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Enhanced rhamnolipid production of *Pseudomonas aeruginosa* SG by increasing copy number of *rhlAB* genes with modified promoter

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Abstract: Rhamnolipid is a potent natural biosurfactants for industrial and environmental applications such as enhanced oil recovery, bioremediation, and biodegradation. The complex regulation network of key genes involved in biosynthesis of rhamnolipid in *Pseudomonas aeruginosa* represents a challenge to industrial production of rhamnolipid. In this study, *rhlAB* genes with native promoter were cloned from *P. aeruginosa* SG and inserted into plasmid pBBR1MCS-5 to construct recombinant plasmid pBBRPrhlAB. And *rhlAB* genes fused with the strong promoter of *oprL* gene from *P. aeruginosa* SG were used to construct recombinant plasmid pBBRPoprAB. Two recombinant plasmids were transformed to strain SG to construct the engineered strains PrhlAB and PoprAB. Both strains PrhlAB and PoprAB have higher yield of rhamnolipid than wild strain SG under both aerobic and anaerobic conditions. Increasing the copy number of *rhlAB* genes with indigenous strong promoter of *oprL* gene, engineered strain PoprAB has the highest yield of rhamnolipid (1.83-fold of strain SG and 1.19-fold of strain PrhlAB) under aerobic conditions. It is more efficient, economic and commercially feasible to use the engineered strain PoprAB for rhamnolipid production. Using the promoter of *oprL* gene to enhance production of other desirable product in *P. aeruginosa* may also be feasible.

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Keywords: rhamnolipid, *Pseudomonas aeruginosa*, enhanced production, gene manipulation, promoter.

Introduction

Rhamnolipid as a natural biosurfactants has been extensively studied.[1] It is a series of congeners, including one or two L-rhamnoses attached to β-hydroxyalkanoic fatty acid with different length of carbon chains.[2] The rhamnolipid compound was firstly reported in 1949, and the rhamnolipid biosynthetic pathway was proposed in 1963.[3,4] The key genes involved in rhamnolipid biosynthesis were isolated and analyzed in the 1990s.[5-8]

Rhamnolipid has broad potential applications in various fields.[1,9] Rhamnolipid as a potent oil-displacing agent was applied to enhance oil recovery[10-12] due to its biodegradability, ecological safety, high surface activity, and low critical micelle concentration (CMC). Moreover, rhamnolipid could increase the bioavailability of hydrocarbon and can form complexes with metals at the interface, resulting in rhamnolipid application in environmental remediation.[13,14] Industrial applications of rhamnolipid include different areas from bioremediation to food additives.[9]

Rhamnolipid was almost produced by *Pseudomonas aeruginosa*. However, the complex gene regulation network involved in biosynthesis of rhamnolipid in *P. aeruginosa* represents a challenge to industrial production, which has been the object of a growing number of studies.[4,15] Over 65 years have passed since the first rhamnolipid was described; studies on rhamnolipid production by *P. aeruginosa* are not a few. And conceivable strategies for rhamnolipid production improvement were studied, such as metabolic engineering,[16] production in heterologous hosts[17-19] and fermentation approaches.[20,21]

In order to improve production of desirable product by microorganisms, researchers tend to use genetic modulation methods, such as overexpression of the key genes and increase the copy numbers of key genes.[22-25] *oprL* is the coding gene of peptidoglycan-associated lipoprotein which plays an

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important role in maintaining cell structure and integrity.[26,27] The promoter of *oprL* gene is constitutive promoter in *P. aeruginosa* and *P. putida*.[26,28] Herein, to enhance rhamnolipid production of *P. aeruginosa* SG, the copy number of *rhlAB* genes was increased, and the native promoter of *rhlAB* genes was replaced by another *Pseudomonas* promoter of *oprL* gene.[26]

Results and discussion

Construction of recombinant plasmids and engineered strains

Through general PCR and overlap PCR, the DNA fragment rhlAB (*rhlAB* genes with native promoter) and DNA fragment Popr-rhlAB (*rhlAB* genes with the promoter of *oprL* gene) were obtained (Fig. 1). As shown in Fig. 1, based on plasmid pBBR1MCS-5, recombinant plasmid pBBRPrhlAB containing the 2.6 kb Kpn I-rhlAB-Hind III fragment and recombinant plasmid pBBRPoprAB containing the 2.6 kb Kpn I-Popr-rhlAB-Hind III fragment were constructed, respectively. The recombinant plasmids were confirmed correct by sequence analysis of the inserted genes using the primers M13-47 and RV-M. In the pBBRPrhlAB construction, the key enzyme complex, rhamnosyltransferase RhlAB, was expressed from the rhlAB genes with its native promoter. And in the construction of recombinant plasmids pBBRPoprAB, rhamnosyltransferase RhlAB was expressed from the rhlAB genes regulated by the promoter of the *oprL* gene. The recombinant plasmids were transformed into the wild type strain *P. aeruginosa* SG to construct the engineered bacterial strains *P. aeruginosa* PrhlAB and *P. aeruginosa* PoprAB. The copy numbers of *rhlAB* genes increased in both engineered strains *P. aeruginosa* PrhlAB and *P. aeruginosa* PoprAB, which may enhance the rhamnolipid production.

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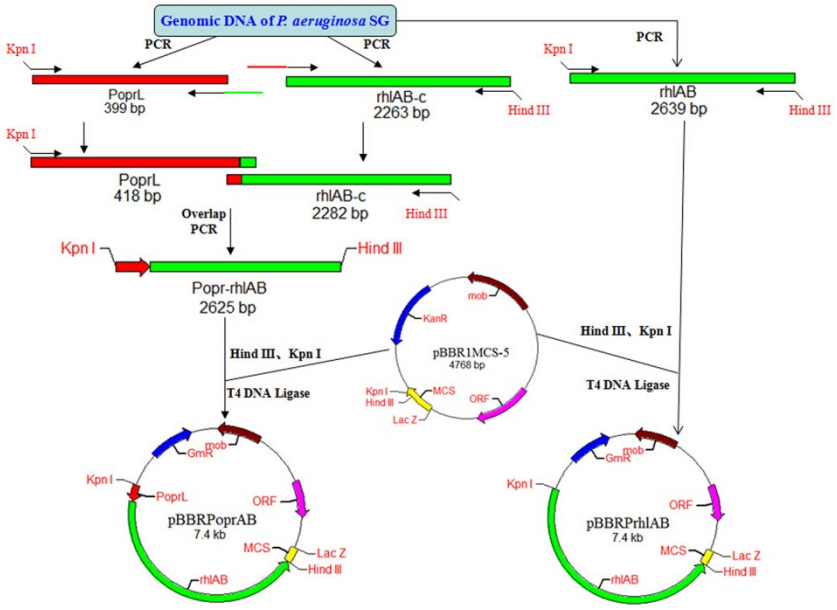

Fig. 1 Schematic diagram of the construction of recombinant plasmids pBBRPrhlAB containing2.6 kb *rhlAB genes* with native promoter and pBBRPoprAB containing 2.6 kb *rhlAB genes* with promoter of *oprL* gene from *P. aeruginosa* SG.

Copy numbers and stability of recombinant plasmids

The recombinant plasmids were extracted and digested to obtain linear plasmids fregments. The concentration of linear plasmid pBBRPrhlAB is 39.1 ng/ul. And the concentration of linear plasmid pBBRPoprAB is 38.4 ng/ul. Furthermore, the DNA length of pBBRPrhlAB is 7399 bp, and the DNA length of pBBRPoprAB is 7385 bp. And the cell concentrations of the engineered strains PrhlAB and PoprAB in LB culture containing gentamicin are 4.72×10^8 cells/ml and 5.69×10^8 cells/ml, respectively. All the experiments and measurements were performed at the same conditions. Therefore, the copy numbers of plasmid pBBRPrhlAB (5.11 copies/cell) and plasmid pBBRPoprAB (4.18 copies /cell) are nearly equal in two engineered strains. In the plasmids construction, the broad host range plasmid pBBR1MCS-5 was used as a gene vector. Plasmid pBBR1MCS-5 and recombinant plasmids (pBBRPrhlAB and pBBRPoprAB) possess the same capacity to replicate in *P. aeruginosa* cells, which result in the nearly equal copies of plasmid pBBRPrhlAB and pBBRPoprAB in engineered strains.

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Colonies of engineered strains from LB plates were continuously inoculated on fresh LB plates and LB plates containing 50 ug/ml of gentamicin every 24 h for 12 times, and the growth percentage of colonies on LB plates containing gentamicin maintained 100%. Results revealed that recombinant plasmids exhibited a good stability in the engineered strains. Plasmid pBBR1MCS-5 is a broad host range vector, it has been tested and found to stably replicate in *Pseudomonas fluorescens*, *P. stutzeri* and *P. putida*.[29] Therefore, the recombinant plasmids derived from pBBR1MCS-5 can also stably replicate in *P. aeruginosa*. Plasmid pBBR1MCS-5 would be a good gene vector for genetic manipulation in *P. aeruginosa*.

Rhamnolipid production by wild strain and engineered strains

Rhamnolipid production by the wild strain and engineered strains was comparatively investigated under aerobic conditions. As shown in Fig. 2, there was a similar kinetics of rhamnolipid production with respect to the wild strain and engineered strains. The surface tension of the culture medium decreased from 63.4 mN/m to 26.2 mN/m in 1 d. Rhamnolipid was fast produced at the end of the logarithmic growth phase, and the yield of rhamnolipid reached maximum at the end of the stationary growth phase. However, the maximum yields of rhamnolipid by three strains were significantly different ($p<0.05$). For the wild type strain, the maximum yield of rhamnolipid was 11.65 g/l, but 17.15 g/l and 20.98 g/l rhamnolipid were determined in cultures of the engineered strains PrhlAB and PoprAB at end of the stationary growth phase, respectively. Through genetic manipulation, the copy numbers of *rhlAB* genes increased in both engineered strains PrhlAB and PoprAB compared to the wild strain SG, which enhanced the rhamnolipid production by engineered strains. Furthermore, the maximum yield of rhamnolipid of strain PoprAB was higher than that of strain PrhlAB. While nearly equal copies of plasmid pBBRPrhlAB and pBBRPoprAB were determined in engineered strains.

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Therefore, the promoter of *oprL* gene has positive effect on rhamnolipid production by *P. aeruginosa*. Compared to the wild strain SG, production of rhamnolipid by engineered strain PoprAB increases 0.83-fold under aerobic conditions. It is efficient, economic and commercial feasibility for using the engineered strain *P. aeruginosa* PoprAB for rhamnolipid production.

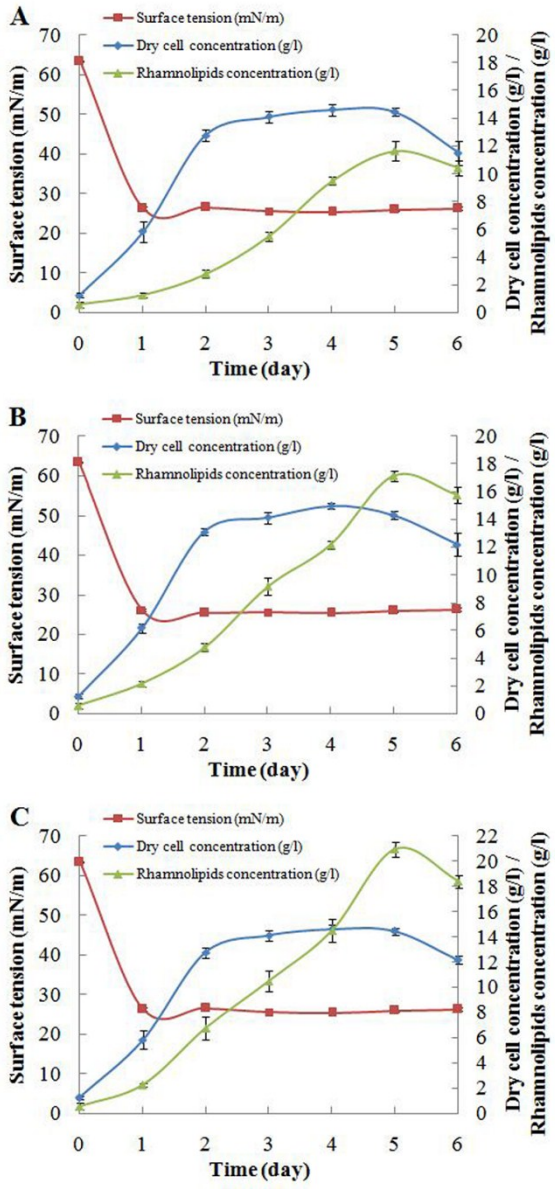

Fig. 2 Cell growth and rhamnolipid production by wild type strain and engineered strains: (■) Surface tension (mN/m); (◆) Dry cell weight (g/l); (▲) Concentration of rhamnolipid (g/l). Fig. 2A: wild type strain *P. aeruginosa* SG; Fig. 2B: engineered strain *P. aeruginosa* PrhlAB with increasing copy number of *rhlAB* genes under native promoter; Fig. 2C: engineered strain *P. aeruginosa* PoprAB with increasing copy number of *rhlAB* genes under promoter of *oprL* gene.

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For three strains, the oil spreading activity, as an indirect characterization of rhamnolipid amount, was measured at the end of the stationary growth phase. For the SG cell-free supernatant (10,000 rpm, 10min) diluted 16-fold, the diameter of formed oil displaced circle was 23±0.67 mm. The cell-free supernatant of engineered strains PrhlAB and PoprAB was also diluted 16-fold, and 35±0.67 mm and 41 mm diameter of oil displaced circles were formed, respectively. The engineered strain PoprAB possessed the highest oil spreading activity, followed by the strains PrhlAB and SG. Results are consistent with data of rhamnolipid production capacity.

Some conceivable strategies for rhamnolipid production improvement have been achieved. Herein, the engineered strain *P. aeruginosa* PoprAB produced 20.98 g/l of rhamnolipid under aerobic conditions, and the wild strain *P. aeruginosa* SG has a yield of 11.65 g/l of rhamnolipid under the same conditions. In other studies, the rhamnolipid yield of engineered strain *P. putida*1067 (pNE2) and the wild strain *P. aeruginosa* EMS1 were 6.97 and 5.18 g/l, respectively,[18] and the rhamnolipid production by the engineered *P. aeruginosa* PEER02 (1.82 g/l) was increased over 50 % compared to the wild type *P. aeruginosa* PAO1 (1.21 g/l).[34] *Pseudomonas aeruginosa* AT10 can produce 18.07 g/l rhamnolipid due to medium optimization.[20] These results revealed that the engineered strain *P. aeruginosa* PoprAB is an efficient strain for rhamnolipid production.

Rhamnolipid production under anaerobic conditions

In our previous study,[30] the wild strain SG can produce 1.08 g/l of rhamnolipid under anaerobic conditions. Herein, rhamnolipid production under anaerobic conditions by engineered strains was investigated. In this study, 2.42 g/l and 3.56 g/l of rhamnolipid were produced by engineered strains PrhlAB and PoprAB in anaerobic medium, respectively. In other studies, we constructed an engineered strain *P. stutzeri* Rhl which produced 1.61 g/l of rhamnolipid under anaerobic conditions;[19] and *P.*

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stutzeri Rhl can produce 3.12 g/l of rhamnolipid under anaerobic conditions after medium optimization.[21] Increasing the copy number of *rhlAB* genes with the modified promoter efficiently enhanced the anaerobic production of rhamnolipid by *P. aeruginosa* SG. Through medium optimization, the anaerobic yield of rhamnolipid by the engineered strain PoprAB will continue to increase.

Rhamnolipid applications in Microbial Enhanced Oil Recovery (MEOR) process and environmental remediation are costly and complex, and large quantities of rhamnolipid are required to produce under oxygen limiting conditions in such applications. In situ production of rhamnolipid is considered to be advantageous in field applications.[31,32] Rhamnolipid-producing bacteria that can efficiently produce rhamnolipid under oxygen limiting conditions could cater the need of in-situ application. The wild strain SG was promising to enhance oil recovery through in situ production of rhamnolipid in simulated core model.[30] Engineered strains can anaerobically produce more rhamnolipid than wild strain SG. Therefore, the engineered strains PrhlAB and PoprAB will also be promising for in situ MEOR applicatiuons.

Discussion and perspectives

In the present work, increasing the copy number of *rhlAB* genes with the modified promoter enhanced the production of rhamnolipid by *P. aeruginosa* under both aerobic and anaerobic conditions. Results also demonstrate that using the promoter of *oprL* gene to enhance production of desirable metabolite in *P. aeruginosa* is feasible. Herein, *rhlAB* genes were deposed on plasmid and not integrated into chromosome of *P. aeruginosa*. Because we want to comparatively study the different effect between increasing the copy number of *rhlAB* genes and replacing the promoter of *rhlAB* genes. Results revealed that both increasing the copy number of *rhlAB* genes and replacing the promoter of *rhlAB*

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genes by the promoter of *oprL* gene increased production of rhamnolipid. Increasing the copy number of *rhlAB* genes with the promoter of *oprL* gene, engineered strain *P. aeruginosa* PoprAB has the highest yield of rhamnolipid (1.83-fold of the wild strain SG and 1.19-fold of the strain PrhlAB) under aerobic conditions.

To enhance production of desirable product by microorganisms, researchers tend to genetically manipulate the strains to over express the involved key genes.[22,23,25] Increasing the copy number of key genes and replacing the native promoter by another indigenous stronger promoter can make the involved key genes overexpression to enhance the production of desirable product by strains.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Table 1 lists the strains and plasmids used in this study. The rhamnolipid-producing bacterial strain *P. aeruginosa* SG[30] was the wild type strain. *Escherichia coli* DH5α (Takara, Japan) was used as the host strain for the plasmids. Except for rhamnolipid production, all these strains were cultured in Luria–Bertani (LB) medium. If necessary, gentamicin is added to the medium to the final concentration of 50 μg/ml. The glycerol-nitrate (GN) medium for rhamnolipid production contained: 45 g/l glycerol; 4.5 g/l $NaNO_3$; 5.2 g/l $K_2HPO_4 \cdot 3H_2O$; 4.0 g/l KH_2PO_4; 0.40 g/l $MgSO_4 \cdot 7H_2O$; 0.10 g/l $CaCl_2$; 1.0 g/l KCl; 1.0 g/l NaCl; 2.0 g/l Yeast Extract. The GN medium pH was adjusted to 6.8. The inoculum amount is 3%. The inoculums were prepared from their 24-hour seed cultures. Seed cultures was centrifuged at 10, 000 *g* for 5 min, and the collected pellets were washed three times with 0.9 % (m/v) NaCl saline water to eliminate the disturbance of residual rhamnolipid produced in seed culture.

Table 1 Bacterial strains and plasmids used in this study

Strain and plasmid	Characteristics	Source
Strains		
E.coli DH5α	F^-,φ80d*lacZ*△M15, △(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-,mk^+), *phoA*, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1	Takara, Japan
P. aeruginosa SG	Rhamnolipid-producing strain; wild-type strain	Laboratory collection [30]

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E. coli PrhlAB	Gm^r; plasmid pBBRPrhlAB was transformed into *E.coli* DH5α	This study
E. coli PoprAB	Gm^r; plasmid pBBRPoprAB was transformed into *E.coli* DH5α	This study
P. aeruginosa PrhlAB	Gm^r; plasmid pBBRPrhlAB was transformed into *P. aeruginosa* SG	This study
P. aeruginosa PoprAB	Gm^r; plasmid pBBRPoprAB was transformed into *P. aeruginosa* SG	This study
Plasmids		
pBBR1MCS-5	Broad host range vector; Gm^r; IncQ; *LacZ*α; mob^+; 4768 bp	Laboratory collection [29]
pBBRPrhlAB	Gm^r; pBBR1MCS-5 containing 2.6 kb *rhlAB* genes with native promoter	This study
pBBRPoprAB	Gm^r; pBBR1MCS-5 containing 2.6 kb *rhlAB* genes with promoter of *oprL* gene	This study

Sequence analysis and PCR primers design

Eight *rhlAB* sequences from different *P. aeruginosa* strains were aligned and analyzed. The GeneBank accession numbers of eight *rhlAB* genes are KC008608, L28170, AE004091, CP000438, AP012280, HM190303, FM209186, and CP002496, respectively. Two pairs of PCR primers (rhlAB-n12 and rhlAB-c12) were designed for amplification of the two different DNA fragments of *rhlAB* genes. The *rhlAB* genes of rhamnosyltransferase I complex RhlAB with native operon promoter were amplified with primers rhlAB-n1 and rhlAB-n2 (Table 2). The coding sequence of rhamnosyltransferase I complex RhlAB (DNA fragment rhlAB-c) was amplified with primers rhlAB-c1 and rhlAB-c2. Sequences of *oprL* gene from *P. aeruginosa* (GeneBank accession number: AE004091, AP012280 and CP002496) were used to design PCR primers Popr-1 and Popr-2 (Table 2). The DNA fragment Popr containing promoter of *oprL* gene as amplified with primers Popr-1 and Popr-2. The primer rhlAB-c1 contained the terminal 19 bp nucleotide sequence of the DNA fragment Popr. The primer Popr-2 contained the initial 18 bp nucleotide sequence of the DNA fragment rhlAB-c.

Table 2 PCR primers used in this study

Primers	Sequences	Characteristics
rhlAB-n1	5'- GGCCTGGTACCCGGTTTTTTCATGCCTT-3'	28 bp, *Kpn I*
rhlAB-n2	5'-ATAGCAAGCTTCAGGACGCAGCCTTCAGC-3'	29 bp, *Hind III*
Popr-1	5'-GACTAGGTACCCTTCATCGGCAACTACAAC-3'	30 bp, *Kpn I*
Popr-2	5'-CAGACTTTCGCGCCGCATATGTAACTCCTAATGAACC-3'	37 bp
rhlAB-c1	5'-GGTTCATTAGGAGTTACATATGCGGCGCGAAAGTCTGTTG-3'	40 bp
rhlAB-c2	5'-ATAGCAAGCTTCAGGACGCAGCCTTCAGC-3'	29 bp, *Hind III*

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Plasmids and strains construction

The genomic DNA of *P. aeruginosa* SG was extracted by TIANamp Bacteria DNA Kit (TIANGEN, China). Using the genomic DNA of *P. aeruginosa* SG as template, DNA fragment rhlAB (*rhlAB* genes with native promoter), DNA fragment rhlAB-c (coding region of *rhlAB* genes) and DNA fragment Popr (containing promoter of *oprL* gene) were amplified by PCR. Using a mixture of equal amount of the DNA fragment rhlAB-c and Popr as template, the DNA fragment Popr-rhlAB (*rhlAB* genes with the promoter of *oprL* gene) was amplified with primers Popr-1 and rhlAB-c2 by overlap PCR. And PCR products of rhlAB and Popr-rhlAB were digested with *Kpn I* and *Hind III* and cloned into pBBR1MCS-5 [29] to construct the recombinant plasmids pBBRPrhlAB and pBBRPoprAB, respectively. The digested DNA fragments and plasmids were purified with a MiniBEST DNA Fragment Purification Kit (Takara, Japan). DNA fragments from the gels were purified with an AXYGEN Gel Extraction Kit (AXYGEN, USA). The recombinant plasmids pBBRPrhlAB and pBBRPoprAB were transformed into *E. coli* DH5α by $CaCl_2$–heat shock transformation method. For recombinant plasmids verification, the inserted genes were sequenced using the sequencing primers M13-47 and RV-M of plasmid pBBR1MCS-5. The recombinant plasmids were extracted using TaKaRa MiniBEST Plasmid Purification Kit Ver.3.0 (TaKaRa, Japan). The concentrations of recombinant plasmids were measured using the Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo, USA).

Competent cells of *P. aeruginosa* SG were prepared by 0.1 M $MgCl_2$ and $CaCl_2$ using a microcentrifuge-based procedure. Using chemical transformation method, the recombinant plasmids pBBRPrhlAB and pBBRPoprAB were transformed into *P. aeruginosa* SG by heat-shocked for 4min at 42 ℃ and incubated for 90 min at 37 ℃. After cultured on LB agar plate containing gentamicin,

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transformants were confirmed by plasmid extraction and PCR verification. After genotypic analysis, engineered strains *P. aeruginosa* PrhlAB and *P. aeruginosa* PoprAB were confirmed and ready for rhamnolipid production.

Copy numbers and stability of recombinant plasmids in engineered strains

The copy numbers of recombinant plasmids in engineered strains was calculated as follows. The recombinant plasmids were extracted from 2 ml LB culture (containing 50 ug/ml gentamicin) of engineered strains using TaKaRa MiniBEST Plasmid Purification Kit Ver.3.0 (TaKaRa, Japan). The recombinant plasmids were firstly digested with *Kpn I* to obtain linear plasmid fragments. Then concentrations of linear plasmid fragments were measured based on A_{260} (the absorbance value of DNA at 260 nm light) using NanoDrop 2000 spectrophotometer (Thermo, USA). Cell concentrations of the engineered strains in LB culture containing gentamicin were measured. Briefly, 1ml of LB culture of engineered strain was serially diluted and dispersed on LB plates (containing 50 ug/ml gentamicin). The plates were incubated for 24 h. The CFU (Colony-Forming Units)/ml were calculated as cell concentration (cells/ml). Copy numbers of recombinant plasmids (CNP) are calculated using the following equation: CNP (copies/cell) = $6.02*10^{23}$ * c / MW / cell number, where c is the concentration of linear dsDNA, and MW is the dsDNA length (bp) multiplied by 660, and the cell number is the cell concentrations multiplied by 2 ml (the volume of culture used for plasmid extraction).

To study the stability of recombinant plasmids in engineered strains, 100 positive single colonies of engineered strains *P. aeruginosa* PrhlAB and PoprAB were picked out from the LB plate, respectively. Then every 100 colonies were point inoculated to fresh LB plates and LB plates containing 50 ug/ml gentamicin, respectively. These plates were incubated for 24 h. And the growth percentage of colonies on LB plates containing gentamicin was calculated for plasmids stability

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determination. Colonies grown on LB plates were used for next point inoculation. Experiments were continuously performed 12 times to detect stability of recombinant plasmid in engineered strains.

Rhamnolipid production by wild strain and engineered strains

Rhamnolipid production by the wild strain SG and engineered strains PrhlAB and PoprAB were conducted in Erlenmeyer flasks (500 ml) containing 200 ml GN medium and incubated on a rotary shaker (200 rpm) at 37 ℃. All experiments were performed in triplicate. Cultures were sampled every day during 6 days' incubation process. The rhamnolipid concentration, surface tension, and biomass (dry cell weight) of samples were determined every day. Oil spreading activity as an indirect characterization of rhamnolipid amount was measured at the end of the stationary growth phase.

The amount of rhamnolipid in samples was quantified by the colorimetric determination of sugars with orcinol.[33,34] The biomass was analyzed by the measurement of dry cell weight. Samples were centrifuged at 12,000 rpm for 5 min to remove the supernatant, and the collected pellets were washed with 0.9 % NaCl saline water for three times. The dry cell weight was determined after drying at 75 °C for 10 h. The surface tension of the culture supernatant (10 000 rpm/min, 10 min) was measured by a BZY-1 automatic surface tension meter (Shanghai equitable Instruments Factory, Shanghai, China). Oil spreading activity was determined as follows: 15 ul of crude oil (density of 0.886 g/cm3 and viscosity of 5.6 mPa·s) was added to the surface of 25 ml of distilled water in a petri dish (90 mm in diameter) to form a thin oil membrane. Then 10 ul of sample was gently dropped onto the center of the oil membrane. The diameter of the clearly formed oil displaced circle was measured.

Rhamnolipid production under anaerobic conditions

The GN medium was boiled under a stream of oxygen-free N2 for 15 min to remove residual oxygen before it was sterilized in an autoclave (121 °C, 20 min). Resazurin (final concentration, 0.0001%

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(wt/vol)) was also added to verify whether or not the reduced medium was obtained. The anaerobic cultivation experiments were conducted in serum bottles (250 ml) sealed with butyl rubber stoppers containing 200 ml GN medium and incubated on a rotary shaker (80 rpm) at 37 ℃.[30] The wild type strain *P. aeruginosa* SG and engineered strains *P. aeruginosa* PrhlAB and PoprAB were used for production of rhamnolipid under anaerobic conditions. After 8 days' incubation, anaerobic culture was analyzed for rhamnolipid concentration, surface tension, and oil spreading activity.

Conclusions

In order to improve rhamnolipid production by P. aeruginosa SG, its key genes *rhlAB* were genetically manipulated. Plasmid pBBR1MCS-5 would be a good gene vector for genetic manipulation in *P. aeruginosa*. Both increasing the copy number of *rhlAB* genes and replacing the promoter of *rhlAB* genes by the promoter of *oprL* gene increased the production of rhamnolipid. Simultaneously increasing the copy number of *rhlAB* genes and using the strong promoter was more efficiently to enhance the production of rhamnolipid by *P. aeruginosa* under both aerobic and anaerobic conditions.

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