BIOSURFACTANT - PRODUCING BACTERIA FOUND IN CONTAMINATED AND UNCONTAMINATED SOILS

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ABSTRACT

Microbially produced surfactants have been studied for microbially enhanced oil recovery (MEOR) and the bioremediation of hydrocarbons. However, most of these studies have used biosurfactants produced by one of a small number of pure-culture microbes isolated in a laboratory. In previous work, we determined that biosurfactant-producing microorganisms were naturally present at two hydrocarbon-impacted sites. In this study, we examine the prevalence of biosurfactant producers in uncontaminated soils. Biosurfactant-producing bacteria were found to constitute a significant proportion (up to 35%) of aerobic heterotrophs. Biosurfactant producers were isolated. Isolates were identified primarily as strains of *Bacillus* and *Pseudomonas*.

Key words: *surfactant, biosurfactant,* Bacillus, *and* Pseudomonas

INTRODUCTION

Biosurfactants are microbially produced surface-active compounds. They are amphiphilic molecules with both hydrophilic and hydrophobic regions causing them to aggregate at interfaces between fluids with different polarities such as water and hydrocarbons (Banat, 1995a; Fiechter, 1992; Georgiou, 1992; Kosaric, 1993; Karanth et al., 1999). These biomolecules may also decrease interfacial surface tension (Lin, 1996; Shafi and Khanna, 1995; Rouse et al., 1994; Volkering et al.,1998; Fiechter, 1992; Georgiou et al., 1992; Karanth et al., 1999). Although the function of biosurfactants in microorganisms is not fully understood, it is known that these secondary metabolites can enhance nutrient transport across membranes, act in various host-microbe interactions, and provide biocidal and fungicidal protection to the producing organism (Lin, 1996; Banat, 1995a; Banat,1995b).

However, it is the ability of the biosurfactant producers to reduce interfacial surface tension, which has important tertiary oil recovery and bioremediation consequences (Lin, 1996; Rouse et al., 1994; Volkering et al., 1998). Many of the known biosurfactant producers are also hydrocarbon-degrading organisms (Rouse et al., 1994; Willumsen and Karlson, 1997; Volkering et al., 1998).

In the past decade, many studies have reported the effects of microbially produced surfactants on bioremediation and enhanced oil recovery (Jack, 1988; Jenneman et al., 1984; Volkering et al., 1998). However, these studies typically involved a single microbe or group of microbes isolated and identified in a laboratory and then applied to either *ex situ* soil core experiments or injected into existing oil reservoirs for observation. In addition, the majority of these studies testing for enhanced biosurfactant production or hydrocarbon recovery were conducted with only a few species such as *Bacillus licheniformis* strain JF-2, *Bacillus subtilis*, or *Pseudomonas fluorescens* (Adkins et al., 1992; Banat, 1995a; Banat, 1995b; Lin, 1998, McInerney et al.,

1990). Few studies, though, have evaluated the presence of natural, indigenous biosurfactant-producing microbes in oil recovery or bioremediation sites.

In a prior study, we reported the surprising presence of a large variety and number of biosurfactant producers isolated from two hydrocarbon-impacted sites (Jennings and Tanner, 1999). In this study, we isolated and identified biosurfactant producers from two additional soils, which were unconnected to any hydrocarbon contamination, and determined the proportion of aerobic heterotrophs which were biosurfactant producers in these soils.

MATERIALS AND METHODS

Sources of environmental samples

Sediments for this study were collected from two different locations. The first sample (RST soil) was taken from a piece of unimproved property located in Garvin County in central Oklahoma. This site is in a pristine, uncontaminated condition and is comprised of a loamy clay soil rich in organic matter. Samples were taken from just below the soil surface and stored at 4° C until use (within 48 hours).

The second sampling location was the Tulsa Rose Garden in Tulsa, Okla. Because the area is a professional, formal botanical garden, the soil system is highly manipulated. This manipulation includes the regular addition of heavy mulch layers, as well to the flower beds. As fungicides and pesticides, samples of the Tulsa Rose Garden soil were taken from where the mulch litter met the soil to a depth of approximately 10 cm deep. Samples were kept at 4° C until use (within 48 hours).

Because comparisons will be made between these two samples and samples collected for the aforementioned study (Jennings and Tanner, 1999), brief descriptions of the prior samples follow. The first sample is from an active natural gas production site near Ft. Lupton, Colo. (Gieg et al., 1999). The soil in this area is classified as a sandy to sandy loam with a low organic content. During the 1970s, gas condensate contamination occurred and, although the source has been removed, residual contamination exists both in the soil and groundwater. Sediments for this study were collected in 1999 from beneath the shallow water table (~ 1.4 meters). Upgradient, uncontaminated sediments were also collected in addition to contaminated sediments.

The second set of soil samples collected for the 1999 study were from within the Tall Grass Prairie Preserve in Osage County, Okla. In January 1999, 70 barrels of dewatered crude oil were spilled into a silty loam clay basin. Sediments from this site, like those from Ft. Lupton, were also collected in March of 1999. Again, both contaminated and neighboring uncontaminated soils were collected for analysis.

Media used

Blood agar plates were used for the screening and isolation of potential biosurfactant-producing bacteria (Bernheimer and Avigad,1970; Banat, 1995a; Banat, 1995b; Lin, 1996).

Full-strength, plate-count agar (PCA; Difco Laboratories, number 0751-17-2) was used for the maintenance of isolated

biosurfactant-producing bacterial colonies and for counting the total number of aerobic heterotrophic bacteria (Atlas and Parks, 1993).

Tryptic soy broth agar (TSBA; Difco product number 0369-17-6) slants were inoculated with colonies for a fatty acid methyl eter (FAME) analysis by Microcheck, Inc. (Sassar, 1990).

Biolog Universal Growth (BUG) medium (Biolog, Inc., Hayward, CA) was used for the preparation of isolates for the Biolog analysis, according to Biolog protocols (Solit, 1999).

METHODS

Isolation of biosurfactant producers

One gram (wet weight) of sediment from the Tulsa Rose Garden site was serially diluted in 0.85% sterile saline. All dilutions were performed in triplicate.

Dilutions were spread-plated on blood agar plates (prepared one day prior) with final dilutions ranging from 10^{-1} through 10^{-4}. These were incubated at 30° C and counted after 24 and 48 hours. After isolation, colonies were maintained at 30°C on PCA plates.

The above screen for beta hemolytic bacteria was repeated for the RST soil sample.

Colony morphology, growth, and microscopic characteristics were recorded for isolates. Similar colonies were grouped together on the basis of microscopic analysis and colony morphology (Tate, 1995; Gerhardt et al., 1994)). For example, isolate A included colonies showing a flat, slightly moist, cream-colored morphology with light feathering at the edges. Microscopic analysis of the bacteria in each of the colonies grouped together and designated "isolate A" revealed non-motile, Gram-positive rods, sometimes linked in short chains. A representative colony of each group was then selected for further analysis.

In addition to the dilution plating of sediments onto blood agar plates, positive and negative controls were also plated. As a positive control, *Bacillus licheniformis* JF-2 (ATCC 39307) was used (McInerney et al., 1990). A mutant form of *Bacillus licheniformis* JF-2 which does not produce biosurfactant was plated as a negative control.

Enumeration of total aerobic heterotrophs

Dilution plating was performed on both the Tulsa Rose Garden and RST soil samples in a similar manner as with the isolation of biosurfactant-producing bacteria, with the exception of substituting PCA for blood agar (Atlas and Parks, 1993). Final plate dilutions ranged from 10^{-2} to 10^{-5}.

Biolog analysis

Representative colonies for each of the above groups were analyzed by the Biolog Microlog System for comparative identification according to Biolog, Inc. protocols (Solit, 1999).

Identification of selected cultures – FAME analysis

Certain colonies which were difficult to identify by the Biolog Analysis were identified by using FAME analysis by Microcheck, Inc. (Northfield, VT) (Sassar, 1990).

RESULTS AND DISCUSSION

Bacterial Numbers

The number of total aerobic heterotrophs were very similar between the Tulsa Rose Garden and RST soil samples, ranging from 9.2 x 10^5 to 1.1 x 10^6, respectively (Table 1). This similarity was not surprising, given that both soil samples contained high amounts of organic matter. Biosurfactant producers ranged from 1.0 x 10^5 to 3.8 x 10^5 (Table 1).

Until recently, biosurfactant producers were thought to be restricted to contaminated soils where conditions would select for microbes with an enhanced ability to utilize hydrocarbons (Rouse et al., 1994; Willumsen and Karlson, 1997; Volkering et al., 1998). However, small levels of biosurfactant production have been demonstrated in unimpacted soils and may be a reflection of the other roles biosurfactants play in a soil ecosystem, functioning as biocides, fungicides, and nutrient transport molecules (Lin, 1996; Banat, 1995a; Banat, 1995b; Jennings and Tanner, 1999). Therefore, detecting a biosurfactant producer population from within the RST and Tulsa Rose Garden soils was not necessarily surprising.

Based on prior observations, we expected to recover a significant population of biosurfactant-producing bacteria (Table 1). However, the extent of the biosurfactant-producing population surpassed expectations in these two hydrocarbon-unimpacted soils. Biosurfactant producers constituted between 10 – 35 % of the aerobic heterotrophic bacterial

Table 1. Numbers of aerobic heterotrophs and biosurfactant-producing bacteria from each of the soil samples.

	Average # Aerobic Heterotrophs	Average # Biosurfactant Producers	Percent Biosurfactant Producers
RST Soil			
Uncontaminated	1.1 x 10^6	3.8 x 10^5	35.0 %
Tulsa Rose Garden			
Uncontaminated	9.2 x 10^5	1.0 x 10^5	10.1 %
Tall Grass Prairie, OK [a]			
Uncontaminated	2.3 x 10^6	1.6 x 10^4	0.7 %
Contaminated	1.0 x 10^6	1.4 x 10^4	1.4 %
Ft. Lupton, CO [a]			
Uncontaminated	1.3 x 10^5	5.5 x 10^2	1.1 %
Contaminated	6.0 x 10^4	1.4 x 10^3	9.1 %

a: These two soils were initially studied as part of a prior project (Jennings and Tanner, 1999).

population (Tulsa Rose Garden and RST soils, respectively) (Table 1). The reason that these numbers are so impressive is that the majority of the Ft. Lupton and Tall Grass Prairie samples had biosurfactant-producing populations at approximately 1 % (Jennings and Tanner, 1999) (Table 1).

The high values for the Tulsa Rose Garden and RST soils may be explained by the relationship between the amount of soil organic matter and the size of the biosurfactant population. For example, Ft. Lupton and Tall Grass Prairie sediments, which are characterized by low organic matter, had low percentages of biosurfactant producers. In contrast, the two soils with the higher organic matter content, the Tulsa Rose Garden and RST soils, had high fractions of biosurfactant producers (Table 1).

In organic-rich soils, microorganisms tend to be metabolically stimulated (Brady and Weil, 1999). As bacterial metabolism is increased, so must be those compounds, such as biosurfactants which aid in transporting various nutrients across cell membranes in order to support this growth (Lin, 1996; Banat, 1995a; Banat,1995b). This may explain why the RST and Tulsa Rose Garden soils have a higher percentage of biosurfactant producers over the Ft. Lupton and Tall Grass Prairie soils. However, it does not explain the discrepancy between the Tulsa Rose Garden and RST soils.

The fraction of biosurfactant producers was three times the level in the RST soil than in the Tulsa Rose Garden soil (Table 1). One of the obvious differences between the two soils is the presence of pesticides at the Tulsa Rose Garden site. Koehler (1994) analyzed the effects of the pesticide Aldicard on various soil mesofauna and microorganisms, finding that application of the pesticide had long-term effects on not only the microbial population within the test site, but also an important class of soil organisms – those which feed upon microbes.

It was found that those organisms which consume bacteria actually increased in numbers after pesticide application. This has implications for the Tulsa Rose Garden site because many biosurfactants are produced as fungicides, and the increase in numbers of biosurfactant producers in comparison to the Ft. Lupton and Tall Grass Prairie soils (which were not treated with pesticides) may be a response to the increased threat by such fungi.

However, this increase due to fungicidal activity may be overshadowed by the biosurfactant's role as a nutrient transporter when microbial growth is as competitive as it may be at the RST site. Whereas at the Tulsa Rose Garden a high percentage of the soil organic material has been manually added in easily degradable forms to enhance flower blooms, the soil organic material at the RST site is primarily in the original form of natural or indigenous plant matter. The high amounts of soil nutrients, including nitrogen and phosphorus, removed from the soil by grasses (which predominated on the site) can easily result in soils depleted of these nutrients (Brady and Weil, 1999; Salisbury and Ross, 1992). This nutrient depletion, even if slight, can decrease the number of microbes in a soil. It has been documented that biosurfactant production is

actually stimulated when certain nutrients are limited, specifically nitrogen and phosphorus (Shafi and Khanna, 1995). Thus, with nonbiosurfactant-producing bacterial populations decreasing in conjunction with selection towards biosurfactant-producing ones, the ratio of producers to nonproducers might increase. Therefore, this may explain why the RST soil has a much higher percentage of biosurfactant producers than the Tulsa Rose Garden soil.

Identification of biosurfactant-producing bacteria

The initial isolation of suspected biosurfactant producers was done on blood agar plates, utilizing the ability of many biosurfactants to lyse erythrocytes, which results in a band of beta hemolysis surrounding biosurfactant-producing bacterial colonies (Bernheimer and Avigad, 1970; Banat, 1995a; Banat, 1995b; Lin, 1996). Such colonies were isolated and then maintained on PCA plates. Colony morphologies, growth patterns on various media, and microscopic analyses indicated that although there were many colonies isolated, there were similarities among many of them. When the isolates were grouped according to these similarities, select colonies were chosen to represent each of these groups.

Of the different colony types analyzed, a total of four different species were identified: *Pseudomonas fluorescens*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus sphaericus*. These results were expected for two main reasons: first, both genera are common soil organisms; and second, the ability for members of both genera to produce biosurfactants is well documented (Banat, 1995a; Banat, 1995b; Georgiou, 1992; Rouse et al., 1994; Shafi and Khanna, 1995).

CONCLUSIONS

In conclusion, biosurfactant-producing bacteria appear to be found in soils which have not been exposed to hydrocarbon contamination, and they seem to predominately be members of the *Bacillus* and *Pseudomonas* genera. In addition, these bacteria appear to be a significant proportion of the aerobic heterotroph population. Finally, the amount of organic matter present in the soil may effect this proportion significantly, as may the availability of the organic matter and other required soil nutrients.

Potential future study of this phenomenon includes the utilization of antibody probes to search not for those bacteria with the potential to produce biosurfactants, but for the biosurfactants themselves within the soil matrix. In addition, further investigations into the effects of soil organic matter as well as pesticide and herbicide application are being considered.

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