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Engineering Bacteria for Production of Rhamnolipid as an Agent for Enhanced Oil Recovery

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ABSTRACT: Rhamnolipid as a potent natural biosurfactant has a wide range of potential applications, including enhanced oil recovery (EOR), biodegradation, and bioremediation. Rhamnolipid is composed of rhamnose sugar molecule and β-hydroxyalkanoic acid. The rhamnosyltransferase 1 complex (RhlAB) is the key enzyme responsible for transferring the rhamnose moiety to the β-hydroxyalkanoic acid moiety to biosynthesize rhamnolipid. Through transposome-mediated chromosome integration, the RhlAB gene was inserted into the chromosome of the *Pseudomonas aeruginosa* PAO1-rhlA$^-$ and *Escherichia coli* BL21 (DE3), neither of which could produce rhamnolipid. After chromosome integration of the RhlAB gene, the constitute strains *P. aeruginosa* PEER02 and *E. coli* TnERAB did produce rhamnolipid. The HPLC/MS spectrum showed that the structure of purified rhamnolipid from *P. aeruginosa* PEER02 was similar to that from other *P. aeruginosa* strains, but with different percentage for each of the several congeners. The main congener (near 60%) of purified rhamnolipid from *E. coli* TnERAB was 3-(3-hydroxydecanoyloxy) decanoate (C_{10}–C_{10}) with mono-rhamnose. The surfactant performance of rhamnolipid was evaluated by measurement of interfacial tension (IFT) and oil recovery via sand-pack flooding tests. As expected, pH and salt concentration of the rhamnolipid solution significantly affected the IFT properties. With just 250 mg/L rhamnolipid (from *P. aeruginosa* PEER02 with soybean oil as substrate) in citrate–Na_2HPO_4, pH 5, 2% NaCl, 42% of oil otherwise trapped was recovered from a sand pack. This result suggests rhamnolipid might be considered for EOR applications.
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KEYWORDS: rhamnolipid; biosurfactant; transposome; chromosomal insertion; interfacial tension; enhanced oil recovery

Introduction

Surfactants pervade our everyday lives. Some surfactants, referred to as biosurfactants, are produced by bacteria or yeast from various substrates including sugars, glycerol, oils, hydrocarbons and agricultural wastes (Lin, 1996). Biosurfactants are classified as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, and polymeric or particulate compounds (Desai and Banat, 1997). The hydrophobic portion of the molecule is long-chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxyl fatty acids. The hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Biosurfactants have been receiving increasing attention as a result of their unique properties, i.e., mild production conditions, lower toxicity, and higher biodegradability, compared to their synthetic chemical counterparts (Rosenberg and Ron, 1999). Thus, it may be possible to reduce the environmental impact by replacing chemical additives with biosurfactants. The initial focus and most current industrial interest in biosurfactants are toward applications in the oil industry to aid in the clean up of oil spills and for enhanced oil recovery from mature oil

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reservoirs. Chemically synthesized surfactants have been used for the same purposes, but they pose environmental problems because of their toxicity and resistance to degradation (Mulligan, 2005).

The rhamnolipid biosurfactants have been studied extensively (Lang and Wullbrandt, 1999). Rhamnolipid is produced as mixtures in various proportions, including one or two rhamnoses attached to β-hydroxyalkanoic acid (Soberon-Chavez et al., 2005). The lengths of the fatty acid chains of rhamnolipid vary significantly, resulting in a multitude of different rhamnolipid congeners. This includes fatty acyl chains with lengths of 8, 10, 12, and 14 carbons, as well as of 12- or 14-carbon chains with a single double-bond. The type of rhamnolipid produced depends on the bacterial strain, the carbon source used, and the process strategy (Mulligan and Gibbs, 1993; Robert et al., 1989). Rhamnolipid induces a remarkably larger reduction in the surface tension of water from 72 to values below 30 mN/m and it reduces the interfacial tension of water/oil systems from 43 to values below 1 mN/m. Rhamnolipid also has an excellent emulsifying power with a variety of hydrocarbons and vegetable oils (Abalos et al., 2001).

The increasing ecological concern with using synthetic chemical surfactants has led us to propose rhamnolipid as environmentally benign substitute, although it will be necessary to reduce production costs. The use of renewable low-cost substrates, such as plant oil and grain starch, and even lignocellulosic biomass, could dramatically increase the economics of rhamnolipid production (Mukherjee et al., 2006; Nitschke et al., 2005). Many bacteria, especially Pseudomonads, can utilize efficiently renewable low-cost substrates, but they either lack the ability to biosynthesize the rhamnolipid, or only have very low yield of rhamnolipid. Therefore, we could use advanced molecular biotechniques to bioengineer renewable substrate-consuming bacteria that produce inexpensive rhamnolipid with high yield. Here, with *Pseudomonas aeruginosa* PAO1-rhlA$^-$ and *Escherichia coli* BL21(DE3) which both are not capable of rhamnolipid biosynthesis, we integrated the key genes of rhamnolipid biosynthesis by the transposome-mediated chromosome insertion technique to tailor bacteria for rhamnolipid production. The studies presented here include: (1) engineering *P. aeruginosa* and *E. coli* strains to modify or improve rhamnolipid production; (2) characterizing the compounds from engineered strains using thin layer chromatography (TLC) and HPLC/MS; (3) determining the interfacial activity of the rhamnolipids produced by the engineered strains; (4) evaluating the possibility of using these biosurfactants as agents for enhanced oil recovery from subsurface oil reservoirs.

Materials and Methods

Bacterial Strain, Media and Chemicals

The strains and plasmids used in this study were summarized in Table S1 (see supplemental materials). Briefly, *P. aeroginosa* PAO1-RhlA$^-$ (Rahim et al., 2001) and *E. coli* BL21(DE3) were the parental strains for engineering rhamnolipid production. *E. coli* DH5 was the host strain for constructing various recombinant plasmids. The *E. coli* strains were commercially available. Except for rhamnolipid fermentation, all these bacteria were grown on Luria-Bertani (LB) media containing suitable antibiotics at 37°C (Sambrook and Russell, 2001). Unless noted otherwise, antibiotics were used at the following concentration: chloramphenicol, 100 mg/mL for *P. aeruginosa* and 25 mg/mL for *E. coli*; ampicillin, 50 mg/mL for *E. coli*; kanamycin, 40 mg/mL for *E. coli*. All enzymes used for DNA manipulation were purchased from New England Biolabs (Beverly, MA). EZ::TN^{TM} Transposase was purchased from EPICENTRE Biotechnologies (Madison, WI).

Fermentation Media and Conditions

Nutrient broth from BD (Franklin Lakes, NJ) was used for seed culture of *P. aeruginosa* and LB for seed culture of *E. coli*. The mineral salt (MS) medium supplemented 0.4% or 2% glucose or 2% soybean oil as carbon source was the rhamnolipid fermentation medium. The MS medium contained (per L): 15 g·$NaNO_3$; 1.1 g·KCl; 1.1 g·NaCl; 0.00028 g·$FeSO_4{\cdot}7H_2O$; 3.4 g KH_2PO_4; 4.4 g K_2HPO_4; 0.5 g $MgSO_4{\cdot}7H_2O$; 0.5 g yeast extract; and 5 mL of a trace element solution containing (per L): 0.29 g $ZnSO_4{\cdot}7H_2O$; 0.24 g $CaCl_2{\cdot}4H_2O$; 0.25 g $CuSO_4{\cdot}5H_2O$; and 0.17 g $MnSO_4{\cdot}H_2O$ (Lindhardt et al., 1989). The trace element solution was filter-sterilized through a 0.2-μm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

P. aeruginosa wild-type and mutant were first grown in nutrient broth for 24 h at 30°C with shaking and then diluted 1:10 into MS medium plus 2% glucose or soybean oil and incubated for 4 days. *E. coli* wild-type and mutant were first grown in LB for 24 h at 30°C with shaking and then diluted 1:10 into MS or LB media plus 0.4% glucose supplemented 50 mM IPTG as inducer and incubated for 24 h. Incubation was carried out in 250-mL Erlenmeyer flask with 25 mL medium and at 30°C with orbital shaking at 250 rpm.

Plasmid and Strain Construction

The pMOD-2C derived from pMOD-2 (EPICENTRE Biotechnologies, Madison, WI) was for constructing artificial transposon. The pACYC184 was digested with *Xba*I and *Sty*I to produce a chloramphenicol resistant cassette. After being blunted with T4 DNA polymerase, this chloramphenicol resistant cassette was inserted into the *Sma*I site of pMOD2 to produce pMOD-2C. Rhamnosyltransferase 1 complex gene RhlAB with native operon promoter was amplified with primers RhlAB-1a (5′-CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3′) and RhlAB-2 (5′-AACCAAGCT TTCAGGACGCAGCCTTCAGCCAT-

CG-3′) and *P. aeruginosa* PA01 genomic DNA (purchased from American Type Culture Collection) as template; PCR product of RhlAB was digested with *Xba*I and *Hin*dIII and cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRABa. 3.5 kb chimeric transposon TnRABa withnative operon promoter was produced by digesting pMOD-2CRABa with *Psh*AI. Coding sequence of rhamnosyltransferase 1 complex RhlAB was amplified with primers RhlAB-1b (5′-AGTTGGTACCATG CGGCGCGAAAGTCTGTTGG-3′) and RhlAB-2 (5′-AACCAAGCTTTCAGGACGCAGCCTTCA GCCATCG-3′); PCR product was digested with *Kpn*I and *Hin*dIII and cloned into pET30a(+) to produce the recombinant plasmid pETRAB. 2.4 kb *Sph*I–*Hin*dIII fragment from pETRAB was cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRABb (In this case, *Sph*I in the fragment and *Xba*I in pMOD-2C were blunted first, then ligated). Chimeric transposon TnRABb with T7 promoter was produced by digesting pMOD-2CRABb with *Psh*AI (Fig. 1A). Transponon TnRABa or TnRABb was incubated with EZ::TN™ Transposase in the absence of Mg^{2+} to produce transposome TnRABasome or TnRABbsome (Fig. 1B) that was transformed into *P. aeruginosa* PAO1-RhlA$^-$ or *E. coili* BL21(DE3) by electroporation (Smith and Iglewski, 1989), respectively (Fig. 1C). After genotypic and phenotypic analysis, engineered strains *P. aeruginosa* PEER02 and *E. coli* TnRAB were confirmed and ready for rhamnolipid production.

Mapping of Transposon Insertion Site

Five micrograms of transposant (*P. aeruginosa* PEER02 or *E. coli* TnERAB) genomic DNA was digested with *Taq*I and then circularized in a ligation reaction using T4 DNA ligase at a DNA concentration of 5 ng/μL. Ligation products were purified with UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) and resuspended in water at 10 ng/μL. Inverse-PCR was performed on 100 ng of ligated DNA using the primers TnAB1(5′-CATAATGAAATAAGATCACTACCGGGC-3′) and TnAB2 (5′-GTGGTCGAACGTTGTCATAGGGA-3′), which face outward from the transposon sequence. The Inverse-PCR products were purified using the QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) and sequenced using the primers of TnAB1 and TnAB2. The resultant sequences were aligned with the complete genome of *P. aeruginosa* PAO1 (Stover et al., 2000) or *E. coli* K-12 (Blattner et al., 1997) to confirm the insertion site of transposon.

Rhamnolipid Quantification

Rhamnolipid was quantified in triplicate by the colorimetric determination of sugars with orcinol (Candrasekaran and Bemiller, 1980). Rhamnolipid was purified by first separating the cells from supernatant by centrifugation (10,000*g*). The supernatant was then extracted with chloroform and ethanol. The 0.5 mL rhamnolipid sample was extracted with 1 mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.5 mL of H_2O was added. To 0.1 mL of each sample with suitable dilution, 0.9 mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80°C the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentration was calculated from standard curves prepared with L-rhamnose (0–50 mg/L) and expressed as rhamnose equivalent.

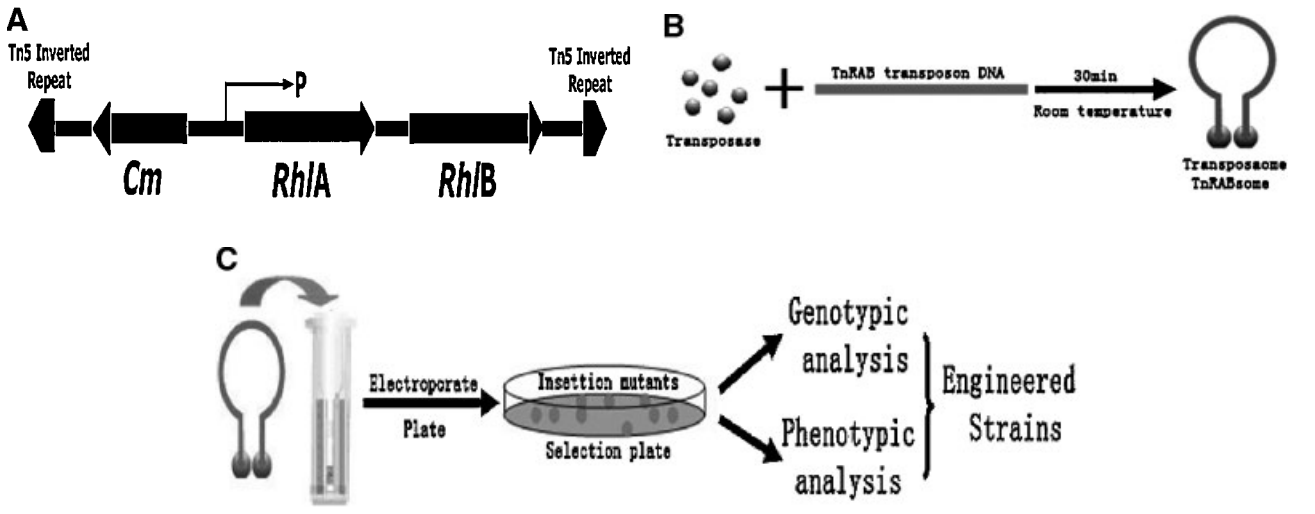

Figure 1. Diagrams for constructing rhamnolipid-producing bacteria. **A**: Structure of rhamnolipid biosynthetic gene RhlAB-containing Transposon (TnRABa or TnRABb); Cm: chloramphenicol resistant cassette; P: RhlAB native promoter or T7 promoter; TnRABa with the native promoter of RhlAB; TnRABb with T7 promoter fused with RhlAB. **B**: Transposome TnRABasome or TnRABbsome construction. **C**: Transposome electroporation and mutant selection.

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Rhamnolipid Purification, TLC and HPLC/MS Analysis

The rhamnolipid was precipitated by acidifying culture supernatant to pH 2 with concentrated HCl and kept at 4°C overnight, then recovered by centrifugation at 10,000*g* for 1 h and dissolved in deionized water. Two volumes of chloroform:ethanol (2:1, v/v) were added to rhamnolipid solution and shaken 30 min for extraction. The organic phase was picked and evaporated to dryness. Finally the rhamnolipid residue was dissolved in deionized water to 500 mg/L.

The purified rhamnolipid was separated, visualized, and compared with known rhamnolipid sample (JBR425, Jeneil Biosurfactant Company, LLC, Saukville, WI) using TLC (silica gel 60 plate). A 10 μL sample (500 mg/L) was loaded into a silica gel 60 plate. After drying at room temperature, the silica gel was developed with a solution of chloroform-methanol-water (65:15:2, v/v/v), then visualized using a 50:1:0.05 (v/v/v) mixture of the solution glacial acetic acid–sulfuric acid–anisaldehyde at 90°C for 30 min. The HPLC/MS analyses were performed according to Schenk's method with minor modification (Schenk et al., 1995). Briefly, a Gemini C18 column (2 mm × 50 mm, 5 μm particle) from Phenomenex and a ThermoFinnigan LCQ Classic ion trap mass spectrometer were used. The HPLC gradient was: starting at 8% solvent B and holding for 1 min, then ramping to 75% solvent B in 20 min, holding at 75% solvent B for 10 min, backing to 8% solvent B in 1 min and holding at 8% solvent B for 5 min. Solvent A is 98:2 (v/v) water:acetonitrile with 0.1% acetic acid, and solvent B is 10:90 water:-acetonitrile with 0.1% acetic acid. A 10 μL sample of 50 mg/L rhamnolipid was applied for HPLC/MS analysis. The mass spectrometer was operated in the negative ion mode scanning 250-950 *m/z* range.

IFT Characteristics of Rhamnolipid

The IFT was determined by using a spinning drop tensiometer (Temco, Inc., Tulsa, OK) as detailed by Cayais et al. (1975). Aqueous rhamnolipid solution was loaded into glass tube (5 mm × 100 mm), followed by injection of 1.5 μL *n*-octane. The glass tube with solution was spun in the tensiometer, and IFT was determined from the *n*-octane drop geometry. After spinning for 10 min, the data were collected for analysis. In these studies, we measured the "fresh" IFT values without pre-mixing rhamnolipid solution and *n*-octane together to reach phase equilibrium. To adjust the pH of rhamnolipid solution, citrate–Na_2HPO_4 buffer (pH 3–8) and boric acid–KCl–NaOH buffer (pH 8–10) were used. Unless noted otherwise, IFT was measured at 30°C.

Sand-Pack Core Flooding Test

A sand-pack core flooding test was performed at room temperature according to Shuler's method with some modifications (Shuler et al., 1989). Dried sand (28–60 mesh) manufactured by Paragon Building Products, Inc. (Norco, CA) was packed into a stainless steel tube (2 cm × 60 cm) to create an artificial core. This sand pack was placed horizontally and the air evacuated from it with a vacuum pump. Next, a buffer-brine solution (citrate–Na_2HPO_4 buffer, with 2 wt% NaCl added) adjusted to pH 5.0 was introduced at 1 mL/min into the pack so that this aqueous phase now occupies all of the pore space. This particular buffer-brine combination was selected because, based on the IFT measurements, rhamnolipid had lowest IFT under this condition. The pore volume (PV) of this pack was calculated as the volume of brine that is imbibed (calculated by weighing the sand-packed core before and after being saturated with this brine of known density.) The porosity of the sand pack is the pore volume divided by its total volume. The brine permeability of the pack is calculated from Darcy's Law by injecting the buffer-brine at a constant flow rate and measuring the pressure gradient. For the sand-pack experiment reported here, the pore volume, porosity, and permeability is 85 mL, 45%, and 17.8 Darcies, respectively. Next, *n*-octane (selected to be the oil phase) displaced the brine in the sand pack until no more water came out. The oil-saturated core was aged for 24 h. Then the buffer-brine was injected into the aged oil-saturated core until no more oil came out.

So at this point, the sand-pack core with trapped oil was ready for flooding with rhamnolipid solution. About 3 PV of 250 mg/L rhamnolipid solution (made up in citrate–Na_2HPO_4 buffer, 2% NaCl, pH 5.0) were injected at 1 mL/min. This same flow rate is used in all the flow steps associated with the sand-pack experiment. Next, the core was shut in and incubated overnight at room temperature. The other 3 PV 250 mg/L rhamnolipid solution were injected at 1 mL/min during the second day. After concluding rhamnolipid flooding, 6 PV of buffer-brine were injected. The produced fraction was collected in a titration tube with 0.1 mL accuracy. After keeping few minutes, the oil phase and water phase would separate naturally, and the oil volume was measured according the graduation in the titration tube with 0.1 mL accuracy. The oil recovery and water cut (percentage of oil- and non-oil phase in each elution fraction of approximately 10 mL) were recorded during the rhamnolipid solution and subsequent buffer-brine injection steps, as well as the cumulative oil recovery as a percent of the trapped oil before rhamnolipid flooding. The IFT and the rhamnolipid concentration for some of the eluted fractions during the rhamnolipid and the buffer-brine flush were measured with the methods mentioned above.

Results

Engineering *P. aeruginosa* and *E. coli* for Rhamnolipid Production

The synthesis of rhamnolipid proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific

rhamnosyltransferase (1 or 2) with TDP-rhamnose acting as a rhamnosyl donor and 3-(3-hydroxyalkanoyloxy) alkanoate acting as acceptor (Fig. S1, Maier and Soberon-Chavez, 2000). L-Rhamnosyl-3-(3-hydroxyalkanoyloxy) alkanoate (Rha–C_m–C_n, m and n: 8, 10, 12, or 14) and L-rhamnosyl–L-rhamnosyl-3-(3-hydroxyalkanoyloxy) alkanoate (Rha–Rha–C_m–C_n) were referred to as mono-rhamnolipid and di-rhamnolipid, respectively. Rhamnosyltransferase 1 is encoded by the RhlA and B (or RhlAB) genes, which are organized in an operon and responsible for biosynthesis of mono-rhamnolipid. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane (Ochsner et al., 1994). Rhamnosyltransferase 2 is encoded by the RhlC gene that is located in another operon with an upstream unknown gene (PA1131) in *P. aeruginosa* PAO1, and not organized with RhlAB (Rahim et al., 2001).

RhlAB is the key enzyme complex in rhamnolipid biosynthesis, but this biosynthesis is modulated by the complicated transcriptional regulatory network in *P. aeruginosa* (Soberon-Chavez and Aguirre-Ramirez, 2005). To achieve the rhamnolipid production, RhlAB is indispensable. Here, we use transposome-mediated chromosomal integration (Fig. 1) to reconstruct the metabolic pathway of rhamnolipid biosynthesis. RhlAB with native promoter and chloramphenicol resistant cassette (Cm) were assembled into chimeric Tn5-derived transposon TnRABa (Fig. 1A) that can bind transposase to produce transposome TnRABasome (Fig. 1B). TnRABasome was electroporated into *P. aeruginosa* PAO1-rhlA$^-$ (Fig. 1C) wild-type strain. Consequently, RhlAB with the native promoter was inserted into the chromosome of *P. areuginosa* PAO1-rhlA$^-$ devoid of rhamnolipid biosynthesis capability, by transposome-mediated chromosomal integration. The constitute strain (*P. areuginosa* PEER02) was confirmed by inverse-PCR amplification and DNA sequencing for the integration of RhlAB. Aligning the sequences with the complete genome of *P. aeruginosa* PAO1 showed that the transposon TnRABa was inserted into a gene of probable metalloprotease (Stover et al., 2000). *P. areuginosa* PEER02 produced rhamnolipid in MS media with either glucose or soybean oil as the carbon source (Table I). Compared the yield of the very wild-type strain PAO1, the insertion of RhlAB in the identified position in PEER02 facilitated the production of rhamnolipid. Due to the unknown properties of the insertion site, it is difficult to speculate what's mechanism resulted in the increase of rhamnolipid production. After 4-day fermentation, substrate (soybean oil or glucose) in the media was also exhausted (data not shown). Therefore, based on the yield and the corresponding conversion rate, soybean oil is better substrate than glucose for rhamnolipids production by *P. areuginosa* PEER02 in the preliminary fermentation condition.

To avoid the complicated metabolic regulation associated with rhamnolipid biosynthesis in *P. aeruginosa*, we attempted to achieve rhamnolipid formation in *E. coli* by the same transposome-mediated chromosomal integration of RhlAB. The native promoter of RhlAB in the transposon TnRABa was replaced with the T7 promoter to produce another artificial transposon TnRABb. Then, TnRABb was inserted into the chromosome of *E. coli* BL21(DE3) to obtain the engineered strain *E. coli* TnERAB. It was also confirmed by Inverse-PCR and sequencing for the RhlAB gene integration. The result of alignment with the complete genome of *E. coli* K12 revealed that transposon TnRABb was inserted into a gene of predicted inner membrane protein (Blattner et al., 1997). With IPTG (isopropyl-beta-D-thiogalactopyranoside) induction, *E. coli* TnERAB could produce rhamnolipid both in LB and MS media with glucose as substrate (Table I).

Table I. Rhamnolipids production by engineered *P. aeruginosa* and *E. coli*.

	Rhamnolipids (mg/L)		
Strains	LB + glucose	MS + glucose	MS + soybean oil
P. aeruginosa PAO1-RhlA$^-$	—[a]	ND[b]	ND
P. aeruginosa PEER02	—	785.4 ± 23.5[c]	1819.1 ± 78.8
P. aeruginosa PAO1	—	501.4 ± 17.2	1211.4 ± 52.1
E. coli BL21(DE3)	ND	ND	—
E. coli TnERAB	175.3 ± 13.2[d]	75.6 ± 11.2	—

[a]Did not test in this condition.
[b]Cannot detect rhamnolipid in this condition.
[c]Results obtained from three independent experiments of 4-day fermentation, average value ± standard deviation.
[d]Results obtained from three independent experiments of 1-day fermentation.

To test the ability of our engineered strains to produce rhamnolipid, we subcultured the engineered strains in LB plate without any antibiotics for 20 generation, and then selected the single colony from each generation to test the rhamnolipid production. The results showed there is no great difference in the yield of rhamnolipid among the strains from different generation. The yield of engineered *P. aeruginosa* PEER02 was 700–800 mg/L with glucose as substrate, and 1,700–1,900 mg/L with soybean oil as substrate, respectively. Engineered *E. coli* TnERAB produced rhamnolipid of 65–80 mg/L in MS plus glucose media, and 150–185 mg/L in LB plus glucose media, respectively.

Rhamnolipid Characterization

Rhamnolipid consists of one or two units of rhamnose linked to one or two fatty acid chains with C_8–C_{14} carbon atoms, which may or may not be saturated. Thin-layer chromatography (TLC) results suggest that the isolated surface-active products from *P. aeruginosa* PEER02 and *E. coli* TnERAB contain rhamnolipid (Fig. 2). The engineered products were separated on a TLC plate alongside a sample of a commercially available rhamnolipid (JBR425, Jeneil Biosurfactant Company). We found that the

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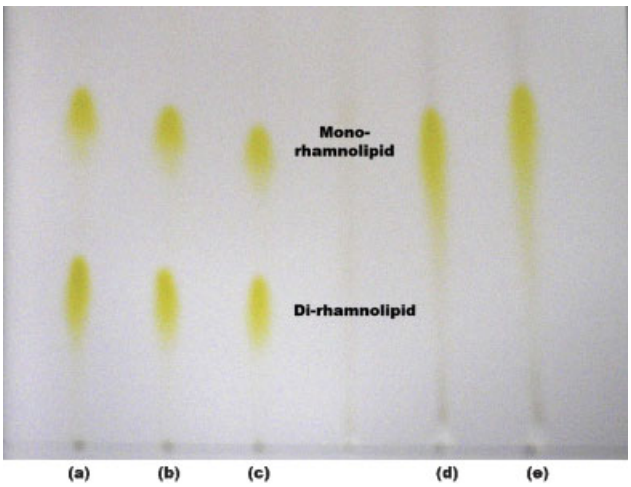

Figure 2. TLC analysis of rhamnolipid from engineered strains. **a**: Commercial rhamnolipid JBR425. **b**: Rhamnolipid produced by *P. aeruginosa* PEER02 with soybean oil as substrate. **c**: Rhamnolipid produced by *P. aeruginosa* PEER02 with glucose as substrate. **d**: Rhamnolipid produced by *E. coli* TnERAB in LB plus glucose media. **e**: Rhamnolipid produced by *E. coli* TnERAB in MS plus glucose media (10 μL sample of 500 mg/L for each lane). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

products from *P. aeruginosa* PEER02 have two spots, which are similar to that of the commercial product. The lower spot is from the di-rhamnolipid structures, while the higher spot comes from mono-rhamnolipid molecules. The products from *E. coli* only have the higher spots, which are the mono-rhamnolipid congeners. Thus, we have demonstrated that, as expected, RhlAB synthesized only mono-rhamnolipid. Di-rhamnolipid biosynthesis requires additional gene(s). *P. aeruginosa* PEER02 can produce di-rhamnolipid because it contains gene RhlC which was confirmed by PCR and sequencing (data not shown). The enzyme encoded by RhlC can transfer the second L-rhamnose to mono-rhamnolipid to synthesize di-rhamnolipid (Rahim et al., 2001).

Then, the products from our engineered strains were analyzed by HPLC/MS analysis to further confirm the presence of rhmanolipid. Figure 3 presents the base peak ion intensity chromatograms of the purified rhamnolipid samples that were produced by our engineered strains with soybean oil or glucose as carbon source. The peak profiles of these chromatograms differ mostly at the later elution times. The proportions of the various congeners of rhamnolipid listed in Table II were obtained from the relative intensities of their corresponding pseudomolecular ions. For rhamnolipid species of the same molecular weight that were chromatographically resolved, this was performed simply by integration of the intensities of their pseudomolecular ions. For isomers that were not chromatographically resolved, their relative proportion was determined by the relative intensities of different fragments ions produced by cleavage of the two molecules at the same position. For example, the rhamnolipid Rha–C_8–C_{10} was not sufficiently separated from Rha–C_{10}–C_8 to allow direct quantification on the basis of the intensity of their pseudomolecular ions at m/z 475. However, their mass spectra differ by the presence of an ion of m/z 305 for the former and an ion of m/z 333 for the latter. These two ions arise from cleavage at the 3-carbon–oxygen bond in both molecules. The relative intensities of the two isomers were calculated by measuring the relative intensities of both ions, in an averaged spectrum obtained from all the spectra presenting the proper pseudomolecular ion, and multiplying these two values with the intensity of their common pseudomolecular ions. This method allows the analysis of very closely related rhamnolipid structures without resorting to a longer and more difficult chromatographic separation.

From the ion intensity chromatograms (Fig. 3), the main structural congeners of the rhamnolipid from each sample were labeled. For the sample from *E. coli*, the main congener is Rha–C_{10}–C_{10} that comprised almost 60% of total amount. The main congeners of the samples from engineered *P. aeruginosa* also have C_{10}–C_{10} carbon chain: Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10}. The percentage of these congeners is more than 30%. Other abundant structure congeners of rhamnolipid from *P. aeruginosa* are Rha–Rha–C_{10}–C_{12} and Rha–Rha–C_{12}–C_{10}. They have the same pseudomolecular ion and their total amount is more than 10%.

IFT Analysis of Rhamnolipid

Like synthetic surfactants, rhamnolipid also reduces interfacial tension between oil and water, thus decreasing the energy required to mobilize trapped oil in subsurface porous rock matrix in an oil reservoir, and displace this oil to a production well. But under what conditions can rhamnolipid does this effectively? Here, rhamnolipid produced by *P. aeruginosa* PEER02 with soybean oil as carbon source was used to examine the effects of salt concentration, pH and temperature on rhamnolipid activity.

First, we investigated the effects of different concentrations of rhamnolipid on IFT to locate a suitable concentration for subsequently analyzing IFT of rhamnolipid at various conditions. As expected (Fig. 4A), the IFT of rhamnolipid solution rapidly decreased with the decrease of rhamnolipid concentration. As a comparison, the IFT of water with no surfactant and the *n*-octane oil phase is approximately 35 mN/m. According to the results of Figure 4A, the critical micelle concentration (CMC) of this product is about 75 mg/L, and so we selected 100 mg/L rhamnolipid for subsequent IFT tests.

From Figure 4B, in the absence of additional salinity, rhamnolipid showed low IFT in the low pH buffer system, and in the range of pH 3–5, there is a minimum value of approximately 0.3 mN/m. If there is any additional salinity, the IFT profile versus pH changes. For example, when 2% NaCl is added into rhamnolipid solution with different pH (square curve in Fig. 4B), the IFT of the rhamnolipid solution decreases dramatically by almost fivefold. However,

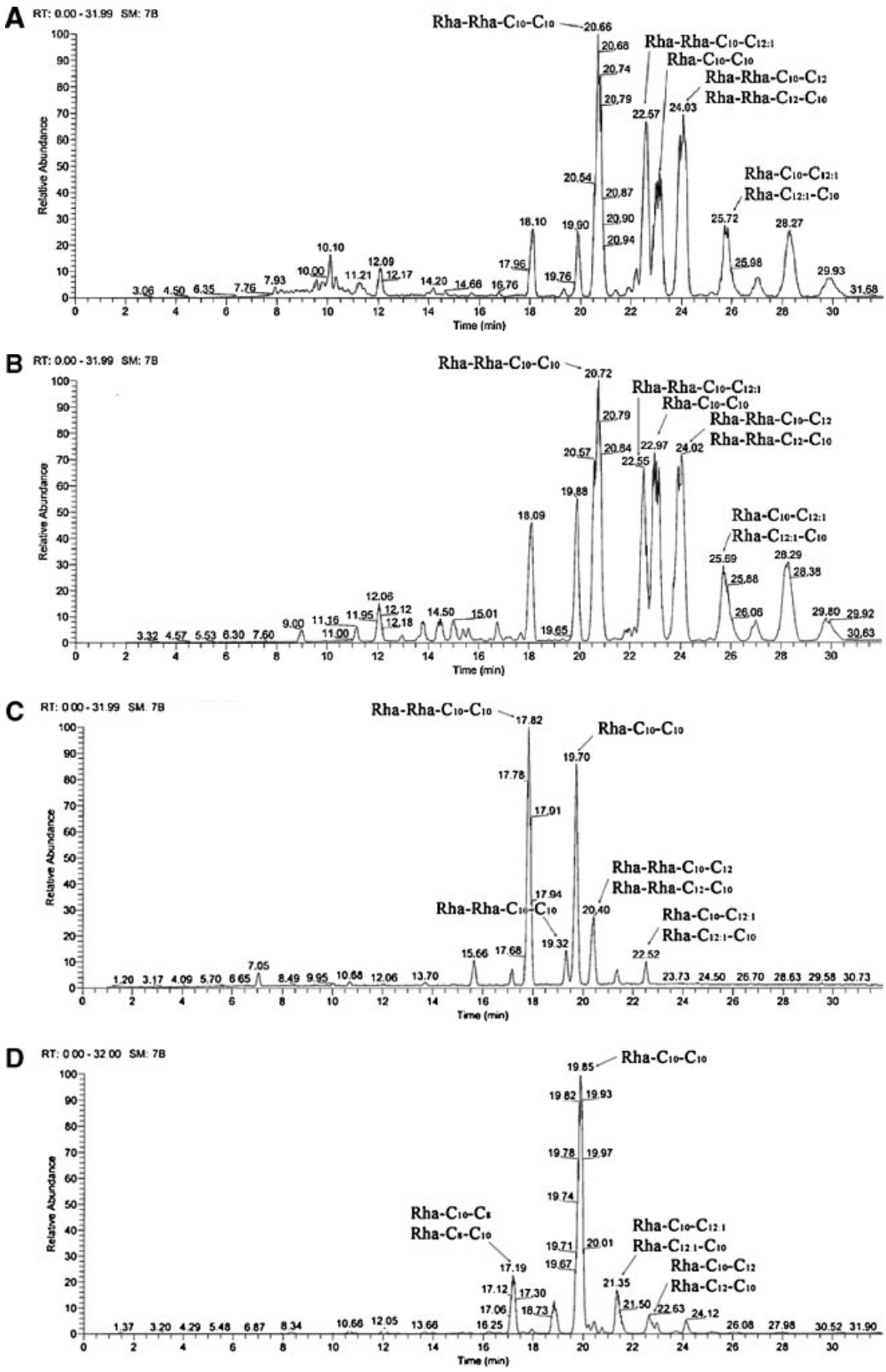

Figure 3. Base peak ion intensity chromatograms of rhamnolipid from various sources. **A**: Rhamnolipid produced from MS supplemented soybean oil by *P. aeruginosa* PEER02. **B**: Rhamnolipid produced from MS supplemented glucose by *P. aeruginosa* PEER02. **C**: Commercial rhamnolipid JBR425. **D**: Rhamnolipid produced from MS supplemented glucose by *E. coli* TnERAB.

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Table II. Structure and relative abundance of the rhamnolipids from various sources.

Structure	Pseudomolecular ion (*m*/*z*)	Relative abundance (%)			
		JBR425	PEER02/G	PEER02/S	TnERAB
Rha–Rha–C_8–C_{10}	621	2.06	2.53	3.77	ND[a]
Rha–Rha–C_{10}–C_8	621	1.37	3.51	0.30	ND
Rha–C_8–C_{10}	475	1.41	4.73	ND	5.65
Rha–C_{10}–C_8	475	0.52	2.21	3.35	5.65
Rha–Rha–C_{10}–C_{10}	649	36.45	19.37	20.20	ND
Rha–Rha–C_{10}–$C_{12:1}$	675	4.08	9.70	13.24	ND
Rha–Rha–$C_{12:1}$–C_{10}	675	ND	ND	ND	ND
Rha–C_{10}–C_{10}	503	38.13	15.33	12.07	57.08
Rha–Rha–C_{10}–C_{12}	677	8.13	10.37	11.59	ND
Rha–Rha–C_{12}–C_{10}	677	2.18	5.76	6.62	ND
Rha–C_{10}–$C_{12:1}$	529	1.15	0.37	6.33	0.14
Rha–$C_{12:1}$–C_{10}	529	0.78	5.25	ND	9.05
Rha–Rha–C_{12}–$C_{12:1}$	703	ND	ND	ND	ND
Rha–Rha–$C_{12:1}$–C_{12}	703	ND	0.87	ND	ND
Rha–Rha–C_{10}–$C_{14:1}$	703	ND	0.16	1.07	ND
Rha–Rha–$C_{14:1}$–C_{10}	703	ND	0.44	0.68	ND
Rha–C_{10}–C_{12}	531	2.15	4.46	3.96	1.50
Rha–C_{12}–C_{10}	531	0.42	2.35	2.64	2.25
Rha–Rha–C_{12}–C_{12}	705	ND	2.15	2.14	ND

JBR425: commercial rhamnolipids; PEER02/G: rhamnolipids from *P. aeruginosa* PEER02 with glucose as substrate in MS media; PEER02/S: rhamnolipids from *P. aeruginosa* PEER02 with soybean oil as substrate in MS media; TnERAB: rhamnolipids from *E. coli* TnERAB with glucose as substrate in MS media and IPTG induction.

[a]ND: none detected.

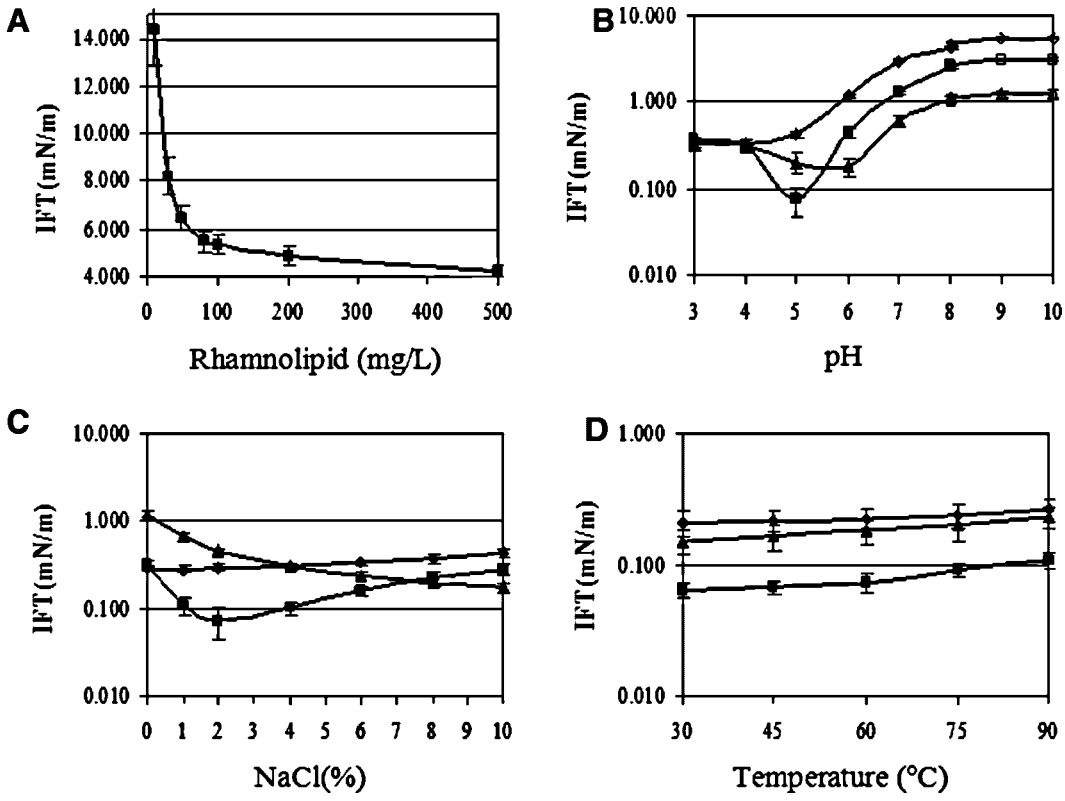

Figure 4. IFT analysis of rhamnolipid in various conditions. **A**: Profile of IFT of different concentration of rhamnolipid in water. **B**: Effects of pH on IFT of rhamnolipid. pH 3–8: citrate–Na_2HPO_4 buffer; pH 8–10: boric acid–KCl–NaOH buffer. Diamond: no NaCl; square: 2% NaCl; triangle: 8% NaCl. **C**: Effects of salinity on IFT of rhamnolipid. Rhmanolipid in citrate–Na_2HPO_4 buffer. Diamond: pH 6; square: pH 5; triangle: pH 4. **D**: Effects of temperature on IFT of rhamnolipid. Diamond: rhmanolipid in citrate–Na_2HPO_4 buffer, pH 4, 1% NaCl; square: rhmanolipid in citrate–Na_2HPO_4 buffer, pH 5, 2% NaCl; triangle: rhmanolipid in citrate–Na_2HPO_4 buffer, pH 6, 8% NaCl. Results obtained from three independent experiments.

with a further increase of salinity (8% NaCl, triangle curve in Fig. 4B), not only does the pH with minimum IFT increase, but absolute values of IFT also increases. In addition, we found that the IFT profiles of rhamnolipid solution with same pH but different salinity differ greatly. For pH 6 (triangle curve in Fig. 4C), IFT decreases with higher salinity, but for pH 4 (diamond curve in Fig. 4C), the behavior is reversed. Increasing salinity increased the IFT in the range of 0–10% NaCl. However, at pH 5 (square curve in Fig. 4C), there is an optimal salinity at 2% NaCl with the lowest IFT of 0.07 mN/m. We also analyzed the IFT profile of rhamnolipid from *E. coli* TnERAB grown in LB plus glucose with the condition of different pH and salinity (Fig. S2). In the condition of pH 8 (boric acid–KCl–NaOH buffer) and 4% NaCl, the IFT reached the lowest value of 0.08 mN/m. The different optimal condition with lowest IFT of the rhamnolipid from *P. aeruginosa* PEER02 and *E. coli* TnERAB showed IFT also depended on the structure of chemicals. Rhamnolipid with different chemical structure (Fig. 3) would show different IFT profile.

Temperature is an important parameter because it can vary substantially in subsurface oil reservoirs. Hence, it is important to determine the temperature sensitivity when evaluating a surfactant for enhanced oil recovery. Our experiments showed that IFT increased only slightly in the range of 30–90°C when rhamnolipid was at different pH and salinity conditions (Fig. 4D).

Core Flooding Test of Rhamnolipid for EOR

After an oil well loses its ability to flow by itself and secondary flow, approximately 2/3 of the original oil still remains in the reservoir. Much of the remaining oil can be recovered by EOR techniques such as polymer flooding or surfactant flooding (Gregory, 2001; Van Hamme et al., 2003). Biosurfactants may be an economical method to recover residual hydrocarbons since they reduce IFT effectively at low concentration. The IFT analysis of our engineered rhamnolipid showed that they can reduce remarkably interfacial tension of oil (octane)/and water at certain combinations of higher salinity and slightly acidic pH. Based on those data from IFT analysis, sand-pack core flooding tests of rhamnolipid produced by constituent *P. aeruginosa* PEER02 with soybean oil as carbon source were examined.

To provide a test for the efficacy of rhamnolipid flooding, we selected *n*-octane as the oil phase. Per the procedure detailed earlier, the sand pack was conditioned with brine, flushed with the oil, and waterflooded to a trapped oil condition (in this case, the trapped oil in sand pack is 17.2 mL). Rhamnolipid injection and subsequent brine flush did recover the trapped oil. The recovered oil was measured and the oil recovery change with injected pore volume of the rhamnolipid and brine flush solution was calculated (Fig. 5A and B).

This two-stage rhamnolipid flooding gave two peaks of oil recovery (Fig. 5A). The first 3 PV of rhamnolipid flooding produced only a small amount of oil recovery. The volume of oil recovered in this stage is 1.95 mL, and the cumulative recovery versus the residual oil is about 12%. But the next 3 PV of rhamnolipid solution injection after the overnight shut-in recovered 4.38 mL oil and dramatically increased the cumulative oil recovery to 37% (Fig. 5B). After rhamnolipid flooding, the 6 PV brine injection obtained another 0.80 mL and increased the cumulative oil recovery further to 42% (Fig. 5B). We also found that the effluent had a low IFT, with the effluent having the lowest IFT coincidental with the effluent exhibiting highest oil cut. Then, as the oil recovery decreased, we found that the IFT increased, and then maintained a relatively stable level. With subsequent brine flooding, the IFT of the effluent increased further because the effluent concentration of the rhamnolipid decreased. Our sand-pack flooding experiment showed that overnight

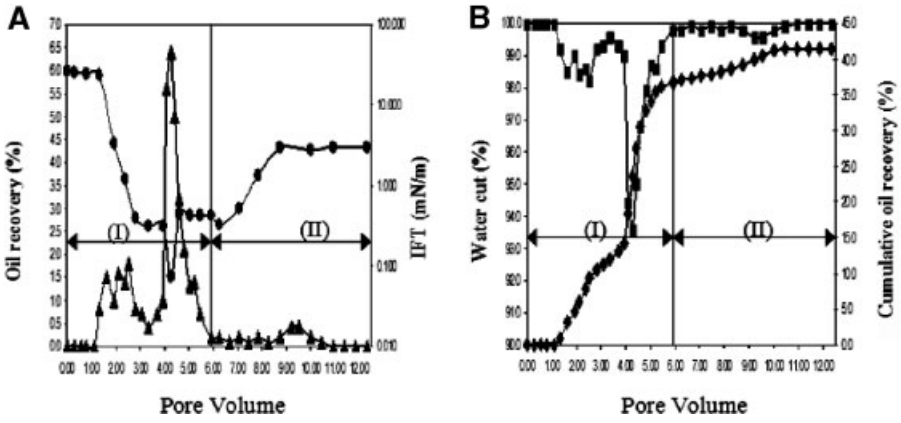

Figure 5. Oil recovery test of a waterflooded sand-pack core by rhamnolipid flooding. **A**: Profile of oil recovery (triangle) and IFT (circle) during flooding. **B**: Water cut (square) and cumulative oil recovery (diamond). (I) rhamnolipid flooding; (II) brine flooding. One pore volume was 85 mL brine solution (50 mM citrate–Na_2HPO_4, 2% NaCl, pH 5.0 buffer). The porosity and brine permeability of the sand-packed core in this experiment was 45% and 17.9 Darcies, respectively.

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incubation of the sand pack with rhamnolipid would increase oil recovery and decrease the water cut (ratio of water phase in each fraction) of effluent. These results are promising towards the application rhamnolipid for the EOR application.

Discussion

Surface-active compounds commonly used by industry are chemically synthesized. However, replacing the synthetic surfactants with biosurfactants could provide advantages such as biodegradability and low toxicity. Currently, the use of biosurfactant has been limited by their relatively high production cost. The main factor limiting commercialization of biosurfactants is associated with non-economical large-scale production. To overcome this obstacle and to compete with synthetic surfactants, inexpensive substrate and effective microorganisms must be developed for biosurfactant production. Agro-industrial wastes are considered as the most promising substrate for low-cost biosurfactant production and can alleviate many processing industrial waste management problems. This would lead to the greater possibility for economical biosurfactant production and reduced pollution caused by those wastes (Maneerat, 2005). Aforementioned, many microorganisms, especially Pseudomonads, can use various low-cost renewable resources as potential carbon sources, but they cannot produce biosurfactants or only have very low yield. Screening high rhamnolipid-producing microorganism from the natural environment is a good choice, but engineering strain for rhamnolipid production give another direction. In this study, we used novel transposome biotechnique to integrate successfully the key genes of rhamnolipid biosynthesis into the chromosome of *P. aeruginosa* and *E. coil* cells (originally devoid of rhamnolipid biosynthesis capability), and made the engineered strains produce rhamnolipid. The use of such transposon–transposase complexes (transposome) would allow us to simply create stable insertion mutation in many other bacteria, including Gram-positive and Gram-negative strains (Hoffman et al., 2000). Unlike plasmid-based engineered strain, transposon-based strains could exist stably under no drug-selection pressure, and the integration site of the targeted gene(s) would easily be confirmed by inverse-PCR, DNA sequencing, and alignment with a vast repository of genome information available from public database. A stable mutant strain would be a great advantage for rhamnolipid production by fermentation and for future microbially enhanced oil recovery (MEOR).

The successes of this biotechnique in engineering rhamnolipid-producing strain implicated that we could apply this technique in other similar study, or further modify the complicated rhamnolipid biosynthesis system in *P. aeruginona* for improving rhamnolipid production. This technique would also result in random mutagenesis due to the transposon insertion while transferring the essential genes to the target strain to modify the related synthetic pathway. The mutagenesis of transcription machinery or some other genes would change the global transcription regulation and elicit cellular phenotypes important for technological application (Alper et al., 2006). Based on the robust screening, the superior desired strain could be identified. In fact, from the fermentation results of *P. aeruginosa* PEER02 and PAO1 (Table I), we already confirmed that screening the superior rhamnolipid-producing strain was possible.

HPLC/MS is a very efficient technology for analysis of complicated rhamnolipid mixtures, allowing a relatively short and simple chromatographic analysis. If using only HPLC to characterize a rhamnolipid mixture, we would have to derivatize the sample with *para*-bromoacetophenone in order to detect the signal with UV (Schenk et al., 1995). Also HPLC could not provide structural information on the various compounds separated. Conversely, mass spectrometry gave useful structural information and allowed quantification of chromatographically unresolved molecules. In recent years, some publications have reported that Rha–C_{10}–C_{10} and Rha–Rha–C_{10}–C_{10} are in fact produced as part of a complex mixture of rhamnolipid (Schenk et al., 1995). Our study results agree with these results (Table II). Identification and quantification of dozens of different rhamnolipid congeners was accomplished using a reverse-phase C_{18} column and a 30 min chromatographic run. Isomeric rhamnolipid species that were not chromatographically resolved were identified by interpretation of their mass spectra and their relative proportions estimated.

As mentioned early, the type of rhamnolipid produced depends on multiple conditions, including the bacterial strain and the carbon source used (Mulligan and Gibbs, 1993; Robert et al., 1989). Our results were coincident with it. The most abundant rhamnolipid produced by engineered *P. aeruginosa* PEER02 on soybean oil or glucose contained two rhamnoses and two 3-hydroxydecanoic acid groups or one 3-hydroxydecanoic acid group. The relative abundances of these two congeners are all about 30%. However, in the commercial product of JBR425 from Jeneil Biosurfactant Company, the most abundant rhamnolipid had two rhamnoses and two 3-hydroxydecanoic acid groups. Its relative abundance is about 40%. Compared with commercial rhamnolipid, a greater percentage of longer carbon chain congeners of the engineered rhamnolipids is obtained from *P. aeruginosa* PEER02. The IFT of engineered rhamnolipid tested is as low as 0.07 mN/m in the optimal condition (Fig. 4). However, the IFT of a commercial sample (JBR425) in the same condition is about 0.2 mN/m. This difference in chemical structures could explain why our rhamnolipid showed better performance in IFT than the commercial one (Jang et al., 2004). In the engineered rhamnolipid from *E. coli*, the most abundant rhamnolipid contained one rhamnose and two 3-hydroxydecanoic acid groups with a ratio near 60%. These results also implicated that we could tailor some bacteria to produce novel rhamnolipids with novel performance (i.e., difference structure or IFT) by the transposome biotechnique that we used in this study.

Substantial work has been carried out towards the characterization of rhamnolipid for its potential application as oil recovery agents (Patel and Desai, 1997). Here, we have analyzed systematically the interfacial activity of rhamnolipid from engineered *P. aeruginosa* with soybean oil as substrate. The desirable capability to create very low IFT conditions (as low as 0.07 mN/m) has been demonstrated. This occurs over a defined set of conditions (mainly controlled by salinity and pH). This sensitivity of IFT to salinity is also confirmed in the synthetic anionic surfactants (e.g. alkyl benzene sulfonates) commonly used by the petroleum industry for enhanced oil recovery (Jang et al., 2004). Experience shows that creating an IFT as low as 0.07 mN/m will mobilize a substantial fraction of oil otherwise not recovered by flushing a porous media with just water (Miller et al., 1991).

The trends of IFT behavior we observe with changes in salinity and pH are similar with that reported elsewhere (Bai et al., 1998). Their paper reports that the IFT between a rhamnolipid solution and hexadecane as the oil phase decrease substantially as NaCl is added to a fresh water biosurfactant solution. These authors also note there is a decrease in the IFT with a change in the pH, and furthermore, there is an optimal pair of salinity and pH conditions to attain the lowest possible IFT.

The change in salinity of course is changing the partitioning of the biosurfactant between the aqueous and oil phases. When you increase the salinity you have a decrease of solubility of the surfactant in that phase; this drives more of the surfactant to the interface and into the oil phase. When there is roughly a balance between the phases, then expect to reach the minimum IFT. If the salinity is increased even further, then too much is driven into the oil and not enough remains in the aqueous (over optimum). This of course is what is expected, given same salinity trends and desire for an "optimal salinity" that occurs with the common sulfonate surfactants used in EOR (Shuler et al., 1989). The change in pH changes the IFT result because the carboxylate group pK_a means a partial dissociation versus pH over our range of interest-pH 4–8 (or 10). With lower pH more of the biosurfactant is in acid form. This change in fraction of bio-surfactant that is dissociated with effect of the pH impacts the IFT.

The sand-pack test data shown in Figure 5 has the results of injection of a 250 mg/L rhamnolipid in a brine with the salinity and pH adjusted to match the lowest IFT conditions shown in Figure 4. This test was designed to create the most favorable conditions for oil recovery from a completely waterflooded sand-pack for this particular rhamnolipid surfactant. After 1 pore volume of injection (resident brine is displaced), the first incremental oil appears in the effluent (Fig. 5). Simultaneously the IFT plummets from the water-oil value of 30 to less then 1 mN/m. Further injection of rhamnolipid solution takes the IFT to near the minimum expected value of 0.07 mN/m and produces nearly half of the oil not recoverable by water injection alone. These observations demonstrate there was a successful propagation of the rhamnolipid chemical, and that a desirable reduced IFT condition was created in situ that was able to displace a significant fraction of the oil remaining after a complete waterflood process. The adsorption of the rhamnolipid during the sand-pack flooding tests was calculated by analyzing for the rhamnolipid concentration in the effluent fractions (data not shown). From these effluent concentrations and volumes, the mass of biosurfactant in the effluent was calculated. Subtracting this from the known mass of biosurfactant injected gives the mass of retained biosurfactant. It is estimated that the retention of the biosurfactant in the sand pack was 0.2 mg/g of sand. This is roughly the same or even lower retention than reported for synthetic surfactants in sandstone coreflooding oil recovery experiments (Shuler et al., 1989).

Based on experience with synthetic surfactants such as alkyl aryl sulfonates, it should be possible to improve even further the efficiency and oil recovery performance of biosurfactant-based chemical formulations for EOR (Miller et al., 1991; Shuler et al., 1989). One common practice is to formulate synthetic surfactants systems to include a so-called co-surfactant. This additive is, for example, a simple alcohol included to improve the fluid properties of the emulsion system, such as adjusting the viscosity of the surfactant formulation or further reducing the low IFT condition over a wider range of process conditions. It is also common practice to include a water soluble polymer to increase the viscosity of the surfactant formulation itself, or the drive water that immediately follows the surfactant solution. This increased viscosity of the displacing aqueous-based fluids invariably improves the oil recovery by increasing the viscous forces that drive oil displacement and increases the stability of the chemical system front driving the bank of displaced oil ahead of it.

Our oil displacement experiment demonstrates that a very low concentration (relative to that of traditional chemical surfactants extensively used EOR, typically at 2,000–10,000 mg/L, Shuler et al., 1989), of the rhamnolipid with the optimum chemical structure and process conditions can recover a significant fraction of trapped oil from a sand pack. Biosurfactants could provide an attractive lower cost option to synthetic surfactants, which typically must be used at chemical concentrations several times higher for the same oil recovery performance.

In addition, the ability to generate rhamnolipids within oil reservoirs by placement of the right microbes might help overcome rhamnolipid adsorption during flow through reservoir rocks and the resultant degradation that would decrease the rhamnolipid concentrations. Using our transposome techniques for integrating rhamnolipids biosynthetic genes to endogenous anaerobic bacteria isolated from oil reservoir to make them produce rhamnolipid in situ could provide a significant break through advance for microbial enhanced oil recovery.

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References

Abalos A, Pinaso A, Infante MR, Casals M, Garcia F, Manresa A. 2001. Physicochemical and antimicrobial properties of new rhamnolipids by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. Langmuir 17:1367–1371.

Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G. 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314:1565–1568.

Bai G, Brusseau ML, Miller RM. 1998. Influence of cation type, ionic strength, and pH on solubilization and mobilization of residual hydrocarbon by a biosurfactant. J Contam Hydrol 30:265–279.

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277(5331):1453–1474.

Candrasekaran EV, Bemiller JN. 1980. Constituent analyses of glycosaminoglycans. In: Whistler RL, editor. Methods in carbohydrate chemistry. New York: Academic Press Inc. p 89–96.

Cayais JL, Schechter RS, Wade WH. 1975. The measurement of low interfacial tension via the spinning drop technique. In: Gould JR, editor. Adsorption at interfaces, ACS Symposium series 8. Washington, DC: American Chemical Society. p 234.

Desai JD, Banat IM. 1997. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 6:47–64.

Gregory A. 2001. Enhanced oil recovery—Ten years on. Petrol Rev 55(655):40–43.

Hoffman LM, Jendrisak JJ, Meis RJ, Goryshin IY, Reznikof SW. 2000. Transposome insertional mutagenesis and direct sequencing of microbial genomes. Genetica 108(1):19–24.

Jang SS, Lin ST, Maiti PK, Blanco M, Goddard PA III, Shuler P, Tang YC. 2004. Molecular dynamics study if a surfactant-mediated decane–water interface: Effect of molecular architecture of alkyl benzene suflonate. J Phys Chem B 108:12130–12140.

Lang S, Wullbrandt D. 1999. Rhamnolipids-biosynthesis, microbial production and application potential. Appl Microbiol Biotechnol 51:22–23.

Lin SC. 1996. Biosurfactant: Recent advances. J Chem Technol Biotechnol 63:109–120.

Lindhardt TJ, Bakhit R, Danies L, Mayerl R, Pickenhagen W. 1989. Microbially produced rhamnolipid as a source of rhamnose. Biotechnol Bioeng 33:365–368.

Maier RM, Soberon-Chavez G. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl Microbiol Biotechnol 54(5):625–633.

Maneerat S. 2005. Production of biosurfactants using substrates from renewable resources. Songklanakarin J Sci Technol 27(3):675–683.

Miller DJ, von Halasz SO, Schmidt M, Pusch G. 1991. Dual surfactant systems for enhanced oil recovery at high salinities. J Petrol Sci Eng 6: 63–72.

Mukherjee S, Das P, Sen R. 2006. Towards commercial production of microbial surfactants. Trends Biotechnol 24(11):509–515.

Mulligan CN. 2005. Environmental applications for biosurfactants. Environ Pollut 133(2):183–198.

Mulligan CN, Gibbs BF. 1993. Factors influencing the economics of biosurfactants. In: Kosaric N, editor. Biosurfactants, production, properties, applications. New York: Marcel Dekker. p 329–371.

Nitschke M, Costa SG, Contiero J. 2005. Rhamnolipid surfactants: An update on the general aspects of these remarkable biomolecules. Biotechnol Prog 21(6):1593–1600.

Ochsner UA, Fiechter A, Reiser J. 1994. Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J Biol Chem 269:19787–19795.

Patel RM, Desai AJ. 1997. Surface-active properties of rhamnolipids from *Pseudomonas aeruginosa* GS3. J Basic Microbiol 37(4):281–286.

Rahim R, Ochsner UA, Olvera C, Graninger M, Messner P, Lam JS, Soberon-Chavez G. 2001. Cloning and functional characterization of the *Pseudomonas aeruginosa* rhlC gene that encodes rhamnosyltransferase 2, an enzyme responsible for di-rhamnolipid biosynthesis. Mol Microbiol 40(3):708–718.

Robert M, Mercade ME, Bosch MP, Parra JL, Espiny MJ, Manresa MA, Guinea J. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44 T1. Biotechnol Lett 11:871–874.

Rodrigues L, Banat IM, Teixeira J, Oliveira R. 2006. Biosurfactants: Potential applications in medicine. J Antimicrob Chemother 57(4): 609–618.

Rosenberg E, Ron EZ. 1999. High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162.

Sambrook J, Russell DW. 2001. Molecular cloning: A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schenk T, Schuphan I, Schmidt B. 1995. High-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*. J Chromatogr A 693:7–13.

Shuler PJ, Kuehne DL, Lerner RM. 1989. Improving chemical flood efficiency with micellar/alkaline/polymer processes. J Petrol Technol 41:80–88.

Smith AW, Iglewski BH. 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res 17(24):10509.

Soberon-Chavez G, Aguirre-Ramirez M. 2005. Is *Pseudomonas aeruginosa* only ''sensing quorum''? Crit Rev Microbiol 31:171–182.

Soberon-Chavez G, Lepine F, Deziel E. 2005. Production of rhamnolipids by *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 68(6):718– 725.

Stover CK, Pham XQT, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim RM, Smith KA, Spencer DH, Wong GKS, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406(6799):959–964.

Van Hamme JD, Singh A, Ward OP. 2003. Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67(4):503–549.