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Emulsan in *Acinetobacter calcoaceticus* RAG-1: Distribution of Cell-Free and Cell-Associated Cross-Reacting Material

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Emulsan is an extracellular polymeric bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1. Antibodies prepared against purified emulsan inhibited the activity of the polymer in a standard emulsification test. These antibodies were used to develop a sensitive enzyme-linked immunosorbent assay to monitor changes in cell-free emulsan throughout the growth cycle. This assay was also used to detect emulsan associated with the cell surface and to monitor changes in the distribution of cell-free and cell-associated emulsan throughout the growth cycle. Cells in the early exponential phase exhibited relatively large amounts of cell-associated emulsan which decreased rapidly between the midexponential and early stationary phases. This drop in cell-associated material was accompanied by a rise in the concentration of extracellular polymer. Moreover, in agreement with previous results (C. Rubinovitz, D. L. Gutnick, and E. Rosenberg, manuscript in press), production of cell-free emulsan was enhanced in the presence of chloramphenicol. The release of this material from the cell surface in the presence of chloramphenicol apparently involved the synthesis of cell-associated cross-reacting material since the relative amount of such cell-bound polymer remained constant during the treatment with the drug.

The growth of microorganisms on hydrocarbons is often accompanied by emulsification of the insoluble carbon source in the aqueous medium (7, 8, 11). In the case of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1, emulsification is brought about by the production of an extracellular, polymeric bioemulsifier termed emulsan. Emulsan (molecular weight, 10^6) is an anionic, D-galactosamine-containing polymer (12) which exhibits substrate specificity with respect to the hydrocarbons it emulsifies (2, 10), yet is excreted into the growth medium even when RAG-1 is grown on a water-soluble substrate such as ethanol or acetate (12).

Previous results from this laboratory suggested that an emulsan-like polymer is also present on the cell surface. For example, cells of RAG-1 were found to be agglutinated by antiserum prepared against partially purified emulsan (1a). In addition, mutants defective in the production of emulsan were found to be incapable of adsorbing a specific bacteriophage, indicating an alteration in the cell surface of such mutants (7).

In this report, we describe a specific quantitative enzyme-linked immunosorbent assay (ELISA) (4) for the determination of cell-free and cell-associated emulsans. The ELISA was used to monitor changes in the two forms of emulsan throughout the growth cycle of RAG-1. The results indicate that a cell-associated form of emulsan accumulates on the surface of the cell before its release into the medium.

MATERIALS AND METHODS

Bacterial strain. The strain used in this study was *A. calcoaceticus* RAG-1 (8).

Medium and growth conditions. Bacteria were grown in minimal medium containing (per liter of water): $K_2HPO_4 \cdot 3H_2O$, 22.2 g; KH_2PO_4, 7.26 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 4 g; ethanol, 20 g. The bacteria were cultivated in shake flasks on a New Brunswick Gyrotory shaker at 30°C (350 rpm) for 72 h, and growth was monitored by absorbance at 540 nm with a Gilford model 2400 spectrophotometer. An absorbance of 1.0 is equivalent to 0.28 mg of cells (dry weight) per ml.

Preparation of extracellular emulsan. The RAG-1 emulsifier was prepared from the cell-free supernatant of a culture of ethanol-grown cells by ammonium sulfate fractionation as previously described (10). The precipitate was suspended in water, dialyzed against distilled water, and freeze-dried. Further purification was achieved by suspending the precipitate in water (1 mg/ml) and precipitating with cetyltrimethylammonium bromide (CTAB), using a CTAB-to-emulsan ratio of 1:1 (wt/wt). The CTAB precipitate was collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min and suspended to the original volume in 50 mM Na_2SO_4. The insoluble fraction was removed by centrifugation as described above; potassium iodide was added to the viscous supernatant fluid to a final concentration of 2 mg/ml to precipitate the remainder of the CTAB. The soluble fraction containing emulsan

was separated from the CTA-iodide precipitate by centrifugation, dialyzed against distilled water, and freeze-dried. Deproteinized emulsan (apoemulsan) was prepared by hot phenol extraction as previously described (12).

Emulsification assay. Emulsan activity was determined by a standard emulsification assay (12) which is based on emulsification of a mixture of 0.1 ml of hexadecane–2-methylnaphthalene (1:1, vol/vol) in 7.5 ml of Tris-magnesium buffer (20 mM Tris-hydrochloride containing 10 mM $MgSO_4$, pH 7.0). After the mixture was shaken for 60 min at 30°C, the turbidity of the stable hydrocarbon-in-water emulsion was measured in a Klett-Summerson colorimeter. One unit of activity is that amount of emulsan which gives rise to a turbidity of 100 Klett units in the standard emulsification assay.

Preparation of anti whole-cell antiserum. Rabbits were injected intravenously with 10^9 washed cells of *A. calcoaceticus* RAG-1 in the midexponential phase of growth. The rabbits were reinoculated in the same manner after 3 and 6 days. Three days after the third injection, the rabbits were bled. Subsequent bleedings were performed 3 days after the rabbits received a booster consisting of the same RAG-1 cell preparation.

Preparation of antisera against emulsan. Antiserum was raised against emulsan (purified by ammonium sulfate and CTAB treatment). Multiple-site intradermal injections of rabbits were made with a 2-ml emulsion containing 1 volume of emulsan (100 and 500 μg) in distilled water (pH 7.0) and 1 volume of Freund complete adjuvant. Two weeks later, two subsequent booster injections with complete adjuvant were carried out as described above; the rabbits were bled on day 10 after the last injection. Antibody titers were determined by the passive hemagglutination method (1). Antiserum preparations exhibiting hemagglutination titers above 2,000 served as the source for the purification of gamma globulin.

Preparation of gamma globulin. The immunoglobulin fraction was purified from the antiemulsan antiserum by ammonium sulfate precipitation followed by chromatography on Whatman DE22 cellulose (3).

Formation of alkaline phosphatase—gamma globulin conjugate. A mixture of 0.3 ml of alkaline phosphatase (Sigma type VII; 5.5 mg of protein per ml) and 0.7 ml of purified immunoglobulin G (IgG) (1 mg/ml) was dialyzed against phosphate-buffered saline (PBS). Freshly prepared glutaraldehyde (60% in water) was added to the mixture to a final concentration of 0.06%, and the reaction mixture was allowed to stand at room temperature for 4 h (or until a yellow-brown color developed). The mixture was dialyzed against PBS, and bovine serum albumin was added to a final concentration of 5 mg/ml. The conjugate was kept at 4°C and diluted 1:100 into PBS before being used in ELISA. The concentrated enzyme gamma globulin conjugate was stable for at least 4 months at 4°C without loss of enzyme activity.

Measurement of free emulsan in ELISA microplates. The ELISA of Engvall and Perlmann (4, 5), modified by Clark and Adams (4), was used. The assay was carried out as follows. (i) The wells of polystyrene microplates were coated with 10 μg of gamma globulin per ml diluted in 0.05 M carbonate buffer (pH 9.6) for 2 h at 37°C and stored overnight at 4°C. The plates were then washed three times with PBS (pH 7.4) containing 0.05% Tween 20 and 0.02 NaN_3. (ii) To each well, 200 μl of the sample to be tested (containing 10 to 100 ng of emulsan per ml) was added. The plates were incubated for 3 h at 37°C and washed as described before. (iii) To each well, 200 μl of diluted gamma globulin-alkaline phosphatase conjugate was added. The plates were incubated for 2 h at 37°C, stored overnight at 4°C, washed, and dried by shaking in air. (iv) A total of 300 μl of alkaline phosphatase substrate solution (0.6 mg of *p*-nitrophenyl phosphate per ml in 1 M Tris buffer, pH 8.8) was added, and the plates were incubated at room temperature. The reaction was stopped after 30 min by the addition of 50 μl of 3 M NaOH. Absorbance at 405 nm (A_{405}) was determined with a Gilford model 2400 spectrophotometer.

Measurements of cell-associated emulsan. (i) ELISA in microplates. RAG-1 cells were washed and diluted to a concentration of between 10^5 and 10^7 cells/ml in PBS containing Tween 20. A 200-μl volume of the suspension was added to the gamma globulin-coated wells, which were then incubated for 24 h at 37°C. The remaining steps in the procedure were performed as described for the cell-free antigen.

(ii) ELISA on filters. Samples (100 ml each) of washed and diluted RAG-1 cells were incubated in test tubes with 100 μl of diluted gamma globulin-enzyme conjugate for 2 h at 37°C. The suspension was filtered through a membrane filter (pore size, 0.45 μm; diameter, 13 mm) (Sartorius 11305 membrane filters) and washed with 20 ml of PBS-Tween 20. The filters were transferred to 5-ml test tubes containing 700 μl of the substrate (0.6 mg/ml in 1 M Tris, pH 8.8). The tubes were incubated at room temperature with continuous shaking. The reaction was stopped after 2 h by the addition of 100 μl of 3 N NaOH, and A_{405} was determined with a Gilford model 2400 spectrophotometer.

Alkaline phosphatase (type VII), phosphatase substrate (*p*-nitrophenyl phosphate disodium 104), and Tween 20 were purchased from Sigma Chemical Co. The polystyrene plates were Dynatech M129 A flat-bottomed Microelisa plates. Complete adjuvants were from Difco Laboratories.

RESULTS

Inhibition of emulsan activity by antiemulsan antiserum. Previous results have described isolation of antibodies prepared against partially purified emulsan (12). Figure 1 shows the effect of increasing concentrations of gamma globulin from antiemulsan antiserum raised against highly purified emulsan on emulsification of a mixture of hexadecane and 2-methylnaphthalene (1:1, vol/vol) in water, according to the standard emulsan activity assay described previously (12). Addition of 40 μg of gamma globulin per ml resulted in a 90% inhibition, whereas gamma globulin from normal rabbit serum had no effect on emulsification. The inhibition of emulsifying activity by the antibodies, using the CTAB-purified deproteinized emulsan (apoemulsan) in the assay, indicates that the principal sites responsible for emulsan activity also interact with the antibody. Thus, these sites must be located

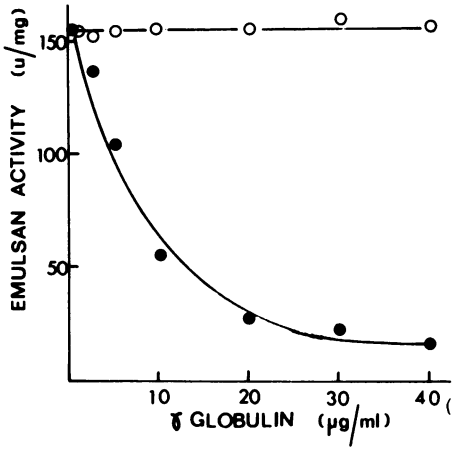

FIG. 1. Inhibition of emulsan activity by specific antibodies. Gamma globulin for antiserum prepared against CTAB-purified emulsan was diluted with PBS to final concentrations of 15, 30, 60, 120, 180, and 240 μg of protein per ml. Samples of 0.1 ml each were added to a series of tubes containing 0.5 ml of emulsan (1 mg/ml in PBS). After incubation for 2 h at 37°C, 200 μl was removed from each tube, and emulsan activity was determined by the standard emulsification assay (●). Control samples received the same samples of gamma globulin from preimmune serum (○) in place of gamma globulin from specific antiserum. Emulsifying activity is expressed in units per milligram (dry emulsan weight).

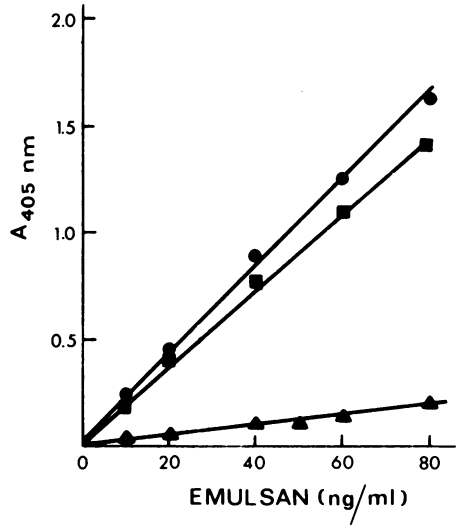

FIG. 2. Titration of different preparations of emulsan by microplate ELISA. Aqueous solutions of different preparations of emulsan (1 mg/ml) were diluted in PBS–Tween 20 to a concentration between 10 and 100 ng/ml. Samples (0.2 ml) were added in duplicate to antibody-coated polystyrene plates, and ELISA was performed as described in the text. The A_{405} values are the average of two independent measurements: (▲) dialyzed lyophilized supernatant fraction obtained from a 48-h culture of RAG-1; (■) CTAB-purified emulsan; (●) apoemulsan.

on the polysaccharide portion of the emulsan molecule. A similar inhibitory effect was observed when gamma globulin from antiserum raised against washed RAG-1 cells was used. Furthermore, these same results were obtained by using antibodies prepared by prior adsorption of the antiserum to cells of mutants which are defective in emulsan production (O. Pines, unpublished data). The apparent specificity of this interaction formed the basis for the quantitative determination of emulsan by using a modification of the ELISA (3).

Determination of cell-free emulsan by ELISA. To titrate various preparations of emulsan by ELISA (Fig. 2), the IgG fraction from antiemulsan antiserum was cross-linked with alkaline phosphatase by using glutaraldehyde as previously described (3). Wells in ELISA microplates were first coated with nonlabeled gamma globulin. Increasing concentrations of the different emulsan preparations were added and allowed to bind to the antibody, and the enzyme-linked gamma globulin was added to the antibody-emulsan-coated wells. The enzymatic activity of the bound phosphatase-labeled antibody (A_{405}) was proportional to antigen concentration in the range 10 to 80 ng/ml. The sensitivity of the ELISA was related to the degree of purity of the emulsan antigens used in the assay. For example, a preparation of unpurified lyophilized supernatant fluid (50 ng/ml) had an A_{405} of 0.16. Further purification of emulsan from the cell-free supernatant fluid by precipitation, first with ammonium sulfate and then with CTAB yielded a product which, when analyzed by ELISA, had an A_{405} of 0.92. Of interest was the observation that when the 15% protein from the CTAB-purified material was first removed by hot phenol, the resulting apoemulsan fraction was about 15% more active in the ELISA, indicating that the antigenic portion of the molecule resided in the polysaccharide backbone.

Determination of emulsan on the cell surface. The fact that cells of *A. calcoaceticus* RAG-1 were agglutinated in the presence of antiemulsan antiserum (Bayer et al., in press) suggested that material which cross-reacts with emulsan (cross-reacting material [CRM]) is located on the surface of the cell. This was established directly by replacing the purified emulsan antigen normally used in the ELISA with washed cells (Fig. 3). The assay of the cell-associated emulsan was linear, with cell concentration between 2×10^5 and 5×10^6/ml. A linear response proportional to cell density was also obtained in the same concentration range when the cells were incu-

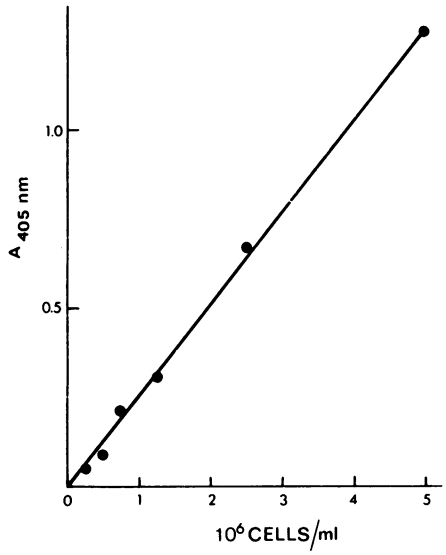

FIG. 3. Determination of emulsan on cells of RAG-1 by microplate ELISA. A logarithmic culture of RAG-1 was concentrated to 10^9 cells per ml, washed, and diluted in PBS–Tween 20 to concentrations between 10^5 and 10^7 cells per ml as described in the text. Samples (0.2 ml) were added in triplicate to the antibody-coated plates. ELISA was performed as described in the legend to Fig. 2. A_{405} is plotted against cell concentration and is the average of three independent measurements.

bated with phosphatase-conjugated antibody, collected on a membrane filter (0.45 μm; Millipore Corp.) to remove unbound enzyme, and allowed to react directly with the phosphatase substrate. The reaction shown in Fig. 3 was performed with cells taken from a culture in the early log phase. Since cells from the stationary phase were relatively inactive (<0.2 $A_{405}/10^7$ cells per ml), it was of interest to monitor the changes in cell-associated and cell-free CRM during growth.

Release of cell-associated emulsan during the growth cycle. When early-log-phase cells of *A. calcoaceticus* RAG-1 were diluted into fresh minimal medium, growth was initially accompanied by an increase in cell-associated emulsan followed by a sharp decrease once the cells had reached a turbidity of 2.0. By 72 h, over 90% of the emulsan had been released into the medium. The rise in emulsifying activity paralleled the rise in the concentration of emulsan, as measured by ELISA. When an inoculum of cells taken from the stationary phase was used in place of a log-phase culture (data not shown), virtually no cell-associated emulsan was detected for the first 8 h of growth. By 12 h, the material on the cell surface had accumulated to about 1.3 $A_{405}/10^7$ cells, after which there was a drop in cell-associated emulsan (Fig. 4).

Effect of CM on release of cell-associated emulsan. Previous results from this laboratory have demonstrated the release of active emulsan into the growth medium in the presence of chloramphenicol (CM) (C. Rubinovitz, D. L. Gutnick, and E. Rosenberg, manuscript in preparation). The CM-induced release was dependent on the addition of a suitable source of carbon, energy, and nitrogen. When washed cells were exposed to CM in the presence of a carbon and nitrogen source, no growth was observed (Fig. 5A), whereas active emulsan accumulated in the medium for a period of up to about 8 h (Fig. 5B and 5D). Very little emulsifier accumulated in the medium in the absence of CM. Even though a net accumulation of emulsan in the cell-free medium was observed in the presence of CM, the cell-associated emulsan remained constant throughout the 8-h period (Fig. 5C). In the absence of CM, the onset of growth was accompanied by a 50% decrease in the cell-associated emulsan.

DISCUSSION

The development of a simple, sensitive, and quantitative immunological assay for emulsan

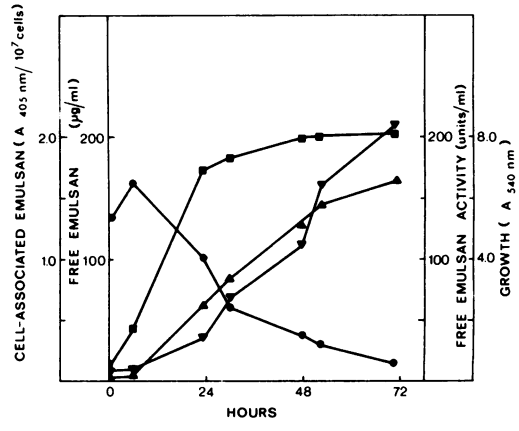

FIG. 4. Distribution of cell-associated and cell-free emulsan during growth of RAG-1. A single colony of RAG-1 was inoculated into 20 ml of minimal medium and allowed to grow overnight (absorbance at 540 nm, 8.2). The inoculum was diluted 1:20 into 40 ml of fresh minimal medium; samples were removed at the indicated times and assayed for growth (■) by absorbance at 540 nm; emulsan activity in the supernatant fraction (▲) as measured by the standard emulsification assay; cell-associated emulsan (●) by microplate ELISA as described in the text; cell-free emulsan (▼) in the supernatant fraction by microplate ELISA as described in the text. Emulsan in the supernatant fraction as determined by ELISA was calculated from a standard curve, using CTAB-purified emulsan. Values obtained for ELISA measurements are the averages of three independent determinations.

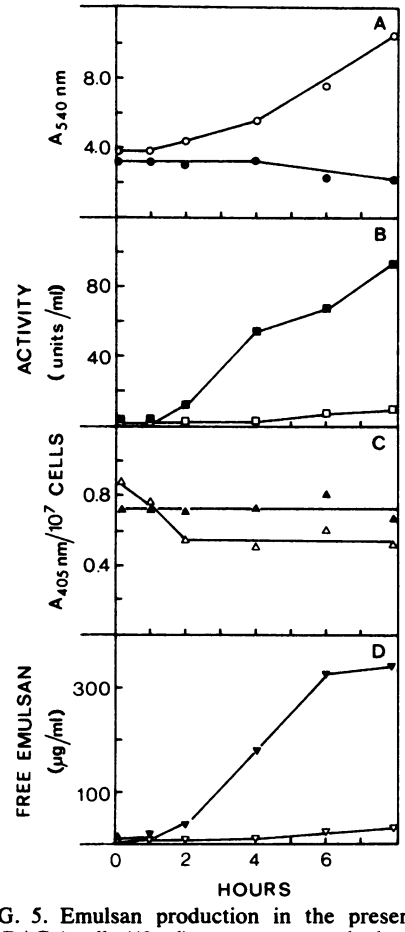

FIG. 5. Emulsan production in the presence of CM. RAG-1 cells (40 ml) were grown to the log phase (absorbance at 540 nm, 3.8), harvested, washed twice with phosphate buffer (0.15 M pH 7.0), suspended in the same volume of fresh growth medium, and divided equally into two flasks. CM was added to one of the cultures to a final concentration of 50 μg/ml (closed symbols), and the second culture served as control (open symbols). Both cultures were shaken at 30°C for 8 h. Samples were removed at the indicated times and assayed for the various parameters as indicated. (A) Growth was followed by measuring absorbance at 540 nm; (B) emulsan activity in supernatant fluid was measured by standard emulsifying assay; (C) cell-associated emulsan was followed by microplate ELISA as described in the legend to Fig. 3; (D) cell-free emulsan was measured by microplate ELISA as described in the legend to Fig. 2.

allowed for the determination of the polymer, both on the cell surface and in the cell-free supernatant fluid. The specificity of the assay resided in the polysaccharide portion of the backbone since (i) the same degree of inhibition of emulsifying activity by the antibody was observed with apoemulsan (deproteinized) as with emulsan itself, and (ii) the ELISA of apoemulsan was at least as sensitive as the assay with emulsan.

The amount of cell-associated CRM appeared to be a function of the growth phase of the cells. Thus, early-log-phase cells exhibited a relatively high level of cell-associated CRM which was released into the medium throughout the later stages of the exponential and stationary phases of growth. Enhanced release of cell-associated emulsan in the absence of growth was also observed in the presence of the protein synthesis inhibitor CM (Fig. 5). This process required the presence of a carbon, energy, and nitrogen source (Rubinovitz et al., in preparation). In addition, in the absence of protein synthesis, there was no change in the amount of cell-associated CRM, despite the accumulation of emulsan in the medium. These results strongly suggest that de novo emulsan synthesis is required for the release of existing cell-associated emulsan. Figure 5 shows that between 2 and 6 h after removal of samples, about 300 μg of emulsan per ml was released into the medium in the presence of CM. The cell concentration was 1.4 mg of cells (dry weight) per ml. The rate of synthesis of emulsan in the presence of CM was, therefore, 300 μg/ml per 4 h per 1.4 mg of cells (dry weight) per ml or 54 μg/h per mg of cells (dry weight).

Although relatively little emulsan was produced under conditions of exponential growth, the results with CM suggest that proteins required for synthesis and release of the bioemulsifier are synthesized and inhibited, rather than repressed. Otherwise, accumulation of active cell-free emulsan would not have been observed in the absence of protein synthesis. One explanation may involve the enhanced channeling of cellular carbon precursors into polysaccharide synthesis under conditions of unbalanced growth. In favor of this hypothesis are previous results (Rubinovitz et al., in preparation) indicating that only about 1/10 the amount of ethanol is required for maximal emulsan production in the presence of CM than is required for production of the same amount of emulsan under normal batch culture conditions. Another characteristic relates to why release of emulsan stops in the presence of CM. We assume that a protein complex participates in the release process, and is itself released together with emulsan. In the presence of CM, this complex would not be regenerated, and the release process would eventually stop. In this regard, an investigation of the properties of mutants which accumulate emulsan on the cell surface, yet do not release active material into the medium, is currently in progress.

It is of interest that cell-associated emulsan, although resembling the cell-free bioemulsifier

antigenically, differs functionally from the isolated polymer. For example, intact logarithmic cells do not emulsify the hydrocarbons in the standard emulsification assay (12). Moreover, *A. calcoaceticus* RAG-1 mutants resistant to a specific phage, ap3, were all defective in active emulsan production and antiemulsan gamma globulin prevented adsorption of ap3 to wild-type cells. Nevertheless, isolated cell-free emulsan was not active in phage adsorption. The cell-associated material, therefore, appears to function in some way as a phage receptor (7).

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