1 mg/ml of protein) was incubated for 2 h at 37°C with purified apoemulsan (0.5 mg/ml). Residual emulsan activity was then determined by the standard emulsification assay.

superantant fluids resembled those in Fig. 1 in that the S1 (RAG-1) and S1b (TL4) antigens were retained, whereas S2 and S3 were eliminated (Fig. 4a and b). In the case of peak S1b (Fig. 4b), the precipitate morphology was significantly altered after adsorption (cf. Fig. 1c). It is possible, therefore, that some of the antigenic determinants which comprise peak S1b are also present on the cell surface of mutant TR3. No CIE peaks were visible in TR3 supernatant fluids, indicating the complete adsorption of the relevant antibodies (data not shown). It is of interest to note that the CIE pattern of purified apoemulsan using Ab-3 (Fig. 5a) was virtually identical to that obtained using unadsorbed antibodies (Fig. 2). Therefore, emulsan cross-reacting material is either missing or present in very low amounts both in the supernatant fluid and on the cell surface of TR3.

When the original antibody preparation was adsorbed onto cells of mutant TL4 and the resulting adsorbed antibody, Ab-4, was tested by CIE against either the purified apoemulsan or the RAG-1 supernatant fluid, the pattern shown in Fig. 5b was obtained. This diffuse, unilateral band apparently represented the component(s) which was missing from the TL4 supernatant fluid, but present in the supernatant fluid of the wild type (Fig. 3b).

**Effect of adsorbed and unadsorbed antibody preparations on emulsan activity and phage adsoprtion.** The results given in Table 2 show the effect of different antibody preparations on the activity of purified apoemulsan. The only IgG preparations which brought about significant inhibition were the unadsorbed and Ab-3 preparations. Similarly, only these two IgG preparations were effective in inhibiting the adsorption of phage ap3 to the cells of the parent RAG-1 (Table 3). Of interest was the finding that antibody Ab-4, prepared by the adsorption of the

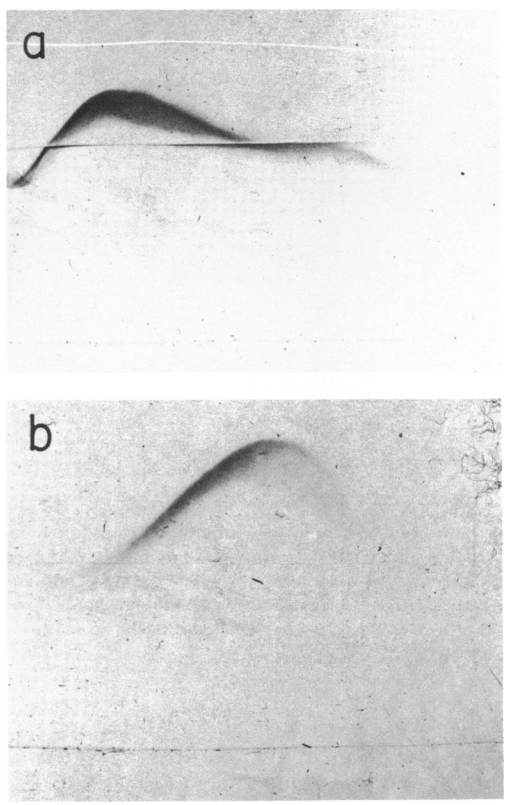


FIG. 4. CIE patterns of supernatant fluids derived from (a) RAG-1 and (b) TL4 using antibodies adsorbed to TR3 cells (antibody preparation Ab-3). Note the absence of components S2 and S3 which characterize the CIE patterns of the native unadsorbed antibody preparation (Fig. 1a and c, respectively). The electrophoretic conditions were as described in the legend to Fig. 1.

minor line (S2) is visible which fails to interact with the emulsan peaks. Note the lack of fusion between the emulsan peaks and the TR3 precipitation lines, whereas the major TL4 antigen partially identical to the emulsan determinants as indicated by the cathodic spur in addition to the fused pattern. The electrophoretic conditions were identical to those described in the legend to Fig. 1; unadsorbed antibodies were used.

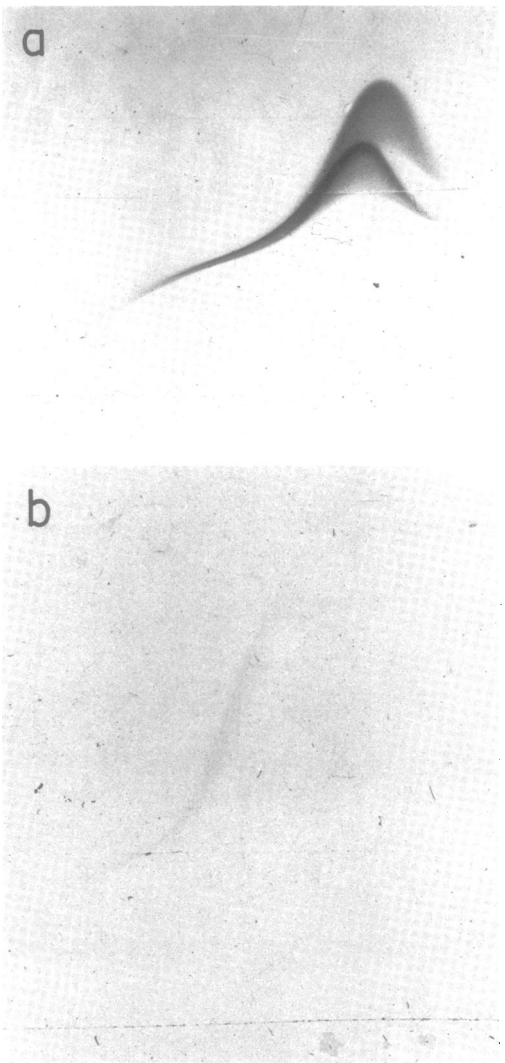


FIG. 5. CIE patterns of purified emulsan. In the second-dimension gel, (a) contained antibodies adsorbed to TR3 cells (Ab-3), and (b) contained antibodies adsorbed to TL4 cells (Ab-4). Compare with the CIE pattern of purified emulsan using unadsorbed antibodies (Fig. 2). The electrophoretic conditions were as described in Fig. 1.

original IgG preparation onto cells of the emulsan-defective mutant TL4, lost its capacity to inhibit either emulsan activity or phage adsorption. The results indicate that the cell surface of mutant TL4 contains both antigens which cross-react with extracellular emulsan and antigens which resemble a component(s) of the phage ap3 receptor. In contrast, both of these antigens are missing from the cell surface of mutant TR3.

**Visualization of emulsan-like antigens on the cell surface.** The antibody adsorption experiments indicated that exponential cells of the wild-type RAG-1 contained emulsan-like antigens on the cell surface. Moreover, previous results from this laboratory suggested that extracellular emulsan production is associated with the release of an emulsan-like polymer from the surface of the cell into the growth medium during the transition from exponential to stationary phase (7, 8, 27). To directly visualize antigens resembling emulsan on the cell surface, we used a modification of immunocytochemical labeling techniques for staining capsular material (5, 14) with cells of RAG-1 (Fig. 6 and 7), mutant TR3 (Fig. 8), and mutant TL4 (Fig. 9). Whereas exponentially growing cells of RAG-1 exhibited a stable capsule of uniform thickness (about 125 nm), stationary-phase cells of the same strain (Fig. 7a and b) were smaller and showed a markedly reduced capsule. Exponential cells of mutant TR3 which were stained with unadsorbed antibody (Fig. 8a and b) were virtually devoid of label, whereas the cells of mutant TL4 (Fig. 9a and b) were characterized by variable amounts of capsular material on the cell surface, always much less than that found either on the exponential- or the stationary-phase cells of RAG-1 (Fig. 6 and 7).

TABLE 3. Effect of adsorbed antibody preparations on phage ap3 adsorption to *A. calcoaceticus* RAG-1[a]

| Antibody | Titer (PFU/ml) | Residual titer (%) |
|---|---|---|
| Control[b] | $3.5 \times 10^7$ | 100 |
| None | $4.0 \times 10^6$ | 11 |
| Unadsorbed | $3.1 \times 10^7$ | 89 |
| Ab-3 | $2.6 \times 10^7$ | 74 |
| Ab-4 | $5.1 \times 10^6$ | 15 |
| RAG-1 adsorbed | $5.5 \times 10^6$ | 16 |
| Preimmune serum | $6.0 \times 10^6$ | 17 |

[a] *A. calcoaceticus* RAG-1 cells were incubated in the presence of the given antibody preparation (0.5 mg/ml of protein) or the appropriate control. Phage ap3 was then added ($3.5 \times 10^7$ PFU/ml), the culture was incubated for an additional 10-min period, the infected culture was centrifuged, and the resulting supernatant fluid was titrated for unadsorbed phage.

[b] Neither RAG-1 cells nor antibodies were added to the preparation of phage ap3 (i.e., no preadsorption of phage to RAG-1 cells).

**Hexosamine content of hydrolyzed cells.** Chemical analyses of washed cells (Table 4) revealed a distinct correlation between the cell-associated hexosamine content and the presence or absence of an emulsan capsule. Early-exponential-phase cells of RAG-1 contained about 5% hexosamine. In contrast, stationary-phase RAG-1 cells, harvested at 50 h, contained only 2.4% hexosamine when grown on 2% (vol/vol) ethanol. This reduction in cellular hexosamine con-

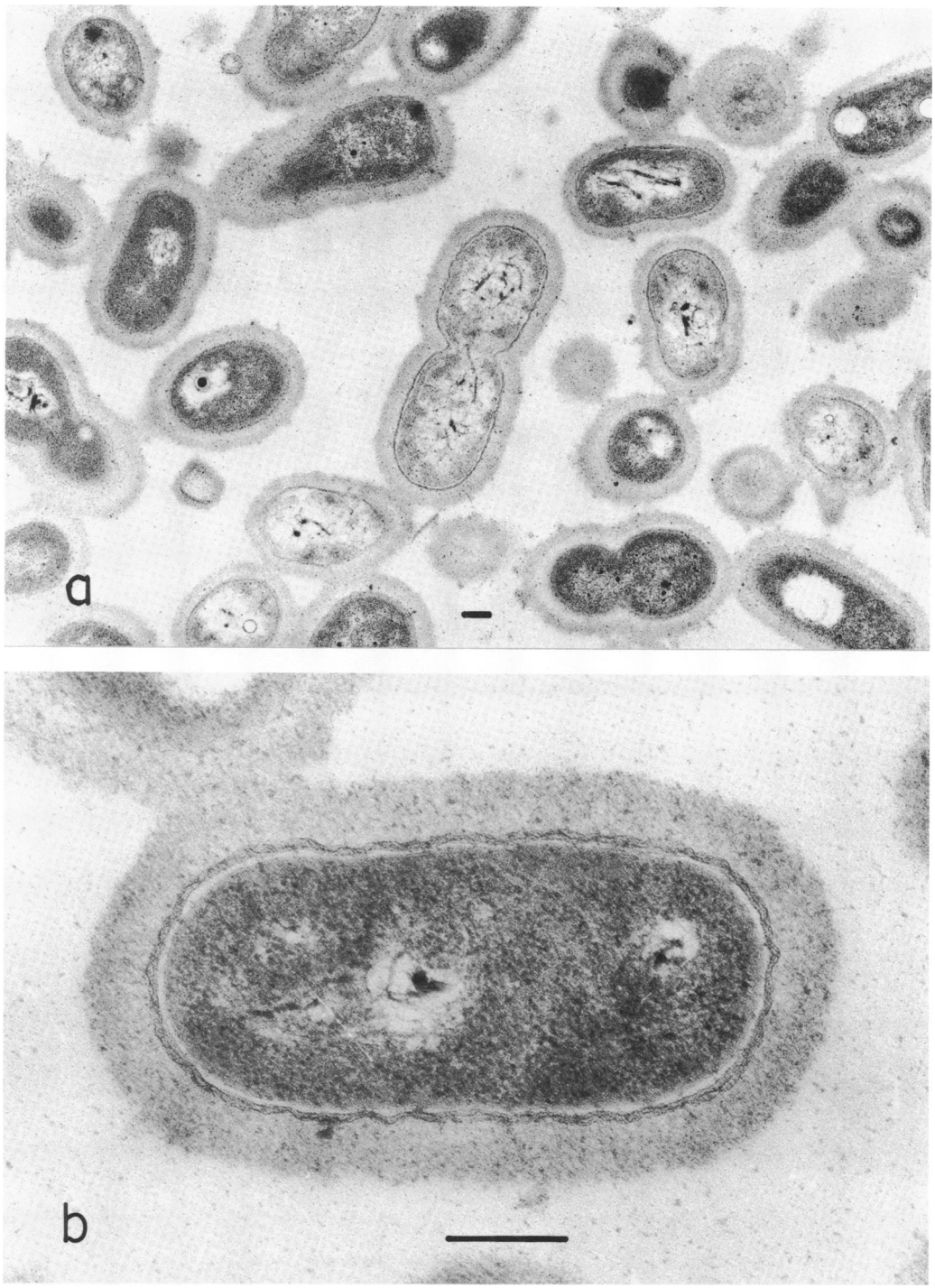


FIG. 6. Transmission electron microscopy of exponential-phase *A. calcoaceticus* RAG-1. (a) The cells were harvested in the exponential phase of growth and exposed to sequential treatments with Ab-3 (TR3-adsorbed) antibody preparation and ruthenium red before processing for thin-section electron microscopy. Note the uniform staining of capsular material which encompasses the cell at an average thickness of approximately 125 nm from the outer cell membrane. (b) A higher magnification of a single exponential-phase RAG-1 cell. The bars represent 250 nm in this and subsequent micrographs.

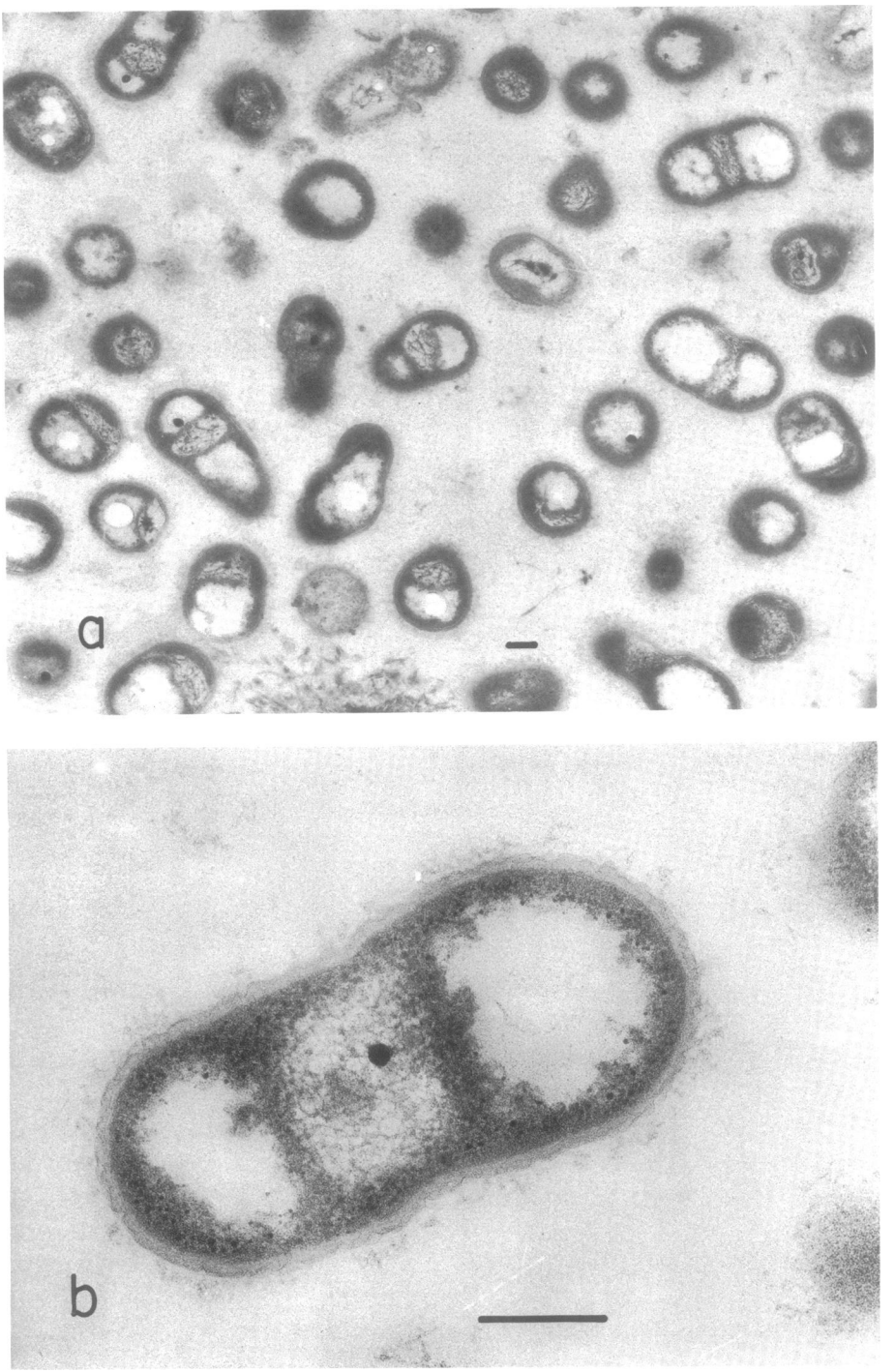


FIG. 7. Transmission electron microscopy of stationary-phase *A. calcoaceticus* RAG-1. (a) The cells were harvested in the stationary phase of growth and treated as described in the legend to Fig. 6. Note the dispersed arrangement of antigenic components on the cell surface and the reduced size of the cells compared with those harvested in the exponential phase. (b) A higher magnification of a single stationary-phase RAG-1 cell.

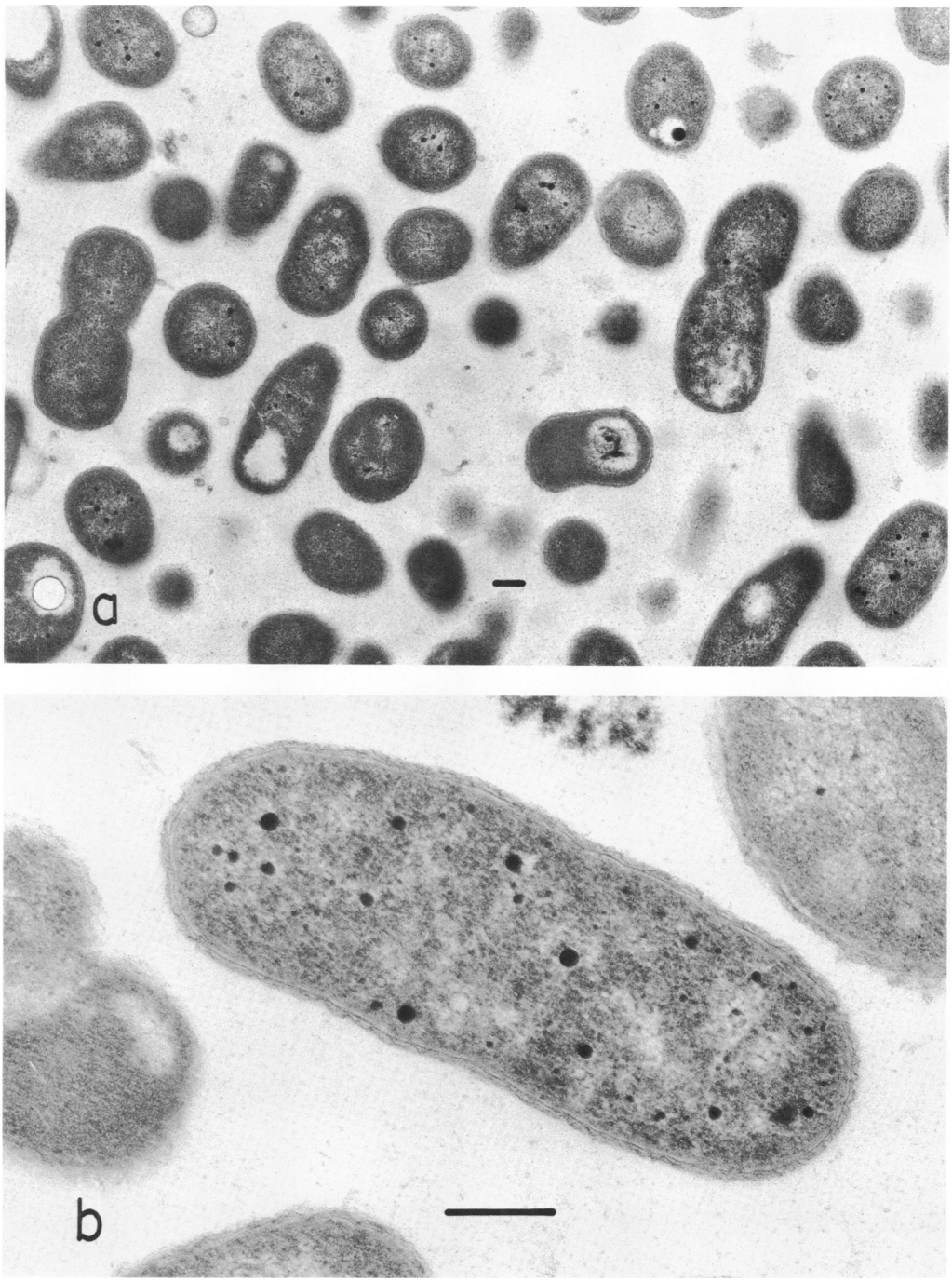


FIG. 8. Transmission electron microscopy of mutant TR3. (a) TR3 cells harvested in the exponential phase of growth and treated as described in the legend to Fig. 7. Note the total absence of capsular material in this mutant. (b) A higher magnification of a single TR3 cell.

tent was accompanied by an accumulation of hexosamine (170 μg/ml) and emulsifying activity (200 U/ml) in the cell-free supernatant. However, when RAG-1 was grown under identical conditions in the presence of 0.5% (vol/vol) ethanol and harvested at 50 h, the hexosamine

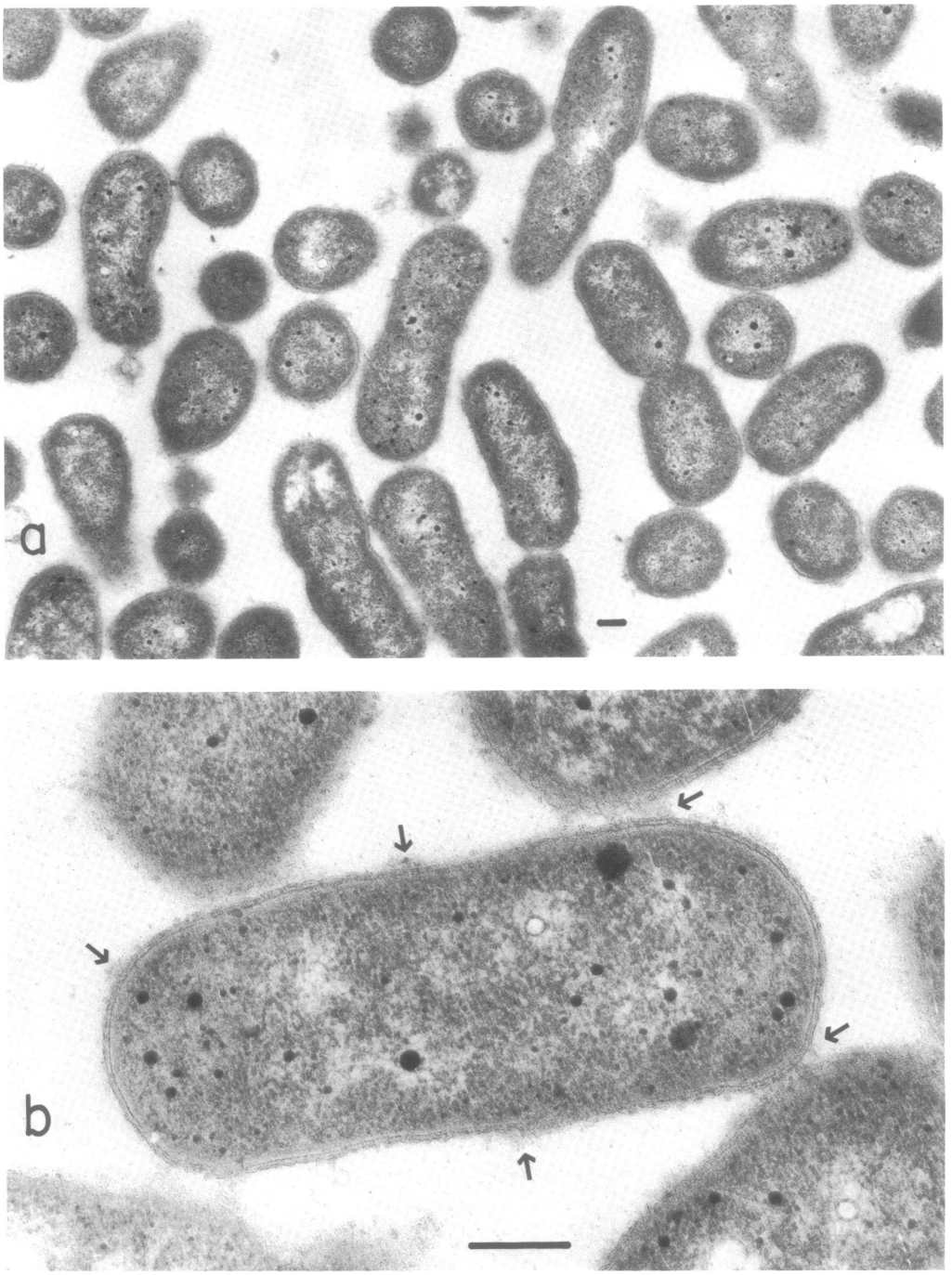


FIG. 9. Transmission electron microscopy of mutant TL4. (a) TL4 cells harvested in the exponential phase of growth and treated as described in the legend to Fig. 7. (b) A higher magnification of a single TL4 cell. Note the isolated accumulation of capsular material (arrows).

content was identical to that of the exponential-phase cells (4.8%). The cell-free supernatant of these stationary-phase cultures grown on low ethanol (0.5% [vol/vol]) contained less than 40 μg of hexosamine per ml and showed emulsifying activity of only 35 U/ml. That high cellular

TABLE 4. Hexosamine content of hydrolyzed cells[a]

| Culture | % Ethanol (vol/vol) | Dry wt (mg/ml) | Hexosamine content | |
|---|---|---|---|---|
| | | | mg/ml | % |
| Log-phase RAG-1 | 2.0 | 3.8 | 0.199 | 5.2 |
| Stationary-phase RAG-1 | 2.0 | 3.5 | 0.084 | 2.4 |
| Log-phase RAG-1 | 0.5 | 4.0 | 0.190 | 4.8 |
| Stationary-phase RAG-1 | 0.5 | 3.6 | 0.172 | 4.8 |
| Log-phase TR3 | 2.0 | 3.9 | 0.030 | 0.8 |
| Stationary-phase TR3 | 2.0 | 3.5 | 0.024 | 0.7 |

[a] Exponentially growing cultures (60 to 70 K.U.) and stationary-phase cultures (800 to 1,000 K.U.) were harvested, washed once in cold water, and suspended in water to about 1,000 K.U. Samples of 5 ml were taken 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.

levels of hexosamine apparently reflect the presence of emulsan is further supported by the analysis of washed cells of mutant TR3, which is defective in emulsan production. The cells of this mutant contained only 0.9% hexosamine, and the supernatant fraction exhibited neither significant emulsifying activity nor hexosamine.

## DISCUSSION

This paper deals with the relationship between active emulsan normally present in the cell-free supernatant fluid of *A. calcoaceticus* RAG-1 and the presence of an emulsan-like polymer on the cell surface. To study this relationship, we developed a CIE analysis to identify antigens present both on the cell surface and in the supernatant fluid, which react with anti-emulsan antibodies. The original antibody preparation used here was raised against purified emulsan, yet showed three distinct components in CIE. The fact that the adsorption of antibodies to the cells of the parent strain (RAG-1) removed these bands from the CIE pattern of supernatant fluids provides evidence that these components are present on the cell surface, as well as in the medium. CIE analysis of supernatant fluids from the parent strain was carried out with a specific immunoglobulin probe adsorbed onto the surface of the cells of the emulsan-defective mutant TR3. Under these conditions, two of the three bands were absent from the CIE pattern, leaving only the major S1 band. Moreover, the adsorbed antibody preparation inhibited both emulsan activity and the adsorption of phage ap3 to the surface of wild-type cells. Adsorption could therefore be used to generate a specific anti-emulsan immunoglobulin preparation.

Apoemulsan gave the same CIE pattern regardless of whether unadsorbed or specific antisera were used. When pure apoemulsan was compared with emulsan in the supernatant fluid of the parent strain (band S1, Fig. 1a and 4a), the two bands were shown to be immunochemically identical (Fig. 3a) but to differ in electrophoretic mobility. The difference is most likely due to the association of emulsan with protein (18, 27), which was subsequently removed during the preparation and purification of apoemulsan. Antigens in the mutant supernatant fluids were either partially identical (TL4) or nonidentical to apoemulsan, strongly suggesting that the mutation(s) in these strains results in the absence of or alterations in the carbohydrate portion of the emulsifier.

Unlike mutant TR3, the adsorption of antibodies onto mutant TL4 essentially abolished the inhibitory effect of the resultant antibody preparation (Ab-4) with respect to emulsan activity and phage adsorption. In addition, Ab-4 exhibited a very weak CIE precipitation pattern (Fig. 5b) with purified apoemulsan or the crude cell-free supernatant fluid of RAG-1. It seems, therefore, that a slight change in the mutant TL4 antigen resulted in a completely different disposition of capsular material on the cell surface (Fig. 9), lack of phage ap3 adsorption, and an inactive cell-free product with respect to emulsifying activity. These observations suggest that small changes in the structure of the exopolysaccharide can abolish functional activities associated with the polymer without the destruction of specific antigenic determinants. In this regard, the defect in mutant TL4 may resemble mutations which lead to a slight change in protein structure and the elimination of activity without affecting the immunological determinants of the molecule (24, 25).

Previous reports from this laboratory have indicated the presence of an emulsan-like polymer on the cell surface of RAG-1: (i) the release of prelabeled polymer into the medium after the exposure of RAG-1 cells to the protein synthesis inhibitor chloramphenicol (19); (ii) the isolation of cell surface mutants defective in emulsan production by selecting for mutants resistant to a specific virulent bacteriophage ap3 (15); and (iii) the gradual loss from the cell surface of emulsan cross-reacting material as detected by an enzyme-linked immunosorbent assay (7, 8). The specific immunocytochemical labeling experiments described in this report are consistent with the above findings and directly demonstrate a reduction in the exocellular capsule between exponential-phase and stationary-phase cells. Chemical analyses of washed cells indicate that prolonged growth in batch culture alone could not account for this difference. A reduction in

capsule size or hexosamine content did not occur when the cells were grown on low ethanol, regardless of the culture conditions. Moreover, no capsule was observed with emulsan-defective mutants, such as mutant TR3 (Fig. 8), and only residual amounts of hexosamine were associated with the cells of this mutant (Table 4). Mutant TR3 was completely devoid of this capsular material, whereas mutant TL4 showed a reduced and altered distribution of emulsan-like antigens on the cell surface.

Bacterial strains that produce capsules may also produce an exopolysaccharide as slime; only a few reports, however, deal with the slime and capsular exopolysaccharides of the same strain (3, 6, 9, 22). In such cases, comparison of the cell-associated polysaccharide and the cell-free form revealed that both had an identical polysaccharide backbone and were similar immunochemically. Although in some cases apparent differences in size and shape were found between the unpurified forms of capsule and slime exopolysaccharide, the purified forms were identical (6, 9). In contrast to many other exopolysaccharides, emulsan from RAG-1 was first recognized by its functional activity (hydrocarbon-emulsifying activity). Only recently has a capsule-like form of this polymer been demonstrated on the cell surface of RAG-1. To date, no chemical or immunochemical differences have been observed between the two forms of the exopolysaccharide. On the other hand, there are functional differences between the cell-associated and cell-free forms of emulsan. Cell-associated emulsan has been implicated as the phage ap3 receptor, whereas the cell-free form does not inactivate or adsorb phage ap3 (15). This phenomenon has been reported for other exopolysaccharide phage receptors that lose their receptor activity upon release from the cell surface (11). Furthermore, cell-free emulsan has strong emulsification activity, whereas RAG-1 cells covered with the polymer in the form of a minicapsule have little or no activity (18). One interpretation of these results is that a change in the conformation of cell-associated exopolysaccharide occurs upon release from the cell surface.

The fact that activities can be described for both the cell-associated and cell-free forms of the polysaccharide clearly indicates that exopolysaccharides are not passive structural components of the bacterial cell surface. The release of emulsan is affected by clearly defined physiological conditions, i.e., emulsan is released as a result of amino acid starvation or chloramphenicol addition or during the unbalanced growth that occurs at the end of the exponential phase (18, 19). In all cases, emulsan release requires an energy and carbon source. Some of the characteristics of emulsan resemble properties generally associated with enzymes, such as substrate specificity (17), cofactor requirements (18), and conformational requirements for the expression of emulsification and receptor activities. The fact that emulsan exhibits these properties supports the concept that complex carbohydrates may play specific roles due to their heterogeneity and unique structural properties (20, 21). In this regard, recent evidence with emulsan-deficient mutants (O. Pines, manuscript in preparation) suggests a role for the cell-associated form of emulsan in the growth of RAG-1 on hydrocarbons.

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