

Reconstitution of Emulsifying Activity of *Acinetobacter calcoaceticus* BD4 Emulsan by Using Pure Polysaccharide and Protein

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Received 11 July 1986/Accepted 19 November 1986

***Acinetobacter calcoaceticus* BD4 and BD413 produce extracellular emulsifying agents when grown on 2% ethanol medium. For emulsifying activity, both polysaccharide and protein fractions were required, as demonstrated by selective digestion of the polysaccharide with a specific bacteriophage-borne polysaccharide depolymerase, deproteinization of the extracellular emulsifying complex with hot phenol, and reconstitution of emulsifier activity with pure polysaccharide and a polysaccharide-free protein fraction. Chemical modification of the carboxyl groups in the polysaccharide resulted in a loss of activity. The protein required for reconstitution of emulsifying activity was purified sevenfold. The BD4 emulsan apparently derives its amphipathic properties from the association of an anionic hydrophilic polysaccharide with proteins.**

The growth of microorganisms on water-insoluble hydrocarbon substrates presents the cell with a unique problem. Because all known hydrocarbon-oxidizing enzymes are cellbound, microorganisms must incorporate these insoluble substrates by direct contact or by pseudosolubilization (6). Emulsification of the hydrocarbon substrate increases the rates of both of these processes. Many hydrocarbon-degrading organisms produce extracellular emulsifiers; these bioemulsifiers can be classified into the following two main groups (17): (i) low-molecular-weight, amphipathic molecules, such as glycolipids, fatty acids, phospholipids, and lipopeptides; and (ii) polymeric bioemulsifiers, such as the lipomannan from *Candida tropicalis* (12), a protein mixture from *Pseudomonas aeruginosa* (7), a polysaccharide-protein-lipid complex from *Phormidium* J-1 (4), and several polysaccharide-containing emulsifiers from *Acinetobacter* strains (10, 20, 21). The extracellular polysaccharide-containing emulsifiers (emulsans) of two *Acinetobacter calcoaceticus* strains, RAG-1 and BD4, have been studied extensively in this laboratory. The RAG-1 emulsan is a heteropolysaccharide containing D-galactosamine, an amino uronic acid, and an unidentified amino sugar (28). Fatty acids, composing 15% of the emulsan dry weight, are attached to the polysaccharide backbone via O-ester linkages (1). Since the chemical structure of the polysaccharide component of *A. calcoaceticus* BD4 has recently been elucidated (11), this bioemulsifier is suitable for studies of structure and function.

A. calcoaceticus BD4 is a heavily encapsulated gram-negative bacterium (24). Strain BD413 is a single-step mini-encapsulated mutant of BD4 (9). Studies on exopolysaccharide production and distribution in these strains (10) showed that BD4 produced four times more total exopolysaccharide (capsular and extracellular) than did BD413. However, BD413 excreted about 50% of the total exopolysaccharide, whereas BD4 excreted only 10% when the strains were grown on glucose-minimal (GM) medium. Furthermore, the BD413 extracellular fraction had emulsifying activity, whereas the capsule polysaccharide obtained from BD4 had no emulsifying activity (10). Emulsifying activity is not due

to differences in the chemical structures of the capsular and extracellular polysaccharides since both polysaccharides are composed of identical heptasaccharide repeating units (rhamnose, glucose, mannose, and glucuronic acid in the molar ratio of 4:1:1:1) and do not contain O-acyl groups or pyruvate (11).

This report defines more precisely the nature of polymeric materials responsible for the emulsifying activity of strains BD4 and BD413. The data demonstrate that emulsifying activity is due to a multicomponent complex consisting of a polysaccharide (referred to as PS-4) and noncovalently bound specific protein(s). Neither component by itself was able to emulsify hydrocarbons in water.

MATERIALS AND METHODS

Bacteria and growth conditions. *A. calcoaceticus* BD4, originally isolated by Taylor and Juni (24), was generously provided by K. Bryn. *A. calcoaceticus* BD413, a mini-encapsulated (9) tryptophan auxotroph (8, 22) of strain BD4, was a gift from E. Juni. All BD4 and BD413 bacteriophage-resistant mutants, including strain BD-R7, were isolated during this study. Strains were maintained on brain heart infusion (BHI) plates (BHI solidified with 1.6% agar, both components from Difco Laboratories, Detroit, Mich.) at 4°C and were transferred at 2-month intervals.

GM medium contained the following per liter of deionized water: 5.0 g of glucose, 9.17 g of $K_2HPO_4 \cdot 3H_2O$, 3.0 g of KH_2PO_4 , 4.0 g of $(NH_4)_2SO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$. Ethanol-minimal medium contained the following per liter of deionized water: 20 ml of ethanol (absolute; Merck & Co., Inc., Rahway, N.J.), 22.2 g of $K_2HOP_4 \cdot 3H_2O$, 7.3 g of KH_2PO_4 , 4.0 g of $(NH_4)_2SO_4$, and 0.2 g of $MgSO_4 \cdot 7H_2O$. All media had a final pH of 7.0. Media for culturing strain BD413 were supplemented with 0.1 mg of L-tryptophan per ml. Minimal agar plates were prepared by solidifying the media described above with 1.6% agar (Difco). Bacterial growth was initiated with a 1% inoculum obtained from starter cultures grown in 2 ml of the same medium in 16-mm test tubes. Flasks, containing 20% their volume of medium, were incubated at 30°C in a New Brunswick Scientific Co. G24 gyratory shaker at 250 rpm. Bacterial growth was

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monitored by determining turbidity in a Klett-Summerson photometer (green filter).

Determination of cellbound and extracellular protein and polysaccharides. Culture samples were centrifuged in the cold at $12,000 \times g$ for 20 min. The cell pellets were suspended in phosphate buffer (62 mM, pH 7.0) at their original volumes. Cell-free supernatant fluid samples were mixed with 4 volumes of ice-cold acetone, left on ice for 30 min, and centrifuged at $12,000 \times g$ for 10 min at 4°C ; the precipitates were dissolved in the phosphate buffer described above. The amount of rhamnose was measured both directly, on the whole-cell suspensions, and on the acetone precipitate fractions by the H_2SO_4 /cysteine procedure for 6-deoxyhexoses (3), by using L-rhamnose as a standard. Polysaccharide content was calculated from the known rhamnose content (55%) of the pure polysaccharide (11). Protein content was measured by a modified procedure of Lowry (14) (with sodium dodecyl sulfate to solubilize amphipathic proteins) by using bovine serum albumin as a standard.

Isolation of BD4 bacteriophages. A fresh sewage sample collected from the Reading Sewage Treatment Plant, Tel Aviv, Israel, was clarified by filtration through Whatman no. 1 filter paper and subsequent centrifugation at $7,000 \times g$ for 20 min at 4°C . The clear supernatant fluid was then treated with a few drops of chloroform, and 0.2 ml of this mixture was inoculated into 20 ml of GM medium in a 100-ml flask together with 0.5 ml of a stationary-phase BD4 culture. After overnight incubation at 30°C , the phage-enriched culture was harvested ($7,000 \times g$, 15 min, 4°C); appropriate dilutions of the supernatant fluid were plated by the soft agar overlay technique onto GM agar plates with BD4 as the indicator strain. After overnight incubation at 30°C , plaques produced by typical capsule-specific phages (small, clear lytic centers with large, turbid halos) were reisolated and purified by several rounds of single-plaque isolation and propagation. One such isolate, termed ϕ SL-1, was chosen for further studies.

Isolation of ϕ SL-1 depolymerase. To an exponential culture (9×10^7 cells per ml) of BD4 in 50 ml of GM medium in a 250-ml flask were added 2×10^8 PFU of ϕ SL-1 per ml. After an additional 20 h of incubation, the culture was harvested ($12,000 \times g$, 15 min). The supernatant fluid (containing 7×10^9 CFU/ml) was centrifuged again as described above, treated with a few drops of chloroform, and filtered through a 0.45- μm -pore-size membrane filter (Millipore Corp., Bedford, Mass.). The clarified lysate was then centrifuged at $100,000 \times g$ for 1 h at 4°C . The depolymerase was recovered from the supernatant fluid by precipitation at 40% ammonium sulfate saturation. The precipitate was dissolved in 1 ml of 20 mM phosphate buffer (pH 7.0) and dialyzed against distilled water.

Depolymerase assay. The depolymerase assay was performed in Eppendorf microcentrifuge tubes with 100 μg of polysaccharide PS-4 as the substrate in a final volume of 200 μl of 10 mM Tris buffer (pH 7.0) containing 1 mM MgSO_4 . The tubes were incubated at 30°C ; at timed intervals, samples (25 to 50 μl each) were removed for determination of reducing sugar content by the ferricyanide method (15) by using glucose as a standard. One unit of depolymerase was defined as the amount of enzyme catalyzing the release of 1 nmol of reducing sugar per h. Concentrations of enzyme were chosen such that the reaction was linear with time for at least 2 h.

Genetic transformation of ϕ R mutants to wild type. Strain BD4 is highly competent for DNA transformation (9). Trans-

formation was performed by the plate method (8). A loopful of the phage-resistant recipient cells (unencapsulated) was mixed with a loopful of donor DNA on a sector of a GM agar plate. After 48 h of incubation, the recipient cells were examined under a phase-contrast microscope by India ink staining. Since this method produces a negative stain, transformed, encapsulated cells with a large white halo were easily detected against the dark background of mutant cells. It was possible to detect one transformant in a field of 10^3 to 10^4 cells.

Isolation of the BD413 emulsifier (EF-55). Each of eight 4-liter flasks, each containing 800 ml of GM medium (supplemented with 0.1 mg of tryptophan per ml), was inoculated with 8 ml of overnight starter cultures of BD413 grown on BHI medium. After 4 days of incubation at 30°C , the cultures were pooled, and the cells were removed by centrifugation at $4,200 \times g$ for 30 min in the cold. To the clarified supernatant fluid was added solid ammonium sulfate to produce 55% saturation. On standing overnight in the cold, a solid precipitate formed. Most of the supernatant was removed by vacuum aspiration; the remainder of the mixture was centrifuged at $4,200 \times g$ for 30 min. The supernatant was discarded, and the pellet was dissolved in deionized water, dialyzed extensively against deionized water, and lyophilized. The yield of this fraction, termed EF-55, was 1.21 g.

Hot phenol treatment of fraction EF-55. In the hot phenol treatment (25), fraction EF-55 (75 mg) was dissolved in 42 ml of deionized water, heated to 70°C , and then stirred with an equal volume of preheated 90% phenol in water; the mixture was stirred at 68 to 70°C for 15 min. After cooling, the mixture was centrifuged, and the lower phenol phase was extracted with an equal volume of water as described above. The phenol phase, together with the interface layer and the pooled viscous aqueous phases, was dialyzed separately against deionized water and lyophilized. The yields were 32 and 25 mg for the water (W-EF-55) and phenol (P-EF-55) phases, respectively.

Isolation of polysaccharide PS-4. Each of four 4-liter flasks, each containing 800 ml of GM medium, was inoculated with 8 ml of BD4 starter cultures that had been grown overnight in BHI medium. After 48 h of incubation at 30°C , the cultures were pooled, and the cells were harvested at $6,000 \times g$ for 1 h at 4°C . The pellet fraction was suspended in water and sheared in a VirTis 45 homogenizer at 28,000 rpm four times for 60 s each time. The viscous mixture was centrifuged at $17,000 \times g$ for 1 h. To the resulting supernatant fluid, 4 volumes of ice-cold acetone were added. After standing in the cold for 1 h, the precipitated polysaccharide was collected by centrifugation, dissolved in water, dialyzed extensively, and then lyophilized. The polysaccharide was dissolved in deionized water and centrifuged at $100,000 \times g$ for 3 h to remove contaminating residual lipopolysaccharide. The final clear supernatant fluid was lyophilized, yielding 0.53 g of polysaccharide (PS-4).

Preparation of the extracellular protein P-R7. Strain BD-R7 was grown in five 1-liter flasks, each containing 200 ml of ethanol-minimal medium, on a gyratory shaker at 30°C . After 48 h of incubation, the cultures were harvested by centrifugation. To the combined, cooled supernatant fluid was added solid ammonium sulfate to yield 60% saturation. The precipitate that formed on standing in the cold was collected by centrifugation and dissolved in 5 mM Tris hydrochloride (pH 7.2) containing 1% NaCl.

Preparation of polysaccharide PS-4 derivatives. The heptasaccharide repeating unit was prepared by treating PS-4 with excess ϕ SL-1 depolymerase and purified by gel

permeation chromatography (P-2; Bio-Rad Laboratories, Richmond, Calif.) as described previously (11). In the polymer, reduction of the glucuronic acid to glucose was achieved by treating the acid form of PS-4 with ethylene oxide and then reducing the ester with sodium borohydride. Esterifications were performed by the carbodiimide method (23). To 100 mg of PS-4 dissolved in 20 ml of water was added 70 μ l of 1 N HCl to yield pH 3.15. After addition of 575 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, the solution was mixed at room temperature; the pH was maintained at 4.75 to 4.80 by the addition of portions (5 to 10 μ l each) of 0.1 N HCl until acid consumption ceased (after 2 h). Total acid consumption was 0.71 mol of H⁺ per mol of glucuronic acid. The solution was then dialyzed and lyophilized, yielding 57 mg of the lactone ester.

Standard emulsification assay. A sensitive assay for determination of emulsifying activity, on both cell-free supernatant fluids and isolated emulsifier fractions, has been described previously (20).

Gel permeation chromatography. Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) chromatography was performed with a 25-mm-internal-diameter, 180-ml-bed-volume column. Fractions (5 ml each) were collected with 20 mM phosphate buffer (pH 7.0) as the eluent at a flow rate of 15 ml/h. The void volume (60 ml) was determined with dextran blue. Protein P-R7 was fractionated on an agarose column (Bio-Gel A-1.5 m, 100/200 mesh; Bio-Rad) 84.5 cm in height and with a 149-ml bed volume. Fractions (3.85 ml each) were collected with 5 mM Tris hydrochloride (pH 7.2) containing 1% NaCl as the eluent at a flow rate of 25 ml/h. The void volume was 50.1 ml.

RESULTS

Characterization of BD4 capsule-specific bacteriophage ϕ SL-1. Electron microscopy of ϕ SL-1 revealed it to be a small phage with a hexagonal head 63 nm in diameter, a 108-nm-long tail, and a base plate 47 nm in diameter. Thus, ϕ SL-1 can be classified into virus group B on the basis of the data of Bradley (2).

When plated with BD4 cells on GM soft agar, ϕ SL-1 produced a characteristic small (1-mm) clear plaque with a surrounding large (5- to 10-mm) turbid halo. Attempts to produce high titers of ϕ SL-1 in liquid culture were initially unsuccessful; after one or two rounds of phage replication, sufficient depolymerase was produced to decapsulate the entire culture (as evidenced by India ink staining). Decapsulated cells are resistant to phage attack. Maximum phage titer was obtained when exponentially growing BD4 (6×10^8 cells per ml) cells were infected with 3×10^7 ϕ SL-1 per ml and incubated for 16 h. The yields were 10^9 to 10^{10} PFU/ml.

Isolation and characterization of ϕ SL-1 depolymerase. The phage depolymerase was isolated from a clarified lysate by precipitation in ammonium sulfate as described in Materials and Methods. Determination of depolymerase activity was performed by two methods. In the first method, a series of twofold dilutions of the enzyme samples was spotted (10 μ l) onto preformed, stationary-phase strain BD4 soft agar lawns. After 16 h of incubation at 30°C, the enzyme dilution still producing an area of decapsulation was noted. The reciprocal of this endpoint dilution represented the enzyme activity; this method yielded only semiquantitative results. In the second method, a colorimetric assay measured the rate of appearance of reducing sugar by using purified strain BD4 sheared capsule (PS-4) as the substrate. The first

TABLE 1. Growth and polysaccharide, protein, and emulsifier production on glucose medium^a

Strain	Growth (Klett units)	Production of:			
		Polysaccharide		Extracellular protein (mg/liter)	Emulsifying activity (U/ml) ^b
		Capsular (mg/liter)	Extracellular (mg/liter)		
BD4	485	450	75	19	9
BD413	230	64	53	71	50
BD4-R7	410	3	0	7	0
BD4-R8	410	3	0	18	0

^a Measurements were performed after 48 h of incubation, at which time all cultures were in stationary phase.

^b On the extracellular fluid.

method was used in evaluating enzyme activity during isolation steps. A prerequisite for the second method is the presence of little or no reducing sugar in the enzyme sample.

Preliminary observations showed that the depolymerase was active in distilled water without added salts or buffer. A slight stimulation of activity (50 to 60%) was obtained in the presence of 1.0 and 10 mM MgSO₄. The stimulating ion was magnesium since (NH₄)₂SO₄ at 10 mM had no effect. Although maximum activity was obtained at pH 7.0 (10 mM Tris buffer), the enzyme showed considerable activity over the entire pH range tested, 5.0 to 8.0. Calcium ion could only partially replace the magnesium ion stimulation. The two polysaccharides PS-4 and PS-413 served equally well as substrates for the depolymerase. Ultrafiltration experiments (Amicon stirred cell fitted with an XM-100 membrane) showed that the depolymerase has a molecular mass greater than 100,000 daltons.

Isolation and characterization of phage-resistant mutants. Mutants resistant to capsule-specific bacteriophages often do not produce exopolysaccharides (13). In order to clarify the role of the capsule polysaccharide in emulsification, phage-resistant mutants were isolated. Plating of GM medium-grown stationary-phase BD4 (ca. 10^7 cells) cells together with 4×10^5 PFU of bacteriophage ϕ SL-1 onto GM medium plates resulted in growth of many apparently phage-resistant mutants after 48 h of incubation at 30°C. These colonies were translucent and flat, as opposed to the round mucoid parental colonies; cells within the translucent colonies were devoid of any capsular material, as evidenced by India ink staining. When these colonies were streaked and tested for phage resistance, however, only a small percentage of them retained their translucent phenotype and phage resistance. The others had resynthesized their capsular material and regained phage sensitivity. (The probable explanation for this latter phenomenon is that enough depolymerase was present on the plate used to select for phage resistance to decapsulate entire colonies, thus rendering them phenotypically, but not genotypically, resistant.) After four sequential isolation and phage-resistant testing steps, two independent ϕ SL-1-resistant clones were isolated. Both mutants, referred to as BD4-R7 and BD4-R8, were devoid of capsular material. DNA from strain BD4 transformed BD4-R7 and BD4-R8 cells to the parental phenotype (i.e., large capsule and ϕ SL-1 sensitivity) at a frequency of about 1%. Controls with no added DNA failed to show any revertants.

Polysaccharide, protein, and emulsifier production by strain BD4 and its derivatives. When grown on GM medium (Table 1), BD4 cells produced a large amount of capsular polysaccharide, BD413 cells produced considerably less, and the two phage-resistant mutants, BD4-R7 and BD4-R8, produced practically none. Strain BD4 extracellular polysaccha-

TABLE 2. Growth and polysaccharide, protein, and emulsifier production on ethanol medium^a

Strain	Growth (Klett units)	Production of:		
		Polysaccharide		Emulsifying activity (U/ml) ^b
		Capsular (mg/liter)	Extracellular (mg/liter)	
BD4	870	74	160	190
BD413	460	16	76	21
BD4-R7	460	6	2	4
BD4-R8	450	6	1	5

^a Measurements were performed after 48 h of incubation, at which time all cultures were in stationary phase.
^b On the extracellular fluid.

ride production was only slightly higher than that of strain BD413, whereas the two phage-resistant strains were completely devoid of the polysaccharide. Examination of extracellular protein production showed that strain BD413 produced 71 µg of protein per ml, whereas strains BD4, BD4-R7, and BD4-R8 produced only 10 to 27% of this amount. These data suggest that, to obtain significant levels of emulsifying activity, production of high levels of both extracellular polysaccharide and protein is necessary.

When grown in 2% ethanol medium (Table 2), BD4 cells produced over 20 times the level of extracellular emulsifying activity and 16 times the amount of extracellular protein but only about 16% the amount of capsular polysaccharide as compared with growth on glucose medium. Strains BD4-R7 and BD4-R8 clumped heavily when grown in ethanol. Total polysaccharide content and emulsifying activity of both strains were ca. 3% that of strain BD4, but extracellular protein levels were 1.7 times higher. The major effect of ethanol medium on the phage-resistant mutants was that extracellular protein concentration was more than 25 times higher than in glucose medium.

Emulsifying activity of purified fractions. All of the BD413 extracellular emulsifying activity precipitated when the growth supernatant fluid was brought to 55% ammonium sulfate saturation. This fraction (EF-55) was applied to a Sepharose-4B column and eluted with phosphate buffer (Fig. 1). The elution profile showed that all of the rhamnose-containing polysaccharide and emulsifying activity, plus some protein, was recovered as a single peak in the void volume. The exclusion limit for Sepharose-4B is 5 × 10⁶ for polysaccharides; therefore, the emulsifier eluted as a high-molecular-weight polysaccharide-protein complex. Most of the protein eluted at a lower molecular weight and had no emulsifying activity. The composition and emulsifying activity of this high-molecular-weight polysaccharide-protein complex (EF-55-4B) were compared with those of the initial emulsifier EF-55 and the highly purified polysaccharide and protein preparations (Table 3). The increased specific emulsifying activity of EF-55-4B was accompanied by a corresponding removal of the lower-molecular-weight inactive protein. Neither highly purified polysaccharide nor protein(s) showed significant emulsifying activity.

Reconstitution of emulsifying activity with polysaccharide and protein. Treatment of EF-55 with the phage depolymerase resulted in a time-dependent loss of emulsifying activity. After 2 days of incubation, only 20% of the original emulsifying activity remained (Table 4). A control lacking depolymerase showed no loss of emulsifying activity during the incubation period. Addition of the purified BD4 capsular polysaccharide (PS-4), which had no emulsifying activity by itself, resulted in restoration of the emulsifying

TABLE 3. Composition and emulsifying activity of purified materials from BD4 and its derivatives

Fraction	Composition (mg/ml) of:		Emulsifying activity (U/mg)
	Polysaccharide	Protein	
EF-55 ^a	3.0	2.2	220
EF-55-4B ^b	3.1	0.55	302
Purified polysaccharide ^c			
W-EF-55	2.8	0.21	25
PS-4	2.9	0.07	0
Protein rich ^d			
P-EF-55	0.1	2.4	5
P-R7	0.04	3.5	0

^aBD413 extracellular emulsifier purified by precipitation at 55% ammonium sulfate saturation and dialysis.

^b Pooled fractions (fractions 12 to 16) eluted from Sepharose-4B (Fig. 1).

^c W-EF-55 is deproteinized (phenol extraction) EF-55; PS-4 is the BD4 capsular polysaccharide isolated by shearing whole cells and purified by acetone precipitation.

^d P-EF-55 is the dialyzed phenol phase of W-EF-55; P-R7 is BD4-R7 extracellular material purified by precipitation at 60% ammonium sulfate saturation and dialysis.

activity of the enzyme-treated EF-55 to its original value. Increasing the PS-4 concentration above the original polysaccharide concentration in EF-55 did not further increase emulsifying activity. Sepharose-4B chromatography of the depolymerase-treated EF-55 indicated only partial depolymerization of the polysaccharide, possibly explaining the residual 20% activity.

Emulsifying activity was reconstituted by mixing purified polysaccharide PS-4 and protein P-R7 (Fig. 2). At a fixed polysaccharide concentration of 70 µg/ml, emulsifying activity increased from 0.1 U/ml with no protein P-R7 to 13.2 U/ml at 40 µg of P-R7 per ml. Similarly, at a fixed P-R7 concentration of 17 µg/ml, the emulsifying activity increased from 0.4 U/ml with no PS-4 to 0.9, 7.7, and 13.0 U/ml with 13, 60, and 70 µg of PS-4 per ml, respectively.

Several chemical derivatives of PS-4 were tested in the emulsifier complementation assay with 93 µg of protein P-R7 per ml. Three of these derivatives were modified via the carboxyl group of the glucuronic acid residue. Abolishing the negative charge of the uronic acid by reduction to glucose or by forming a lactone ester caused reductions of 75 and 69% in the emulsifying activities, respectively. Forming a hydroxypropyl ester, which contains a three-carbon aliphatic residue, caused a loss of 51% of the emulsifying activity. The heptasaccharide repeating unit of PS-4, which

TABLE 4. Treatment of fraction EF-55 with φ SL-1 depolymerase and reconstitution of emulsifying activity

Fraction	Emulsifying activity, U/ml (%)
PS-4 ^a	0
EF-55 ^b	140 (100)
+ Depolymerase ^c	30 (21)
+ Depolymerase + PS-4 ^d	139 (99)

^a The purified polysaccharide PS-4 (Table 3) was used at a final concentration of 0.27 mg/ml.

^b Contained 0.20 mg of protein and 0.27 mg of polysaccharide per ml.

^c EF-55 (1.6 ml) was incubated with 80 µl of the φ SL-1 depolymerase preparation (6.2 U) for 48 h at 30°C.

^d PS-4 (0.27 mg/ml) was added to the enzyme-treated EF-55 just before the assay for emulsifying activity.

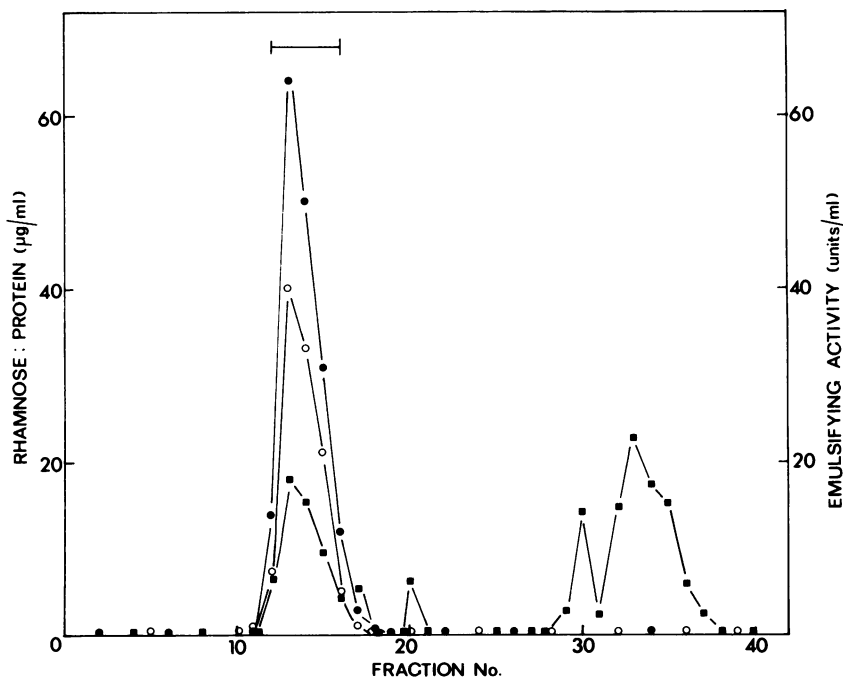


FIG. 1. Sepharose-4B chromatography of fraction EF-55. Fractions (5 ml each) were assayed for rhamnose (●), protein (■), and emulsifying activity (○).

contains the same sugar residues and linkages as the polymeric PS-4, exhibited no emulsifying activity. These results indicate that both the negative charge carried by the uronic acid and the polymeric, high-molecular-weight form of PS-4 are necessary for full emulsifying activity.

The protein(s) necessary for reconstituting emulsifying activity was purified about sevenfold by agarose chromatography (Table 5). Essentially all of the activity was eluted in the void volume (46 to 54 ml; peak 1). Thus, the active protein(s) probably exists as large aggregates (even in the absence of the polysaccharide). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%) of peak 1 showed at least four polypeptides of molecular masses 22.6, 23.7, 48.5, and 76 kilodaltons. Most of the P-R7 protein eluted from the gel at 127 to 147 ml (peak 2). This relatively low-molecular-mass material (a single major 25-kilodalton band) was inactive in the reconstitution assay.

DISCUSSION

Growth of *A. calcoaceticus* BD4 and BD413 on different carbon sources results in the production of various amounts of extracellular emulsifying activity. In general, there was a good correlation between emulsifying activity and the presence of both extracellular protein and polysaccharide. For example, when grown on glucose medium, strain BD4 produced high levels of extracellular polysaccharide but low levels of protein and emulsifying activity; on ethanol medium, on which BD4 produced high levels of emulsifying activity, the extracellular fluid contained high amounts of both protein and polysaccharide.

Isolation of the capsule-specific bacteriophage ϕ SL-1 made possible direct tests for the involvement of polysaccharide and protein in the strain BD4 emulsifying activity. The phage served as the source for a specific endo- β -D-glucosidase that hydrolyzes the bond β -D-glucose-(1-3)-L-

rhamnose (11). When this phage-induced depolymerase was added to the BD413 emulsifier (fraction EF-55), activity decreased over time of incubation. Activity could be restored by addition of pure capsule polysaccharide (PS-4), further indicating that the polysaccharide was required for emulsifying activity. The polysaccharide component of the emulsifier complex was easily obtained in a pure form by either purification of the BD4 capsular polysaccharide or hot phenol deproteinization of EF-55. However, the protein prepared by depolymerase treatment of EF-55 or by phenol extraction of EF-55 contained depolymerase and fragments of polysaccharide or was denatured. Therefore, a "clean" source of extracellular protein was needed. Mutants resistant to capsule-specific bacteriophages, often defective in exopolysaccharide synthesis (13), were considered to be a source of such proteins. By using phage ϕ SL-1 as a selective agent, strain BD4 phage-resistant mutants were isolated. When grown on glucose medium, the BD4 phage-resistant mutants produced neither extracellular polysaccharide nor emulsifying activity and only low levels of extracellular protein. However, when grown on 2% ethanol medium, the mutants produced large amounts of extracellular protein and only ca. 3% the amount of exopolysaccharide and emulsifying activity as the parental BD4. One of these mutants, BD4-R7, was the source of extracellular protein (P-R7) used in the emulsifier reconstitution studies.

When protein P-R7 was used in the emulsifier complementation assay, together with polysaccharide PS-4, a complete reconstitution of emulsifying activity was obtained. Reconstitution of emulsifying activity was also achieved by addition of P-R7 to the deproteinized BD413 extracellular polysaccharide. Addition of the purified polysaccharide (PS-4) to depolymerase-treated EF-55 also resulted in reconstitution of the emulsifying activity value to that of the original BD4 emulsan.

It is interesting to compare the chemical structure and

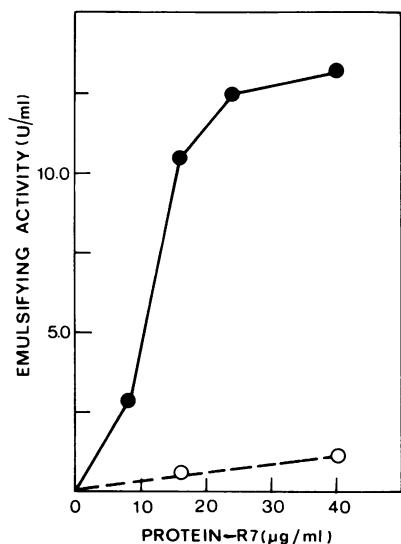


FIG. 2. Reconstitution of emulsifying activity by addition of protein to the pure polysaccharide PS-4. The emulsifying activity of mixtures containing various concentrations of protein P-R7 and 70 µg of PS-4 per ml (●) and of R7 only (○) was determined by the standard emulsification assay.

emulsifying activity of the BD4 and RAG-1 emulsans. The chemical structure of each of the extracellular emulsans is identical to that of the capsular polysaccharide of the corresponding strain (5, 11). Moreover, in capsule form, both of the BD4 and RAG-1 emulsan polysaccharides prevent cell adhesion to hydrocarbons (18). How then do the RAG-1 and BD413 polysaccharide emulsans assist in emulsification? When emulsans are released from the capsular structures, they contain noncovalently linked complexed proteins. In addition, RAG-1 emulsan contains covalently linked fatty acids. As reported here, the protein component plays a crucial role in the emulsifying activity of the BD4 emulsan. In the case of the RAG-1 emulsan, the protein is not absolutely required for emulsifying activity, presumably because hydrophobic sites, covalently linked fatty acids, contribute significantly to its activity. Characteristic of both emulsans is the fact that pure polysaccharide backbones, lacking hydrophobic fatty acid esters and complexed proteins, show little or no emulsifying activity. Therefore, the ability of the emulsans to adsorb hydrocarbon-water interfaces and to form stable interface films is due to amphipathic

TABLE 5. Protein requirement for reconstitution of emulsifying activity

Amt of P-R7 (µg/ml)	Emulsifying activity, ^a U/ml (U/mg of protein)
16	7.7 (480)
6 (peak 1) ^b	20.0 (3,300)
44 (peak 2) ^b	0.5 (11)

^a The standard emulsification assay contained 70 µg of polysaccharide PS-4 per ml.

^b P-R7 was fractionated on a Bio-Gel A-1.5 m column. Peak 1 corresponds to the void volume and contained 12% of the input protein and 95% of the emulsifying activity. Peak 2, corresponding to a molecular weight of less than 50,000, contained 85% of the input protein and less than 3% of the emulsifying activity.

properties derived from a unique combination of a hydrophilic polysaccharide backbone with covalently or noncovalently hydrophobic components. Accordingly, substrate specificity shown by different *Acinetobacter* emulsans (10, 19, 21) should be dependent on their hydrophobic components. Moreover, the polysaccharide backbone either must be bound covalently to the hydrophobic components or must be able to form stable complexes with them. The polysaccharide component conveys specific properties to the outer surface of the emulsified droplets, such as the ability to bind uranyl ions and specific phages (16, 26, 27).

The concept of hydrophilic polysaccharides attaching to hydrocarbons via specific linkers might help to explain how polysaccharide capsules are attached to the outer membrane of gram-negative bacteria. To date, no covalent linkages between these capsular polysaccharides and other cell envelope components have been found. It is possible that the same specific linkers in RAG-1 (fatty acids) and in BD413 (proteins) play a role in stabilizing the hydrophilic capsules around the hydrophobic outer cell membrane. If this is the case, then capsules and other hydrophilic structures which are bound to the lipid membrane might be functional as amphiphilic proteins when released from the cell surface together with amphipathic proteins. In this regard, we show here that an *Escherichia coli* lipoprotein is as active as protein P-R7 with PS-4 when added to PS-4 in the emulsifier complementation assay.

ACKNOWLEDGMENTS

We thank S. Inouye for a sample of *E. coli* lipoprotein and A. Bogin for performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

This work was sponsored in part by a grant from Petroleum Fermentations, Curacao, Netherlands Antilles.

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