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DEVELOPMENT OF MORE EFFECTIVE BIOSURFACTANTS FOR ENHANCED OIL RECOVERY

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ABSTRACT

Bacillus mojavensis JF-2 produces a lipopeptide biosurfactant that lowers the interfacial tension between oil and water to values ≤ 0.01 mN/m. This organism grows in the absence of air and at elevated temperatures and salinities. These are the environmental conditions found in many domestic oil reservoirs. For these reasons, we chose this organism for our studies. Core displacement experiments at elevated pressure showed that about 23% of the residual oil remaining in Berea sandstone cores was recovered. Oil recovery was highly correlated to the amount of biosurfactant produced. In sand-packed columns, substantial amounts of residual oil (up to 43%) were recovered when about 20 g/l of the biosurfactant was used in conjunction with a mobility control agent. A microbially enhanced oil recovery simulator was used to relate oil recovery to biosurfactant concentration. From the model and our data, we found that the minimum biosurfactant concentration needed to recovery substantial amounts of residual oil was about 1 mg/l. At this concentration, about 12% of the residual oil is recovered. With this information and the yield values for the amount of biosurfactant produced from glucose determined from experimental analyses, we estimated that residual oil could be recovered at a cost of about 0.40 to 3.90 dollars per barrel, depending on the size of the amount of nutrients injected in the oil reservoir.

We found that the addition of peptide supplements to a mineral medium improved both the anaerobic growth and biosurfactant production of *B. mojavensis* JF-2. The presence of glass beads to simulate a porous matrix increased the total amount of biosurfactant recovered. The amount of biosurfactant produced in the presence of glass beads was about 3 mg/l. This concentration exceeded the minimum biosurfactant concentration needed for sufficient for substantial oil recovery based on reservoir simulation studies. The addition of Proteose peptone to mixed microbial communities obtained from groundwater and inoculated with *Bacillus mojavensis* strain JF-2 resulted in the production of 2,3-butanediol. 2,3-Butanediol is a fermentation end product characteristic of *Bacillus* species and a cosurfactant which may increase the efficacy of the biosurfactant for oil recovery. In groundwater microcosms amended with glucose and 27 mM nitrate or Proteose peptone and 27 mM nitrate, up to 90% of the viable microbial population contained genes for the production of the lipopeptide biosurfactant. Thus, it is possible to control the dynamics of natural microbial populations during microbial oil recovery

processes by nutrient manipulations. Our studies indicate that biosurfactant-mediated oil recovery is an economically attractive technology for enhanced oil recovery.

EXECUTIVE SUMMARY

Oil reservoirs contain diverse and metabolically active microbial communities. Knowledge of the microbial ecology of oil reservoirs can be used to stimulate the beneficial activities of microorganisms to enhance oil recovery. Microorganisms produce a variety of products that are potentially useful for enhancing oil production. Several products have become commercial viable technologies such as paraffin control. However, the mechanism by which microorganisms improve oil recovery is poorly understood and not all applications have been effective. This is probably why microbial treatments are viewed with a great deal of skepticism in the petroleum industry. Many microorganisms produce detergent-like molecules called biosurfactants. Microorganisms that are members of the genus *Bacillus* make a biosurfactant, which has a protein and lipid component. Such biosurfactant molecules are called lipopeptides. Lipopeptide biosurfactants reduce the interfacial tension between oil and water by several factors of ten. The reductions in interfacial tension by lipopeptide biosurfactants are comparable to chemically synthesized surfactants used in enhanced oil recovery. The results in two field trials in Oklahoma conducted by scientists at the National Institute for Petroleum Research in the 1980's showed that additional oil was produced and the water-to-oil ratio decreased after a lipopeptide-producing bacterium and carbohydrate-based nutrients were injected into the reservoir. These data indicated that biosurfactant-mediated oil recovery may be a promising technology for widespread application for oil recovery. However, quantitative information on how much biosurfactant must be produced to obtain significant recovery of residual oil is not available. Also, it is not clear what nutrient sources are best to grow the microorganisms that make these biosurfactants, and whether the production of the biosurfactant would occur at elevated pressures found in many reservoirs.

GOALS

1. How biosurfactant-producing genes organized?
2. What nutrients are needed to grow biosurfactant-producing organisms and stimulate biosurfactant production?
3. What are the quantitative relationships among nutrient consumption, biosurfactant production and oil recovery? and
4. What are the economics of biosurfactant-mediated oil recovery?

BIOSURFACTANT IS REQUIRED FOR ENHANCEMENT OF OIL RECOVERY FROM POROUS MODEL SYSTEMS (QUESTION 3).

Bacillus mojavensis JF-2 produces a lipopeptide biosurfactant that lowers the interfacial tension between oil and water to values ≤ 0.01 mN/m. This organism grows in the absence of air and at elevated temperatures and salinities. These are the environmental conditions found in many domestic oil reservoirs. For these reasons, we chose this organism for our studies.

First, we wanted to know whether this organism could grow at the pressures found in many oil reservoirs. Core displacement experiments showed that *in situ* biosurfactant production occurred at elevated pressure and resulted in the recovery of about 23% of the residual oil. Next, we wanted to know whether oil was recovered due to the production of the biosurfactant or by some other mechanism. To test these possibilities, we isolated a mutant strain of *B. mojavensis* strain JF-2 that did not make a biosurfactant. The biosurfactant-deficient mutant of strain JF-2 did not recover residual oil. Residual oil was recovered when the parental strain (original strain) was used and oil recovery was highly correlated to the amount of biosurfactant produced. In sand-packed columns, we found that concentrations in excess of 2 g/l grams of biosurfactant were needed for oil recovery. Substantial amounts of residual oil (up to 43%) were recovered from sand-packed columns when about 10 g/l of the biosurfactant was used in conjunction with a mobility control agent. These conditions are similar those used in chemical enhanced oil recovery. Our studies with model porous systems showed that biosurfactant production resulted in substantial residual oil recovery and was effective at elevated pressures.

A microbially enhanced oil recovery simulator was used to relate oil recovery to biosurfactant concentration. We found that the minimum biosurfactant concentration needed to recover residual oil was about 1 mg/l. At this concentration, about 12% of the residual oil is recovered according to model predictions. This is the first time that an important engineering design criterion has been obtained. Such data are critical to the assessment of the economics of the process.

GENES FOR BIOSURFACTANT PRODUCTION CAN BE EXPLOITED (QUESTION 2)

A molecular biology study of the biosurfactant-producing *B. mojavensis* strain JF-2 was carried out to gain a better understanding of its metabolism for MEOR purposes. Molecular phylogenetic analyses showed that strain JF-2 was found to be most closely related to *Bacillus*

mojavensis, and therefore is redesignated as a member of this species, and not a species *Bacillus licheniformis* to which it had previously been assigned. DNA probes from the surfactin synthesis and production genes (*srfA* and *sfp*) of *Bacillus subtilis*, designated SrfA1 and Sfp1, were designed and used to isolate genes related to biosurfactant production in strain JF-2. We isolated more than 50 recombinants encoding the presumed genes for biosurfactant production from strain JF-2. A cosmid clone of 40 kilobases (kb) containing the putative surfactant synthetase cluster was subjected to sequence analysis. To date, about 40% (15kb) of the cluster has been satisfactorily sequenced. Analysis of the assembled partial sequences yielded some useful information. A portion (*ca*. 3.6kb) of the *B. mojavensis* cosmid sequence exhibited highest homology (>80% identity) with the *B. subtilis srfA-sfp* region of surfactin synthetase. Another portion of the deduced amino acid sequences of the JF-2 gene products located distal to the *srfA-sfp* region showed considerable identity with the *B. subtilis* YckE-TlpC gene sequence (*ca*. 93% identity). Four open reading frames in this region of the *B. mojavensis* cosmid sequence corresponded to proteins involved in membrane functions and carbohydrate metabolism. These analyses should be exploitable in the manipulation of growth and biosurfactant production in the field, as well to monitor the progress of MEOR processes as they are applied in the reservoir. Most importantly, DNA probes can be used in field tests to determine whether nutrient additions stimulate the growth of biosurfactant-producing populations.

GROWTH OF BIOSURFACTANT PRODUCERS AND PRODUCTION OF THE BIOSURFACTANT UNDER RESERVOIR CONDITIONS (QUESTION 2)

Studies were conducted to improve growth and biosurfactant production under anaerobic conditions. The addition of peptide supplements to a mineral medium improved both the anaerobic growth and biosurfactant production of *B. mojavensis* JF-2. The addition of peptide supplements increased the dry weight of the culture by about 7 fold. Fractionation of components present in the peptide supplements showed that a compound of about 3900 Da molecular weight was responsible for the enhancement of anaerobic growth. The presence of glass beads to simulate a porous matrix increased the total amount of biosurfactant recovered. The amount of biosurfactant produced in the presence of glass beads was about 3 mg/l. This concentration exceeded the minimum biosurfactant concentration needed for sufficient for substantial oil recovery based on reservoir simulation studies.

One major question is whether our nutrient formulation would selectively enrich for lipopeptide-producing microorganisms in mixed microbial communities. We used groundwater from a local aquifer as our model subsurface microbial community. The addition of Proteose peptone to groundwater microcosms inoculated with *Bacillus mojavensis* strain JF-2 resulted in the production of 2,3-butanediol, a fermentation end product characteristic of *Bacillus* species. This metabolite was also detected in microcosms that did not receive an inoculum of with *B. mojavensis* strain JF-2 so long as Proteose peptone was present. In groundwater microcosms amended with glucose and 27 mM nitrate or Proteose peptone and 27 mM nitrate that were inoculated with *B. mojavensis* strain JF-2, up to 90% of the viable microbial population contained genes for the production of the lipopeptide biosurfactant. Further analyses of these microbial cells showed that they grew anaerobically at high salt concentrations. Some of them made biosurfactants in liquid culture. All of these are characteristics of *B. mojavensis* strain JF-2. These studies showed that it is possible to control the dynamics of natural microbial populations during microbial oil recovery processes by nutrient manipulations. These studies also demonstrated the utility of the DNA probes that we developed.

BIOSURFACTANT-MEDIATED MEOR SHOULD BE SIGNIFICANTLY REVENUE POSITIVE (QUESTION 4)

The analyses performed during this project provided critical information to conduct a preliminary analysis of the economics of oil recovery. We showed that the *in situ* production of a biosurfactant is the most important mechanism for residual oil recovery. Our experiments take into account the production of other microbial products and alterations in permeability that could have influenced oil recovery. Also, our experiments were conducted at elevated pressures. Thus, biosurfactant production occurs under conditions that simulate actual oil reservoirs. Computer simulations provided us with an estimate of the critical biosurfactant concentration needed for residual oil recovery. Biosurfactant concentrations in excess of about 1 mg/l result in residual oil recoveries of about 10%. Our biosurfactant-producing bacterium, *Bacillus mojavensis* strain JF-2, is able to generate biosurfactant concentrations in excess of this value when growth in the presence of a surface (e. g., glass beads). Assuming that all of the glucose was completely utilized, we can calculate a specific yield of 1 milligram of biosurfactant per gram of glucose used.

We determined the amount of oil recovered and the revenue generated from a small domestic oil reservoir after a biosurfactant-mediated process has been conducted (single well pattern area of 10 acres, thickness of 35 feet, and a porosity of about 18%). Commonly, the oil saturation after waterflooding is between 0.2 and 0.4 in well-managed, mature floods. We assumed that the implementation of the MEOR process in the field would only be 50% efficient. The amount of oil recovered from the pattern by MEOR could be between 4.5 and 9.0 [mstb]. At an oil price of 20 per [stb], the incremental revenue from the MEOR process could range from $90,000 to $180,000 for the 10-acre pattern. The cost of the glucose was calculated from mass of biosurfactant made in the oil reservoir and the yield of biosurfactant per glucose used. Three values for the pore volume of nutrients injected were used, 0.1, 0.5 and 1.0 and three values for biosurfactant yields were considered, 1mg/g, 10 mg/g and 100 mg/g (mg of biosurfactant per g of glucose used). Our analyses showed that MEOR process is economic if implemented today. Given the current specific yield for biosurfactant, the recoverable value ranged from 51 to 5 dollars for every dollar expended to purchase glucose when 0.1 and 1.0 pore volumes were injected, respectively. This amounts to an incremental nutrient cost of about 0.39 to 3.90 dollars per barrel of oil recovered when 0.1 and 1.0 pore volumes were injected, respectively. This is very close to the economies for actual MEOR field trials that have been published in the literature. We realize that there are other costs such as increased pumping charges and water treatment and labor costs. However, it is likely that nutrients themselves will be the main financial cost.

SUMMARY

We have made the following significant contributions to the understanding of biosurfactant-mediated oil recovery:

1) We have determined the critical biosurfactant concentration needed for substantial oil recovery. One milligram of the biosurfactant per liter will recover about 10% of the residual oil.

2) We showed that biosurfactant production is one of the most important mechanisms for microbially-mediated oil recovery.

3) We developed of a nutrient formulation that supports luxurious anaerobic growth of biosurfactant-producing bacteria and biosurfactant production.

4) We demonstrated that selective nutrient addition enriches biosurfactant-producing bacteria in mixed microbial communities.

5) We developed molecular tools to monitor biosurfactant-producing bacteria in mixed microbial communities.

6) We demonstrated that biosurfactant-mediated oil recovery is economical.

CHAPTER 1. CRITICAL ASSESSMENT OF THE USE OF MICROORGANISMS FOR OIL RECOVERY

ABSTRACT

Oil reservoirs contain diverse and metabolically active microbial communities. Knowledge of the microbial ecology of oil reservoirs can be used to stimulate the beneficial activities of microorganisms to enhance oil recovery. Microorganisms produce a variety of products that are potentially useful for oil recovery. Microbial processes to control paraffin deposition in and around the well-bore have had the most commercial success. However, the mechanism for improved oil recovery with these products is not known and may be the reason why these products show variable technical performance when applied to different oil reservoirs. The addition of nitrate to oil field injection systems reduces the sulfide levels in produced water and stimulates the growth of sulfide-oxidizing denitrifiers within the reservoir, indicating that nitrate addition is an effective strategy to control the detrimental activities of sulfate-reducing bacteria in oil reservoirs. The addition of nutrients to stimulate the growth of indigenous microorganisms reduces permeability variation within the reservoir, slows the rate of decline in oil production, and extends the operational life of marginal oil fields. The production of acids, gas and solvents by the addition of carbohydrate-based nutrients results in increased oil production in carbonate and carbonaceous sandstone reservoirs. Lipopeptide biosurfactants can lower the interfacial tension between oil and water by several factors of ten and the organisms that produce these biosurfactants grow under the conditions that are found in most oil reservoirs. These data indicate that biosurfactant-mediated oil recovery may be a promising technology for widespread application for oil recovery. The results in two field trials in Oklahoma, U. S. showed that additional oil was produced and the water to oil ratio decreased after a biosurfactant-producing bacterium and carbohydrate-based nutrients were injected into the reservoir. However, the lack of information on reaction stoichiometries and rates of production makes it difficult to determine whether biosurfactants can be produced in the reservoir in quantities sufficient to recover oil. Microbial technologies will only gain widespread application when quantitative measures of microbial performance such as reaction stiochiometries and rates and required

product concentrations are obtained and when the mechanisms by which microbial processes recover oil are thoroughly understood.

INTRODUCTION

Oil, being an essential energy source, is both the lifeblood and a liability of many industrialized nations. The use of crude oil as an energy source has allowed many nations to develop a high standard of living. Continued economic growth will increase the demand for oil, which must be met by current production technologies or by new discoveries. For many countries like the United States, domestic oil production is in decline and the likelihood of discovering large, new oil reserves is low. These countries must then rely on foreign imports, which can slow economic growth and employment and aggravate trade deficits. Current production technologies are able to recover only about one-third of the oil originally in place and it is estimated that more than 300 billion barrels of oil remain in U. S. reservoirs after conventional technologies reach their economic limit [1]. New technologies that can recovery the rest of the oil offer the most timely and cost effective solution to reverse the decline in domestic oil production and increase the oil reserves of the United States. This is the objective of enhanced oil recovery (EOR) research and development. Although the long-term economic potential for enhanced oil recovery is large, actual EOR production in the United States has never been very large, less than 5% of the total U. S. production. This has been the case even though a variety of economic incentives have been provided to stimulate the development and application of EOR processes [1]. Often, the large capital or high chemical/energy costs of EOR technologies limit their application to a few reservoirs with very favorable conditions. Thus, the development of more cost-effective technologies is clearly needed.

The low oil prices that prevailed during the last 10 to 15 years lead to a shift in focus from EOR to improved oil recovery (IOR). The objective of IOR is to increase the rate of oil production or extend the life of a well without necessarily increasing the ultimate recovery of oil from the reservoir. The distinction EOR and IOR is critical since the performance of a process must be based on the objectives that one wished to achieve. The data that one needs to collect to determine whether IOR has occurred are often not as extensive as that needed to determine whether the EOR has occurred. To determine if EOR has occurred, extensive reservoir

characterization and modeling is often needed while assessment of IOR can be made by monitoring oil production rates and operating costs. Many commercial microbial technologies function marketed as both EOR and IOR processes. Thus, the goals that one wished to obtain are often not articulated which makes it difficult to assess the performance of many of the products. The results of many of the field trials of microbial processes support the conclusion that IOR occurred. However, it is less clear whether microbial processes have been successful at EOR.

Microbially processes have several unique advantages that may result in the development of economically attractive technologies. Microbial processes do not consume large amounts of energy as do thermal processes, nor do microbial processes depend on the price of crude oil as do many chemical processes. Because microbial growth occurs at exponential rates, it should be possible to produce large amounts of useful products rapidly from inexpensive and renewable resources. Microbial processes have the potential to be more cost effective than conventional IOR and EOR processes [2]. In fact, economic analyses of recent microbial field trials show that incremental oil can be produced for less than three dollars (U. S.) per barrel [3-5]. Given these advantages, it is reasonable to ask why microbial IOR and EOR technologies are not more widely implemented? The inconsistent technical performance [6-8] the lack of understanding of the mechanism of oil recovery, and lack of quantitative measures of microbial performance (rates of reaction, stoichiometry, and required product concentrations) make it difficult to extrapolate the results of one microbial field trial to other reservoirs. The goal of this review is assess the potential of microbial process for oil recovery from a microbial science and petroleum engineering perspective in order to delineate where further research is needed.

MICROBIOLOGICAL PERSPECTIVE

Microorganisms produce a variety of products that are potentially useful for oil recovery (Table 1.1). The use of microbial byproducts as means to recover oil has been an intensively studied [9, 10]. However, there are only a few instances where sufficient information has been obtained to determine whether microbial processes actually the cause of the change in oil production [3, 5, 11, 12]. The effectiveness of a microbial process is often difficult to assess

Table 1.1. Types of microbial processes for oil recovery

Process	Production problem	Type of activity or product needed
Well bore cleanup	Paraffin and scale deposits	Emulsifiers, biosurfactants, solvents, acids, hydrocarbon metabolism
Well stimulation	Formation damage, poor drainage	Gas, acids, solvents, biosurfactants
	High water production (coning)	Biomass and polymer production
Enhanced waterfloods	Poor displacement efficiency	Biosurfactants, solvents, polymers
	Poor sweep efficiency	Biomass and polymer production
	Souring (hydrogen sulfide production)	Nitrate reduction, hydrogen sulfide oxidation

since many factors, either associated with the well itself or within the reservoir, can affect oil production. These may include the cessation of production from a well, the replacement of worn or old equipment, changes in the stroke rate of the pump, etc. The changes in production or injection rates in other wells may also affect oil production from the well under study. These factors leave uncertainties as to the cause of the change in oil recovery unless well-designed field experiments are conducted to control for these factors. Control wells that use the same equipment and are operated in an identical fashion, without receiving a key component of the microbial process, should be included to demonstrate a cause and effect relationship between the microbial process and the parameter under study. The goal of some microbial approaches such as paraffin removal is often to reduce operating expenses rather than to increase oil production. For these cases, fuel consumption, electricity usage, or the amount and frequency of chemical treatments rather than the oil production should be measured. Preferably, wells with complete production histories should be chosen to obtain accurate estimates of the rate of change in these parameters over time. For reservoir-wide processes, tracer experiments should be conducted to

verify flow paths. Unless such analyses are done, it will be difficult to convince the petroleum industry that microbial processes provide a reliable technology.

The mechanisms for MEOR remain essentially unchanged from those originally proposed by ZoBell [13, 14]. Although these mechanisms are often discussed independently, it is likely that microbial processes act synergistically to affect oil recovery. Their relative importance will depend on conditions that limit oil production within a given reservoir, the strains of microorganism involved, and the protocols used for injection of nutrients and inocula. The different mechanisms by which microorganisms could enhance oil recovery are:

1. Biotransformation of crude oil to remove paraffinic and asphaltic fractions.

2. Acid production leading to the dissolution of the rock matrix and the increases in pore size and permeability. This will be particularly important in limestone or carbonaceous sandstone formations.

3. Gas production resulting in decreased oil viscosity, repressurization of the reservoir, and dissolution of the reservoir matrix in the case of limestone or carbonaceous sandstone reservoirs.

4. Solvent production where the solvent acts directly to reduce oil viscosity or reduce wettability, or indirectly as a co-surfactant to decrease interfacial tension.

5. Polymer production to increase the viscosity of the aqueous phase and improve the mobility ratio and sweep efficiency.

6. Selective plugging to block the dominant flow channels or reduce permeability variation within a reservoir to improve sweep efficiency.

7. Surfactant production to decrease interfacial tension and improve microscopic displacement efficiency.

8. Alteration of the viscosity of hydrocarbons by breaking long chain hydrocarbons into less viscous, smaller molecular weight hydrocarbons or by the production of emulsifiers.

The review will deal exclusively with processes that involve the in situ growth and metabolism of microorganisms within the reservoir. When implementing an in situ microbial process it is important to realize that petroleum reservoirs contain diverse and metabolically active microbial communities even in extremely thermophilic and hypersaline oil reservoirs [9, 15-20]. These organisms are active in the cycling of carbon and sulfur in the reservoir [9, 15] and

have the potential to use the hydrocarbon itself as the main electron donor for anaerobic respiration [21, 22]. Thus, if a common microbial activity such as hydrocarbon metabolism or common microbial metabolite such as gas or polymer is needed to recover oil, it is likely that a microbial population already exists in the reservoir that can catalyze this process. However, if a specific microbial metabolism such as biosurfactant production is needed to recover oil, this activity may not be present in all reservoirs and an inoculum may be needed. Microbial process dependent on the use of inocula will be problematic since the normal homeostatic mechanisms within the microbial community will act to prevent the establishment of introduced species unless the environmental conditions are altered to select for the introduced organism. Given that oil reservoirs have diverse and metabolically active microbial communities, care must be taken to ensure that the nutrient regime used to stimulate beneficial activities does not also stimulate detrimental ones such as souring and corrosion. A thorough understanding of what organisms are present and the factors that influence their growth and activity will allow operators to better exploit the natural microbial activities present in the reservoir.

ENGINEERING PERSPECTIVE

To fully realize the potential of microbial process to improve or enhance oil production, one must first understand the factors that limit oil production within a given reservoir and then develop specific microbial processes to recover that oil. It is only when the reservoir engineering standpoint is understood that an effective process for oil recovery can be developed. There is no average oil reservoir, and the chemical and physical properties of oil reservoirs are quite variable, as are that factors that entrap oil. Thus, a generic microbial process will probably not be successful when applied to a specific reservoir. Also, the mechanism of the microbial process must be understood in terms which petroleum engineers can use to evaluate the performance of the process. This is important since ultimately it will be the engineer who must make the decision whether a given microbial process will be effective for use in a given reservoir. The following sections will provide a brief synopsis of the factors that limit oil recovery and will analyze the relationship between the microbial performance and reservoir engineering principles.

An understanding of the multi phase flow properties of reservoir rock and the mechanisms that entrap oil is important for the success of any EOR project. The rate of oil and

water production from a well during primary and secondary recovery is governed by capillary pressure near the well bore [23, 24]. The respective saturations for water and oil in this region are functions of the capillary pressure between the two fluids. An important factor governing capillary pressure behavior is pore size distribution in the rock. If the pore entrance size distribution decreases, a shift in the capillary pressure occurs which causes oil production to stop at higher residual oil saturation.

The most common use of microbial technology has been to alleviate or prevent well damage. The migration of small particles of clay or minerals, the precipitation of paraffins and asphaltenes from the crude oil, and the compaction of sand can block the main drainage routes for oil to the well and change the pore size distribution. As a consequence of these processes, a considerable amount of mobile oil is left unrecovered a short distance away from the well. This oil can be recovered if the drainage routes can be re-established. Technologies aimed at improving the drainage patterns near the well bore can lead to stimulation of oil production. This is probably one of the main reasons why microbial paraffin treatments have been successful in improving oil production from individual wells [25-28]. It may also explain the stimulation of oil recovery that is sometimes observed after the growth of fermentative anaerobes in the vicinity of the well bore [6, 29]. The removal of particulates from the main drainage channels, either by the transformation of paraffinic or asphaltic materials, or by the dislodgement of the particles by the production of gases, solvents, acids, and/or surface active agents would change the pore size distribution to one that is more favorable to oil flow [24]. While mechanistically attractive, there is no direct evidence to support this hypothesis.

The efficiency of oil recovery is defined by the following equation [30]:

$$E_r = E_d \cdot E_v$$

where E_r is the recovery efficiency expressed as a fraction of the original oil in place, E_d is the microscopic oil displacement efficiency expressed as a fraction of the total volume of oil displaced from a segment of rock, and E_v is the volumetric sweep efficiency expressed as fraction of the total reservoir that is contacted by the recovery fluid.

The microscopic displacement efficiency is a measure of the amount of oil that remains in small pores or dead-end pores after a recovery process. The viscous and capillary forces that hold this oil in place are expressed as a ratio called the capillary number (N_{ca}) [31]:

$$N_{ca} = (\mu_w v_w)/(s_{ow})$$

where μ_w is the viscosity, v_w is the flux of fluid, and s_{ow} is the oil-water interfacial tension. Chemical methods such as surfactant, micellar polymer, or caustic flooding attempt to reduce interfacial tension thereby increasing the capillary number. Thermal methods aim at reducing the viscosity (μ) of the entrapped oil while the addition of polymers to the brine acts to increase the viscous pressure gradients in the flowing phase. Significant oil recovery occurs when there are large changes in the capillary number; estimates suggest that changes of 1000-fold in the capillary number are needed for substantial oil recovery [32]. Since large changes in viscous forces are only possible for the recovery of heavy oil, the reduction in interfacial tension by surfactants seems to be one of the few ways to achieve such a large change in capillary number needed to recover lighter oils. Chemical flooding techniques have very high microscopic displacement efficiencies in laboratory studies [33], but economics and other concerns have prevented widespread use of these technologies. Microorganisms make a variety of biosurfactants [34], but it has been believed that biosurfactant would not be able to generate the ultra-low interfacial tensions required for economic oil recovery and that it would not be possible to turn-on biosurfactant production in the reservoir. However, recent studies have shown that one group of biosurfactants can generate ultra-low interfacial tensions [35, 36] and the rapid advances in regulation of biosurfactant production suggests that it may be possible to control biosurfactant production in the reservoir.

The efficiency of oil recovery is often dominated by the volumetric sweep efficiency [30]. One factor resulting in poor volumetric sweep efficiencies is the difference between the mobilities of the oil and aqueous phases. Compared to oil, water moves more rapidly through the reservoir. This results in an irregular front with water pushing through the oil and reaching the production well first. The relative mobility of these two phases is expressed as the mobility ratio:

$$M = k_w \mu_o / \ k_o \mu_w$$

where M is the mobility ratio, k_w is the relative permeability of water in the water flooded zone, k_o is the relative permeability to oil in the oil saturated zone, μ_w is the viscosity of water, and μ_o is the viscosity of oil. Mobility ratios less than one are favorable and result in uniform displacement of the oil front. Polymers such as xanthan gum are used in the waterfloods to increase the viscosity of water which decreases the mobility ratio and thus and improves the sweep efficiency of the waterflood.

Another major factor resulting in poor sweep efficiency is the permeability variation in the reservoir [23, 37]. Variations in permeability are commonplace in reservoirs and dramatically affect the ultimate oil recovery. Water will preferentially flow through zones of high permeability and the oil contained in low permeability zones will not be recovered by waterflooding. Permeability profile modification is thus an important area in petroleum engineering and one where microbial processes has been successfully applied [4, 38].

It is important to note that microbial processes aimed at alleviation or prevention of well damage control will to improve the rate of oil production but not the ultimate amount of oil recovered and thus are IOR and not EOR processes. Microbial processes aimed at improving the microscopic displacement efficiency (biosurfactant production) or the sweep efficiency (microbial selective plugging) will increase the ultimate oil recovery factor and are thus EOR.

MICROBIAL TECHNOLOGIES

MEOR processes can be grouped into four main categories depending on the type of production problem to be treated and whether the process is localized to the well bore, or the near well bore environment, or the process is reservoir-wide (Knapp et al., 1990).

Well-bore clean up processes. Most of the microbial field trials can be classified as well bore clean-out or well stimulations where the purpose of the treatment is to increase the production of oil from an individual well [6, 8, 39]. Well bore clean-out processes generally involve the use of hydrocarbon or scale-removing bacteria to remove deposits from the tubing, rods, or other surfaces in the well itself. These processes have become a mature commercial technology with thousands of wells being treated on a regular basis [25, 27, 28]. Although there is still much controversy over whether oil production is improved and the mechanisms by which

this occurs, the fact that this technology has survived in the market place for many years attests to its effectiveness. Wells treated with hydrocarbon-degrading bacteria have less paraffin deposition on sucker rods and other production equipment [27] and do not require as frequent treatment with "hot oil" to maintain the drainage patterns in the well-bore vicinity [40-42]. Both of these effects greatly reduce the operating costs and extent the lifetime of economic operation of wells. There is also some circumstantial evidence that oil production from individual wells and in waterflooded reservoirs increases as a result of the action of hydrocarbon-degrading bacterial [25-28, 40, 42, 43]. These claims come from analyses of production decline curves where actual or predicted improvements in oil production are noted after microbial treatment. However, given the high variable nature of the data used in the generation of most decline curves, it is difficult to predict oil production with much certainty. While the evidence that the application of hydrocarbon-degrading bacteria substantially affects oil recovery is subject to interpretation, there is fairly good evidence that these products can result in a significant reduction in the operating costs and extend the operational life of wells.

Well stimulation technologies. In well stimulation processes, a well close to its economic limit is injected with a mixed anaerobic culture and an aqueous solution of a fermentable carbohydrate, usually molasses. The well is closed for a period of 10 to 15 days before fluid is allowed to flow. Because of its simplicity, this type of approach has been used in many early field trials [6, 44, 45]. Hitzman [6] states that these processes were most effective in carbonate wells with an API gravity of 15-30^{o}, salinity less than 100,000 ppm, and a temperature around 35 to 40^{o}C. Of the 24 wells treated by Petrogen, Inc., 75% showed an increase in well-head pressure and an increase in oil production for a period of 3 to 6 months. The Hardin-Simmons University team has treated over 80 wells and obtained pressure increases in 64 of them [45]. More than 40 of these wells showed some increase in oil production. More recently, workers in China have reported continued increases in oil production by the repeated treatment of well with fermentative bacteria and nutrients [29]. Although this is a simple technology to implement, very inconsistent results have been obtained in many field trials and there seems to be little change in oil production in sandstone reservoirs [6, 7]. The lack of technical performance and reproducibility has mostly likely limited the continued use of this technology.

More recently, Sheehy [12] developed a well stimulation process that relies on the stimulation of indigenous bacteria with inorganic nutrients rather than using injected bacteria and

a carbohydrate-based nutrient. Information on the mechanisms underlying this technology has not been made public. However, it is one of the few processes where data from untreated control wells is reported to demonstrate the feasibility of the process. After treatment, oil production from the well increased by 40% over the control well and was maintained for more than a year. Economic analysis indicated that additional oil was recovered at less than two Australian dollars per barrel. Another type of well stimulation method that has been proposed involves the use of ultramicrobacteria to plug selectively thief zones and reduce coning in heavy oil production [46].

Microbially enhanced waterflooding processes. Microbial enhanced water flooding processes are displacement processes that are done late in the life of a water flood. Nutrients with or without a bacterial innoculum are injected into the reservoir in order to stimulate microbial activity throughout the reservoir to mobilize residual oil, or to alter the flow paths of a water flood. The number of reported field trials of this type of MEOR process is much fewer than individual well treatment processes. However, several of these projects have been well documented and it is clear from the information provided that additional oil was recovered [47-50]. Analysis of two studies suggest that about one barrel of oil was recovered for every gallon of molasses used [47, 48], which would indicate that microbial enhanced water flooding is economically feasible.

Fermentative microbially enhanced waterflooding processes. Analogous to well stimulation technologies, fermentative bacteria and carbohydrate-based nutrient usually molasses is injected deep into the reservoir rather than near the well. Fluid injection is stopped to provide time for the microbes to grow and metabolism of the injected nutrients, mainly to acids, solvents and gases. In many of the field trials, the increase in oil production occurred after an increase in microbial activity suggesting a casual relationship. This technology seems to be most effective in carbonate formations and carbonaceous formations, with additional oil production lasting for periods from months to years [6, 7, 51]. In carbonate formations, the production of organic acids would alter the pore structure of the matrix by dissolution of the carbonate materials. Many MEOR field trials have reported a lowering in the pH of the reservoir by 1-2 units [50, 52, 53] which shows that sufficient amounts of microbial acids can be produced in the reservoir. The production of carbon dioxide, either through microbial fermentation or from the dissolution of the rock matrix, would act to repressurize the reservoir, reduce oil viscosity, and possibly aid in the dissolution of the rock matrix. Engineering calculations suggest that it is unlikely that

sufficient amounts of carbon dioxide can be produced to alter the pressure of a reservoir [54, 55]. However, many field trials provide ample evidence that microbial trials does increase carbon dioxide production and decrease oil viscosity. A field project in the Romashkino reservoir, Bashkir horizon of the Tatarien oilfield showed a 100 % increase in the recovery of oil from the reservoir [49]. This was done in a carbonate field so perhaps the acid produced with the carbonate to produce carbon dioxide.

The microbial production of solvents (butanol, acetone and isopropanol) may also have a beneficial affect on the oil recovery since these oil compounds are miscible in crude oil and may decrease its viscosity and improve mobility. Davidson and Russell [56] showed that large amounts of butanol were generated after the injection of fermentative anaerobes and carbohydrate-based nutrients. Thus, it is possible to generate large amounts of a microbial metabolite in an oil reservoir. However, no improvement in oil production was observed suggesting that solvent production alone may not be a viable approach for MEOR. With many studies, the lack of detailed information on the operation of the oil field makes it impossible to determine whether other factors influenced oil recovery. Such is the case for the 16-well treatment at Last Chance, Colorado [57] or with several field trials in Russia where the cessation of oil production during the six month incubation period was not taken into consideration [58].

One of the best-documented field trials was done by Mobile Oil Company in 1954 in the Upper Cretaceous Nacatoch Formation in Union County, Arkansas [50]. *Clostridium acetobutylicium* was injected and then fed a 2% beet molasses. The rate of oil production increased by 250 %. Unfortunately, the reservoir had been extensively water flooded and the residual oil saturation was very low, 4.5 to 8.5%. Thus, the total amount of oil recovered was low. Successful field trials have also been reported in carbonaceous sandstone reservoirs in Romania [53], Hungary [6], and Germany [49]. Lazar et al. [53] report incremental costs less than three dollars per barrel of oil which shows that not only was additional oil recovered as a consequence of microbial activity, but that the process was economical.

Another field test conducted in the Fuyu Oilfield in China [59] showed that gas and acid production might have a beneficial effect on oil recovery. But the lack of stoichiometric information makes this study difficult to interpret.

Microbial control of hydrogen sulfide production. The prevention of souring and corrosion caused by elevated levels of H_2S is a major concern in the petroleum industry. Most of

the efforts to date involve the use of biocides to control the activity of sulfate-reducing bacteria. This approach assumes that the sulfide is biologically produced which may not always be the case. Several alternative biological processes to control sulfide production have been developed. In the first [60], nitrate is added to the brines as the terminal electron acceptor. When nitrate is present, denitrifying bacteria will out compete sulfate-reducing bacteria for organic electron donors and the flow of electrons will shift from sulfate reduction to nitrate reduction. Also, if nitrate levels are high, then denitrifiers will produce nitrous oxide, which will increase the oxidation/reduction potential of the environment and inhibit or kill the growth of the strictly anaerobic sulfate-reducing bacteria. Reisel et al. [61] found that nitrate addition to sand columns containing an active population of thermophilic sulfate-reducing bacteria resulted in the accumulation of nitrite which inhibited the further production of sulfide. The second process [62] involves the use of a sulfide-resistant strain of *Thiobacillus denitrificans* that can oxidize the sulfide to sulfate by reducing nitrate to ammonium. Laboratory studies showed that the sulfide-resistant strain of *T. denitrificans* prevented sulfide accumulation by sulfate-reducing bacteria in liquid cultures and in sandstone and carbonaceous sandstone cores [62, 63]. Telang et al. [64] found that the injection of ammonium nitrate (400 mg l^{-1}) and sodium phosphate (12 mg l^{-1}) at an average flow rate of 38 liters day^{-1} for fifty days reduced the sulfide concentration in two production wells to less that 25% of the pretreatment values. Reverse sample genome probing showed that a sulfide-oxidizing, nitrate-reducing bacterium, strain CVO, became the dominant member of the microbial community immediately after nutrient injection. This study shows that it is possible to enhance selectively a specific microbial activity if the ecological conditions that control the activity are understood. A third process involves the addition of nitrate along with inhibitors of sulfate reducers (molybdate and nitrite) to not only inhibit sulfide production but also to stimulate oil recovery [39]. Results of the first field trials show promising results with this technology.

Permeability profile modification or selective plugging. The growth of microorganisms in high permeability zones of the reservoir or in the dormant channel will effectively reduce the mobility of water in these regions and divert the water into regions of the reservoir with higher oil saturations. McInerney et al. [65] devised a simple and elegant process using indigenous microorganisms that already exist in the reservoir. The basis of the selectivity is that most permeable regions are those that will receive a greater proportion of the nutrients since these

regions receive most flow. Thus, most of the microbial growth is will be in the more permeable regions. Since the process does not depend on the production of a specific chemical or the growth of a specific bacterium, it is applicable in any reservoir where an improvement of the volumetric sweep efficiency is required.

Studies have shown that the in situ growth of bacteria in sandstone cores or other reservoir model systems can lead to significant reductions in permeability [46, 66-72]. Raiders et al. [72] showed that the preferential plugging of the high permeability regions by microbial growth diverted fluid into the low permeability zones and increases oil recovery from these regions. A recent field trial of this process showed that in situ growth of microorganism can block a major channel and reduce permeability variations in the reservoir [38]. The recovery of residual oil was also observed in this field trial. Several other microbial processes to improve sweep efficiency of water floods have been developed [33, 68, 73, 74]. Some of these processes have not generated the permeability reductions required for significant flow diversion or oil recovery.

A recent field trial of a microbial plugging involved the injection of nitrate and phosphate into portions of an oil field undergoing waterflooding [4]. This process relies on the stimulation of indigenous hydrocarbon-degrading bacteria in the reservoir by the addition of nitrate and phosphate. Eleven of the fifteen production wells that received nutrients had an increase oil production while only two production wells of the control patterns that did not receive nutrients showed an increase in oil production. Most of the production wells in the control patterns exhibited their normal decline in oil production or were shut-in because of low oil production during the test. The production of oil with a different composition in the treated wells compared to control wells verified that incremental oil was recovered. The authors estimate that this process can increase the life of a reservoir by a period of five to twelve years and result in additional oil recovery of about 69,000 bbl in the first 42 months of the project. A field test in the Fuyu oil field in China [59] has shown improved recovery and it has been suggested that one of the recovery mechanisms was biomass production in the reservoir that altered the flow paths and improved the sweep efficiency of the reservoir.

Biosurfactant enhanced waterflooding. Many microorganisms are known to produce biosurfactants [34], but only a few are known to significantly reduce the interfacial tension between oil and brine to the level needed for substantial oil recovery [35, 36, 75]. Many

biosurfactant-producing microorganisms are aerobic bacteria making them unsuitable for in situ applications since most oil reservoirs are usually devoid of any oxygen. Moreover, the effectiveness of many of the biosurfactants produced by these organisms in high salt environments typical of the mid-continent region of the United States has not been determined. Several anaerobic bacteria are known to produce biosurfactants but the degree of surface tension reduction is much less than that reported for other organisms [76-80]. This is not the case for lipopeptide surfactants produced by several species of *Bacillus*, especially the lipopeptide produced by *Bacillus* strain JF-2.

Bacillus strain JF-2 produces a salt tolerant, heat stable, lipopeptide surfactant that reduces interfacial tension of oil brine mixtures to less than 0.01 mN/m [35, 36, 81, 82]. Interfacial tensions as low as 0.006 mN/m have been observed with as little as 10 to 25 mg/l of the lipopeptide in the absence of a cosurfactant. *Bacillus* strain JF-2 grows and produces its biosurfactant anaerobically, at salt concentrations up to 8% NaCl, and temperatures up to 45^0 C [35, 83, 84]. Thus, this organism is able to grow and produce its biosurfactant under the conditions found in many reservoirs in the United States, making it an ideal candidate for MEOR. Laboratory studies have shown that oil is recovered when strain JF-2 or related strains are grown in sandstone cores [85, 86]. Cumulative oil recovery from the sandstone cores was highly correlated to cumulative surfactant oil production (r^2 = 0.979) and very little oil was recovered from cores that were inoculated with strains from a mutant strain of JF-2 that had lost the ability to reduce surface tension [85]. However the amount of residual oil recovered was highly variable. However, the results of laboratory core flood experiments show that, while residual oil is recovered by the in situ biosurfactant production, oil recoveries were low [81, 85, 86]. However, residual oil recoveries ranging from 14 to 39% have been reported when using biosurfactant-producing *Bacillus* strains [87]. Lin et al. [35] found that biosurfactant production depended on the growth rate of the organism and the dissolved oxygen tension. The lack of appropriate control of the environmental factors needed for biosurfactant production may explain the poor reproducibility of oil recoveries in core experiments. If such problems can be overcome, biosurfactant-based technologies probably hold the greatest potential for enhanced oil recovery of any of the microbial processes [54, 55].

ENGINEERING ANALYSIS

For a proper application of MEOR in the field a thorough understanding of reservoir engineering is needed. Since MEOR is affected by many factors, it is important that each of these factors be considered. Bryant and Lockhart [11] pointed out to a number of factors that can adversely affect the effectiveness of microbial treatment. They include microbial reaction rate kinetics, adsorption losses, flow rates, nutrient concentration, source of nutrients and finally whether the microbes are exogenous or indigenous. Reaction and adsorption kinetics suggest that the concentration of nutrient would dissipate as the nutrients travel through the reservoir due to microbial consumption and adsoprtion to the rock matrix [11]. Within a short distance from the injection point, the concentration of nutrients is predicted be below that needed to support growth or elicit sufficient product formation. However, the results of several field trials suggest that the sugars found in molasses and phosphate can travel considerable distances in oil reservoirs [4, 38]. This indicates the microbial activity and adsoprtion phenomena may be lower than predicted from laboratory studies.

An important question is whether a microbial metabolite such as a biosurfactant can be produced in sufficient amounts to enhance oil recovery [11]. Laboratory experiments [81, 82, 85, 86] show that biosurfactant producing strains of *Bacillus* do recover residual oil, but oil recoveries are low (about 15% of the residual oil saturation). The authors speculate that low concentrations of surfactant and adsorption losses may be the cause of the low oil recoveries. Further research on the factors that control biosurfactant production may lead to nutrient formulations that can stimulate biosurfactant production. However, whether these processes will be able to stimulate the production of a specific metabolite such as a biosurfactant in the presence of a diverse natural population of microorganisms that inhabit oil reservoirs remains to be determined. Secondly, not all reservoirs may contain indigenous populations of biosurfactant producers. Whether the appropriate organisms can be injected into oil reservoirs in sufficient concentrations and contact a sufficient fraction of the reservoir matrix will be the major constraint that may prevent the application of microbially mediated oil recovery [11].

While microbial processes show great promise as cost-effective technologies for oil recovery, there are still a number of questions concerning the technical feasibility, the mechanisms for oil recovery, and stoichiometry and kinetics of in situ microbial processes that

prevent the widespread application of these technologies for oil recovery. Critical information concerning the rates and stoichiometries of product formation and growth in porous media are needed so reservoir simulations can be conducted to estimate oil recoveries and predict economic returns. Until this information is obtained, practicing reservoir engineers will view these technologies with considerable skepticism.

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CHAPTER 2. IDENTIFICATION AND ANALYSIS OF BIOSURFACTANT GENES IN *BACILLUS MOJAVENSIS* STRAIN JF-2

ABSTRACT

A molecular biology study of the biosurfactant-producing *Bacillus* strain JF-2 was carried out to gain a better understanding of its metabolism for MEOR purposes. From 16S rRNA sequence analysis and DNA-DNA similarity, strain JF-2 was found to be most closely related to *Bacillus mojavensis*, and therefore is redesignated as a member of this species, and not of species *Bacillus licheniformis* to which it had previously been assigned. DNA probes from the surfactin synthesis and production genes (*srfA* and *sfp*) of *Bacillus subtilis*, designated SrfA1 and Sfp1, were designed and used to isolate genes related to biosurfactant production in strain JF-2. We isolated more than 50 recombinants encoding the presumed genes for biosurfactant production from strain JF-2. A cosmid clone of 40 kb containing the putative surfactant synthetase cluster was subjected to sequence analysis. To date, about 40% (15kb) of the cluster has been satisfactorily sequenced. Analysis of the assembled partial sequences yielded some useful information. A portion (*ca*. 3.6kb) of the *B. mojavensis* cosmid sequence exhibited highest homology (>80% identity) with the *B. subtilis srfA-sfp* region of surfactin synthetase. Another portion of the deduced amino acid sequences of the JF-2 gene products located distal to the *srfA-sfp* region showed considerable identity with the *B. subtilis* YckE-TlpC gene sequence (*ca*. 93% identity). Four open reading frames in this region of the *B. mojavensis* cosmid sequence correspond to proteins involved in membrane functions and carbohydrate metabolism. This analysis may be deemed to confirm the role of the isolated *B. mojavensis* genes in biosynthesis or secretion of the surfactant. Together with further sequence information, our new understanding of the phylogenetic status of JF-2, and our physiological studies (later chapters), should be exploitable in the manipulation of growth and biosurfactant production in the field, as well to monitor the progress of MEOR processes as they are applied in the reservoir.

INTRODUCTION

Biosurfactants are surface-active compounds produced by a variety of microorganisms. Their broad range of potential industrial applications includes enhanced oil recovery and bioremediation of pollutants. Biosurfactants are being investigated as replacements for synthetic

surfactants in enhanced oil recovery because they are easily biodegradable, more active under the extreme conditions of some subsurface environments, and can be produced *in situ* from renewable substrates. Biosurfactants are amphiphilic compounds produced on cell surfaces, or excreted extracellularly, and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual water molecules at the surface and interface between water and oil, respectively.

Among the many classes of biosurfactants, lipopeptides are of interest because of their high surface activities. Lipopeptides consist of a short cyclic peptide (3-12 amino acids) attached to a lipid moiety. Many strains of *Bacillus* are known to produce lipopeptides with remarkable surface-active properties. The most studied of these lipopeptides is surfactin from *B. subtilis* (Sullivan, 1998). Surfactin is an acylated cyclic heptapeptide that reduces the surface tension of water from 72 to 27 mN m^{-1} even at concentrations below 0.05%. Chapter 1 of this report provides a review of the potential utility of microbial biosurfactants in MEOR.

B. mojavensis strain JF-2 (ATCC 39307), isolated from oil well produced waters, has been shown to produce a surface-active lipopeptide under aerobic, microaerophilic and anaerobic conditions (McInerney *et al.*, 1990; Lin *et al.*, 1994). This biosurfactant is of interest for a variety of applications, particularly for microbially-enhanced oil recovery (Yakimov *et al.*, 1997). The lipopeptide surfactant produced by *B. mojavensis* strain JF-2 is one of the few biosurfactants that can generate ultra-low interfacial tensions needed for enhanced oil recovery. Also, the organism can grow can grow at salt concentrations up to 8% and temperatures up to 50°C which are conditions found in many reservoirs in Oklahoma. In Chapters 3 and 4 of this report, studies are presented which show that certain nutrients (e.g. choice of carbon source, addition of nitrate, and a peptide source) can be provided in the culture medium to maximize both growth of *B. mojavensis* and production of biomass.

The current knowledge concerning the genetics of biosurfactant production and its regulation by *B. mojavensis* strain JF-2 is limited. Our central aims in this portion of the work was to use molecular genetic tools: 1) to obtain a better understanding of lipopeptide biosynthesis and its regulation; and 2) to monitor the surfactant-producing microbial populations as MEOR proceeds. This information can then be used to improve the production of the biosurfactant in order to improve oil recovery . Here, we describe the cloning and partial

nucleotide sequence of genes involved in biosurfactant production in *B. mojavensis* strain JF-2. We also report the phylogenetic status of this organism.

MATERIALS AND METHODS

Bacterial strains, culture conditions. *Bacillus mojavensis* JF-2 (formerly *Bacillus licheniformis* ATCC39307), *Bacillus mojavensis* ATCC51516^T, *Bacillus subtilis* ATCC6051^T, *Bacillus licheniformis* ATCC14580^T, *Bacillus subtilis* ATCC21332, *Bacillus* sp. 018 , *Escherichia coli* EPI405 (Epicentre Technologies, Madison, WI) and *Escherichia coli* DH10B (Life Technologies, Rockville, MD) were used in this study. All the strains were routinely grown in Luria Bertani (LB) Nutrient Broth (NB) medium at 37°C. All procedures were carried out with Aerosol Resistant Tips (Molecular Bio-Products) to prevent any DNA contamination from the chambers of the pipeter.

DNA extraction. Total DNA from *Bacillus* strains was obtained by lysozyme treatment and phenol-chloroform extraction. *Bacillus* strains were cultured on LB medium for overnight at 37°C in shaker (200 rpm) and 40 ml of the culture was centrifuged for 5 min at 12,000 x *g*. The cell pellet was resuspend in 10 ml of 50 mM Tris (pH 8.0) and centrifuged again. To the resuspended sample, 0.4 ml of 0.4 M EDTA was added and the sample was incubated for 20 min at 37ºC. Next, a freshly prepared solution of lysozyme (0.4 ml of 20 mg/ml) was added and the sample was incubated for 10-20 min at 37ºC. Proteinase K (to final concentration of 100 µg/ml) and sodium dodecyl sulfate (SDS, to final concentration 0.05%) were then added and the sample was incubated at 50ºC for at least 1 h until clear. RNase (10 µl of 10 mg/ml) was added and the sample was then incubated for 15 min at 65ºC. Hot phenol (5 ml) was added and the sample was centrifuged (4°C, 20 min, 12,000 x *g*). The top aqueous phase was transferred with a wide-bore pipette tip to another tube and the remaining phenol layer was discarded. Five milliliters of a solution of phenol:chloroform:isoamyl alcohol (25:24:1; v:v:v) were added to the aqueous fractions and mixed gently for 1 min. The mixture was centrifuged at 4°C for 20 min at 12,000 x *g*. The top aqueous phase was removed with a wide-bore pipette tip. Five milliliters of chloroform:isoamyl alcohol (24:1) were added to the sample and this suspension was centrifuged for 10 min at 4°C. The top aqueous phase was removed to a new tube. Two volumes of cold 100% ethanol were added to the aqueous phase and the tube was inverted slowly to precipitate DNA. DNA was spooled and rinsed in 70% ethanol. Ethanol was drained from the

DNA and the DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA). The dissolved DNA was heated at 65ºC for 10 min with the lid open to evaporate ethanol.

16S rRNA gene amplification, sequencing and phylogenetic analysis. PCR amplification of the 16S rRNA gene of *Bacillus* strains was performed with a GeneAmp PCR system 2400 as described previously (Sambrook *et al.*, 1989). The complete sequence of the 16S rRNA gene was obtained by using internal 16S rRNA oligonucleotide sequencing primers u27f(5'-AGAGTTTGATCCTGGCTCAG) and u1495r(5'-TACGGCTACCTTGTTACGAC)and an automated sequencer (Perkin Elmer). Sequencing was performed by the University of Oklahoma Health Sciences Center Molecular Biology Service Facility. Sequences were provided as electronic data files.

DNA-DNA hybridization. DNA-DNA hybridization was performed with ^{32}P-CTP-labelled genomic DNAs of strain JF-2 by using the S1 nuclease method at a reannealing temperature of 55°C (Johnson, 1994). DNA similarities of strain JF-2 were estimated by comparison with DNAs of *B. mojavensis* ^{T}, *B. subtilis* ^{T}, *B. licheniformis* ^{T} and *Bacillus* sp. 018.

Genomic library construction, cloning and sequencing. After cloning of *B. mojavensis* strain JF-2 genomic DNA into cosmids, more than two thousand cosmid clones were constructed by the recombinant DNA procedures described below. First, genomic DNA was randomly sheared by passing it through a syringe needle. Next, the ends of the sheared DNA were repaired by using standard reagents (The pWEB::TNC™ Deletion Cosmid Cloning Kit, Epicentre Technologies), and then fragments of about 40 kb were selected by comparison of the size of the cosmids with a 40 kb standard on agarose gel. Finally, the size-selected DNA was ligated into the linearized and dephosphorylated Vector, packaged using Lambda Packaging Extracts, and plated onto the EPI305/100™ cells. From the gene libraries containing 40-kb fragments of *B. mojavensis* strain JF-2 DNA, more than fifty positive colonies were identified by colony hybridization with digoxigenin-dUTP labelled oligonucleotides SrfA1 and Sfp1 described below.

The specific primers were designed from known sequences of the *B. subtilis* surfactin synthetase/competence gene *srfA* (Nakano *et al.*, 1991) and surfactin production gene *sfp* (Cosmina *et al.*, 1993; Nakano *et al.*, 1992). These primers allow amplification of conserved regions in *srf*A and *sfp* genes of *B. subtilis*. A schematic of the organization of these genes is presented in Fig. 2.3. For oligonucleotides SrfA1, the forward primer (5-GCGGTAGAAAAACTGCTTGC-3) corresponds to the region from 394276 bp to 394295 bp of *B.*

subtilis genome, and the reverse primer (5-ACAGGTTCGTCTGCTTTGCT-3) corresponds to region from 394506 bp to 394525 bp of *B. subtilis* genome. For oligonucleotides Sfp1, the forward primer (5-CACCTGAAAAACGGGAGAAA-3) corresponds to region from 407627 bp to 407646 bp of *B. subtilis* genome, and the reverse primer (5-TGTGAATCAAACGCACCAAT-3) corresponds to the region from 407415 bp to 407434 bp of *B. subtilis* genome (A-1).

Once the primary cosmid libraries were obtained, a set of randomly-nested deletion clones was generated from the primary positive cosmid clones by incubating each cosmid clone with EZ::TN™ Transposase and transforming competent *E. coli* with the modified cosmid DNA. Successful transposition reactions which generated deletions or inversions resulted in the loss of the chloramphenicol resistance gene (Chl^R) from the pWEB::TNC cosmid. Transposition clones were ampicillin resistant (Amp^R) and chloramphenicol sensitive (Chl^S). The primary parental pWEB::TNC cosmid clones were Amp^R/Chl^R that had not undergone the loss of the chloramphenicol resistant gene. Size screening was then accomplished by preparing lysate cosmid DNA directly from Amp^R/Chl^S colonies and then sizing the cosmids by agarose gel electrophoresis. Deletion clones migrated with a range of sizes (1 to 44.1 kb) depending on the size of the deletion. Based on agarose gel results, deletion clones were chosen that provided sufficient sequence overlap to allow assembly of the DNA sequence of the cloned DNA insert. The nucleotide sequences of the cloned fragments were determined by the dideoxy chain termination method with fluorescently-labeled nucleotides. The more than four hundred chosen randomly-nested deletion clones derived from the selective primary clones were sequenced using the pWEB::TNC sequencing primers. Thirty-eight of the primary clones were also sequenced using the M13 Forward Primer and T7 Promoter Primer. Each successful DNA sequence reaction yielded about 600 nucleotides of readable sequence. Sequence data were provided as electronic files from the OUHSC facility.

Using assembled sequences, which covered a portion of the cosmid (see results), a search for homologies with other known sequences was carried out. The BLAST program was used to compare the sequences with the genes present in the GenBank database; multiple alignments of DNA sequences were done with CLUSTAL W. Analyses and assembly of the nucleotide sequences of the clones were performed with Vector NTI Suite computer programs (InforMax).

RESULTS AND DISCUSSION

Reclassification of *B. licheniformis* ATCC 39307 (JF-2) as *B. mojavensis.* In 1983, Jenneman et al. described surfactant-producing strain JF-2 as a member of the species *Bacillus licheniformis* based on a limited set of characteristics, including anaerobic growth and certain other metabolic tests. Because of the variety of biosurfactant-producing *Bacillus* species, molecular biological techniques were employed to determine more fully the relationship of strain JF-2 to other strains.

SSCP analysis of 16S rRNA genes of Bacillus *species.* First, the PCR-amplified 16S rRNA genes of strain JF-2 and four comparator species were analyzed by single strand conformation polymorphism (SSCP). This technique uses restriction fragment pattern analysis to qualitatively compare the relatedness of strains, (similar to the tests forensic analysts do with human DNA samples). To obtain fragments of the appropriate length for analysis, the amplified fragments were digested with the restriction enzymes *Alu*I, *Hinf*I and *Mbo*I. In Figure 2.1, no apparent differences were observed in the band patterns of the five strains after digestion with with *Hinf*I and *Mbo*I, which suggested that JF-2 is a member of the genus *Bacillus*. However, the restriction patterns with *Alu*I in lanes 1, 2, and 4, respectively, grouped three strains together (JF-2, *B. mojavensis, B. subtilis*) and two strains together (*Bacillus licheniformis*, *Bacillus* sp. SP018; Lanes 3 and 5) (Fig. 2.1).

16S rRNA gene sequence analysis and phylogenetic analysis. The nearly complete sequence of the 16S rRNA of strain JF-2 (1529 bp) was determined and compared to sequences of *B. mojavensis* (1526 bp), *B. licheniformis* (>1466 bp), *B. subtilis* (>1419 bp), and other bacteria (>1409 bp). The 1529 bp sequence from JF-2 is given in Appendix 2-I. The results of the comparisons of the rRNA sequences are presented in Figure 2.2. The 16S rRNA sequence of strain JF-2 and B. *mojavensis* (strain ATCC 51516; GenBank Accession No. AB021191) were nearly identical (similarity value of 99.9%). The similarity between JF-2 and B. *subtilis* ATCC 6633 (AB018486) was 99.7%, while between JF-2 and *B. licheniformis* (AY017347) was 98.0%. Another, more intuitive way to consider these data is based on total number of nucleotide changes between strains. There were two nucleotide differences in the approximately 1500 positions compared between JF-2 and *B. mojavensis*, and about 30 differences between JF-2 and *B. licheniformis*. Between the strains of the diverse species *B. subtilis* and JF-2 there ranged were from about four changes to about ten.

As noted above, strain JF-2 was originally described as a strain of *B. licheniformis* (Jenneman *et al.,* 1983). This study, however, reveals higher ribosomal DNA homology between JF-2 and *B. mojavensis* than JF-2 and *B. licheniformis*, which is consistent with the results of SSCP analysis of the 16S rRNA genes.

DNA-DNA hybridization. The genomic DNA homology values of strain JF-2 with *Bacillus* strains, obtained by the S1-nuclease DNA hybridization method are listed in Table 2.1. Strain JF-2 exhibited about 75% DNA homology with *B. mojavensis* ^{T} and 35% genomic DNA homology with *B. subtilis* ^{T}, but it had significantly less DNA homology with *B. licheniformis* ^{T} (10%).

Reassignment of strain JF-2 to the species Bacillus mojavensis. In summary, on the basis of restriction analysis of 16S rDNA, sequence analysis of 16S rRNA, and DNA-DNA hybridization with closely-related comparator species, it can be concluded that strain JF-2, previously named *B. licheniformis* ATCC strain 39307, should be reclassified as a member of the species *B. mojavensis.* This phylogenetic reassignment of strain JF-2 may have implications in its employment in MEOR. First, the strain is a natural isolate from oil reservoir produced waters. One would assume that it was best-adapted to subsurface conditions (saline, temperature, *etc*.) in an Oklahoma reservoir. However, much less is known about the physiology, genetics, and molecular biology of *Bacillus mojavensis* than is known about *B. subtilis* or *B. licheniformis*. Other investigators' results with other *B. licheniformis* strains may not be directly applicable to *B. mojavensis* strain JF-2, and *vice versa*. Two, the fact that *B. mojavensis* is a closer relative to *B. subtilis* than to *B. licheniformis* (see Fig. 2.2 and Table 2.1) means that the *B. subtilis* biosurfactant surfactin system may be the best model for the JF-2 biosurfactant. Much data is available on the surfactin system (Sullivan, 1998). As the following sections show, this data was quite useful in successfully cloning the *B. mojavensis* JF-2 biosurfactant synthesis genes. It is also to be hoped that the huge body of information on *B. subtilis* nutrition, physiology, sporulation, and molecular biology can be exploited in the JF-2 system to maximize production of its biosurfactant.

Design and specificity of the probes for biosurfactant production genes. The most thoroughly studied biosurfactant system from a molecular biology standpoint is that of *Bacillus subtilis* (whose entire genome has been sequenced; Kunst et al., 1997). The lichenysin A biosurfactant system of *Bacillus licheniformis* strain BNP20 has also been studied, and exhibits

some sequence homology with the surfactin synthetase genes of *B. subtilis*. Figure 2.3 shows the chromosomal location of the *B. subtilis* surfactin synthetase region as well as an expanded diagram of these genes. The figure also shows a diagram of the *B. licheniformis* lichenysin A region. The homology in location and transcriptional direction is noteworthy for the genes *srfAC* and *lchAC*; *srfAB* and *lchAB;* and *srfAA* and *lchAA*, from *B. subtilis* and *B. licheniformis*, respectively.

B. subtilis was chosen as sequence model for the design of molecular probes to detect *B. mojavensis* JF-2 genes. This choice was supported by the newly-recognized phylogeny of *B. mojavensis* JF-2, which was more closely related to *B. subtilis* than to the species *B. licheniformis* to which it had been previously assigned (see above, and Fig. 2.2 and Table 2.1).

Probes for identifying the biosurfactant production genes were designed for two conserved regions of the biosurfactant operon (Fig. 2.3). Then the protein sequences of the probes SrfA1 and Sfp1 were aligned *in silico* with sequences from other bacteria. The specificity of these probes for biosurfactant is confirmed by the results shown in Fig. 2.4. Comparative sequence analysis revealed that the most specific target site of SrfA1 was the surfactin synthetase gene (100% identity, as expected)(Fig. 2.4). Homologies of the probe sequences with related genes, while significant, were much lower than for surfactin synthetase. Identity values for the probes compared to some antibiotic genes were about 50%: with gramicidin S synthetase (57% identity), lichenysin synthetase (54%) and peptide synthetase (42 to 54%) (Fig. 2.4). The probe Sfp1 also showed high amino acid homology with the surfactin production site (100%) but not to the same extent with other potential lipopeptide synthetases (Fig. 2.4). The next highest homology (69%) observed was between Sfp1 and the Iturin A antibiotic-producing genes from *B. subtilis*.

The PCR product from strain JF-2 DNA amplified with SrfA1 and Sfp was labeled with a digoxigenin (DIG) (Roche Molecular Biochemicals) tag to permit chemiluminescent detection of hybrids. The probes (SrfA1 and Sfp1) derived from the surfactin synthetase gene of *B. subtilis srfA-sfp* were then used to screen a JF-2 genomic DNA library for fragments containing the biosurfactant gene sequence. The specificity of the oligonucleotide probes was evaluated by hybridization techniques. First of all, dot blots were used for the qualitative screening of target DNA in *B. mojavensis* JF-2 compared to *B. subtilis*. Similar dot intensities were detected with two bacterial genomic DNAs and both probes (SrfA1 and Sfp1). When more genomic DNA was

used, higher intensities were generated (Fig. 2.5). However, no hybridization was observed when *E. coli* (negative control) was used (data not shown).

Southern blot analysis of digested chromosomal DNA of *B. mojavensis* JF-2 also revealed fragments that strongly hybridized with labelled oligonucleotide probes SrfA1 and Sfp1 (Fig. 2.6). The banding intensities and patterns of hybridization for all seven restriction enzymes and two partial digests indicate the presence of sequences *in B. mojavensis* that are homologous to *srf*A and *sfp* genes in *B. subtilis*, which code for the surfactin synthetase and other activities of biosurfacant production.

Partial sequence analysis of *B. mojavensis* JF-2 biosurfactant producing genes. We have isolated the fragment of the DNA containing genes that appear to be involved in biosurfactant production in *B. mojavensis* strain JF-2. By analogy with *B. subtilis* surfactant synthesis, the complete set of biosurfactant genes is quite large, over 20,000 base pairs. The size of the cosmid containing the biosurfactant genes is 40 kB. The large size and the presence of many repetitive sequences in the cosmid, have made the sequencing of this region and identification of its genes a formidable task. To date, we have sequenced about 15,000 base pairs of the 40,000 base pair fragment. The sequences that have been satisfactorily completed (that is, covered by two or more sequencing readouts, preferably one or more in each direction), do not yet cover the entire surfactant synthesis operon shown above (Fig. 2.3, bottom). The most thorough sequences cover much of the right hand portion of the operon, from *SrfAB* to the right. On the left hand portion of the cosmid, the sesquence of the region from approximately *YckE* to *TlpC* has been completed.

***SrfAB* to *sfp*.** Preliminary analysis of the sequences which cover portions of the region from *srfAB* to *sfp* was carried out. Although this analysis is being carried out rather prematurely, owing to the incomplete nature of the sequence to date, it has been informative. For example, homology searches using GenBank alignment revealed that the longest stretch of our assembled protein sequence is about 67% identical to the surfactin synthetase, SrfAB, of *Bacillus subtilis* (GenBank accession No. Q04747) (Fig. 2.7). The second-highest region of homology was with the lichenysin synthetase LicB of *Bacillus licheniformis* (47% similarity; GenBank accession no. AAD04758) (Table 2.2). Two other assembled DNA sequences in the srfAB to sfp region (of 4703 base pairs and 2557 based pairs), are 87% similar to the *srfAA* gene and 86% similar to the *srfAD* gene of *B. subtilis* (GenBank accession no. Z99105), respectively.

From the preliminary analysis of the deduced amino acid sequences (for part of the region), it is clear that the putative biosurfactant operon of JF-2, the surfactin synthetase operon of *B. subtilis* and the lichenysin operon of *B. licheniformis* code for strongly homologous peptides (Fig. 2.8 and Table 2.3). Therefore, it can be expected that the biosyntheses of these substances follow similar pathways in the three organisms. The detailed analysis of the three peptides in Table 2.3 (comparing their size, functionality of amino acids, amino acid analysis, *etc*.) again suggests that the JF-2 gene is more closely related to the *B. subtilis* gene than to the *B. licheniformis* gene. This is in conformance with the phylogenetic relationships among these species discussed above.

yckE to tlpC. We have sequenced much of a region which is in close physical proximity to the biosurfactant genes. This 4363 base pair region, upstream from the *srfA* locus contains four putative genes, identified as open reading frames (ORFs) (Fig. 2.9 and Appendix 2-II). One of these ORFs (*orf1*, 1225 bp) was found to be very similar to a gene involved in carbohydrate metabolism in *Bacillus subtilis* (93% identity), *Lactococcus lactis* (51% identity), *Fusobacterium mortiferum* (46% identity) and *Bacillus halodurans* (33% identity) (Fig. 2.10 and Table 2.4.A). The predicted gene product from the second ORF (*orf2*, 389 bp) in the opposite transcriptional direction encodes a protein of 129 amino acid residues that has a mass of 15160 m.w. (Table 2.5). Database alignments of this gene product showed 85% identity to the DNA-entry nuclease inhibitor found in the corresponding 5' flanking region of *srfAA* of the *Bacillus subtilis* (Fig 2.9 and Table 2.4.B). The third putative open reading frame (*orf3*, 435 bp, 145 aa) is 78% similar to a gene for membrane-associated nuclease in *B. subtilis* (Fig 2.9 and Table 2.4.C). The derived ORF3 protein sequence also shares homology with sporulation-specific extracellular nuclease precursor in *B. subtilis* (63% identity). The homology searches of the fourth ORF (*orf4*, 1722 bb, 574 aa) by databank alignment revealed that the amino acid sequence exhibits very high homology with a methyl-accepting chemotaxis protein (TlpC) from *B. subtilis* (75% identity) (Fig 2.10 and Table 2.4.D). However, the ORF4 sequence demonstrated very low homology with *Bacillus licheniformis* DNA sequences in the database.

The common features and uniqueness of the sequences among all sequences in GenBank indicate that strain JF-2 and *B. subtilis* share similar functions, including biosurfactant synthesis as well as the four other activities of ORFs 1 to 4. In *B. subtilis,* the membrane-associated nuclease, the extracellular nuclease, and possibly the ß-glucosidase are probably involved in

sporulation. The DNA-entry nuclease is probably involved in genetic competence, DNA transformation, and/or recombination. The chemotaxis protein might be involved in recombination, or with escape of the cell prior to or committing to sporulation. The homologies between the activities found in the biosurfactant regions of chromosomes of the two species are good evidence that they serve similar functions in each.

One hopes that the extensive knowledge about genetic control in *B. subtilis* can be utilized to control biosurfactant production in *B. mojavensis*; the completed sequence might be mined to find regulatory sequences whose control switches are known in the former organism, and then use this to regulate biosurfactant production by the latter.

CONCLUSIONS

We have described the large gene cluster around the putative biosurfactant production genes of *B. mojavensis*. We have also analyzed the amino acid sequences of the corresponding proteins and compared them with those of other proteins. Examination of the sequence similarities between *B. mojavensis* strain JF-2 and *B. subtilis* shows that these bacteria share close homology in the biosurfactant peptide synthesis gene and adjacent genes.

The study of the biosurfactant biosynthesis has suffered from the lack of well-characterized genetic systems with which to identify and isolate biosynthesis genes and to examine mutationally altered 'biosurfactant biosynthetases'. Because *B. mojavensis* strain JF-2 can be manipulated by a variety of genetic techniques, including specialized and generalized transduction, plasmid-cloning vehicles, transformation and transposon mutagenesis, this sequence information holds great promise for future work. A genetic approach will be important and for improving the production of the biosurfactant. In particular, a strategy based on the isolation of biosurfactant mutant strain followed by genetic complementation using a *B. mojavensis* strain JF-2 wild-type gene library will be used to study genes involved in biosurfactant biosynthesis. Our goals are to develop a modified biosurfactant that can be effective in oil recovery over a wide range of environmental conditions, and to increase the amount of the biosurfactant made. Such results complement physiological studies on nutrient amendments and biosurfactant production, and it will be possible to use this information to develop probes and PCR primers to monitor surfactant-producing microorganisms during MEOR field operations.

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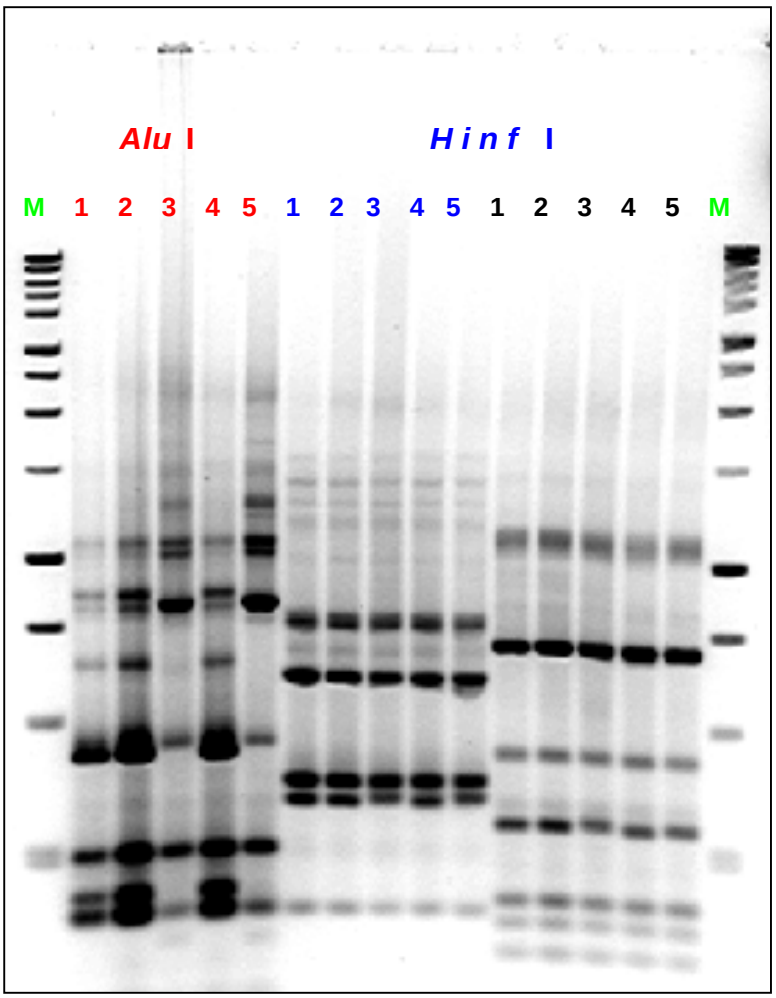


Figure 1.1. SSCP analysis of the 16s rDNA digested with *Alu*I, *Hinf*I and *Mbo*I from 5 species of *Bacillus*. Lanes: 1-5, JF-2, *B. mojavensis* ATCC51516 ^{T}, *B. licheniformis* ATCC14580^{T}, *B. subtilis* ATCC21332 ^{T}, *Bacillus* sp. SP018

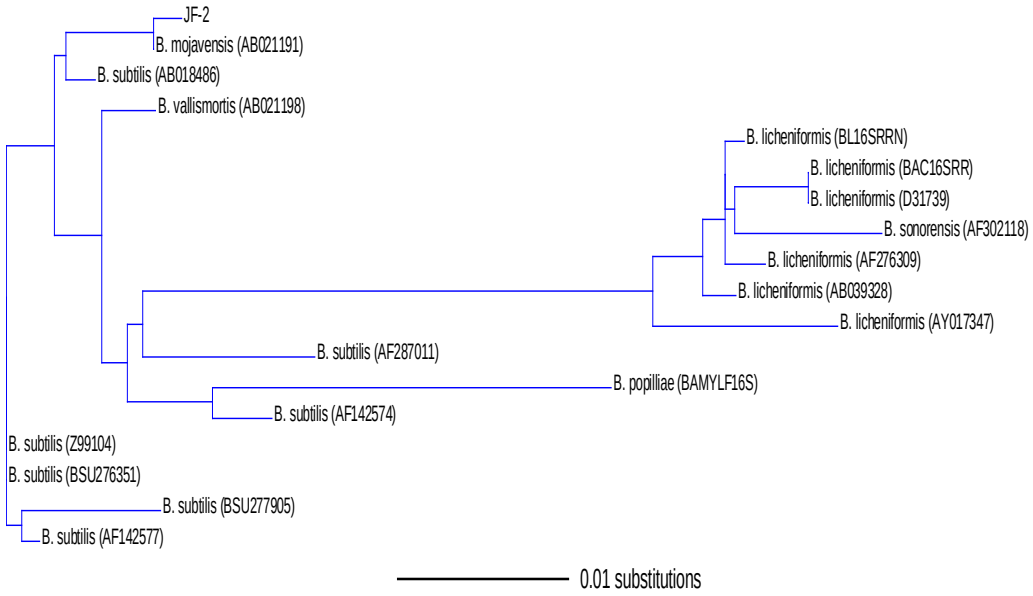


Figure 2. Phylogenetic relationship of strain JF-2 with other bacilli strains. Phylogenetic tree is based on 16S rRNA gene sequence similarities and constructed using the neighbor-joining method. GenBank accession numbers are provided for each strain.

Table 2.1. Percentage of DNA-DNA similarity between JF-2 and other bacilli strains.

Strain	% similarity
B. mojavensis ^{T}	75
B. licheniformis ^{T}	11
B. subtilis ^{T}	35
Bacillus strain 018*	27

The DNA-DNA similarity values are the means of least two determination. **Bacillus* strain 018 was obtained from Dr R. Tanner, Dept. of Botany and Microbiology, The University of Oklahoma.

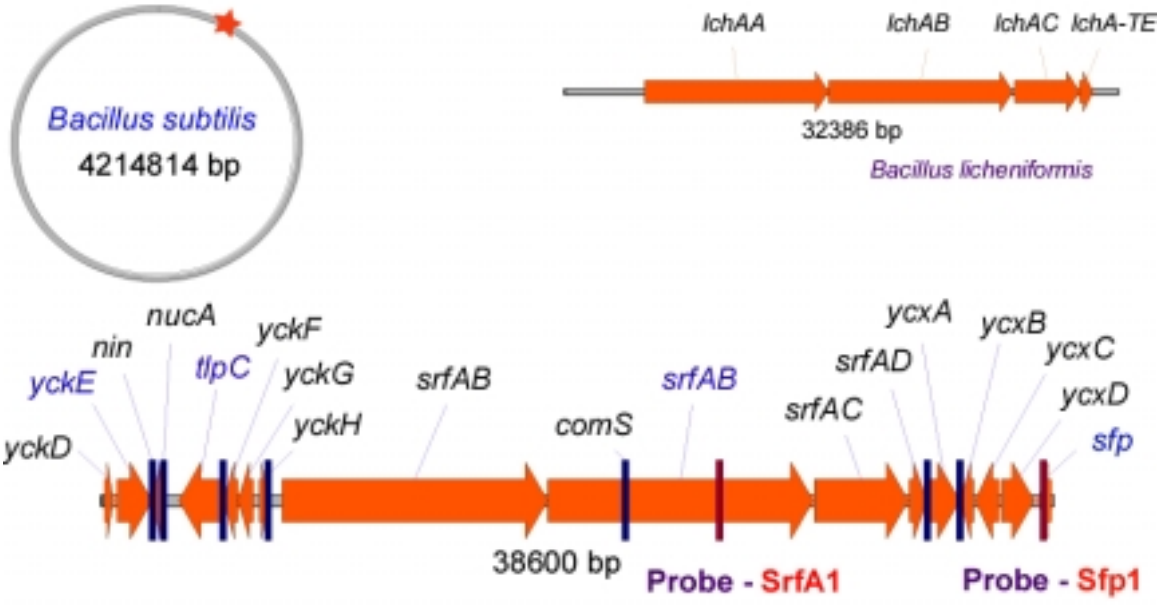
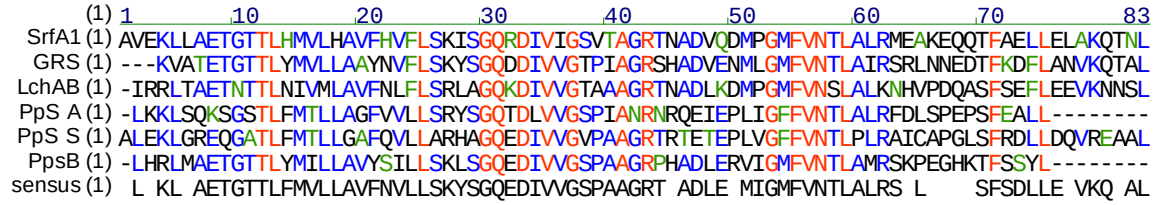
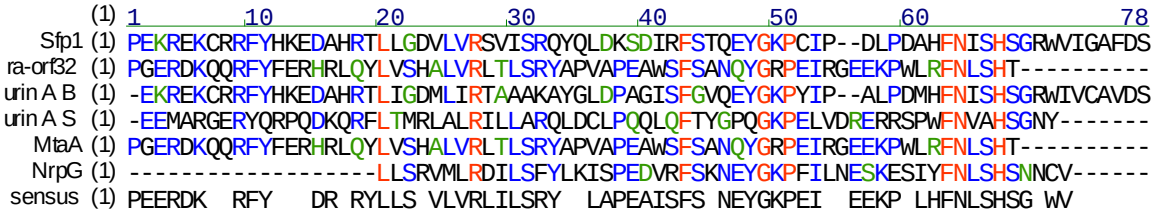


Figure 2.3. Diagram of surfactin synthetase (*srfA-sfp*) gene region of *B. subtilis* complete genome (GenBank accession number: Z99105) (Kunst *et al.*, 1997) and lichenysin A synthetase operon of *Bacillus licheniformis* BNP29 (GenBank accession number: BLAJ5061). Shown are the locations of surfactin synthetase gene of *B. subtilis srfA-sfp* and the probes (SrfA1 and Sfp1).



	organism	protein	accession #	Identities
SrfA1	*Bacillus subtilis*	surfactin synthetase	Q04747	83/83 (100%)
GRS	*Bacillus brevis*	gramicidin S synthetase	JX0340	46/80 (57%)
LchA B	*Bacillus licheniformis*	lichenysin synthetase	CAA06324	45/82 (54%)
PpS A	*Anabaena sp.*	peptide synthetase	CAC01604	33/74 (44%)
PpS S	*Streptomyces verticillus*	peptide synthetase	AF210249	35/83 (42%)
PpsB	*Bacillus subtilis*	peptide synthetase	I40457	40/74 (54%)



	organism	protein	accession #	Identities
Sfp1	*Bacillus subtilis*	surfactin production	BAA08991	76/76 (100%)
Gra	*Streptomyces violaceoruber*	granaticin biosyntheis	JX0340	20/63 (31%)
Iturin A B	*Bacillus subtilis*	lipopeptide antibiotic iturin A	CAA06324	52/75 (69%)
Iturin A S	*Synechocystis sp.*	lipopeptide antibiotic iturin A	BAA04883	18/70 (25%)
MtaA	*Stigmatella aurantiaca*	Ppant transferase	AF188287	23/68 (33%)
NrpG	*Proteus mirabilis*	biosynthetase	I40457	21/53 (39%)

Figure 2.4. Alignment of *B. subtilis* Probe SrfA1 and Sfp1 with homologous regions from other organisms. Identical/conserved amino acids are highlighted. Identities between probes (SrfA1 and Sfp1) and related organisms are shown on tables.

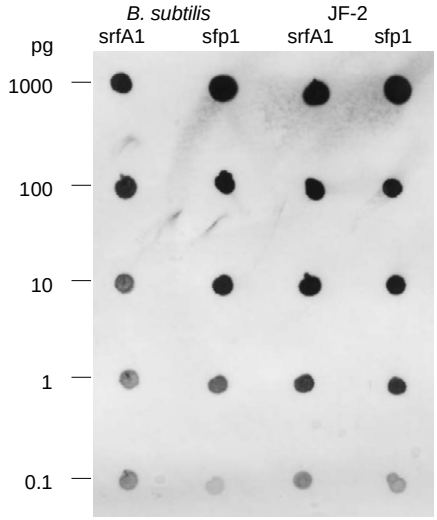


Figure 2.5. Dot blot hybridizations with DIG-labeled probes with different concentrations of DNA from JF-2 and *B. subtilis*.

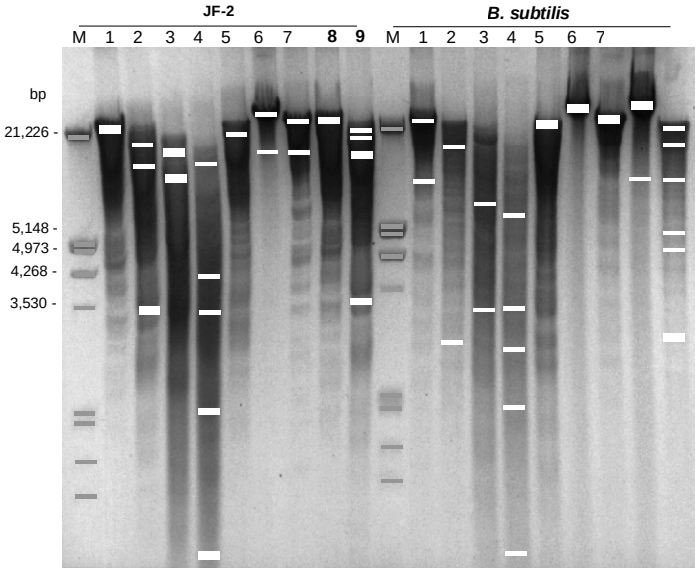


Figure 2.6. Southern blot analysis of the digested genomic DNA of JF-2 and *B. subtilis* hybridized with probe SrfA1. Lanes: 1-7, *BamH*I, *Bgl*II, *Eco*RI, *Hind*III, *Nco*I, *Sma*I and *Xho*I respectively; 8-9, partially digested *BamH*I and *Bgl*II.

Figure 2.7. Alignment of amino acid sequences deduced from JF-2 DNA assembled fragments and *B. subtilis srfAB* (accession number Z99105) (CLUSTAL X). Sequences were retrieved from GenBank. Identical and/or similar amino acids are shaded; similar amino acids are in boldface within the shaded regions.

```
                      1                                                  50
          Consensus    (1)  VAVS LRKTLSQSLPDYMVPAHLIQMDSLPLTPNGKINKKELPAPQSDA
      Query sequence   (53) KVSVSGLRKTLSQSLPDYMVPAHLIQMDSLPLTPNGKINKKELPAPQSDA
gi|3041735|sp|Q04747  (909) EVAVSELRKTLSQSLPDYMVPAHLIQMDSLPLTPNGKINKKELPAPQSEA
                      51                                                 100
          Consensus   (51) VQ EYAAPKTESE KLAEIWEGILGVKAGVTDNFFMIGGHSLKAMMLTAK
      Query sequence  (103) VQSEYAAPKTESETKLAEIWEGILGVKAGVTDNFFMIGGHSLKAMMLTAK
gi|3041735|sp|Q04747  (959) VQPEYAAPKTESEKKLAEIWEGILGVKAGVTDNFFMIGGHSLKAMMMTAK
                      101                                                150
          Consensus  (101) IQEHFHKE PIKVLFEKPTIQ              QTFEPIRQA YQQHY
      Query sequence  (153) IQEHFHKEXPIKVLFEKPTIQXXXXXXXXXXXXXXQTFEPIRQAPYQQHY
gi|3041735|sp|Q04747 (1009) IQEHFHKEVPIKVLFEKPTIQELALYLEENESKEEQTFEPIRQASYQQHY
                      151                                                200
          Consensus  (151) PVSPAQRRMYILNQLGQA TSYNV AVLLLEGEV KDRLE AIQQLI RH
      Query sequence  (203) PVSPAQRRMYILNQLGQASTSYNVRAVLLLEGEVNKDRLEHAIQQLIDRH
gi|3041735|sp|Q04747 (1059) PVSPAQRRMYILNQLGQANTSYNVPAVLLLEGEVDKDRLENAIQQLINRH
                      201                                                250
          Consensus  (201) EIL  SFDMIDG VVQTVHKNISF LEAAKGREEDAEEIIKAFVQPFELN
      Query sequence  (253) EILPPSFDMIDGKVVQTVHKNITFQLEAAKGREEDAEELIKSFVQPFELN
gi|3041735|sp|Q04747 (1109) EILRTSFDMIDGEVVQTVHKNISFHLEAAKGREEDAEEIIKAFVQPFELN
                      251                                                300
          Consensus  (251) RAPLVRSKLVQLEE RHLLLIDMHHIITDGSSTGILIGDLAKIYQGADLE
      Query sequence  (303) RAPLVRSKLVQLEEERHLLLIDMHHIITDGSSTGILIGDLAKIYQGADLE
gi|3041735|sp|Q04747 (1159) RAPLVRSKLVQLEEKRHLLLIDMHHIITDGSSTGILIGDLAKIYQGADLE
                      301                                                349
          Consensus  (301) LPQIHYKDYAVW KE   HQKDE YWLD FKGELPILDLPADF RPAER
      Query sequence  (353) LPQIHYKDYAVWQKEHADHQKDEAYWLDTFKGELPVLDLPADFPRPAER
gi|3041735|sp|Q04747 (1209) LPQIHYKDYAVWHKEQTNYQKDEEYWLDVFKGELPILDLPADFERPAER

                      1                                                  50
          Consensus    (1) VMDRSA  M  ILGVMKAGAAFLPIDPDTP ERIRYSLEDSGA F VVNE
      Query sequence    (1) VMDRSADLMTAILGVMKAGAAFLPIDPDTPEERIRYSLEDSGARFTVVNE
gi|3041735|sp|Q04747 (1552) VMDRSAEVMVSILGVMKAGAAFLPIDPDTPGERIRYSLEDSGAKFAVVNE
                      51                                                 100
          Consensus   (51) RNMTAIGQY G     D   WRNE K RP   SG  NLAY IYTSGTTGK
      Query sequence   (51) RNMTAIGQYQGTTINIDQADWRNENKKRPELMSGPQNLAYIIYTSGTTGK
gi|3041735|sp|Q04747 (1602) RNMTAIGQYEGIIVSLDDGKWRNESKERPSSISGSRNLAYVIYTSGTTGK
                      101                                                150
          Consensus  (101) PKGVQIEHRNLTNYVSWFS EAGLTK      DK VLLSSYAFDLGYT
      Query sequence  (101) PKGVQIEHRNLTNYVSWFSKEAGLTK-----TDKSVLLSSYAFDLGYTSI
gi|3041735|sp|Q04747 (1652) PKGVQIEHRNLTNYVSWFSEEAGLTKRRADGNDKTVLLSSYAFDLGYTCM
                      151                                                200
          Consensus  (151) FPVL  GGELHIV KETYTAPD I  YIKEHGITYIKLTPSLFHT VNTA
      Query sequence  (151) FPVLQAGGELHIVPKETYTAPDQIGRYIKEHGITYIKLTPSLFHTMVNTA
gi|3041735|sp|Q04747 (1702) FPVLLGGGELHIVQKETYTAPDEIAHYIKEHGITYIKLTPSLFHTIVNTA
                      201                                                250
          Consensus  (201) SF     FESLRLIVLGGEKIIP DV AF K Y HTEFINHYGPTEATIG
      Query sequence  (201) SFTKEPHFESLRLIVLGGEKIIPADVLAFSKVYRHTEFINHYGPTEATIG
gi|3041735|sp|Q04747 (1752) SFAFDANFESLRLIVLGGEKIIPTDVIAFRKMYGHTEFINHYGPTEATIG
                      251                                                300
          Consensus  (251) AIAGRVDL EP  FAKRPTIGRPIAN GALVLNE LKLVPPGASGQLYIT
      Query sequence  (251) AIAGRVDLTEPGTFAKRPTIGRPIANTGALVLNESLKLVPPGASGQLYIT
gi|3041735|sp|Q04747 (1802) AIAGRVDLYEPDAFAKRPTIGRPIANAGALVLNEALKLVPPGASGQLYIT
                      301                                                350
          Consensus  (301)   G        P          P          G        S G
      Query sequence  (301) RTG------GLPEDISAVLSSHPRNSLKTRMRRGAXCIKPEMSSGDWRTV
gi|3041735|sp|Q04747 (1852) GQGLARGYLNRPQLTAERFVENPYSPGSLMYKTGD--VVRRLSDG-----
                      351                                                400
          Consensus  (351)    FIGRADDQ        R  R   G       S SG
      Query sequence  (351) PVEFIGRADDQGENPWLPHRAERNXNGHAXPQRHSRSGSACGFXGRASRA
gi|3041735|sp|Q04747 (1902) TLAFIGRADDQ-----VKIRGYRIELGEIETVMLSLSGI------QEAVV
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                          401                                                 450
          Consensus  (401) L V  GG          S      A        T     I  PAF  QVD
      Query sequence  (401) LRVLYGG---------SSHXKSRAP---ETAFTNTCRLIXSPAFXXQVDV
gi|3041735|sp|Q04747 (1952) LAVSEGGLQELCAYYTSDQDIEKAELRYQLSLTLPSHMI--PAFFVQVDA
                          451                                                 500
          Consensus  (451) IPLTANGKT        PNAAQS   G K L      L     RIWQKTL
      Query sequence  (451) IPLTANGKTRQKRIARXPNAAQSRIXGVKLLR--KQHLKKAFGRIWQKTL
gi|3041735|sp|Q04747 (2002) IPLTANGKTDRNALPK-PNAAQS---GGKALAAPETALEESLCRIWQKTL
                           501                                          550
          Consensus  (501) GIEAIGIDDNFFDLGGHSLKGMMLIANIQAELEK VPLKALFEQPTVRQL
      Query sequence  (501) GIEAIGIDDNFFDLGGHSLKGMMLIANIQAELEKTVPLKALFEQPTVRQL
gi|3041735|sp|Q04747 (2052) GIEAIGIDDNFFDLGGHSLKGMMLIANIQAELEKSVPLKALFEQPTVRQL
                          551                                                 600
          Consensus  (551) AAYME SA SGG   LKPADKQD YPLSSAQKRMYVLNQLDRQTISYNMP
      Query sequence  (551) AAYMEESAASGGYHMLKPADKQDVYPLSSAQKRMYVLNQLDRQTISYNMP
gi|3041735|sp|Q04747 (2102) AAYMEASAVSGGHQVLKPADKQDMYPLSSAQKRMYVLNQLDRQTISYNMP
                          601                                                 650
          Consensus  (601) SVLLMEGEL I   R    QLVNRHESLRTSF EA GEPVQRI E A  D
      Query sequence  (601) SVLLMEGELHISRLRESLNQLVNRHESLRTSFTEADGEPVQRIVEEASID
gi|3041735|sp|Q04747 (2152) SVLLMEGELDIWPARLT-PQLVNRHESLRTSFMEANGEPVQRIIEKAEVD
                          651                                                 700
          Consensus  (651) LHVF A E EA QKIKEFIRPFDL  AP            KHLLLLDMHH
      Query sequence  (651) LHVFDAEEGEAEQKIKEFIRPFDLSNAPXXXXXXXXXXDQKHLLLLDMHH
gi|3041735|sp|Q04747 (2202) LHVFEAKEDEADQKIKEFIRPFDLNDAPLIRAALLRIEAKKHLLLLDMHH
                          701                 727
          Consensus  (701) IIADGVSRGIFVKELALLYKGEQLPEP
      Query sequence  (701) IIADGVSRGIFVKELALLYKGEQLPEP
gi|3041735|sp|Q04747 (2252) IIADGVSRGIFVKELALLYKGEQLPEP

                          1                                                    50
          Consensus    (1)   AD EALKS LKETLPDYMIPAFWV LNELPVTANGKVDRK LPEPDIE
      Query sequence (1438) KXSDXEALKSALKETLPDYMIPAFWVXLNELPVTANGKVDRKXLPEPDIE
gi|3041735|sp|Q04747 (2981) ETADIEALKSTLKETLPDYMIPAFWVTLNELPVTANGKVDRKALPEPDIE
                          51                                                  100
          Consensus   (51)  GSGEYKAPSTDMEELLAGIWQDVLGISEVGV DNFFSLGGDSIKGIQMA
      Query sequence (1488) XGSGEYKAPSTDMEELLAGIWQDVLGISEVGVXDNFFSLGGDSIKGIQMA
gi|3041735|sp|Q04747 (3031) AGSGEYKAPTTDMEELLAGIWQDVLGMSEVGVTDNFFSLGGDSIKGIQMA
                          101                  129
          Consensus  (101) SRLNQHGWKLEMKDLFQHPTIEELTQYVE
      Query sequence (1538) SRLNQHGWKLEMKDLFQHPTIEELTQYVE
gi|3041735|sp|Q04747 (3081) SRLNQHGWKLEMKDLFQHPTIEELTQYVE
```

Table 2.2. Homology of the identified gene products with other surfactant production bacteria

Protein	Organism	Identities	Positives	GenBank #
surfactin synthetase	*Bacillus subtilis*	305/349 (87%) 492/727 (67%) 118/129 (91%)	321/349 (91%) 552/727 (75%) 122/129 (94%)	Q04747
lichenysin synthetase	*Bacillus licheniformis*	357/747 (47%)	455/747 (60%)	AAD04758
tyrocidine synthetase	*Brevibacillus brevis*	238/708 (33%)	380/708 (53%)	O30409
Gramicidin S synthetase	*Brevibacillus brevis*	248/718 (34%)	377/718 (52%)	P14688
peptide synthase	*Bacillus subtilis*	234/710 (32%)	354/710 (49%)	P39845
bacitracin synthetase	*Bacillus licheniformis*	228/712 (32%)	369/712 (51%)	O68008
peptide synthetase	*Bacillus subtilis*	236/717 (32%)	355/717 (49%)	AAC36721
microcystin synthetase	*Microcystis aeruginosa*	215/717 (29%)	344/717 (47%)	AAK61390
lysobactin synthetase	*Lysobacter sp.*	218/733 (29%)	346/733 (47%)	T18545
pristinamycin synthase	*Streptomyces pristinaespiralis*	211/720 (29%)	319/720 (44%)	T30289
CDA peptide synthetase	*Streptomyces coelicolor*	216/721 (29%)	338/721 (46%)	T36248
siderophore non-ribosomal peptide synthetase	*Pseudomonas putida*	216/781 (27%)	341/781 (43%)	CAC32046
virginiamycin S synthetase	*Streptomyces virginiae*	210/720 (29%)	314/720 (43%)	T30874
peptide synthetase	*Mycobacterium smegmatis*	209/743 (28%)	320/743 (43%)	T14165
peptide-synthetase	*Amycolatopsis mediterranei*	207/718 (28%)	311/718 (43%)	T17468

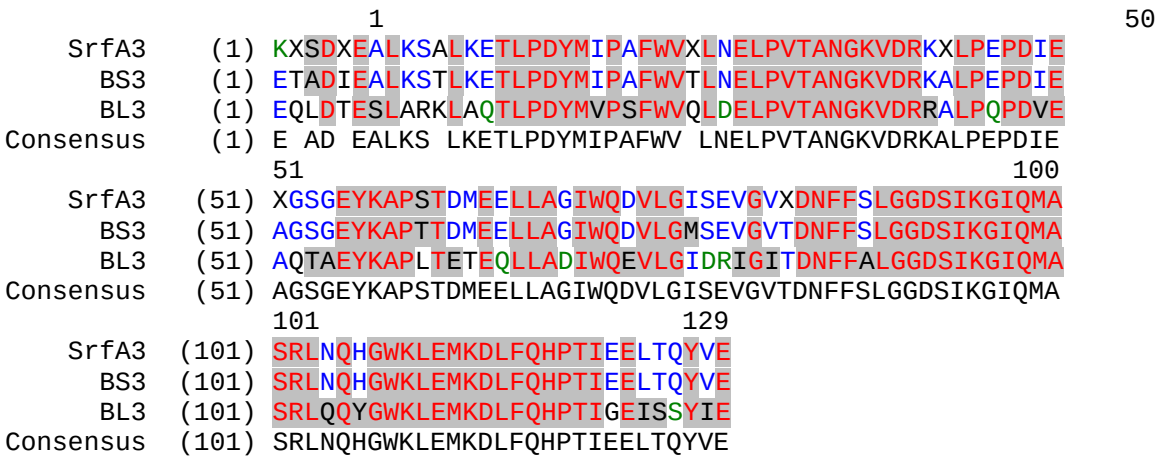

Figure 2.8. Comparison of amino acid sequence of the predicted biosurfactant production region (*srfAB*) from JF-2 (SrfA3) with that from *B. subtilis* (BS3, GenBank accession number Q04747) and *B. licheniformis* (BL3, GenBank accession number AAD04758).

Identical and/or similar amino acids are shaded; similar amino acids are in boldface within the shaded regions

Table 2.3. Comparison and analysis of amino acid sequence (129 aa) (Figure 2.8) from strain JF-2 with that from *B. subtilis* and *B. licheniformis*.

Analysis	Protein		
	JF-2	*B. subtilis*	*B. licheniformis*
Molecular Weight	14416.64 m.w.	14356.39 m.w.	14595.59 m.w.
1 microgram =	69.364 pMoles	69.655 pMoles	68.514 pMoles
A[280] of 1 mg/ml	1.45 AU	1.46 AU	1.52 AU
Isoelectric Point	4.46	4.34	4.40
Charge at pH 7	-11.03	-13.03	-10.12
Amino Acid(s)	Number count		
	JF-2	*B. subtilis*	*B. licheniformis*
Charged (RKHYCDE)	38	38	37
Acidic (DE)	22	23	21
Basic (KR)	11	10	11
Polar (NCQSTY)	25	28	31
Hydrophobic (AILFWV)	42	44	47
A Ala	7	9	10
C Cys	0	0	0
D Asp	9	9	10
E Glu	13	14	11
F Phe	4	4	4
G Gly	10	10	8
H His	2	2	1
I Ile	7	7	9
K Lys	9	8	6
L Leu	14	14	15
M Met	4	5	3
N Asn	4	4	2
P Pro	7	7	7
Q Gln	5	5	11
R Arg	2	2	5
S Ser	8	6	6
T Thr	5	10	8
V Val	7	7	6
W Trp	3	3	3
Y Tyr	3	3	4
B Asx	13	13	12
Z Glx	18	19	22
X Xxx	6	0	0

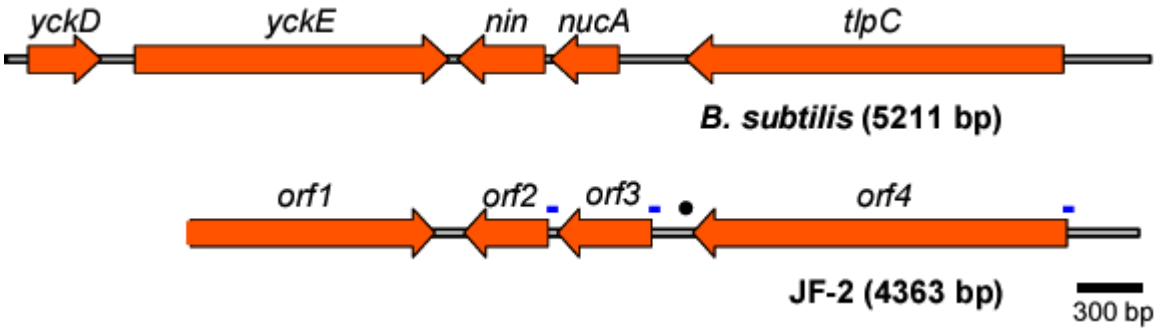

Figure 2.9. Schematic representation of homologous regions in the nucleotide sequence of upstream region from the *srfA* locus of *B. subtilis* (GenBank accession number: Z99105) and JF-2 DNA assembled fragment.

Small bar (–) indicate ribosomal binding site. Round-like (•) mark indicate Tandem stop codon.

Table 2.4. Homology of identified gene products with other bacteria protein

A. Orf1

Protein	**Organism**	**Identities**	**Positives**	**GenBank #**
beta-glucosidase	*Bacillus subtilis*	171/182 (93%)	178/182 (97%)	BGL2_BACSU
beta-glucosidase A	*Lactococcus lactis*	95/183 (51%)	122/183 (66%)	AE006255
phospho-beta-glucosidase	*Fusobacterium mortiferum*	85/181 (46%)	107/181 (59%)	AAB49339
Beta_glucosidase	*Clostridium acetobutylicum*	60/149 (40%)	80/149 (53%)	NP_149175
beta-glucosidase	*Bacillus halodurans*	47/141 (33%)	79/141 (56%)	BAB07637
phospho-beta-glucosidase BglB	*Enterococcus faecium*	88/339 (25%)	142/339 (41%)	AF121254
glycosyl hydrolase, family 1	*Streptococcus pneumoniae*	53/156 (33%)	85/156 (54%)	AAK76086
phospho-beta-galactosidase I	*Lactobacillus gasseri*	50/154 (32%)	82/154 (53%)	JE0395
beta-glucosidase	*Listeria monocytogenes*	51/146 (34%)	75/146 (51%)	CAC20633

B. Orf2

Protein	**Organism**	**Identities**	**Positives**	**GenBank #**
DNA-entry nuclease inhibitor	*Bacillus subtilis*	82/96 (85%)	87/96 (90%)	P12669
BH3298~unknown	*Bacillus halodurans*	15/46 (32%)	23/46 (50%)	BAB07017
Predicted dehydrogenase	*Thermoplasma volcanium*	17/46 (36%)	23/46 (50%)	NP_110909
putative V-type Na+ -ATPase	*Streptococcus pyogenes*	19/53 (35%)	27/53 (50%)	AAK33259
pesticin	*Yersinia pestis*	12/50 (24%)	29/50 (58%)	NP_046432

Table 2.4 continued.
C. Orf3

Protein	Organism	Identities	Positives	GenBank #
membrane-associated nuclease *nucA*	*Bacillus subtilis*	113/144 (78%)	121/144 (84%)	S61274
DNA entry nuclease	*Bacillus subtilis*	93/103 (90%)	98/103 (95%)	BAA06431
Sporulation-specific extracellular nuclease precursor	*Bacillus subtilis*	73/115 (63%)	94/115 (81%)	P42983
endo-exonuclease	*Armillaria mellea*	18/39 (46%)	22/39 (56%)	AF134483
hypothetical protein	*Streptomyces coelicolor*	24/86 (27%)	38/86 (44%)	T28698
nuclear distribution protein	*Emericella nidulans*	16/83 (19%)	39/83 (46%)	AAC35556
mitochondrial Aldehyde Dehydrogenase	*Saccharomyces cerevisiae*	17/42 (40%)	24/42 (57%)	NP_010996
multidrug resistance protein	*Leishmania tarentolae*	15/32 (46%)	19/32 (59%)	DVLNS

D. Orf4

Protein	Organism	Identities	Positives	GenBank #
methyl-accepting chemotaxis protein TlpC	*Bacillus subtilis*	420/556 (75%)	451/556 (81%)	BAA06432
metyl-accepting chemotaxis protein	*Bacillus halodurans*	110/360 (30%)	190/360 (52%)	BAA75352
transducer protein	*Halobacterium salinarum*	88/358 (24%)	158/358 (44%)	T44253
methyl-accepting chemotaxis protein	*Thermotoga maritima*	87/361 (24%)	163/361 (45%)	F72288
methyl-accepting chemotaxis protein	*Pyrococcus horikoshii*	91/374 (24%)	159/374 (42%)	NP_142424
methyl-accepting chemotaxis protein (*tlpC*-2)	*Archaeoglobus fulgidus*	79/360 (21%)	156/360 (43%)	NP_069878
chemotaxis transducer	*Pseudomonas aeruginosa*	81/342 (23%)	142/342 (41%)	E83067
putative chemotaxis protein	*Ralstonia sp*	86/345 (24%)	138/345 (40%)	AAG13635

Figure 2.10**.** Alignment of amino acid sequences deduced from a JF-2 DNA assembled fragments and a *B. subtilis*. Sequences were retrieved from GenBank. Amino acid identity and limited conserved substitutions. Identical and/or similar amino acids are shaded; similar amino acids are in boldface within the shaded regions

Orf1

```
                        1                                                  50
          Consensus    (1) VKYWVTLNEQNYNFNHGFITAMHPPGVKDRKRFYEANHIAFLANAKAIES
      Query sequence   (1) VKYWVTLNEQNYNFNHGFLTAMHPPGVKDRKRFYEANHIAFLANAKAIES
gi|1168655|sp|P42403  (162) VKYWVTLNEQNYNFNHGFITAMHPPGVKDRKRFYEANHIAFLANAKAIES
                        51                                                 100
          Consensus   (51) FR YVPEGKIGPSFAYSPAYPLSSHPEDI AFENAEEF NNWWLDMYCWG
      Query sequence  (51) FRKYVPEGKIGPSFAYSPAYPLTSHPEDITAFENAEEFMNNWWLDMYCWG
gi|1168655|sp|P42403  (212) FREYVPEGKIGPSFAYSPAYPLSSHPEDILAFENAEEFTNNWWLDMYCWG
                        101                                                150
          Consensus  (101) TYPQIPFR LEKQGWAPTIE GDMDLLAKGKPDFVGVNYYQTITYERNPL
      Query sequence (101) TYPQIPFRYLEKQGWAPTVEPGDMELLAKGKPDFVGVNYYQTITYERNPL
gi|1168655|sp|P42403  (262) TYPQIPFRCLEKQGWAPTIEAGDMDLLAKGKPDFVGVNYYQTITYERNPL
                        151                                                200
          Consensus  (151) DGVSEGKMNTTGQKGTNQETGIPGLFKTKKNP-LTTSNWDWTIDP GLRI
      Query sequence (151) DGVSEGKMNTTGQKGTNQETGMPGLFKTKKNP-LTTSNWDWTIDPVGLRI
gi|1168655|sp|P42403  (312) DGVSEGKMNTTGQKGTNQETGIPGVFKTKKNP-LTTSNWDWTIDPIGLRI
                        201                                                250
          Consensus  (201) GLRRIT RYQLPVFITENGLGEFDKVEDGT  DDYRIDYL SHLEQCRQA
      Query sequence (201) GLRRITTRYQLPVFITENGLGEFDKVEDGTIHDDYRIDYLQSHLEQCRQA
gi|1168655|sp|P42403  (362) GLRRITSRYQLPVFITENGLGEFDKVEDGTVQDDYRIDYLRSHLEQCRQA
                        251                                                300
          Consensus  (251) ISDGVDLIGYCSWSFTDLLSWLNGYQKRYGFVYVNRDEE   DLKRLKKK
      Query sequence (251) ISDGVDLIGYCSWSFTDLLSWLNGYQKRYGFVYVNRDEENEHDLKRLKKK
gi|1168655|sp|P42403  (412) ISDGVDLIGYCSWSFTDLLSWLNGYQKRYGFVYVNRDEESTSDLKRLKKK
                        301          316
          Consensus    (1) SFYWYQDVIKTNGE L
      Query sequence  (300) SFYWYQDVIKTNGENV
gi|1168655|sp|P42403  (462) SFYWYQDVIKTNGESL
```

Orf2

```
                        1                                                  50
          Consensus    (1) EKGYA  D AISFEAQRN K  IL RLNSSETV SYEKKVTVPFHVT  G
      Query sequence   (5) EKGYAETDEAISFEAQRNSKVYILLRLNSSETVASYEKKVTVPFHVTTDG
gi|112708|sp|P12669|  (37) EKGYAAADGAISFEAQRNTKAFILFRLNSSETVNSYEKKVTVPFHVTENG
                        51                                              96
          Consensus   (51) I IESIMSKRLSFDLPKGDYQ TC TVPAE SDLHADTYIIDAVSV
      Query sequence  (55) IEIESIMSKRLSFDLPKGDYQFTCYTVPAELSDLHADTYIIDAVSV
gi|112708|sp|P12669|  (87) IHIESIMSKRLSFDLPKGDYQLTCWTVPAEMSDLHADTYIIDAVSV
```

Orf3

```
                        1                                                  50
          Consensus    (1) DIIKT                KGDFFS  QKTSQT EYDET AF   RYP
      Query sequence   (2) DIIKTXXXXXXXXXXXXXXXXKGDFFSDEQKTSQTEEYDETLAFLFERYP
gi|1171856|sp|P12667   (4) DIIKTILLVIVIIAAAAVGLIKGDFFSADQKTSQTKEYDETMAFPSDRYP
                        51                                                 100
          Consensus   (51) ETA HIKDAI EGHS VCTIDRD AEERREQSLKDVPSK GYDRDEWPMA
      Query sequence  (52) ETANHIKDAISEGHSDVCTIDRDRAEERREQSLKDVPSKTGYDRDEWPMA
gi|1171856|sp|P12667  (54) ETAKHIKDAINEGHSEVCTIDRDGAEERREQSLKDVPSKKGYDRDEWPMA
                        101                                          144
          Consensus  (101) MCKEGGEGASVEYISPADNRGAGSWVGH LTDYPDGTKVLFTIQ
      Query sequence (102) MCKEGGEGASVEYISPADNRGAGSWVGHQLTDYPDGTKVLFTIQ
gi|1171856|sp|P12667  (104) MCKEGGEGASVEYISPADNRGAGSWVGHRLTDYPDGTKVLFTIQ
```

Orf4

```
                        1                                                   50
          Consensus    (1) VF VILLFS SVG VMLK IT SMK MAT KAKGDLALSSTYIDDV  G
      Query sequence   (17) VFAVILLFSLSVGAVMLKDITSSMKKMATDKAKGDLALSSTYIDDVILGN
gi|730960|sp|P39209|   (18) VFVVILLFSASVGTVMLKEITESMKQMATEKAKGDLALSSTYIDDVMSGD
                        51                                                  100
          Consensus   (51) W VKN KLYKG TQINGNEDIVDLLG KTGDT TIFQGDTRVATNVMK G
      Query sequence   (67) WEVKNDKLYKGETQINGNEDIVDLLGKKTGDTVTIFQGDTRVATNVMKDG
gi|730960|sp|P39209|   (68) WQVKNNKLYKGQTQINGNEDIVDLLGEKTGDTITIFQGDTRVATNVMKNG
                        101                                                 150
          Consensus  (101) ERAVGTQAS EV  AVLK GK FYGQADVAGSSYQTAYMPLKD  GNIIG
      Query sequence  (117) ERAVGTQASAEVTDAVLKNGKQFYGQADVAGSSYQTAYMPLKDKEGNIIG
gi|730960|sp|P39209|  (118) ERAVGTQASSEVIAAVLKKGKRFYGQADVAGSSYQTAYMPLKDQNGNIIG
                        151                                                 200
          Consensus  (151) MLYTGANQSILASLTQSLFTQFA                FTR IN RLN
      Query sequence  (167) MLYTGANQSILASLTQSLFTQFAXXXXXXXXXXXXXXXXFTRRINLRLNV
gi|730960|sp|P39209|  (168) MLYTGANQSILASLTQSLFTQFAIVLVIVIMVSVILVLVFTRKINKRLNA
                        201                                                 250
          Consensus  (201) LK AFESAGNGDMTIEVSDK  DEL ELSVYYNKMRMKLN TIQTV
      Query sequence  (217) LKYAFESAGNGDMTIEVSDKSADELAELSVYYNKMRMKLNHTIQTVXXXX
gi|730960|sp|P39209|  (218) LKSAFESAGNGDMTIEVSDKTGDELSELSVYYNKMRMKLNDTIQTVQQSA
                        251                                                 300
          Consensus  (251)             GAEETNQASEKITEAVQ IANGA  QITRIENSE SL
      Query sequence  (267) XXXXXXXXXXXXGAEETNQASEKITEAVQHIANGAKLQITRIENSEKSLE
gi|730960|sp|P39209|  (268) LQLASASQQLSAGAEETNQASEKITEAVQQIANGAQDQITRIENSESSLK
                        301                                                 350
          Consensus  (301) Q   DIR IS NT AIA KGQLAQSKADIGQKEI NVQAQMD IH SI K
      Query sequence  (317) QTTVDIRQISSNTVAIAEKGQLAQSKADIGQKEISNVQAQMDTIHHSIEK
gi|730960|sp|P39209|  (318) QASADIRDISANTAAIADKGQLAQSKADIGQKEIANVQAQMDAIHQSIQK
                        351                                                 400
          Consensus  (351)         LDGR KQIEQIL VITQIADQTN             GE GKG
      Query sequence  (367) RRXDHSGSLDGRFKQIEQILFVITQIADQTNXXXXXXXXXXXXXGEHGKG
gi|730960|sp|P39209|  (368) SG-EIIHQLDGRSKQIEQILSVITQIADQTNLLALNAAIEAARAGEQGKG
                        401                                                 450
          Consensus  (401) FAVVADEVRKLAEESQ S GQISKLI EI  DM  SA SVEHVKTEAAEG
      Query sequence  (417) FAVVADEVRKLAEESQRSVGQISKLIAEIKNDMTVSAQSVEHVKTEAAEG
gi|730960|sp|P39209|  (418) FAVVADEVRKLAEESQQSAGQISKLIIEIQKDMNRSARSVEHVKTEAAEG
                        451                                                 500
          Consensus  (451) V MI RTRDAFK             SDLSASVTNISAS   IN SFAAN
      Query sequence  (467) VNMIHRTRDAFKXXXXXXXXXXXXXSDLSASVTNISASTQKINESFAANP
gi|730960|sp|P39209|  (468) VTMIQRTRDAFKEIAAATGEISAEISDLSASVTNISASAHQINDSFAANT
                        501                                                 550
          Consensus  (501)  DIKEST NTRQAAALTEEQFAAMEEITAASETLS LAEELTG ISQFKM
      Query sequence  (517) PDIKESTENTRQAAALTEEQFAAMEEITAASETLSHLAEELTGFISQFKM
gi|730960|sp|P39209|  (518) ADIKESTKNTRQAAALTEEQFAAMEEITAASETLSQLAEELTGIISQFKM
                        551
          Consensus  (551) INQ EN
      Query sequence  (567) INQPEN
gi|730960|sp|P39209|  (568) INQAEN
```

Table 2.5. Analysis of amino acid sequence from strain JF-2 (Figure 10).

Analysis	Protein		
	Orf2	Orf3	Orf4
Length	129 aa	145 aa	574 aa
Molecular Weight	15160.07 m.w.	16129.76 m.w.	62344.19 m.w.
1 microgram =	65.963 pMoles	61.997 pMoles	16.040 pMoles
Molar Extinction coefficient	8970	18020	17210
1 A[280] corr. to	1.69 mg/ml	0.90 mg/ml	3.62 mg/ml
A[280] of 1 mg/ml	0.59 AU	1.12 AU	0.28 AU
Isoelectric Point	10.39	4.52	5.22
Charge at pH 7	17.05	-12.96	-15.35
Amino Acid(s)	Number count		
	Orf2	Orf3	Orf4
Charged (RKHYCDE)	41	53	150
Acidic (DE)	6	28	74
Basic (KR)	23	15	58
Polar (NCQSTY)	35	33	164
Hydrophobic (AILFWV)	45	48	218
A Ala	6	12	66
C Cys	6	2	0
D Asp	3	14	29
E Glu	3	14	45
F Phe	9	5	16
G Gly	2	11	32
H His	4	3	9
I Ile	8	10	49
K Lys	12	8	41
L Leu	18	9	50
M Met	4	2	14
N Asn	4	2	28
P Pro	7	5	4
Q Gln	6	5	38
R Arg	11	7	17
S Ser	8	9	47
T Thr	9	10	42
V Val	3	10	36
W Trp	1	2	1
Y Tyr	2	5	9
B Asx	7	16	57
Z Glx	9	19	83
X Xxx	3	0	1

Appendix 2.1. DNA sequence of the16S rRNA gene from *B. mojavensis* strain JF-2.

```
   1     TGATCCTGGC TCAGGACGAA CGCTGGCGGC GTGCCTAATA CATGCAAGTC
         ACTAGGACCG AGTCCTGCTT GCGACCGCCG CACGGATTAT GTACGTTCAG
  51     GAGCGGACAG ATGGGAGCTT GCTCCCTGAT GTTAGCGGCG GACGGGTGAG
         CTCGCCTGTC TACCCTCGAA CGAGGGACTA CAATCGCCGC CTGCCCACTC
 101     TAACACGTGG GTAACCTGCC TGTAAGACTG GGATAACTCC GGGAAACCGG
         ATTGTGCACC CATTGGACGG ACATTCTGAC CCTATTGAGG CCCTTTGGCC
 151     GGCTAATACC GGATGCTTGT TTGAACCGCA TGGTTCAAAC ATAAAAGGTG
         CCGATTATGG CCTACGAACA AACTTGGCGT ACCAAGTTTG TATTTTCCAC
 201     GCTTCGGCTA CCACTTACAG ATGGACCCGC GGCGCATTAG CTAGTTGGTG
         CGAAGCCGAT GGTGAATGTC TACCTGGGCG CCGCGTAATC GATCAACCAC
 251     AGGTAACGGC TCACCAAGGC AACGATGCGT AGCCGACCTG AGAGGGTGAT
         TCCATTGCCG AGTGGTTCCG TTGCTACGCA TCGGCTGGAC TCTCCCACTA
 301     CGGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG
         GCCGGTGTGA CCCTGACTCT GTGCCGGGTC TGAGGATGCC CTCCGTCGTC
 351     TAGGGAATCT TCCGCAATGG ACGAAAGTCT GACGGAGCAA CGCCGCGTGA
         ATCCCTTAGA AGGCGTTACC TGCTTTCAGA CTGCCTCGTT GCGGCGCACT
 401     GTGATGAAGG TTTTCGGATC GTAAAGCTCT GTTGTTAGGG AAGAACAAGT
         CACTACTTCC AAAAGCCTAG CATTTCGAGA CAACAATCCC TTCTTGTTCA
 451     ACCGTTCGAA TAGGGCGGTA CCTTGACGGT ACCTAACCAG AAAGCCACGG
         TGGCAAGCTT ATCCCGCCAT GGAACTGCCA TGGATTGGTC TTTCGGTGCC
 501     CTAACTACGT GCCAGCAGCC GCGGTAATAC GTAGGTGGCA AGCGTTGTCC
         GATTGATGCA CGGTCGTCGG CGCCATTATG CATCCACCGT TCGCAACAGG
 551     GGAATTATTG GGCGTAAAGG GCTCGCAGGC GGTTCCTTAA GTCTGATGTG
         CCTTAATAAC CCGCATTTCC CGAGCGTCCG CCAAGGAATT CAGACTACAC
 601     AAAGCCCCCG GATCAACCGG GGAGGGTCAT TGGAAACTGG GGAACTTGAG
         TTTCGGGGGC CTAGTTGGCC CCTCCCAGTA ACCTTTGACC CCTTGAACTC
 651     TGCAGAAGAG GAGAGTGGAA TTCCACGTGT AGCGGTGAAA TGCGTAGAGA
         ACGTCTTCTC CTCTCACCTT AAGGTGCACA TCGCCACTTT ACGCATCTCT
 701     TGTGGAGGAA CACCAGTGGC GAAGGCGACT CTCTGGTCTG TAACTGACGC
         ACACCTCCTT GTGGTCACCG CTTCCGCTGA GAGACCAGAC ATTGACTGCG
 751     TGAGGAGCGA AAGCGTGGGG AGCGAACAGG ATTAGATACC CTGGTAGTCC
         ACTCCTCGCT TTCGCACCCC TCGCTTGTCC TAATCTATGG GACCATCAGG
 801     ACGCCGTAAA CGATGAGTGC TAAGTGTTAG GGGGTTTCCG CCCCTTAGTG
         TGCGGCATTT GCTACTCACG ATTCACAATC CCCCAAAGGC GGGGAATCAC
 851     CTGCAGCTAA CGCATTAAGC ACTCCGCCTG GGGAGTACGG TCGCAAGACT
         GACGTCGATT GCGTAATTCG TGAGGCGGAC CCCTCATGCC AGCGTTCTGA
 901     GAAACTCAAA GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT
         CTTTGAGTTT CCTTAACTGC CCCCGGGCGT GTTCGCCACC TCGTACACCA
 951     TTAATTCGAA GCAACGCGAA GAACCTTACC AGGTCTTGAC ATCCTCTGAC
         AATTAAGCTT CGTTGCGCTT CTTGGAATGG TCCAGAACTG TAGGAGACTG
1001     AATCCTAGAG ATAGGACGTC CCCTTCGGGG GCAGAGTGAC AGGTGGTGCA
         TTAGGATCTC TATCCTGCAG GGGAAGCCCC CGTCTCACTG TCCACCACGT
1051     TGGTTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA
         ACCAACAGCA GTCGAGCACA GCACTCTACA ACCCAATTCA GGGCGTTGCT
1101     GCGCAACCCT TGATCTTAGT TGCCAGCATT CAGTTGGGCA CTCTAAGGTG
         CGCGTTGGGA ACTAGAATCA ACGGTCGTAA GTCAACCCGT GAGATTCCAC
1151     ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AATCATCATG
         TGACGGCCAC TGTTTGGCCT CCTTCCACCC CTACTGCAGT TTAGTAGTAC
1201     CCCCTTATGA CCTGGGCTAC ACACGTGCTA CAATGGACAG AACAAAGGGC
         GGGGAATACT GGACCCGATG TGTGCACGAT GTTACCTGTC TTGTTTCCCG
1251     AGCAAAACCG CGAGGTTAAG CCAATCCCAC AAATCTGTTC TCAGTTCGGA
         TCGTTTTGGC GCTCCAATTC GGTTAGGGTG TTTAGACAAG AGTCAAGCCT
1301     TCGCAGTCTG CAACTCGACT GCGTGAAGCT GGAATCGCTA GTAATCGCGG
         AGCGTCAGAC GTTGAGCTGA CGCACTTCGA CCTTAGCGAT CATTAGCGCC
1351     ATCAGCATGC CGCGGTGAAT ACGTTCCCGG GCCTTGTACA CACCGCCCGT
         TAGTCGTACG GCGCCACTTA TGCAAGGGCC CGGAACATGT GTGGCGGGCA
1401     CACACCACGA GAGTTTGTAA CACCCGAAGT CGGTGAGGTA ACCTTTATGG
         GTGTGGTGCT CTCAAACATT GTGGGCTTCA GCCACTCCAT TGGAAATACC
1451     AGCCAGCCGC CGAAGGTGGG ACAGATGATT GGGGTGAAGT CGTAACAAGG
         TCGGTCGGCG GCTTCCACCC TGTCTACTAA CCCCACTTCA GCATTGTTCC
1501     TAGCCGTATC GGAAGGTGCG GCTGGATCA
         ATCGGCATAG CCTTCCACGC CGACCTAGT
```

Appendix 2.2 DNA sequence of *orf1 (yckE)* - *orf4 (tlpC)* region of *B. mojavensis* strain Jf-2.

```
   1     TGAGATGGGG TGAAGGCATA TCGATTCTCT GTTAGCTGGC CGCGTATTTT
         ACTCTACCCC ACTTCCGTAT AGCTAAGAGA CAATCGACCG GCGCATAAAA
  51     TCCAAAAGGA AAAGGAGAAA TCAATGAAGC CGGTCTGCAT TTTACGATAA
         AGGTTTTCCT TTTCCTCTTT AGTTACTTCG GCCAGACGTA AAATGCTATT
 101     CCTGATTGAT GAATTGCTTT CTCATAACAT AGAACCGGTT TTGACTTTAT
         GGACTAACTA CTTAACGAAA GAGTATTGTA TCTTGGCCAA AACTGAAATA
 151     ATCACTGGGA TTTGCCTCAG GCGCTTATGG CGAATATGGC GGATTTGAGT
         TAGTGACCCT AAACGGAGTC CGCGAATACC GCTTATACCG CCTAAACTCA
 201     CGAGAAACAT CATAGAGGAT TTTAATCATT ACTGCATTAC TCTTTATAAA
         GCTCTTTGTA GTATCTCCTA AAATTAGTAA TGACGTAATG AGAAATATTT
 251     CGCTTTGGGC AGAAAGTGAA ATATTGGGTA ACGTTAAATG AACAAAACTA
         GCGAAACCCG TCTTTCACTT TATAACCCAT TGCAATTTAC TTGTTTTGAT
 301     CAATTTTAAT CACGGCTTTC TAACAGCTAT GCATCCGCCT GGCGTGAAGG
         GTTAAAATTA GTGCCGAAAG ATTGTCGATA CGTAGGCGGA CCGCACTTCC
 351     ACAGAAAACG ATTTTACGAA GCAAATCATA TTGCGTTTCT GGCAAATGCG
         TGTCTTTTGC TAAAATGCTT CGTTTAGTAT AACGCAAAGA CCGTTTACGC
 401     AAAGCCATTG AGTCCTTCAG AAAATATGTG CCCGAAGGCA AAATAGGACC
         TTTCGGTAAC TCAGGAAGTC TTTTATACAC GGGCTTCCGT TTTATCCTGG
 451     AAGCTTTGCT TATTCTCCCG CATACCCTTT AACCAGTCAT CCAGAGGACA
         TTCGAAACGA ATAAGAGGGC GTATGGGAAA TTGGTCAGTA GGTCTCCTGT
 501     TTACGGCATT TGAAAATGCT GAAGAATTTA TGAATAATTG GTGGCTGGAT
         AATGCCGTAA ACTTTTACGA CTTCTTAAAT ACTTATTAAC CACCGACCTA
 551     ATGTACTGCT GGGGAACCTA CCCGCAAATT CCTTTCCGCT ATTTAGAAAA
         TACATGACGA CCCCTTGGAT GGGCGTTTAA GGAAAGGCGA TAAATCTTTT
 601     ACAGGGATGG GCACCGACAG TCGAACCGGG TGATATGGAG CTGCTTGCCA
         TGTCCCTACC CGTGGCTGTC AGCTTGGCCC ACTATACCTC GACGAACGGT
 651     AAGGGAAGCC GGATTTTGTA GGTGTCAACT ATTATCAAAC GATTACTTAC
         TTCCCTTCGG CCTAAAACAT CCACAGTTGA TAATAGTTTG CTAATGAATG
 701     GAACGAAATC CGCTCGACGG TGTCTCAGAA GGGAAAATGA ATACGACGGG
         CTTGCTTTAG GCGAGCTGCC ACAGAGTCTT CCCTTTTACT TATGCTGCCC
 751     CCAAAAAGGG ACCAATCAGG AAACAGGGAT GCCGGGACTA TTTAAAACTA
         GGTTTTTCCC TGGTTAGTCC TTTGTCCCTA CGGCCCTGAT AAATTTTGAT
 801     AGAAAAACCC CGAACCCTCA CAACGAGCAA CTGGGATTGG ACGATTGATC
         TCTTTTTGGG GCTTGGGAGT GTTGCTCGTT GACCCTAACC TGCTAACTAG
 851     CGGTAGGATT GCGTATCGGG CTTCGCCGTA TTACGACACG TTATCAGCTT
         GCCATCCTAA CGCATAGCCC GAAGCGGCAT AATGCTGTGC AATAGTCGAA
 901     CCTGTGTTTA TTACAGAAAA CGGTTTAGGA GAATTCGATA AAGTTGAAGA
         GGACACAAAT AATGTCTTTT GCCAAATCCT CTTAAGCTAT TTCAACTTCT
 951     CGGCACTATA CATGATGATT ATAGAATTGA TTATTTGCAA TCGCATCTTG
         GCCGTGATAT GTACTACTAA TATCTTAACT AATAAACGTT AGCGTAGAAC
1001     AGCAATGCAG ACAGGCCATT AGTGATGGAG TCGATTTGAT TGGATATTGC
         TCGTTACGTC TGTCCGGTAA TCACTACCTC AGCTAAACTA ACCTATAACG
1051     AGCTGGTCAT TTACTGATCT GTTAAGCTGG CTGAACCGGT TATCAAAAAA
         TCGACCAGTA AATGACTAGA CAATTCGACC GACTTGGCCA ATAGTTTTTT
1101     GATACGGCTT TGTTTATGTG AATCGTGACG AAGAGAATGA ACATGACTTA
         CTATGCCGAA ACAAATACAC TTAGCACTGC TTCTCTTACT TGTACTGAAT
1151     AAACGATTGA AGAAAAAAAG CTTTTATTGG TATCAGGATG TCATTAAGAC
         TTTGCTAACT TCTTTTTTTC GAAAATAACC ATAGTCCTAC AGTAATTCTG
1201     AAATGGAGAA AATGTATAAA GAGTCCCTGA GAGTATTCCT CTCAGGGCTT
         TTTACCTCTT TTACATATTT CTCAGGGACT CTCATAAGGA GAGTCCCGAA
1251     TTCATTAGAC GGAAACAGCA TCAATGATAT AAGTGTCAGC GTGCAGATCT
         AAGTAATCTG CCTTTGTCGT AGTTACTATA TTCACAGTCG CACGTCTAGA
1301     GACAGCTCAG CCGGCACAGT GTAGCATGTG AATTGATAGT CTCCTTTAGG
         CTGTCGAGTC GGCCGTGTCA CATCGTACAC TTAACTATCA GAGGAAATCC
1351     AAGGTCAAAG GACAGCCTCT TGGACATGAT GCTTTCAATT TCAATTCCGT
         TTCCAGTTTC CTGTCGGAGA ACCTGTACTA CGAAAGTTAA AGTTAAGGCA
1401     CTGTTGTGAC GTGAAAAGGA ACAGTCACTT TTTTTTCATA GGATGCAACT
         GACAACACTG CACTTTTCCT TGTCAGTGAA AAAAAAGTAT CCTACGTTGA
1451     GTTTCTGAAC TGTTAAGGCG CAAAAGAATA TATACCTTTG AATTCCGCTG
         CAAAGACTTG ACAATTCCGC GTTTTCTTAT ATATGGAAAC TTAAGGCGAC
1501     TGCCTCAAAC GAAATGGCTT CGTCCGTTTC CGCATATCCT TTTTCAAATT
         ACGGAGTTTG CTTTACCGAA GCAGGCAAAG GCGTATAGGA AAAAGTTTAA
1551     GCTTCATCTG TCCCAATCGA TGACTGGCGG TGCGGAAACC CTTTTGAAAT
         CGAAGTAGAC AGGGTTAGCT ACTGACCGCC ACGCCTTTGG GAAAACTTTA
1601     ACAGTAAATT GATGATATGA AATCGTCAGT TTCTTGCGGC TTCCATGATT
         TGTCATTTAA CTACTATACT TTAGCAGTCA AAGAACGCCG AAGGTACTAA
1651     TGATCAATGT GCTGTTCCTT CCTCTTTTAA CTGTTTACTG AATCGTAAAT
```

```
        ACTAGTTACA CGACAAGGAA GGAGAAAATT GACAAATGAC TTAGCATTTA
1701    AAAACCTTTG TGCCGTCAGG GTAATCGGTC AGCTGATGCC CGACCCAGGA
        TTTTGGAAAC ACGGCAGTCC CATTAGCCAG TCGACTACGG GCTGGGTCCT
1751    GCCTGCGCCG CGATTGTCAG CGGGAGAAAT ATATTCTACA GAAGCCCCTT
        CGGACGCGGC GCTAACAGTC GCCCTCTTTA TATAAGATGT CTTCGGGGAA
1801    CACCGCCTTC TTTGCACATG GCCATTGGCC ATTCATCTCG GTCATACCCT
        GTGGCGGAAG AAACGTGTAC CGGTAACCGG TAAGTAGAGC CAGTATGGGA
1851    GTTTTGGAAG GTACGTCTTT TAATGATTGT TCGCGGCGTT CCTCAGCTCG
        CAAAACCTTC CATGCAGAAA ATTACTAACA AGCGCCGCAA GGAGTCGAGC
1901    GTCTCTGTCT ATGGTGCACA CATCTGAATG CCCCTCACTT ATTGCGTCCT
        CAGAGACAGA TACCACGTGT GTAGACTTAC GGGGAGTGAA TAACGCAGGA
1951    TGATATGATT TGCTGTCTCA GGATAACGCT CAAAAAGGAA GGCCAGTGTT
        ACTATACTAA ACGACAGAGT CCTATTGCGA GTTTTTCCTT CCGGTCACAA
2001    TCGTCATATT CTTCTGTTTG AGACGTTTTT TGTTCGTCTG AGAAAAAGTC
        AGCAGTATAA GAAGACAAAC TCTGCAAAAA ACAAGCAGAC TCTTTTTCAG
2051    TCCCTTAATC AGGCCAACAG CTGCAGCTGC TATGACAACT ATTACGAGAA
        AGGGAATTAG TCCGGTTGTC GACGTCGACG ATACTGTTGA TAATGCTCTT
2101    GTAAAGTTTT TATAATGTCC ACTCGTGATG ATCACCTCCC GCGTCAGCAA
        CATTTCAAAA ATATTACAGG TGAGCACTAC TAGTGGAGGG CGCAGTCGTT
2151    CTTTTAAAAC TGCTCCAAGA GTAATGCAGA AAGGGCTTGG TGTAAAGATT
        GAAAATTTTG ACGAGGTTCT CATTACGTCT TTCCCGAACC ACATTTCTAA
2201    AACAAATTTT GTTTTGCCCT AGGTTTATTG CAGGAATCGC ATTTTAGCAG
        TTGTTTAAAA CAAAACGGGA TCCAAATAAC GTCCTTAGCG TAAAATCGTC
2251    ATCGAAATAT TCATTTTTAA AAAAATAACG TTATCAAATT GAAATATTAA
        TAGCTTTATA AGTAAAAATT TTTTTATTGC AATAGTTTAA CTTTATAATT
2301    TACGAGATTA TAAGCTGTTT TCAGGTTGAT TAATCATTTT AAATTGGCTT
        ATGCTCTAAT ATTCGACAAA AGTCCAACTA ATTAGTAAAA TTTAACCGAA
2351    ATGAAACCGG TAAGCTCTTC AGCTAAATGG GATAACGTTT CCGAGGCTGC
        TACTTTGGCC ATTCGAGAAG TCGATTTACC CTATTGCAAA GGCTCCGACG
2401    AGTAATCTCT TCCATTGCAG CAAATTGCTC TTCTGTTAAA GCTGCTGCCT
        TCATTAGAGA AGGTAACGTC GTTTAACGAG AAGACAATTT CGACGACGGA
2451    GCCGGGTATT TTCTGTTGAT TCTTTAATGT CTGGTGGATT GGCGGCAAAG
        CGGCCCATAA AAGACAACTA AGAAATTACA GACCACCTAA CCGCCGTTTC
2501    GATTCATTTA TTTTTTGCGT ACTGGCTGAA ATGTTGGTAA CAGATGCTGA
        CTAAGTAAAT AAAAAACGCA TGACCGACTT TACAACCATT GTCTACGACT
2551    TAGATCGCTG ATTTCCGCAG TAATTTCATC TGTTGCGGCT GCAATTTCTT
        ATCTAGCGAC TAAAGGCGTC ATTAAAGTAG ACAACGCCGA CGTTAAAGAA
2601    TAAATGCGTC TCTGGTGCGG TGGATCATGT TTACACCTTC AGCAGCTTCT
        ATTTACGCAG AGACCACGCC ACCTAGTACA AATGTGGAAG TCGTCGAAGA
2651    GTTTTCACAT GTTCAACAGA CTGCGCAGAT ACAGTCATAT CATTTTTTAT
        CAAAAGTGTA CAAGTTGTCT GACGCGTCTA TGTCAGTATA GTAAAAAATA
2701    CTCAGCGATT AATTTGGAAA TCTGTCCGAC AGACCGTTGA GATTCCTCTG
        GAGTCGCTAA TTAAACCTTT AGACAGGCTG TCTGGCAACT CTAAGGAGAC
2751    CTAATTTGCG AACTTCATCA GCAACAACGG CAAAGCCTTT GCCGTGTTCT
        GATTAAACGC TTGAAGTAGT CGTTGTTGCC GTTTCGGAAA CGGCACAAGA
2801    CCGGCTCGGG CAGCTTCAAT TGCCGCATTA AGGGCAAGCA GGTTTGTCTG
        GGCCGAGCCC GTCGAAGTTA ACGGCGTAAT TCCCGTTCGT CCAAACAGAC
2851    ATCGGCAATT TGGGTGATGA CAAATAAAAT TTGTTCAATT TGTTTAAAAC
        TAGCCGTTAA ACCCACTACT GTTTATTTTA AACAAGTTAA ACAAATTTTG
2901    GGCCGTCCAG ACTGCCTGAA TGATCTCACC GCCTTTTTTC AATCGAATGG
        CCGGCAGGTC TGACGGACTT ACTAGAGTGG CGGAAAAAAG TTAGCTTACC
2951    TGAATGGTAT CCATTTGTGC CTGAACATTT GAAATTTCTT TTTGGCCAAT
        ACTTACCATA GGTAAACACG GACTTGTAAA CTTTAAAGAA AAACCGGTTA
3001    GTCTGCTTTA GATTGCGCAA GCTGTCCTTT CTCAGCAATG GCAACAGTAT
        CAGACGAAAT CTAACGCGTT CGACAGGAAA GAGTCGTTAC CGTTGTCATA
3051    TTGAAGAAAT TTGCCGTATG TCAACCGTTG TTTGCTCCAG TGATTTTTCA
        AACTTCTTTA AACGGCATAC AGTTGGCAAC AAACGAGGTC ACTAAAAAGT
3101    CTGTTTTCAA TTCGTGTGAT TTGAAGCTTT GCTCCGTTTG CAATATGCTG
        GACAAAAGTT AAGCACACTA AACTTCGAAA CGAGGCAAAC GTTATACGAC
3151    AACAGCTTCT GTGATTTTTT CTGAAGCTTG ATTTGTTTCT TCTGCTCCGG
        TTGTCGAAGA CACTAAAAAA GACTTCGAAC TAAACAAAGA AGACGAGGCC
3201    CTGAAAGCTG CTGAGAGGCT GATGCAAGCT GAAGGGCCGA TTGCTGGACG
        GACTTTCGAC GACTCTCCGA CTACGTTCGA CTTCCCGGCT AACGACCTGC
3251    GTTTGGATTG TGTGATTCAA CTTCATTCTC ATCTTATTAT AGTAGACACT
        CAAACCTAAC ACACTAAGTT GAAGTAAGAG TAGAATAATA TCATCTGTGA
3301    CAGTTCTGCT AACTCATCAG CGGACTTGTC TGAAACTTCT ATTGTCATAT
        GTCAAGACGA TTGAGTAGTC GCCTGAACAG ACTTTGAAGA TAACAGTATA
3351    CCCCGTTTCC GGCGCTTTCA AAGGCGTATT TTAACACGTT TAGCCGCAGA
        GGGGCAAAGG CCGCGAAAGT TTCCGCATAA AATTGTGCAA ATCGGCGTCT
3401    TTGATTCTTC TGGTGAAAAG CAGAACAAGT ATGACAGATA TAATAATAAC
        AACTAAGAAG ACCACTTTTC GTCTTGTTCA TACTGTCTAT ATTATTATTG
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3451      TTCAGCGAGA ACGATAGCAA ATTGAGTGAA CAGCGATTGG GTAAGCGATG
          AAGTCGCTCT TGCTATCGTT TAACTCACTT GTCGCTAACC CATTCGCTAC
3501      CTAAAATGCT TTGATTGGCT CCTGTATACA GCATACCGAT AATGTTGCCT
          GATTTTACGA AACTAACCGA GGACATATGT CGTATGGCTA TTACAACGGA
3551      TCCTTATCTT TTAAGGGCAT ATATGCTGTT TGATAAGAGG AGCCCGCCAC
          AGGAATAGAA AATTCCCGTA TATACGACAA ACTATTCTCC TCGGGCGGTG
3601      ATCTGCTTGT CCGTAGAATT GCTTCCCGTT TTTTAAAACA GCGTCAGTGA
          TAGACGAACA GGCATCTTAA CGAAGGGCAA AAAATTTTGT CGCAGTCACT
3651      CTTCAGCAGA AGCTTGAGTG CCGACAGCTC TTTCGCCATC TTTCATTACG
          GAAGTCGTCT TCGAACTCAC GGCTGTCGAG AAAGCGGTAG AAAGTAATGC
3701      TTGGTTGCCA CCCGTGTATC TCCCTGAAAA ATGGTTACGG TGTCACCTGT
          AACCAACGGT GGGCACATAG AGGGACTTTT TACCAATGCC ACAGTGGACA
3751      TTTTTTGCCA AGTAGATCAA CAATGTCTTC ATTTCCGTTT ATTTGTGTCT
          AAAAAACGGT TCATCTAGTT GTTACAGAAG TAAAGGCAAA TAAACACAGA
3801      CGCCTTTATA CAGCTTATCG TTTTTCACCT CCCAGTTCCC TAAAATCACG
          GCGGAAATAT GTCGAATAGC AAAAAGTGGA GGGTCAAGGG ATTTTAGTGC
3851      TCGTCAATAT ACGTGCTGCT TAAAGCAAGA TCGCCTTTCG CCTTGTCAGT
          AGCAGTTATA TGCACGACGA ATTTCGTTCT AGCGGAAAGC GGAACAGTCA
3901      GGCCATTTTT TTCATGCTGC TTGTAATGTC CTTAAGCATC ACGGCGCCGA
          CCGGTAAAAA AAGTACGACG AACATTACAG GAATTCGTAG TGCCGCGGCT
3951      CTGATAGAGA AAACAATAGA ATAACCGCGA AGACCAAGCC GAGAATTTTA
          GACTATCTCT TTTGTTATCT TATTGGCGCT TCTGGTTCGG CTCTTAAAAT
4001      GTTCCAAGCT TCAGTTTTAT TTGCTTTTTC AATATAATTC CTCCCTTTGT
          CAAGGTTCGA AGTCAAAATA AACGAAAAAG TTATATTAAG GAGGGAAACA
4051      ATATTTGTAG GTCATATTAC TTATCGGGTT TTTGGGTTTA AAGTTTTATT
          TATAAACATC CAGTATAATG AATAGCCCAA AAACCCAAAT TTCAAAATAA
4101      TTAGAGGAAT ATCAAAAACC GGCCTGATCT CCAGGCCGGT TATGTGACGC
          AATCTCCTTA TAGTTTTTGG CCGGACTAGA GGTCCGGCCA ATACACTGCG
4151      TATTCAAGGT TTGCGTGGTA AGTGAACATG GTTTCAGAAT CGAGTCCTTT
          ATAAGTTCCA AACGCACCAT TCACTTGTAC CAAAGTCTTA GCTCAGGAAA
4201      TTTCTCCATG AGTTTTAAAA TGACTGCATC ATAGAATAGC AGCAATGTTT
          AAAGAGGTAC TCAAAATTTT ACTGACGTAG TATCTTATCG TCGTTACAAA
4251      GCTCAAATAA TGAACCCATT GGCTGTATGG TTGATAAGCT TCCGTTGACC
          CGAGTTTATT ACTTGGGTAA CCGACATACC AACTATTCGA AGGCAACTGG
4301      GGCCTTAGGA GAACCGGCAT TTGATGATGA GATCCGATTG TTTTCCAATG
          CCGGAATCCT CTTGGCCGTA AACTACTACT CTAGGCTAAC AAAAGGTTAC
4351      CTCGAATCGG ATT
          GAGCTTAGCC TAA
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CHAPTER 3. GROWTH AND BIOSURFACTANT PRODUCTION BY BACILLUS MOJAVENSIS STRAIN JF-2

ABSTRACT

Bacillus mojavensis JF-2 anaerobically produces biosurfactant that can significantly lower interfacial tension. Studies were conducted to improve growth and biosurfactant production under anaerobic conditions. The addition of peptide supplements to a mineral medium improved both the anaerobic growth and biosurfactant production of B. mojavensis JF-2. The addition of peptide supplements such as Tryptone and Proteose Peptone increased the dry weight of the culture about 7 fold although the doubling time did not change significantly. A number of enzymatic digests of protein did improve the anaerobic growth of B. mojavensis JF-2 but acid digests of protein, such as Casamino acids, did not. The addition of vitamins at concentrations found in commercial sources of Proteose Peptone did not enhance anaerobic growth. Fractionation of Proteose Peptone by size exclusion chromatography indicated that a 3900 Da fraction supported anaerobic growth similar to controls with Proteose Peptone. Addition of single amino acids and single amino acid polypeptides such as polyglutamate, polyglutamine or polytryptophan did not improve growth over that observed in unamended controls. The presence of glass beads in Proteose Peptone supplemented medium increased the total amount of biosurfactant recovered and most of the recovered biosurfactant was found associated with the glass beads. The amount of biosurfactant produced in the presence of glass beads was sufficient for substantial oil recovery based on reservoir simulation studies.

INTRODUCTION

Surfactants are surface-active agents that contain both hydrophilic and hydrophobic components. As a result, they can be useful for enhanced oil recovery (EOR). Conventional production technologies are only able to recover approximately 30% to 50% of the oil originally in place. However the large capital, chemical, energy and/or environmental cost of current EOR technologies limits their application. A promising technology for enhanced oil recovery is microbially enhanced oil recovery (MEOR). MEOR exploits naturally occurring processes and

products. Thus, gas, solvents or acid production by microbial metabolism or production of secondary metabolites such as polymers or biosurfactants, could compete with similar industrial chemicals currently employed in EOR. Environmentally friendly and economical feedstocks such as molasses might replace the bulk chemicals trucked into the oil field.

This study was designed to enhance the anaerobic growth and anaerobic biosurfactant production of *B. mojavensis* JF-2, an organism isolated from oil-field production water. *B. mojavensis* JF-2 produces a lipopeptide biosurfactant, which is capable of lowering the surface tension of water to near its theoretical limit (1). This strain grows optimally under conditions of salt concentration, temperature and pH found in many oil reservoirs. Although anaerobic growth of *B. mojavensis* JF-2 has been reported previously (2), anaerobic growth and biosurfactant production was variable and influenced by unknown nutritional factors. In this study, both anaerobic growth and biosurfactant production were improved and anaerobic growth was reproducible. Continual research is necessary to elucidate further the nutritional influences of protein hydrolysates on growth and biosurfactant production.

Here, sugar sources supporting optimal growth and biosurfactant production were determined and a novel growth stimulatory factor from commercial peptide hydrolysates was discovered. We discuss how to extrapolate such findings to the field for economical MEOR.

METHODS AND MATERIALS

Medium: A modified Medium E (ME_2) was used (3). ME_2 contained the following components per 900 mls: TES buffer (N-tris(hydroxymethyl)methyl–2- aminoethansulfonic acid) (22.9g) (or a 100 mM phosphate buffer); the pH was adjusted to 6.8; sodium chloride (50g); sucrose (20 mM); yeast extract (1g); sodium nitrate (1g); dibasic potassium phosphate (1.0g); ammonium sulfate (1g); magnesium sulfate (0.25g) and 100 ml of a metal solution. The metal solution was a modification of Wolin's metal solution (4) and was composed of the following components per liter: EDTA (1g); $MnSO_4 \cdot H_2O$ (3g); $FeSO_4 \cdot 7H_2O$ (0.1g); $CaCl_2 \cdot 2H_2O$ (0.1g); $CoCl_2 \cdot 2H_2O$ (0.1g); $ZnSO_4 \cdot 7H_2O$ (0.1g); $CuSO_4 \cdot 7H_2O$ (0.01g); H_3BO_4 (0.01g); $Na_2MO_4 \cdot 2H_2O$ (0.01g); $AlK(SO_4)_2$ (0.01g). ME_2 also contained 3% Proteose Peptone #2. This same media without Proteose Peptone was referred to as ME_1. Cysteine hydrochloride was added in the concentration of 0.025%, when reductant was required. Stock solutions of glucose and other

sources of carbohydrate were made ten times the intended final concentration. All medium and stock solutions were anaerobically prepared by boiling the medium or stock solution under nitrogen and dispensing under nitrogen. The headspace of all tubes and bottles was composed of 100% nitrogen (5).

Experimental setup: An anaerobically prepared serum bottle (100% N_2 headspace) was inoculated directly from a freshly grown plate of *B. mojavensis* JF-2. This was incubated for 12 to 24 hours and this culture was used to inoculate the experimental set up. A 1% liquid inoculum was used. All tubes and bottles were incubated at 37^{o}C and growth was measured as absorbance at 600nm.

Surface tension measurements: The surface tension was measured using a De Nuoy ring tensiometer. Three milliliters of the culture supernatant was allowed to equilibrate at room temperature in small plastic weigh pans and the surface tension was recorded. Nanopure water was used as the high surface tension standard (~ 73 dynes/cm) and a 10% solution of Micro-90 detergent was used as the low surface tension standard (~ 27 dynes/cm).

Total carbohydrate analysis: Carbohydrate analysis was performed using glucose-phenol sulfuric method (6).

Biosurfactant Quantification: The JF-2 biosurfactant was quantified by high performance (pressure) liquid chromatography (HPLC). A C18 column was used. The mobile phase was composed of 70% methanol and 30% 10 mM phosphate buffer at a pH of 6.8. The flow rate was 1 ml/min and the injection volume was 20 ul. A UV detector was used with the wavelength set at 210 nm. Samples for HPLC analysis were prepared by acid precipitation of the biosurfactant and extraction with methanol. A 10ml cell free sample was acidified with 50% HCl to a pH of 2.0. This was allowed to refrigerate over night. The sample was then centrifuged at 8000 rpm for 20 minutes. The collected precipitate was extracted with 2 ml of methanol. The methanol/precipitate solution was shaken for one hour and then centrifuged at 15000 rpm for 5 minutes. Twenty micro liters of the methanol extraction was injected on the HPLC.

RESULTS

Various nutrient supplements were tested and the optimum concentration for each was determined if possible. Some enzymatic digests such as Soytone were inhibitory if supplied in

concentrations above 2%; others, such as Proteose Peptone, required a concentration of 3% or greater to produce an effect. It was necessary therefore, to compare the supplements at different concentrations.

Nutrient supplements that contained mixtures of individual amino acids such as Casamino acids, did not support the level of growth that Proteose Peptone did (Table 3.1, Figure 3.1). Supplementing Casamino acids with tryptophan or a mixture of tryptophan, methionine, and glutamate (e.g., amino acids known to be deficient in Casamino acids) resulted in absorbances of 0.1 to 0.2. These were much lower than observed in medium with Proteose Peptone. When Media E without Proteose Peptone (ME_1) was supplemented with a vitamin mixture that emulated the known vitamin components in Proteose Peptone, this did not replace the growth supporting nutrients present in Proteose Peptone. Since Proteose Peptone is known to contain peptides in addition to single amino acids and vitamins, several polypeptides were tested to determine if they could replace the requirement for Proteose Peptone. Neither polyglutamate (glu_7), polyglutamine (gln_9) nor polytryptophan (try_{10}) supported growth of *B. mojavensis* JF-2 (Table 3.1).

Other than peptides, amino acids, and vitamins, Proteose Peptone contains some fatty acids and potentially other unidentified growth factors. It was found that neither Tween 80 (3%) nor rumen fluid (up to 50%) replaced the requirement for Proteose Peptone. Tween 80 provides fatty acids and rumen fluid potentially contains many growth factors.

To clarify further the properties of the growth-stimulating component, Proteose Peptone #3 was fractionated with a Sephadex-G25 column to determine the average molecular size of the component that supported anaerobic growth of *B. mojavensis* JF-2 (Figure 3.2). Three peaks of material that absorbed at 280nm eluted from the column. The fractions within each peak were pooled and lyophilized. The powdered material was added to culture medium to determine which supported anaerobic growth of *B. mojavensis* JF-2. Table 3.2 shows that the fractions with a size range of about 3,900 Da (fractions 12-18) contained most of the growth-supporting activity originally present in Proteose Peptone #3.

Once consistent growth was established, it was important to verify that the medium could support anaerobic growth of *B. mojavensis* JF-2 under strictly anaerobic conditions. In the course of this research, it has been observed that even a small amount of oxygen will lead to substantial growth of *B. mojavensis* JF-2 (even in medium that is not

supplemented with Proteose Peptone). *B. mojavensis* JF-2 is facultative and growth under conditions with small amounts of oxygen is not likely to be the same as under reduced conditions. Further, our application to MEOR requires strict anaerobic conditions (reduced conditions) and it was necessary that the medium support growth under strict anaerobic conditions. Previous reports of anaerobic growth of *B. mojavensis* JF-2 and similar stains of *B. licheniformis* have differed in how they have defined anaerobic conditions. (*B. mojavensis* JF-2 was previously identified as a *B. licheniformis*). Occasionally stationary flasks were used (7) rather than carefully sealed pre-reduced medium (5). Stationary flasks have oxygen present in the medium initially and receive a consistent (even if low) supply of oxygen at the surface during growth. Sealed, pre-reduced medium has been purged of nearly all oxygen and reduced with sulfide or other reductant to remove any trace oxygen left. Figure 3. 3 3 shows that *B. mojavensis* JF-2 grew as well under strictly anaerobic conditions as in anaerobically prepared medium with out a chemical reductant.

Once growth under strictly anaerobic conditions was clearly established, the effect of the addition of Proteose Peptone on biosurfactant production was determined. Table 3.3 shows that little or no biosurfactant was produced unless Proteose Peptone was added to the medium. Cultures of *B. mojavensis* JF-2 were incubated for 19 days before the amount of biosurfactant was quantified.

Simply improving anaerobic growth and biosurfactant production under anaerobic conditions was not sufficient to meet the requirements of this application. MEOR requires an economical approach to field application. As a result, different carbon sources were tested as at to their role in anaerobic growth and biosurfactant production. The addition of glucose as the carbon source resulted in the highest maximum growth rate and highest yield of biosurfactant (Table 3.4). The other sugars were not satisfactory sources of carbon except for molasses and Maltrin 250.

Since the application of this work was to increase *in situ* production of biosurfactant in the oil field, the effect of surface area available for growth on biosurfactant production was determined. The presence of glass beads led to an increase in the total amount of biosurfactant produced by *B. mojavensis* JF-2 in Proteose Peptone–supplemented medium (Figure 3.4). Aerobically grown cultures bead-free cultures synthesized about the same amount of biosurfactant, as did anaerobically grown, bead-free cultures. A biofilm was observed on and

about the surface of the beads and most of the biosurfactant produced was associated with the beads.

DISCUSSION

Importance of Proteose Peptone: Proteose Peptone #3 significantly stimulated growth and biosurfactant production by anaerobic cultures (Table 3.4). The increased biosurfactant production might simply be the result of improved growth with Proteose Peptone or it could be due to a factor that Proteose Peptone provides that is, directly or indirectly, used in biosurfactant biosynthesis.

It is not known why the various enzymatic digests enhanced anaerobic growth of *B. mojavensis* JF-2. These digests consist of a mixture of oligopeptides, amino acids, vitamins and possibly some fatty acids or other unknown growth factors. Any one or a combination of these factors could enhance growth. However, from this work, vitamins, individual amino acids, and some poly amino acids can be eliminated as possible candidates. Fatty acids provided by Tween 80 and growth factors in rumen fluid can also be eliminated. It is likely that a protein, a peptide, or lipopeptide, is involved in enhancing growth under these conditions.

The biochemical nature of the anaerobic growth-promoting factor from Proteose Peptone is not yet known but its average molecular size has been determined. The activity eluted from a Sephadex G-25 column in fractions corresponding to globular proteins of 3900 Da. This fraction contained the large majority of the activity present in Proteose Peptone (Table 3.3). The results suggest that a protein of about 3900 is involved in enhancing anaerobic growth. There are several reports on the utilization of peptides by anaerobes (8,9,10). The requirement of an oligomeric form of amino acids may be the result of the lack of a transport system for one or more amino acids. Peptide uptake would function then as an uptake system for a particular amino acid in the case where individual amino acids cannot be taken up.

Carbon sources for anaerobic growth of *B. mojavensis* JF-2: Glucose sucrose, and fructose were most effective at supporting anaerobic growth of *B. mojavensis* JF-2 (Table 3.4). Galactose, maltose, and mannitol on the other hand did not support growth above the unamended control. Growth rates on the three sugars were identical. However, cell yields with fructose (74 mg/mmol) were twice those with glucose or sucrose (32-37 mg/mmol). Biosurfactant yields, on

the other hand, were highest with glucose (0.20 mg/mmol), less on fructose (0.13 mg/mmol), and much less on sucrose (0.040 mg/mmol). The explanation for these differences is not known at this time. However, it does show that growth and biosurfactant production can be controlled independently. One can use sucrose to support rapid growth and biomass production. Biosurfactant production can then be enhanced by the addition of glucose.

Evaluating commercial sources of carbohydrates: Since the application of pure sugars on an oil field-scale may not be practical, commercial sources of these sugars were evaluated (Table 3.3). Both molasses and Karo syrup, excellent sources of glucose, fructose and sucrose, supported extensive anaerobic growth of *B. mojavensis* JF-2. Among the maltodextrans tested, Maltrin M180, M200 and M250, only M250 supported significant improvements in growth and growth rate above unamended controls. According to the commercial analysis of these products, M250 contained the highest proportion of simple sugars. These results suggest that commercial sources of simple sugars, particularly molasses, hold the most promise for stimulating the growth of *B. mojavensis* JF-2.

Stimulation of anaerobic biosurfactant production by growth in the presence of glass beads: When *B. mojavensis* JF-2 was grown for 5 days in cultures with glass beads of various sizes, at least twice as much biosurfactant was produced in cultures with beads as opposed to those without beads (Figure 3.4). Visual inspection showed that the beads were covered by a biofilm of bacterial cells. From Table 3.4 it is clear that the presence or absence of beads was a greater influence on biosurfactant production than the nutritional/redox condition, a very significant finding for MEOR. The natural condition of the application *in situ* will provide an extensive surface area for biofilm formation that will encourage biosurfactant production. Complicated nutritional stimulation of biosurfactant production per say may not be necessary.

Although Figure 3. 4 suggests that the amount of surfactant produced increases with bead size and decreases with increasing surface area, further experiments are required to clarify this finding. In the case of higher surface area, more biosurfactant may be bound to the surface area, leading to a decreased detection of biosurfactant.

A concentration of 3mg/l biosurfactant is in the range where significant oil recovery will occur based on reservoir simulations (see chapter of this report). The simulations demonstrated that a concentration of 2.25 mg/l would lead to 12% recovery of residual oil. With beads, a concentration of 10 mg/l can be reached, well above the amount required for MEOR.

CONCLUSIONS

Proteose Peptone enhanced anaerobic growth and biosurfactant production by *B. mojavensis* JF-2. The nature of the stimulatory component has not yet been identified. Field application of strain JF-2 in MEOR appears promising with an inexpensive source of simple sugars such as molasses. For batch production of biosurfactant, or in employing nutrient pulses in the field, providing fructose or sucrose to increase cell mass, followed by glucose to increase biosurfactant production, might be a feasible control scenario. The concentration of biosurfactant produced is sufficient for enhanced oil recovery.

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Table 3. 1. The effect of various medium additions on anaerobic growth of *Bacillus mojavensis* strain JF-2.

ADDITION TO ME_1	MAXIMUM OD AT 600 NM
5% Tryptone	.085
1% Soytone	0.20
1% Peptone	0.20
3% Proteose Peptone	0.60
3% Proteose Peptone #3	0.98
3% Neopeptone	0.55
6% Casaminoacid	0.18
1% Yeast Extract	0.10
Casaminoacid plus 0.2% tryptophan	0.10
Casaminoacid plus try, met, glu. (0.2%)	0.20
0.2% Poly Glutamate	0.21
0.2 % Poly Glutamine	0.25
0.2% Poly tryptophan	0.24
3% Tween 80	0.10
50% Rumen fluid	0.10
No addition	0.10

Table 3.2. The amount of activity found in the different fractions of Proteose Peptone when fractionated on a Sephadex G-25 column.

CONDITION	UNITS OF ACTIVITY *
Unamended Medium	0.0
Medium with Proteose Peptone	4.2
Fractions 7-11	1.8
Fractions 12-18	5.8
Fractions 19-99	1.2
Fractions 23-32	0

*One unit of activity is defined as the amount of component that results in an increase in absorbance of 0.05. The activity of each fraction was calculated by dividing the maximum change in absorbance by the 0.05 OD/unit ($(OD_f–OD_i)$/0.05 OD/unit).

Table 3.3. The effect of Proteose Peptone on biosurfactant production by *Bacillus mojavensis* strain JF-2 when grown under anaerobic conditions after 19 days incubation.[1]

Medium Additions	Biosurfactant concentration[2]	Surface tension[3]	Yield[4]	Sucrose consumed
Proteose Peptone	0.065 ± 0.011	43.5 ± 0.71	3.7 mg/mol	15 mmol
None	ND[5]	58.5 ± 2.12	ND	17 mmol
Uninoculated	ND	66.5 ± 2.12	ND	ND

[1] The medium used was Media E without Proteose Peptone (ME_1)

[2] mg/ml

[3] Dynes/cm

[4] Biosurfactant per mmol of sucrose

[5] Not detected

Table 3.4. The effect of different carbon sources on the anaerobic growth of *Bacillus mojavensis* strain JF-2.

Carbon source [1]	OD_{600} [2]	Growth rate constant (hr^{-1})	Biosurfactant yield[3]	Cell yield [4]
Glucose	1.10	0.17	0.20	36.8
Fructose	0.98	0.16	0.13	73.7
Galactose	0.11	-[5]	-	-
Sucrose	0.98	0.16	0.04	31.5
Maltose	0.08	-	-	-
Mannitol	0.10	-	-	-
Molasses	1.00	-	-	-
Karo syrup	0.50	-	-	-
Maltrin M180	0.10	0.01	-	-
Maltrin M200	0.12	0.01	-	-
Maltrin M250	0.19	0.10	-	-

[1]Cultures with no added carbon source grew to less than 0.10 A_{600}.

[2]Max OD at times ranging from 48 to 60 hours.

[3]mg/ mmol glucose equivalent.

[4]mg dry weight/ glucose equivalent.

[5]Not determined

Figure 3. 1. Media E_1 (media E without Proteose Peptone) with vitamins, Casamino acids and Proteose Peptone.

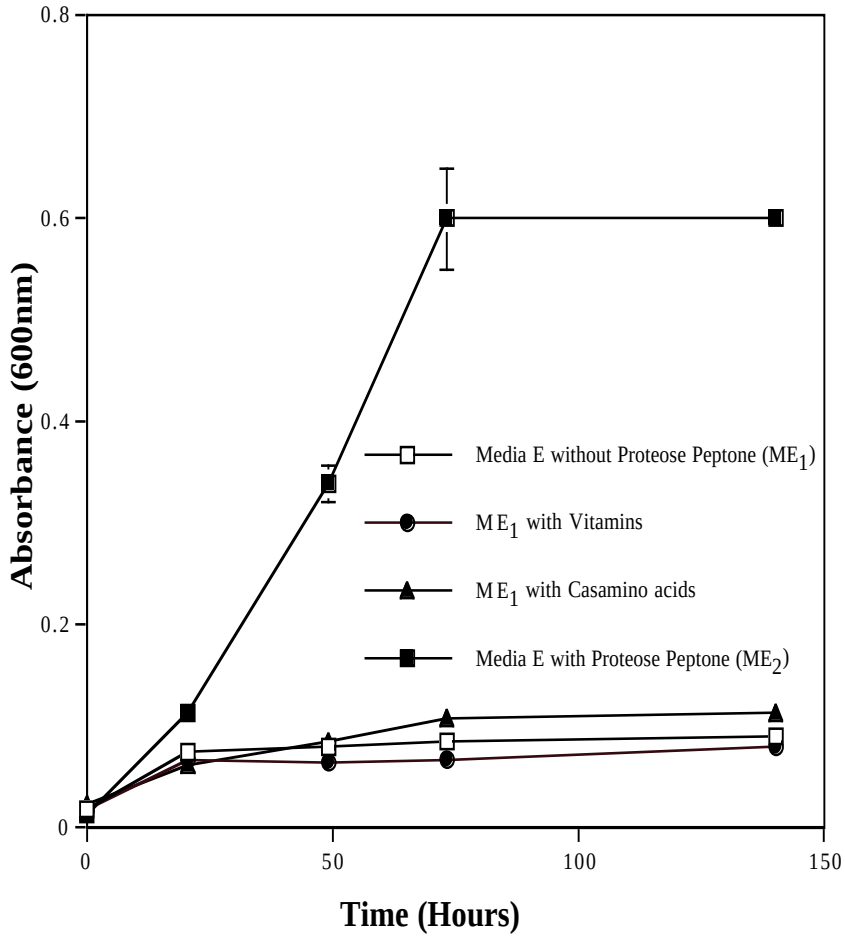


Figure 3. 2. Fractionation of Proteose Peptone by size-exclusion chromatography. The standards, aprotinen, cyanocobalamin, and tryptophan are shown at their point of elution.

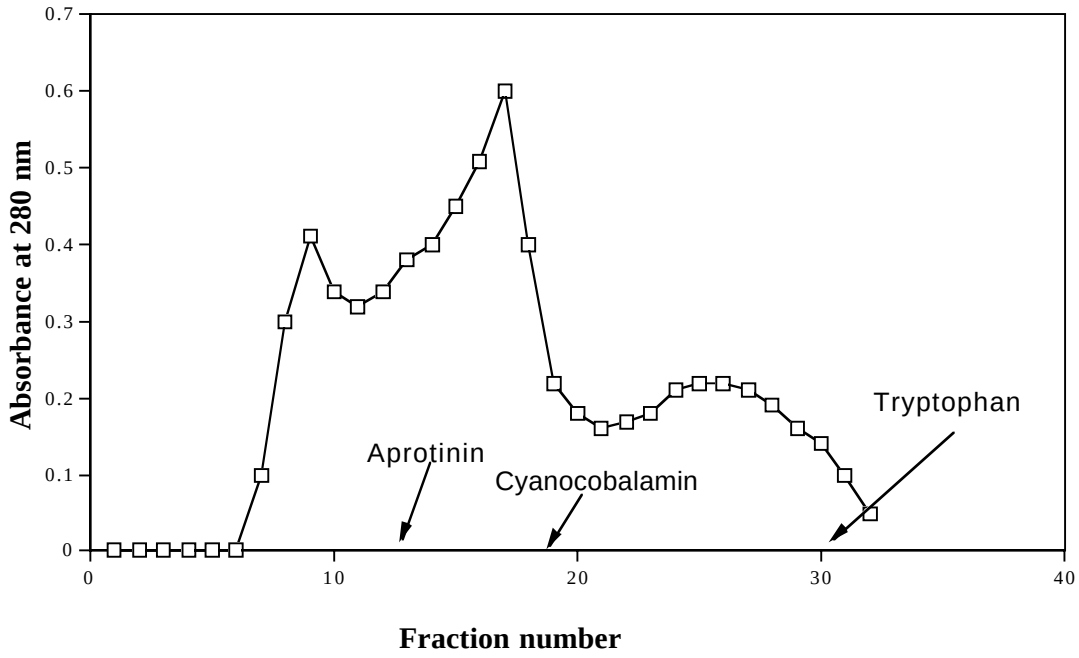


Figure 3. 3. Growth of *B. mojavensis* in Media E with and without Proteose Peptone and with and without chemical reducing agents.

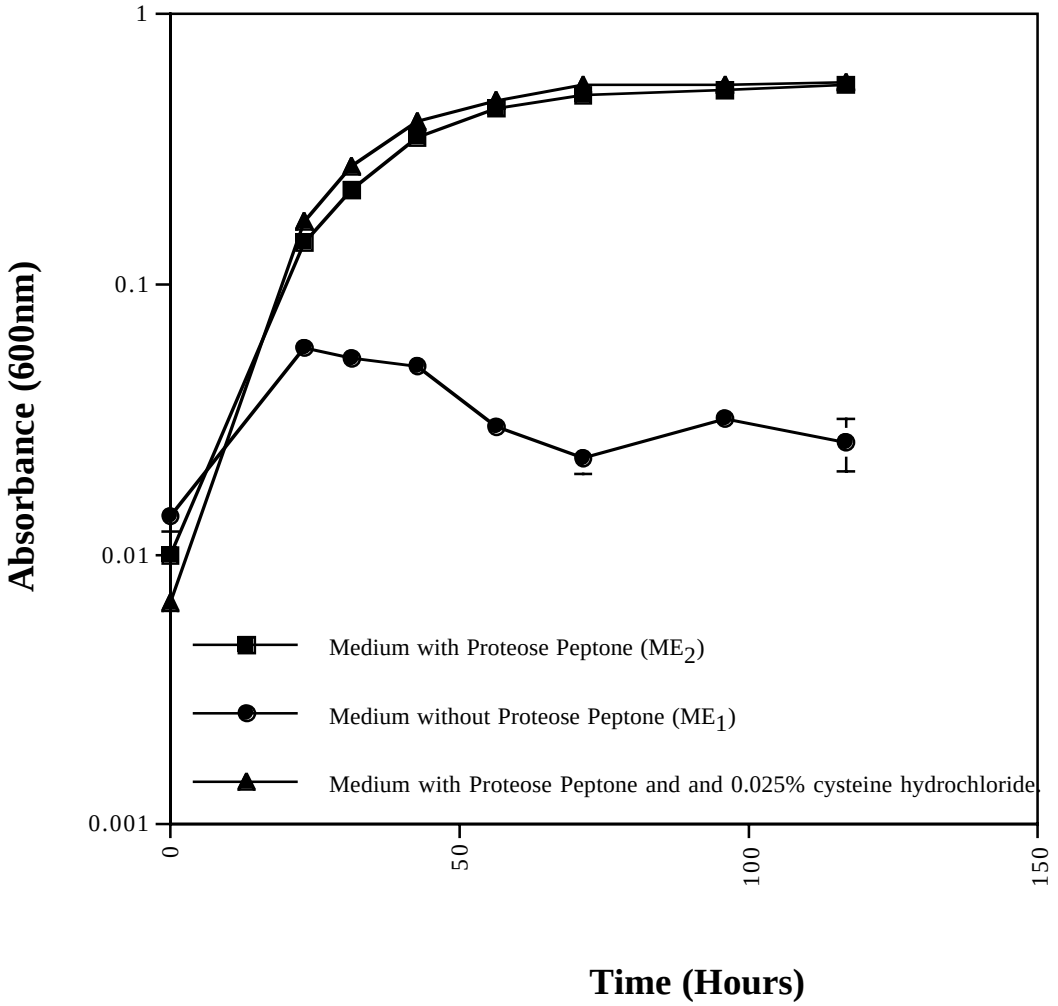
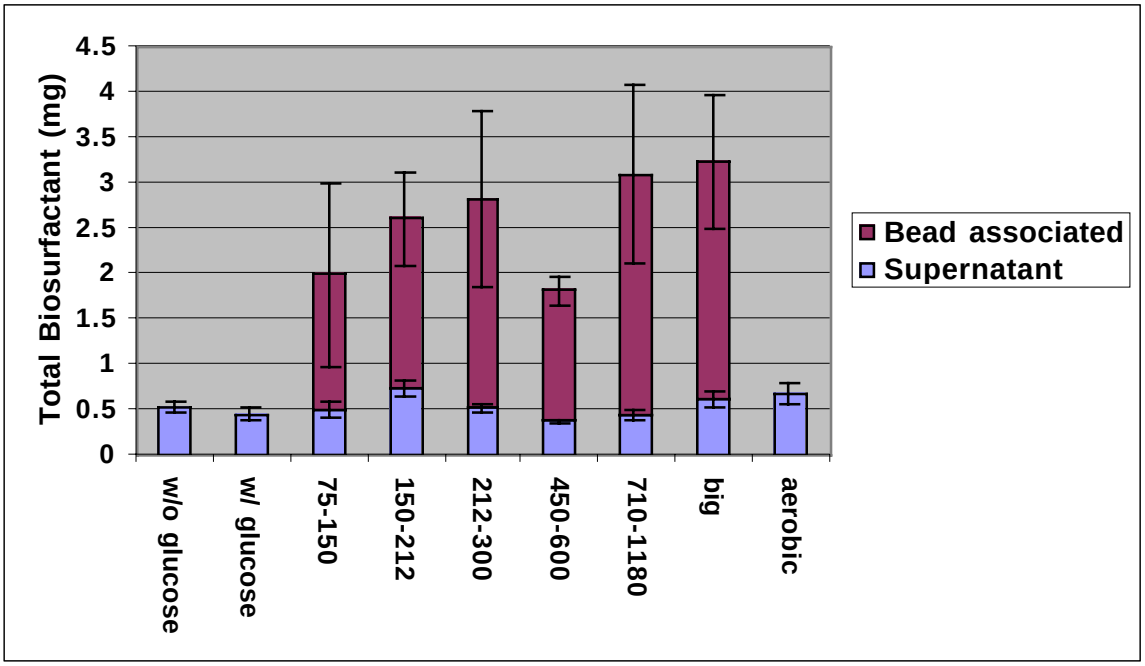


Figure 3. 4. The distribution of biosurfactant in anaerobic culture with glass beads. Error bars represent standard deviation.



CHAPTER 4. COMPETITION OF *BACILLUS MOJAVENSIS* JF-2 WITH NATURAL MICROBIAL POPULATION

ABSTRACT

The addition of Proteose peptone to groundwater microcosms inoculated with *Bacillus mojavensis* strain JF-2 resulted in the production of 2,3-butanediol, a fermentation end product characteristic of *Bacillus* species. This metabolite was also detected in microcosms that did not receive an inoculum of with *B. mojavensis* strain JF-2 so long as Proteose peptone was present. In groundwater microcosms amended with glucose and 27 mM nitrate or Proteose peptone and 27 mM nitrate that were inoculated with *B. mojavensis* strain JF-2, up to 90% of the viable microbial population contained genes for the production of the lipopeptide biosurfactant. Further analyses of the microbial cells that contained the biosurfactant genes showed that they grew anaerobically at high salt concentrations and some made biosurfactants in liquid culture. All of these are characteristics of *B. mojavensis* strain JF-2. These studies showed that it is possible to enhance the growth of bacteria with the potential to produce lipopeptide biosurfactants by selective nutrient additions. In fact, nearly all of the cells in the population had the genetic potential to make lipopeptide biosurfactants with certain nutrient amendments. It is possible to control the dynamics of natural microbial populations during microbial oil recovery processes by nutrient manipulations.

INTRODUCTION

It is becoming increasingly important to develop enhanced oil recovery (EOR) techniques to recover additional oil from existing wells (Yonebayashi et al., 2000). The microbial EOR process has some promise. The biosurfactants produced by microorganisms are naturally occurring substances and thus should not persist for long period of time in the environment. Because microbial growth occurs at exponential rates, it should be possible to produce large amounts of useful products rapidly from inexpensive and renewable resources. Lastly, some microbial biosurfactants are known to significantly decrease the interfacial tension

between oil and water (see Chapter 1 for review). The lipopeptide biosurfactant produced by *Bacillus mojavensis* strain JF-2 reduces the surface tension from 72 to 27 mN m^{-1} and the interfacial tension to between oil and water to less than 10^{-3} mN/m (Jahaveri et al., 1985; McInerney et al., 1990; Lin et al., 1998). This organisms can grow and produce the lipopeptide biosurfactant under anaerobic conditions in the presence of high salt concentrations and at elevated temperatures. These are the environmental conditions found in many oil reservoirs.

There are several different strategies for employing biosurfactants in MEOR. One approach might be the production of biosurfactant in bioreactors ex-situ and subsequent injection of the biosurfactant into the reservoir. This approach would require the development of cultures that make very large concentrations of biosurfactants in order for product recovery to be economically feasible. The injection of a biosurfactant-producing microorganism into a reservoir and the subsequent stimulation of its growth and metabolism in the reservoir is another approach. This approach has been shown to be successful in a previous MEOR field trial (Bryant et al., 1987). Localized production of biosurfactant might be an efficient mechanism to recover residual oil. However, this claim has never been tested. A third approach would be to selectively stimulate biosurfactant-producing bacteria that occur naturally in oil reservoirs. However, it is not know whether organisms that make biosurfactants are commonly found in oil reservoirs. These last two approaches both require mechanisms to stimulate selectively a) the growth of biosurfactant-producing organisms and b) the production of their biosurfactant. Previous studies have shown that the injection of nutrients in oil reservoirs will stimulate microbial metabolism (Bryant et al.,1987, Jenneman et al., 1983; Lin et al., 1996). The presence of gases, solvents, acids, and surface active agents have been detected in field stuides. However, most of these products are a consequence of the centrl energy metabolism of the microorganism. Biosurfactants are secondary metabolites that may not be needed for the growth of cells. Thus, it is not yet clear whether it is possible to stimulate the production of such molecules selectively.

The objective of this work to determine if the nutrient formulation shown in Chapter 3 to allow the anaerobic growth of *Bacillus mojavensis* strain JF-2 will allow this organism to establish itself in a mixed microbial community. The experiment was designed to simulate the conditions that might occur during the application of a MEOR process. A readily available source of subsurface microorganisms was groundwater obtained from a local anaerobic aquifer whose microbiology has been studies in detail (Cozzarelli et al., 2000).

MATERIALS AND METHODS

Source of groundwater. The groundwater was collected in May 2001 from the aquifer underlying the landfill in Norman, OK. The groundwater was extracted from well #40 at 3.75-m depth by using a peristaltic pump (Cozzarelli et al., 2000). The groundwater was flushed with 100% nitrogen gas and then stored in the anaerobic chamber at room temperature until used.

Microorganisms and growth conditions. *Bacillus mojavensis* JF-2 (ATCC # 39307) was grown aerobically in Medium E at 37^{o}C for 24 hours. Medium E contained (per liter): 2.7g KH_2PO_4, 13.9 K_2HPO_4, 50g NaCl, 10g glucose, 1 g yeast extract, 1 g $NaNO_3$, 1 g $(NH_4)_2SO_4$, 30 g Proteose peptone #2, and 10 ml of a metal solution. The metal solution (a modified Wolin's trace metal solution) contained: 1 g EDTA, 3 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CoCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 2H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g AlK $(SO_4)_2$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4.7H_2O$, 25 g $MgSO_4$.

Flasks with 200 ml of medium E were prepared and autoclaved (121^{o}C; 15 min). The flasks were inoculated with *B. mojavensis* strain JF-2 and then grown aerobically at 37°C for 20 hours. After growth, the cells were harvested by centrifugation (11,300 x g; 15min; at 10°C) and the cell pellet was resuspended in a sterile 50 mM of TES buffer (pH 7.0). One ml of this cell suspension was used to inoculate each microcosm.

Microcosm preparation. Each microcosm was prepared by adding 50 ml of groundwater to sterile serum bottles inside of the anaerobic chamber (Balch and Wolfe, 1976). The serum bottles were capped with rubber stoppers and sealed with aluminum caps and taken out of the anaerobic chamber. The gas phase of each serum bottle was exchanged with 100% nitrogen by evacuation under vacuum and respressurization with the above gas phase (Balch and Wolfe, 1976). Nutrients were added to the serum bottles in different combinations by adding 0.5 ml of a sterile, anaerobically prepared stock solution using a sterile syringe and needle flushed with nitrogen gas in order to maintain anaerobic conditions (Balch et al., 1976). The stock solutions used were: 2.7 M nitrate, 0.12 M nitrate, 30% Proteose peptone #2, 57.4 mM K_2HPO_4 and 0.55 M glucose. The final concentrations of the respective nutrient in the microcosm were: 27 mM or 1.2 mM of nitrate, 0.3% of Proteose peptone, 0.574 mM of K_2HPO_4 and 5.5 mM of glucose. The stock solutions were sterilized by autoclaving at 121°C for 20 minutes, except for

glucose, which was sterilized by passage through a 0.22μm filter. After nutrients were added to the microcosms, 1 ml of the JF-2 cell suspension was added to each serum bottle. Each nutrient treatment was done in triplicate. The serum bottles were incubated at 23^{o}C inside the anaerobic chamber. Samples were taken by using syringes with needles previously flushed with nitrogen gas after 1, 10 and 30 days of incubation.

A second set of microcosms was prepared as above but it did not receive an inoculum of JF-2 and only 20 ml rather than 50 ml of groundwater was added to each serum bottle. These microcosms were sampled after immediately after preparation and after 10 days of incubation.

Analytical techniques. Bacterial enumeration was performed by plating onto 1/10X Plate Count Agar containing (per liter): 5 g of tryptone, pancreatic digest of casein USP, 2.5 g of yeast extract, 1 g of dextrose-glucose, 15 g of agar. The medium was diluted 10-fold and 13.5 g of agar was added prior to preparation of the plates. A 1:10 serial dilution until 1:10^{7} dilution was obtained was performed with samples from the microcosm using sterile, Nanopure water. Each agar plate received 0.1ml of the diluted cell suspension. The plates were incubated at 23^{o}C inside the anaerobic chamber.

The surface tension of microcosms and cultures was measured using a Fisher Tensiometer model 215. Nanopure water was used as a standard (73 mN/m). All the samples were measured at room temperature.

The concentration of glucose was measured by the phenol sulfuric method (Gerhart et al., 1981). The absorbance was read at 488 nm against the blank prepared without glucose. The concentration of glucose was determined from a standard curve prepared by plotting the absorbance versus the concentration of glucose of standards.

Nitrate concentration was determined by using a Dionex Ion Chromatography system with an AS4A-SC 4-mm particle-size column, a model CD 20 conductivity detector, and a mobile phase of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate delivered at 2ml/min.

Acetate and butanediol concentration were determined using a gas chromatograph (GC) equipped with a flame ionization detector (FID) and a 6' glass column packed with Carbopack^{TM} B-DA 80/120 4% Carbowax 20M resin. The GC was set to a flow rate of 24 ml/min of helium and operated with an injector temperature of 200°C and a detector temperature of 200°C. A thermal gradient from 155°C to 185°C with temperature increasing at 3^{o}C per min was used. and.

Samples as well as standards were diluted in a solution of 30 mM of oxalic acid prior to injection.

The concentration of biosurfactant in the samples was quantified by high pressure liquid chromatography analysis. A C_{18} column was used with a mobile phase of 70% methanol and 30% of 10 mM phosphate buffer (pH 6.8). The HPLC was run at a flow rate of 1 ml/min and the injection volume was 20 µl. A UV detector was used with a wavelength set at 210 nm (Lin et al., 1994). Sample preparation is described in Chapter 3 of this report.

The ammonium concentration was measured by using the indophenol blue reaction (Gerhart et al., 1981). The ammonium standard was prepared by dissolving 381.9 mg of anhydrous NH_4Cl in 1 liter of Nanopure water. One ml of this solution contained 122 µg of NH_3. The samples were centrifuged (11,300 x g; 15min; at 10°C) to remove cells and debris. The supernatant was removed and diluted in nanopure water to a final volume of 5 ml. To the diluted samples, the following reagents were added: 25µl of 0.003 M $MnSO_4$, 0.25 ml of hypochlorite reagent (10 ml of 5% sodium hypochlorite solution in 40 ml of Nanopure water), and 0.30ml of phenate reagent (2.5 g of NaOH and 10 g of phenol in 100 ml solution). The absorbance was read at 630 nm against the blank prepared with the above reagents. The concentration of ammonium was determined from a standard curve prepared by plotting the absorbance of standards versus their concentration.

DNA blot analysis. Cells were transferred to a nylon membrane from agar plates by placing the nylon membrane on the surface of the agar plates. The cells were then lysed by placing the membrane in a solution of 0.5 N of NaOH for 10 minutes at room temperature. The membrane was transferred first to a filter paper soaked with 0.5 M Tris HCl (pH 7.5) for 5 minutes, second to a filter paper soaked with a solution containing 0.5 M Tris HCl (pH 7.5) and 1.5 M NaCl for 5 minutes, and finally transferred to a filter paper soaked with 2xSSC solution (2xSSC solution contained 17.33 g of NaCl and 8.82 g of sodium citrate per liter, pH 7.8). The membrane was baked under vacuum for 2 hours at 80^{o}C to immobilize the DNA onto the membrane.

The membrane with immobilized DNA was placed in a hybridization glass tube (Fisher Biotech) containing 20 ml prehybridization solution (DIG Eazy Hyb, Boehringer Mannheim) per 100 cm^2 of membrane surface area, and incubated in a hybridization incubator (Fisher Biotech) at 37-42°C for 2 hours. The prehybridization solution was discarded and the prehybridization

solution containing the labeled probe *srfA1* (5-25 ng/ml) was added (see Chapter 2 of this report). The membrane was then incubated in a hybridization incubator at 37-42°C overnight. The hybridization solution was discarded into a tube. The membrane was washed in 2x washing solution (300 mM NaCl, 30 mM sodium citrate, 0.1% SDS, pH 7.0) at room temperature for 10 min. The membrane was washed again in 0.5x washing solution (75 mM NaCl, 7.5 mM sodium citrate, 0.1% SDS, pH 7.0) at 68°C for 30 min.

Chemiluminescent Detection. After hybridization and post-hybridization washes, the membrane was equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween®20) for 1 minute. The membrane was blocked by gently agitating it in blocking solution (100 mM maleic acid, 150 mM NaCl; pH 7.5; 1% (w/v) Blocking reagent [Boehringer Mannheim]) for 30-60 minutes. The blocking solution was removed and the membrane was incubated in the antibody solution (the Anti-Digoxigenin-AP in blocking solution [1:100000 v/v], Boehringer Mannheim) for 30 minutes. After the antibody solution was discarded, the membrane was washed in washing buffer for 30 minutes. The washing buffer was removed and the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for two minutes. The membrane was placed between two sheets of acetate (PhotoGene Development Folders, Gibco BRL) and 0.5 ml (per 100 cm^2) of the Chemiluminescent substrate (CSPD® 1:100 in detection buffer, Boehringer Mannheim) was then added on top of the membrane by scattering the drops over the surface of the membrane. With a damp tissue, the top sheet of plastic was wiped gently to remove any bubbles present under the sheet and to create a liquid seal around the membrane. The filter was incubated for 5 minutes. The semi-dry membranes were sealed in acetate sheets. The membrane was incubated at 37°C for 15 minutes. For detection of the chemiluminescent signal, the membrane was exposed to Lumi-Film (Boehringer Mannheim) for 15-20 minutes..

RESULTS

Anaerobic metabolism in groundwater microcosms inoculated with *B. mojavensis* strain JF-2. The ability of *Bacillus mojavensis* strain JF-2 to establish itself in the presence of a natural microbial population was evaluated by using groundwater as a model subsurface microbial population. Groundwater amended with nutrients known to support the growth of

strain JF-2 was utilized. The depletion of nutrients, the production of characteristic products and the presence of the biosurfactant genes in bacteria isolated from the groundwater incubations were used as indicators that JF-2 or physiologically similar bacteria established themselves in the mixed microbial community. In Chapter 3, we showed that the addition of Proteose Peptone to Medium E stimulated anaerobic growth of JF-2. We also found that 2,3- butanediol was produced by strain JF-2 under these conditions. Many species of the genus *Bacillus* species produce 2,3-butanediol as a product of carbohydrate fermentation as well as use nitrate as electron acceptor in the absence of oxygen (Shariati et al., 1995). We used two nitrate concentrations (1.2 and 27 mM) since indications in the literature suggest that high concentrations of nitrate selectively enrich for *Bacillus licheniformis* and related species, such as *B. mojavensis*.

The initial glucose concentration in the microcosms was approximately 5 mM. Glucose depletion was evident in many of the microcosms that contained Proteose peptone and were inoculated with strain JF-2 (Table 4.1). After 10 days of incubation, glucose consumption in excess of 90% of the initial amount added was evident in all of the microcosms. Acetate was produced after 10 days of incubation. Glucose-amended microcosms without Proteose peptone and without nitrate or with low nitrate concentrations (1.2 mM) produced about 18 mM acetate. About 11 mM acetate would be expected from the anaerobic degradation of 5.5 mM glucose. Production of acetate at concentrations above that expected from the amount of glucose added suggested that additional carbon sources were present in the groundwater to support anaerobic metabolism. Large amounts of acetate were produced (in excess of 30 mM) when Proteose peptone was added to glucose-amended microcosms with low nitrate concentrations. The increase in acetate production may have been due to the metabolism of Proteose peptone itself since microcosms amended with only Proteose peptone had an acetate concentration of about 19 mM after 10 days of incubation (Table 4.1). In microcosms with high nitrate (27 mM), acetate concentrations were 0.7 mM in glucose-amended microcosms, 4.9 in microcosms in microcosm with glucose and Proteose peptone, 11 mM in microcosms with glucose, Proteose peptone and phosphate, and 6.0 mM in microcosms with Proteose peptone (Table 4.1). The lower acetate concentrations in microcosms amended with high nitrate compared to those with low nitrate suggests that nitrate respiration accounted for more of the reducing equivalents when nitrate levels were high.

Interestingly, 2,3-butanediol, a fermentation product known to be synthesized by *Bacillus* species, was produced in the enriched groundwater only when Proteose peptone was added to the solutions.

The nitrate was added to the groundwater as an electron acceptor in the absence of oxygen. It can be also used as a nitrogen source for some microorganisms. Previous work done by Cozzarelli et al. (2000) showed that the nitrate is reduced to ammonium in groundwater from the landfill aquifer. Analysis of the microcosms for these two compounds showed depletion in the nitrate and an increase in ammonium concentration (Table 4.2). It was expected that one mole of nitrate would be reduced to one mole of ammonium. The fact that the amount of ammonium produced was less than stoichiometrically predicted from nitrate consumption suggests that the nitrate may have been transformed to another nitrogen compound, either nitrite or to nitrogen gas. These latter two compounds were not quantified in our experiment. However, the fact that nitrate was consumed and ammonium was produced showed that anaerobic nitrate metabolism did occur in our microcosms.

The surface tension was measured in samples collected from each microcosm. However no significant decrease of surface tension (<55 Nm/cm) was detected (data not shown).

Anaerobic metabolism in uninoculated groundwater microcosms. A control was carried out by amending nutrients to groundwater without inoculating it with JF-2. After 10 days of incubation, the glucose concentration in glucose-amended microcosms ranged from 2.8 to 3.6 mM, regardless of the nitrate concentration (Table 4.3). When microcosms were inoculated with JF-2, glucose concentrations were all below 1 mM (Table 4.1). Acetate and 2,3-butanediol concentrations were lower in microcosms that were not inoculated with JF-2 (Table 4.3) compared to the respective microcosms that were inoculated with JF-2 (Table 4.1).

The presence of Proteose peptone enhanced the metabolism of microorganisms naturally present in the groundwater. The concentration of acetate and 2,3-butanediol was higher in microcosms that received Proteose peptone compared to the respective microcosm that did not received Proteose peptone (Table 4.3). Up to 0.76 mM 2,3-butanediol was detected, which is similar to levels found in microcosms that were inoculated with JF-2 (Table 4.1). Evidently, the microorganisms naturally present in groundwater have the ability to produce 2,3-butanediol. Large amounts of nitrate were consumed in microcosms that were not inoculate with JF-2 (Table

4.4). Ammonium production was limited, precluding major conclusions concerning the mechanism of metabolism of nitrate by the indigenous microorganisms.

Presence of microorganisms with biosurfactant genes. Molecular analysis was used to determine which nutrient amendment allowed the establishment of JF-2 or a similar microorganism that contained biosurfactant genes. The gene probe designed in Chapter 2 of this report was used to determine the percentage of the total viable cell population that contained genes for biosurfactant production.

A sample of amended groundwater from each bottle was plated on diluted PCA medium to allow the growth of the most numerous microorganisms in the microcosms. A dot blot was done on these plated to determine which of the colonies had cells that contained the biosurfactant genes. The probe used to hybridize to the DNA extracted from the colonies corresponded to a region of one of the genes involved in the synthesis of *Bacillus subtilis* biosurfactant, a lipopeptide almost identical to the biosurfactant synthesized by *Bacillus mojavensis* (Lin et al., 1992). Chapter 2 shows that the probe would be specific to *B. mojavensis* biosurfactant genes. The dot blot analysis revealed that, in microcosms amended with glucose and 27 mM of nitrate and microcosms amended with the Proteose peptone and 27 mM of nitrate, 90% of the total viable microbial population contained microorganisms that had surfactin synthetase-like genes (Table 4.5).

The colonies that hybridized with the biosurfactant gene probe and colonies that did not hybridize with the gene probe were further analyzed to determine if they had physiologies similar to *B. mojavensis* strain JF-2 (Table 4.6). A total of 15 positive colonies that contained the biosurfactant gene as revealed by the dot blot analysis and 15 colonies which did not hybridize with the probe were inoculated into Medium E and Nutrient Broth. These cultures were incubated under aerobic and anaerobic conditions at two temperatures, 23°C and 37°C. Medium E contains high salt concentrations which is selective for *B. mojavensis*-like organisms. Also, *B. mojavensis* and related organisms are known to grown anaerobically in this medium. Only colonies that contained the biosurfactant genes as revealed by dot blot analysis grew in Medium E or Nutrient Broth (Table 4.6). Colonies that did not hybridize with the probe did not grow in either of these two media at either temperature, but they did grow on dilute PCA agar. In addition, the surface tension was measured from cultures that grew in these media. Those cells that were obtained from microcosms with Proteose peptone or glucose and 27 mM of nitrate

lowered the surface tension of the medium to 49 Nm/m. Thus, microbial cells that grew in the microcosms amended with glucose and 27 mM nitrate or with Proteose peptone and 27 mM nitrate were predominately cells that contained biosurfactant genes similar to those needed to produce a lipopeptide biosurfactant. These cells also grew anaerobically in high salt medium and at elevated temperatures (37^{o}C), both characteristics of *B. mojavensis* and related organisms.

DISCUSSION

The depletion of nitrate and glucose and the production of acetate, 2,3-butanediol and ammonium showed that the nutrient amendments stimulated bacterial metabolism in microcosms inoculated with JF-2 and in those that did not receive JF-2. The butanediol, a product known to be synthesized by members of the genus *Bacillus* (Shariati et al., 1995) was detected in the groundwater supplemented by Proteose peptone. Butanediol was detected when only glucose was added. These data show the importance of Proteose peptone and nitrate concentration in enhancing the production of a specific microbial product, 2,3- butanediol. Thus, nutrient additions can lead to the selective stimulation of particular microbial metabolism. The analysis of the microbial fermentation products in the microcosms showed that acetate and butanediol are the major compounds synthesized with glucose as carbon source. The amount of acetate and butanediol produced in the groundwater supplemented by Proteose peptone alone showed that the addition of this nutrient is sufficient to support bacterial growth and bacterial metabolism. Butanediol is of particular importance for MEOR, because it can serve as a co-surfactant, a solvent, enhancing the activity of biosurfactants.

Molecular analysis showed the importance of either Proteose peptone or a high level of nitrate (27 mM) in enhancing the growth of microorganisms that contain genes for the synthesis of lipopeptide biosurfactants even in a mixed microbial population where many different kinds of microorganisms are present. It is remarkable that this approach resulted microbial population where almost all of the viable microbial cell (90%) have the potential to make lipopeptide biosurfactants. Further investigations confirmed that the cells that tested positive for the biosurfactant genes were physiologically similar to *B. mojavensis* strain JF-2. Several of these strains did produce a biosurfactant when grown in liquid culture. Our work does show that we can manipulate mixed microbial communities to selectively enhance for physiologies that are

needed for enhanced oil recovery. However, further investigations should be done to determine minimal concentrations of each nutrient needed to select for biosurfactant-producing bacilli.

A puzzling result is that a decrease of surface tension and the lipopeptide biosurfactant were not detected in any of the microcosms, even those where 90% of the cultivable cells contained the biosurfactant genes. Previous publications noticed the loss of the biosurfactant during aging of the culture (Jenneman et al., 1983, Lin et al., 1996, 1998). Due to sample size limitations, we were only able to take one sample for surface tension measurements from each microcosm. This was done after microbial metabolism and growth ceased (30 days). In many cases, viable cell numbers had already begun to decline by 30 days. It is entirely possible that the biosurfactant was synthesized and disappeared from the culture by the time we sampled. However, we do know that the most numerous organisms isolated from these microcosms did produce a biosurfactant when grown in liquid culture.

This work confirms our studies with sand-packed columns that showed the growth of JF-2 and the production of 2.3-butanediol and biosurfactant after inoculation and addition of glucose, nitrate and Proteose peptone. These sand-packs were not sterilized before inoculation, but this shows that our nutrient regime will allow strain JF-2 to establish itself in a mixed microbial community under the salinities seen in many oil reservoirs. This is an important point since many oil reservoirs may not contain an indigenous population of microorganisms that make biosurfactants. Our work shows that, in model systems, we can inoculate with *B. mojavensis* strain JF-2 and that it can maintain itself and synthesize its biosurfactant when specific nutrients: glucose, Proteose peptone and nitrate are added.

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Table 4.1. Glucose consumption and acetate and 2,3-butanediol production in microcosms inoculated with *B. mojavensis* strain JF-2.[1]

Treatment	Glucose (mM)		Acetate (mM)		Butanediol (mM)	
	Day 1	Day 10	Day 1	Day 10	Day 1	Day 10
Glucose	5.0±1.95	0.2±0.05	0.4±0.4	18±5.3	0.0±0.00	0.0±0.00
Glucose + high NO_3^-	5.2±0.04	0.2±0.05	0.4±0.07	0.7±0.4	0.0±0.00	0.0±0.00
Glucose + low NO_3^-	5.3±0.24	0.2±0.3	0.3±0.3	18±0.4	0.0±0.00	0.0±0.00
Glucose + high NO_3^- + PP[2]	1.8±0.7	0.4±0.04	12±5.2	4.9±3.7	0.34±0.2	0.33±0.1
Glucose + low NO_3^- + PP[2]	2.3±2.6	0.3±0.03	13±1.1	32±0.2	0.46±0.2	0.69± 0.4
Glucose + high NO_3^- + PP + $PO_4^=$	1.5±0.5	0.30.03	13±2.1	11±6.6	0.44±0.1	0.76± 0.4
Glucose + low NO_3^- + PP + $PO_4^=$	0.6±0.1	0.3±0.06	14±2.5	33±1.6	0.69±0.4	0.65± 0.03
PP + high NO_3^-	1.6±0.1	0.3±0.03	1.6±0.2	6.0±1.1	0.43±0.6	0.22± 0.02
PP + low NO_3^-	0.6±0.02	0.2±0.01	2.9±1.6	12±5.3	0.1±0.03	0.41± 0.2
PP	0.5±0.06	0.3±0.04	3.6±0.4	19±3.2	0.2±0.16	0.71± 0.04
Unamended	0.0±0.0	0.0±0.00	0.1±0.2	1.7±0.4	0.0±0.00	0.0±0.00

[1]The data are means ± standard deviations of triplicate incubations. Microcosms were incubated anaerobically at 23°C.

[2]Abbreviations: PP, Proteose peptone.

Table 4.2. Nitrate utilization and ammonium production in microcosms inoculated with *B. mojavensis* strain JF-2.[1]

Treatment	Nitrate (mM)		Ammonium (mM)	
	Day 1	Day 10	Day 1	Day 10
Glucose	0.5±0.01	0.5±0.00	2.4±1.7	3.0±0.4
Glucose + high NO_3^-	27±2.6	4.2±1.5	2.3±0.6	2.4±2.1
Glucose + low NO_3^-	1.4±0.1	0.5±0.00	2.3±0.9	3.8±0.5
Glucose + high NO_3^- + PP[2]	8.2±5.0	0.5±0.00	3.6±1.6	11±1.0
Glucose + low NO_3^- + PP	0.5±0.005	0.5±0.00	4.9±1.4	11±2.6
Glucose + high NO_3^- + PP + $PO_4^=$	16±7.9	0.5±0.00	3.7±2.4	9.2±2.1
Glucose + low NO_3^- + PP + $PO_4^=$	0.5±0.01	0.5±0.00	5.5±1.3	9.4±1.3
PP + high NO_3^-	13±3.8	0.5±0.00	2.4±2.1	10±3.4
PP + low NO_3^-	0.5±0.004	0.5±0.00	4.3±1.0	12±2.9
PP	0.5±0.003	0.5±0.00	4.6. ±04	8.6±2.1
Unamended	0.5±0.04	0.5±0.00	1.4±1.2	2.4±2.1

[1]The data are means ± standard deviations of triplicate incubations. Microcosms were incubated anaerobically at 23^{o}C.

[2]Abbreviations: PP, Proteose peptone.

Table 4.3. Acetate and 2,3-butanediol production and glucose consumption in groundwater that was not inoculated with *B. mojavensis* strain JF-2.

Treatment	Glucose (mM)		Acetate (mM)		Butanediol (mM)	
	Initial	Day 10	Initial	Day 10	Initial	Day 10
Glucose	3.4±0.7[2]	2.8±1.4	0.02±0.03	4.5±0.03	ND[1]	0.07±0.09
Glucose + high NO_3^-	3.8±0.04	3.7±0.2	0.2±0.3	ND	ND	0.0±0.0
Glucose + low NO_3^-	3.5±0.5	3.6±0.2	0.09±0.01	0.7	ND	0.01±0.02
PP[1]	0.3±0.03	0.1±0.01	0.1±0.05	22±1.1	ND	0.77±0.08
PP + high NO_3^-	0.7±0.02	0.2±0.01	0.07±0.03	8.5±1.2	ND	0.07±0.08
PP + low NO_3^-	0.5±0.02	0.2±0.01	0.1±0.03	19±0.6	ND	0.45±0.2
Unamended	0.08±0.0	0.1±0.01	0.1±0.01	ND	ND	0.0±0.0

[1] Abbreviations: PP, Proteose peptone; ND, not detected.

[2]The data are means ± standard deviations of triplicate incubations. Microcosms were incubated anaerobically at 23^{o}C.

Table 4.4. Effect of nutrient additions on nitrate and ammonium concentrations in groundwater that was not inoculated with *B. mojavensis* strain JF-2.

Treatment	Nitrate (mM)		Ammonium (mM)	
	Initial	Day 10	Initial	Day 10
Glucose	0.0 ± 0.0	0.14 ± 0.01	5.3 ± 0.2	5.7 ± 0.4
Glucose + high NO_3^-	35.6 ± 4.0	0.1 ± 0.02	5.6 ± 0.6	5.6 ± 0.6
Glucose + low NO_3^-	0.1 ± 0.02	0.05 ± 0.05	4.7 ± 0.5	6.2 ± 1.1
PP[1]	0.1 ± 0.0	0.007 ± 0.00	3.3 ± 2.1	4.7 ± 4.3
PP + high NO_3^-	36.2 ± 3.5	0.003 ± 0.00	3.9 ± 4.3	6.1 ± 2.7
PP + low NO_3^-	0.1 ± 0.02	0.007 ± 0.00	4.6 ± 3.6	4.8 ± 0.9
Unamended	0.1 ± 0.01	0.004 ± 0.00	5.2 ± 0.7	6.0 ± 0.4

[1] Abbreviations: PP, Proteose peptone.

Table 4.5. Viable cell concentration and number of organisms that contained biosurfactant genes in groundwater inoculated with *B. mojavensis* strain JF-2.

Treatment	Cell concentration (viable cells per ml)		Percentage of cells with biosurfactant genes[1]
	Day 2	Day 2	
Glucose	1.20E+08	TNTC[2]	-[3]
Glucose + 2.7% NO_3	8.40E+08	1.48E+09	90
Glucose + 0.1%NO_3	2.75E+08	8.12E+07	ND[2]
Glucose + 2.7% NO_3 + PP[2]	TNTC	1.32E+09	-
Glucose + 0.1%NO_3 + PP	1.38E+07	1.27E+06	4.3
Glucose + 2.7% NO_3 +PP + PO_4	3.37E+08	8.50E+07	-
Glucose + 0.1%NO_3 + PP + PO_4	3.35E+06	ADL	ND
PP + 2.7% NO_3	1.62E+09	2.87E+07	90
PP + 0.1% NO_3	2.61E+08	1.11E+07	ND
PP	1.19E+08	5.83E+07	15
Unamended	9.15E+06	ADL	-

[1] Cells of colonies from plate count agar were transferred to a membrane and then hybridized with a probe for the biosurfactant gene (*srfA*). The percentage corresponds to the number of total culturable cells that contained the biosurfactant gene.

[2] Abbreviations: PP, proteose peptone; TNTC, too numerous to count; ND, not detected.

[3] -, not determined.

Table 4.6. Confirmation that colonies that tested positive with the biosurfactant gene probe were physiologically similar to *B. mojavensis* strain JF-2.

Colony Type	Medium E				Nutrient Broth		
	Aerobic		Anaerobic		Aerobic		Anaerobi
	23°C	37°C	23°C	37°C	23°C	37°C	23°C
Positive[1] colonies	10/15	10/15	10/15	8/15	11/15	12/15	3/15
Negative colonies	0/15	0/15	0/15	0/15	0/15	0/15	0/15

[1]The positive colonies were those that hybridized with the probe *srf*A corresponding to the biosurfactant genes. The negative colonies were those that did not hybridize to the above probe, indicating that they did not carry the biosurfactant genes.

CHAPTER 5. BIOSURFACTANT-MEDIATED OIL RECOVERY IN MODEL POROUS SYSTEMS

ABSTRACT

Core displacement experiments at elevated pressures were conducted to determine whether microbial processes are effective under conditions that simulate those found in an actual oil reservoir. The *in situ* growth of *Bacillus mojavensis* strain JF-2 resulted in the recovery of about 23% of the residual oil remaining in Berea sandstone cores after waterflooding. Oil recovery by *B. mojavensis* strain JF-2 was highly correlated to surfactant production. A biosurfactant-deficient mutant of strain JF-2 did not recover residual oil. Substantial amount of residual oil (up to 43%) was recovered when sufficient concentration of the biosurfactant was used in conjunction with a mobility control agent.

INTRODUCTION

Enhanced oil recovery due to microbial activity is a commercially viable technology in the petroleum industry, with thousands of wells treated on a yearly basis in the United States (1, 2). Several well-controlled field trials have shown that additional oil is recovered and that the economics of microbially enhanced oil recovery (MEOR) are very attractive (3,4). Although these results are promising, the implementation of MEOR still suffers from a lack of understanding of how microorganisms can recover oil. In order to develop MEOR as a commercially viable technology more fully, a greater understanding of the mechanisms of MEOR processes is needed. Chapter 1 of this report reviews this problem in detail.

After a reservoir has been waterflooded, brine, gas, and small droplets of oil remain trapped within pores of the reservoir medium. Many tertiary processes currently being employed can increase the recovery of this trapped oil. These processes include *in situ* combustion, CO_2 and chemical flooding, and selective plugging. In MEOR, the growth and metabolism of indigenous bacteria are stimulated in the reservoir. The bacteria produce products similar to those used in chemical flooding such as carbon dioxide, solvents, surfactants, and polymers. Since microorganisms use relatively inexpensive materials to synthesize these products, MEOR

may provide a cost-effective alternative to the above methods. Laboratory studies conducted by Bryant and Douglas (5) suggest that the production of gas, solvents, organic acids, and surfactants are important mechanisms for microbially enhanced oil recovery in sandstone cores. However, it is not clear whether all of these products need to be produced, and which of these products is the most effective for the recovery of oil (6).

One of the main mechanisms that limits the ultimate recovery of oil is the entrapment of oil in microscopic pores by capillary forces. Only when the interfacial tension between oil and water is lowered by a factor of about a thousand-fold will significant amounts of residual oil be recovered. Biosurfactants are a potentially important mechanism for oil recovery since they can significantly reduce the interfacial tension between the oil and brine. In chemical surfactant flooding, much of the surfactant adheres to the surface of the reservoir rock near the well-bore. This decreases the concentration of the surfactant to levels below that needed to recover residual oil. A potential advantage of microbial processes is that the surfactant is produced *in situ*, near the site where it is needed to recover residual oil. However, since biosurfactant-producing organisms have other activities that could also result in oil recovery, it is difficult to determine whether surfactant production alone is a major mechanism for oil recovery by microorganisms.

The objectives of our work were first to determine whether microbial processes could recover residual oil at pressures that exist in actual oil reservoirs. Second, the importance of biosurfactant production for the recovery of residual oil was studied. In these studies, a biosurfactant-producing, microorganism called *Bacillus mojavensis* strain JF-2 was used. This bacterium produces a cyclic peptide biosurfactant that significantly reduces the interfacial tension between oil and brine (7). Chapters 2, 3, and 4 of this report cover the molecular biology, physiology and ecology of this organism. The use of a mutant deficient in surfactant production and a mathematical MEOR simulator were used to determine the major mechanisms of oil recovery by these two strains. Lastly, we studied the effect of biosurfactant concentration and mobility control on the effectiveness of biosurfactant-mediated oil recovery.

MATERIALS AND METHODS

Organisms. *Bacillus mojavensis* strain JF-2 was obtained from our culture collection.

Media and Conditions of Cultivation. Composition of the medium used to grow *B. mojavensis* strain JF-2 in liquid culture and in the sandstone cores is shown in Table 5.1. The medium contained inorganic minerals and organic growth factors, a buffer, and glucose as the sole energy source. Anaerobic growth of *B. mojavensis* strain JF-2 also required the addition of sodium nitrate as an electron acceptor, and maximal surfactant production by this strain required high amounts of sodium chloride. *B. mojavensis* strain JF-2 and a non-surfactant-producing mutant of this strain were also grown in medium E (see 8 for composition) that was modified by the addition of 0.5 g/l each of yeast extract and sodium nitrate.

Procedures for the preparation and use of anaerobic media and solutions were those of Byrant (9) and Balch and Wolfe (10).

Isolation of non-surfactant-producing mutant. A spontaneous mutant of *B. mojavensis* strain JF-2 that no longer lowered the surface tension of the medium was obtained by selection for non-hemolytic colonies on blood agar plates (11). The parent strain of JF-2 was grown aerobically in modified medium E at 37^{O}C, and transferred to sterile medium when the culture reached the stationary phase of growth. The culture was serially transferred in this manner twenty times to enrich for non-surfactant-producing cells. The non-surfactant-producing mutant was isolated by serial dilution and inoculation of blood agar plates. Non-hemolytic colonies were picked and restreaked to obtain a pure culture.

Sandstone core flow apparatus. Berea sandstone cores were steam-cleaned for two weeks to remove humic acids and other organic materials. After steam cleaning, the cores were dried at 125^{O}C for 24 hours, and then placed in a vacuum dessicator to cool. Each core was wrapped with Teflon™ tape, and then inserted into a rubber sleeve. Liquid gasket material was applied to the ends of the rubber sleeve to prevent leaks. The rubber sleeve with the core was placed in a stainless steel cylindrical core holder. The core holder was connected to the core flow apparatus using stainless steel tubing and compression fittings. The components of the system were rated to operating pressures of about 35,000 kPa.

The core holder was placed inside of a constant temperature oven to maintain the temperature at 36^{o}C. Pressure gauges were attached to monitor the confining pressure and the pore pressure. A back-pressure regulator located on the effluent side of the core was used to control the pore pressure. A stainless steel transfer vessel operated at a pressure equal to that of the pore pressure of the core was used to inject fluids into the core. A piston-driven pump was

used to displace the fluid from the transfer vessel into the core. A plastic syringe located downstream of the back-pressure regulator was used to collect liquid and gas samples. Prior to use, the core apparatus was pressurized with nitrogen for several days to check for leaks.

Sandstone core flow experiments. Petrophysical data for the cores used in this study are given in Table 5.2. To determine whether the *in situ* generation of microbial products at reservoir pressures enhances oil recovery, cores were incubated at an initial pore pressure of about 7,000 kPa. A 50 g/l sodium chloride solution was used to waterflood each core after the incubation period.

Each core was vacuum-saturated with the 50 g/l sodium chloride solution. After saturation, core was inserted into the rubber sleeve and then placed inside of the core holder. The core holder was connected to the flow system, and the core was flooded with ten pore volumes of the 50 g/l sodium chloride solution containing 0.1 M $CaCl_2$ to stabilize clay particles, and 50 ml/l of methanol to disinfect the core apparatus. The core apparatus was incubated for twenty-four hours with the 50 g/l sodium chloride solution containing calcium chloride and methanol. The apparatus was then flushed with ten pore volumes of the respective brine solution without methanol and calcium chloride. The core was flooded with oil to connate water saturation, and then flooded with the respective brine to residual oil saturation (12).

To determine whether the disinfection procedure was effective, an uninoculated core was aseptically flooded with sterile medium and incubated. Neither the presence of viable cells in the effluent nor an increase in pore pressure was observed after five days of incubation.

A series of nutrient treatments were performed on each core after the core was flooded to residual oil saturation. Each core received from three to five pore volumes of culture medium containing a 5% (vol/vol) inoculum of a culture of *B. mojavensis* strain JF-2. The core was incubated without fluid flow until no further change in the pore pressure was observed. The core was then flooded with about five pore volumes of the respective brine solution. The amounts of oil, gas, and brine collected after each treatment were measured volumetrically.

Table 5.3 shows the actual amounts of culture medium and brine that each core received and the incubation times of each treatment.

Preparation of the biosurfactant solution. *B. mojavensis* strain JF-2 was grown anaerobically in Medium E with Proteose peptone in one-liter volumes using 2-liter Schott bottles. The composition of Medium E with Proteose peptone and the procedures for anaerobic

growth are described in Chapter 3 of this report. After growth ceased, the cells were removed from the medium by centrifugation (10, 000 x g; 20 min; 10^{o}C). The supernate was removed and the pH of the cell-free medium was brought to less than 2 by the addition of 50% HCl. The acidified cell-free medium was left at 4^{o}C overnight. This procedure resulted in the precipitation of the biosurfactant. This precipitated material was removed by centrifugation as described above. The pH of the biosurfactant-free, cell-free medium was adjusted to 7.0 by the addition of pellets of NaOH. This neutralized, biosurfactant-free, cell-free medium was used to prepare the biosurfactant solution used in the column studies.

Large amounts of the biosurfactant were obtained by growing *B. mojavensis* strain JF-2 aerobically in carboys containing 8 liters of Medium E without Proteose peptone. After growth ceased, the pH of the medium was adjusted to less than 2 by the addition of concentrated HCl. The acidified medium was kept overnight at 4^{o}C to precipitate the biosurfactant. The medium was centrifuged as described above. The supernate was discarded and the pellet was dissolved in 200 ml of methanol. The methanol solution was centrifuged as above to remove particulate material. The concentration of the biosurfactant in the methanol solution was measured by high pressure liquid chromatography as described in Chapter 3. This value was used to determine the volume of the methanol solution that had to be added to the neutralized, biosurfactant-free, cell-free medium to give needed biosurfactant concentration. This solution is designated as the biosurfactant solution.

Preparation of sand-packed columns. Plexiglas columns approximately 4.5 cm (inside diameter) by 40 cm long were used. The end plates of the column had an opening for a threaded fitting that was sealed with a rubber septum. A fine mesh nylon screen was placed over the opening on the inside of the end plate to prevent sand from plugging the passageway. The column pieces were assembled and then weighed. The top end plate was removed and the column were filled with quartz sand (approximately 100 mesh grain size). The side of the column was gently tapped during filling to ensure homogeneous packing. After packing, the top end plated was inserted and the column was weighed. The weight of the sand was calculated from the difference of the two measurements. The internal volume of the column was calculated from the internal diameter and length. The pore volume and porosity of each column was calculated from the internal volume, the weight of sand, and the density of the sand (ρ_{sand} = 2.65 gm/cc).

Air was removed from each column by connecting the column to a vacuum source for 10 minutes. A syringe needle attached to nylon tubing was inserted into the septum of the bottom end of the column. The tubing was connected to the vacuum source. A 5% NaCl brine solution was used to saturate and flood all columns. The vacuum line was removed and a syringe needle attached to nylon tubing connected to a reservoir of 5% NaCl was inserted into the bottom septum. The brine reservoir was placed under positive pressure by inserting a syringe needle attached to nylon tubing connected to nitrogen gas cylinder. The brine flowed into the column by positive displacement. Once the brine reached the top of the column, a syringe needle was inserted into the top septum to allow brine to be discharged from the column. One pore volume of brine was flushed through the column to ensure that it was completely saturated. At this time, the flow rate of brine was measured with a stopwatch and a graduated cylinder. The injection pressure was obtained from a pressure gauge attached between the brine reservoir and the column. The initial permeability of the column to brine was calculated from the flow rate and injection pressure according to Darcy's law. The column was then weighed and the volume of brine inside the column was calculated from the difference in the wet and dry weight of the column and the brine density.

An oil reservoir with a nitrogen gas phase was connected to nylon tubing attached to a syringe needle. The syringe needle was inserted into the bottom septum of the column as described above and a syringe needle attached to nylon tubing was inserted into the top septum to serve as the discharge point. Oil flowed upward by positive displacement by keeping the oil reservoir pressurized with nitrogen gas. The displaced water was collected in a graduated cylinder and the volume measured. After only oil was displaced from the column, the flow rate and injection pressure were determined as described above. These data were used to calculate the effective permeability of the column to oil at residual water saturation. The amount of residual water present in the column was calculated from the amount of water displaced from the column during oil flooding and the amount of water present after brine saturation. The weight of the column was measured and the amount of residual water saturation was determined from the differences in weight before and after oil saturation and from the densities of oil and brine.

The column was then flooded to residual oil saturation. The syringe needle attached to tubing connected to the oil reservoir was removed. A syringe needle attached to tubing connected to the brine reservoir was inserted into the bottom septum of the column. Brine was

injected into the column by positive displacement as described above. The amount oil displaced from the column was determined volumetrically using a graduated cylinder. This value was used to calculate the residual oil saturation from difference in oil volume before and after brine flooding. After water breakthrough, the flow rate of brine and injection pressure were determined as described above. These data were used to calculate the effective permeability of the column to brine at residual oil saturation. Six pore volumes of brine were injected through the column to ensure that it was at residual oil saturation. Permeabilities of the sand-packed columns are shown in Table 5.4.

Effect of biosurfactant concentration on oil recovery. The sand-packed column was flooded with a biosurfactant solution using the procedures for brine flooding. A syringe needle attached to nylon tubing connected to a reservoir of the biosurfactant solution was inserted into the bottom septum of the column. The biosurfactant solution was injected into the column by positive displacement as described above. All columns were flooded with 200 ml of the biosurfactant solution. This amount was approximately 2 pore volumes. Effluent from the columns was collected in 50-ml syringes that were held in a vertical position. This allowed the separation of oil and brine into separate phases in the syringe so that the volumes of each phase could be measured. The effect of biosurfactant concentration on oil recovery was determined by injecting different concentrations of the biosurfactant into the column. Duplicate columns were used for each biosurfactant concentration tested.

Some columns received 60-ml of a biosurfactant solution that contained 0.1 % by weight of partially hydrolyzed polyacrylamide (PHPA). Five ml of the PHPA solution was injected into the column. Next, 60 ml of the biosurfactant solution was injected into the column. This was followed by the injection of 25 ml of a 0.1% PHPA solution in 5% NaCl. Each column was then flooded with 150 ml of 5% NaCl and the effluent was collected in 30-ml aliquots. As a control, duplicate columns were treated as described above, but received the PHPA solution without the biosurfactant added.

Analyses. Absolute permeability, porosity, pore volume, connate water saturation, residual oil saturation, and effective permeabilities to oil and water were determined as described (12, 13, 14, 17). The permeability reduction factor (in percent) was calculated from the change in permeability after each treatment (15). Pore pressure was measured using a calibrated gauge.

The amount of gas produced was estimated volumetrically by using a plastic syringe connected to the effluent flow line. Organic acids and alcohols were quantified by using high pressure liquid chromatography (HPLC) and gas chromatography, respectively (15, 16). Carbon dioxide was measured by gas chromatography, and hydrogen was measured using a gas chromatograph equipped with a mercury reduction detector (18). Glucose was measured using the glucose oxidase method (Sigma, Inc., St. Louis, MO). Since the HPLC method did not separate lactate and succinate, the succinate dehydrogenase assay (Sigma, Inc., St. Louis, MO) was used to check for the presence of succinate. Succinate was not detected.

The amounts of products produced by liquid cultures were corrected for the small amounts of these products present in the medium at the start of incubation, and for the amounts of these products produced in control cultures that lacked glucose. The amounts of fermentation products produced during *in situ* growth in cores were corrected for the small amounts of the products in the influent medium.

Growth of liquid cultures was measured spectrophotometrically by following the change in absorbance with time. Cell concentration was determined by quantifying the amount of whole cell protein. Samples were centrifuged (12, 000 x *g*) for 2 minutes to collect the cells. The cell pellet was washed twice by resuspending the pellet in a 10 mM sodium/potassium phosphate buffer (pH 7.2) and recentrifuging. The final cell pellet was resuspended in 0.1 N NaOH and incubated at 70^{o}C. Protein was determined colorimetrically using bovine serum albumin as the standard (19).

Surface tension of cultures and core effluents was measured using a DeNoy ring and an automated tensiometer (7). The relative amount of surfactant produced was estimated from the number of units of surfactant activity as described previously (7) or measured quantitatively by the HPLC method (Chapter 3).

RESULTS

Fermentation Studies. The fermentation balance for *B. mojavensis* strain JF-2 was incomplete since this strain produced a large amount of an unknown metabolite. Of the identified products, 47.2 mmoles of lactate, 21.8 mmoles of acetate, 13 mmoles of propionate, and 21.8 mmoles of CO_2 (calculated) were produced from 100 mmoles of glucose. The carbon recovery

was 41%. The unidentified product migrated between isobutyrate and butyrate on both gas chromatography and HPLC. Assuming that the unidentified peak has four carbons and has a similar detector response as butyrate, about 80 mmol were produced per 100 mmoles of glucose. This would give a carbon recovery of about 95%.

A conversion factor that relates the units of surfactant activity to the moles of biosurfactant was calculated (7). We found that the most purified fraction of the biosurfactant contained 1090 units of activity per mg (dry weight) (8). Lin et al. (20) reported a molecular weight of the JF-2 biosurfactant of 1035 g/mole. Assuming that our most pure fraction contains only the JF-2 biosurfactant, then one mole of the biosurfactant would have 1.1×10^9 units of activity. This conversion factor can be used to estimate the molar concentration of the JF-2 biosurfactant produced in core experiments by determining the number of units of surfactant activity present.

Isolation of a biosurfactant mutant. Cultures of strain JF-2 lost the ability to reduce the surface tension of the medium when they were repeatedly transferred in liquid medium. We noticed that the cultures that had been repetitively transferred had a large number of smooth colonies, while those that had been inoculated directly from an agar colony had rough colonies, with only a very small percentage of smooth colonies (<0.2%). We hypothesized that the ability to produce the surfactant was not a stable trait of JF-2, and that smooth colonies were mutants of JF-2 that did not produce the biosurfactant (21). We tested this hypothesis by determining whether smooth colonies were hemolytic and whether they had surfactant activity.

The number of rough and smooth colonies in cultures that had been transferred 15 times was compared to that found in cultures that were inoculated directly from a rough colony. Cultures that had been transferred 15 times in liquid medium had about 1.5×10^9 smooth colonies per milliliter. No rough colonies were observed ($<10^7$ colonies/ml). The surface tension of these cultures was greater than 40 mN/m. With some of the cultures, the surface tension was as high as 60 mN/m. Cultures that were inoculated directly from a rough colony had predominantly rough colonies (about 2.4×10^9 colonies/ml) and very few smooth colonies (about 1×10^7 colonies/ml). These cultures always had surface tensions below 30 mN/m. One culture that had been repetitively transferred and one that had been inoculated directly from a rough colony were plated onto blood agar medium to determine the number of hemolytic and

non-hemolytic colonies. All of the colonies that grew on plates inoculated with culture that had had not been transferred were hemolytic. Blood agar plates inoculated with cultures that had been transferred 15 times had a large number of non-hemolytic colonies; about 69% of all of the colonies were non-hemolytic. These data suggest that selection for rough colony morphology was required to maintain biosurfactant production by strain JF-2, and that repetitive transfer in liquid medium is not recommended for the maintenance of the strain.

One of these non-hemolytic colonies was picked and restreaked onto blood agar medium to obtain a pure culture of a biosurfactant-deficient mutant of JF-2. When grown in liquid culture, the JF-2 mutant did not lower the surface tension of the medium below 60 mN/m, which was close to the surface tension of uninoculated growth medium. As a comparison, the wild-type strain of JF-2 consistently lowered the surface tension of the medium below 30 mN/m and had a critical micelle dilution of 16. The biosurfactant-deficient mutant of JF-2 was non-hemolytic and formed smooth colonies. Otherwise, it had the same physiological properties as the wild-type strains. The mutant grew anaerobically at 45^{o}C in medium with 50 g/l NaCl. Fermentation products of the mutant were similar to those of the wild-type strain of JF-2 as were the morphology and Gram reaction. These data show that a spontaneous, biosurfactant-deficient mutant of strain JF-2 was obtained. The mutant strain will be useful as a negative control to determine the importance of surfactant production in MEOR.

Requirement for biosurfactant production for oil recovery in Berea sandstone cores. The biosurfactant-deficient mutant strain of JF-2 was used to determine the importance of biosurfactant production for oil recovery. Two cores (cores 3 and 4) were inoculated with the wild-type strain of JF-2 that had not been repetitively transferred (Table 5.5). One core was inoculated with a culture of JF-2 that had been transferred about 15 times (core 5) and one core was inoculated with a culture of the biosurfactant-deficient mutant strain (core 6). When the wild-type strain of JF-2 was used, 23 and 21% of the residual oil was recovered from cores 3 and 4, respectively. Analysis of the core effluents showed that small amounts of acetate (0.1 mM), butyrate (1.1 mM) and lactate (0.8 mM) were produced. Surface tensions of the effluents were below 30 mN/m. Microscopic analysis of the effluent showed that most of the turbidity in the aqueous phase was due to very small drops of oil, about the size of a bacterial cell. In contrast, effluent samples from the core inoculated with the serially transferred culture of JF-2 had a thin film of oil. When the JF-2 mutant was used, a small amount of oil (about 1 ml) was produced

after the first treatment. In the second treatment, only a thin film of oil was detected. Less than 6% of the residual oil was recovered when the mutant strain was used.

Figure 5.1 shows that oil recovery was highly correlated (r^2=0.979) to surfactant production for core 4. A similar trend was observed for core 3.

Relationship between oil recovery and biosurfactant concentration in Berea core experiments. Oil recovery from Berea sandstone cores was correlated to the cumulative amount of biosurfactant produced. In order to obtain a quantitative relationship between biosurfactant concentration and oil recovery, oil-saturated, sand-packed columns were flooded with different biosurfactant concentrations. The first experiment used biosurfactant concentrations ranging from 0 to 0.3% (wt/vol) (Table 5.6). Some oil was recovered during the first 50 ml of effluent collected for the columns. This was attributed to oil that was partially mobilized by the brine flood and may have represented oil the remained in the dead volume of the column or the tubing. For these reasons, the oil the was collected with the first 50 ml of effluent was not used to calculate oil recovery due to the action of the biosurfactant. Biosurfactant-mediated oil recoveries were low, ranging from 0.7 to 3.2 of the residual oil saturation (Table 5.6). The volume of oil recovered did increase when the biosurfactant concentration increased from 0.1 to 0.17%. However, the amount of oil recovered when the biosurfactant concentration was 0.3% was nearly identical to that when the biosurfactant concentration was 0.17%. Overall, the amount of oil recovered as a percentage of the residual oil saturation was low regardless of the biosurfactant concentration.

In order to determine if oil recovery was to due metabolically active cells that may have been inadvertently injected along with the biosurfactant solution, two of the columns were treated with nutrients to stimulate microbial growth and activity. In this experiment, the two columns that received the 0.3% biosurfactant solution were each flooded with 100 ml of medium E with Proteose Peptone (see Chapter 3 for composition). The two columns that did not receive any biosurfactant were each flooded with 100 ml of 5% NaCl solution. The two columns that received the 0.17% biosurfactant solution were incubated without any further treatment. All six of the columns were incubated at 37^{o}C for 2 days and then at 23^{o}C for 8 days. Each column was then flooded with 200 ml of 5% NaCl brine solution. Some additional oil was recovered from the two columns that initially received 0.17% biosurfactant solution (Table 5.7). However, oil was also recovered from the control columns that only received 5% NaCl. No additional oil was

recovered from the two columns that initially received 0.3% biosurfactant solution and then received nutrient medium. These data showed that the oil recovery was due to the biosurfactant activity and not to the production of other metabolic products by the bacteria or from the injection of cells into the column.

The low recoveries and drop in oil production after flooding with one pore volume of the biosurfactant solution may be due to the loss of biosurfactant within the column. As the solution is injected into the column, some loss of the biosurfactant would be expected due to adsorption onto the surface of the sand. Low biosurfactant concentrations would not lead to substantial oil recoveries. To test this hypothesis, a second series of columns was flooded with higher biosurfactant concentrations than used in the first experiment. The expectation was that the high biosurfactant concentrations would overcome any loss of biosurfactant due to adsorption. In this experiment, the biosurfactant concentration ranged from 0 to 1.23% (wt/vol). The latter value is comparable to the concentration of chemically synthesized surfactants used in EOR floods. The data in Table 5.8 show that increased biosurfactant concentrations did not lead to increased oil recoveries. Even when a 1.23% biosurfactant concentration was used, the oil recovery was substantially not different from that of columns that received only the 5% NaCl solution.

Effect of mobility control on oil recovery. In the columns that received the 1.23% biosurfactant solution, a visible oil bank formed as the biosurfactant solution entered the column. However, with continued flooding, the oil bank dissipated and the amount of residual oil recovered was not substantially different than control columns. This observation lead us to hypothesize that mobility control was required to maintain the stability of the oil bank. We tested this hypothesis by using a viscosifying agent to control the mobility of the biosurfactant flood (Table 5.9). Flooding the columns with the viscous biosurfactant solution (1.23% biosurfactant; 11.0 centipoise) resulted in the formation of a very noticeable oil bank. In one column, the oil recovery was 43% while, in the second column, the oil recovery was 25%. In the second column, the oil bank dissipated as it moved through an area that contained visible air pockets. This may have been the reason why oil recovery was lower in this column. Control columns that were flooded with the polymer only did not recover residual oil (data not shown). Figure 5.2 shows that almost all of the oil was recovered within small percentage of the total effluent volume collected.

DISCUSSION

While field trial indicate that MEOR processes are cost-effective technologies for oil recovery (2-4, 23), there are still a number of questions concerning the technical feasibility and the mechanisms by which oil is recovered by MEOR. Bryant and Lockhart (24) pointed to a number of factors that can adversely affect MEOR. Reaction and adsorption kinetics suggest that the concentration of nutrients and the products needed to recover oil would dissipate as the microbial front moves through the reservoir. Second, the mechanism by which microorganisms recover oil is unclear as is whether the required microbial metabolites would be produced in sufficient amounts to enhance oil recovery. Lastly, laboratory studies suggest that large volumes of nutrients and brine may be needed for substantial oil recovery (Table 5.5) (1-6). Our work directly addresses several of these concerns.

We have unequivocally shown that biosurfactant production is required for oil recovery by *B. mojavensis* strain JF-2. A mutant that lacks the ability to produce the lipopeptide biosurfactant did not recover substantial amounts of residual oil from Berea sandstone cores (Table 5.5). Also, cumulative oil production was highly correlated with cumulative oil recovery (Fig. 5.1). Use of the biosurfactant-producing strain of *B. mojavensis* resulted in oil recoveries of about 23%. Evidently, the in situ production of the biosurfactant is sufficient to result in substantial oil recovery. At least for this process, a clear mechanism for oil recovery has been shown and this mechanism does result in significant recovery of residual oil.

One problem with the sandstone core studies was the large volumes of nutrients and brine that were used during the experiment. While the design of the experiment was to test the mechanism of oil recovery, it is clear that nutrient volumes in excess of one pore volume would not be economical for oil recovery from actual reservoirs. Our work with sand-pack columns does show that realistic volumes of the biosurfactant (e.g., one pore volume or less) will result in substantial oil recovery if mobility of the biosurfactant slug is controlled. The addition of a viscosity agent together with the biosurfactant resulted in recovery of up to 43% of the residual oil. Such efficiencies mirror the results obtained within the more mature chemical flooding technologies (25). Secondly, when a mobility control agent present, most of the oil was recovered with a very small percentage of the total effluent volume (Fig. 5.2). Thus, large

volume of water would not have to be processed during a field application of biosurfactant-mediated oil recovery. This will make the technology more economically attractive.

We used sand-packed columns in order to have a large model system to study the relationship between oil recovery and biosurfactant concentrations. The large pore volume of the sand-pack columns provided a marked increase in the scale of the experimental system compared to Berea sandstone cores. Because of this the amounts of oil that were produced were easily quantified volumetrically. However, this system had high porosity and permeability, which made mobility control issues a dominant factor in controlling the effectiveness of the process. Once the viscosity of the biosurfactant solution was adjusted to match that of the oil phase, residual oil recoveries markedly increased. Oil recovery factors of 43% have not been previously reported in laboratory studies of biosurfactant-mediated oil recovery (see Chapter 1). We feel that our work represents a substantial breakthrough not only in the understanding of the mechanisms involved in MEOR but also in the technical feasibility of MEOR.

CONCLUSION

Our data show that the main mechanism for oil recovery by *B. mojavensis* strain JF-2 is biosurfactant production. Oil recovery is lost when the ability to produce the biosurfactant is lost. We also show that there is a strong correlation between the amount of biosurfactant produced by *B. mojavensis* strain JF-2 and the amount of oil recovered. Thus, factors that lead to greater production of the biosurfactant will result in increased oil recovery. Large amounts of residual oil are recovered (up to 43%) when sufficient concentrations of the biosurfactant are used in conjunction with a mobility control agent.

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Table 5.1. Medium components used for the growth of *Bacillus mojavensis* strain JF-2 in liquid culture and in sandstone cores.

Component	(g or ml/l)
Tanner's Minerals[a]	20.0 ml
Tanner's Metals[a]	10.0 ml
Glucose	1.8 g
Yeast Extract	5.0 g
NaCl	50.0 g
Piperazine-N,N'-bis[2-ethanesulfonic acid]	4.0 g
Balch Vitamins[a]	5.0 ml
$NaNO_3$	1.0 g

[a] Composition of solutions is given in reference 22.

Table 5.2. Petrophysical properties of Berea sandstone cores[a]

Core Number	3	4	5	6
Porosity (%)	19.0	14.0	17.0	17.5
Pore volume (ml)	113	85	103	105
Absolute permeability (μm^2)	0.18	0.10	0.29	0.26
Oil permeability (μm^2)	0.09	0.07	0.20	0.15
Connate water (ml)	41	30	33	35
Connate water saturation (%)	36	35	32	33
Water permeability (μm^2)	0.02	0.02	0.06	0.07
Residual oil (ml)	35	26	30	26
Residual oil saturation (%)	31	31	29	24

[a]All cores had a diameter of 5.08 cm and a length of 28.42-29.85 cm. These cores were used for experiments described in Tables 5.3 and 5.5.

Table 5.3. Treatment regimes for microbially enhanced oil recovery experiments in Berea sandstone core experiments.

Core No.	Organism	Treatment No.	Nutrient Volume (ml)	Brine Flood Volume (ml)	Incubation Time (days)
3	*B. mojavensis* (wild-type)	1	300	400	8
		2	300	400	9
		3	300	400	4
4	*B. mojavensis* (wild-type)	1	300	400	4
		2	300	400	5
		3	300	400	4
		4	300	400	5
		5	300	400	5
5	*B. mojavensis* (wild-type) serially transferred	1	250	400	5
		2	250	400	6
		3	250	320	2
6	*B. mojavensis* (mutant)	1	300	350	4
		2	300	350	5
		3	300	350	4

Table 5.4.The permeabilities of sand-pack columns used with high biosurfactant concentrations after brine saturation, oil saturation and brine flooding.[a]

Pack No.	1	2	3	4	5	6	7	8
$K_{abs,\ water}$ (D)	1.42	1.43	0.79	1.14	1.61	0.60	1.07	1.34
$K_{eff,\ oil}$ (D) at S_{wc}	0.87	1.33	0.69	0.60	1.29	0.12	1.14	1.00
$K_{eff,\ water}$ (D) at S_{wor}	0.50	0.25	0.53	0.86	0.92	0.23	0.90	0.88

[a] See methods for details.

Table 5.5. Experimental results obtained for Berea sandstone cores inoculated with *Bacillus mojavensis* strain JF-2.

Core No.	Treat-ment No.	Culture	Initial Pore Pressure (kPa)	Volume of Oil recovered (ml)	Residual Oil Recov-ery (%)	Gas Product-ion (ml)
3	1	Wild-type	7,770	3		<0.1
	2		7,840	2		1
	3		7,490	3	23	<0.1
4	1	Wild-type	7,350	1		<0.1
	2		7,490	2		<0.1
	3		7,350	2		<0.1
	4		7,350	0.5		<0.1
	5			<0.5	24	<0.1
5	1	Wild-type	7,350	<0.5		<0.1
	2	serially transfer-red	7,350	<0.5		<0.1
	3		7,350	ND	<3	<0.1
6	1	Mutant	7,350	1		<0.1
	2		7,350	<0.5		<0.1
	3		7,420	ND	<6	<0.1

[a] See Tables 5.1 for medium, 5.2 for the petrophysical properties of the cores, and 5.3 for treatment details.

[b]ND, not determined.

Table 5.5. continued.

Core No.	Surfactant (units)	PRF (%)[a]	Influent pH	Effluent pH
3	180	79	6.8	7.3
	120	90	6.9	7.3
	60	0.7	6.8	7
4	132	105	7	7.1
	116	118	6.8	7.2
	79	109	6.8	7.2
	57	96	6.9	7.2
	46	91	7.1	7.4
5	ND[b]	262	6.8	7.5
	165	257	6.9	7.4
	6	245	6.9	7
6	53	131	6.5	7
	ND	140	6.8	7
	ND	140	6.9	7.1

[a] PRF, permeability reduction factor is the percent decrease in initial permeability.

[b]ND, not determined.

Table 5.6. The effect of biosurfactant concentration on oil recovery from sand-packed columns.

Treatment	Residual oil (ml)	Residual oil (%)	Volume of oil (ml) in effluent fraction number				Oil[1] (ml)	Percent residual oil recovery (%)
			1	2	3	4		
5% NaCl	10.0	11.0	0.00	0.01	0.00	0.00	0.01	0.1
	9.80	10.0	0.25	0.01	0.00	0.00	0.01	0.1
0.1% LP[2]	7.90	9.0	0.1	0.01	0.1	0.02	0.13	1.6
	16.2	17.0	0.30	0.01	0.10	0.01	0.12	0.7
0.17% LP	11.8	14.0	0.14	0.05	0.10	0.15	0.30	2.5
	14.7	18.0	0.15	0.05	0.25	0.00	0.30	2.0
0.30% LP	18.5	22.0	0.20	0.02	0.10	0.15	0.27	1.4
	11.0	12.0	0.25	0.01	0.20	0.15	0.36	3.2

[1] Oil recovered: was corrected for oil collected with the first effluent fraction, which was not considered the result of biosurfactant action.

[2] Abbreviation: LP, lipopeptide biosurfactant concentration.

Table 5.7. The amount of oil recovered from sand-packs after nutrient addition and incubation.

Treatment	Volume of oil (ml) in effluent fraction number				Oil[1] (ml)	Percent recovery (%)
	1	2	3	4		
Brine	0.01	0.0	0.0	0.0	0.01	0.01
	0.1	0.0	0.0	0.0	0.1	1.0
None	0.15	0.1	0.0	0.0	0.25	3.2
	0.2	0.0	0.0	0.0	0.2	1.2
Medium	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0

[1] Oil recovered: was corrected for oil collected with the first effluent fraction, which was not considered the result of biosurfactant action.

Table 5.8. Oil recovery when high biosurfactant concentrations were used with sand-packed columns.

Treatment	Residual oil (ml)	Residual oil (%)	Volume of oil (ml) in effluent fraction number				Oil [1] (ml)	Oil recovery (%)
			1	2	3	4		
5.00 % NaCl	13.0	14.9	0.7	0.6	0.6	0.1	1.3	10.5
	22.0	24.4	1.0	0.4	0.4	0.2	1.0	4.8
Spent medium	21.5	23.1	2.0	0.1	0.3	0.5	1.0	5.1
	26.0	27.1	1.5	0.05	-[3]	-	-	-
0.17% LP[2]	20.0	22.2	0.5	0.1	0.1	0.1	0.3	1.5
	25.9	32.0	2.0	0.3	0.0	0.1	0.4	1.6
1.20% LP	21.0	23.5	0.6	1.0	0.2	0.05	1.2	6.4
	18.0	20.1	2.0	0.3	0.15	0.05	0.5	3.1

[1] Oil recovered: was corrected for oil collected with the first effluent fraction, which was not considered the result of biosurfactant action.

[2] Abbreviation: LP, lipopeptide biosurfactant concentration.

[3] Fluid could not be injected into the column.

Table 5.9. Oil recovery when a mobility control agent was used in conjunction with the biosurfactant in sand-packed columns

Column	Residual Oil (ml)	Residual Oil (%)	Volume of oil (ml) in effluent fraction number					Oil recovery (%)
			1	2	3	4	5	
1	11	2.5	0.2	2.0	0.2	0.2	5.1	46.3
2	20	2.5	0.1	2.0	0.2	0.2	5.0	25.0

Figure 1. Correlation between the cumulative amount of oil recovered and the cumulative amount of biosurfactant produced from a Berea sandstone core.

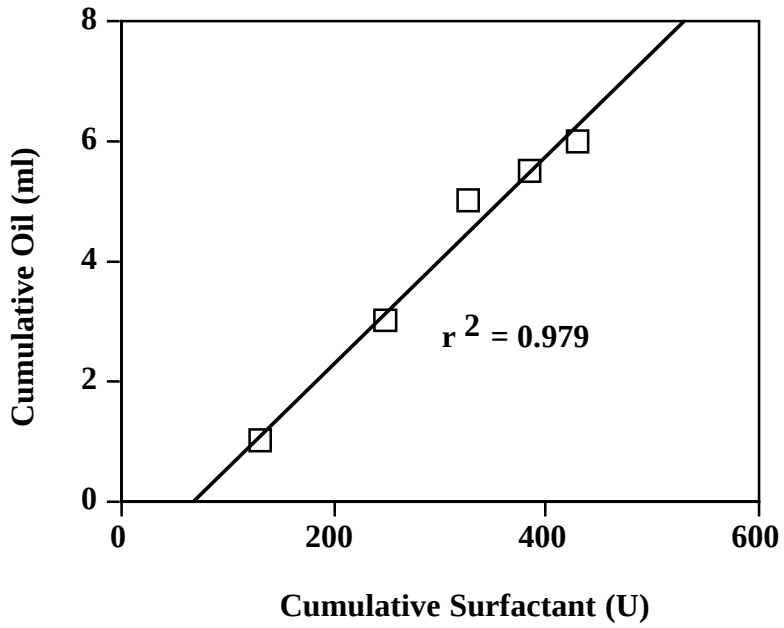
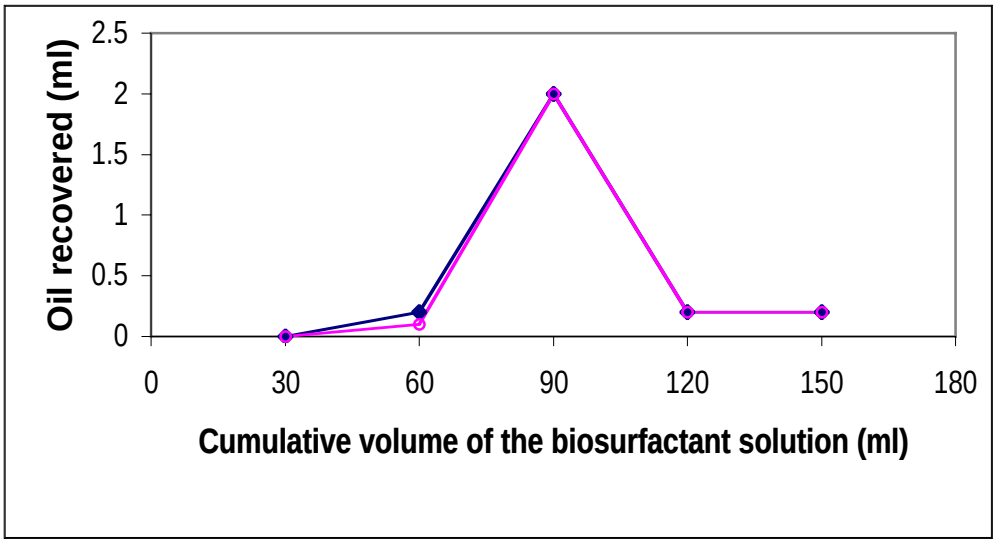


Figure 5.2. Oil production as a function of effluent volume when mobility control agent was used in conjunction with a high biosurfactant concentration in sand-packed columns.



CHAPTER 6. COMPUTER SIMULATIONS OF BIOSURFACTANT-MEDIATED OIL RECOVERY.

ABSTRACT

A microbially enhanced oil recovery simulator was used to study the effects of glucose concentration and inoculum size on the recovery of residual oil. First, a series of simulations were conducted at a constant glucose concentration with different concentrations of bacteria in the medium injected in the core. Next, a series of simulations were run where the inoculum concentration of the bacteria was constant and the glucose concentration was varied. The amount of oil recovered (about 89 ml) and the amount of biosurfactant produced (about 27 mg) did not vary when the inoculum size of the bacteria ranged from 1.1 to 9.5 ng/ml of cells when the glucose concentration was held constant at 20 g/l. The amount of oil recovered and the amount of biosurfactant produced increased with increasing glucose concentrations when the bacterial inoculum concentration was held constant at 9.5 ng of cells per ml. Increasing the glucose concentration from 6 to 10 g/l resulted in an almost 2-fold increase in oil recovery (47 to 80 ml). However, further increases in the glucose concentration (10 to 20 g/l) did not result in as dramatic increases in oil recovery (80 to 89 ml). This suggests that there is an optimal glucose concentration for oil recovery. Maximal oil recoveries approached 12% of the residual oil saturation at the higher glucose concentrations (10 to 20 g/l). Biosurfactant concentrations needed for residual oil recovery were estimated by computer simulations to be about 1 mg/l. These results indicate that biosurfactant-mediated oil recovery can lead to significant increases in oil recovery.

INTRODUCTION

To study the efficacy of microbially enhanced oil recovery (MEOR), a mathematical model that simulates the growth, transport and metabolism of microorganisms and nutrients in porous media was developed (1, 2) . It simulates bacterial transport, growth and the metabolism involved in MEOR. The mathematical model is a three-phase, multiple species, one-dimensional model. Convection and dispersion equations are incorporated into the model to characterize and quantify biomass production, product formation and nutrient utilization in the MEOR process.

The mathematical model includes component transport equations, a black oil model, microbial growth kinetic equations, a permeability reduction model and models of oil recovery processes.

A series of simulator runs were done to study the recovery of oil during a batch process at the laboratory scale. In these runs, nutrients and microorganisms were injected into the core and flow was stopped to allow time for microbial growth and metabolism to occur in the core. The purpose of these runs was to understand the effects of different glucose and cell concentrations on the amount of oil recovered. This information can be used to develop optimal injection protocols for MEOR processes and to determine the optimal conditions for oil recovery.

MATERIALS AND METHODS.

Mathematical Model used for the Simulation. The description of the model used for these studies has been previously described (1,2). For the simulations reported here, growth and metabolism were modeled as being limited by a single nutrient, e. g. glucose. The mechanism for oil recovery for this series of simulations was interfacial tension reduction by surfactants. Biosurfactants produced during the growth and metabolism of bacteria reduce the interfacial tension between the oil and water phases. This can allow the mobilization of residual oil trapped within the porous media (1). A 50-block linear core model was selected for this simulation. The core was assumed to have been initially flooded to residual oil saturation prior to the MEOR treatment. The inoculum injected into the core consisted of bacteria and the nutrients required for their growth. The simulation consisted of an injection period, an incubation period and a post incubation water flood. During the incubation period, microbial growth and metabolism resulted in biosurfactant production. The biosurfactant altered the interfacial tension between the water and the hydrocarbon phases and mobilized hydrocarbons. The hydrocarbons were flushed from the core by the water flood. The simulator output during the simulated water flood included effluent concentrations of different products as well as the amount of oil mobilized as the result of biosurfactant production. Based on these values, the total amount of biosurfactant produced and the oil recovery factor could be estimated.

Specification of the Simulation System.

Simulator model selected: 50 Block Linear Model

Dimensions of the model: 50 (3.04)(9.00)(9.00) ml with dimensions given as DX, DY, DY

Dimension of each block in the X-axis, DX: 3.04 cm

Dimension of each block in the Y-axis, DY: 9.004 cm

Dimension of each block in the Z-axis, DZ: 9.004 cm

Total Volume of the core: 12,250 ml.

Porosity of the core (ϕ): 20% or 0.20

Residual Oil saturation in the core before the start of the MEOR process: S_o = 30%

Water Saturation: S_w = 70%

Initial Oil in Core: 0.30(.20) (12253 ml) = 740 ml

Durations of the MEOR simulator runs.

Injection : 16 hours at 150 ml/hr

Incubation : 24 hours (No injection into the system)

Post Incubation Water Flood: 24.7 hours at 150 ml /hr of water

Model development. The mathematical model uses a three-dimensional reservoir model to simulate the recovery of residual oil from a waterflooded reservoir by using biosurfactants. The oil in the reservoir model is at or close to residual oil saturation. The model simulates the recovery of oil using biosurfactant through three sequential mechanisms.

1. The injection of nutrients and bacteria into the reservoir model. The transport of microorganisms, nutrients and metabolic products takes place in the aqueous phase due to viscous, capillary, gravity and dispersion forces.
2. The incubation of microorganisms inside the reservoir under reservoir conditions. During this stage, the production of surfactants occurs.
3. The injection of water with or without nutrients into the reservoir. Water under the influence of low oil-water interfacial tension will recover additional oil from the reservoir.

Model variables include:

a) Concentration of microorganisms in the injected solution.

b) Concentration of carbon nutrient in the solution

c) Concentration of nitrogen nutrient in the solution.

d) Amount of microorganisms and nutrient flooded into the porous medium.

e) Duration of incubation.

f) Post incubation flooding medium.

g) Reservoir heterogeneities – porosity, permeability and saturations.

The mechanism being studied for the recovery of residual oil was oil recovery by reduction in the interfacial tension between the injected water and the oil by the presence of a biosurfactant. The biosurfactant is produced in the reservoir during the incubation stage. First, microorganisms and nutrients are injected into the reservoir. The variables that could be controlled are the concentration and volume of nutrients and microorganisms flooded into the reservoir. The next stage is an incubation period where no injection of fluids into the reservoir occurs. During this time, the microorganisms grow and convert the nutrients into microbial cells and products, one of which is the biosurfactant. The specific yield (grams of biosurfactant per gram of substrate used) used in the simulation was obtained from experimental data. After incubation, a incubation, a waterflood is initiated.

Some of the relationships that were used in the recovery modeling are described below.

$$\text{Capillary Number, } N_{cp} = \frac{v\mu}{\sigma} \qquad (1)$$

Capillary number is the ratio of the viscous to capillary forces. Order of magnitude increases in capillary number increases are needed for significant recovery of the residual oil. In the biosurfactant-based microbial oil recovery process, the interfacial tension between the hydrocarbon and aqueous phase is reduced, which increases the magnitude of the capillary number. Once the waterflood is initiated, the water can now displace the mobile hydrocarbon phase. This mechanism is not as simple as has been described above. There are several issues that have to be considered. These include:

a) The dissipation of the surfactant as function of distance traveled,

b) The velocity of the water to be injected. The velocity depends on the injection pressure used, which is dictated by the parting pressure of the formation and the capabilities of the surface handling facilities.

c) The degree to which the interfacial tension has been lowered by the surfactant. This has a direct bearing on the amount of recoverable oil.

The surfactant is generated by the metabolism of carbon nutrients by the bacteria. An empirical equation for the rate of generation of products is given by:

$$R_p = \mu_{pm} \frac{C_s - C_{sc}}{K_{p/s} + C_s - C_{sc}} (C_b + \text{ó}\tilde{\text{n}}_b) \qquad (2)$$

where μ_{pm} is the maximum specific production rate of product '**p**'; $K_{p/s}$ is the saturation constant for formation of product p by consumption of substrate '**s**'; C_{sc} is the critical concentration of substrate '**s**', or the minimum amount of substrate required before the microorganisms start producing the biosurfactant.

In our study, the only product considered was the biosurfactant. The model has the potential to simulate the production of nitrogen gas, carbon dioxide, acetic acid, alcohol and polymer. Carbon dioxide is always produced irrespective of the substance under consideration. The substrate is considered to be the compound that is consumed by the microorganisms to produce the biosurfactant.

The following relation relates the interfacial tension to the concentration of the biosurfactant:

$$\text{Log}(\sigma_{ow}) = \text{Log}(\sigma_{ow}) + [\text{Log}(\frac{\text{ó}_{max}}{\text{ó}_{min}})] \left(\frac{C_{6,max} - C_6}{C_{6,max} - C_{6,min}} \right)^{e_s} \qquad (3)$$

where σ_{ow} is the instantaneous interfacial tension between the oil and aqueous phase;σ_{max} is the maximum interfacial tension between the oil and aqueous phase; and σ_{min} is the minimum interfacial tension between the oil and aqueous phase.

C_6 is the instantaneous biosurfactant concentration; $C_{6,max}$ is the maximum surfactant concentration; and $C_{6,min}$ is the minimum surfactant concentration; e_s is an exponent parameter.

The subscript '6' is used for the surfactant because in the model, the surfactant is numbered as component 6. This will make it easier to discuss the model in subsequent sections.

The reduction in interfacial tension increases the capillary number that makes oil mobile. The residual oil saturation is a function of the capillary number,

$$S_{or} = \left(S_{or}^{h}\right) + \left(S_{or}^{w} - S_{or}^{h} \right) T_{o1} \left[\log(N_{co}) + T_{o2}\right] \qquad \text{for} \quad S_{or}^{h} \le S_{or} \le S_{or}^{w} \qquad (4)$$

S_{or}^{w} , S_{or}^{h} are the residual oil saturation's at low and high capillary numbers; T_{o1} and T_{o2} are parameters related to the capillary desaturation curve and are defined as

$$T_{o1} = \left[\text{Log} \ \frac{N_{co}^{w}}{N_{co}^{h}} \ \right]^{-1} \qquad (5)$$

$$T_{o2} = -\text{Log}\left(N_{co}^{h}\right) \qquad (6)$$

where N_{co}^{w} and N_{co}^{h} are the low and high capillary numbers. These numbers are function of rock wettability and pore size distribution.

The following is the relation for the capillary number for the oil phase

$$N_{co} = \frac{\left|K \ \ \nabla \Phi_o\right|}{\sigma_{ow}} = \frac{\sqrt{K_x \frac{f\nabla\Phi_o}{fx} + K_y \frac{f\nabla\Phi_o}{fy} + K_z \frac{f\nabla\Phi_o}{fz}}}{\sigma_{ow}} \qquad (7)$$

where, K is the diagonal permeability tensor; Φ_o is the potential for the hydrocarbon phase; and σ_{ow} is the interfacial tension between the hydrocarbon and aqueous phases.

A linear model is used to define the relation between the oil-water capillary pressure and the oil water interfacial tension,

$$p_{cow} = p_{cow}^{w} \ \frac{\sigma_{ow} - \sigma_{min}}{\sigma_{max} - \sigma_{min}} \qquad (8)$$

where, p_{cow}^{w} is the oil water capillary pressure at low capillary number.

The residual oil saturation from the equation 4 is used to determine the phase relative permeabilities. Relative oil permeabilities to the water and oil phases are determined using the relation,

$$K_{ro}(S_o) = K_{ro}^{w}(S_o) + \frac{S_{or}^{w} - S_{or}}{S_{or}^{w} - S_{or}^{h}} K_{ro}^{h}(S_o) - K_{ro}^{h}(S_o) \quad (9)$$

$$K_{rw}(S_w) = K_{rw}^{w}(S_w) + \frac{S_{wr}^{w} - S_{wr}}{S_{wr}^{w} - S_{wr}^{h}} K_{rw}^{h}(S_w) - K_{rw}^{h}(S_w) \quad (10)$$

where, subscript w and h represent the conditions at high and low capillary numbers respectively. Relative permeabilities for oil and water phases at high capillary numbers are given by straight line models,

$$K_{ro}^{h} = S_o$$
$$K_{rw}^{h} = S_w$$

Treatments. Two studies were made. The first study used a constant bacterial concentration and varied the glucose concentration in the inoculum and the second study used a constant nutrient concentration and varied bacterial concentration in the inoculum 1) The first series of runs kept the bacterial concentration in the inoculum constant at 9.5 ng of cells/ml and varied the glucose concentration from 6 to 20 g/l .The injection of the inoculum was done at a rate of 150 ml/hr for 16 hours to ensure that one pore volume of fluid was injected into the core. The inoculum injection was followed by an incubation period of 24 hours and then a water flood was simulated and oil and biosurfactant produced were washed out of the core.

The second series of runs kept the concentration of the glucose in the injection fluid constant at 20 mg/ml (110 millimolar).The inoculum bacterial concentration varied from 1.1 to

9.5 ng of cell per ml. The injection period was for 16 hours at the rate of 150 ml/hr to ensure that 1.0 pore volume was injected into the core. This injection period was followed by incubation for 24 hours and then a water flood was simulated where the oil and surfactant were produced from the from the core.

RESULTS AND DISCUSSION

The amount of oil recovered and the amount of biosurfactant produced increased with increasing glucose concentrations when the bacterial inoculum concentration was held constant at 9.5 ng of cells per ml. Increasing the glucose concentration from 6 to 10 g/l resulted in an almost 2-fold increase in oil recovery (47 to 80 ml) (Table 6.1, Figure 6.1). However, further increases in the glucose concentration (10 to 20 g/l) did not result in as dramatic increases in oil recovery (80 to 89 ml). This suggests that there is an optimal glucose concentration for oil recovery. Maximal oil recoveries approached 12% of the residual oil saturation at the higher glucose concentrations (10 to 20 g/l). The surfactant produced varied linearly with glucose concentration in the inoculum.

Figure 6.2 shows the relationship between oil recovery and biosurfactant concentration. The data suggest that concentrations of about 1 to 3 mg/l will result in residual oil recoveries of about 10%.

The amount of oil recovered (about 89 ml) and the amount of biosurfactant produced (about 27 mg) did not vary when the inoculum size of the bacteria ranged from 1.1 to 9.5 ng of cells per ml when the glucose concentration was held constant at 20 g/l (Table 6.2). These data suggest that the amount of cells present in the inoculum is not critical to oil recovery. Apparently, enough time and nutrients were present to allow sufficient growth of the organism even though the initial concentration of bacterial cells varied.

CONCLUSIONS

From the observations that the amount of oil produced did not vary markedly when the glucose concentration was 10 g/l or greater suggests that there may be an optimum nutrient concentration to maximize the oil recovery factor. From the observations that the amount of oil produced and the total surfactant reached did not vary when different inoculum concentrations of cells were used suggests that if sufficient incubation time is allowed then the initial bacterial

concentrations will not be critical. Computer simulations indicate that the critical biosurfactant concentration that must be reached is about 1 mg/l. When this concentration is reached residual oil recovery occurs. The microbes will continue to grow until all the nutrients are consumed. These results can be used to develop a injection protocol that might be followed when injecting bacteria and nutrients into the core and to improve recovery of the trapped hydrocarbons on a field scale.

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Table 6.1. Simulation of microbial oil recovery at different glucose concentrations.

Glucose (mg /ml)	Glucose (μM)	Total Oil Produced (ml)	Recovery Factor (%)	Surfactant Produced (μg)
6.0	33.0	46.78	6.30	8045.0
8.0	44.0	72.63	9.80	10980.0
10.0	55.0	80.07	10.80	13680.0
12.0	66.0	83.75	11.29	16570.0
20.0	110.0	89.39	12.00	27540.0

Table 6.2. Simulation of Microbial Oil Recovery at different microbe concentrations.

Cell Concentration (ng/ml)	Total Oil Produced (ml)	Recovery Factor (%)	Total Surfactant Produced (μg)
8.50	89.78	12.10	27610
9.50	89.39	12.05	27540
1.05	89.71	12.09	27610
1.15	87.62	11.80	27570

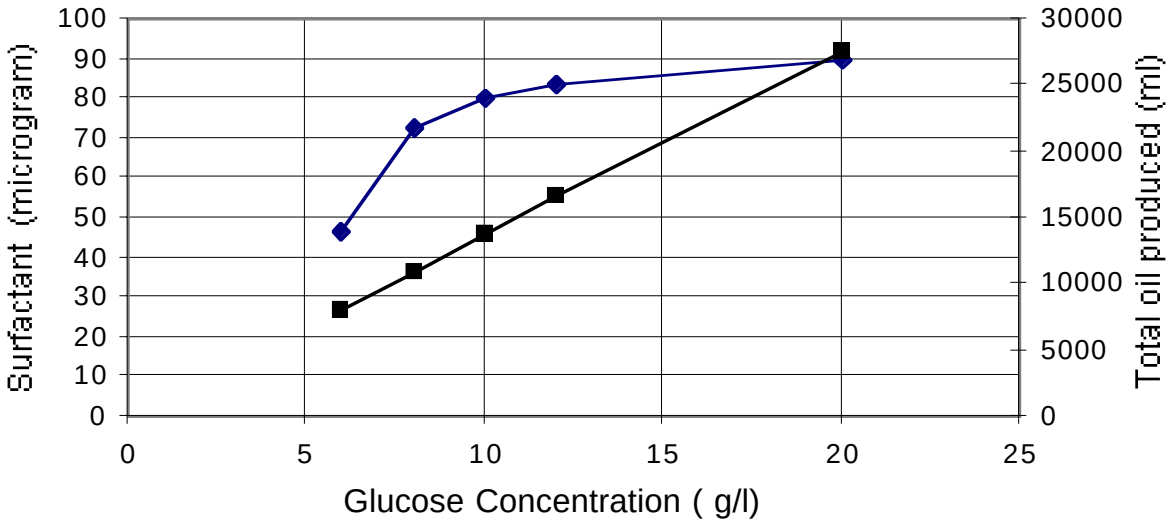
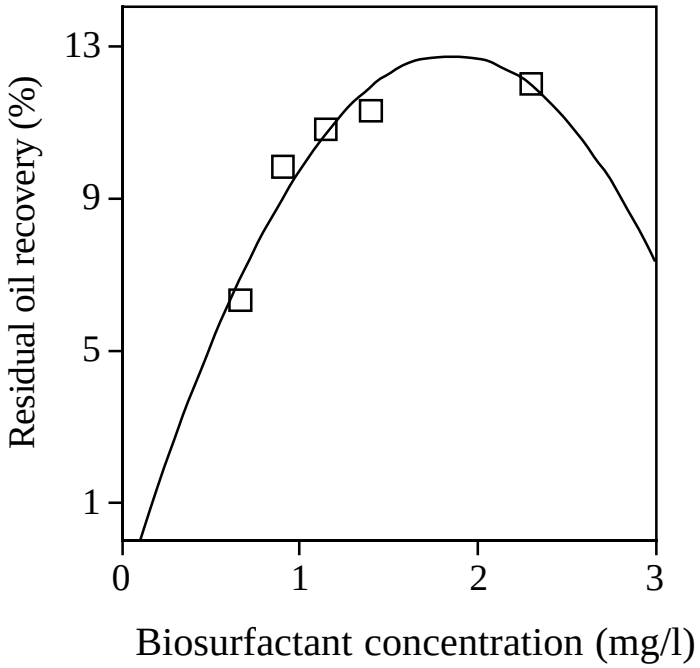


Figure 6.1. Plot of the oil and surfactant produced when nutrient concentration is varied and microbe concentration is kept constant in the inoculum (see Table 6.1). Symbols: Diamonds, oil produced; Squares, surfactant in the effluent.

Figure 6.2. Simulation results relating biosurfactant concentration to residual oil recovery. Data are from Table 6.1.



CHAPTER 7. ECONOMIC ANALYSIS OF THE MEOR TREATMENT ON FIELD SCALE: INTEGRATION OF THE INFORMATION OBTAINED FROM MICROBIAL AND ENGINEERING ANALYSES.

The analyses performed during this project have provided critical information required to conduct a preliminary analysis of the economics of oil recovery. In Chapter 5, we showed that the *in situ* production of a biosurfactant is the most important mechanism for residual oil recovery from Berea sandstone cores. These experiments take into account the production of other microbial products and alterations in permeability that could have influenced oil recovery. Also, these experiments were conducted at elevated pressures. This shows that biosurfactant production can occur under conditions that simulate actual oil reservoirs. Our subsequent work focused on quantifying biosurfactant production and its effect on oil recovery.

Computer simulations conducted in Chapter 6 provided us with an estimate of the critical biosurfactant concentration needed for residual oil recovery. Biosurfactant concentrations in excess of about 1 mg/l will result in residual oil recoveries of about 10% (Figure 6.2). In chapter 3, we showed that our biosurfactant-producing bacterium, *Bacillus mojavensis* strain JF-2, was able to generate biosurfactant concentrations in excess of this value when growth in the presence of a surface (e. g., glass beads) (Figure 3.4). Assuming that all of the glucose that was present in these incubations was completely utilized, we can calculate a specific yield that relates the amount of biosurfactant made to the amount of glucose used. This value is 1 milligram of biosurfactant per gram of glucose used. A second yield value was obtained from cultures of *B. mojavensis* strain JF-2 growing in liquid culture (Table 3.3). This value was about 0.02 milligrams of biosurfactant per gram of glucose used. The low yield in liquid culture supports our conclusion that a surface enhances biosurfactant production. Since the porous rock matrix provides a large surface area, we will use the specific biosurfactant yield value obtained when strain JF-2 is grown in the presence of a surface for the economic calculations below.

We should note that yield information for nitrogen sources and Proteose peptone have not been determined. Proteose peptone is an expensive material. If needed in large amounts, this would dramatically affect the economics of the process. However, our data in Chapter 5 indicate that biosurfactant production occurs in situ in Berea sandstone cores without Proteose peptone and Chapter 6 shows that indigenous biosurfactant-producing bacteria can be enriched with the

addition of glucose and nitrate. Based on these experimental findings, we have not included the cost of Proteose peptone in our analysis. Further research will hopefully result in the discovery of a low-cost substitute for Proteose peptone.

OIL REVENUE FROM A SIMULATED BIOSURFACTANT-MEDIATED OIL RECOVERY PROCESS

We will determine the amount of oil recovered and the revenue generated from a small domestic oil reservoir after a biosurfactant-mediated process has been conducted. We assume that the oil reservoir has been waterflooded to residual oil saturation. From Chapter 6, we predict that if the critical biosurfactant concentration is reached in the reservoir, we will recover about 10% of the residual oil (Figure 6.2).

$$\Delta Np = 0.10 \, (Vp \, Sowf) / Bo \, \eta$$

Assume that a single pattern in the field is 10 acres. The formation has a thickness of 35 feet and a porosity of 18.5%. These figures are representative of a single pattern in many mature, shallow, water-flooding projects in the United States. A typical, low-shrinkage oil might have an oil formation volume factor, Bo, of 1.1 [bbl/stb] at flooding conditions. The pattern has a pore volume, Vp, of 500,000 [bbls]. Also assume that the implementation of the MEOR process in the field will only be 50% efficient, η. From this information, the additional oil recovered by the biosurfactant-mediated MEOR process is:

$$\Delta Np = 0.10 \, (500{,}000[bbls] \, Sowf) / (1.1 \, [bbl/stb]) \, 0.5$$

or $$\Delta Np = 23{,}000 \, Sowf \, [stb].$$

Commonly, the oil saturation after water flooding is between 0.20 and 0.40 in well-managed mature floods. The amount of oil recovered from the pattern could be between 4.5 and 9.0 [mstb]. At an oil price of $20 per [stb], the incremental oil revenue from the MEOR process could range from $90,000 to $180,000 for the 10-acre pattern.

COST OF GLUCOSE FOR THE BIOSURFACTANT-MEDIATED MEOR PROCESS

The cost of the glucose was calculated from mass of biosurfactant made in the oil reservoir and the yield of biosurfactant per glucose used. The mass of biosurfactant, Msurf [kg] needed to attain the critical biosurfactant concentration was calculated as the product of the

number of reservoir pore volumes injected, NPV, the pore volume Vp [bbl], and the critical biosurfactant concentration, Xsurf, divided by the water formation volume factor, Bw, using the following relationship:

Msurf = NPV * Vp * Xsurf / Bw

Three values for the pattern pore volume injected, NPV, were used, 0.1, 0.5 and 1.0. The critical biosurfactant concentration of 1 mg/l was obtained from Figure 6.2 and was converted to a per barrel of reservoir fluid basis (160 mg/bbl). Bw was assumed to be 1.0 [bbl/stb].

Msurf = NPV * 500,000[bbl] * 160 [mg/stb] / (10^6 [mg/kg] *1.0 [bbl/stb])

Calculations showed that the injection of 0.1, 0.5 and 1.0 pore volumes of glucose would result in the production of 8, 40 and 80 kg of biosurfactant.

The cost of the glucose needed to make the above quantities of biosurfactant was calculated as follows:

Cost = [Msurfactant (kg) / Yield] x Cost of glucose ($/kg)

For the calculations shown in Table 7.1, three yield values were considered, 1mg/g, 10 mg/g and 100 mg/g (mg of biosurfactant per g of glucose used). The first value is similar to the data we obtained from our glass bead experiment (Figure 3.4). The other two values were used to determine the effect that improved production or better strains would have on the economics of the MEOR process. The glucose was assumed to cost $0.22/kg. The recoverable value from the MEOR process was calculated according to the following relationship:

Recoverable Value = (Value of oil recovered) / (Cost of glucose)

Our analyses suggest that a biosurfactant MEOR process is economic if implemented today (Table 7.1). Even if a complete pore volume of glucose nutrient solution is injected into the reservoir, the operator would receive a return on investment of $5 for every dollar expended to purchase glucose with the low yield from laboratory experiments. This amounts to an additional cost of about $3.90 dollars per barrel of oil recovered. This is very close to the economies for actual MEOR field trials (1-3). We realize that there are other costs such as increased pumping charges and water treatment and labor costs. However, it is likely that nutrients themselves will be the main financial cost.

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Table 7.1 Oil revenue and cost of nutrients for a biosurfactant-mediated oil recovery process.

Yield (mg/g)	Pore Volume	Recoverable value ($/$)[1]	Cost of nutrient per bbl of oil ($/bbl)[2]
1	0.1	51	0.39
	0.5	10	1.95
	1.0	5	3.90
10	0.1	510	0.04
	0.5	100	0.19
	1.0	51	0.39
100	0.1	5,100	0.004
	0.5	1,000	0.02
	1.0	510	0.04

(Assume Sowf = 0.20, and glucose price = $ 0.22 per kg.)

[1] Oil revenue is the amount of revenue generated from the additional oil recovered by biosurfactant production from small domestic oil reservoir described in the text divided by the cost of glucose needed to conduct the MEOR process.

[2] Cost of glucose needed to conduct the MEOR process divided by the amount of oil recovered.

CHAPTER 8. TECHNOLOGY TRANSFER, OUTREACH AND SCIENTIFIC TRAINING

We communicated our results in several local and national venues, e. g., local technology exchange forums, the American Society for Microbiology annual meetings, discussions with several small businesses and oil companies, informal departmental seminars, and in the semiannual and final reports to the Department of Energy. Chapter 1 of this report, "Critical assessment of the use of microorganisms for oil recovery" is in press in *Research Signpost: Recent Developments in Microbiology*. Part of Chapter 2, the phylogenetic analysis of strain JF-2 will be submitted for publication in a microbiology journal. Chapters 3 and 4 will comprise major portions of the dissertations of Ms. Martha Folmsbee and Ms. Housna Mouttaki, respectively, and will be published in applied microbiology journals. Chapter 5 will be part of a major paper.

The promising laboratory and modeling results in Chapters 3, 4, 5, 6 and 7 have been recognized by a local independent oil producer. Arrow Oil and Gac, Inc. of Norman, OK will provide assess to a reservoir, field and technical support, and production data. If our funding permits, they will allow a test of the use of nutrients and strain JF-2 to demonstrate biosurfactant-mediated MEOR.

The multidisciplinary nature of this project provided an excellent educational opportunity for the students and staff involved. In weekly group meetings, information ranging from state-of-the-art molecular biology techniques and results of computer simulations was routinely presented. The microbiologists were able learn from the engineers and vice versa, and ideas from diverse disciplines were often combined to drive experiments that followed (e. g., see Chapters 3 and 5).

Students involved in the project.

Microbiology Undergraduates:

Mr. Warren Frey studied the nutrition of strain JF-2 as part of his honors research project. He is now a medical student at the Uniformed Services University.

Ms. Michelle Staudt studied the nutrition and growth kinetics of strain JF-2, which resulted in her Senior Capstone Thesis. She is now a graduate student in microbiology at

the University of Oklahoma Health Science Center. She received an Undergraduate Research Opportunity Program award from the University of Oklahoma and presented her work at our annual Undergraduate Research Day.

Both Michelle and Warren presented their findings at our department's annual undergraduate research symposium.

Ms. Deepu Madduri is studying the metabolism of organisms that live in oil reservoirs. This work will be part of her Honors Thesis.

Graduate Students:

Ms. Martha Folmsbee studied the nutrition of JF-2 and biosurfactant production. This work will be used for her dissertation to receive a Ph. D in microbiology.

Ms. Housna Mouttaki studied the competition of JF-2 with indigenous microorganisms in subsurface habitats. This work will be part of her dissertation for her Ph. D in microbiology

Mr. Saikrishna Maudgalya modeled biosurfactant-mediated oil recovery and conducted oil displacement experiments. This work will be used for his M. S. thesis in petroleum engineering.

Mr. Anh Din conducted oil displacement experiments as part of his training for his masters degree in petroleum engineering.

Mr. Shihong Shu initiated the modeling studies of biosurfactant-mediated oil recovery and received support during his studies for his masters degree in petroleum engineering.

Postdoctoral Research Associate:

Dr. Sung Ok Han received his doctoral degree from University of Melbourne, Australia. He performed the molecular biology work described in Chapter 2 and worked with Ms. Mouttaki on the competition experiments in Chapter 4. He is now with the Department of Microbiology at the University of California, Davis.