Biosurfactants and oil bioremediation

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Oil pollution is an environmental problem of increasing importance. Hydrocarbon-degrading microorganisms, adapted to grow and thrive in oil-containing environments, have an important role in the biological treatment of this pollution. One of the limiting factors in this process is the bioavailability of many fractions of the oil. The hydrocarbon-degrading microorganisms produce biosurfactants of diverse chemical nature and molecular size. These surface-active materials increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation.

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Abbreviation
PAH polycyclic aromatic hydrocarbon

Introduction

Microorganisms produce a large variety of surface-active materials, or surfactants for short. Bioemulsifiers are often produced by bacteria capable of growing on hydrocarbons and have been shown to stimulate the growth of these bacteria and to accelerate bioremediation. This review will deal with the general types of bioemulsifiers and their presumed mode of action. More information can be found in several recent reviews [1•,2••,3,4,5••,6].

Oil-degrading bacteria

Petroleum bioremediation is carried out by microorganisms capable of utilizing hydrocarbons as a source of energy and carbon [7,8,9•]. These microorganisms are ubiquitous in nature and are capable of degrading the various types of hydrocarbons — short-chain, long-chain and numerous aromatic compounds, including polycyclic aromatic hydrocarbons. All these compounds have low solubility in water. This fact, coupled to the fact that the first step in hydrocarbon degradation involves a membrane-bound oxygenase, makes it essential for bacteria to come in direct contact with the hydrocarbon substrates. One biological strategy that can enhance contact between bacteria and water-insoluble hydrocarbons is emulsification of the hydrocarbon. Therefore, it is not surprising that bacteria growing on petroleum usually produce potent emulsifiers. These surfactants help to disperse the oil, increase the surface area for growth, and help detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted [10].

Types of bacterial biosurfactants

Bacteria make low molecular weight molecules that efficiently lower surface and interfacial tensions and high molecular weight polymers that bind tightly to surfaces [2••,3,4,11••].

The low molecular weight biosurfactants are generally glycolipids in which carbohydrates are attached to a long-chain aliphatic acid or lipopeptides. Glycolipid bioemulsifiers, such as rhamnolipids, trehalose lipids and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids. One of the best-studied glycolipids is rhamnolipid, produced by several species of *Pseudomonads*, which consists of two moles of rhamnose and two moles of β-hydroxydecanoic acid [12]. Recently, a new class of glycolipids, glucose lipids, produced by *Alcanivorax borkumensis* has been described [13–15]. These consist of an anionic glucose lipid with a tetrameric oxyacyl sidechain.

The high molecular weight bacterial surfactants are produced by a large number of bacterial species from different genera and are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. The high molecular weight surfactants are less effective in reducing interfacial tension, but are efficient at coating the oil droplets and preventing their coalescence. These are highly efficient emulsifiers that work at low concentrations (0.01%–0.001%), representing emulsifier-to-hydrocarbon ratios of 1:100–1:1000. These high molecular weight bioemulsifiers exhibit considerable substrate specificity. For example, some emulsify efficiently mixtures of aliphatic and aromatic (or cyclic alkane) hydrocarbons, but will not emulsify pure aliphatic, aromatic or cyclic hydrocarbons; others can also emulsify pure hydrocarbons but only of a high molecular weight. The best-studied biosurfactants are the bioemulsans produced by different species of *Acinetobacter* [16]. Among them is the emulsan from *Acinetobacter* RAG-1, which is a complex of an anionic heteropolysaccharide and protein whose surface activity results from the presence of fatty acids that are attached to the polysaccharide backbone via *O*-ester and *N*-acyl linkages. *Acinetobacter calcoaceticus* BD4, initially isolated and characterized by Taylor and Juni [17], produces a surface-active extracellular polysaccharide–protein complex. Alasan, produced by a strain of *Acinetobacter radioresistens*, is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 10^6 Da [18]. 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 [19,20]. Recently, one of the alasan proteins, with an apparent molecular weight of 45 kDa, was studied at the molecular level. This protein has an amino acid sequence homologous

to that of *Escherichia coli* OmpA and is highly effective in stabilizing oil-in-water emulsions and in solubilizing hydrocarbons, including polycyclic aromatic hydrocarbons [21•].

Production of bacterial biosurfactants

The bioemulsifiers are usually produced as cultures reach the stationary stage of growth. In several cases it was shown that emulsifier production is induced by molecular signals involved in quorum sensing. This regulatory feature appears to be general, and probably applies to the production of both low and high molecular weight emulsifiers, as in all cases emulsifier production is concurrent with the increase in cell density and the onset of the stationary phase of growth [3,22–28].

Because oil-degrading bacteria can utilize only a limited group of hydrocarbons, bacteria attached and growing on an oil droplet become nutrient-starved once this group of hydrocarbons is depleted. If the biosurfactant is cell-bound it can cause the microbial cell surface to become more hydrophobic, depending on its orientation. For example, the cell-surface hydrophobicity of *Pseudomonas aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid [29], whereas the cell-surface hydrophobicity of *Acinetobacter* strains was reduced by the presence of its cell-bound emulsifier [30]. These data suggest that microorganisms can use their biosurfactants to regulate their cell-surface properties to attach or detach from surfaces according to need. This has been demonstrated for *A. calcoaceticus* RAG-1 growing on crude oil [10]. During exponential growth, emulsan is cell-bound in the form of a minicapsule. This bacterium utilizes only relatively long chain n-alkanes for growth. After these compounds are utilized, RAG-1 becomes starved, although it is still attached to the oil droplet, which is enriched in aromatics and cyclic paraffins. Starvation of RAG-1 causes release of the minicapsule of emulsan. It was shown that this released emulsan forms a polymeric film on the n-alkane-depleted oil droplet, thereby desorbing the starved cell [30]. In effect, the 'emulsifier' frees the cell to find fresh substrate. At the same time, the depleted oil droplet has been 'marked' as used, because it now has a hydrophilic outer surface to which the bacterium cannot attach. The detachment of bacteria from the depleted oil drop enables them to move to other drops where they metabolize the specific group of utilizable hydrocarbons. Therefore, detachment of bacteria from oil drops results in a more efficient bioremediation.

Involvement of biosurfactants in oil bioremediation

There are at least two ways in which biosurfactants are involved in bioremediation: increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic compounds.

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 [31]. 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 effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water and not be 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 cluster of cells creates its own microenvironment and no overall cell-density dependence would be expected.

Increasing the bioavailability of hydrophobic water-insoluble substrates

The low water solubility of many hydrocarbons, especially the polycyclic aromatic hydrocarbons (PAHs), is believed to limit their availability to microorganisms, which is a potential problem for bioremediation of contaminated sites. It has been assumed that surfactants would enhance the bioavailability of hydrophobic compounds. Several non-biological surfactants have been studied, and both negative and positive effects of the surfactants on biodegradation were observed. For example, the addition of the surfactant Tergitol NP-10 increased the dissolution rate of solid-phase phenanthrene and resulted in an overall increase in the growth of a strain of *Pseudomonas stutzeri* [32]. A similar effect was obtained by the addition of Tween 80 to two *Sphingomonas* strains — the rate of fluoranthene mineralization was almost doubled. By contrast, the same surfactant inhibited the rate of fluoranthene mineralization by two strains of *Mycobacterium* [33], and no stimulation was observed in other studies using several surfactants [34,35].

Biosurfactants are more effective than chemical surfactants in increasing the bioavailability of hydrophobic compounds. In addition, they are selective, environmentally friendly, and generally less stable than most synthetic surfactants. The high molecular weight bioemulsifier Alasan was recently shown to significantly increase the rate of biodegradation of several PAHs [11••].

One of the major reasons for the prolonged persistence of high molecular weight hydrophobic compounds is their low water solubility, which increases their sorption to surfaces and limits their availability to biodegrading

microorganisms. When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited [36]. Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility [37]. Surfactants that lower interfacial tension dramatically are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Low molecular weight biosurfactants that have low critical micelle concentrations (CMCs) increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles [38]. Data have been reported which indicate that biosurfactants can stimulate, inhibit or have no effect on biodegradation of hydrocarbons, as reviewed by Bruheim [34]. In this regard, Arino [39] reported that a rhamnolipid-producing strain of *P. aeruginosa* is involved in the degradation of PAHs by a bacterial community. Much less is known on how polymeric biosurfactants increase apparent solubilities of hydrophobic compounds. Recently, it has been demonstrated that alasan increases the apparent solubilities of PAHs 5–20-fold and significantly increases their rate of biodegradation [11••,40••].

Utilizing biosurfactants for bioremediation

Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g. nutrients and oxygen), conditions (e.g. pH and moisture content), and prevailing microorganisms. Thus, bioremediation usually consists of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, if necessary, supplying air and often adding bacteria. The addition of emulsifiers is advantageous when bacterial growth is slow (e.g. at cold temperatures or in the presence of high concentrations of pollutants) or when the pollutants consist of compounds that are difficult to degrade, such as PAHs. Bioemulsifiers can be applied as an additive to stimulate the bioremediation process; however, with advanced genetic technologies it is expected that the increase in bioemulsifier concentration during bioremediation would be achieved by the addition of bacteria that overproduce bioemulsifiers. This approach has been recently used successfully in the cleaning of oil pipes. Cultures of *A. radioresistens* [18], which produce the bioemulsifier alasan but are unable to use hydrocarbons as a carbon source, were added to a mixture of oil-degrading bacteria to enhance oil bioremediation (EZ Ron, E Rosenberg, unpublished results).

Bacteria that overproduce bioemulsifiers can also participate in oil degradation. Alternatively, they can function in a bacterial consortium, supplying the emulsifier for other bacteria that carry out the degradation of the hydrocarbons. In this latter case, the bioemulsifier can diffuse in the soil or can even be transferred to the other bacteria on close contact, such as in biofilms. Recently, horizontal transfer of capsule polysaccharide has been demonstrated in bacteria [41•], resulting in bacteria coated with emulsifying polysaccharide capsule produced by bacteria of another species. The effect of these phenomena on oil bioremediation remains to be further investigated. Clearly, optimization of this process would involve selecting the best oil-degrading microorganisms, the most suitable biosurfactant, the best bioemulsifier producers and the most effective combination of these.

Conclusions

Biosurfactants are produced by a variety of oil-degrading microorganisms. These biosurfactants can be of low molecular weight, acting by decreasing the oil–water interfacial tension, or high molecular weight and act as biodispersants by preventing coalescence of oil drops in water. The high molecular weight bioemulsifiers are heteropolysaccharides, and the active components are lipids or proteins. The activity of bacterial biosurfactants in bioremediation stems from their ability to increase the surface area of hydrophobic water-insoluble substrates and to increase the solubility and bioavailability of hydrocarbons. They can be added to bioremediation processes as purified materials or in the form of bioemulsifier-overproducing bacteria. In either case, they can stimulate the growth of oil-degrading bacteria and improve their ability to utilize hydrocarbons.

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