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Alasan, a New Bioemulsifier from *Acinetobacter radioresistens*

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Acinetobacter radioresistens **KA53, isolated by enrichment culture, was found to produce an extracellular, nondialyzable emulsifying agent (referred to as alasan) when grown on ethanol medium in a batch-fed reactor. The crude emulsifier was concentrated from the cell-free culture fluid by ammonium sulfate precipitation to yield 2.2 g of emulsifier per liter. Alasan stabilized a variety of oil-in-water emulsions, including *n*-alkanes with chain lengths of 10 or higher, alkyl aromatics, liquid paraffin, soybean and coconut oils, and crude oil. Alasan was 2.5 to 3.0 times more active after being heated at 100°C under neutral or alkaline conditions. Emulsifying activity was observed over the entire pH range studied (pH 3.3 to 9.2), with a clear maximum at pH 5.0. Magnesium ions stimulated the activity both below (pH 3.3 to 4.5) and above (pH 5.5 to 9.3) the pH optimum. Alasan activity was higher in 20 mM citrate than in 20 mM acetate or Tris-HCl buffer. Preliminary chemical characterization of alasan indicated that it is a complex of an anionic, high-molecular-weight, alanine-containing heteropolysaccharide and protein.**

Bioemulsifiers have received increasing attention in recent years because of their role in the growth of microorganisms on water-insoluble hydrophobic materials such as hydrocarbons and because of their commercial potential in the cosmetics, food, and agricultural industries (16).

Bioemulsifiers are amphipathic molecules that can be divided into (i) low-molecular-weight compounds such as glycolipids and phospholipids, which lower the interfacial tension between hydrophobic liquids and water and thus reduce the energy required to form emulsions, and (ii) polymers which stabilize emulsions (16). Most probably because of their high surface-to-volume ratio, microorganisms are an excellent source of potentially useful amphipathic biopolymers. Although several emulsion-stabilizing polymers derived from microorganisms have been reported (see, e.g., references 14, 16, and 17), only a few have been well characterized with regard to both surface activity properties and chemical composition.

In *Acinetobacter* species, production of extracellular bioemulsifiers is a widespread phenomenon (17). Of 16 different *Acinetobacter* strains examined, 8 produced high levels of extracellular emulsifiers when grown on minimal salt media containing ethanol as the carbon source (20). All the emulsifiers were nondialyzable and contained polysaccharide as their major component. Two of these *Acinetobacter* emulsifiers have been studied extensively: RAG-1 and BD4 emulsans.

The RAG-1 emulsan is a noncovalently linked complex of a lipoheteropolysaccharide and a protein. The polysaccharide, referred to as apoemulsan, has a molecular weight of about 9.9×10^5. The major sugar components of apoemulsan are D-galactosamine, D-galactosaminuronic acid, and diaminodideoxy glucosamine (23). Fatty acids are covalently linked to the polysaccharide backbone through O-ester and N-acyl linkages (3). The fatty acids constitute about 12% of the biopolymer and contribute to the amphipathic behavior of apoemulsan. *Acinetobacter calcoaceticus* BD4 produces a large polysaccharide capsule. When released into the medium, the capsular polysaccharide forms a complex with proteins, which then becomes an effective emulsifier (10). The BD4 emulsan polysaccharide consists of a repeating heptasaccharide unit containing L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose in molar ratios of 4:1:1:1 (11). Extracellular protein fractions of BD4, free of polysaccharide, and the polysaccharide by itself were not surface active. However, the original emulsifying activity could be reconstituted by mixing the purified polysaccharide and protein fractions (12).

In this paper, we present data on the isolation and growth characteristics of a newly isolated *Acinetobacter* strain which produces a new and interesting emulsion stabilizer, referred to as alasan. One of the unique features of alasan is that its emulsifying activity increased greatly after exposure to high temperature under neutral or alkaline conditions. Preliminary physical and chemical properties of alasan are presented.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Acinetobacter* sp. strain KA53 was isolated in this study by a standard enrichment culture technique with acetate as the carbon and energy source (2). The strain was maintained on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). After incubation at 30°C for 3 days, the plates were stored at 4°C.

Growth and emulsifier production experiments were carried out in ethanol medium (EM) containing (per liter of deionized water) 5 ml of ethanol, 1.8 g of urea, 13.7 g of Na_2HPO_4, 7.26 g of KH_2PO_4, 0.4 g of $MgSO_4 \cdot 7H_2O$, and 1 ml of a trace elements solution. The trace elements solution contained (per 10 ml) 3.68 mg of $CaCl_2 \cdot 2H_2O$, 6.24 mg of $CuSO_4 \cdot 5H_2O$, 6.04 mg of $FeSO_4 \cdot 7H_2O$, 5.94 mg of $MnSO_4 \cdot 4H_2O$, 4.22 mg of $ZnSO_4 \cdot 7H_2O$, 7.88 mg of $CoCl_2 \cdot 6H_2O$, and 6.96 mg of Na_2MoO_4. Additional nutrients were added during the fermentation, as described in Table 1.

Growth and emulsifier production were carried out at 30°C in a 2.5-liter fermentor (Multigen; New Brunswick Scientific Co. Inc.) equipped with an oxygen electrode. Bacterial growth was initiated by introducing a 0.1% inoculum obtained from a starter culture that was grown in 5 ml of EM in a 125-ml flask at 30°C for 24 h. Growth was followed by determination of the turbidity (A_{600}) in a Gilford spectrophotometer (model 240) and by determination of viable counts.

Petroleum products and other oils used as emulsifier substrates. Crude oil ($d = 0.864$) was obtained from the Haifa Israel Refinery. Food grade soybean, coconut, and vegetable oils were obtained from Florum Co., Tel Aviv, Israel. Hexylbenzene, heptylbenzene, and 2-methylnaphthalene were products of Fluka Chemical Co., Buchs, Switzerland. The remaining hydrocarbons used were purchased from Merck Chemicals.

Determination of emulsifying activity. The standard emulsification assay (18) was used to measure emulsifying activity. Samples to be tested (0.1 to 0.5 ml) were introduced into a 125-ml flask containing TM buffer (20 mM Tris-HCl

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TABLE 1. Growth and emulsifier production by strain KA53[a]

Time (h)	pH	Turbidity (A_{600})	Dry wt (mg/ml)		Emulsifying activity (U/ml)	
			Cells	Extracellular	Total	Extracellular
0	7.0	0.01	ND[b]	ND	<5	<5
20[c]	7.2	5.38	ND	0.60	30	19
24[d]	7.3	12.2	6.7	0.63	35	11
38[e]	8.2	15.5	13.7	1.10	47	35
43	8.1	19.6	13.9	1.60	87	65
48[f]	8.2	23.0	14.6	2.33	90	70
64[g]	7.8	26.2	16.5	2.53	120	98
68[d]	7.9	30.7	19.0	2.90	170	170
72[d]	8.1	32.1	19.0	3.25	180	150
87	8.3	33.0	19.0	4.60	220	190

[a] Strain KA53 was grown in a 2.5-liter fermentor containing 1.4 liters of EM. The initial aeration conditions were 0.5 liter/min and 300 rpm. When the dissolved oxygen fell below 10% saturation, the air flow and rpm values were increased, so that between 24 and 87 h, they were 2.5 liters/min and 500 rpm, respectively. Samples were taken at the time intervals indicated, and the different parameters were determined as described in Materials and Methods. Additional nutrients were provided as detailed in footnotes *c* to *g*.

[b] ND, not done.

[c] 0.5% ethanol and 0.18% urea.

[d] 1.0% ethanol.

[e] 1.0% ethanol, 0.18% urea, 0.04% $MgSO_4 \cdot 7H_2O$, and 0.1% trace elements.

[f] 0.5% ethanol.

[g] 0.5% ethanol, 0.18% urea, and 0.1% trace elements.

buffer [pH 7.0], 10 mM $MgSO_4$) to a final volume of 7.5 ml, and then 0.1 ml of a 1:1 (vol/vol) mixture of hexadecane and 2-methylnaphthalene was added. The samples were incubated at 30°C with reciprocal shaking (160 strokes per min) for 1 h. Turbidity was then determined in a Klett-Summerson photometer (fitted with a green filter). One unit of emulsifying activity per milliliter was defined as the amount of biopolymer that yielded 100 Klett units (KU) in the assay mixture. Emulsion turbidity was directly proportional to the alasan concentration between 20 and 500 μg of alasan per ml. All emulsifying activity measurements were performed in duplicate or triplicate. The average standard deviation of the measurement was 9%.

The hydrocarbon substrate specificity of alasan was determined as described above, except that the 0.1 ml of standard hydrocarbon mixture was substituted by other hydrocarbons, hydrocarbon mixtures, and oils. As coconut oil is solid at room temperature, its ability to be emulsified was determined at 60°C.

The ability of alasan and apo-alasan to stabilize oil-in-water emulsions was tested by exposing the mixtures to ultrasonication (Braun Labsonic, fitted with a standard probe) at 70 to 75 W for 100 s.

Preparation of alasan and apo-alasan. Alasan was obtained by ammonium sulfate precipitation of 1.0 liter of cell-free extracellular fluid following an 87-h fermentation run as described in Table 1. The supernatant was filtered through a 0.45-μm-pore-size membrane filter and brought to 65% ammonium sulfate saturation. After standing overnight at 4°C, the turbid suspension was centrifuged at 10,000 × *g* for 20 min. The pellet was dissolved in water, dialyzed extensively against deionized water, and lyophilized, yielding 2.35 g of alasan.

Apo-alasan (deproteinized alasan) was obtained by the hot-phenol method (22). Alasan (500 mg in 100 ml of water) was 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, cooled to 0°C, and centrifuged. The lower, phenol phase was extracted with water as above. The combined viscous aqueous phases were dialyzed against distilled water and lyophilized. The white, fluffy material (containing approximately 2% residual protein) was termed apo-alasan.

Analytical methods. Cell dry weight and extracellular biopolymer were determined on culture samples that were centrifuged at 4°C at 12,000 × *g* for 15 min. The cell pellets were washed twice with deionized water and suspended in distilled water. Cell dry weight values were determined by weighing samples of the cell suspensions after drying them to constant weight in tared aluminum foil cups at 90°C. The supernatant fluids were dialyzed overnight and then dried to constant weight for determination of the extracellular material. Protein was determined by a modified Lowry procedure (13) in which sodium dodecyl sulfate was used to solubilize amphipathic proteins. Bovine serum albumin was used as the standard.

The carbohydrate content was estimated by hydrolyzing the samples in 1 N H_2SO_4 for 4 h at 100°C in sealed ampoules, neutralizing the sample with NaOH, and then estimating reducing sugar by the ferricyanide procedure (21) with glucose as the standard.

The viscosity of 1.8-ml samples was measured in an Ostwald-Fenske microviscometer (average buffer value, 53.5 s) at 30°C. The average of three determinations was used in all calculations. The concentration of the biopolymer was 2 mg/ml.

Potentiometric titrations were carried out with a pH meter fitted with a microelectrode, in which 0.01-ml portions of 0.1 N HCl or 0.1 N NaOH were added, with thorough mixing and exclusion of carbon dioxide, to 3.0-ml solutions to be titrated.

Thin-layer chromatography (TLC) was carried out on 20- by 20-cm precoated cellulose plates (Sigma). Hydrolyzed samples of the biopolymer were developed with ethyl acetate-pyridine-water-acetic acid (5:5:3:1 by volume). After air drying, the chromatograms were stained for (i) reducing sugar with alkaline silver nitrate (5) and (ii) amino acids and amino sugars by spraying the plates with 0.2% ninhydrin in acetone followed by heating at 105°C for 5 min.

RESULTS

Isolation and characterization of *Acinetobacter* sp. strain KA53. By using a standard enrichment culture technique for the isolation of acinetobacters (2), 12 different strains were isolated from oil-polluted soil samples taken near a gas station in Kenya. The pure cultures were grown to stationary phase in EM and centrifuged, and the extracellular fluids were screened for emulsifying activity by the standard emulsification assay. The strain that yielded the highest emulsification value, referred to as KA53, was chosen for further study.

Strain KA53 was identified as a member of the genus *Acinetobacter* by physiological and genetic characterization (8, 9). India ink staining indicated that the exponentially growing cells had a minicapsule. The strain grew on ethanol, proline, DL-4-aminobutyrate, glutarate, malonate, azelate, DL-lactate, phenylacetate, L-phenylalanine, and acetate as carbon sources but failed to grow on glucose, sucrose, *trans*-aconitate, citrate, aspartate, β-alanine, L-histidine, D-malate, histamine, serine, or tryptophan. Strain KA53 did not hydrolyze gelatin and grew in EM agar at 37 and 41°C but not at 44°C. It was not sensitive to BD4-specific (10) or RAG-1-specific (15) bacteriophages. On the basis of all these tests and the identification scheme of Grimont and Bouvet (7), KA53 appears to be a strain of *Acinetobacter radioresistens.*

***Acinetobacter* KA53 growth and emulsifier production.** The growth characteristics and emulsifier production during a batch-fed fermentation run are summarized in Table 1. *A. radioresistens* KA53 had an approximate doubling time of 2.4 h during the initial 20-h exponential phase of growth. The viable count (data not shown) decreased slowly after reaching a maximum of 8.0×10^9 cells per ml at 68 h. The total dry weight (cell biomass and extracellular material) at the end of the fermentation was 23.6 g/liter.

The ratio of emulsifying activity (units per milliliter) to cell biomass (milligrams per milliliter) increased from 5.3 to 7.3 to 11.6 at 24, 64, and 87 h, respectively. The extracellular activity was 190 U/ml after 87 h, indicating that the majority of emulsification activity was extracellular. The extracellular biopolymer as a fraction of the cell biomass increased during growth from 9.8% at 20 h to 24% at 87 h.

Effect of heat and alkali treatment on the emulsifying activity of alasan. Treating the supernatant fluid (obtained after centrifugation and filtration of the growth medium) at 100°C, either at neutral pH or in 0.1 N NaOH, resulted in an increase in its emulsifying activity (Table 2). This phenomenon was termed activation. At neutral pH, the emulsifying activity increased by a factor of 2.5. Samples heat activated at neutral pH retained their activity following dialysis. Activation at alkaline pH was different from activation at neutral pH in that dialysis (molecular weight cutoff 6,000 to 8,000) of the alkali-treated samples caused a large decrease in the activity.

Emulsifying properties of alasan. Aliphatic hydrocarbons ranging from pentane to octadecane were assayed for their ability to serve as substrates for emulsification (Table 3). The

TABLE 2. Effect of heat and alkali treatment on the emulsifying activity of alasan[a]

Duration of treatment at 100°C (min)	Emulsifying activity (U/ml)		Relative activity[b]
	Before dialysis	After dialysis	
0	600	585	1.0
At pH 7			
10	1,350	1,730	2.3
15	1,500	1,340	2.5
45	1,140	980	1.9
60	1,500	1,340	2.5
In 0.1 N NaOH			
2	1,260	680	2.1
5	1,710	620	2.9
10	1,530	510	2.6
15	1,530	650	2.6
30	1,800	260	3.0
45	1,370	83	2.3
60	1,080	45	1.8

[a] Samples of the filtered supernatant fluid following growth of strain KA53 on EM were heated for different times at 100°C, either at neutral pH or in 0.1 N NaOH. After the alkali treatment, the sample was neutralized to pH 7. Samples were assayed for emulsifying activity before and after overnight dialysis (Molecular weight cutoff, 6,000 to 8,000).

[b] Relative activity is the activity after heat treatment compared with the untreated control (none of the samples were dialyzed).

higher the molecular weight of the alkane, the more effective alasan was in stabilizing the emulsion. Pentane, hexane, cyclohexane, heptane, and iso-octane were emulsified poorly or not at all, whereas C_{10} to C_{18} compounds were emulsified effectively. The emulsified C_{10} to C_{18} compounds were stable for at least 24 h (82 ± 15% of the original values).

Benzene, alkylbenzenes, and 2-methylnaphthalene were assayed for their ability to be emulsified by alasan (Table 3). Benzene derivatives containing methyl groups, i.e., toluene and the xylenes, were emulsified slightly. Methylnaphthalene was clearly a better substrate than the methylbenzenes. Alkylbenzenes containing three or more carbons in the side chain were emulsified efficiently. Maximum emulsifying activity was obtained with heptylbenzene.

Mixtures containing an aromatic and an aliphatic hydrocarbon, such as hexadecane and 2-methylnaphthalene, were excellent substrates for alasan emulsification. At a ratio of 2 volumes of 2-methylnaphthalene to 1 volume of hexadecane, the emulsion turbidity was 1,400 KU compared with 950 KU for a 1:1 mixture and less than 600 KU for each of the pure hydrocarbons.

The ability of alasan to stabilize highly dispersed oil-in-water emulsions is demonstrated in Table 4. Emulsions of liquid paraffin and soybean oils, prepared by ultrasonication in the absence of an emulsifier, broke in a few hours, leaving less than 1% of the oil dispersed in the aqueous phase. On the other hand, emulsions prepared in the presence of 0.005% alasan were stable for at least 10 days. The partial drop in turbidity was due exclusively to creaming and not coalescence of oil drops, because gentle hand mixing resulted in restoration of the initial turbidity. The deproteinized preparation of the emulsifier, apo-alasan, was almost as effective as alasan. Alasan was also effective in stabilizing emulsions prepared with food grade vegetable and coconut oils.

Emulsifying activity was observed over the entire pH range studied (pH 3.3 to 9.2), with a maximum at pH 5.0 (Fig. 1). At pH 5.0 without Mg^{2+} ions in the assay solution, alasan showed a specific activity of 325 U/mg compared with 290 U/mg in the presence of Mg^{2+} ions. The specific activity at pH 7.0 (the pH used in the standard assay) was 109 U/mg without Mg^{2+} and 160 U/mg with Mg^{2+}. Magnesium ions enhanced the emulsifying activity both below (pH 3.3 to 4.5) and above (pH 5.5 to 9.3) the pH optimum. NaCl (0.5 to 3.0%) had no effect on the emulsifying activity of alasan (data not presented). Activity in

TABLE 3. Hydrocarbon substrate specificity of alasan

Hydrocarbon substrate[a]	Emulsion turbidity (KU)
Alkanes	
n-Pentane	<20
n-Hexane	27
Cyclohexane	<20
n-Heptane	43
iso-Octane	24
Decane	240
Tetradecane	290
Pentadecane	310
Hexadecane	330
Heptadecane	410
Octadecane	570
Aromatics	
Benzene	<20
Toluene	120
o-Xylene	100
m-Xylene	170
Ethylbenzene	220
iso-Propylbenzene	540
Butylbenzene	340
Hexylbenzene	420
Heptylbenzene	1,180
Octylbenzene	450
2-Methylnaphthalene	590
Mixtures	
Hexadecane + 2-methylnaphthalene[b]	360
Hexadecane + 2-methylnaphthalene[c]	950
Hexadecane + 2-methylnaphthalene[d]	1,200
Hexadecane + 2-methylnaphthalene[e]	1,400
Cyclohexane + hexadecane[c]	320
Benzene + cyclohexane[c]	<20
Toluene + cyclohexane[c]	30
Crude oil	1,900

[a] The standard emulsification assay was used with 0.1 mg of alasan per ml and 0.1 ml of the indicated hydrocarbon substrates.

[b] Ratio of 2:1 (vol/vol).

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

[d] Ratio of 1:1.5 (vol/vol).

[e] Ratio of 1:2 (vol/vol).

TABLE 4. Stabilization of light paraffin and soybean oil-in-water emulsions with alasan[a]

Oil	Emulsifier	Turbidity (KU) at:			
		$t = 0$	1 day	6 days	10 days
Liquid paraffin	None	3,700	30	0	0
Liquid paraffin	Alasan	11,600	8,200	4,300	4,000
Liquid paraffin	Apo-alasan	8,600	6,500	3,200	2,700
Soybean	None	7,200	70	0	0
Soybean	Alasan	10,500	9,000	8,950	8,900
Soybean	Apo-alasan	9,400	7,700	7,100	6,700

[a] Mixtures containing 160 mg of liquid paraffin oil or 180 mg of soybean oil and 0.5 mg of alasan or 0.5 mg of the deproteinized apo-alasan (see Materials and Methods for details of the preparation) in 10 ml of 30 mM Tris-Mg buffer (pH 7.2) were emulsified by ultrasonication. Turbidities were determined immediately ($t = 0$) and after standing for 1, 6, and 10 days.

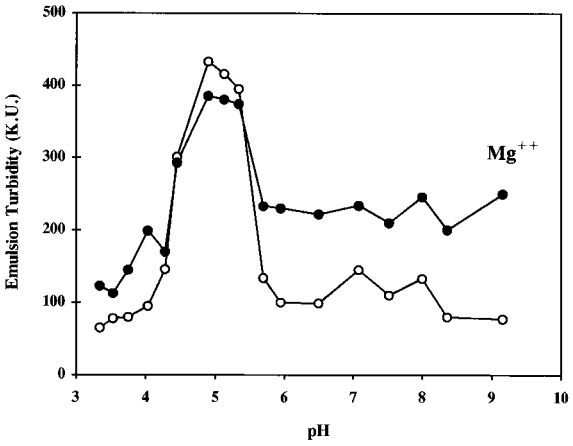

FIG. 1. Effect of pH and magnesium ions on the emulsifying activity of alasan. A modification of the standard emulsification assay was carried out with 20 mM acetate buffer for the pH range 3.3 to 5.3 and 20 mM Tris-HCl buffer for the pH range 5.4 to 9.2. Activity was measured at the different pH values with (●) and without (○) the addition of 10 mM $MgSO_4$. Alasan was present at 13.3 μg/ml.

the presence of 20 mM citrate buffer was approximately 20% higher than in the presence of 20 mM acetate over the entire pH range studied.

Preliminary chemical characterization of alasan. Gel filtration of apo-alasan on Biogel A-1.5 indicated that the average molecular weight was 9×10^5 and that over 90% had a molecular weight greater than 7×10^5. The reduced viscosities of alasan and apo-alasan were 245 and 380 cm^3/g, respectively.

Titration of apo-alasan between pH 2.5 and 11.0 showed an inflection point in the acid range, corresponding to a pK of 3.8. Between pH 3 and 6, 1.28 meq was required to titrate 1 g of polymer. Between pH 7 and 11, only 0.25 meq was necessary. After strong acid hydrolysis (4 N HCl at 100°C for 4 h) and evaporation to remove volatile compounds (e.g., acetic acid), 1.8 meq/g was needed to titrate the hydrolysate in the acid range and 4.0 meq/g was needed in the basic range. These data suggest that the unhydrolyzed polysaccharide had 5 times more carboxylic groups than free amino groups and that the hydrolyzed apo-alasan contained 2.5 times more amino groups than carboxyl groups.

Hydrolysis products following treatment of apo-alasan with 4 N HCl at 100°C were subjected to cellulose thin-layer chromatography. From their mobilities and staining characteristics, five monomers were tentatively identified: glucosamine, galactosamine, alanine, glucose (minor), and galactose. In addition, there was an unidentified aminouronic acid. D-Glucose and L-alanine were identified independently by enzymatic procedures.

Data from ^1H and ^{13}C nuclear magnetic resonance spectra of apo-alasan and mild-acid-treated apo-alasan demonstrated the presence of (i) six carbonyl carbon atoms (177.1 to 167.8 ppm) consistent with the presence of *N*-acyl amino sugars, uronic acid, and alanine; (ii) at least six anomeric carbons between 95 and 103 ppm; (iii) four C-2 carbons of amino sugars between 50 and 56 ppm; (iv) the CH of alanine at 49.5 and 4.2 ppm; and (v) five or six methyl groups between 14.8 and 21.9 ppm, including the methyl of alanine (17.4 and 1.25 ppm).

Amino acid analysis of hydrolyzed alasan and apo-alasan indicated the presence of only one major amino acid, alanine (Table 5). Alanine represented 7% of the total dry weight of apo-alasan, corresponding to 0.79 meq/g. Alanine was the only amino acid whose specific concentration increased after phenol treatment. According to the total amino acids found, the protein contents of alasan and apo-alasan were 17 and 4%, respectively.

DISCUSSION

The data presented here demonstrate three important features of alasan which differ from the previously studied emulsans RAG-1 and BD4: hydrocarbon substrate specificity, activation by heat under neutral and alkaline conditions, and the presence of covalently bound alanine. In contrast to RAG-1 (18) and BD4 (10) emulsans, which require both aliphatic and aromatic hydrocarbons for emulsifying activity, alasan was able to emulsify long-chain aliphatics and methylnaphthalene separately. For example, under standard emulsifying conditions and with the activity toward the 1:1 mixture of hexadecane and methylnaphthalene set at 100%, the emulsifying activity values for methylnaphthalene alone were 1, 3, and 62% for RAG-1 emulsan, BD4 emulsan, and alasan, respectively. Similarly, octadecane was emulsified efficiently by alasan (Table 3), whereas RAG-1 and BD4 emulsans were unable to emulsify that substrate. Of 14 *Acinetobacter* strains that produced extracellular emulsifying agents (20), none were able to emulsify both pure aliphatics and aromatics. Thus, alasan appears to be unusual in its ability to emulsify both long-chain aliphatics and aromatics containing at least 10 and 9 carbon atoms, respectively.

To our knowledge, heat activation of polymeric emulsifiers has not been reported previously. The increased emulsifying activity of heat-treated alasan (Table 2) was stable for at least 3 months at −12°C, 4°C, and room temperature. The mechanism of the heat activation is unknown, but rather large conformational changes in alasan do take place from 30 to 100°C, as shown by viscosimetry (data not presented). The stability of alasan to alkali indicates that ester linkages are not required for its emulsifying activity. In contrast, RAG-1 emulsan completely loses its activity when treated with alkali (3).

Stability to heat and alkalis and the ability to emulsify long-chain alkanes are properties of alasan with potential practical applications. For example, in the food and cosmetics indus-

TABLE 5. Amino acid analysis of alasan and apo-alasan[a]

Amino acid	Content (%) in:	
	Alasan	Apo-alasan
Ala	4.5	7.0
Arg	0.6	0.2
Asp	2.5	0.5
Glu	2.1	1.1
Gly	1.3	1.2
His	0.4	ND[b]
Ile	0.1	0.3
Leu	1.3	0.3
Lys	2.5	0.3
Met	0.1	ND
Phe	0.8	0.2
Pro	0.4	0.4
Ser	ND	0.5
Thr	1.4	0.3
Tyr	1.0	0.5
Val	1.6	0.4

[a] Amino acid analysis was carried out on 10 μg of each sample following hydrolysis at 100°C in 6 N HCl for 24 h. The sensitivity of the detection was 0.3%.
[b] ND, not detected.

tries, most of the oil-in-water emulsions that require stabilization involve long-chain alkanes such as mineral and coconut oils. The heat stability of alasan should facilitate the formation of sterile, stable oil-in-water emulsions.

No systematic attempt was made to optimize media or fermentation parameters for alasan production. Under the conditions used, 47.4 g of ethanol per liter and 7.2 g of urea per liter produced 23.6 g of total biomass per liter. Thus, the yield from ethanol was 49.8%. Assuming that biomass contained 14% nitrogen, all the urea-nitrogen was incorporated into biomass. The yield of alasan was 4.6 g/liter, or 9.7% of the input ethanol. In addition to the extracellular alasan, considerable amounts of emulsifying activity remained cell bound. This was apparent (i) from a comparison of total and extracellular activity (Table 1), (ii) from the observation that washed cell suspensions heated to 100°C released large amounts of emulsifying activity (data not shown), and (iii) by analogy with RAG-1 emulsan (6, 19). To quantitatively determine the amount and distribution of alasan accurately, it will be necessary to develop specific chemical or immunological assays for the purified material. Emulsifying-activity measurements can be misleading because of the large effects of temperature, pH, and protein content on the results.

The polysaccharide component of alasan appears to have a molecular weight close to 10^6 and consists of a complex polysaccharide containing covalently bound alanine. Assuming alanine (0.79 meq/g of polysaccharide) to be present in stoichiometric amounts (moles per mole of repeating unit), the molecular weight of the repeating unit would be 1,266. A repeating unit consisting of Glc, Gal, GlcNAC, GalNAC, Ala and a disaccharide containing two *N*-acyl hexosamines would have a molecular weight of 1,243. All of the amino groups must be N-acylated in the polymer, since potentiometric titration revealed only 0.25 meq/g of free amino groups. After acid hydrolysis of apo-alasan, 4.0 meq/g of amino groups was present per g, corresponding to 5.0 amino groups per repeating unit. These could be accounted for by Ala, GlcNAC, GalNAC, and the postulated diaminodisaccharide. The presence of one carboxyl group per repeating unit in the polymer and a second one generated by acid hydrolysis could be explained by the linkage of alanine to a uronic acid through its amino group (one free carboxyl); after hydrolysis, the second carboxyl would be generated. Amide linkages between amino acid and uronic acid residues of polysaccharide have been described previously (1, 4).

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REFERENCES

1. **Anderson, M., S. Ratnayake, L. Kenne, L. Ericson, and R. J. Stack.** 1993. Structural studies of the extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain X6C61. Carbohydr. Res. **246:**291–301.
2. **Bauman, P.** 1968. Isolation of *Acinetobacter* from soil and water. J. Bacteriol. **96:**34–42.
3. **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.
4. **Dengler, T., B. Jann, and K. Jann.** 1986. Structure of the serine-containing capsular polysaccharide K40 antigen from *E. coli* O8:K40:H9. Carbohydr. Res. **150:**233–240.
5. **Gal, A.** 1968. Separation and identification of monosaccharides from biological materials by thin-layer chromatography. Anal. Biochem. **24:**452–461.
6. **Goldman, S., Y. Shabtai, C. Rubinovitz, E. Rosenberg, and D. L. Gutnick.** 1982. Emulsan in *Acinetobacter calcoaceticus* RAG-1: distribution of cell-free and cell-associated cross-reacting material. Appl. Environ. Microbiol. **44:** 165–170.
7. **Grimont, P. A. D., and P. J. M. Bouvet.** 1991. Taxonomy of *Acinetobacter*, p. 28–35. *In* K. J. Towner, E. Bergogne-Berezin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Press, New York.
8. **Juni, E.** 1978. Genetics and physiology of *Acinetobacter*. Annu. Rev. Microbiol. **32:**349–371.
9. **Juni, E., and A. Janick.** 1969. Transformation of *Acinetobacter calcoaceticus*. J. Bacteriol. **98:**281–288.
10. **Kaplan, N., and E. Rosenberg.** 1982. Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. Appl. Environ. Microbiol. **44:**1335–1341.
11. **Kaplan, N., E. Rosenberg, B. Jann, and K. Jann.** 1985. Structural studies of the capsular polysaccharide of *Acinetobacter calcoaceticus* BD4. Eur. J. Biochem. **152:**453–458.
12. **Kaplan, N., Z. Zosim, and E. Rosenberg.** 1987. Reconstitution of emulsifying activity of *Acinetobacter calcoaceticus* BD4 emulsan by using pure polysaccharide and protein. Appl. Environ. Microbiol. **53:**440–446.
13. **Markwell, M. A. K.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
14. **Neu, T. R., and K. C. Marshall.** 1990. Bacterial polymers: physiochemical aspects of the interactions at interfaces. J. Biomater. Applic. **5:**107–133.
15. **Pines, O., and D. L. Gutnick.** 1981. Relationship between phage resistance and emulsan production: interaction of phages with the cell surface of *Acinetobacter calcoaceticus* RAG-1. Arch. Microbiol. **130:**129–133.
16. **Rosenberg, E.** 1986. Microbial surfactants. Crit. Rev. Biotechnol. **3:**109–132.
17. **Rosenberg, E., and N. Kaplan.** 1986. Surface-active properties of *Acinetobacter* exopolysaccharides, p. 311–341. *In* M. Inouye (ed.), Bacterial outer membranes as model systems. John Wiley & Sons, Inc., New York.
18. **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.
19. **Rubinovitz, C., D. L. Gutnick, and E. Rosenberg.** 1982. Emulsan production by *Acinetobacter calcoaceticus* in the presence of chloramphenicol. J. Bacteriol. **152:**126–132.
20. **Sar, N., and E. Rosenberg.** 1983. Emulsifier production by *Acinetobacter calcoaceticus* strains. Curr. Microbiol. **9:**309–314.
21. **Spiro, R. G.** 1966. Analysis of sugars found in glycoproteins. Methods Enzymol. **8:**7–9.
22. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Carbohydrate chemistry. Academic Press, Inc., New York.
23. **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.