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Microbial Production of Surfactants and Their Commercial Potential

JITENDRA D. DESAI[1]* AND IBRAHIM M. BANAT[2]

Applied Biology and Environmental Sciences Division, Research Center, Indian Petrochemicals Corporation Limited, Vadodara 391 346, India,[1] and Department of Biology, Faculty of Science, United Arab Emirates University, Al-Ain, Abu-Dhabi, United Arab Emirates[2]

"By which one sees an unperishable entity in all beings
and undivided among the divided then that knowledge is pure.
But if one merely sees the diversity of things with their divisions and limitations, without the truth, then that knowledge is merely an ignorance."

The Bhagavad Gita, chapter XVIII

INTRODUCTION

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which makes surfactants some of the most versatile process chemicals (71, 72).

Current worldwide surfactant markets are around $9.4 billion per annum (226), and their demand is expected to increase at a rate of 35% toward the end of the century (71). Almost all surfactants currently in use are chemically derived from petroleum; however, interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries (10, 11, 60, 118, 155, 257).

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates for enhancing oil recovery (219, 227, 234) and

* Corresponding author. Fax: 91-265-372098.

deemulsification processes (28). Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity; higher biodegradability (266); better environmental compatibility (65); higher foaming (203); high selectivity and specific activity at extreme temperatures, pH, and salinity (126, 257); and the ability to be synthesized from renewable feedstocks. Earlier work on biosurfactants centered mainly on the properties, biosynthesis, and chemistry and has been reviewed by many workers (34, 49, 50, 209a, 244). However, in the last few years, significant work on the fermentative production, genetics, and commercial applications of biosurfactants has been done; this, along with a brief account on the recent developments in microbial screening for biosurfactants, forms the subject matter of the present review.

RECENT ANALYTICAL METHODS

Screening of Potential Biosurfactant-Producing Microorganisms

Recent advances in the field of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential. Van der Vegt et al. (254) developed an axisymmetric drop shape analysis (ADSA) by profile for the assessment of potential biosurfactant-producing bacteria. In this technique, drops of culture broth are placed on a fluoroethylene-propylene surface and the profile of the droplet is determined with a contour monitor. Surface tensions are calculated from the droplet profiles by ADSA. Only biosurfactant-producing bacterial suspensions show reduction in surface tensions. Shulga et al. (231) described a colorimetric estimation of biosurfactants based on the ability of the anionic surfactants to react with the cationic indicator to form a colored complex. Development of other simple methods include the following: (i) a rapid drop-collapsing test (105), in which a drop of a cell suspension is placed on an oil-coated surface, and drops containing biosurfactants collapse whereas non-surfactant-containing drops remain stable; (ii) a direct thin-layer chromatographic technique for rapid characterization of biosurfactant-producing bacterial colonies as described by Matsuyama et al. (143); (iii) colorimetric methods described by Siegmund and Wagner (232) and Hansen et al. (79) for screening of rhamnolipid-producing and hydrocarbon-degrading bacteria, respectively; and (iv) estimation of the emulsification index value (*E*-24) by vigorously shaking culture broth samples with an equal volume of kerosene and measuring the percent emulsification after 24 h by the method of Cooper and Goldenberg (35), which is most suitable for emulsifying biosurfactants.

Estimation of Biosurfactant Activity

Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic-lipophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m, and addition of surfactant lowers this value to 30 mN/m. When a surfactant is added to air/water or oil/water systems at increasing concentrations, a reduction of surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supramolecular structures like micelles, bilayers, and vesicle. This value is known as the critical micelle concentration (CMC). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. Microbial culture broth or biosurfactants are diluted severalfold, surface tension is measured for each dilution, and the CMC is calculated from this value. The values of the surface tension, interfacial tension, and CMC of some known biosurfactants are listed in Table 1.

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase. Biosurfactants may stabilize (emulsifiers) or destabilize (deemulsifiers) the emulsion. The emulsification activity is assayed by the ability of the surfactant to generate turbidity due to suspended hydrocarbons such as a hexadecane–2-methylnaphthalene mixture (47, 210) or kerosene (35), etc., in an aqueous assay system. The deemulsification activity is derived by determining the effect of surfactants on a standard emulsion by using a synthetic surfactant (209a, 266).

The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion by comparing it with surfactants with known HLB values and properties. The HLB scale can be constructed by assigning a value of 1 for oleic acid and a value of 20 for sodium oleate and using a range of mixtures of these two components in different proportions to obtain the intermediate values. Emulsifiers with HLB values less than 6 favor stabilization of water-in-oil emulsification, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification.

BIOSURFACTANT CLASSIFICATION AND THEIR MICROBIAL ORIGIN

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants.

Although there are a number of reports on the synthesis of biosurfactants by hydrocarbon-degrading microorganisms, some biosurfactants have been reported to be produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol (35, 74, 92, 184, 186). The biosurfactant-producing microbes are distributed among a wide variety of genera. The major types of biosurfactants, with their properties and microbial species of origin, are listed in Table 1 and are described briefly in the following section. For more details, readers are referred to Desai and Desai (50), Rosenberg (209a), Kosaric et al. (124), and Banat (11).

Glycolipids

Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids.

Rhamnolipids. Rhamnolipids, in which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid, are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (108). L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Fig. 1A) and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate,

TABLE 1. Microbial source and properties of important types of microbial surfactants

Biosurfactant	Organisms	Surface tension (mN/m)	CMC	Interfacial tension (mN/m)	Reference(s)
Glycolipids					
Rhamnolipids	*P. aeruginosa*	29		0.25	74, 208
	Pseudomonas sp.	25–30	0.1–10	1	88, 128, 185
Trehalolipids	*R. erythropolis*	32–36	4	14–17	200
	N. erythropolis	30	20	3.5	140, 142
	Mycobacterium sp.	38	0.3	15	40
Sophorolipids	*T. bombicola*	33		1.8	40, 68
	T. apicola	30		0.9	93, 250
	T. petrophilum				38
Cellobiolipids	*U. zeae*, *U. maydis*				24, 242
Lipopeptides and lipoproteins					
Peptide-lipid	*B. licheniformis*	27	12–20	0.1–0.3	109, 263
Serrawettin	*S. marcescens*	28–33			143
Viscosin	*P. fluorescens*	26.5	150		176
Surfactin	*B. subtilis*	27–32	23–160	1	3, 20
Subtilisin	*B. subtilis*				20
Gramicidins	*B. brevis*				139
Polymyxins	*B. polymyxa*				240
Fatty acids, neutral lipids, and phospholipids					
Fatty acids	*C. lepus*	30	150	2	40, 43
Neutral lipids	*N. erythropolis*	32		3	136
Phospholipids	*T. thiooxidans*				17, 121
Polymeric surfactants					
Emulsan	*A. calcoaceticus*				210, 270
Biodispersan	*A. calcoaceticus*				211, 213
Mannan-lipid-protein	*C. tropicalis*				112
Liposan	*C. lipolytica*				32, 33
Carbohydrate-protein-lipid	*P. fluorescens*	27	10		47, 189
	D. polymorphis				236
Protein PA	*P. aeruginosa*				87, 89
Particulate biosurfactants					
Vesicles and fimbriae	*A. calcoaceticus*				76, 113
Whole cells	Variety of bacteria				58, 209a

referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa* (57, 88, 102, 103). The formation of rhamnolipid types 3 and 4 containing one β-hydroxydecanoic acid with one and two rhamnose units, respectively (242), methyl ester derivatives of rhamnolipids 1 and 2 (86), and rhamnolipids with alternative fatty acid chains (128, 185, 206) has also been reported. Rhamnolipids from *Pseudomonas* spp. have been demonstrated to lower the interfacial tension against *n*-hexadecane to 1 mN/m and the surface tension to 25 to 30 mN/m (74, 128, 185). They also emulsify

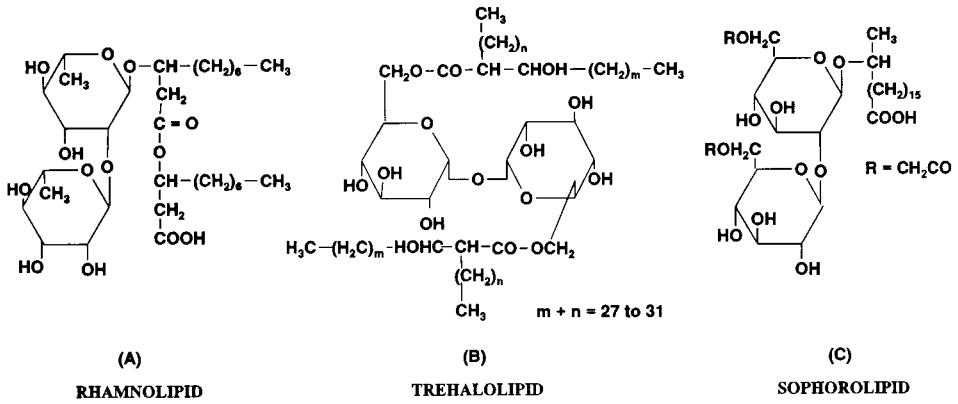

FIG. 1. Structure of some common glycolipid biosurfactants. (A) Rhamnolipid type 1 from *Pseudomonas aeruginosa* in which two rhamnose subunits are linked to two β-hydroxydecanoic acids in a side chain. (B) Trehalose dimycolate from *Rhodococcus erythropolis*, in which disaccharide trehalose is linked to two long-chain α-branched β-hydroxy fatty acids. (C) Sophorolipid from *Torulopsis bombicola* in which dimeric sophorose is linked to a long-chain (C_{18}) hydroxy fatty acid.

alkanes and stimulate the growth of *P. aeruginosa* on hexadecane (88). Itoh and Suzuki (102) isolated two mutants of *P. aeruginosa*, PU-1 and PU-2, which grew poorly on alkanes due to their inability to produce rhamnolipids. These mutants grew normally when the growth medium was supplemented with rhamnolipid.

Trehalolipids. Several structural types of microbial trehalolipid biosurfactants have been reported (128, 133). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium*, *Nocardia*, and *Corynebacterium*. Mycolic acids are long-chain, α-branched-β-hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation (5, 40, 128, 244). Trehalose dimycolate produced by *Rhodococcus erythropolis* (Fig. 1B) has been extensively studied (126, 200). *R. erythropolis* also synthesizes a novel anionic trehalose lipid (207). Trehalose lipids from *R. erythropolis* and *Arthrobacter* sp. lowered the surface and interfacial tensions in the culture broth to 25 to 40 and 1 to 5 mN/m, respectively (128, 133, 200).

Sophorolipids. Sophorolipids, which are produced mainly by yeasts such as *Torulopsis bombicola* (39, 68, 99), *T. petrophilum* (38), and *T. apicola* (250), consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid (Fig. 1C). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides. Similar mixtures of water-soluble sophorolipids from several yeasts have also been reported (93). Cutler and Light (44) showed that *Candida bogoriensis* produces glycolipids in which sophorose is linked to docosanoic acid diacetate. *T. petrophilum* produced sophorolipids on water-insoluble substrates such as alkanes and vegetable oils (38). These sophorolipids, which were chemically identical to those produced by *T. bombicola*, did not emulsify alkanes or vegetable oils. When *T. petrophilum* was grown on a glucose-yeast extract medium, however, sophorolipids were not produced, but an effective protein-containing alkane emulsifying agent was formed (38). These results appear to contradict the conventional belief that microbial emulsifiers and surfactants are produced to facilitate the uptake of water-insoluble substrates. Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents (39). Both lactonic and acidic sophorolipids lowered the interfacial tension between *n*-hexadecane and water from 40 to 5 mN/m and showed remarkable stability toward pH and temperature changes (38, 128).

Lipopeptides and Lipoproteins

A large number of cyclic lipopetides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *Bacillus brevis* (139) and *B. polymyxa* (240), respectively, possess remarkable surface-active properties. Ornithine-containing lipids from *P. rubescens* (265) and *Thiobacillus thiooxidans* (120), cerilipin, an ornithine- and taurine-containing lipid from *Gluconobacter cerinus* IFO 3267 (246), and lysine-containing lipids from *Agrobacterium tumefaciens* IFO 3058 (245) also exhibit excellent biosurfactant activity. An aminolipid biosurfactant called serratamolide has been isolated from *Serratia marcescens* NS.38 (164). Studies on serratamolide-negative mutants showed that the biosurfactant increased cell hydrophilicity by blocking the hydrophobic sites on the cell surface (14).

The cyclic lipopeptide surfactin (Fig. 2), produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.005% (3). *B. licheniformis* produces

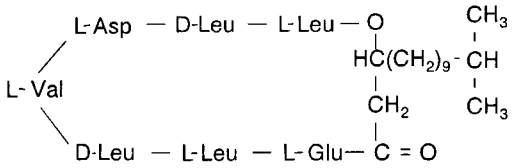

FIG. 2. Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis*.

several biosurfactants which act synergistically and exhibit excellent temperature, pH, and salt stability (147, 263). The surfactant BL-86, produced by *B. licheniformis* 86, is capable of lowering the surface tension of water to 27 mN/m and the interfacial tension between water and *n*-hexadecane to 0.36 mN/m and promoting excellent dispersion of colloidal β-silicon carbide and aluminum nitride slurries (94, 95). Recent structural analysis revealed that it is a mixture of lipopeptides with major components ranging in size from 979 to 1,091 Da. Each molecule contains seven amino acids and a lipid portion which is composed of 8 to 9 methylene groups and a mixture of linear and branched tails (96). Another important characteristic of this compound is its ability to lyse mammalian erythrocytes and to form spheroplasts (3, 20); this property has been used to detect surfactin production through hemolysis on blood agar.

Recently, Yakimov et al. (263) have shown production of a new lipopetide surfactant, lichenysin A, by *B. licheniformis* BAS-50 containing long-chain β-hydroxy fatty acids. Lichenysin A reduces the surface tension of water from 72 to 28 mN/m with a CMC of as little as 12 μM, comparing favorably with surfactin (24 μM). The detailed characterization of lichenysin A showed that isoleucine was the C-terminal amino acid instead of leucine and an asparagine residue was present instead of aspartic acid as in the surfactin peptide. Addition of branched-chain α-amino acids to the medium caused similar changes in lipophilic moieties of lichenysin-A and lowering of surface tension activity (263a).

Fatty Acids, Phospholipids, and Neutral Lipids

Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on *n*-alkanes (5, 33, 43, 208). The HLB is directly related to the length of the hydrocarbon chain in their structures. In *Acinetobacter* sp. strain HO1-N phosphatidylethanolamine (Fig. 3), rich vesicles are produced (113), which form optically clear microemulsions of alkanes in water. The quantitative production of phospholipids has also been detected in some *Aspergillus* spp. (113) and *Thiobacillus thiooxidans* (17). *Arthrobacter* strain AK-19 (259) and *P. aeruginosa* 44T1 (208) accumulate up to 40 to 80% (wt/wt) of such lipids when cultivated on hexadecane and olive oil, respectively. Phosphatidylethanolamine produced by *R. erythropolis* grown on *n*-alkane caused a lowering of interfacial

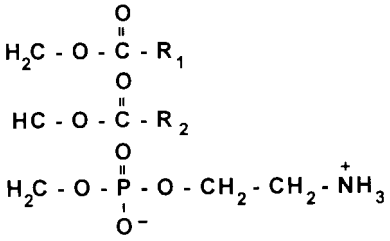

FIG. 3. Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R_1 and R_2 are hydrocarbon chains of fatty acids.

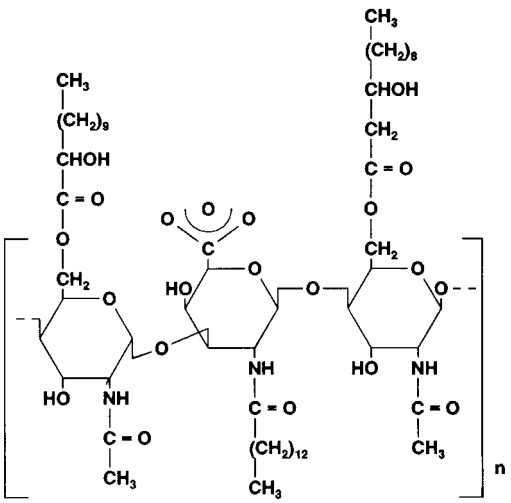

FIG. 4. Structure of emulsan, produced by *Acinetobacter calcoaceticus*, in which fatty acids are linked to a heteropolysaccharide backbone.

tension between water and hexadecane to less than 1 mN/m and a CMC of 30 mg/liter (126).

Polymeric Biosurfactants

The best-studied polymeric biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. *Acinetobacter calcoaceticus* RAG-1 produces a potent polyanionic amphipathic heteropolysaccharide bioemulsifier (Fig. 4) called emulsan (210). The heteropolysaccharide backbone contains a repeating trisaccharide of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid, and an unidentified *N*-acetyl amino sugar (271). Fatty acids are covalently linked to the polysaccharide through *o*-ester linkages (18, 225, 271). Emulsan is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001 to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (18, 76, 270). On long standing, this emulsion separates into two layers. The upper cream layer, which is known as emulsanosol, contains 70 to 75% oil (270). Biodispersan is an extracellular, nondialyzable dispersing agent produced by *A. calcoaceticus* A2 (213). It is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and contains four reducing sugars, namely, glucosamine, 6-methylaminohexose, galactosamine uronic acid, and an unidentified amino sugar (211). Recently, Navonvenezia et al. (173) described the isolation of alasan, an anionic alanine-containing heteropolysaccharide-protein biosurfactant from *Acinetobacter radioresistens* KA-53, which was found to be 2.5 to 3 times more active after being heated at 100°C under neutral or alkaline condition.

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* (32, 110) and is composed of 83% carbohydrate and 17% protein (32). The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid. Palejwala and Desai (184) reported the production by a gram-negative bacterium of a potent bioemulsifier with carbohydrate as a major component. Sar and Rosenberg (218) demonstrated that polysaccharide had no emulsification activity alone but became a potent emulsifier when combined with some proteins released during growth on ethanol.

Cameron et al. (29) recently reported the production of large amounts of mannoprotein by *Saccharomyces cerevisiae*; this protein showed excellent emulsifier activity toward several oils, alkanes, and organic solvents. The purified emulsifier contains 44% mannose and 17% protein. Kappeli et al. (111, 112) have isolated a mannan-fatty acid complex from alkane-grown *Candida tropicalis*; this complex stabilized hexadecane-in-water emulsions. *Schizonella malanogramma* and *Ustilago maydis* produce biosurfactant which has been characterized as erythritol- and mannose-containing lipid (59). Recently, Kitamoto et al. (117) demonstrated the production of two kinds of mannosylerythritol lipids in *Candida antarctica* T-34. Hisatsuka et al. (87, 89) described the isolation from *P. aeruginosa* of a protein-like activator that was involved in emulsification of hydrocarbons. It has a molecular weight of 14,300 and contains 147 amino acids, of which 51 are serine and threonine (89). The production by *P. aeruginosa* P-20 of a peptidoglycolipid bearing 52 amino acids, 11 fatty acids, and a sugar unit has been described previously (123). An emulsifying and solubilizing factor containing protein and carbohydrate from hexadecane-grown *Pseudomonas* spp. has also been reported (17, 153). Desai et al. (47) demonstrated the production of bioemulsifier by *P. fluorescens* during growth on gasoline. This bioemulsifier is composed of 50% carbohydrate, 19.6% protein, and 10% lipid. Trehalose and lipid-*o*-dialkyl monoglycerides were the major components of the carbohydrate and lipid, respectively. Similarly, an extracellular bioemulsifier composed of carbohydrate, protein, and lipids was isolated from *C. tropicalis* (236) and *Phormidium* strain J1 (58).

Particulate Biosurfactants

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion which plays an important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* sp. strain HO1-N with a diameter of 20 to 50 nm and a buoyant density of 1.158 g/cm^3 are composed of protein, phospholipid, and lipopolysaccharide (113). The membrane vesicles contain about 5 times as much phospholipid and about 350 times as much polysaccharide as does the outer membrane of the same organism.

Surfactant activity in most hydrocarbon-degrading and pathogenic bacteria is attributed to several cell surface components, which include structures such as M protein and lipoteichoic acid in the case of group A streptococci, protein A in *Staphylococcus aureus*, layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *Bacillus brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1 (49, 58, 128, 209a, 262).

PHYSIOLOGY AND GENETICS

Physiological Role

Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible substrates (17, 88, 114, 122, 210, 235). Biosurfactant-negative mutants of *P. aeruginosa* KY-4025 (102) and *P. aeruginosa* PG-201 (122) showed poor growth compared to the parent strains on *n*-paraffin and hexadecane, respectively, and addition of rhamnolipid to the medium restored growth on these hydrocarbons. From a physiological point of view, production of such a large amount of polymer will be a waste if it has no function. The genetic system also loses the expression of the gene over a long period by mutation and selection if the gene product has no specific advantage for survival. The function of biosurfactant in

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