APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1982, p. 1335–1341
0099-2240/82/121335-07$02.00/0

Vol. 44, No. 6

Exopolysaccharide Distribution of and Bioemulsifier Production by *Acinetobacter calcoaceticus* BD4 and BD413

NACHUM KAPLAN AND EUGENE ROSENBERG*

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

Received 15 April 1982/Accepted 16 August 1982

The heavily encapsulated *Acinetobacter calcoaceticus* BD4 and the "mini-encapsulated" single-step mutant *A. calcoaceticus* BD413 produced extracellular polysaccharides in addition to the capsular material. The molar ratio of rhamnose to glucose (3:1) in the extracellular BD413 polysaccharide fraction was similar to the composition of the capsular material. In both strains, the increase in capsular polysaccharide was parallel to cell growth and remained constant in stationary phase. The extracellular polysaccharides were detected starting from mid-logarithmic phase and continued to accumulate in the growth medium for 5 to 8 h after the onset of stationary phase. Strain BD413 produced one-fourth the total rhamnose exopolysaccharide per cell that strain BD4 did. Depending on the growth medium, 32 to 63% of the rhamnose polysaccharide produced by strain BD413 was extracellular, whereas in strain BD4 only 7 to 14% was extracellular. In all cases, strain BD413 produced more extracellular rhamnose polysaccharide than strain BD4 did. In glucose medium, strain BD413 also produced approximately 10 times more extracellular emulsifying activity than strain BD4 did. The isolated capsular polysaccharide obtained after shearing of BD4 cells showed no emulsifying activity. Thus, strain BD413 either produces a modified extracellular polysaccharide or excretes an additional substance(s) that is responsible for the emulsifying activity. Emulsions induced by the ammonium sulfate-precipitated BD413 extracellular emulsifier require the presence of magnesium ion and a mixture of an aliphatic and an aromatic hydrocarbon.

Acinetobacter calcoaceticus RAG-1 was isolated and characterized as a hydrocarbon-degrading microorganism which produces a potent extracellular bioemulsifier (11, 13). This emulsifier, termed emulsan, is an anionic heteropolysaccharide with an average molecular weight of 9.9×10^5 (19). The major sugar components of emulsan are *N*-acetyl-D-galactosamine and an unidentified amino uronic acid (19). Fatty acids are linked via O-ester bonds to the polysaccharide backbone (2).

RAG-1 was initially incorrectly identified as an *Arthrobacter* species (11). Subsequently, it was demonstrated by a variety of conventional biochemical tests (O. Pines and R. Gherna, unpublished data), including DNA base analysis, that strain RAG-1 is in fact an *Acinetobacter* species. This was confirmed by DNA transformation of the highly competent *A. calcoaceticus* BD413 (3, 8) with RAG-1 DNA (N. Kaplan, M.Sc. thesis, Tel Aviv University, 1980).

Because it was demonstrated that RAG-1 DNA could transform strain BD413 to prototrophy, the possibility of transforming the genes for synthesis of emulsan into strain BD413 was considered. However, preliminary studies revealed that strain BD413 also produced potent extracellular emulsifier(s). Although this finding eliminated the possibility of straightforward screening for the proposed transformants, it indicated that emulsifier production may be a general property of *Acinetobacter* species.

A. calcoaceticus BD413 is a "mini-encapsulated" mutant derived from the heavily encapsulated *A. calcoaceticus* BD4 (10). The capsular polysaccharide of strain BD4 is composed of L-rhamnose and D-glucose in a molar ratio of 4 to 1 (18). The observation that strains BD4 and BD413 produced rhamnose-containing extracellular polysaccharides in addition to the capsular material led to an examination of the production and distribution of these exopolysaccharides. The single mutational event that led to the formation of the reduced capsule in strain BD413 resulted in both enhanced extracellular rhamnose polymer and bioemulsifier activity.

MATERIALS AND METHODS

Microorganisms and growth conditions. *A. calcoaceticus* BD4, originally isolated by Taylor and Juni (18),

was generously provided by K. Bryn. *A. calcoaceticus* BD413 trpE27, a mini-encapsulated (10) tryptophan auxotroph (8, 14) derived from strain BD4, was a gift from E. Juni. Both strains were maintained on nutrient agar plates (nutrient broth solidified with 1.5% agar, both from Difco Laboratories, Detroit, Mich.). All growth experiments, unless stated otherwise, were performed in 100-ml flasks containing 20 ml of the following media. (i) LPMS medium contained (per liter of deionized water): 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 $MgSO_4 \cdot 7H_2O$ (final pH 7.0). (ii) HPMS medium was the same as LPMS medium, except that the amount of phosphate salts was increased to 22.2 g of $K_2HPO_4 \cdot 3H_2O$ and 7.26 g of KH_2PO_4 per liter. D-Glucose or lactic acid (a variable mixture of D- and L-lactic acids, BDH, Poole, England) was added from 25% (wt/vol) stock solutions to LPMS and HPMS media to a final concentration of 0.5%. All media for culturing strain BD413 were supplemented with 0.1 mg of L-tryptophan per ml. Brain heart infusion was a product of Difco. Bacterial growth was initiated by introducing a 1% inoculum obtained from starter cultures grown in 2 ml of the identical medium in 16-mm test tubes. Flasks were incubated at 30°C in a New Brunswick G24 Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 250 rpm. Bacterial growth was followed by determining turbidity in a Klett-Summerson photometer equipped with a green filter.

Determination of cell-bound and extracellular polymeric rhamnose. Culture samples were centrifuged in the cold at 12,000 × *g* for 20 min. The cell pellets were suspended in phosphate buffer (62 mM, pH 7.0) to the original sample volume. Cell-free supernatant fluid samples (0.5 ml) were mixed with 4 volumes of ice-cold acetone, left on ice for 30 min, and centrifuged at 12,000 × *g* for 10 min at 4°C. The white precipitates obtained were dissolved in 0.5 ml of the phosphate buffer described above. The amount of rhamnose was measured both directly on the whole-cell suspensions (9) and on the acetone precipitate fractions by the sulfuric acid-cysteine procedure for 6-deoxyhexoses (4), using L-rhamnose as a standard. The rhamnose content of acetone precipitates of fresh brain heart infusion (24 μg per ml of original brain heart infusion) was subtracted from all determinations of rhamnose in extracellular samples obtained from brain heart infusion-grown cultures.

Control experiments indicated that all of the extracellular rhamnose was non-dialyzable and could be recovered in the pellet fraction after acetone precipitation.

Determination of total cell numbers and dry weights. *A. calcoaceticus* BD4 and BD413 were grown in 100-ml flasks containing 20 ml of LPMS medium supplemented with 0.5% glucose. After 48 h of incubation, the cultures were harvested by centrifugation at 12,000 × *g* for 30 min at 4°C. The cell pellet was washed once in phosphate buffer (62 mM, pH 7.0) and then was suspended in the same buffer to the original culture volume. The washed cells were then dialyzed extensively in the cold against distilled water. After dialysis, the turbidity of the cell suspension was determined in a Klett-Summerson photometer (green filter), and the total cell number was determined by using a Petroff-Hausser bacteria counter. A comparison of the total cell number determined before and after dialysis showed that cell lysis did not occur during dialysis. For determination of cell dry weight, triplicate 10-ml samples of the dialyzed cell suspensions were treated in tared aluminum foil cups at 80°C for 16 h.

Standard emulsification assay. A sensitive assay for determination of emulsifying activity, both on cell-free supernatant fluids and on isolated emulsifier fractions, has been developed previously in this laboratory (13). In brief, 0.1 to 0.5 ml of the sample to be tested and 0.1 ml of a mixture of hexadecane–2-methylnaphthalene (1:1, vol/vol) were introduced into a 100-ml flask containing Tris-magnesium buffer (Tris [20 mM, pH 7.0] containing 2 mM $MgSO_4$) to a final volume of 7.5 ml. The assay mixture was incubated at 30°C with reciprocal shaking (150 strokes per min) for 1 h. The turbidities of the assay mixtures were then determined in a Klett-Summerson photometer (green filter). The turbidities resulting from mechanical emulsification of the assay mixture without added emulsifier (5 to 20 Klett units [K.U.]) and from the initial turbidities of the samples tested were subtracted from the experimental results. One unit of emulsifying activity per milliliter is defined as that concentration of activity that yields 100 K.U. in the assay mixture.

Isolation of an extracellular emulsifier fraction from *A. calcoaceticus* BD413. Strain BD413 cells were grown in 250-ml flasks containing 50 ml of HPMS medium supplemented with 0.5% lactic acid. After incubation at 30°C (150 rpm) for 48 h, these cultures were used as an inoculum (2%) for four 4-liter flasks, each containing 1.1 liters of the same medium. After 92 h of incubation, the cultures were harvested by centrifugation in the cold at 4,230 × *g* for 15 min. The supernatant fluids were pooled (total volume, 4,380 ml), and 712 g of ammonium sulfate was added to reach 30% saturation. No visible precipitate formed after the solution was left overnight in the cold. Ammonium sulfate was then added sequentially to reach 35% (842 g), 40% (981 g), 45% (1,121 g), and 50% (1,263 g) saturation. After each step, the solution was left overnight in the cold. A visible precipitate appeared at 45 to 50% saturation. The turbid suspension was centrifuged at 4,230 × *g* for 15 min at 4°C. The yellow-brown precipitate was dissolved in 100 ml of deionized water, dialyzed extensively in the cold against distilled water, and lyophilized. The lyophilized fraction, termed EF-50, was dried to constant weight in a desiccator, yielding 463 mg.

Emulsification requirements of emulsifier fraction EF-50. To determine the optimal pH for emulsifying activity, Tris buffer was omitted from the standard emulsification assay, and the pH was adjusted with NaOH or HCl. The reported pH values are those in the reaction mixture containing 50 μg of EF-50 per ml. To study the cation requirements for emulsification, the standard emulsification assay containing 50 μg of EF-50 per ml was modified to contain varying amounts of $MgSO_4$ or varying amounts of NaCl.

Shearing of capsular polysaccharide from *A. calcoaceticus* BD4. Strain BD4 was grown in 100-ml flasks containing 20 ml of HPMS medium supplemented with 0.5% lactic acid. After incubation at 30°C (150 rpm) for 24 h, this culture was used as an inoculum (1%) for four 1-liter flasks, each containing 200 ml of the same medium. After 24 h of incubation (200 K.U.), the cultures were pooled and harvested by centrifugation in the cold at 17,000 × *g* for 20 min. The pellet was

washed twice in distilled water and suspended in distilled water to a final volume of 40 ml. The washed cell suspension was passed under pressure twice through a glass chromatogram sprayer (9). The sheared cell suspension was then centrifuged as described above, and the supernatant fluids were pooled. The cell pellet was resuspended in distilled water to a final volume of 26 ml, and the pellet and supernatant fractions were assayed for rhamnose content separately.

Hydrocarbons and petroleum products. Hexadecane (Purum, >99%) was from Fluka AG, Switzerland. Octane (analytical grade) was obtained from Merck, Darmstadt, West Germany. 2-Methylnaphthalene was purchased from the Aldrich Chemical Co., Milwaukee, Wis. Darius crude oil was obtained from the Ashkelon-Eilat Pipeline Co., Israel. Gach-Saran gas oil and Agha-Jari kerosene were obtained from the Haifa Refinery, Haifa, Israel.

RESULTS

Growth and exopolysaccharide production. The kinetics of growth and exopolysaccharide production of strains BD4 and BD413 on glucose-containing medium are shown in Fig. 1 and 2, respectively. *A. calcoaceticus* BD4 had a doubling time of 2.7 h (Fig. 1) and reached stationary phase after 17 h of incubation. The pH of the growth medium decreased from an initial value of 7.0 to a minimum of 6.0 at 10 h and then increased to 6.4. Rhamnose-containing exopolysaccharides increased parallel to culture turbidity. The fraction of rhamnose that was cell associated reached a maximum value at the same time that cells reached stationary phase, whereas extracellular rhamnose reached only 50% of its maximal value at 17 h. The value for extracellular rhamnose at 9 h (not shown) was 0.003 g per liter. The total rhamnose production after 33 h was 0.58 g per liter. The extracellular rhamnose represented only a small fraction (14%) of this total.

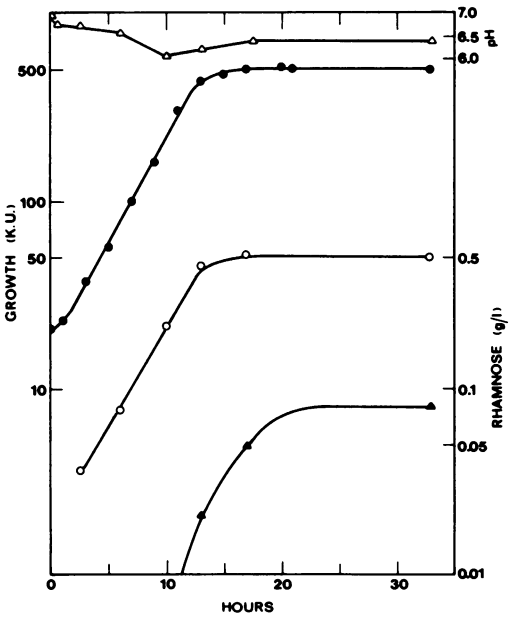

FIG. 1. Kinetics of growth and exopolysaccharide production by *A. calcoaceticus* BD4. An overnight culture in glucose-LPMS medium was inoculated (0.2 ml) into a 100-ml sidearm flask containing 20 ml of the same medium. At the time intervals indicated, the culture turbidity (●) was determined, and 3-ml samples were removed for determination of pH (△), cell-bound rhamnose (○), and extracellular rhamnose (▲) content.

A. calcoaceticus BD413 had an average doubling time of 4.5 h (Fig. 2) during the initial 20 h of incubation and reached stationary phase after 22 h. The pattern of pH change was similar to that for strain BD4, except that the minimal value (5.75) was lower and prevailed for a longer time period. Cell-associated rhamnose reached a maximum value at the same time that cell growth ceased, and extracellular polymeric rhamnose reached only 75% of its maximal value at 22 h. The total rhamnose produced by *A. calcoaceticus* BD413 at 33 h was 0.23 g per liter. The largest difference between the strains was that *A. calcoaceticus* BD413 excreted a much larger portion (43%) of the total polymeric rhamnose it produced as compared with strain BD4.

Distribution of rhamnose exopolysaccharides in *A. calcoaceticus* BD4 and BD413. To determine quantitatively the amount of cell-bound and extracellular exopolysaccharides per cell for the two strains, the following experiment was performed: *A. calcoaceticus* BD4 and BD413 were grown under the conditions described in the legends to Fig. 1 and 2; after 48 h of growth, the cultures were harvested and total cell numbers and cell dry weights were determined. The values obtained, standardized for 100 K.U., were 5.8×10^8 cells per ml and 0.46 g per liter for *A. calcoaceticus* BD4 and 1.2×10^9 cells per ml and 0.38 g per liter for *A. calcoaceticus* BD413. By using these values together with the rhamnose values at 33 h in Fig. 1 and 2, and assuming that rhamnose represents 80% of the capsular polysaccharide of *A. calcoaceticus* BD4 (18), the amount of exopolysaccharide per cell and the weight fractions of these polysaccharides were calculated. The dry weight per cell for *A. calcoaceticus* BD4 (7.9×10^{-13} g) was 2.5 times greater than that for *A. calcoaceticus* BD413 (3.2×10^{-13} g). Total exopolysaccharide production per cell was 3.9 times greater in *A. calcoaceticus* BD4 (2.5×10^{-13} g) than in strain BD413 (0.64×10^{-13} g). This exopolysaccharide represented 31% of the dry cell weight in *A. calcoaceticus* BD4, with 87% of it being cell associated. In *A. calcoaceticus* BD413, the total

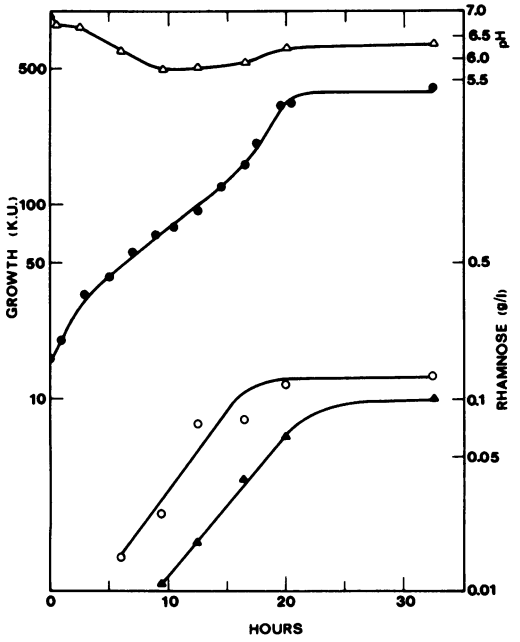

FIG. 2. Kinetics of growth and exopolysaccharide production by *A. calcoaceticus* BD413. The experiment was performed as described in the legend to Fig. 1, except that the growth medium was supplemented with 0.1 mg of tryptophan per ml. The culture turbidity (●), pH (△), and cell-bound (○) and extracellular (▲) rhamnose contents were determined at timed intervals.

exopolysaccharide represented 20% of the dry cell weight, with an almost equal distribution between cell-bound and extracellular forms of the polysaccharide.

Effect of carbon source on growth and exopolysaccharide production. It had previously been demonstrated that *A. calcoaceticus* BD4 capsule diameter varied with growth substrate, including glucose and lactic acid (18); however, the extracellular polysaccharide was not examined. Glucose proved to be a better carbon source than lactic acid for total exopolysaccharide production in *A. calcoaceticus* BD4 and BD413 (Table 1). Exopolysaccharide production by strain BD4 was strongly inhibited during growth on brain heart infusion. No such inhibitory effect was found for *A. calcoaceticus* BD413. Depending on the carbon source, *A. calcoaceticus* BD413 produced a total of between 142 and 205 mg of polymeric rhamnose per liter and excreted between 32 and 63% of this total, whereas *A. calcoaceticus* BD4 produced a total of 202 to 593 mg per liter and excreted between 7 and 14% of this total. With all three substrates, *A. calcoaceticus* BD413 produced less capsular polysaccharide but more extracellular polysaccharide than did strain BD4.

Production of extracellular emulsifying activity. Cell-free supernatant fluids of strains BD4 and BD413 were assayed for emulsifying activity (Fig. 3). Significant amounts of extracellular emulsifying activity were produced by *A. calcoaceticus* BD413 after 10 h of growth, reaching a value of 55 U/ml after 33 h of growth. In comparison, *A. calcoaceticus* BD4 showed a slow release of extracellular emulsifying activity that reached a value of only 7 U/ml after 33 h of growth.

The cell-free supernatant fluid fractions assayed for rhamnose content (Table 1) were also assayed for emulsifying activity, using the standard emulsification assay. The results obtained for cultures grown on lactic acid and glucose media were 25 and 4 U/ml, respectively, for *A. calcoaceticus* BD4, and 61 and 70 U/ml, respectively, for strain BD413. No emulsifying activity was detected in the brain heart infusion cultures. However, a control experiment showed that both brain heart infusion and acetone-precipitat-

TABLE 1. Effect of carbon source on growth and exopolysaccharide production[a]

Strain	Carbon source[b]	Growth (K.U.)	pH[c]	Exocellular rhamnose polysaccharide[d]	
				Cell-associated (mg/liter)	Extracellular (mg/liter)
BD4	Lactic acid	560	8.55	313	42
BD4	Glucose	460	6.38	534	59
BD4	Brain heart infusion	580	8.64	188	14
BD413	Lactic acid	300	8.47	53	89
BD413	Glucose	325	6.12	116	79
BD413	Brain heart infusion	530	8.54	140	65

[a] Cells were grown in 100-ml flasks containing 20 ml of medium with gyratory shaking at 30°C. After 48 h of incubation (24 h for brain heart infusion medium), the cultures were harvested by centrifugation; supernatant fluids and resuspended pellets were assayed separately. Each experiment was repeated at least twice with similar results; data from a single experiment are presented.

[b] Lactic acid and glucose cultures were grown in HPMS and LPMS media, respectively.

[c] The initial pH in all cases was 7.0 ± 0.1.

[d] The exopolysaccharide concentration is expressed as rhamnose equivalents.

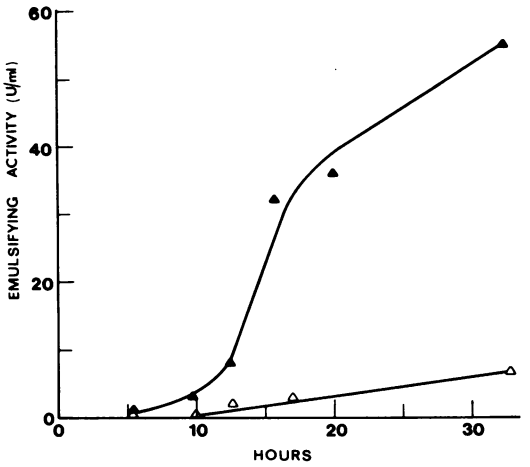

FIG. 3. Extracellular emulsifier production by *A. calcoaceticus* BD4 and BD413. Samples of cell-free supernatant fluid taken during the growth experiments described in the legends to Fig. 1 and 2 from strains BD4 (△) and BD413 (▲) were assayed by the standard emulsification assay.

ed brain heart infusion caused a 99% inhibition of the emulsifying activity of a cell-free supernatant fraction (containing 75 U/ml) obtained from an *A. calcoaceticus* BD413 culture grown on glucose. Therefore, the amount of emulsifying activity, if any, during growth on brain heart infusion is not measurable by this technique.

Requirements for the extracellular emulsifier of *A. calcoaceticus* BD413. A partially purified emulsifier, termed EF-50, was obtained from the cell-free medium of *A. calcoaceticus* BD413, as described in Materials and Methods. Table 2 summarizes the results of using different hydrocarbon substrates for emulsification by fraction EF-50. A mixture of both an aliphatic (e.g., hexadecane) and an aromatic (e.g., 2-methylnaphthalene) hydrocarbon was required for maximum emulsion formation. In mixtures of hexadecane and 2-methylnaphthalene, a volume fraction of hexadecane of 0.2 to 0.7 was necessary for effective emulsification (Fig. 4). Neither of these two hydrocarbons alone was significantly emulsified. Similarly, a mixture of octane and 2-methylnaphthalene was emulsified better than either hydrocarbon alone. Similar data presented for octane were obtained with alkanes from C_9 to C_{12}, whereas hexadecane was typical of the C_{14} to C_{16} aliphatics.

An examination of the optimal conditions for the emulsifying activity of fraction EF-50 demonstrated that maximal emulsifying activity was obtained in the pH range of 6.5 to 7.5 in the presence of 2 to 5 mM magnesium ion. Higher concentrations of magnesium ion (20 to 100 mM) caused a 60% inhibition of maximal activity. Sodium ion stimulated the activity over a wide concentration range (2 to 50 mM); however, the maximum sodium-stimulated activity was only 63% of the activity obtained with the optimal concentration of magnesium. Higher concentrations of sodium ion (100 mM) caused a sharp inhibition of the maximal activity.

Examination of emulsifier activity of the rhamnose polymer obtained by shearing *A. calcoaceticus* BD4. The specific emulsifying activity determined on whole cells before or after shearing was less than 10% of that of the extracellular

TABLE 2. Hydrocarbon substrate specificity of the extracellular emulsifier of *A. calcoaceticus* BD413[a]

Hydrocarbon substrate	Relative emulsifying activity (%)[b]
Hexadecane–2-methylnaphthalene[c]	100
Hexadecane	8
2-Methylnaphthalene	3
Octane–2-methylnaphthalene[c]	74
Octane	26
Crude oil	9
Gas-oil	43
Kerosene	74

[a] The extracellular emulsifier of BD413 used in these experiments was partially purified by precipitation at 50%, ammonium sulfate saturation, and dialysis.

[b] The standard emulsification assay was used with 50 μg of the emulsifier fraction per ml and 0.1 ml of the indicated hydrocarbon substrate. Emulsifying activity is expressed as the percentage of the activity when hexadecane–2-methylnaphthalene was used.

[c] Ratio of 1:1 (vol/vol).

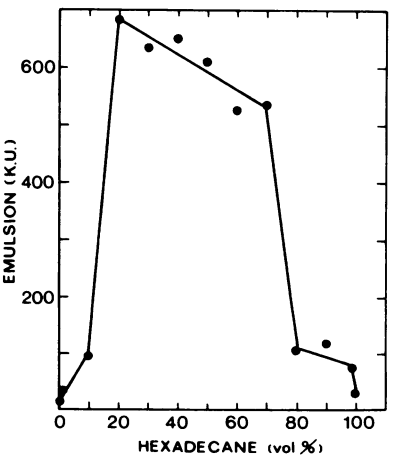

FIG. 4. Emulsification of mixtures of hexadecane and 2-methylnaphthalene by fraction EF-50. The standard emulsification assay, containing 50 μg of EF-50 per ml, was modified to contain the indicated mixtures of hexadecane and 2-methylnaphthalene.

TABLE 3. Shearing of capsular polysaccharide from *A. calcoaceticus* BD4

Fraction	Vol (ml)	Rhamnose (mg/ml)	Emulsifying activity (U/ml)	Specific emulsifying activity (U/mg of rhamnose)
Washed pellet	40	0.90	44	49
Cell pellet after shearing	26	0.85	28	33
Supernatant fraction after shearing	49	0.33	0	0
Emulsifier EF-50	—	1.00	518	518

emulsifier fraction EF-50 (Table 3). To see whether the rhamnose capsular polysaccharide had emulsifying activity when solubilized, the following experiment was performed. *A. calcoaceticus* BD4 cells grown on lactic acid were thoroughly washed in distilled water and passed under pressure twice through a glass chromatogram sprayer to remove the capsular polysaccharide from the cells (9). This treatment resulted in a release of 44% of the cell-bound rhamnose polysaccharide (Table 3). The viscous soluble preparation containing the sheared capsular polysaccharide showed no emulsifying activity. As a control, the extracellular emulsifier of BD413 was exposed to shearing conditions similar to those for the BD4 cells. No loss in emulsifying activity was found.

DISCUSSION

This paper deals with the relationships between the cell-associated rhamnose capsule, extracellular rhamnose polysaccharides, and bioemulsifiers of *A. calcoaceticus* BD4 and BD413. These strains produced both extracellular and capsular rhamnose-containing exopolysaccharides when grown in lactic acid, glucose, or brain heart infusion media. Although there is no direct evidence that the capsular and extracellular polysaccharides share a common biosynthetic pathway, the following data suggest this. Rhamnose and glucose in a molar ratio of 3 to 1 were the only major sugar components found in the partially purified extracellular polysaccharide fraction obtained from strain BD413 (N. Kaplan, M.Sc. thesis, Tel Aviv University, 1980); this is very similar to the reported monosaccharide composition of the capsule of BD4 (18). In addition, the same single-gene mutation which led to a reduced capsule in BD413 also led to increased extracellular polysaccharide. Comparison of the cell-bound and extracellular rhamnose-glucose polymers by ^{13}C-nuclear magnetic resonance (15) should provide more detailed information on the structural relationship between the two polysaccharide fractions.

In the case of emulsan produced by *A. calcoaceticus* RAG-1, the extracellular galactosamine-containing polymer is produced from the capsular material, as demonstrated by a variety of techniques, including immunological experiments (6) and radioactive tracer experiments (C. Rubinovitz, D. L. Gutnick, and E. Rosenberg, submitted for publication). RAG-1 cells accumulated capsular material on the cell surface during logarithmic phase and then released this polymeric material in the form of an active emulsifier in stationary phase or during conditions of unbalanced growth.

In strains BD4 and BD413, no decrease in capsular polysaccharides was found. The extracellular rhamnose polysaccharides were detected starting from mid-logarithmic phase and accumulated until approximately 5 h after the onset of stationary phase (Fig. 1 and 2). In strain BD413, the appearance of extracellular emulsifying activity was parallel to the kinetics of the extracellular polysaccharide (Fig. 2 and 3). In contrast, strain BD4 produced approximately the same amount of extracellular rhamnose polysaccharide but only 15% of the emulsifying activity (Fig. 3).

It is of interest to compare the extracellular emulsifier of BD413 with emulsan produced by *A. calcoaceticus* RAG-1 (12, 13, 19). Although the emulsifiers have completely different chemical compositions, they both show similar pH optima and magnesium ion requirements. They also have an absolute requirement for a mixture of an aliphatic and an aromatic hydrocarbon for efficient hydrocarbon emulsification in water. However, the BD413 emulsifier showed a higher specificity for short-chain than for long-chain aliphatics, whereas emulsan showed a greater efficiency in emulsifying the higher-molecular-weight fractions (12). This probably explains the observation that crude oil was only weakly emulsified by the BD413 emulsifier, whereas crude oil is the best substrate for emulsan.

The finding that the capsular polysaccharide of strain BD4 showed no emulsifying activity when released artificially by shearing (Table 3)

suggests that strain BD413 either produces a modified extracellular polysaccharide or produces an additional substance that is responsible for the emulsifying activity. Certain exopolysaccharide-producing bacteria can perform post-polymerization modifications such as acylation and ketalation that might confer different charge and hydrophobic properties on the modified polymers (17). Such minor chemical differences might explain the altered exopolysaccharide distribution and emulsifying properties in strain BD413. This is consistent with the findings that capsular and extracellular polysaccharides produced by the same bacterial strain (e.g., 1, 5, 7) exhibit no differences in monosaccharide composition and immunological properties, but in certain cases show differences in the size and gross morphology of these polysaccharides (16).

Recently, we have isolated a bacteriophage specific for strain BD4 that possesses an enzymatic activity that hydrolyzes the capsule of BD4. Characterization of the substrate specificity of this phage-induced enzyme, together with further detailed chemical studies, should allow for determination of the molecule(s) responsible for emulsification and the chemical relationships between the capsular and extracellular polysaccharides of strains BD4 and BD413.

LITERATURE CITED

1. **Bayer, M., and H. Thurow.** 1977. Polysaccharide capsule of *Escherichia coli*: microscope study of its size, structure, and sites of synthesis. J. Bacteriol. **130**:911–936.
2. **Belsky, I., D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: determination of emulsifier-bound fatty acids. FEBS Lett. **101**:175–178.
3. **Brooks, K., and T. Sodeman.** 1974. Clinical studies on a transformation test for identification of *Acinetobacter* (Mima and Herellae). Appl. Microbiol. **27**:1023–1026.
4. **Dische, Z., and L. B. Shettles.** 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
5. **Dudman, W. F., and J. F. Wilkinson.** 1956. The composition of the extracellular polysaccharides of *Aerobacter-Klebsiella* strains. Biochem. J. **62**:289–295.
6. **Goldman, S., Y. Shabtai, C. Rubinovitz, E. Rosenberg, and D. L. Gutnick.** Emulsan in *Acinetobacter calcoaceticus* RAG-1: distribution of cell-free and cell-associated cross-reacting material. Appl. Environ. Microbiol. **44**:165–170.
7. **Hungerer, D., K. Jann, B. Jann, F. Orskov, and I. Orskov.** 1967. Immunochemistry of K antigens of *Escherichia coli*. 4. The K antigen of *E. coli* 09:K30:H12. Eur. J. Biochem. **2**:115–126.
8. **Juni, E.** 1972. Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. J. Bacteriol. **112**:917–931.
9. **Juni, E., and G. A. Heym.** 1964. Pathways for biosynthesis of a bacterial capsular polysaccharide. IV. Capsule resynthesis by decapsulated resting-cell suspensions. J. Bacteriol. **87**:461–467.
10. **Juni, E., and A. Janick.** 1969. Transformation of *Acinetobacter calcoaceticus (Bacterium anitratum)*. J. Bacteriol. **98**:281–288.
11. **Reisfeld, A., E. Rosenberg, and D. Gutnick.** 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. **24**:363–368.
12. **Rosenberg, E., A. Perry, D. T. Gibson, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: specificity of hydrocarbon substrate. Appl. Environ. Microbiol. **37**:409–413.
13. **Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick.** 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. **37**:402–408.
14. **Sawula, R. V., and I. P. Crawford.** 1972. Mapping of the tryptophan genes of *Acinetobacter calcoaceticus* by transformation. J. Bacteriol. **112**:797–805.
15. **Seymor, F. R.** 1977. Nuclear magnetic resonance and mass spectroscopy of polysaccharides, p. 114–127. *In* P. A. Sanford and A. Laskin (ed.), Extracellular microbial polysaccharides. American Chemical Society, Washington, D.C.
16. **Sutherland, I. W.** 1977. Bacterial exopolysaccharides—their nature and production, p. 27–96. *In* I. Sutherland (ed.), Surface carbohydrates of the prokaryotic cell. Academic Press, London.
17. **Sutherland, I. W.** 1979. Microbial exopolysaccharides: control of synthesis and acylation, p. 1–34. *In* R. C. W. Berkeley, G. W. Gooday, and D. C. Ellwood (ed.), Microbial polysaccharides and polysaccharases. Academic Press, London.
18. **Taylor, W. H., and E. Juni.** 1961. Pathways for biosynthesis of a bacterial capsular polysaccharide. I. Characterization of the organism and polysaccharide. J. Bacteriol. **81**:688–693.
19. **Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg.** 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. **37**:414–420.