4 Bioemulsans: Surface-active Polysaccharide-containing Complexes

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Introduction

In general, microorganisms are specialists, and in any particular ecological niche one microorganism or a limited number of strains will dominate. These microorganisms have adapted through evolution to be able to survive in this niche during the long periods when growth is impossible, but when nutrients do become available they can outgrow their competitors. The rapid growth of microorganisms depends largely on their high surface-to-volume ratio, which allows for the efficient uptake of nutrients and release of waste products. The price that the microorganism pays for the high surface-to-volume ratio is that it is totally exposed. All of the components on the outside of the cell must be able to function under the specific conditions of the ecological niche. It is probably for this reason that the "diversity of the microbial world" is best expressed on the outside of the cell. Because of their high surface-to-volume ratio and the diversity of their exocellular polymers, microorganisms are a rich source of potentially useful polymers. One group of such polymers, referred to as emulsans, is amphipathic polysaccharides and/or proteins that stabilize oil-in-water emulsions.

2 Historical Outline

Research on microbial bioemulsifiers can be traced to pioneering attempts to: (1) solve practical problems in the petroleum industry; and (2) understand how microorganisms grow on water-insoluble hydrocarbons.

2.1 The Petroleum Industry Connection

The two major problems of the petroleum industry that involved surface-active agents (surfactants) were the secondary recovery of oil, and the production of single-cell protein (SCP) from hydrocarbons. One of the earliest studies of the microbial production of surfactants was carried out by La Riviere (1955a,b), who showed that eight different microbial cultures, including Aspergillus nii-
ger, Psuedomonas aeruginosa, Candida lipolytica, Desulfovibrio desulfuricans and Mycobacterium phlei, lowered surface tension by 14 to 34 mN m⁻¹. Updegraff and Wren (1954) patented a process for the release of oil from petroleum-bearing materials using sulfate-reducing bacteria. However, subsequent studies demonstrated that only small increases (ca. 5%) in oil recovery were obtained with microbial surfactants (Dostalek and Spurny, 1958). The early work on the use of microorganisms for secondary recovery of oil has been reviewed by Davis (1967) and Zajic and Panchal (1976).

During the early 1960s, British Petroleum announced that it intended to manufacture microbial protein from hydrocarbons on a large scale. This stimulated many oil companies to support in-house as well as university research on the optimization of the microbial conversion of n-paraffins to what came to be called SCP. It soon became clear that the growth of microorganisms at high cell density in fermentors on n-paraffins was limited by the low solubility of hydrocarbons in water (Johnson, 1968; Erickson and Nakahara, 1975). For example, the solubilities of decane, dodecane and tetradecane in water at 25 °C are 3.1×10⁻⁷ M, 1.7×10⁻⁸ M, and 9.8×10⁻¹⁰ M, respectively (Goldberg, 1985). Accordingly, the growth rate of microorganisms on hydrocarbons is limited by the transfer rate of the substrate into solution, and then across the cell membrane. Numerous observations were reported indicating that the dispersion of hydrocarbons in oil increased during fermentation as a result of increased interfacial area between the oil drops and the aqueous culture broth. This led to the discovery that microorganisms, in this case yeast, produce extracellular bioemulsifiers, such as fatty acids and various polymers (Iguchi et al., 1969; Abbot and Gledhill, 1971).

2.2 The Emulsan Story

By means of the enrichment culture technique, a mixed population of microorganisms was obtained which catalyzed the emulsification of crude oil in supplemented seawater (Reisfeld et al., 1972). From this mixed culture, one strain (RAG-1) was isolated that degraded ca. 60% of the oil and efficiently dispersed the nondegraded oil into small droplets (2–5 μm diameter). The initial studies indicated that the material responsible for emulsification was present in the extracellular fluid of cultures grown on crude oil as the carbon and energy source. The emulsifier-producing RAG-1 strain was initially mis-classified as a member of the genus Arthrobacter. Subsequently, the strain was identified as an Acinetobacter (Pines and Gutnick, 1981) and referred to as A. calcoaceticus RAG-1 (ATCC 31012).

RAG-1 was initially isolated with the goal of using it to treat oil pollution in the sea. However, it soon became clear that this goal was impracticable because growth of this bacterium, as with any known microorganism, requires utilizable nitrogen (N) and phosphorus (P) sources in addition to the crude oil. The oceans are “deserts” when it comes to N and P compounds, and crude oil contains very little N and P. The addition of utilizable N and P compounds (e.g., urea, nitrates and phosphates) to an open system such as an ocean is of no use because these water-soluble compounds diffuse away from the oil and are not available to the bacteria adhering to the oil spill. Consequently, Rosenberg et al. (1974) used RAG-1 in a closed system, the cargo compartment of an oil tanker during its ballast voyage, in order to prevent oil pollution resulting from discharge of oily ballast water.

The oil tanker/RAG-1 experiment was performed in January, 1973 on a ballast
voyage from Eilat in the Gulf of Aqaba to Khargh Island in the Persian Gulf. One of the slop tanks containing 100 m³ of oily ballast water over a layer of sludge was supplemented with urea and phosphate. After inoculation with RAG-1, the slop tank was oxygenated with air at 3 m³/min⁻¹. A nonaerated slop tank served as a control. Samples removed from the tanks during the first 3 days of the voyage showed that the number of bacteria in the tank that was supplemented with urea and phosphate, aerated and inoculated with RAG-1, reached over 10⁸ bacteria per mL, whereas the nonaerated control tank contained ca. 10⁴ bacteria per mL.

Furthermore, the nondegraded oil in the experimental tank was thoroughly emulsified. After 4 days, the tanks were emptied, whereupon the experimental tank was completely free of the thick layer of oily sludge that remained in the control tank. In this experiment, removal of the sludge was made possible by providing conditions that favored bioemulsification rather than optimum cell growth. This microbiological cleaning method has the advantage that cleaning takes place while the tank is full of ballast water, thereby decreasing the danger of explosion. Moreover, all submerged components of the tank are in contact with the cleaning agent.

The potential of using RAG-1 to clean the cargo compartments of oil tankers during their ballast voyage (Gutnick and Rosenberg, 1977) led to an initial patent on the process (U.S. patent No. 3,941,692) and more than 20 subsequent patents throughout the world on RAG-1 and emulsans. The oil tanker experiment reached the attention of Leslie Misrock, a patent attorney and pioneer biotechnology entrepreneur, who obtained the rights from Tel Aviv University and established a company, Petroferm USA, to exploit these discoveries. Between 1975 and 1990, most of the research on emulsans was supported by Petroferm. RAG-1 emulsan was the first of a number of emulsans that are produced by microorganisms. During the past few years, several reviews have been published on different aspects of emulsans: their role in bacterial adhesion (Neu, 1996); growth on hydrocarbons (Rosenberg and Ron, 1996); surface-active polymers from the genus Acinetobacter (Rosenberg and Ron, 1998a); production (Wang and Wand, 1990); molecular genetics (Sullivan, 1998); commercial applications (Fiechter, 1992); enhanced oil recovery (Banat, 1995); bioemulsans (Rosenberg and Ron, 1997); high and low molecular mass microbial surfactants (Rosenberg and Ron, 1999); and their natural roles (Ron and Rosenberg, 2001).

3 Occurrence and Chemical Properties

A large number of bacterial species from different genera produce exocellular polymeric surfactants composed of polysaccharides, protein, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers (Table 1). Xanthan, although it has some surfactant properties, is primarily a thickener, and as such, will not be discussed in this chapter.

3.1 RAG-1 Emulsan

The best-studied polymeric bioemulsifiers are the emulsans produced by different species of Acinetobacter (Rosenberg and Ron, 1998a). RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein (Rosenberg and Kaplan, 1987). Its surface activity is due to the presence of fatty acids, comprising 15% of the emulsan dry weight, that are attached to the polysacchar-
ride backbone via O-ester and N-acyl linkages (Belsky et al., 1979). The chemical and physical properties of RAG-1 emulsan are summarized in Table 2. RAG-1 cells excrete maximum amounts of emulsan in shake flasks when grown in a minimal medium containing 2% ethanol as the sole carbon and energy source. Under these conditions, approximately 80% of the emulsan produced is released when the cells are in stationary phase (Goldman et al., 1982). Emulsan is an effective emulsifier at low concentrations (0.01–0.001%), representing emulsan-to-hydrocarbon ratios of 1:100 to 1:1000, and exhibits considerable substrate specificity (Rosenberg et al., 1979a). RAG-1 emulsan does not emulsify pure aliphatic, aromatic, or cyclic hydrocarbons; however, all mixtures that contain an appropriate mixture of an aliphatic and an aromatic (or cyclic alkane) are emulsified efficiently. Maximum emulsifying activity occurs over a wide pH range and requires the presence of divalent cations (Rosenberg et al., 1979b).

Tab. 1  Selected microbially produced emulsans

<table>
<thead>
<tr>
<th>Emulsan</th>
<th>Producing microorganisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG-1 emulsan</td>
<td>A. calcoaceticus RAG-1</td>
<td>Rosenberg et al. (1979a,b)</td>
</tr>
<tr>
<td>BD4 emulsan</td>
<td>A. calcoaceticus BD413</td>
<td>Kaplan and Rosenberg (1982)</td>
</tr>
<tr>
<td>Alasan</td>
<td>A. radioresistent KA53</td>
<td>Navon-Venezia et al. (1995)</td>
</tr>
<tr>
<td>Biodispersan</td>
<td>A. calcoaceticus A2</td>
<td>Rosenberg et al. (1988a,b)</td>
</tr>
<tr>
<td>Mannan-lipid-protein</td>
<td>C. tropicalis</td>
<td>Keppeli et al. (1984)</td>
</tr>
<tr>
<td>Liposan</td>
<td>C. lipolytica</td>
<td>Cirigliano and Carman (1984)</td>
</tr>
<tr>
<td>Emulsan 378</td>
<td>P. fluorescens</td>
<td>Persson et al. (1988)</td>
</tr>
<tr>
<td>Protein complex</td>
<td>M. thermoautotrophium</td>
<td>De Acvedo and McInerney (1996)</td>
</tr>
<tr>
<td>Insecticide emulsifier</td>
<td>P. tralacida</td>
<td>Appaiah and Karanth (1991)</td>
</tr>
<tr>
<td>Thermophilic emulsifier</td>
<td>B. stearothermophilus</td>
<td>Gunjar et al. (1995)</td>
</tr>
<tr>
<td>Acetylheteropolysaccharide</td>
<td>S. paucimobilis</td>
<td>Ashitaputre and Shah (1995)</td>
</tr>
<tr>
<td>Food emulsifier</td>
<td>C. utilis</td>
<td>Shepherd et al. (1995)</td>
</tr>
<tr>
<td>Sulfated polysaccharide</td>
<td>H. euribalinia</td>
<td>Calvo et al. (1998)</td>
</tr>
</tbody>
</table>

Tab. 2  Chemical and physical properties of RAG-1 emulsan (Data from Rosenberg et al., 1979b)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical composition</td>
<td>d-galactosamine, 25%</td>
</tr>
<tr>
<td>Intrinsic viscosity (cm³·g⁻¹)</td>
<td>550</td>
</tr>
<tr>
<td>Diffusion constant (cm³·g⁻¹)</td>
<td>5.3×10⁻⁸</td>
</tr>
<tr>
<td>Partial molar volume (cm³·g⁻¹)</td>
<td>0.71</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td>980</td>
</tr>
<tr>
<td>Dimensions (nm)</td>
<td>3×200</td>
</tr>
</tbody>
</table>
3.2 BD4 Emulsan

A. calcoaceticus BD4, which was initially isolated and characterized by Taylor and Juni (1961), produces a large polysaccharide capsule. Under certain growth conditions (e.g., 2% ethanol as the carbon and energy source), an enhanced release of the capsules was obtained (Sar and Rosenberg, 1983). However, in contrast to RAG-1, no decrease in capsular polysaccharide was observed; rather, capsular polysaccharide levels remained constant when enhanced levels of extracellular polysaccharide were obtained. When the crude capsular material was applied to a Sepharose 4B column, it eluted in a single peak containing a polysaccharide–protein complex. The polysaccharide component was obtained by deproteinization and its chemical structure elucidated (Figure 1). The protein component was obtained from the extracellular supernatant fluid of strain BD4-R7, a capsule-negative mutant of BD4 that produces no extracellular polysaccharide. The polysaccharide and protein components had no emulsifying activity by themselves. However, mixing the protein and polysaccharide fractions led to a reconstitution of the emulsifying activity (Kaplan et al., 1987). Apparently, the protein (which is hydrophobic) binds to the hydrocarbon initially in a reversible fashion. The polysaccharide then attaches to the protein and stabilizes the oil-in-water emulsion.

3.3 Alasan

Alasan, produced by a strain of Acinetobacter radioresistens, is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 10^3 kDa (Navon-Venezia et al., 1995). However, the polysaccharide component of alasan is unusual in that it contains covalently bound alanine. The protein component of alasan appears to play an important role in both the structure and activity of the complex (Navon-Venezia et al., 1998). Deproteinization of alasan with hot phenol or treatment with specific proteinases caused a loss in most of the emulsifying activity. When a solution of alasan was exposed to increasing temperatures, there were large changes in the viscosity and emulsifying activity of the complex (Figure 2). Between 30 and 50 °C, the viscosity increased 2.6-fold, but with no change in activity. However, between 50 and 90 °C the viscosity decreased 4.8-fold while the activity increased 5-fold. None of these changes occurred with the protein-free polysaccharide, indicating that they were due to the interactions of the protein and polysaccharide portions of the complex. Alasan lowers interfacial tension from 69 to 41 dynes cm⁻¹ at 20 °C and has a critical micelle concentration (CMC) of 200 μg/ml (Barkay et al., 1999). Although alasan, as well as the other emulsans, is not as effective as some of the lower molecular-weight bioemulsifiers at lowering interfacial tension, the emulsans are extremely effective at stabilizing oil-in-water emulsions.

\[ \rightarrow 3 \cdot \alpha-L-Rha-(1 \rightarrow 3) \cdot \alpha-D-Man-(1 \rightarrow 3) \cdot \alpha-L-Rha-(1 \rightarrow 3) \cdot \beta-D-Glc-(1 \rightarrow 2) \]

\[ \alpha-L-Rha-(1 \rightarrow 4) \cdot \beta-D-GlcUA \]

**Fig. 1** The chemical structure of the *Acinetobacter calcoaceticus* BD4 capsular polysaccharide (Kaplan et al., 1985).
Recently, alasan has been fractionated into three proteins (of 16, 31 and 45 kDa) and the alasan polysaccharide (Toren et al., 2001). Each of the three alasan proteins showed emulsifying activity: the 45-kDa protein had the highest specific activity, 11% higher than the intact alasan complex. The N-terminal amino acid sequence of the 45-kDa protein showed high similarity to the OmpA protein of several Gram-negative bacteria. The function of the alasan polysaccharide is not clear, but it may play a role in the release of the proteins into the medium and in protecting the protein complex against proteolytic activities. It was interesting to note that the purified 45-kDa protein was readily hydrolyzed by trypsin, whereas the protein that was bound to the polysaccharide was resistant.

3.4 Biodispersan

*A. calcoaceticus* A2 produces an extracellular anionic polysaccharide surfactant of molecular mass 51,400 Daltons that effectively disperses limestone and titanium dioxide (Rosenberg et al., 1988a,b). The biopolymer, referred to as biodispersan, binds to powdered calcium carbonate and changes its...
surface properties in a way that allows for better dispersion in water. In addition to being a surfactant, biodispersan acts also as a surfactant, aiding in the fracturing of limestone during the grinding process (Rosenberg et al., 1989).

3.5 Other High Molecular-mass Bioemulsifiers

Sar and Rosenberg (1983) reported that the majority of *Acinetobacter* strains produce extracellular nondialyzable emulsifiers. These strains included both soil and hospital isolates. Marin et al. (1996) have reported the isolation of a strain of *A. calcoaceticus* from contaminated heating oil which emulsifies that substrate. Neufeld and Zajic (1984) demonstrated that whole cells of *A. calcoaceticus* 2CA2 have the ability to act as emulsifiers, in addition to producing an extracellular emulsifier.

A large number of high molecular-weight complex bioemulsifiers have been reported, though in general little is known about these other than the producing organism and the overall chemical composition of the crude mixture. An alkane-oxidizing *Rhodococcus* sp. produces a high molecular-weight complex that when released into the medium stabilizes oil-in-water emulsions (Bredholt and Eihmjen, 1999). *Halomonas eurihalina* produces an extracellular sulfated heteropolysaccharide (Calvo et al., 1998), while *Pseudomonas tralucida* produced an extracellular acetylated polysaccharide that was effective in emulsifying several insecticides (Appaiah and Karanth, 1991). Several recently reported bioemulsifiers are effective at high temperature, including the protein complex from *Methanobacterium thermoautotrophium* (De Acevedo and McInerney, 1996) and the protein–polysaccharide–lipid complex of *Bacillus stearothermophilus* ATCC 12980 (Gunnar et al., 1995).

Yeasts produce a number of emulsifiers, which is particularly interesting because of the food-grade status of several yeasts. Liposan is an extracellular emulsifier produced by *Candida lipolytica* (Cirigliano and Carman, 1985) and is composed of 83% carbohydrate and 17% protein. Mannanprotein emulsifiers are produced by *Saccharomyces cerevisiae* (Cameron et al., 1988), while a variety of polymeric bioemulsifiers for potential use in foods was reported by Shepard et al. (1995).

4 Natural Role of Emulsans

Although bioemulsifiers are produced by a large number of microorganisms and are clearly significant in many aspects of growth, it is difficult to generalize on their role in microbial physiology. To begin with, relevant experiments have been performed only with few emulsifier-producing microbes. In addition, with increasing numbers of identified microbial emulsifiers it has become clear that microbial surfactants have very different structures, are produced by a wide variety of microorganisms, and have very different surface properties. Thus, it is expected that bioemulsifiers have various roles, some of which are unique to the physiology and ecology of the producing microorganisms. At this stage it is impossible to draw any generalizations or to identify one or more functions that are clearly common to all microbial surfactants. Thus, most of the concepts have been derived from a consideration of the surface properties of the biosurfactants and experiments in which biosurfactants are added to microorganisms growing on water-insoluble substrates. In the following discussion, we will present a few natural roles for emulsans that have been suggested or demonstrated.
4.1 Increasing the Surface Area of Hydrophobic Water-insoluble Substrates

For bacteria growing on hydrocarbons the growth rate can be limited by the interfacial surface area between water and oil. When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect, and there are certain conceptual difficulties in understanding how emulsification can provide an (evolutionary) advantage for the microorganism producing the emulsifier. Stated briefly, emulsification is a cell density-dependent phenomenon; that is, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to emulsify oil effectively. Furthermore, any emulsified oil would disperse in the water and not be any more available to the emulsifier-producing strain than to competing microorganisms. One way to reconcile the existing data with these theoretical considerations is to suggest that the emulsifying agents do play a natural role in oil degradation, but not in producing macroscopic emulsions in the bulk liquid. If emulsion occurs at or very close to the cell surface, and no mixing occurs at the microscopic level, then each cell creates its own micro-environment and no cell density dependence would be expected.

4.2 Increasing the Bioavailability of Hydrophobic Water-insoluble Substrates

One of the major reasons for the prolonged persistence of high molecular-weight hydrophobic compounds is their low water solubility that increases their sorption to surfaces and limits their availability to biodegrading microorganisms. When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited (Van Delden et al., 1998). Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Deziel et al., 1996). Surfactants that lower interfacial tension are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Recently, it has been demonstrated that alasan increases the apparent solubilities of polycyclic aromatic hydrocarbons (PAHs) between 5- and 20-fold, and also significantly increases their rate of biodegradation (Barkay et al., 1999; Rosenberg et al., 1999).

4.3 Binding of Toxic Heavy Metals

Emulsans, like most anionic polysaccharides, bind cations. In some cases, the uronic acid residues of the polysaccharide are arranged so that divalent cations are bound avidly. RAG-1 emulsan is particularly efficient at binding uranyl ions, partially because of its negative charge and partially due to hydrophobic interactions (Zosim et al., 1983).

4.4 Regulating the Attachment-detachment of Microorganisms to and from Surfaces

One of the most fundamental survival strategies of microorganisms is their ability to locate themselves in an ecological niche where they can multiply. This is true not only for microbes that live in or on animals and plants, but also for those that inhabit soil and aquatic environments. The key elements in this strategy are cell surface structures that