Arch Microbiol (1981) 130:129–133

Archives of
Microbiology

Relationship Between Phage Resistance and Emulsan Production, Interaction of Phages with the Cell-Surface of *Acinetobacter calcoaceticus* RAG-1

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Abstract. The hydrocarbon-degrading strain *Acinetobacter calcoaceticus* RAG-1 produces an extracellular emulsifying agent capable of forming stable oil-in-water emulsions. The bioemulsifier, termed emulsan, is a polyanionic heteropolysaccharide (M.W. 10^6) composed mainly of N-acyl D-galactosamine and an N-acyl hexosamine uronic acid. In order to probe the interaction of emulsan with the cell surface prior to its release into the growth medium, two new virulent bacteriophages for *A. calcoaceticus* RAG-1 were isolated from sewage and the properties of phage resistant mutants were studied. The two phages, ap-2 and ap-3, were differentiated on the basis of plaque morphology, electron microscopy and buoyant density. Isolated mutants of *A. calcoaceticus* RAG-1 which were resistant to one of the two phages retained sensitivity to the other phage. Resistance to phage ap-3 was accompanied by a severe drop in emulsan production. Independently isolated derivatives of *A. calcoaceticus* RAG-1 with a defect in emulsan production also turned out to be resistant towards phage ap-3. Antibodies prepared against purified emulsan specifically inhibited phage ap-3 adsorption to the cell surface of the parental strain.

Key words: *Acinetobacter calcoaceticus* – Emulsan – Emulsifying agent – Cell-bound/cell-free heteropolysaccharide – Phage receptor

The growth of microorganisms on hydrocarbons is frequently accompanied by the emulsification of the insoluble substrate in the aqueous medium (Erickson and Nakahara 1975; Gutnick and Rosenberg 1977; Zajic and Panchal 1977). In some cases this process has been shown to be due to the production of either exo- or extra-cellular emulsifying agents (Gutnick and Rosenberg 1977). Previously, we have described the isolation and partial characterization of an extracellular polymeric emulsifying agent, termed emulsan, produced by *Acinetobacter calcoaceticus* RAG-1 (Zuckerberg et al. 1979). Emulsan is a polyanionic heteropolysaccharide (M.W. 10^6) containing 1. a polysaccharide backbone composed of N-acyl D-galactosamine, and an N-acyl hexosamine uronic acid, 2. esterified fatty acids (0.5 μmol/mg) consisting mainly of α and β hydroxydodecanoic acid and 3. protein which can be removed by phenol treatment without destruction of emulsifying activity or loss of viscosity. In addition to the appearance of active emulsan in the cell free medium following growth, recent immunological and physiological evidence from our laboratory has demonstrated that the bioemulsifier is also present on the surface of the cells themselves (Gutnick et al. 1980). This latter conclusion emerged from studies on 1. the agglutinability of emulsan-producing *A. calcoaceticus* RAG-1 cells by anti-emulsan antiserum, 2. the reactivity of such cells in an enzyme-linked immunosorbent assay (ELISA) using IgG prepared from this antiserum, and 3. the excretion of prelabelled cell-associated emulsan into the medium by non-growing cells in the presence of chloramphenicol or amino acid starvation.

Another approach to probing the outer surface of bacterial cells involves the interaction of phages with specific cell-associated receptors (Datta et al. 1977; Lindberg 1977; Mutoh et al. 1978; Osborn and Wu 1980). In this report we describe the isolation of two *A. calcoaceticus* phages, ap-2 and ap-3. Evidence is presented suggesting that a cell-bound form of emulsan is involved in receptor activity for phage ap-3.

Materials and Methods

Bacterial Strains and Growth Conditions

Acinetobacter calcoaceticus RAG-1 (ATCC 31012) was isolated previously and has been described (Reisfeld et al. 1972). *A. calcoaceticus* strain RAG-92 is a lysine auxotroph isolated from the wild-type *A. calcoaceticus* RAG-1. Strain RAG AG-1 was isolated from RAG-1 by selecting fast-growing derivatives on minimal medium supplemented with a low (0.25% v/v) concentration of ethanol by A. Gottlieb. Strain *A. calcoaceticus* RAG-10 was isolated from *A. calcoaceticus* RAG-92 by selecting a derivative which grew poorly on minimal agar plates containing 1% hexadecane as carbon source, by R. Avigad. Unless otherwise stated, bacteria were cultivated in a minimal medium containing 22.2 g K_2HPO_4, 7.26 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7\ H_2O$ in 1,000 ml distilled water and 2% ethanol (v/v) as the carbon source. Starter cultures were prepared by innoculating single colonies into 2 ml minimal medium and incubating overnight on a New Brunswick gyrotory incubator shaker model G25 at 30° C, 350 rpm. Unless otherwise stated, the overnight starter was diluted 1:100 into fresh minimal medium (20 ml in 100 ml flasks) and grown under the same conditions. Growth was followed turbidometrically by measuring optical density at 540 nm on a Gilford model spectrophotometer or with a Klett Summerson colorimeter fitted with a green filter.

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0302-8933/81/0130/0129/$01.00

General Phage Techniques

The standard agar overlay method of Adams (1959), was used to enumerate phage. *A. calcoaceticus* RAG-1 phages were grown and assayed on RAG-1 cells cultivated in broth medium containing 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, 2.3 g $MgSO_4 \cdot 7\ H_2O$ in 1,000 ml distilled water.

Isolation of Phages and Phage Resistant Mutants of A. calcoaceticus RAG-1

Isolation of Phages. Samples of sewage from the municipal treatment plant in the Tel Aviv area were clarified by centrifugation ($8{,}000 \times g$ for 30 min) and subsequent filtration of the supernatant fluid (Millipore HAWP-45). Portions of this filtrate were added to logarithmic broth cultures of RAG-1, and the infected cultures were allowed to grow overnight. After centrifugation, the supernatant fluid was filtered, chloroform added and the phage enrichment process repeated. The supernatant preparation was then titered on lawns of RAG-1. Isolated plaques which developed after overnight incubation were picked and the phages purified by three sequential rounds of single plaque isolation, propagation and titering.

Isolation of A. calcoaceticus RAG-1 Phage Resistant Mutants. A logarithmic broth culture of *A. calcoaceticus* RAG-1 ($0.4\,OD_{540}$) was mixed with 1 ml phage suspension (10^9 PFU/ml) and incubated for 5 min at 30 °C without shaking. Dilutions of the infected culture were plated by the soft agar overlay technique. Individual colonies appearing on the plates were isolated and streaked onto minimal plates and tested for phage resistance. Mutant strains TR1, TR2, TR3, TR4 and 312 are derivatives of *A. calcoaceticus* RAG-1 and are resistant to phage ap-3. Mutant strain 215 is a derivative of *A. calcoaceticus* RAG-1 and is resistant to phage ap-2. Mutant strains TL1, TL2, and TL3 are resistant to phage ap-3 and were derived from the lysine auxotrophic strain *A. calcoaceticus* RAG-92.

Phage Growth Curve. Logarithmic broth cultures of RAG-1 (10 ml, $0.4-0.8\,OD_{540}$) growing at 30° C with shaking were infected with phage at a multiplicity of 0.08 to 0.25 phages per bacterium. The infected cultures were incubated 10 min without shaking at 30° C, sedimented at $8{,}000 \times g$ for 5 min, resuspended in fresh medium and diluted 1×10^{-4} into fresh broth medium and further incubated at 30° C with shaking. Under these conditions, between 70 – 80 % of input phage ap-2 and 90 – 95 % of input phage ap-3 were adsorbed by the cells. To determine intracellular phage growth, chloroform treated samples of the infected culture taken at different times after infection, were serially diluted and titered.

Phage Purification

1. Large lysates for phage purification were prepared by infecting a 700 ml RAG-1 log phase culture ($0.4\,OD_{540}$) and incubating with shaking until lysis occurred. Crude lysates were centrifuged at $10{,}000 \times g$ in a Sorvall GSA rotor for 10 min to remove cell debris.

2. Concentration of phage lysates was performed by the method described by Yamamoto and Alberts (1970). NaCl concentration was adjusted to 0.5 M and polyethylene glycol (M.W. 6,000) was added to a final concentration of 10 %. The mixture was allowed to stand for 60 min at 4° C and then centrifuged at $8{,}000 \times g$ for 10 min. The pellet was resuspended in 1.5 ml phosphate buffer (150 mM, pH 7.0).

3. CsCl-Density Gradient Centrifugation. CsCl density gradients were performed as follows: solid CsCl was added to the phage preparation to a final concentration of 44 % (w/w) and run in a Beckman L-2-50 ultracentrifuge with a type 65 fixed angle rotor at 30,000 rpm for 20 h at 7° C. Fractions of 0.2 ml were collected, assayed for phage titer and refractive index (Bausch & Lomb 3-L refractometer).

The density of CsCl in each fraction was calculated from the refractive index. Peak fractions were pooled and dialyzed against phosphate buffer.

Electron Microscopy

Phage suspensions of the dialyzed pooled peak fractions described above were mounted on carbon coated grids and stained with 2 % uranyl acetate. Phages were observed with a JEOL 100 B electron microscope.

Emulsan Preparation and Assay for Emulsifying Activity

The standard emulsifying activity assay for RAG-1, emulsan, has been described previously (Rosenberg et al. 1979). The assay is performed by shaking mixtures of hexadecane and 2-methylnaphthalene in buffer with appropriate amounts of culture supernatants or purified emulsan preparations. In this test, turbidity is a linear function of emulsan concentration. One unit of emulsan activity per ml gives rise to a turbidity of 100 Klett Units in the emulsifying activity assay. Emulsan was prepared as described previously (Rosenberg et al. 1979).

Preparation of Anti-Emulsan Antiserum

Anti-emulsan antiserum was prepared against highly purified emulsan by S. Goldman according to the method described previously (Zuckerberg et al. 1979). The antibody preparations used in this study were prepared by precipitation of antisera in the presence of 40 % saturated ammonium sulfate.

Phage Adsorption and Inactivation

Overnight broth cultures (10 ml) were infected with either phage ap-2 or ap-3 to a final titer of 5×10^7 PFU/ml. After 10 min incubation at 30° C, the cells were removed by centrifugation (10,000 rpm for 10 min in a Sorvall SS-34 rotor), and the supernatant fluid was titrated. Inactivation of ap-3 by purified emulsan was attempted by incubating phage ap-3 (5×10^7 PFU/ml) with emulsan (1 mg/ml) for 15 min at 30° C and subsequently titrating the mixture. Experiments testing the effect of anti-emulsan antibodies on phage adsorption were performed as follows: One milliliter logarithmic cultures (10^7 cells/ml – $0.04\,OD_{540}$) of RAG-1 were incubated together with 0.2 ml anti-emulsan antibodies and incubated for 30 min at 30° C, prior to addition of phage. Phage adsorption was assayed as described above.

Coating of Red Blood Cells with Emulsan

Coating of sheep red blood cells with emulsan was performed by glutaraldehyde cross-linking and the presence of emulsan on the surface of the sheep red blood cells was checked by hemagglutination with anti-emulsan antibodies (Avrameas et al. 1969).

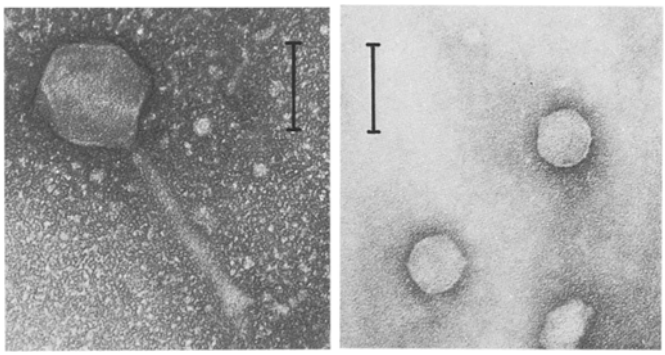

Fig. 1. Electron micrograph of *Acinetobacter calcoaceticus* RAG-1 phages ap-2 (**a**) and ap-3 (**b**) stained with 2% uranyl acetate (magnification 120,000). Bar marker is 0.1 μm

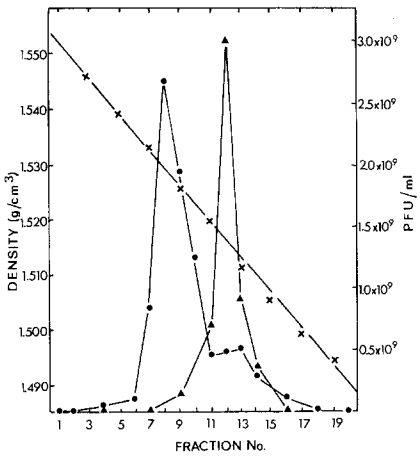

Fig. 2. CsCl density gradient centrifugation of bacteriophages ap-2 and ap-3. Symbols: ap-2 (●) and ap-3 (▲) titers, fraction density (×). Phage lysates with a concentration of 44% CsCl (w/w) were centrifuged in a Beckman L-2-50 ultracentrifuge with a type 65 fixed angle rotor for 20 h at 7° C. The refractive index and phage titer were determined for each fraction. The density of CsCl in each fraction was calculated from the refractive index

Results

Isolation of Bacteriophages ap-2 and ap-3

In order to probe the interaction of emulsan with the cell surface prior to its release into the growth medium, two new virulent bacteriophages with lytic activity towards *Acinetobacter calcoaceticus* RAG-1, designated ap-2 and ap-3, were isolated from sewage. The phages were readily distinguished on the basis of characteristic plaque morphologies. The clear plaques of phage ap-2 were small (diameter 1 mm) and lacked a halo. Phage ap-3 yielded clear plaques 2–3 mm in diameter surrounded by a small turbid halo. Differences in phage morphology were observed under the electron microscope as shown by negative staining in Fig. 1. Phage ap-2 was characterized by a hexagonal head 125 nm wide and a tail 240 nm long (Fig. 1 a). Phage ap-3 exhibited a smaller hexagonal head (68 nm wide) with no observable tail or base plate (Fig. 1 b).

The buoyant density of phage ap-2 was 1.528 g/cm^3 and that of phage ap-3 1.518 g/cm^3, as determined by CsCl equilibrium centrifugation (Fig. 2). Both the size and density of the two phages are consistent with characteristics of double stranded DNA phages (Akerman et al. 1973).

The intracellular growth curves of phages ap-2 and ap-3 are presented in Fig. 3. The eclipse period for ap-2 was between 40–45 min and an average burst size estimated at 45 PFU/infected cell. For phage ap-3 a shorter eclipse period was observed (25–30 min) with an average burst size of 30 PFU/infected cell.

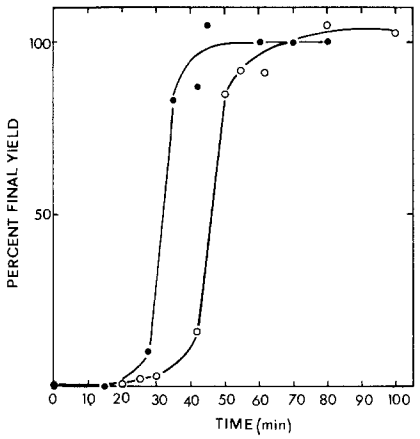

Fig. 3. Intracellular growth curves of *A. calcoaceticus* RAG-1 bacteriophages ap-2 (○) and ap-3 (●). Logarithmic cultures of *A. calcoaceticus* RAG-1 were infected with phage and incubated without shaking at 30° C for 10 min. The infected culture was washed and diluted 1×10^{-4} into fresh broth medium and incubated at 30° C with shaking. Samples of this culture were taken at different times, treated with chloroform and titrated

Mutants of A. calcoaceticus RAG-1 Resistant to ap-2 and ap-3

It was of interest to characterize the possible effects of cell surface modification on emulsan production. Since such changes in cell surface would be likely to arise as a result of mutation to phage resistance, spontaneous mutants of *A. calcoaceticus* RAG-1 resistant to either ap-2 or ap-3 were isolated. Such mutants appeared in the population of *A. calcoaceticus* RAG-1 at frequencies ranging between 7×10^{-6} and 1×10^{-5}. Mutants resistant to one of the phages were isolated at the same frequency regardless of whether the parent strain was wild type or resistant to the other phage. Sensitivity to the two phages appeared to be due to different receptors: 1) 67 out of 67 mutants resistant to phage ap-3 retained sensitivity to ap-2, while 53 out of 53 mutants resistant to phage ap-2 were still sensitive to ap-3; 2) the same percent adsorption of phage ap-3 was obtained when either wild type or phage ap-2 resistant *A. calcoaceticus* RAG-1 strains were examined (Table 1). Overnight cultures of RAG-1, the lysine auxotroph *A. calcoaceticus* RAG-92 and mutant strain *A. calcoaceticus* RAG-215 (resistant to phage ap-2) all adsorbed over 99% of the infecting phage ap-3. Virtually no adsorption of phage ap-3 by ap-3 resistant mutant strains TL1, TL2, TL3 or 312 were observed under these conditions. These results demonstrate that the mutation leads to a loss of phage receptor activity. Of interest was the finding that RAG-1 derivatives such as *A. calcoaceticus* RAG-10 and *A.*

Table 1. Adsorption of phage ap-3 to wild-type and mutant *Acinetobacter calcoaceticus* strains

Strain	Residual titer (PFU/ml $\times 10^{-7}$)	% Adsorption
RAG-1	0.0028	99.5
92 (lys$^-$)	0.0012	99.7
215 (ap-2 resistant)	0.0021	99.6
TL 1 (ap-3 resistant)	4.2	N.S.[a]
TL 2 (ap-3 resistant)	4.1	N.S.
TL 3 (ap-3 resistant)	4.8	N.S.
312 (ap-3 resistant)	4.5	N.S.
AG-1 (emulsan-defective)	4.1	N.S.
10 (emulsan-defective)	5.0	N.S.

Overnight broth cultures (10 ml) were infected with phage ap-3 such that the initial titer of the infecting phage was 5.0×10^7 PFU/ml. After 10 min incubation at 30°C, the cells were removed by centrifugation and the supernatant fluid titrated for residual unadsorbed phage

[a] N.S. = not significant

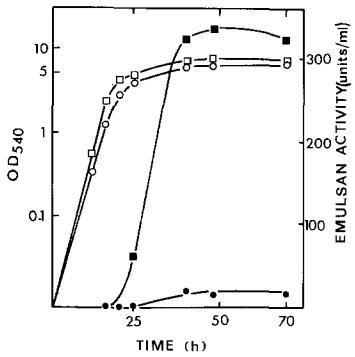

Fig. 4. Kinetics of growth and emulsan production by *A. calcoaceticus* RAG-1 and the ap-3 resistant mutant TR3. Symbols: culture turbidity of RAG-1 (□) and TR3 (○), and emulsan activity of RAG-1 (■) and TR3 (●). 20 ml cultures were grown in minimal medium supplemented with 2% ethanol. At different times 0.5 ml samples of the growing culture were assayed for emulsan activity and the culture turbidity (OD_{540}) was determined, as described in Materials and Methods

Table 2. Final growth yields and emulsan production of wild type and mutant *A. calcoaceticus* strains

Strain	Turbidity of 72 h culture (OD_{540})	Emulsan activity (units/ml)	Specific emulsan (units/ml/ 10 OD_{540})
RAG-1	8.24	338	410
92 (lys$^-$)	8.40	375	446
215 (ap-2 resistant)	7.60	275	362
TR 1 (ap-3 resistant)	6.48	15	23
TR 2 (ap-3 resistant)	8.16	30	37
TR 3 (ap-3 resistant)	6.96	20	29
TR 4 (ap-3 resistant)	6.48	18	28
TL 1 (ap-3 resistant)	6.87	9	13
TL 2 (ap-3 resistant)	5.90	18	31
TL 3 (ap-3 resistant)	10.38	27	26
314 (ap-3 resistant)	6.22	25	40
312 (ap-3 resistant)	6.55	12	18
AG-1 (ap-3 resistant)	7.71	27	35
10 (ap-3 resistant)	7.73	9	12

Cultures were grown in 20 ml minimal media supplemented with 2% ethanol for 72 h. Turbidity (OD_{540}) and emulsan activity were determined as described in Materials and Methods

calcoaceticus RAG-AG-1 were also unable to adsorb phage ap-3. These latter two mutants which were not selected on the basis of phage resistance have been shown to be defective in the production of the RAG-1 extracellular emulsifying agent, emulsan. It was of interest, therefore, to examine directly the extracellular emulsifying activity of phage ap-3 resistant mutants.

Emulsan Production by Mutants of A. calcoaceticus RAG-1 Resistant to Phage ap-3

As illustrated in Table 2, ap-3 resistant mutants of either strain RAG-1 or the lysine auxotroph strain RAG-92 all exhibit much lower extracellular emulsan activity than the corresponding parent. Whereas the parental strains showed emulsan activities between 300 and 400 units per ml per 10 OD_{540} of culture, the corresponding activities in supernatants of the mutants were never higher than 40 units/ml per 10 OD_{540}. In contrast, mutant strains resistant to phage ap-2 exhibited parental levels of emulsan activity. The kinetics of growth and appearance of emulsan activity of the parental strain RAG-1 and the ap-3 resistant mutant strain TR3 are shown in Fig. 4. It can be seen that the growth of both mutant and the parent strain were similar. Emulsan activity in the parent strain began to appear in the cell free supernatant after about 24 h and reached a maximum level of approximately 300 units per ml, between 48 and 72 h. In sharp contrast, however, maximal emulsan activity for the mutant TR3 was only 20 units per ml by 72 h.

Specific Inhibition of ap-3 Adsorption by Anti-Emulsan Antibodies

Since mutant strains resistant to phage ap-3 were defective with regard to emulsan production, it was of interest to examine the possible role of emulsan as phage ap-3 receptor. Attempts to inactive phage ap-3 with either crude or purified emulsan preparations were unsuccessful. Moreover, when emulsan was bound covalently to the surface of sheep red blood cells, no adsorption of phage ap-3 was found. However, as illustrated in Fig. 5, when cells of strain RAG-1 were preincubated with anti-emulsan antibodies, a significant inhibition of phage ap-3 adsorption was observed. Incubation of RAG-1 cells with normal rabbit antiserum IgG showed no effect on phage adsorption. Furthermore, anti-emulsan antibodies did not significantly change the adsorption of phage ap-2 under the same conditions.

Discussion

In this paper we describe the interaction of two new virulent bacteriophages, ap-2 and ap-3, with cells of the host strain *Acinetobacter calcoaceticus* RAG-1 and its derivatives. The two phages were differentiated on the basis of 1. plaque morphology, 2. buoyant density, 3. visualization by electron microscopy after negative staining and 4. the fact that

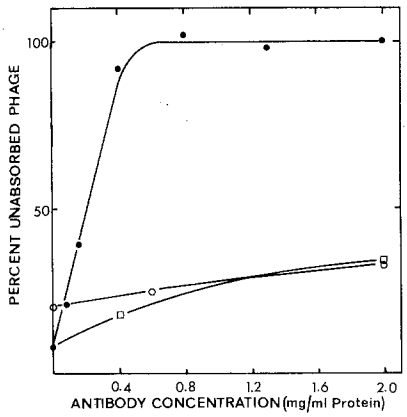

Fig. 5. Specific blocking of phage ap-3 adsorption to *A. calcoaceticus* RAG-1 cells by anti-emulsan antibodies. Symbols: percent unadsorbed phage ap-3 as a function of anti-emulsan antibody (●) and normal rabbit serum antibody (□) concentrations. Percent unadsorbed phage ap-2 in the presence of anti-emulsan antibodies (○). Logarithmic RAG-1 cells (10^7 cells/ml, $0.04\,OD_{540}$) were incubated in the presence of the appropriate antibody preparation for 30 min at 30°C. The appropriate phage was then added (5×10^7 PFU/ml) and the culture was incubated for another 10 min at 30°C. The infected culture was centrifuged and the supernatant titrated for unadsorbed phage.

derivatives of *A. calcoaceticus* RAG-1 resistant to one of the phages retain sensitivity to the other phage. The fact that mutants resistant to either ap-2 or ap-3 were defective only in the adsorption of the corresponding phage indicates that resistance to the two phages is the result of different cell-surface modifications.

Two general lines of evidence suggest a role for cell-bound emulsan in phage ap-3 receptor activity. The first involves the correlation between ap-3 resistance and the defect in emulsan production in a variety of mutants of the host strain RAG-1. Not only were mutants selected directly for ap-3 resistance defective in emulsan production, but emulsan-deficient mutants isolated by other techniques were found to be ap-3 resistant. Phage ap-2 resistant mutants exhibited normal emulsan production.

The second set of results demonstrates the specific inhibition of ap-3 adsorption to wild-type cells of *A. calcoaceticus* RAG-1 by anti-emulsan antibodies. In this regard, it is of interest that neither cell-free emulsan preparations nor emulsan-coated sheep red blood cells adsorbed phage ap-3. One explanation may be that an additional factor(s) on the cell-surface of the bacterial host strain is required for adsorption of phage ap-3. Such requirements for additional factors have been observed for protein, lipopolysaccharide (LPS) and carbohydrate-capsular phage receptors (Datta et al. 1977; Lindberg 1977; Mutoh et al. 1978). One interesting example involves the interaction of phage T4 with *Escherichia coli* K_{12} in which in vitro phage-receptor activity was observed only when isolated LPS from *E. coli* K_{12} was mixed with a specific outer membrane protein (Mutoh et al. 1978).

In contrast, isolated LPS from *E. coli* B can serve as a receptor for T4 in the absence of added factors (Wilson et al. 1970).

An alternative explanation for the inactivity of cell-free emulsan in phage AP-3 adsorption stems from the fact that cell-associated emulsan shows very little, if any, emulsifying activity in contrast to the cell-free material (Rosenberg et al. 1979). Thus, cell-free emulsan preparations may not exist in a conformation appropriate for phage adsorption. Experiments designed to differentiate between these two hypotheses are currently in progress.

Acknowledgements. The authors are indebted to Rina Avigad for excellent technical assistance, to Sarah Goldman for the antibody preparations and assistance with the covalent attachment of emulsan to sheep red blood cells, and to Eugene Rosenberg and E. A. Bayer for their critical reading of the manuscript.

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Received April 16, 1981