CALIFORNIA INSTITUTE
OF TECHNOLOGY

DOE CONTRACT **DE-FC26-04NT15525**
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Bio-Engineering High Performance Microbial Strains for MEOR by Directed Protein Evolution Technology

- **Date of Report: October 2005**
- **Covering the period October 2004 – September 2005, Year one of the Project Period**
- Sections written by Xiangdong Fang, Qinhong Wang, Patrick Shuler and Baojun Bai

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Bio-Engineering High Performance Microbial Strains for MEOR by Directed Protein Evolution Technology

DOE Project: DE-FC26-04NT15525

Progress Report July 2005

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California Institute of Technology

Year one Annual Report October 2004 – September 2005

1.0 EXECUTIVE SUMMARY

This report summarizes the research work performed and results of the work for the year one of this 3-year project. The main objectives are 1) to employ the latest bioengineering technologies to develop manufacturing methods at the surface that create more cost-effective surfactants for the chemical flood EOR process; 2) to improve the surfactant activity and the thermal stability of bio-surfactant systems deployed subsurface for MEOR; and 3) to develop improved laboratory methods and tools that screen quickly candidate bio-systems for EOR.

During the first year of the project period,

1. We have successfully cloned the genes involved in the rhamnolipid and surfactin bio-synthesis.
2. By using the Transposon containing Rhamnosyltransferase gene rhlAB, we have successfully produced rhamnolipds in both *P. aeroginosa* PAO1-RhlA$^-$ strain and *P. fluorescens* ATCC15453 strain, with the increase of 55 to 175 fold in rhamnolipid production comparing with wild type bacteria strain.
3. We have completed the first round direct evolution studies using Error-prone PCR technique and have constructed the library of RhlAB-containing Transposon to express mutant gene in heterologous hosts.
4. Several methods, such as colorimetric agar plate assay, colorimetric spectrophotometer assay and oil spreading assay, have been established to detect and screen rhamnolipid and surfactin production.
5. The behavior of the rhamnolipid and surfactin as EOR agents have been characterized by surfactant adsorption assay, Interfacial Tension (IFT) assay and wettability tests Using calcite flotation methods.

2.0 BACKGROUND

In MEOR processes it is thought that the bacteria in place may mobilize trapped oil by several mechanisms. Some of the best candidate microbes produce gases, low molecular weight acids, and polymers in addition to surfactants (Bryant, 1987, 1989, 1996, Premuzic, 1991). This proposed work will focus on the biological generation of surfactants because the ability of these chemicals to reduce interfacial tension (IFT) between the water and oil phases offers the best chance of mobilizing substantial amounts of hydrocarbons not otherwise recovered by waterflooding. It has been reported that a 30% increase in total oil recovery from underground sandstone by using trehalolipids from *Nocardia rhodochrous.* More typically, bio-systems in MEOR laboratory tests recover of the order of 10 – 20% of water flood incremental oil (Yonebayashi, 1997).

The chemical material cost is perhaps the main factor controlling the profitability of conventional surfactant flooding EOR (Wu 1996, Taber 1996). An inherent potential advantage of a biological approach is that we can make surfactants whose cost is decoupled from the price of crude oil. The nutrients and other raw materials to create bio-surfactants do not have to be petroleum-based, and in fact potentially may come from waste streams. Commercial synthetic surfactants invariably are more expensive than the crude oil from which they are derived.

For EOR application, chemical surfactants cost in range of $1 to $3 per pound, whereas lignin-based sulfonates are cheaper. But the current cost of bio-surfactants may be about 3 to 10 times higher using current technology (Desai and Banat, 1997). Attempts have been made to improve overall process economics in bio-surfactant production. Those efforts have been focused on searching for cheap substrates, increasing the productivity by manipulating physiological conditions, mixing multiple microbial cultures, and modifying the downstream recovery processes. Potential overall improvements via these strategies are quite limited.

Results from our study will open the door to manufacture of bio-surfactants suitable for EOR at a reasonable price. First and foremost, we will employ advanced bioengineering methods to have bacteria with the capability to create surfactant with orders of magnitude greater activity than at present. Secondly, because these materials are to used in the oilfield, there is much less concern with the purity, color, etc. of the

produced product. We can save on much of the expense normally associated with product separation and purification by accepting a less refined product for oilfield use. Especially if the bio-surfactant manufacture is at or near a target oil field, then the supernatant solution containing the chemical may be a sufficiently concentrated product as is (there is little or no transportation cost, hence excess water in the product is not a factor.) Thirdly, one of our strategies is to engineer the bacteria to be more robust in their nutritional requirements. Potentially, the nutrients for the bacteria creating the bio-surfactant may come from waste streams such as waters containing dissolved and dispersed oil components, further driving down the cost of their manufacture.

Another major challenge to deploying MEOR technology where the bacteria are introduced into the subsurface is that they have only limited activity to produce bio-surfactant (and other desirable products). A second concern is also the "care and feeding" of injected bacteria. Once these bacteria are introduced they must receive continued nutrition to survive. They also are subject to decreased activity or even extinction under the high reservoir temperatures or other changes in their environment.

Our proposed approach will address both of these concerns. We will clone the mutated gene responsible for extraordinary growth of bio-surfactant from a host organism into candidate bacteria that have a demonstrated ability to thrive in oil reservoir environments. Thermophillic, chemotropic organisms are prime host candidates as they 1) function at high temperatures, 2) use constituents indigenous to the substrate, 3) tolerant of pH and salinity extremes.

3.0 RESULTS AND DISCUSSION

Briefly, the overall strategy for the study is:

1) Start with microbes that make bio-surfactants by a well-defined mechanism where the responsible gene is well established. This knowledge makes it possible to begin immediately to carry out directed evolution studies to create mutant candidates.

2) Clone promising mutant candidates from the above study into thermophilic, oil field adapted microbes. The objective is to create strains that make a lot of one of these bio-surfactants, and in a host microbe that functions well in-situ in an oil reservoir.
3) Conduct studies to evaluate these bio-surfactants as candidates for conventional chemical flood EOR, as compared to common synthetic surfactants. Also determine if the oilfield adaptable microbes now with "supermutants" are superior for in-situ, MEOR.

3.1 Summary of the Tasks for this Reporting Period

Task 1.0 Directed evolution studies for *P. aeruginosa* and *B. subtilis* (24 months)
These studies will identify the first-round of "super-mutants" with one or more desirable characteristics, 1) "super-activity", 2) thermal stability, or 3) use of cheap substrates. We will write a report documenting in particular the detailed laboratory procedures to arrive at the successful mutant candidates.

Subtask 1.1 (Month 1 - 8) -- Develop screening methods to carry out an evaluation of the performance of candidate mutants, based on one of four approaches: axisymmetric drop shape analysis, rapid drop-collapsing test, thin-layer chromatography, or colormetric analysis. Conduct the first round mutagenesis tests via Error-prone PCR. Perform "base-case" fermentation with the natural, wild form of the two study bacteria.

Subtask 1.2 (month 9 to 22) -- Second round mutation studies (via in vitro DNA shuffling) to upgrade to a smaller number of better candidate mutants. Perform batch fermentation on selected mutated strains to demonstrate achieve a desired effect.

Task 2.0 Genetic cloning of "supermutants" into thermophilic oilfield strains (29 months)
The purpose of these studies is to clone the mutants with high activity and thermal stability into microbes adaptable to in-situ, oilfield reservoir conditions. Part of this task is to document the detailed laboratory procedures and results in our efforts to create a robust MEOR microbe.

Subtask 2.1 (Month 1 - 6) -- Literature review of thermophilic microbes that are candidates for oil reservoirs.

Subtask 2.2 (Month 7 - 20) -- Develop laboratory methods to add bio-surfactant function to these target microbes. Initially use wild, natural versions of *P. aeruginosa* and *B. subtilis* as the genome source.

Task 3.0 Evaluation of Bio-Surfactants and Bio-Systems for EOR (21 months)

The purpose of these studies is to 1) characterize and optimize the target bio-surfactants (rhamnolipids and surfactin) as candidate chemicals for conventional chemical EOR, and 2) evaluate in-situ, MEOR performance of oil reservoir microbes now mutated to produce prodigious amounts of rhamnolipids or surfactin. One activity will be to document the surfactant performance screening procedures and results in a report.

Subtask 3.1 (month 4 - 9) -- Micro-scale tests with purified rhamnolipid and surfactin. Develop analytical methods to measure bio-surfactant concentrations in solution. Using whole cell lysate generated from the wild bacteria strains; determine phase behavior and IFT versus a series of n-alkanes to characterize the behavior of the 2 bio-surfactants as EOR agents. Also conduct screening tests for bio-surfactant adsorption onto reservoir materials such as kaolinite.

3.2 Highlights of Efforts and Details of Results – Task 1, Directed evolution studies for *P. aeruginosa* and *B. subtilis*; and Task 2, cloning of "supermutants" into thermophilic oilfield strains

3.2.1 Overview the molecular mechanism of the rhamnolipid bio-synthesis

The goal of the studies in this Task is to identify the "super-mutant-genes" involved in the rhamnolipid or surfactin biosynthesis with one or more desirable characteristics, 1) "super-activity", 2) thermal stability, or 3) use of cheap substrates.

P. aeruginosa produces extracellular glycolipids named rhamnolipid which is composed of L-rhamnose and 3-hydroxyalkanoic acid. In liquid cultures, they are produced as a complex mixture of congeners containing one or two 3-hydroxy fatty acids

of various lengths, linked to a mono- or dirhamnose moiety. In general, the two more abundant rhamnolipids are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate. According to the biosynthetic pathway proposed by Burger et al, rhamnolipid synthesis proceeds by two sequential glycosyl transfer reactions, each catalysed by a different rhamnosyltransferase. The first rhamnosyltransferase, which catalyses the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA), is encoded by the rhlAB operon (Figure 1). Both genes, co-expressed from the same promoter, are essential for rhamnolipid synthesis. Environmental factors, especially nutritional conditions, influence rhamnolipid production. This quorum sensing system is composed of rhlI, the N-butyrylhomoserine lactone autoinducer synthase gene, and rhlR, which encodes the transcriptional activator. The second rhamnosyltransferase, encoded by rhlC, has been characterized and its expression shown to be co-coordinately regulated with rhlAB by the same quorum sensing system.

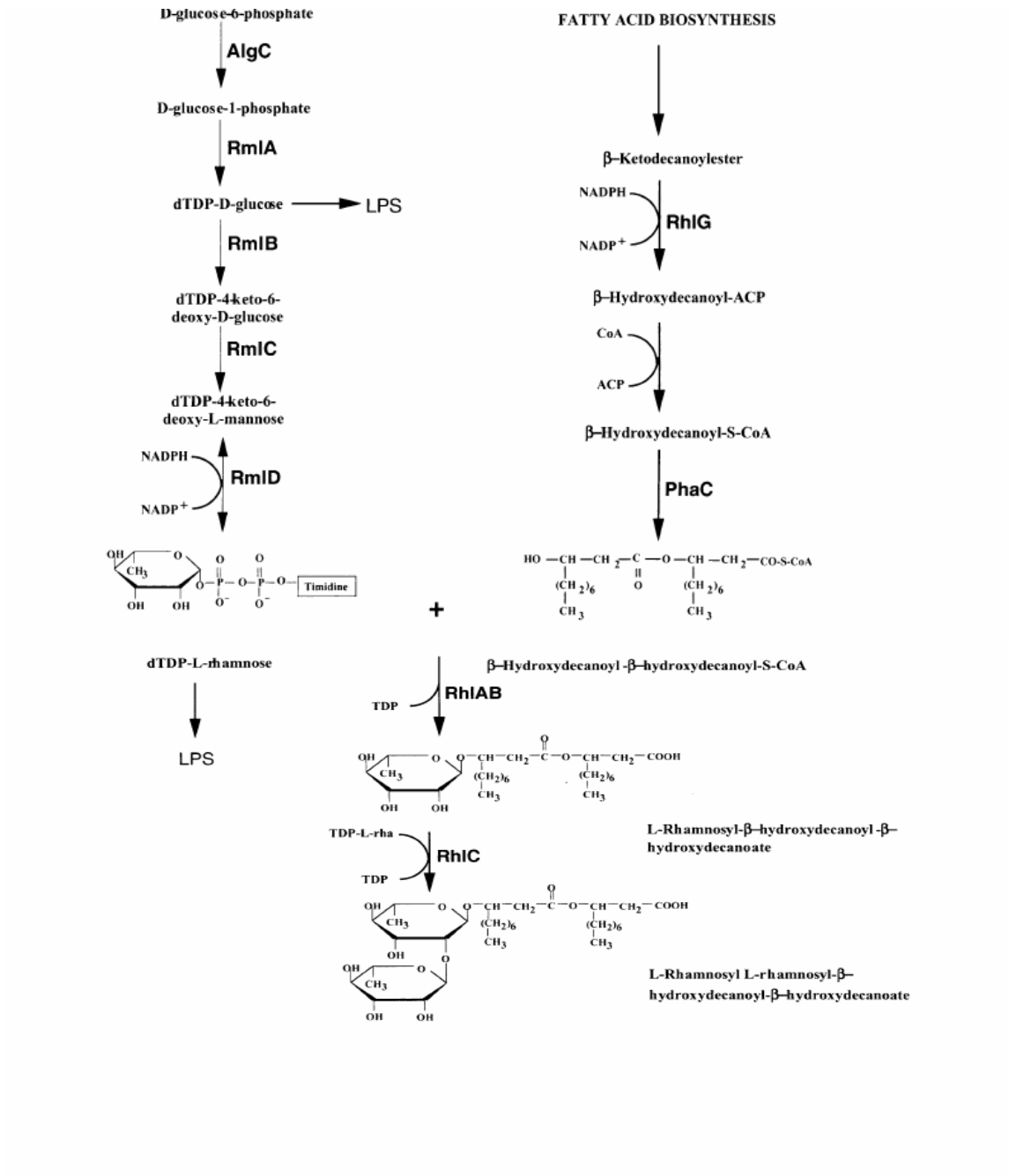


Figure 1. Putative synthetic pathway of rhamnolipids in P. aeruginosa

3.2.2 Vector preparation for gene cloning

1) The following commercial vectors were used in our rhamnosyltransferase gene cloning study.

a) pUC19

pUC19 (Invitrogen) is a small, high-copy number *E. coli* plasmid cloning vector containing multiple cloning site and Ampicillin resistance gene (Fig. 2a).

b) pACYC177

pACYC177 (New England Biolabs) is an *E. coli* plasmid cloning vector containing double drug resistance gene (amicillin and kanamycin).(Fig. 2b).

c) pACYC184

pACYC184 (New England Biolabs) is an *E. coli* plasmid cloning vector containing double drug resistance gene (chloramphenicol and tetracycline).(Fig. 2c).

d) pBluescript SK(+) II

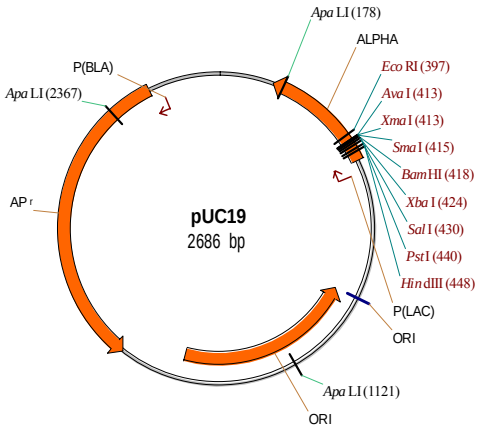
pBluescript SK(+) (Stratagene) II is a small, high-copy number *E. coli* phagemid cloning vector containing multiple cloning site and ampicillin resistance gene (Fig. 2d).

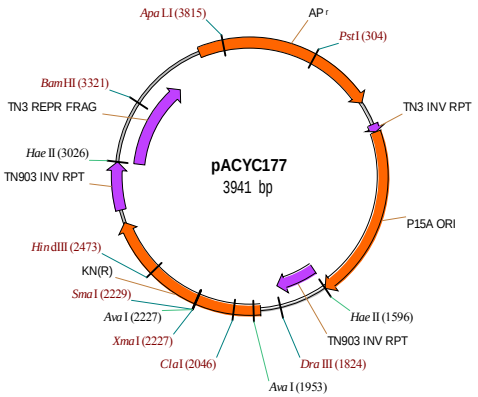
e) pET30a(+)

pET30a(+) (Novagen) is an *E. coli* plasmid expression vector containing multiple cloning site and kanamycin resistance gene (Fig. 2e).

f) pMOD-2 Transposon Construction Vector

pMOD-2 Transposon Construction Vector (Takara) is a pUC-based vector containing multiple cloning site and ampicillin resistance gene (Fig. 2f). After *Pvu*II or *Psh*AI digestion, a Tn5 transposon was produced.





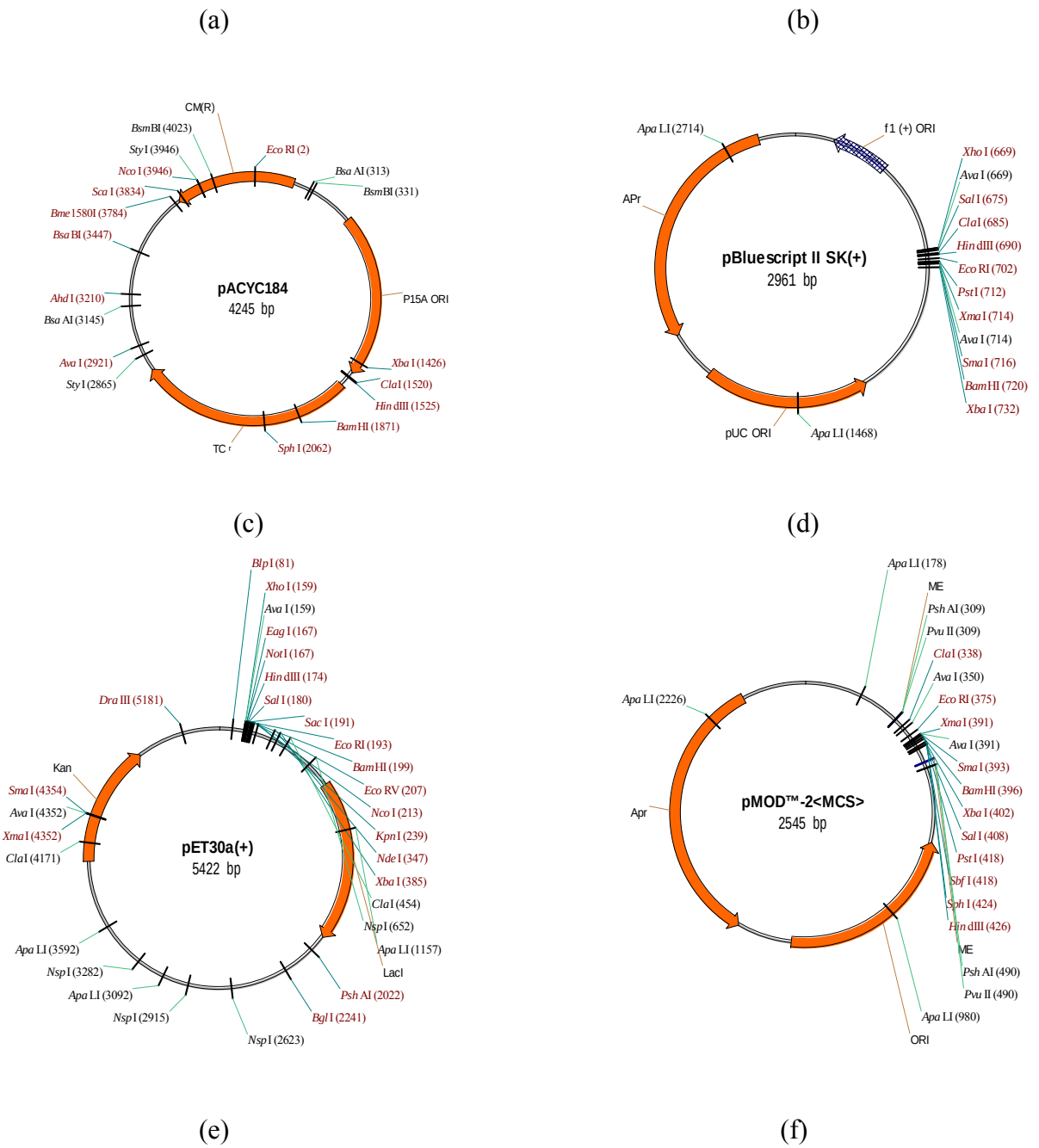


Fig. 2 Maps of commercial vectors used in our gene-cloning study

2) Vectors specially constructed for our rhamnosyltransferase gene cloning study

a) pUC19C

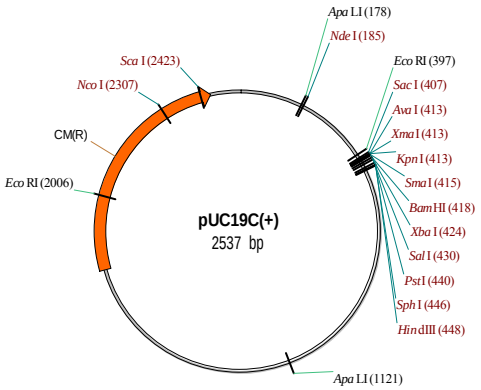
pUC19 was digested with *Aat*II and *Adh*I and then treated with T4 DNA polymerase to produce a 1.7kb double blunted-end fragment (Fig. 3a). pACYC184 was digested *Bsa*AI and *Bme*1580I and then treated with T4 DNA polymerase to produce a 750bp double blunted-end fragment which contains chloramphenicol resistance cassette (Fig. 3c). These two fragments were ligated with T4 DNA ligase and transformed into *E. coli* to produce recombinant plasmid pUC19C (Fig. 3a).

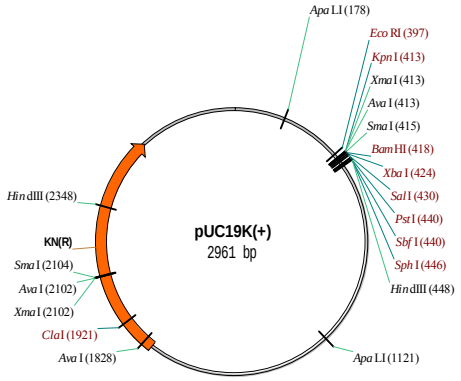
b) pUC19K

pUC19 was digested with *Aat*II and *Adh*I and then treated with T4 DNA polymerase to produce a 1.7kb double blunted-end fragment (Fig. 2a). pACYC177 was digested *Dra*III and *Hae*II and then treated with T4 DNA polymerase to produce a 1.2kp double blunted-end fragment which contains kanamycin resistance cassette (Fig. 2b). These two fragments were ligated with T4 DNA ligase and transformed into *E. coli* to produce recombinant plasmid pUC19K (Fig. 3b).

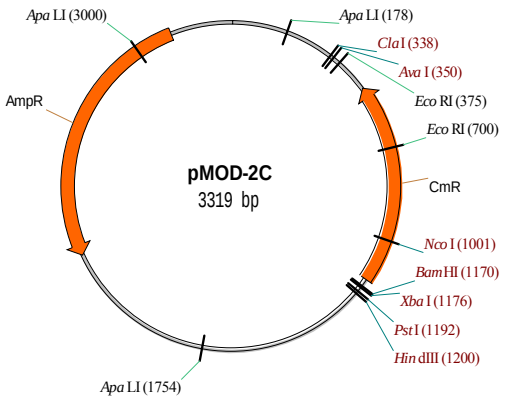
c) pMOD-2C

pMOD-2 Transposon Construction Vector was digested with *Sma*I and then treated with alkaline phosphatase to produce linear fragment. pACYC184 was digested *Bsa*AI and *Bme*1580I and then treated with T4 DNA polymerase to produce a 750bp double blunted-end fragment which contains chloramphenicol resistance cassette (Fig. 2c). These two fragments were ligated with T4 DNA ligase and transformed into *E. coli* to produce recombinant plasmid pMOD-2C (Fig. 3c). Thus, the recombinant plamid pMOD-2C can produce Tn5-derived tranposon which containing chloramphenicol resistance gene after *Pvu*II or *Psh*AI digestion.





(a) (b)



(c)

Fig.3 Vectors specially constructed in the gene-cloning study

3.2.3 Cloning Genes Involved in Rhamnolipid Biosynthesis

1) Cloning and Identification of Rhamnolipid Biosynthesis Gene Cluster from *Pesudomonas aeruginosa* PAO1 in *Esecherichia coli* DH5α

Designing PCR Primers

The DNA sequences of the rhamnosyltransferase genes rhlA, rhlB, rhlI and rhlR were pulled out from Genebank and list in attachment A of this report. DNA primers were designed and synthesized as listed in Table 1. Rhamnosyltransferase genes were then amplified by PCR with those primers, and then purified and cloned into the vectors described in the section 3.2.2.

Table 1 Oligonucleutide Primers used in this study

Name	Sequence (5'→3')
RhlABRL-1	CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG*
RhlABRL-2	CAGCCGAAGCTTGAAGCGGAAAAAAGTGCGCGAAACG

RhlAB-2	AATTGAATTCAGGACGCAGCCTTCAGCCATCG

* Sequences with underlines were restricted endonuclease sites which were needed for cloning.

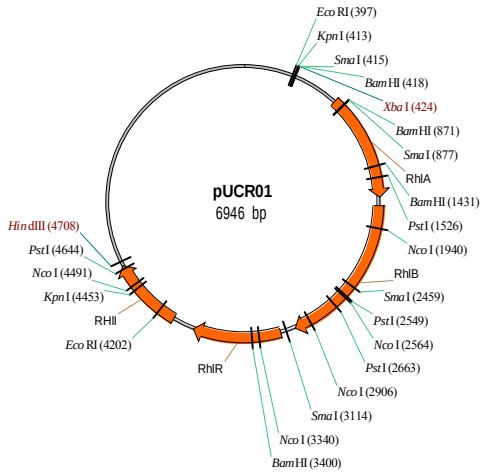
a) Whole rhamnolipid biosynthesis gene cluster RhlABRI with native operon promoter (containing upstream 400bp sequence) was amplified with primer RhlABRL-1 and RhlABRL-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template;

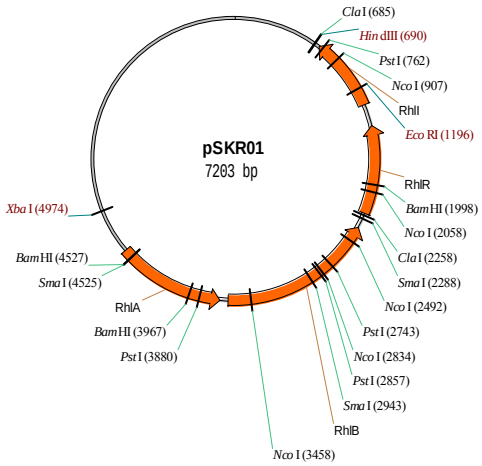
b) Rhamnosyltransferase 1 complex RhlAB with native operon promoter (containing upstream 400bp sequence) was amplified with primer RhlABRL-1 and RhlAB-2 and *P. aeruginosa* PA01genomic DNA as template;

Gene Cloning by PCR

a) PCR product of RhlABRI was digested with *Xba*I and *Hind*III and cloned into pUC19 and pBluescript II SK(+), respectively. The recombinant plasmid pUCR01 and pSKR01 were produced, respectively (Fig 4a and 4b).

b) PCR product of RhlAB was digested with *Xba*I and *Eco*RI and cloned into pUC19 and pBluescript II SK(+), respectively. The recombinant plasmid pUCRPAB and pSKRPAB were produced, respectively (Fig 4c and 4d).





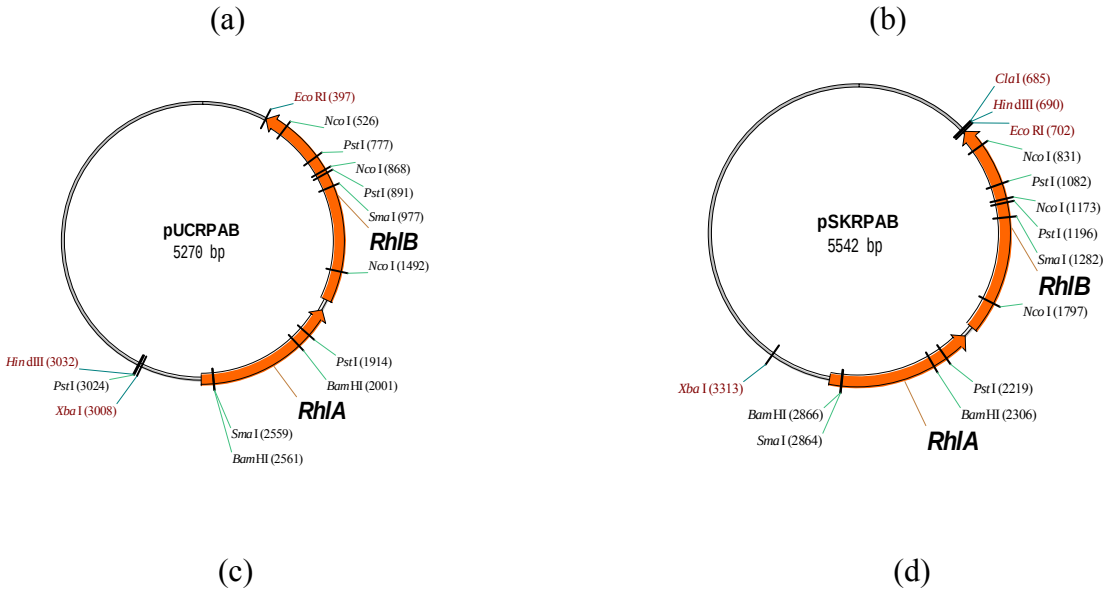


Fig. 4 Recombinant plasmids containing rhamnolipid biosynthesis genes

3.2.4 Cloning and Identification of dTDP-L-rhamnose Biosynthetic Gene Cluster from *Pesudomonas aeruginosa* PAO1 in *Esecherichia coli* DH5α

Table 2 Oligonucleutide Primers used in this study

Name	Sequence (5'→3')
PaRml-1	CGCATCTAGACTTGTAATCAGTAGGTC CCG*
PaRml-2	GGCTAAGCTTGCTTTCAAGACGAACGAACC

* Sequences with underlines were restricted endonuclease sites which were needed for cloning

Whole dTDP-L-rhamnose Biosynthetic Gene Cluster RmlABCD with native operon promoter (containing upstream 300bp sequence) was amplified with primer PaRml-1 and PaRml-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template; PCR product of RmlABCD was digested with *Xba*I and *Hind*III and cloned into pUC19C. The recombinant plasmid pUCCPaRml was produced and identified by restriction digestion analysis (Fig 5).

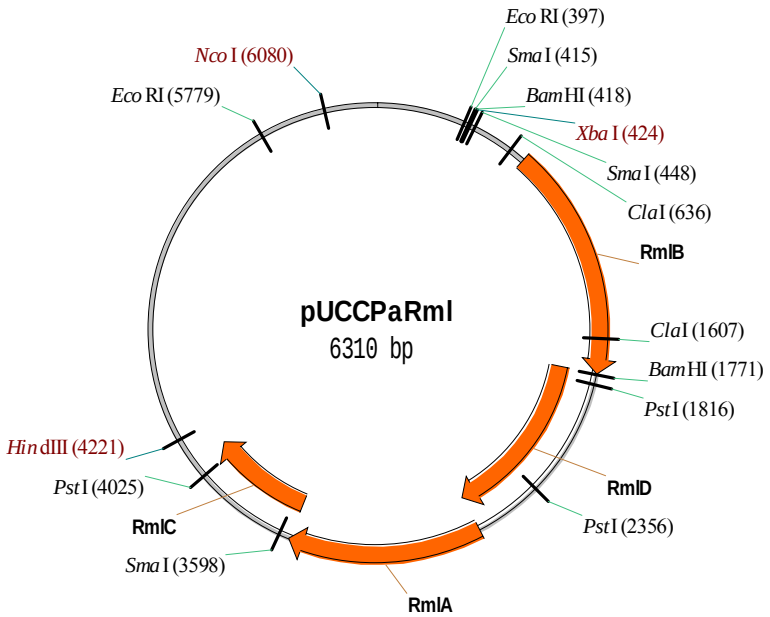


Fig.5 Recombinant plasmids containing dTDP-L-rhamnose Biosynthetic Gene Cluster

3.2.5 Cloning and Identification of polyhydroxyalcanoate synthase PhaC and ketoacyl reductase RhlG from *Pesudomonas aeruginosa* PAO1 in *Esecherichia coli* DH5α

Table 3 Oligonucleutide Primers used in this study

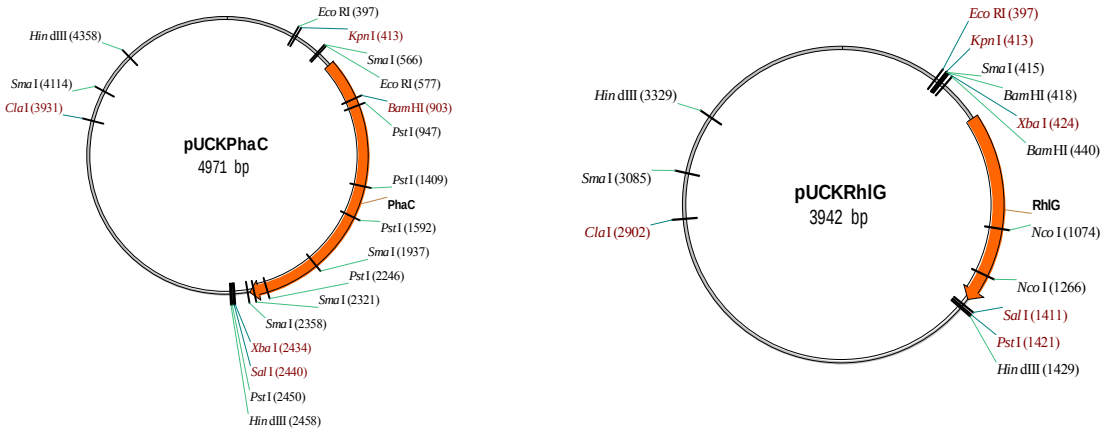
Name	Sequence (5' – 3')
PaPhaC-1	CAG TGGTACCGC ATA GTC AAT GTT CCG CAA CG*
PaPhaC-2	CCA GTCTAGAGA GCA TTC GAG GAT TCG GTC
PaRhlG-1	CGT CTCTAGACG GTG CAA TGG ATC CTT GGC
PaRhlG-2	CTG AGTCGACCG TCA TGT TAC CCG GAT TGG

* Sequences with underlines were restricted endonuclease sites which were needed for cloning

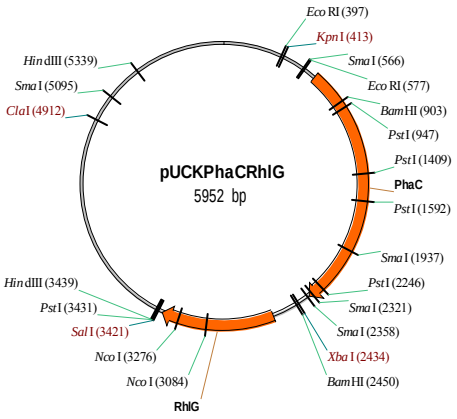
a) Polyhydroxyalcanoate synthase PhaC with native operon promoter (containing upstream 300bp sequence) was amplified with primer PaPhaC-1 and PaPhaC-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template; PCR product of PhaC was digested with *Kpn*I and *Xba*I and cloned into pUC19K. The recombinant plasmid pUCKPaPhaC was produced and identified by restriction digestion analysis (Fig 6a).

b) Ketoacyl reductase RhlG with native operon promoter (containing upstream 300bp sequence) was amplified with primer PaRhlG-1 and PaRhlG-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template; PCR product of RhlG was digested with *Xba*I and *Sal*I and cloned into pUC19K. The recombinant plasmid pUCKPaRhlG was produced and identified by restriction digestion analysis (see Fig 6b).

c) PCR product of RhlG after *Xba*I and *Sal*I digestion was also cloned into pUCKPaPhaC. The recombinant plasmid pUCKPaPhaCRhlG was produced and identified by restriction digestion analysis (Fig 6c).



(a) (b)



(c)

Fig. 6 Recombinant plasmids containing Polyhydroxyalcanoate synthase and Ketoacyl reductase

3.2.6 Gene Cloning for Surfactin Biosynthesis

Table 4 Oligonucleutide Primers used in this study

Name	Sequence (5' – 3')
SrfAA-1	CCAGGGTACCAT GGA AAT AAC TTT TTA CCC TTT AAC GGA TGC AC *
SrfAA-2	GCTGCTCGAGGACTCATATGTTTCGCTCCCGCCTTCTGT
SrfAB-1	GAGCCCATGGCTATG AGC AAA AAA TCG ATT CAA AAG GTG TAC GC
SrfAB-2	GCTGCTCGAGGA CTC ATA TGT TTC GCT CCC GCC TTC TGT
SrfAC-1	CCAGGGTACCATGAGTCAATT TAG CAA GGA TCA GGT TCA AG
SrfAC-2	CGTCCTCGAGGG CTG TCT CCT CCT TTC ATC ACT TC
SrfAD-1	CCCGGGTACCATGAG CCA ACT CTT CAA ATC ATT TGA

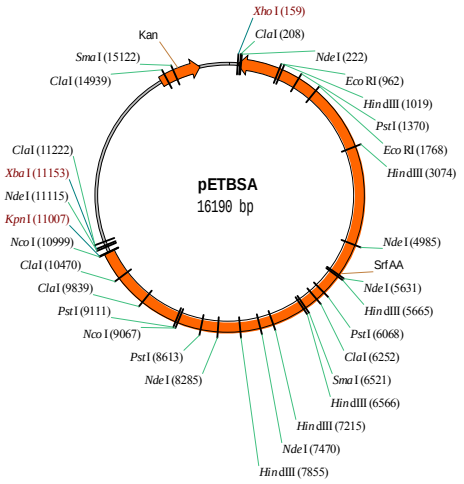
	TGC GTC
SrfAD-2	CGTCCTCGAGGCCGA AGC TGT CCG CTT TTG ATC

* Sequences with underlines were restricted endonuclease sites which were needed for cloning

a) Surfactin synthase SrfAA was amplified with primer srfAA-1 and SrfAA-2 and *Bacillus subtilis* ATCC 21332 genomic DNA as template; PCR product of SrfAA was digested with *Kpn*I and *Xho*I and cloned into pET30a(+). The recombinant plasmid pETBSA was produced and identified by restriction digestion analysis (see Fig 7a).

b) Surfactin synthase SrfAB was amplified with primer srfAB-1 and SrfAB-2 and *Bacillus subtilis* ATCC 21332 genomic DNA as template; PCR product of SrfAB was digested with *Nco*I and *Xho*I and cloned into pET30a(+). The recombinant plasmid pETBSB was produced and identified by restriction digestion analysis (see Fig 7b).

c) Surfactin synthase SrfAC was amplified with primer srfAC-1 and SrfAC-2 and *Bacillus subtilis* ATCC 21332 genomic DNA as template; PCR product of SrfAC was digested with *Kpn*I and *Xho*I and cloned into pET30a(+). The recombinant plasmid pETBSC was produced and identified by restriction digestion analysis (see Fig 7c).
d) Thiseterase SrfAD for sufactin biosynthesis was amplified with primer srfAD-1 and d) SrfAD-2 and *Bacillus subtilis* ATCC 21332 genomic DNA as template; PCR product of SrfAD was digested with *Kpn*I and *Xho*I and cloned into pET30a(+). The recombinant plasmid pETBSD was produced and identified by restriction digestion analysis (see Fig 7d).
e) Surfactin biosynthetic gene SrfACD was amplified with primer srfAC-1 and SrfAD-2 and *Bacillus subtilis* ATCC 21332 genomic DNA as template; PCR product of SrfACD was digested with *Kpn*I and *Xho*I and cloned into pET30a(+). The recombinant plasmid pETBSCD was produced and identified by restriction digestion analysis (see Fig 7e).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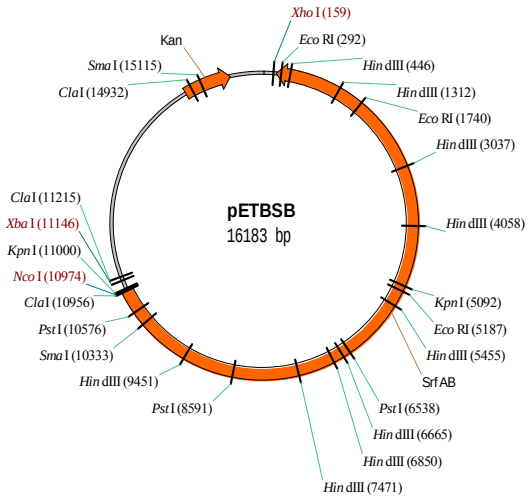
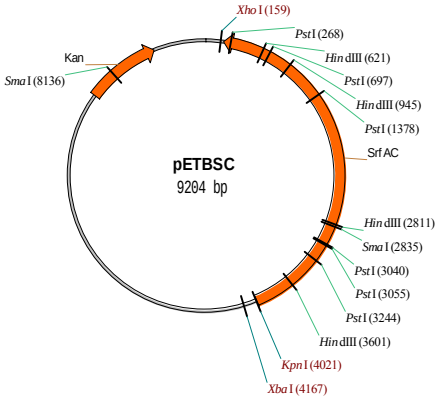
16190 bp
Xho I (159)
Cla I (208)
Nde I (222)
Eco RI (962)
Hin dIII (1019)
Pst I (1370)
Eco RI (1768)
Hin dIII (3074)
Nde I (4985)
Srf AA
Nde I (5631)
Hin dIII (5665)
Pst I (6068)
Cla I (6252)
Sma I (6521)
Hin dIII (6566)
Hin dIII (7215)
Nde I (7470)
Hin dIII (7855)
Pst I (8613)
Nde I (8285)
Nco I (9067)
Pst I (9111)
Cla I (9839)
Cla I (10470)
Nco I (10999)
Kpn I (11007)
Nde I (11115)
Xba I (11153)
Cla I (11222)
Sma I (15122)
Cla I (14939)
Kan

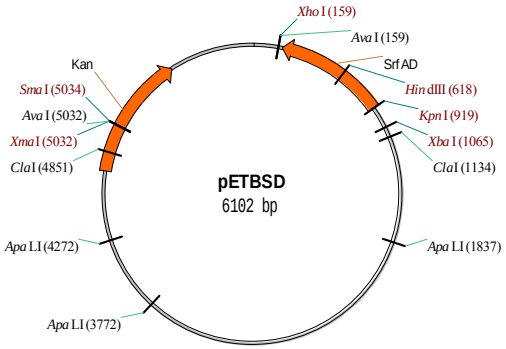
(a)


pETBSB
16183 bp
Xho I (159)
Eco RI (292)
Hin dIII (446)
Hin dIII (1312)
Eco RI (1740)
Hin dIII (3037)
Hin dIII (4058)
Kpn I (5092)
Eco RI (5187)
Hin dIII (5455)
Srf AB
Pst I (6538)
Hin dIII (6665)
Hin dIII (6850)
Hin dIII (7471)
Pst I (8591)
Hin dIII (9451)
Sma I (10333)
Pst I (10576)
Cla I (10956)
Nco I (10974)
Kpn I (11000)
Xba I (11146)
Cla I (11215)
Sma I (15115)
Cla I (14932)
Kan

(b)


pETBSC
9204 bp
Xho I (159)
Pst I (268)
Hin dIII (621)
Pst I (697)
Hin dIII (945)
Pst I (1378)
Srf AC
Hin dIII (2811)
Sma I (2835)
Pst I (3040)
Pst I (3055)
Pst I (3244)
Hin dIII (3601)
Kpn I (4021)
Xba I (4167)
Sma I (8136)
Kan

(c)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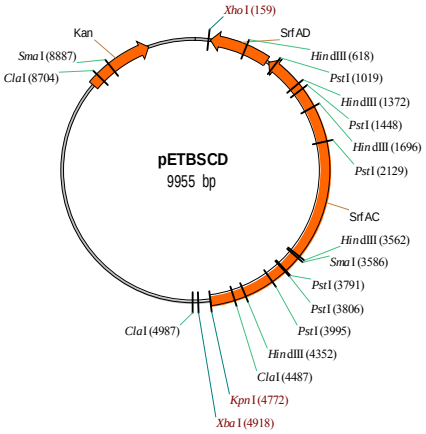
6102 bp
Xho I (159)
Ava I (159)
Srf AD
Hin dIII (618)
Kpn I (919)
Xba I (1065)
Cla I (1134)
Apa LI (1837)
Apa LI (3772)
Apa LI (4272)
Cla I (4851)
Xma I (5032)
Ava I (5032)
Sma I (5034)
Kan

(d)



(e)

Fig.7 Recombinant plasmids containing Surfactin Biosynthesis genes

3.2.7 Construction of Engineered *E. coli* for producing rhamnolipids

The different combination of plasmids which containing rhamnolipid biosynthetic genes was transformed into *E. coli* DH5α to obtain the following engineered strains (Table 5).

Table 5 Engineered *E. coli* for producing rhamnolipids

Strain	Phenotype
E. coli ABMSR01	containing pSKR01; Amp
E. coli ABMUR01	containing pUCR01; Amp
E. coli ABMSRAB	containing pSKRAB; Amp
E. coli ABMURAB	containing pUCRAB; Amp
E. coli ABMR71	containing pUCRAB and pUCCRml; Amp, Cm
E. coli ABMR701	containing pUCRAB, pUCCRml and pUCKPhaC; Amp, Cm, and Kan
E. coli ABMR702	containing pUCRAB, pUCCRml and pUCKRhlG; Amp, Cm, and Kan
E. coli ABMR703	containing pUCRAB, pUCCRml and pUCKPhaCRhlG; Amp, Cm, and Kan

Amp: ampicillin resistance; Cm: Chloramphenicol resistance; Kan: kanamycin resistance.

3.2.8 Construction of RhlAB-containing Transposon insertion mutants

Rhamnosyltransferase 1 complex RhlAB with native operon promoter (containing upstream 400bp sequence) was amplified with primer RhlABRL-1(5'-CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3') and RhlB-2 (5' - AAC CAA GCT TTC AGG ACG CAG CCT TCA GCC ATC G - 3') and *P. aeruginosa* PA01genomic DNA as template; PCR product of RhlAB was digested with XbaI and HindIII and cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRAB (Fig. 8). 3.5kb RhlAB-containing Transposon was produced by digesting pMOD-2CRAB with *Psh*AI. RhlAB-containing Transposon (TnCRPAB) was transformed into transposome after adding transposase (Fig. 9).

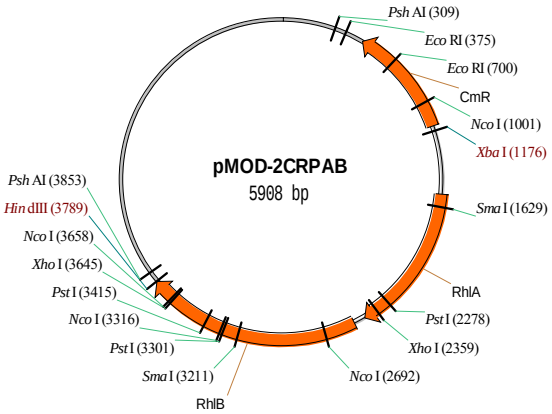
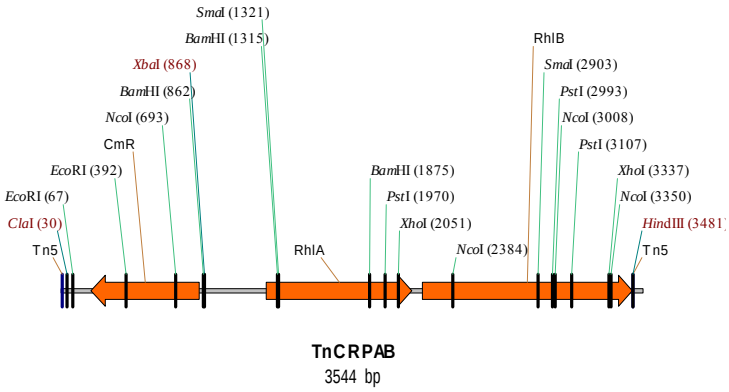
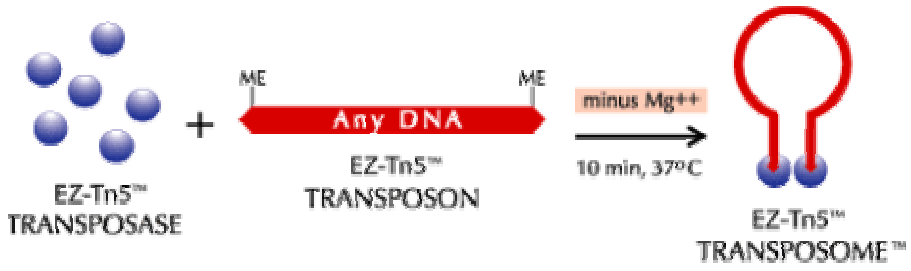


Fig.8 Recombinant plasmids with RhlAB-containing Transposon



(a)



(b)

Fig.9 Structure of RhlAB-containing Transposon (a) and diagram of transposome construction (b)

RhlAB-containing Transposome then was transformed into *P. aeroginosa* PAO1RhlA- (a gift from Dr. Joseph S. Lam) and *P. fluorescens* ATCC15453 by electroporation. Insertion mutants was selected by antibiotics resistance and confirmed by PCR (Fig. 10).

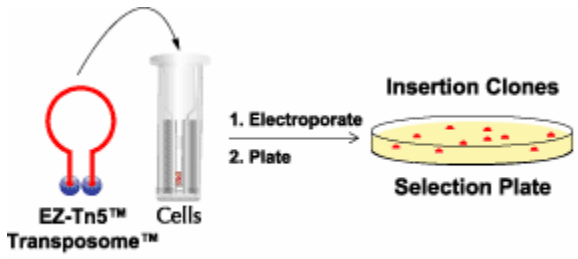


Fig.10 Diagram of Insertaion mutant construction and selection

3.2.9 Rhamnolipid production by engineered *Esecherichia coli*

Strains

Esecherichia coli DHα stains with different combination of plasmids which contained genes from rhamnolipid biosynthetic pathway were tested for rhamnolipid production (see Table 5). *Esecherichia coli* DHα with plasmid pUC19 was as control.

Media

LB (5g/L Yeast extract, 10g/L tryptone, 10g/L NaCl) media was used in the seed culture. The mineral salts medium and a 2% glucose or soybean oil as carbon source was as rhamnolipd fermentation media. The mineral salts medium used throughout this study was made according to Lindhardt *et al.*(1) contained (g/L): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g/L): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4$. $5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17. The trace element solution was filter-sterilized through a 0.22-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

Cultivation conditions

Engineered *E. coli* was first grown in LB medium for 24h at 30ºC with shaking and then diluted 1:10 into mineral salts medium and 2% glucose or soybean oil and incubated for 5 days. Incubation was carried out in 18X150mm test tube with 6mL medium and at 30ºC with orbital shaking at 250rpm.

Analytical method for rhamnolipid

Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g). The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.2mL of H_2O was added. To 0.1mL of each sample 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added (2). After heating for 30min at 80ºC the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L) and expressed as rhamnose equivalents.

Results

Table 6 Rhamnolipid fermentation by Engineered *E. coli*.

Stains	Rhamnolipid (mg/L)	
	Glucose	Soybean oil
Esecherichia coli DHα/pUC19	4.40	0.71
E. coli ABMSR01	7.01	0.80
E. coli ABMUR01	5.85	1.42
E. coli ABMSRAB	7.18	2.13
E. coli ABMURAB	10.32	2.50
E. coli ABMR71	6.11	1.67
E. coli ABMR701	7.28	2.02
E. coli ABMR702	10.07	1.68
E. coli ABMR703	11.74	2.16

3.2.10 Rhamnolipid production by RhlAB-insertion mutants of *P. aeroginosa* and *P. fluorescens*

Strains

P. aeroginosa PAAB02 and PAAB06 and *P. fluorescens* PFAB01 with RhlAB-insertion in chromosome were tested for rhamnolipid production. *P. aeroginosa* PAO1-RhlA$^-$ (from the gift from Dr.)and *P. fluorescens* ATCC15453 were as controls.

Media

Nutrient broth (NB) (Difco) was used in the seed culture. The mineral salts medium and a 2% glucose or soybean oil as carbon source was as rhamnolipd fermentation media. The mineral salts medium used throughout this study was made according to Lindhardt *et al.* contained (g/L): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g/L): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4$. $5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17. The trace element solution was filter-sterilized through a 0.22-µm

membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

Cultivation conditions

Psedonomas wildtype and mutants were first grown in NB medium for 24h at 30ºC with shaking and then diluted 1:10 into mineral salts medium and 2% glucose or soybean oil and incubated for 5 days. Incubation was carried out in 18X150mm test tube with 6mL medium and at 30ºC with orbital shaking at 250rpm. For time course of rhamnolipid fermentation, incubation was carried out in 250mL Erlenmeyer flaks with 25mL medium and at 37ºC with orbital shaking at 250rpm.

Analytical method for rhamnolipid

Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g). The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.2mL of H_2O was added. To 0.1mL of each sample 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30min at 80ºC the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L) and expressed as rhamnose equivalents.

Results

Table 7 Rhamnolipid fermentation by *Pseudonomas* mutants

Stains	Rhamnolipid (mg/L)	
	Glucose	Soybean oil
P. aeroginosa PAO1-RhlA$^-$	4.4	11.2
P. aeroginosa PAAB02	757.6	1746.7
P. aeroginosa PAAB06	785.4	1819.1
P. fluorescens ATCC15453	6.6	19.4
P. fluorescens PFAB01	847.9	1076.8

The results shows that two of our *P. aeroginosa* engineered strains, PAAB02 and PAAB 06, comparing with *P. aeroginosa* PAO1-RhlA$^-$ strain, both gave about 175 fold increase in rhamnolipid production when grew in Glucose medium; and about 160 fold increase in rhamnolipid production when grew in soybean oil medium. When *P. fluorescens* strain is used, our engineered strain PFAB01 produced about 128 times more rhamnolipids than wild type strain when grew in glucose medium, and 55 times more rhamnolipids when grew in soybean oil medium.

3.2.11 Error-prone PCR of Rhamnosyltransferase complex RhlAB

Error-prone PCR of Rhamnosyltransferase 1 complex RhlAB was conducted with GeneMorph II Random Mutagenesis Kit (Stratagene) accoding to instruction manual. The primers for PCR were RhlABRL-1 (5'-CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3') and RhlB-2 (5' - AAC CAA GCT TTC AGG ACG CAG CCT TCA GCC ATC G - 3'). Plamid pUCRPAB (Fig 3c) was as DNA template.

3.2.12 Library construction of RhlAB-containing Transposon insertion mutants of P. aeroginosa

Error-prone PCR product of RhlAB was liagted with 3.3kb XbaI-HindIII fragment from plasmid pMOD-2C (Fig. 3c) with T4 DNA liagse. Ligation product was digested with *Psh*AI and a 3.5kb *Psh*AI-fragment which is RhlAB-containing transposon was purified from digestion product. RhlAB-containing transposon was transformed into *P. aeroginosa* PAO1-RhlA$^-$ by electroporation. Electroporated *P. aeroginosa* PAO1-RhlA$^-$ was plated in LB plus chloramphenicol (50μg/L) and incubated at 30ºC for 3 days. Those colonies appeared on LB agar generally were RhlAB-containing Transposon insertion mutants. We finally construct a library of 3500 mutants for further screening for rhamnolipid production and structure analysis by HPLC-MS.

3.3 Highlights of Efforts and Details of Results – Task 1, Develop screening methods to carry out an evaluation of the performance of candidate mutants

3.3.1 Analytical method for rhamnolipid (Oricinol Method)

Methods

1) Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g);
2) The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v);
3) The organic phase was evaporated to dryness and 0.2mL of H_2O was added;
4) To 0.1mL of each sample 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added;
5) After heating for 30min at 80ºC the samples were cooled at room temperature (15-20min) and the OD_{421} was measured;
6) The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L) and expressed as rhamnose equivalents.

Results

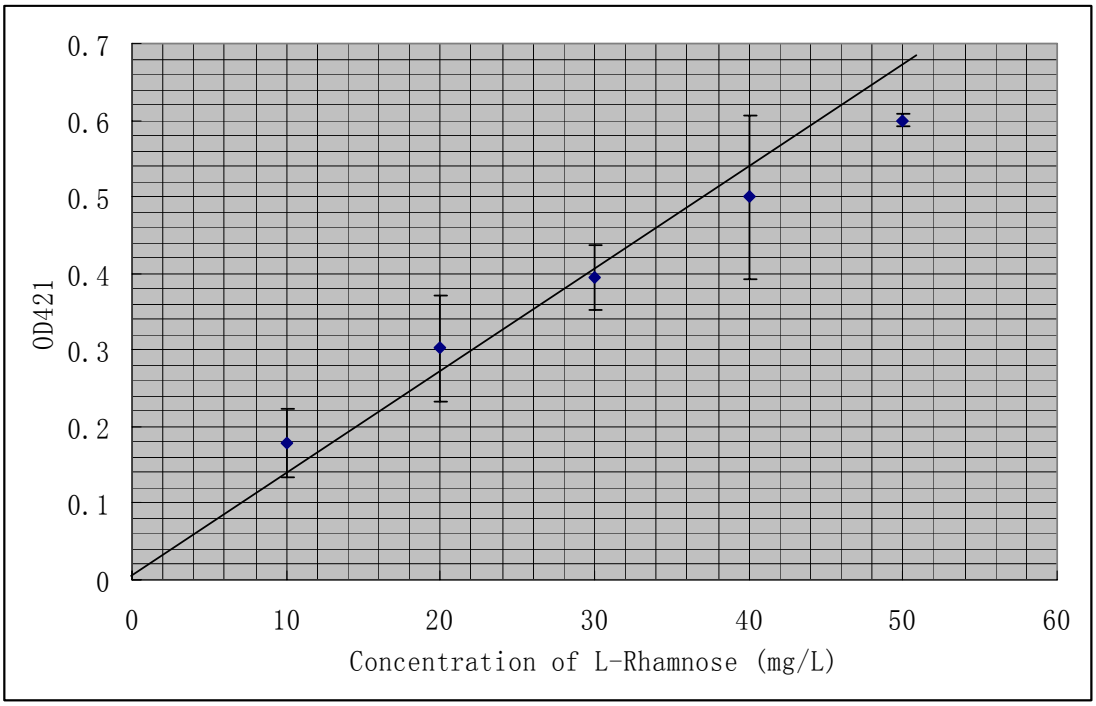


Fig. 11 Standard Curve of L-Rhamnose assay by Orcinol method

3.3.2 semi-quantitative agar plate assay to detect the extracellular rhamnolipids.

Methods

On the light blue agar that contains CTAB and basic dye methylene blue, rhamnolipid-productive pseudomonas colonies are surrounded by dark blue halos. And the diameter of the dark blue halo is proportional to the rhamnolipid concentration.

Results

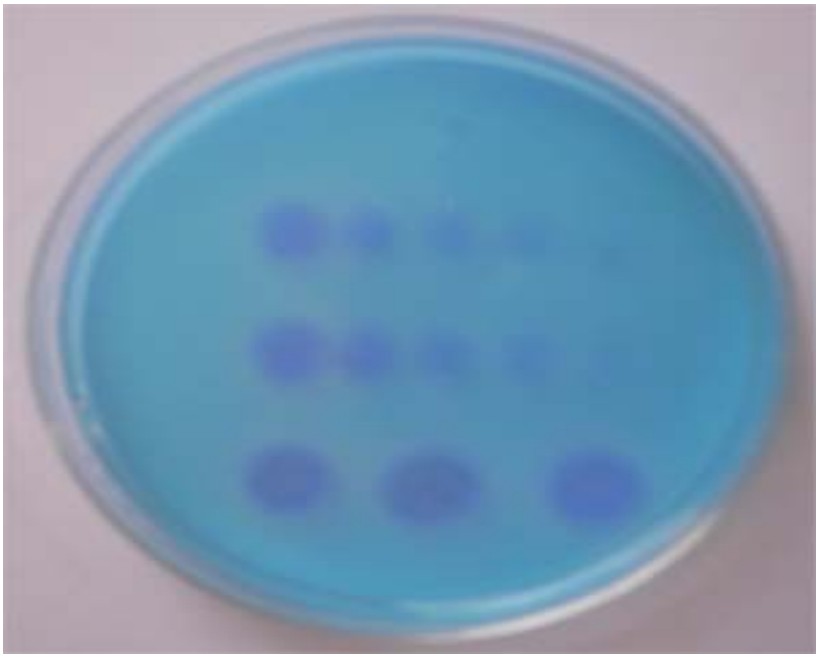


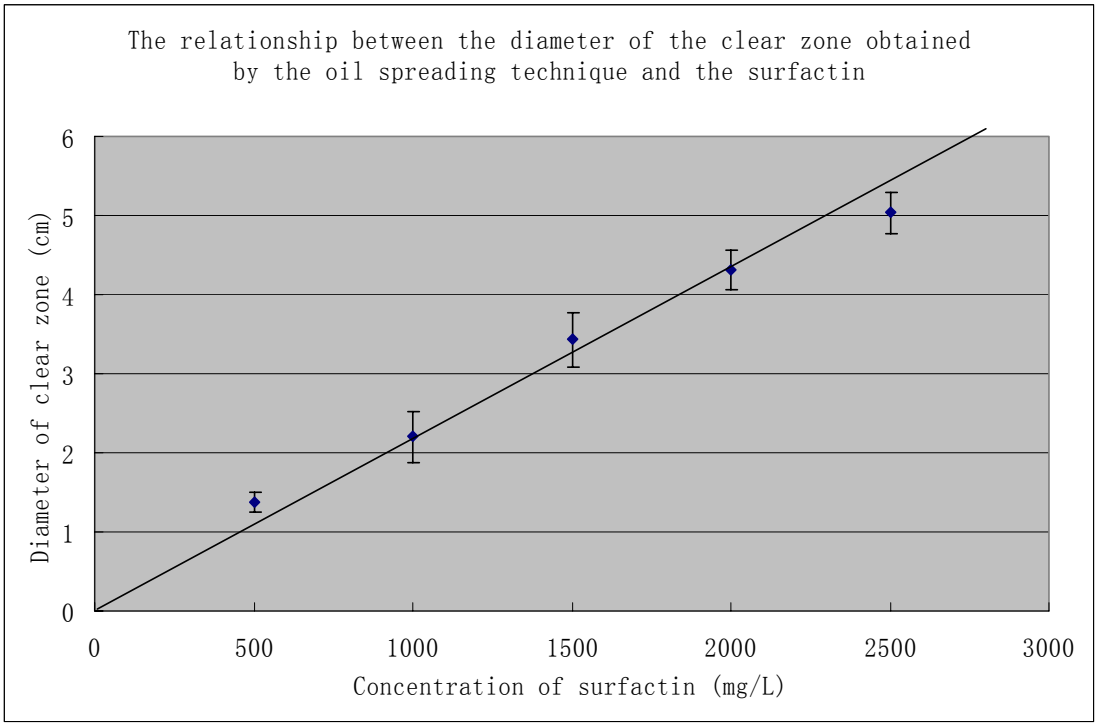
Fig 12. Rhamnolipids form blue halo on agar that contains CTAB and basic dye methylene blue

3.3.3 Quantification of biosurfactant by Oil Spreading Technique

Methods

1) Add 50mL of distilled water to a large Petri dish (15cm diameter);
2) Add 50μL of petroleum crude oil (CRC53352-1, McElroy field, Chevron Texaco) to the surface of the water;
3) Add 10μL (for different biosurfactant, there is different concentration range) to the surface of oil;
4) Measure the diameter of the clear zone on the oil surface after 2 minutes;
5) Make plot of standard curve. The diameters of triplicate samples were determined.

Results



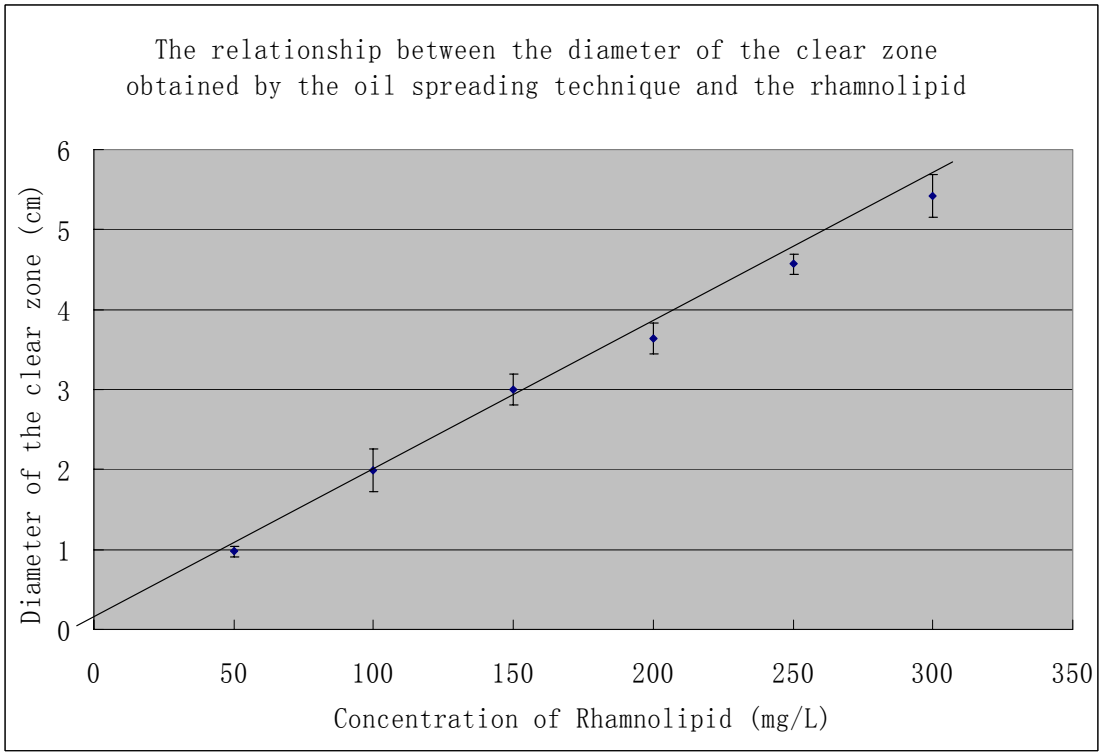


Fig 13. The relationship between the diameter of the clear zone obtained by the oil spreading technique and the concentration of biosurfactant. Error bars indicate the standard deviation of the three independent measurements. Surfactin was from Sigma (St. Louis, MO). Rhamnolipid was from bench-top fermentation by *Pseudonomas fluroscrens* PFAB01 and the concentration was quantified by Orcinol method.

3.4 Highlights of Efforts and Details of Results – Task 3, Evaluation of Bio-Surfactants and Bio-Systems for EOR

3.4.1 Adsorption of Rhamnolipid onto Mineral Powder

Materials

Biosurfactant. Rhamnolipid JBR425 was used in this study. A colorimetric method was used to determine its concentration. 1.9% Orcinal solution in 53% H_2SO4 was used as an indicator. To calculate rhamnolipid concentration, a standard calibration curve of JRB425 at distilled water was established, shown in Fig. 14. Rhamnolipid has to be diluted to less than 100 mg/L to have a linear relationship between concentration and absorbance at the wavelength of 421 nm.

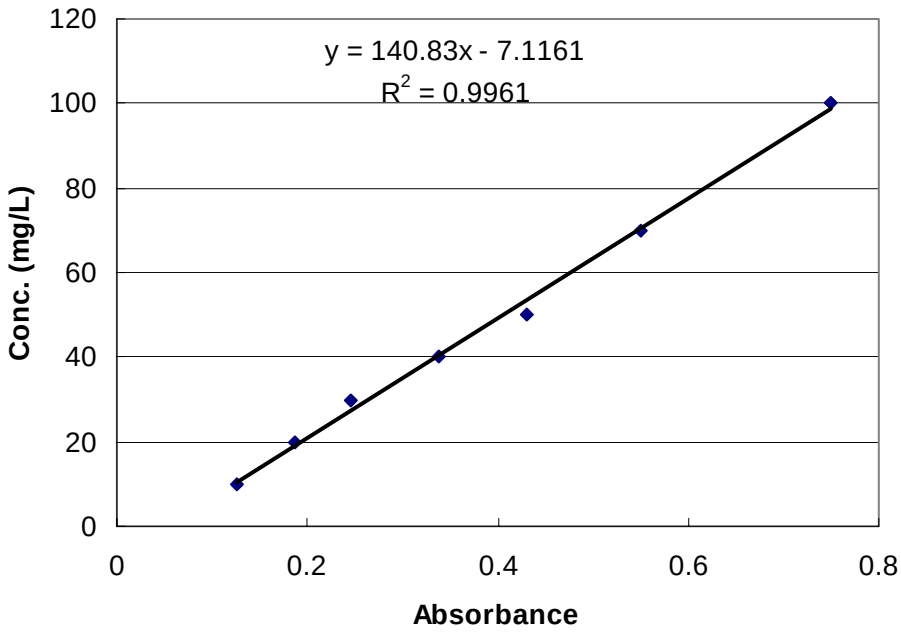


Fig. 14 Standard curve of JBR425 at distilled water

Adsorbents: Three minerals common in reservoir rocks are used as adsorbents: silica, kaolinite, montmorillonite. Table 1 lists their sources and their chemical compositions.

Table 8. Sources and main compositions of five minerals

Mineral	Source	Chemical composition
Sodium Montmorillonite	University of Missouri-Columbia, Source Clay Minerals Respository	$4SiO_2 \cdot Al_2O_3 \cdot 2H_2O$
Kaolinite	Source Caly Repositiry, The Clay Minerals Society, Purdue University	$Al_2Si_2O_5(OH)_4$ or $SiO_4 \cdot Al_2O_3 \cdot 2H_2O$
Silica	N/A	SiO_2

Silica. Silicate minerals are oxides of silicon and a small number of elements from the first three columns of the periodic table and the transition elements. The silica mineral is constructed of SiO_4 tetrahedra which share all four corners with other SiO_4 tetrahedra.[8]

Kaolinite. The chemical composition of kaolinite is $2Al_2Si_2O_5(OH)_4$ or $2SiO_4 \cdot Al_2O_3 \cdot 2H_2O$ per unit cell. Kaolinite consists of sheets of SiO_2 tetrahedra bonded to sheets of Al_2O_3 octahedra. Figure 15 shows the structure of kaolinite.

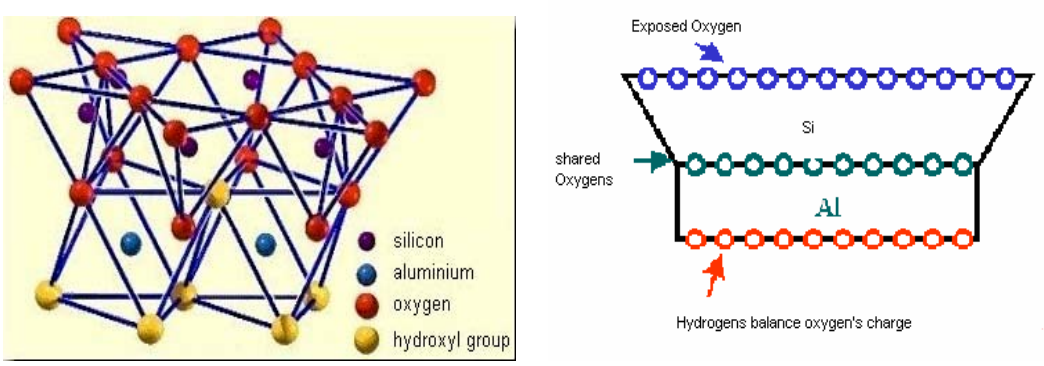


Fig. 15. Crystal lattice of kaolinite. Kaolinite consists of an octahedron layer (coordinates around Al) and a tetrahedron layer (coordinates around Si).

Montmorillonite. The montmorillonite used in this study is Wyoming bentonite, which is composed primarily of sodium montmorillonite. The term is usually reserved for

hydrous aluminum silicates approximately represented by the formula: $4SiO_2 \cdot Al_2O_3 \cdot 2H_2O$+water; but with some of the aluminum cations, Al^{3+}, being displaced by magnesium cations, Mg^{2+}. This replacement of Al^{3+} by Mg^{2+} causes the montmorillonite structure to have an excess of electrons. This negative charge is satisfied by loosely held cations from the associated water. The name sodium montmorillonite refers to clay minerals in which the loosely held cation is the Na^+ ion. As seen in Fig. 16, sodium bentonite has a 2:1 expanding crystal lattice. It consists of superimposed layers composed of two Si-O tetrahedral sheets framing an AL-O-OH octahedral sheet. The structure of bentonite is due to the isomorphic substitution of Si and Al in the crystalline lattice with cations of lower valence. In aqueous dispersion, water can penetrate into the infra-layer space, causing swelling of bentonite. The result of the swelling process is a dispersion of colloidal, plate-shaped particles bearing two clearly differentiated interfaces:[12] the faces, which are always negatively charged and neutralized by a layer of mobile and exchangeable cations (usually Na^+), and the edges, which constitute ~5% of total surface area and carry a positive, negative or neutral charge as a function of pH, ions in solution, etc.

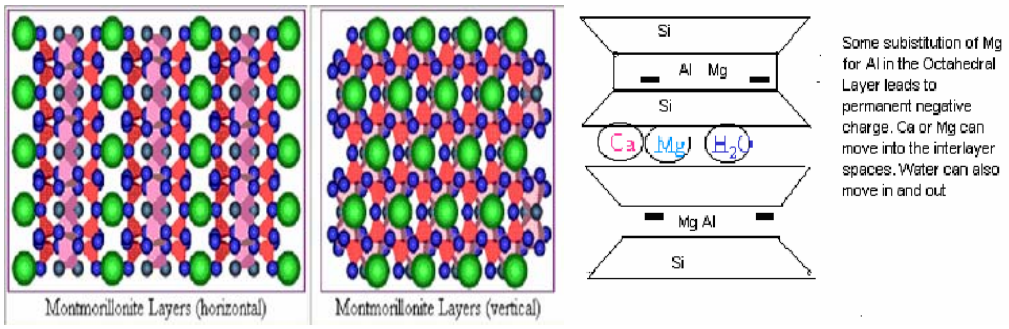


Fig. 16. Bentonite (montmorillonite) structures

Adsorption Experimental Method

Static adsorption experiments were performed to study rhamnolipid adsorption onto minerals common in reservoirs. In the experiments, Ten mL of rhamnolipid solution of known concentration was pipetted in to a test tube. Then one gram of tested mineral was added into the test tube and was vigorously shaken to make them homogenous. The test tube was shaken for 24 h and then was centrifuged at 3500 rpm for 20 minutes, and then to decant the supernatant solution to another test tube for concentration determination. The concentration difference between stock and the supernatant sample was used to calculate adsorption density. Mass adsorption density was used in the study and the unit is mg/g. All experiments were carried out at room temperature, and all rhamnolipid solutions were prepared by distilled water.

Results

1. **Biosurfactant Adsorption Isotherm on Montmorilonite**

   Figure 17 shows the rhamnolipid adsorption isotherm onto montmorilonite. The adsorption densites increase with equilibrated concentration. It appears that the adsorption reach a plateau when equilibrated concentration is above 800 mg/L. Further experiment will be carried out to determine if the transition point is related to CMC of the rhamnolipid. Figure 18 compares the rhamnolipid initial and equilibrated concentration and concentration loss caused by its adsorption onto montmorilonite. The rhamnolipid concentration loss is from 3.61% to 38.26% with highest concentration loss at the initial concentration of 1500 mg/L over measured range.

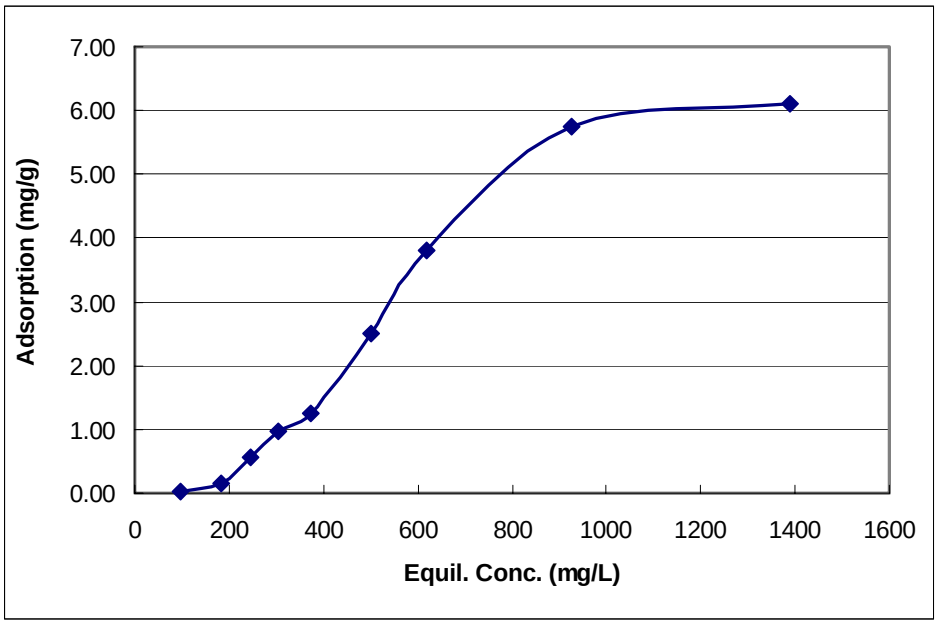


Fig.17 Adsorption isotherm of rhamnolipid onto Montmorillonite

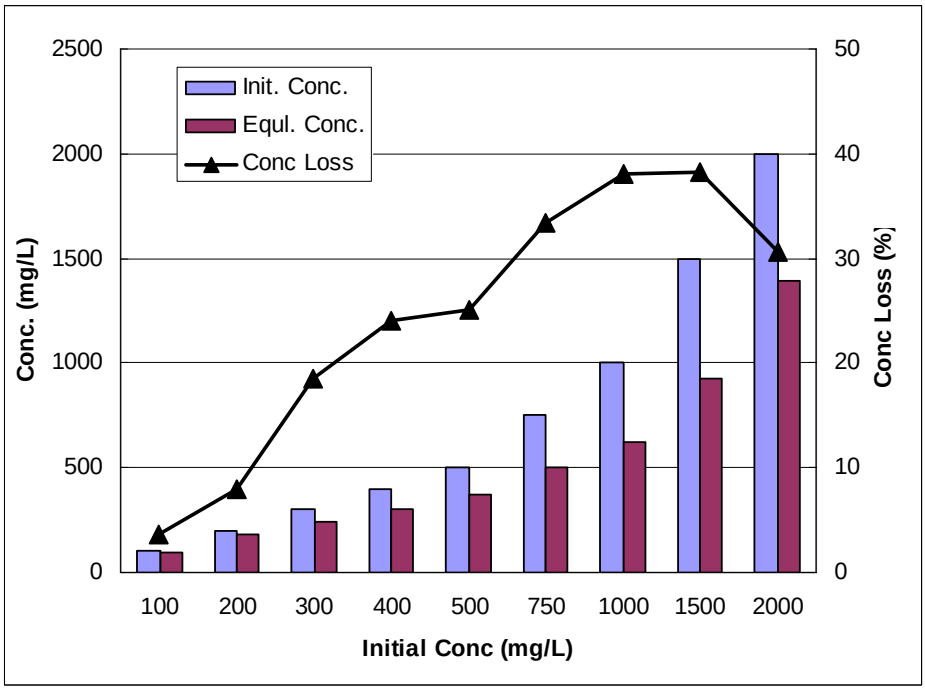


Fig. 18. Concentration change and loss due to adsorption

2. Rhamnolipid Adsorption Isotherm on Kaolinite

Figure 19 shows the rhamnolipid adsorption isotherm onto kaolinite. The adsorption densities increase with equilibrated concentration. Figure 20 compares the

rhamnolipid initial and equilibrated concentration and concentration loss caused by its adsorption onto kaolinite. The rhamnolipid concentration loss is from 49.94% to 32.32% with the percentage loss decreasing with increasing initial concentration over the measured range while total adsorption increased.

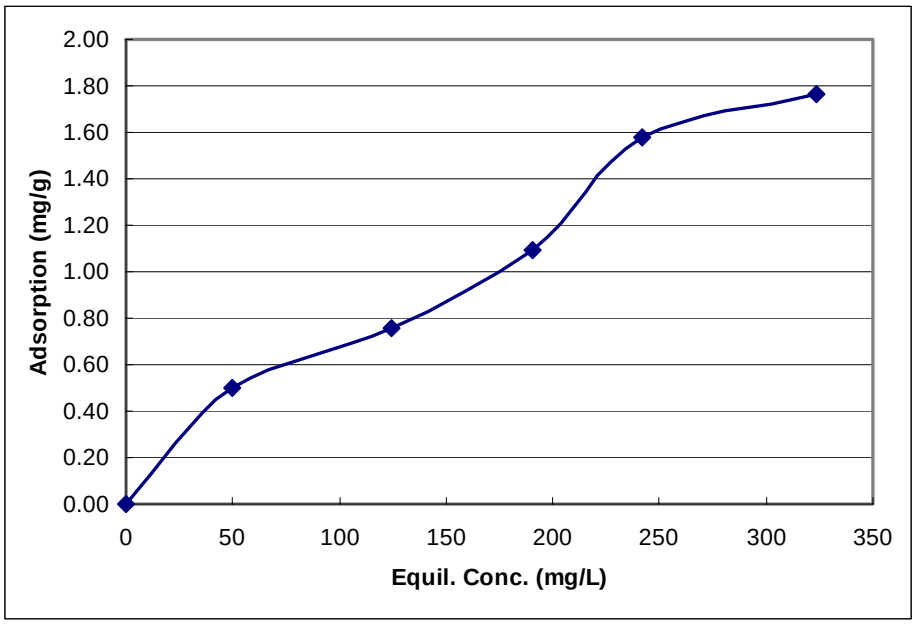


Fig. 19. Adsorption isotherm of rhamnolipid onto kaolinite.

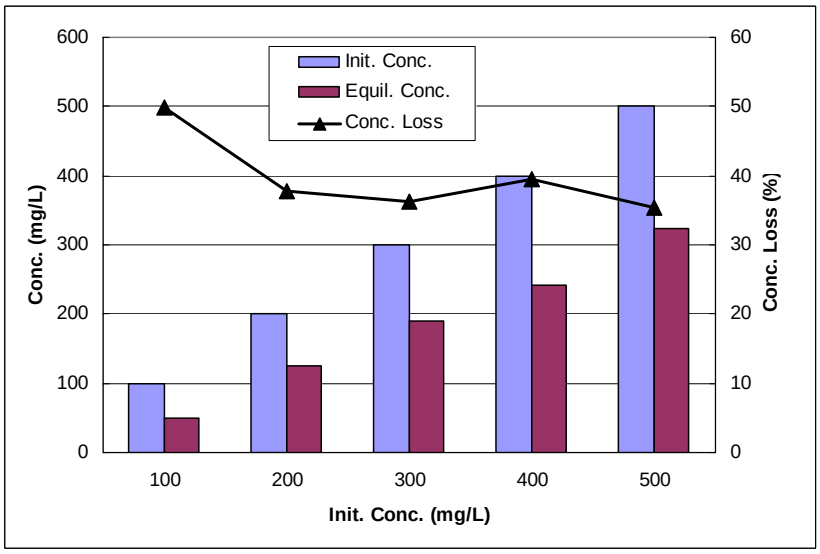


Fig. 20. Concentration change and loss due to rhamnolipid adsorption onto kaolinite.

3. Rhamnolipid Adsorption Isotherm on Silica

Fig. 21 shows the rhamnolipid adsorption isotherm onto silica. Like the rhamnolipid adsorption as previous two clays, the adsorption densities increase with equilibrated concentration. Fig. 22 compares the rhamnolipid initial and equilibrated concentration and concentration loss caused by its adsorption onto montmorilonite. The rhamnolipid concentration loss is from 10.13% to 18.41% with highest concentration loss at the initial concentration of 100 mg/L over measured range.

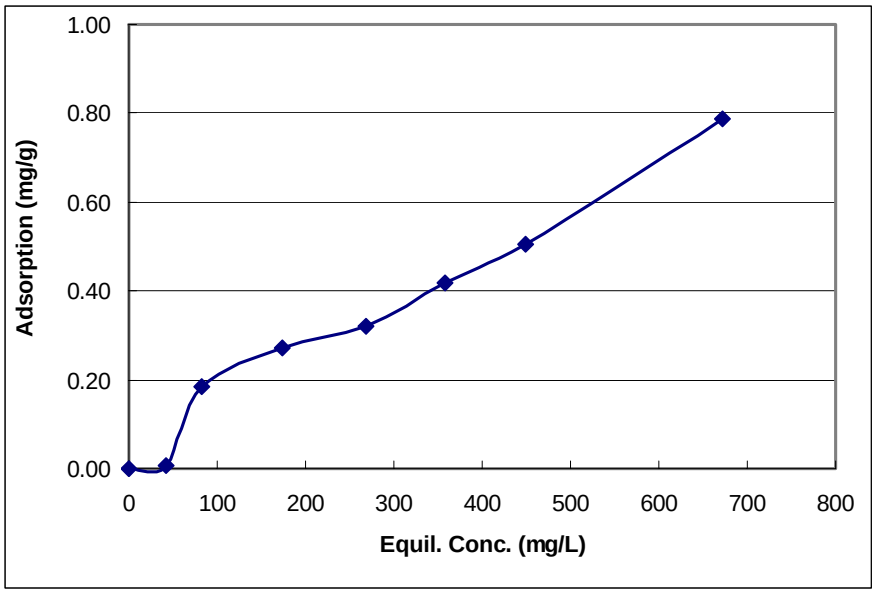


Fig. 21. Adsorption isotherm of rhamnolipid onto Silica

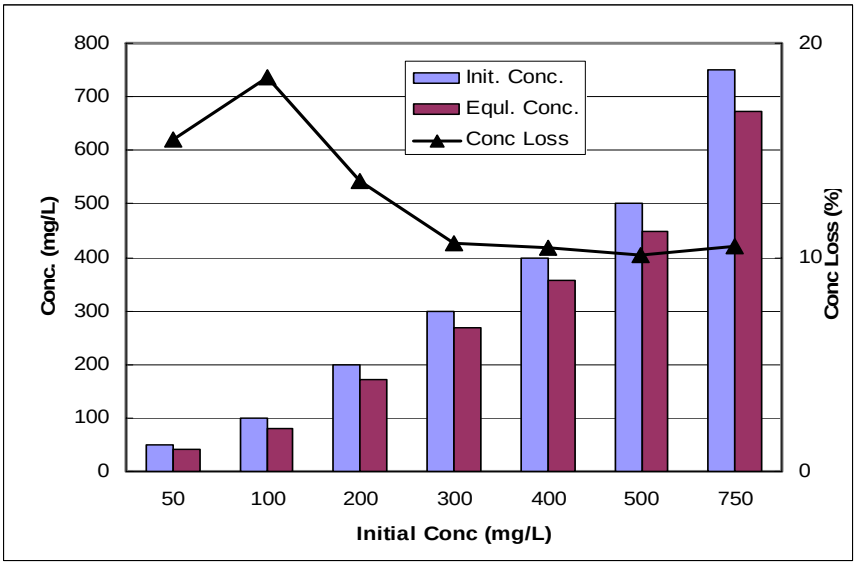


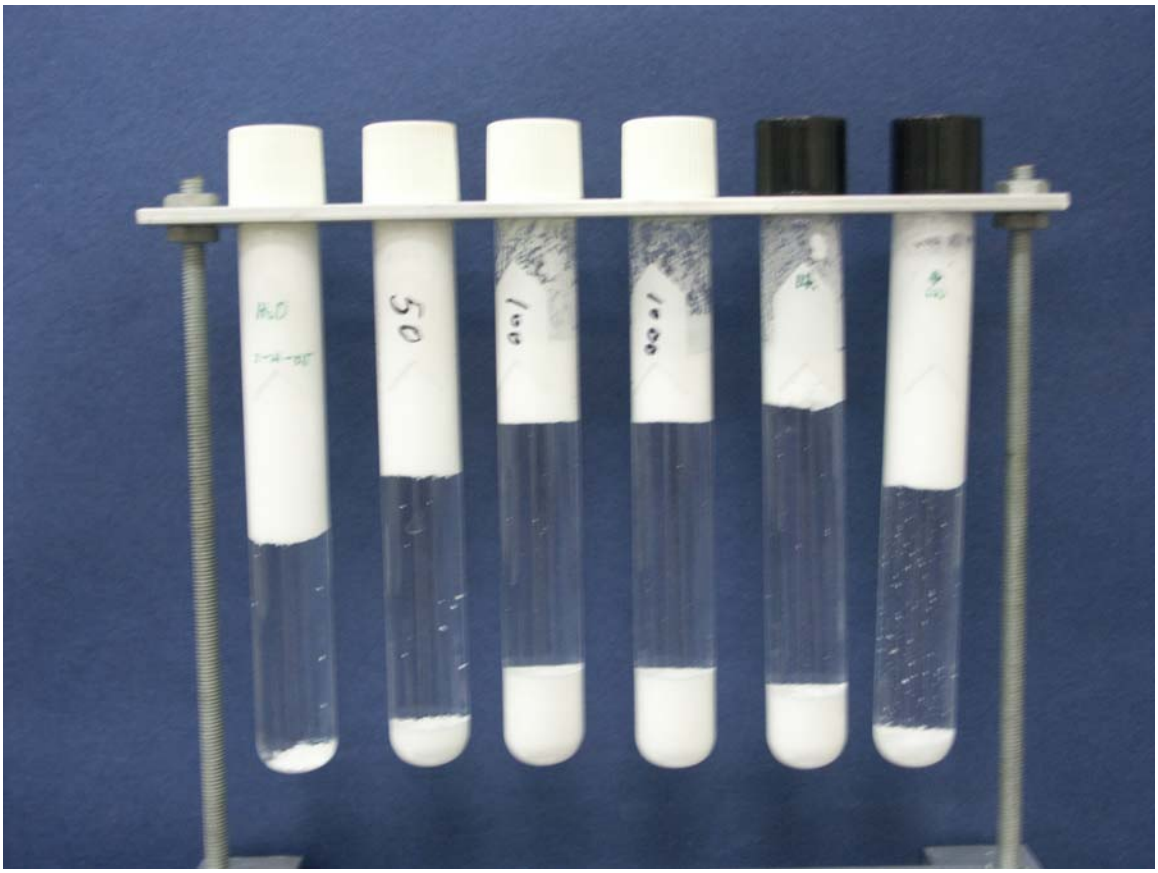
Fig. 22. Concentration change and loss due to rhamnolipid adsorption onto silica.

3.4.2 Rhamnolipids Wettability Tests Using Calcite Flotation Methods

Method

1) Calcite powder was treated with 0.066 M cyclohexanepentanonic acid solutions in decane and vacuum dried. This makes the calcite surfaces oil-wet.
2) Add 1gram of treated calcite powder to 10ml test tube.
3) Add 10ml of distilled water as blank control or aqueous surfactants solutions and shake vigorously.
4) Place test tubes on rack and allow settling over night.
5) Take the final reading of the percent of solid calcite powder floating or now sinking at the bottom of test tubes. The powder that sinks has been made water-wet by that surfactant.

Results



1 2 3 4 5 6

Fig 23. Tube 1 – no rhamnolipid; tubes 2-4, 50ppm, 100ppm, 1000ppm rhamnolipid respectively; tubes 5 and 6, synthetic chemical surfactants.

3.4.3 HPLC-MS analysis of rhamnolipids produced by our engineered strains

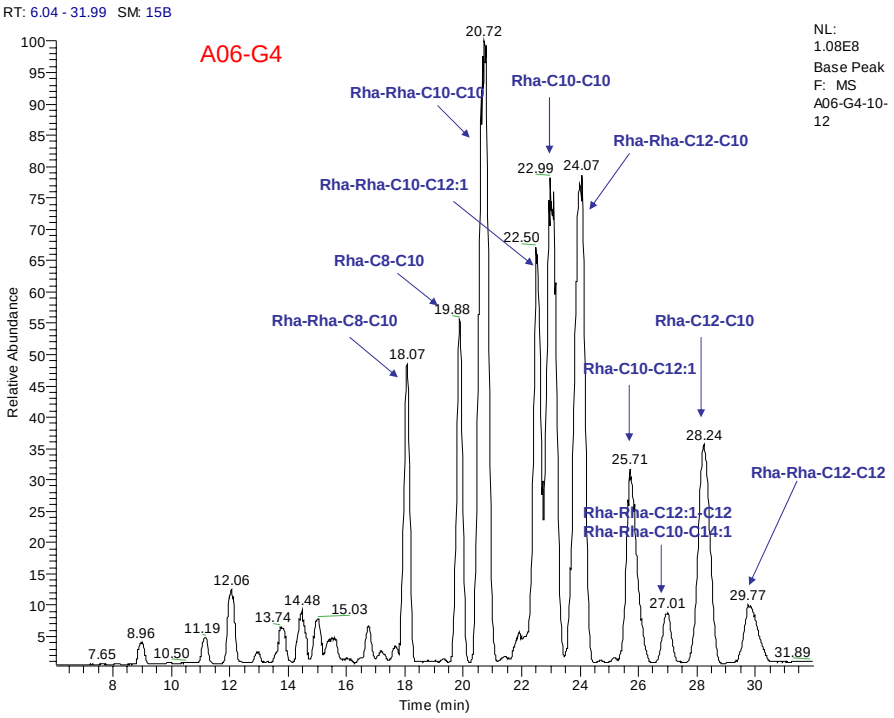
Methods

Bacteria strains and cultivation conditions are as described in section 3.2.10. The rhamnolipids were precipitated by acidifying culture supernatant to PH 3 with 12M HCl and kept at $4^{o}C$ overnight, and recovered by centrifugation at 15000g for 30 minutes and dissolved in ethyl acetate. The oily residue was then dissolved in 0.05M sodium bicarbonate and washed three times with ethyl acetate. Finally, the aqueous solution was retreated with 12M HCl and rhamnolipis were extracted with ethyl acetate and dried. The rhamnolipid residue was then dissolved to 500mg/l final concentration with a 10% acetonitrile (in water) solution containing 2mM ammonium acetate.

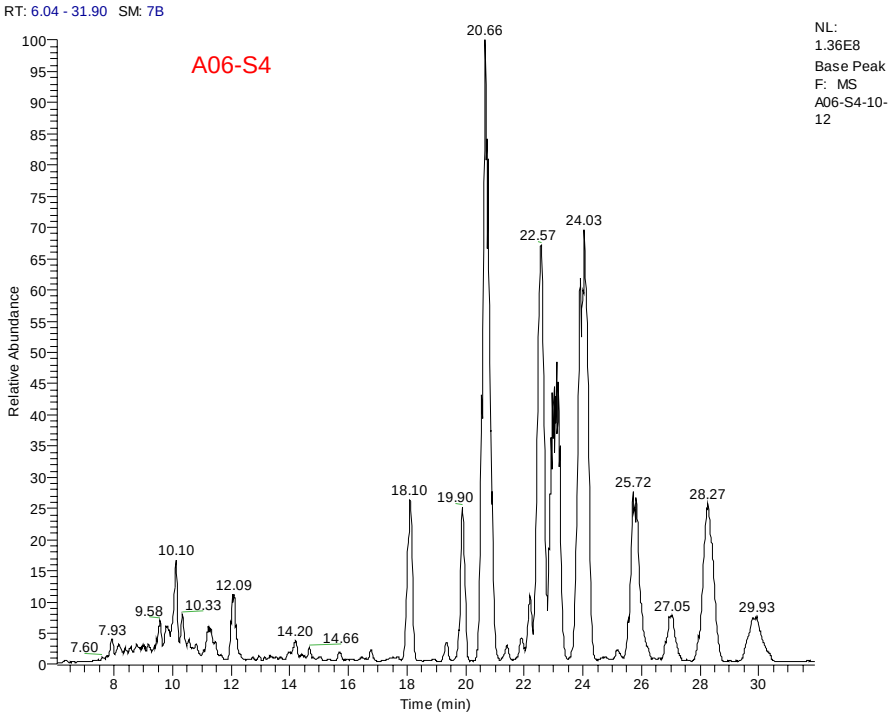
The HPLC-MS analyses were performed with a Gemini C18 column(2 mm x 50 mm, 5 u particle) from Phenomenex and a ThermoFinnigan LCQ Classic ion trap mass spectrometer. The LC gradient was: starting at 8%B and holding for 1 minute, then ramp to 75%B in 20 minutes, hold at 75%B for 10 minutes, back to 8%B in 1 minute and hold at 8%B for 5 minutes. Solvent B is 10:90 water:acetonitrile with 0.1% acetic acid. Solvent A is 98:2 (v/v) water:acetonitrile with 0.1% acetic acid. The mass spectrometer was operated in the negative ion mode scanning 250-950 m/z range. Data dependent MS/MS was set to perform MS/MS of the more abundant ions in the mass range.

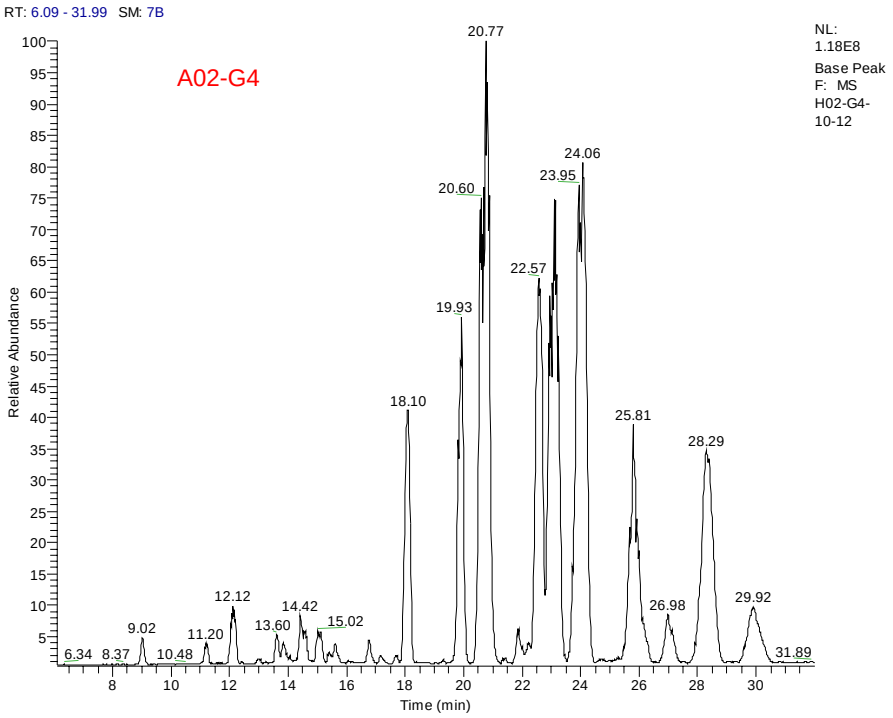
Results

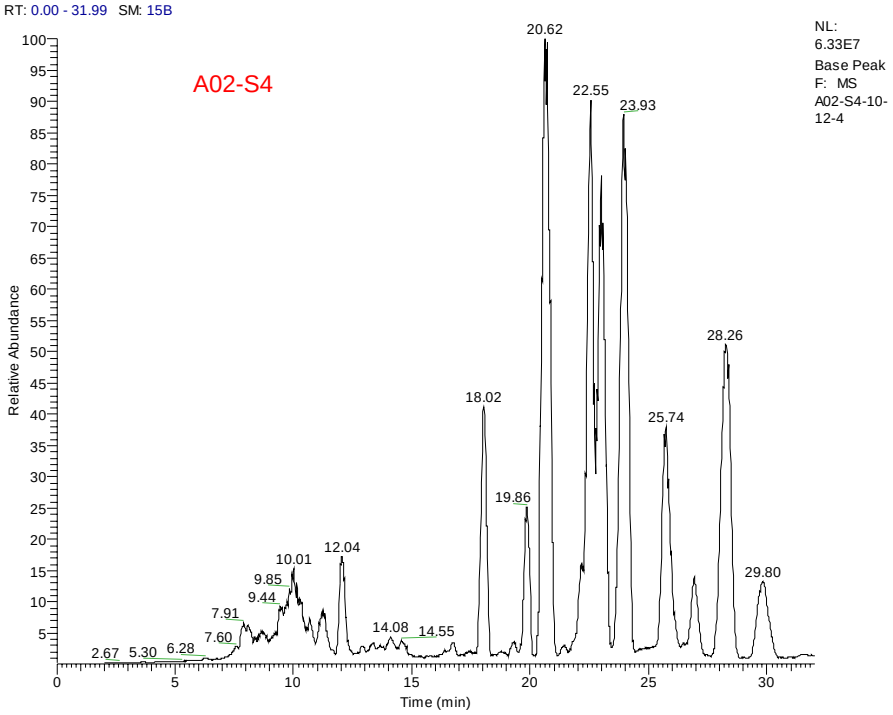
Figure 24 presents the base peak ion intensity chromatograms for the 3 rhamnolipid samples produced by our engineered strains, as described in section 3.2.10. Those 3 strains were each grown at either glucose (A02-G4, A06-G4 and F01-G4) or soybean oil (A02-S4, A06-S4 and F01-S4) containing medium. They showed that rhamnolipids produced by our engineered stains are actually a complex mixture of compounds that all contain one or two rhamnose groups linked to one or two 3-hydroxy fatty acids with different chain length, mainly from C_8 to C_{14}. The major rhamnolipid compounds are marked on the A06-G4 chromatogram.


RT: 6.04 - 31.99 SM: 15B
A06-G4
NL:
1.08E8
Base Peak
F: MS
A06-G4-10-12
Rha-Rha-C10-C10
Rha-C10-C10
Rha-Rha-C12-C10
Rha-Rha-C10-C12:1
Rha-C8-C10
Rha-Rha-C8-C10
Rha-C12-C10
Rha-C10-C12:1
Rha-Rha-C12-C12
Rha-Rha-C12:1-C12
Rha-Rha-C10-C14:1
20.72
22.99
24.07
22.50
19.88
18.07
28.24
25.71
29.77
27.01
12.06
14.48
15.03
13.74
11.19
8.96
7.65
10.50
31.89
Relative Abundance
Time (min)

3.4


RT: 6.04 - 31.90 SM: 7B
A06-S4
NL:
1.36E8
Base Peak
F: MS
A06-S4-10-12
20.66
22.57
24.03
18.10
19.90
25.72
28.27
10.10
12.09
10.33
9.58
7.93
7.60
14.20
14.66
27.05
29.93
Relative Abundance
Time (min)


RT: 6.09 - 31.99 SM: 7B
A02-G4
NL:
1.18E8
Base Peak
F: MS
H02-G4-
10-12
Relative Abundance
Time (min)
20.77
24.06
23.95
20.60
22.57
19.93
18.10
25.81
28.29
12.12
14.42
13.60
15.02
9.02
11.20
26.98
29.92
6.34
8.37
10.48
31.89


RT: 0.00 - 31.99 SM: 15B
A02-S4
NL:
6.33E7
Base Peak
F: MS
A02-S4-10-
12-4
Relative Abundance
Time (min)
20.62
22.55
23.93
28.26
18.02
25.74
19.86
12.04
10.01
9.85
9.44
29.80
7.91
14.08
14.55
7.60
2.67
5.30
6.28

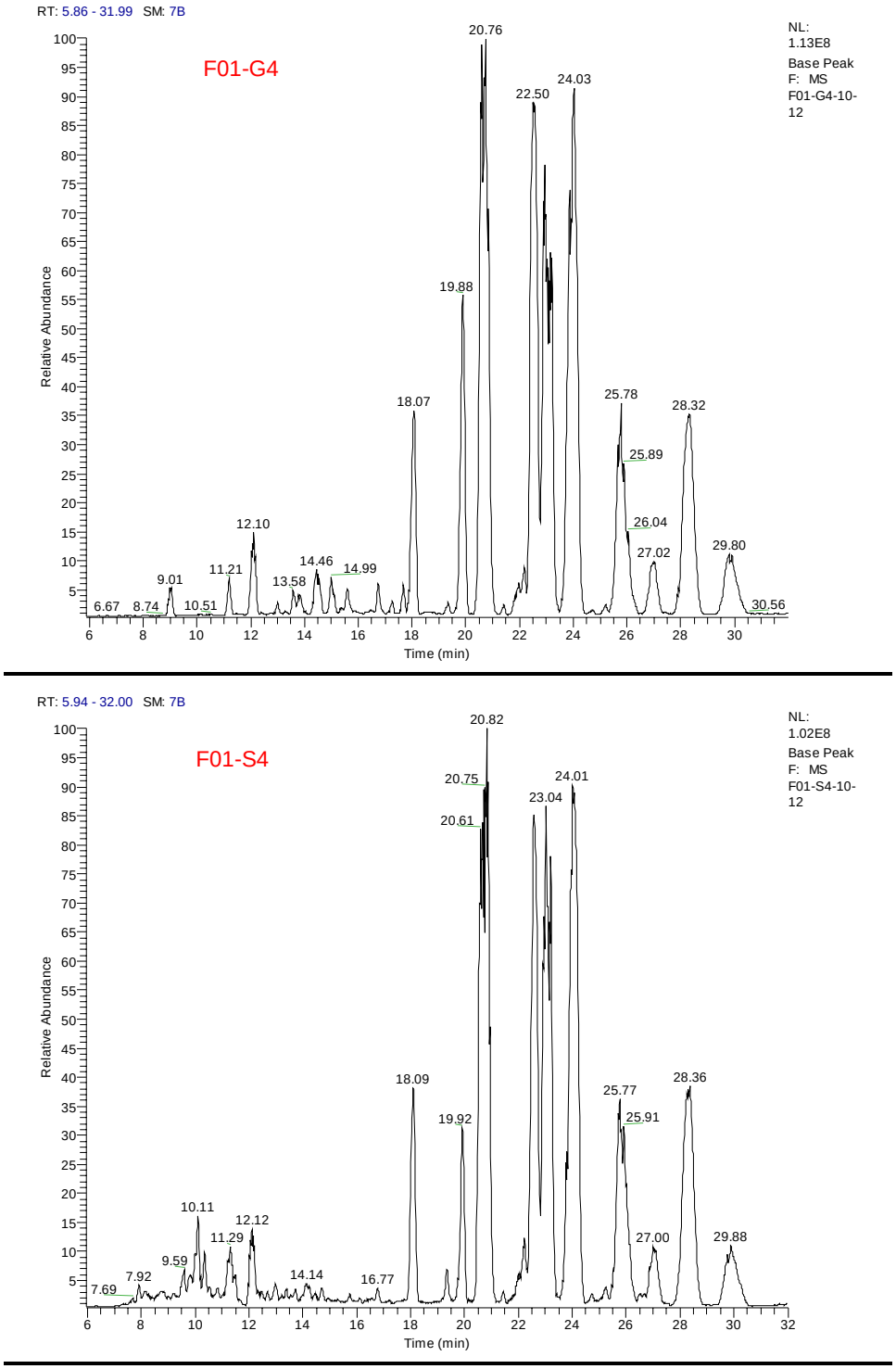


Fig 24. Base peak ion intensity chromatograms of rhamnolipids extracted for 3 engineered bacteria strains, each grown at either Glucose or Soybean Oil containing medium

3.4.4 Phase Behavior and IFT Analysis of rhamnolipids produced by our engineered strains

Methods

The rhamnolipids samples were produced and purified as described in section 3.4.3. The Interfacial Tension (IFT) was determined by using a spinning drop tensiometer (Temco Inc.) as detailed by Cayais, 1977. For our rhamnolipid samples, the glass tube was filled with aqueous phase, followed by injection of 2 micro liters of octane. The galss tube was spun in the tensiometer at 30°C, and IFT was determined from the octane drop geometry. In these studies, we measured the "fresh" IFT values without pre-mixing aqueous and oil phases together to reach phase equilibrium.

Results

Table 9 shows the IFT measurements of our 3 engineered strains in comparing with the IFT values of the rhamnolipids produced by wild type *P. aeroginosa* PAO1-RhlA^{-} and *P. fluorescens* ATCC15453 strains. Each wild type and mutant strains was grown on both glucose medium and soybean oil medium. The IFT of the rhamnolipids from engineered strains are considerably lower that those from wild type strains because of the much higher production rate. Also it is noticed that IFT of the rhamnolipid produced by cells grown in soybean oil medium is somehow lower that IFT of those rhamnolipids produced by cells grown on glucose medium, especially the A06 strain which gave almost 3 time lower IFT when cells are grown in soybean oil medium.

Table 9. IFT measurements of Rhamnolipid samples produced by either wild type or engineered bacteria strains

Rhamnolipid Sample	Concentration (g/l)	IFT (dyne/cm)
A-rhlA(-)-G4	0.004	7.3
A-rhlA(-)-S4	0.011	6
A02-G4	0.5	1.7
A02-S4	0.5	1.48
A06-G4	0.5	1.7
A06-S4	0.5	0.62
Fw-G4	0.01	17
Fw-S4	0.02	18.5
F01-G4	0.5	2.05
F01-S4	0.5	1.75

* A-rhlA(-)-G4: *P. aeroginosa* PAO1-RhlA$^-$ strain grown in glucose medium

* A-rhlA(-)-S4: *P. aeroginosa* PAO1-RhlA$^-$ strain grown in soybean oil medium

* A02-G4 & A06-G4: 2 engineered *P. aeroginosa* PAO1 strain grown in glucose medium

* A02-S4 & A06-S4: 2 engineered *P. aeroginosa* PAO1 strain grown in soybean oil medium

* Fw-G4: *P. fluorescens* strain grown in glucose medium

* Fw-S4: *P. fluorescens* strain grown in soybean oil medium

* F01-G4: engineered *P. fluorescens* strain grown in glucose medium

* F01-S4: engineered *P. fluorescens* strain grown in soybean oil medium

3.4.4 The effect of salt concentration on IFT value of rhamnolipids

Figure 25 shows the IFT measurements of the rhamnolipid JBR425 under different NaCl concentrations, from0.2% to 2%. 1000ppm of rhamnolipid aqueous solution was used in the measurements. The results indicate that the IFT decreases when NaCl salt concentration increases.

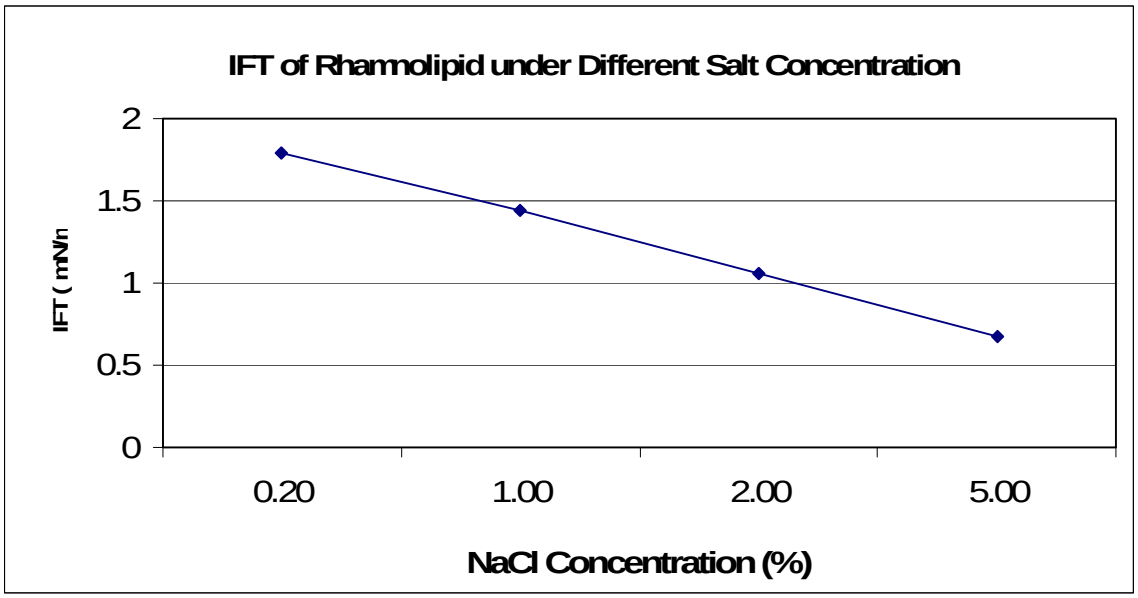


Figure 25. The effect of the salt concentration on IFT value of the rhamnolipids

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4.0 Cost and Schedule and Milestone Status

During the first year of study, we have met the following milestones for the project:

1. We have developed the rapid screening procedures for mutation studies and have characterized rhamnolipid and surfactin as EOR surfactants. (month 9)
2. We have completed 1st round of directed evolution tests and demonstrate superior batch fermentation performance versus the natural, wild strains. (month 12)
3. We have started the studies of evaluation of rhamnolipid and surfactin as surfactant products for conventional chemical EOR applications. (month 21)
4. We are on our way to develop lab methods to clone a bio-surfactant mutant into candidate thermophilic oil field microbes. (month 20)

The budget information and status for the project are shown below:

Total Approved Budget: $445,871(DOE share $356,697, Non-Fed share $89,174)

Funding Received from DOE: $356,697

Actual Costs incurred for period 10/1/04 - 9/30/05: $419,623

Cost Sharing for Period: $104.906

Total Costs for Period: $524,529

5.0 Summary of Significant Accomplishments

1. We have successfully cloned the genes involved in the rhamnolipid and surfactin bio-synthesis.

2. By using the Transposon containing Rhamnosyltransferase gene rhlAB, we have successfully produced rhamnolipds in both *P. aeroginosa* PAO1-RhlA$^-$ strain and *P. fluorescens* ATCC15453 strain, with the increase of 55 to 175 fold in rhamnolipid production comparing with wild type bacteria strain.

3. We have completed the first round direct evolution studies using Error-prone PCR technique and have constructed the library of RhlAB-containing Transposon to express mutant gene in heterologous hosts.

4. Several methods, such as colorimetric agar plate assay, colorimetric spectrophotometer assay and oil spreading assay, have been established to detect and screen rhamnolipid and surfactin production.

5. The behavior of the rhamnolipid and surfactin as EOR agents have been characterized by surfactant adsorption assay, Interfacial Tension (IFT) assay and wettability tests Using calcite flotation methods.

6.0 Future Work / Potential Problems

The main focus for the next year of project period will be:

1. Continue our directed evolution studies, screen and improve our mutant bacteria strains for better MEOR candidate.
2. Continue to search for better oilfield microbe strains that could be carrier of our improved mutant genes for potential in-situ oilfield EOR applications.
3. Further improve the methods to evaluate rhamnolipid and surfactin for their EOR characteristics.

7.0 Technology Transfer Activities

We have discussed our research work and potential technology value with several major oil companies, such as Chevron, Exxon-Mobil and Shell. We also presented some of our research results as a poster at the SPE workshop in Daqing, China in September 2005 (the traveling expenses were funded by another non-DOE project).

8.0 Acknowledgements

We would like to thank DOE for their financial support and guidance throughout this project.

Appendix A

DNA and protein sequences of rhlA and rhlB genes.

```
        XbaI
       ~~~~~~
  1    TCTAGATGCC TTTTCCGCCA ACCCCTCGCT GTTCCCCGCC GGCCGCTCTG GCACGCCTTA TCGCGGGCGG
       GCAGGGGCTT ATGCGCAGGC GGCCGCCCGT
101    CCTGTGAAAT CTGGCAGTTA CCGTTAGCTT TCGAATTGGC TAAAAAGTGT TCATCGGCTA CGCGTGAACA
       CGGACGCCAA TCGTTTGCGC AGGCCGATCT
201    GCAAGACCCA CACAAGCCCC TCGCCTGAAG GGGTACGCAT CCGCCGTGGC TGGTCCGCGC GGATGGCCGC
       TGAGTTACTT GTCTGCCGTT CGAACAATAA

       M   R  R  E   S  L  L
301    GAACGAACTC TACGTAATGC CGGGATACCC GTGGCAGCGA TAGCTGTTTG CCTGTTCGAA AATTTTTGGG
       AGGTGTGAAA TGCGGCGCGA AAGTCTGTTG
                                                          BamHI
                                                          ~~~~~~~
                                                               SmaI
                                                             ~~~~~~
                                                               XmaI
                                                             ~~~~~~
                                                               AvaI
                                                             ~~~~~~
        V  S  V  C   K  G  L   R  V  H   V  E  R  V   G  Q  D   P  G  R   S  T  V  M
       L  V  N   G  A  M   A  T  T  A ·
401    GTATCGGTTT GCAAGGGCCT GCGGGTACAT GTCGAGCGCG TTGGGCAGGA TCCCGGGCGC AGCACGGTGA
       TGCTGGTCAA CGGCGCGATG GCGACCACCG
       ·  S  F  A   R  T  C   K  C  L  A   E  H  F   N  V  V   L  F  D  L   P  F  A
       G  Q  S   R  Q  H  N   P  Q  R ·
501    CCTCGTTCGC CCGGACCTGC AAGTGCCTGG CCGAACATTT CAACGTGGTG CTGTTCGACC TGCCCTTCGC
       CGGGCAGTCG CGTCAGCACA ACCCGCAGCG
       · G  L  I   T  K  D  D   E  V  E   I  L  L   A  L  I  E   R  F  E   V  N  H   L
       V  S  A   S  W  G   G  I  S
601    CGGGTTGATC ACCAAGGACG ACGAGGTGGA AATCCTCCTG GCGCTGATCG AGCGCTTCGA GGTCAATCAC
       CTGGTCTCCG CGTCCTGGGG CGGTATCTCC
        T  L  L  A   L  S  R   N  P  R   G  I  R  S   S  V  V   M  A  F   A  P  G  L
       N  Q  A   M  L  D   Y  V  G  R ·
701    ACGCTGCTGG CGCTGTCGCG CAATCCGCGC GGCATCCGCA GCTCGGTGGT GATGGCATTC GCCCCTGGAC
       TGAACCAGGC GATGCTCGAC TACGTCGGGC
       ·  A  Q  A   L  I  E   L  D  D  K   S  A  I   G  H  L   L  N  E  T   V  G  K
       Y  L  P   Q  R  L  K   A  S  N ·
801    GGGCGCAGGC GCTGATCGAG CTGGACGACA AGTCGGCGAT CGGCCATCTG CTCAACGAGA CCGTCGGCAA
       ATACCTGCCG CAGCGCCTGA AAGCCAGCAA
       · H  Q  H   M  A  S  L   A  T  G   E  Y  E   Q  A  R  F   H  I  D   Q  V  L   A
       L  N  D   R  G  Y   L  A  C
901    CCATCAGCAC ATGGCTTCGC TGGCCACCGG CGAATACGAG CAGGCGCGCT TTCACATCGA CCAGGTGCTG
       GCGCTCAACG ATCGGGGCTA CTTGGCTTGC
              BamHI
       PstI
              ~~~~~~~
       ~~
        L  E  R  I   Q  S  H   V  H  F   I  N  G  S   W  D  E   Y  T  T   A  E  D  A
       R  Q  F   R  D  Y   L  P  H  C ·
1001   CTGGAGCGGA TCCAGAGCCA CGTGCATTTC ATCAACGGCA GCTGGGACGA ATACACCACC GCCGAGGACG
       CCCGCCAGTT CCGCGACTAC CTGCCGCACT
       PstI                                                                    ApaLI
       AvaI
       ~~~~
       ~~~~~~~          ~~~~~~
       ·  S  F  S   R  V  E   G  T  G  H   F  L  D   L  E  S   K  L  A  A   V  R  V
       H  R  A   L  L  E  H   L  L  K ·
1101   GCAGTTTCTC GCGGGTGGAG GGCACCGGGC ATTTCCTCGA CCTGGAGTCC AAGCTGGCAG CGGTACGCGT
       GCACCGCGCC CTGCTCGAGC ACCTGCTGAA
       · Q  P  E   P  Q  R  A   E  R  A   A  G  F   H  E  M  A   I  G  Y   A
1201   GCAACCGGAG CCGCAGCGGG CGGAACGCGC GGCGGGATTC CACGAGATGG CCATCGGCTA CGCCTGAACC
       CTTGACCTGC GAAGACCCGG CCTGGCCGGG
                                          M  H  A   I  L  I   A  I  G  S   A  G  D
       V  F  P   F  I  G  L   A  R  T ·
1301   CTTTGCGGTT GCATAACGCA CGGAGTAGCC CCATGCACGC CATCCTCATC GCCATCGGCT CGGCCGGCGA
       CGTATTTCCC TTCATCGGCC TGGCCCGGAC
       · L  K  L   R  G  H  R   V  S  L   C  T  I   P  V  F  R   D  A  V   E  Q  H   G
       I  A  F   V  P  L   S  D  E
1401   CCTGAAACTG CGCGGGCACC GCGTGAGCCT CTGCACCATC CCGGTGTTTC GCGACGCGGT GGAGCAGCAC
       GGCATCGCGT TCGTCCCGCT GAGCGACGAA
                         NcoI
                        ~~~~~~~
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      L  T  Y  R   R  T  M   G  D  P   R  L  W  D   P  K  T   S  F  G   V  L  W  Q
      A  I  A   G  M  I   E  P  V  Y ·
1501  CTGACCTACC GCCGGACCAT GGGCGATCCG CGCCTGTGGG ACCCCAAGAC GTCCTTCGGC GTGCTCTGGC
      AAGCCATCGC CGGGATGATC GAGCCGGTCT
      ·  E  Y  V   S  A  Q   R  H  D  D   I  V  V   V  G  S   L  W  A  L   G  A  R
      I  A  H   E  K  Y  G   I  P  Y ·
1601  ACGAGTACGT CTCGGCGCAG CGCCATGACG ACATCGTGGT GGTCGGCTCG CTATGGGCGC TGGGCGCACG
      CATCGCTCAC GAGAAGTACG GGATTCCCTA

      AvaI

      ~~~~~~
      · L  S  A   Q  V  S  P   S  T  L   L  S  A   H  L  P  P   V  H  P   K  F  N   V
      P  E  Q   M  P  L   A  M  R
1701  CCTGTCCGCG CAGGTCTCGC CATCGACCCT GTTGTCGGCG CACCTGCCGC CGGTACACCC CAAGTTCAAC
      GTGCCCGAGC AGATGCCGCT GGCGATGCGC
       K  L  L  W   R  C  I   E  R  F   K  L  D  R   T  C  A   P  E  I   N  A  V  R
      R  K  V   G  L  E   T  P  V  K ·
1801  AAGCTGCTCT GGCGCTGCAT CGAGCGCTTC AAGCTGGATC GCACCTGCGC GCCGGAGATC AACGCGGTGC
      GCCGCAAGGT CGGCCTGGAA ACGCCGGTGA
      ·  R  I  F   T  Q  W   M  H  S  P   Q  G  V   V  C  L   F  P  A  W   F  A  P
      P  Q  Q   D  W  P  Q   P  L  H ·
1901  AGCGCATCTT CACCCAATGG ATGCATTCGC CGCAGGGCGT GGTCTGCCTG TTCCCGGCCT GGTTCGCGCC
      GCCCCAGCAG GATTGGCCGC AACCCCTGCA
                                          SmaI
                                         ~~~~~~
                                          XmaI
                                         ~~~~~~
                                          AvaI
                                         ~~~~~~
      · M  T  G   F  P  L  F   D  G  S   I  P  G   T  P  L  D   D  E  L   Q  R  F   L
      D  Q  G   S  R  P   L  V  F
2001  CATGACCGGC TTCCCGCTGT TCGACGGCAG TATCCCGGGG ACCCCGCTCG ACGACGAACT GCAACGCTTT
      CTCGATCAGG GCAGCCGGCC GCTGGTGTTC
                            PstI                NcoI
                           ~~~~~~              ~~~~~~
       T  Q  G  S   T  E  H   L  Q  G   D  F  Y  A   M  A  L   R  A  L   E  R  L  G
      A  R  G   I  F  L   T  G  A  G ·
2101  ACCCAGGGCT CGACCGAACA CCTGCAGGGC GACTTCTACG CCATGGCCCT GCGCGCGCTG GAACGCCTCG
      GCGCGCGTGG GATCTTCCTC ACCGGCGCCG
                                            PstI
                                           ~~~~~~~
      ·  Q  E  P   L  R  G   L  P  N  H   V  L  Q   R  A  Y   A  P  L  G   A  L  L
      P  S  C   A  G  L  V   H  P  G ·
2201  GCCAGGAACC GCTGCGCGGC TTGCCGAACC ACGTGCTGCA GCGCGCCTAC GCGCCACTGG GAGCCTTGCT
      GCCATCGTGC GCCGGGCTGG TCCATCCGGG
      · G  I  G   A  M  S  L   A  L  A   A  G  V   P  Q  V  L   L  P  C   A  H  D   Q
      F  D  N   A  E  R   L  V  R
2301  CGGTATCGGC GCCATGAGCC TAGCCTTGGC GGCGGGGGTG CCGCAGGTGC TGCTGCCCTG TGCCCACGAC
      CAGTTCGACA ATGCCGAACG GCTGGTCCGG

      AvaI           NcoI

      ~~~~~~~        ~~~~~~
       L  G  C  G   M  R  L   G  V  P   L  R  E  Q   E  L  R   G  A  L   W  R  L  L
      E  D  P   A  M  A   A  A  C  R ·
2401  CTCGGCTGCG GGATGCGCCT GGGCGTGCCG TTGCGCGAGC AGGAGTTGCG CGGGGCGCTG TGGCGCTTGC
      TCGAGGACCC GGCCATGGCG GCGGCCTGTC
      ·  R  F  M   E  L  S   Q  P  H  S   I  A  C   G  K  A   A  Q  V  V   E  R  C
      H  R  E   G  D  A  R   W  L  K ·
2501  GGCGTTTCAT GGAATTGTCA CAACCGCACA GTATCGCTTG CGGTAAAGCG GCCCAGGTGG TCGAACGTTG
      TCATAGGGAG GGGGATGCTC GATGGCTGAA
                 HindIII
                 ~~~~~~
      · A  A  S
2601  GGCTGCGTCC TGAAAGCTT
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