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研究报告

Chromosomal integration of red fluorescent protein gene in rhamnolipids-producer *Pseudomonas aeruginosa* SG and its application in petroleum reservoir

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Abstract: [Background] Microbial transfer in oilfiled production facilities is closely related to reservoir microbial community compositions and microbiologically improved oil production process. However, it is hard to distinguish the targeted microorganisms from the numerous indigenous microorganisms containing the same specific genes. Therefore, constructing microorganisms with specific gene marks is necessary. **[Objective]** In order to effectively track microbial transfer in oilfiled production facilities, an engineering strain *Pseudomonas aeruginosa* SG-rfp containing specific mark gene was constructed. **[Methods]** The red fluorescent protein gene (*rfp*) with a constitutive promoter was inserted into *amp* gene encoding β-lactamase on chromosome of rhamnolipids-producer *P. aeruginosa* SG isolated from petroleum reservoir by homologous recombination using integration vector pEX18Gm-URD and *amp* gene. **[Results]** *P. aeruginosa* SG-rfp constitutively expressing *rfp* gene was constructed. Strain SG-rfp is intolerant of ampicillin, kanamycin, streptomycin, and gentamicin. As well as the wild-type strain SG, SG-rfp can produce biosurfactants in aerobic and anaerobic conditions and significantly enhance oil recovery efficiency from oil-bearing core. Using strain SG-rfp, diffusion-limited microbial transfer in oil-bearing porous media was investigated and demonstrated via core-flooding test. **[Conclusion]** This study provides a powerful tool for in-depth investigation of microbial transfer in oilfiled production facilities and microbiologically improved oil production process.

Keywords: Petroleum reservoir, Microbial transfer, *Pseudomonas*, Red fluorescent protein gene

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红色荧光蛋白基因标记产鼠李糖脂铜绿假单胞菌 SG 及其在油藏中的应用

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摘 要：【背景】微生物在油田注采系统中的迁移直接影响到油藏微生物群落组成及其在油田生产中的应用。然而，由于缺少特异性标记，很难将目标微生物同众多的土著微生物区分开。因此，需要构建携带特异性基因的微生物菌株。【目的】为了有效追踪定位微生物在油田注采系统中的迁移，本文构建一株红色荧光蛋白标记假单胞菌。【方法】运用染色体同源重组的方法，将带有组成型表达启动子的红色荧光蛋白编码基因(red fluorescent protein gene，*rfp*)插入到一株分离自油藏环境且产鼠李糖脂的铜绿假单胞菌 SG 染色体上编码 β-内酰胺酶基因内部，获得标记菌株 SG-rfp。【结果】构建的菌株 SG-rfp 能够在非诱导条件下表达红色荧光蛋白，而且对氨苄青霉素、卡那霉素、链霉素和庆大霉素不具有耐受性。与野生型菌株 SG 相比，构建的 SG-rfp 菌株也能够在有氧和缺氧条件下产生鼠李糖脂，在岩芯驱油实验中能够较好地提高原油采收率。此外，应用菌株 SG-rfp，本文研究并证实了微生物在含油多孔介质中的迁移扩散及所受限制。【结论】本文所构建的菌株 SG-rfp 为深入研究微生物在油田注采系统中的迁移及微生物在油田生产中的应用提供了有力工具。

关键词：油藏，微生物迁移，假单胞菌，红色荧光蛋白基因

Microorganisms occurring in petroleum reservoir have great significances for oil production process because their metabolisms and metabolites can enhance oil recovery from oil-bearing rocks, prevent reservoir souring or equipment corrosion[1]. To date, microorganisms that produce biosurfactants, gases, and polymers have been widely employed to enhance oil recovery for high water-cut reservoirs[1-2]. Microbial technique that uses isolated microorganisms associated with their metabolites to recover entrapped oil from oil-bearing rocks is known as exogenous microbiologically enhanced oil recovery (EMEOR), and that use indigenous microorganisms occurring in oil reservoir via nutrients stimulation is called indigenous microbiologically enhanced oil recovery (IMEOR)[2]. Recently, MEOR has been proposed as an effective alternative way for depleted oil reservoirs[2-3].

No matter EMEOR or IMEOR process, selected exogenous microorganisms and or nutrients are delivered into petroleum reservoir via water-injection well, resulting in biologically active zones within reservoir, wherein the growing microorganisms and produced metabolites mobilize entrapped oil from oil-bearing rocks. A critical process of EMEOR is whether the injected microorganisms actually grow and metabolize in oil reservoir[4]. Another is the transport ability of the injected microorganisms in oil-bearing strata, which directly influences the sweep efficiency of EMEOR process[2]. To track microbial transfer, it seems to be practicable to detect specific genes that can represent the existence of injected microorganisms by PCR or quantitative PCR (qPCR), such as, *srfA* for surfactin-producers, *licA* for lichenysin-producers, and *rhlR* for rhamnolipid-producers. Nonetheless, it is hard to distinguish the injected microorganisms from the greater number of indigenous microorganisms containing the same specific genes. Therefore, constructing microorganisms with specific gene marks is necessary and attractive.

Indigenous microorganisms are fed by injected nutrients, and then grow throughout petroleum reservoir in IMEOR process[5]. Before diving further into the implementation, one must first dissect the microorganisms inhabiting in the petroleum reservoir

and then decide on the proper nutrients used to stimulate the indigenous microorganisms. However, water-flooding seems like an inoculated process because injected water generally contains a large diversity of exogenous microorganisms with dissolved oxygen and inorganic ions[6-7]. Whether the microorganisms in injected water could enter oil-bearing strata, and how they influence the subsurface microbial communities, afterwards, the application of IMEOR process? To reveal the question, microbial microorganisms with specific gene marks are needed, because it is difficult to accurately reflect whether the microbial strains in injected water can enter the deep environment of petroleum reservoir by solely relying on analysis of microbial 16S rRNA gene[8].

MEOR has been proven as an energy-efficient and environmentally friendly alternative oil recovery method for depleted petroleum reservoir, yet it still has not been widely implemented for oil industry due to the paucity of detailed MEOR mechanisms, including the foresaid issue. In this study, *Pseudomonas aeruginosa* SG Δ*amp*(*amp*::*rfp*) that can stably constitutively red fluorescent protein gene (*rfp*) in the absence of antibiotic selection was constructed via chromosome integration of *rfp* gene into chromosome of rhamnolipids-producing *P. aeruginosa* SG, which was isolated from petroleum reservoir[9]. *P. aeruginosa* is a common rhamnolipids producer, belongs to aerobic or facultative bacteria, and are frequently detected and isolated from petroleum reservoirs[10-13]. In addition, *in-situ* production of rhamnolipids by *P. aeruginosa* has been proven as a promising MEOR approach[12-13].

1 Materials and Methods

1.1 Main reagents and equipments

AxyPrep™ Genomic DNA Miniprep Kit and Plasmid Miniprep Kit were purchased from Corning Life Sciences (Wujiang) Co., Ltd. The endonucleases, PrimerSTAR Max DNA Polymerase, T4 DNA ligase were purchased from TaKaRa Biomedical Technology (Beijing) Co., Ltd. The main instruments include MyCycler PCR (Bio-Rad Laboratories) and UV-5500 ultraviolet and visible spectrophotometer (Shanghai Metash Instruments Co., Ltd.).

1.2 Strains, plasmids, primers and cultivation

In this study, *P. aeruginosa* SG was isolated from produced water of an oil production well[9]. *Escherichia coli* DH5α was used as the host strain for plasmid construction. *E. coli* S17 serving as transformation host was used to transfer plasmid pCom8-rfp or pEX18Gm-URD into *P. aeruginosa* SG by bi-parental mating. All strains were grown in Luria-Bertani (LB) medium at 37 °C. When required, media were supplemented with ampicillin, kanamycin, streptomycin, and gentamicin. For biosurfactants production, the wild-type strain SG and constructed strain SG-rfp were cultured in fermentation medium (FM) with pH of 7.2, containing, g per liter of distilled water, Na_2HPO_4 0.6, KH_2PO_4 0.2, $NaNO_3$ 4.0, $CaCl_2$ 0.01, $FeSO_4$ 0.01, $MgSO_4$ 0.3, yeast extract 0.01, and glycerol 20. Cultivation was performed at 37 °C and 180 r/min. The plasmids and primers used in this study are listed in Table 1.

1.3 Bacterial transformation

Competent *E. coli* cells were prepared and transformed according to Douglas Hanahan's method[14]. Plasmid pCom8-rfp and pEX18Gm-URD were introduced into *P. aeruginosa* SG by bi-parental mating using *E. coli* S17. The detailed protocol was described in the following sections.

1.4 Preparation of *rfp* gene containing a constitutive promoter

A 782-bp DNA fragment carrying *rfp* gene with 5′ artificial constitutive promoter Prfp was generated by PCR amplification with plasmid pDsRed-Express-N1 as a templet using primers R1-F and R1-R. The fragment was cloned in pMD19-T, resulting in pMD19-rfp, and then transformed in *E. coli* DH5α for plasmid amplification. The DNA sequence of the promoter Prfp is shown in Figure 1. The DNA upstream of the transcriptional start site (+1) was derived from PtacI promoter[15]. Differing with the PtacI, *lac* operator that can be repressed by *lac* repressor and derepressed by isopropyl *β*-D-thiogalactoside was removed from the DNA downstream of position +1. Prfp contains Shine-Dalgarno sequence allowing protein synthesis to start at the ATG of *rfp* gene.

Table 1 Plasmids and primers used in this study
表 1 本文用到的质粒和引物

Item	Description	Source
Plasmids		
pMD19-T vector	Cloning vector, Amp^R	TaKaRa, Dalian
pDsRed-Express-N1	The source of the red fluorescent protein (*rfp*) gene	Lab stock
pCom8-aacc1	*E. coli-Pseudomonas* shuttle vector, Gm^R, *oriT*, P_{alkB}, *alkS*	Lab stock
pCom8-rfp	Gm^R, *oriT*, Pfrp, *rfp*	This work
pEX18Gm	Gene replacement vector, Gm^R, *oriT*, *sacB*,	Lab stock
pEX18Gm-URD	Gm^R, *oriT*, *sacB*, Pfrp, *rfp*	This work
Primers	(5′→3′)	
R1-F	GAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAAGTTCACGTCACTAAG GAGAATACCATATGGTGAGCAAGGGCGAGGA	This work
R1-R	AGCTTACTTGTACAGCTCGTCCATGC	This work
R2-F	GATACGGAATTCGAGCTGTTGACAATTAATCATCGGCTC	This work
R2-R	ATGACGGGATCCGCTTACTTGTACAGCTCGTCCATGC	This work
R3-F	ATGGTGAGCAAGGGCGAGGA	This work
R1-R	AGCTTACTTGTACAGCTCGTCCATGC	This work
ampUP-F	GATACGGAATTCGATGCGGTCGAAGGAGTCACACAG-3	This work
ampUP-R	TACGAGCCGATGATTAATTGTCAACAGCTCTTAGGCGAGGGTGGCGGTGAAGGTCT	This work
rfp-F	AGACCTTCACCGCCACCCTCGCCTAAGAGCTGTTGACAATTAATCATCGGCTCGTA	This work
rfp-R	CTTGTCCTGGGTCAGGGCATAGCCAGCTTACTTGTACAGCTCGTCCATGC	This work
ampDN-F	GCATGGACGAGCTGTACAAGTAAGCTGGCTATGCCCTGACCCAGGACAAG	This work
ampDN-R	ATGACGGGATCCTGAGGATGGCGTAGGCGATCTTCA	This work
sacB-F	TGTTCAAGGATGCTGTCTTTG	This work
sacB-R	CAAAAGCCATATAAGGAAACATAC	This work
SG-F	ATGCCGCCAGCCTGGGAGTGAC	This work
SG-R	GGGGCGTGCGTCTGCTGGTAGT	This work

1.5 Construction of pCom8-rfp and transformation in SG

Plasmid pCom8-aacc1, an *E. coli-Pseudomonas* shuttle vector[16], was first linearized with *Eco*R I and *Bam*H I, and ligated to a PCR fragment containing Prfp and *rfp* gene that was amplified from plasmid pMD19-rfp using primers R2-F and R2-R and digested with *Eco*R I and *Bam*H I. The recombinant plasmid called pCom8-rfp was verified by sequencing with primers R3-F and R1-R, then, was transformed into *E. coli* S17, and finally transformed into SG by bi-parental mating via conjugation transfer as follows. The donor *E. coli* S17 containing plasmid pCom8-rfp and receptor SG were inoculated into 5 mL LB liquid medium containing 10 μg/mL gentamicin and 50 μg/mL kanamycin, respectively, and cultured at 37 °C, 180 r/min overnight in a shaker. The cultures were harvested by centrifugation at 6 000×*g* for 2 min to collect cells, which were washed twice using 2 mL of 10 mmol/L sterile magnesium sulfate solution, respectively. The collected donor and recipient bacterial cell pellets

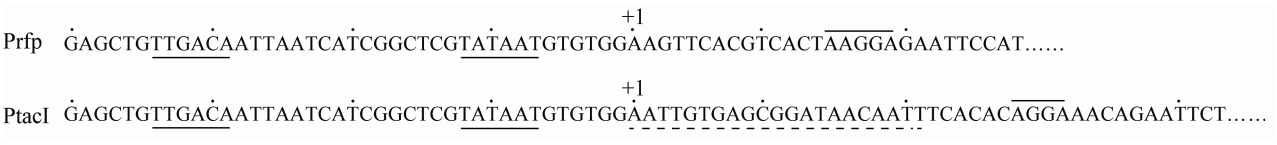

Figure 1 DNA sequences of promoter Prfp modified based on PtacI promoters
图 1 由 PtacI 启动子改造的 Prfp 的 DNA 序列信息
Note: The −35 sequences and the Pribnow box sequence of the promoters are underlined; The Shine-Dalgarno sequence of ribosome site is overlined; The *lac* operator is indicated with broken lines; The transcription start sites are indicated with +1; Dots indicated every tenth nucleotide.
注：下划线所示为−35 区和 Pribnow 框；上划线所示为核糖体结合位点 SD 序列；虚线所示为 *lac* 操纵子序列；+1 为转录起始位点；上标点所示为第 10 个碱基.

were suspended by adding 100 μL sterile magnesium sulfate (10 mmol/L). Subsequently, inoculating the cell suspension in a sterile filter membrane placed on LB agar plate, and then, was incubated at 37 °C for 16 h. The bacteria were collected from the filter membrane by washing with 200 μL 10 mmol/L sterile magnesium sulfate, and were evenly coated on LB agar plate containing 10 μg/mL gentamicin and 50 μg/mL kanamycin, and then, were incubated at 37 °C until colonies developed. The transformants containing pCom8-rfp were verified by PCR amplification and DNA sequencing using primers R3-F and R1-R.

1.6 Construction of recombinant plasmid pEX18Gm-URD

A DNA fragment containing *amp* gene was first obtained from *P. aeruginosa* SG using primer pairs SG-F/SG-R. Then, the upstream and downstream homologous arms (approximate 700 bp and 850 bp, respectively) of *amp* gene were amplified by PCR from the above DNA fragment using primer pairs ampUP-F/ampUP-R and ampDN-F/ampDN-R, respectively. The *rfp* gene with Prfp was amplified from pMD19-rfp using primers rfp-F/rfp-R. Then, overlapping PCR was performed to splice the upstream and *rfp* fragments, and was subsequently spliced with downstream fragment, resulting in DNA fragment URD. After digestion by *Eco*R I and *Bam*H I, the fragment was ligated into pEX18Gm digested with *Eco*R I and *Bam*H I, resulting in recombinant plasmid pEX18Gm-URD (Figure 2A), which was verified by enzyme digestion and PCR amplification (Figure 2B).

1.7 Chromosomal integration of *P. aeruginosa* SG

The recombinant plasmid pEX18Gm-URD was first transformed into *E. coli* S17, and then, transformed into *P. aeruginosa* SG via conjugation transfer by bi-parental mating according to the method described above, with modifications as follows. After conjugation and transfer on sterile filter membrane, the suspended cells were spread on LB agar containing 10 μg/mL gentamicin and 50 μg/mL kanamycin, and incubated at 37 °C for the selection of single-crossover recombinant, in which the plasmid pEX18Gm-URD was integrated into the genome of SG through homologous recombination involving either of the homologous arms (Figure 2C). The grown transformants were further verified by PCR amplification using primers sacB-F/sacB-R and R3-F/R1-R. The verified single-crossover colony was cultured in LB agar for 20 generations, and then, spread on LB agar containing 5% sucrose to select second recombinational colonies, followed by screening for ampicillin-sensitive and gentamicin-sensitive colonies. The colony with correct integration was verified by PCR amplification using primers SG-F/SG-R.

1.8 Growth, surfactant production, and oil recovery test for *P. aeruginosa* SG-rfp

The growth and surfactant production curves of *P. aeruginosa* SG and SG-rfp were determined when growing on FM medium with inoculation of 2% at 37 °C and 180 r/min. The inoculated seed liquid was prepared in LB liquid at 37 °C and 180 r/min for 16 h. Samples were collected continuously for determinations of cell concentration (OD_{600}) on ultraviolet-visible spectrophotometer and surface tension using POWEREACH JK99B digital tension-meter at 25 °C.

The enhanced oil recovery efficiency of *P. aeruginosa* SG-rfp was evaluated by core-flooding test using artificial heterogeneous cores. The cores are 29.8 cm in length, with cross-sectional area of 20.1−20.7 cm^2, permeability of 0.319−0.388 μm^2, porosity of 25.91%−26.28%, and pore volume (PV) of 157.4−160.0 mL. The cores were first saturated by crude oil with viscosity of 50.1 mPa·s, subsequently, were water flooded at injection rate of 0.5 mL/min till water content of the effluent reached 95%, which is close to high water-cut oil production wells. To calculate the enhanced oil recovery efficiency (ORE), 0.5 PV of FM medium was injected into one core as the control, and 0.5 PV of fermentation broth of SG-rfp was injected into another core. Then, the cores were sealed and incubated at 37 °C for 7 days. After incubation, the cores were water flooded again till water content of effluent reached 95% to calculate the ORE that means the volume of displaced oil divided the volume of original oil in core.

1.9 Transfer of *P. aeruginosa* SG-rfp in oil-bearing porous medium

Transfer of *P. aeruginosa* SG-rfp in oil-bearing

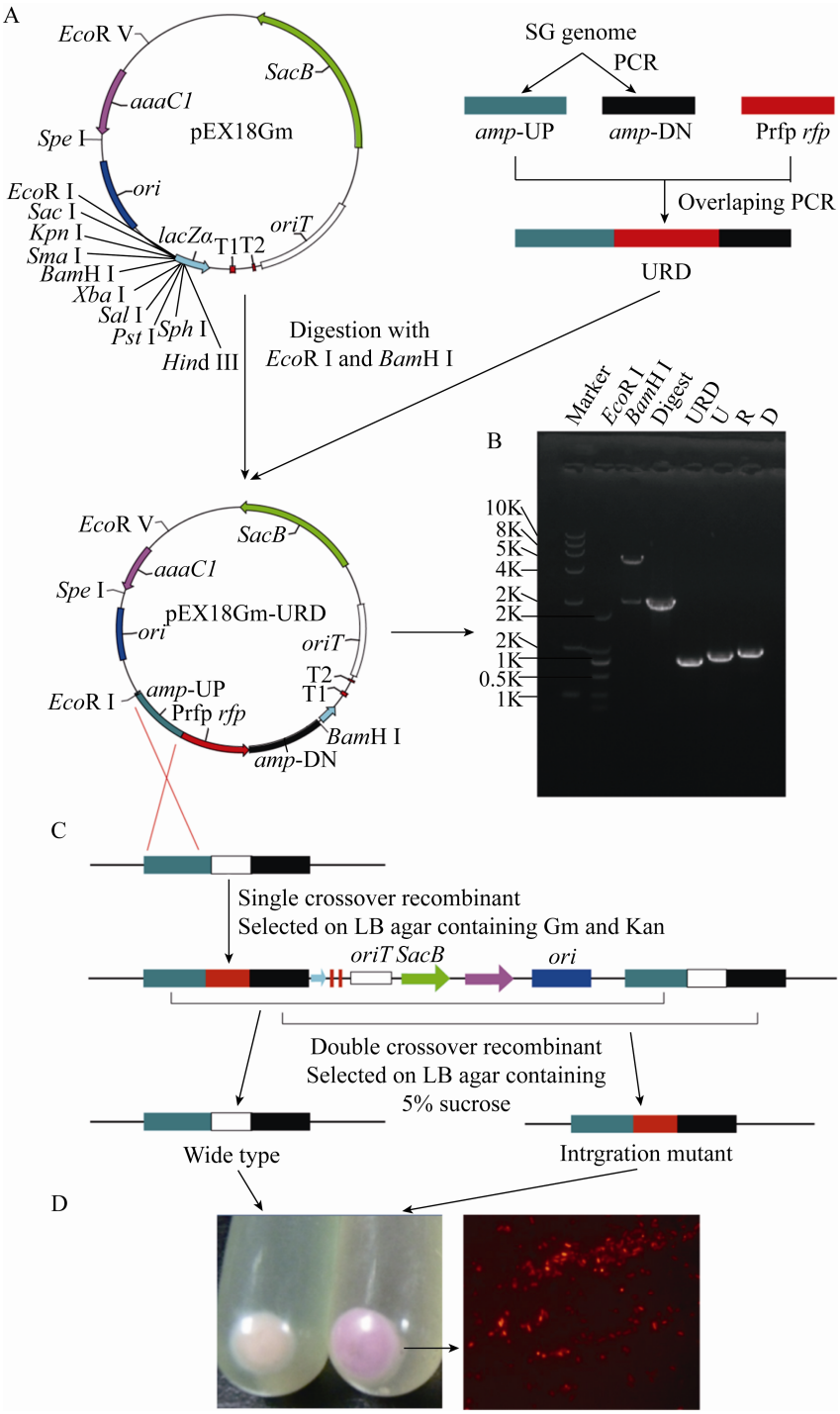

Figure 2 Scheme of chromosomal integration of *rfp* gene in *P. aeruginosa* SG

图 2 红色荧光蛋白基因 *rfp* 插入铜绿假单胞菌 SG 染色体方案

Note: The construction of plasmid pEX18Gm-URD (A) verified by *Eco*R I and *Bam*H I digestion and PCR (B); Scheme of recombination events leading to gene insertion, and chromosome structures of final mutants (C); Color of the cell pellets of wide type stain SG and integration mutant SG-rfp cultured in LB medium, red fluorescence emitted by SG-rfp observed under laser scanning confocal microscope (D).

注：A：质粒 pEX18Gm-URD 构建；B：*Eco*R I/*Bam*H I 酶切和 PCR 验证；C：同源重组及突变子染色体示意图；D：野生型和插入突变型菌株菌体颜色及激光扫描共聚焦显微镜成像分析.

porous medium was tested in core-flooding experiment. The cores are 7.0−7.1 cm in length, with cross-sectional area of 4.91 cm^2, permeability of 0.203 μm^2 and 1.373 μm^2. The cores were prepared as described above. The oil-bearing cores were first water flooded till water content of effluent reached 95%, then, were flooded by displacing water containing 10^7 cells/mL *P. aeruginosa* SG-rfp at flow rate of 0.5 mL/min. The fluorescence of the effluent liquid was determined using a PerkinElmer EnSpire multiscan spectrum with excitation wavelength of 587 nm and emission wavelength of 610 nm.

2 Results and Discussion

2.1 Constitutive expression of *rfp* gene in *P. aeruginosa* SG

In order to constitutively and stably express *rfp* gene in *P. aeruginosa* SG in absence of inducers, an artificial promoter Prfp derived from tacI promoter was first designed[12]. As shown in Figure 1, Prfp has same DNA sequences as the PtacI upstream DNA of the transcriptional start site (+1), but lacks the *lac* repressor binding site. The tacI promoter is about 11 times more efficient than the *lac* UV5 promoter and at least 3 times as strong as the fully derepressed *trp* promoter, because of the consensus −35 sequence (T-T-G-A-C-A) and the consensus Pribnow box sequence (T-A-T-A-A-T)[12]. Due to the absence of *lac* operator, *rfp* can be expressed in absence of inducer isopropyl β-D-thiogalactoside. To test the feasibility of constitutive expression of *rfp* in *P. aeruginosa* SG, *rfp* gene containing promoter Prfp was cloned in expression plasmid pCom8-aacc1, an *E. coli-Pseudomonas* shuttle vector containing oriT[13], resulting in plasmid pCom8-rfp. The recombinant plasmid pCom8-rfp was transferred into SG by bi-parental mating. The transferred plasmid was verified by plasmid extraction and restriction enzyme digestion. The expression of the cloned *rfp* in strain SG was confirmed by the observation of red fluorescence using laser scanning confocal microscope (LSM710, Germany). In addition, the centrifuged cell pellet of the transformant is rosy red that is easy to identify from wild-type strain SG.

2.2 Chromosome integration of *rfp* gene in *P. aeruginosa* SG

The plasmid pCom8-rfp is easily lost in transformed *P. aeruginosa* SG in the absence of selective pressure from antibiotics. However, it is not practically feasible to add a mass of antibiotics in industrial applications. Therefore, *rfp* gene containing promoter Prfp was integrated into the chromosome of *P. aeruginosa* SG via homologous recombination (Figure 2C) using the integration vector pEX18Gm-URD (Figure 2A) and the *amp* gene that encodes β-lactamase, which is responsible for the resistance to β-lactam antibiotics in many Gram-negative bacteria, including *Enterobacter cloacae* and *P. aeruginosa*[17]. The clones with chromosomal mutation were identified by colony PCR using the primers SG-F/SG-R, which generated PCR products of 4.5 kb for the successful inserted mutations that were designated SG-rfp. The color of the centrifuged cell precipitation of SG-rfp is rosy red that was significantly different with that of the wild-type strain SG (Figure 2D). Red fluorescence emitted by SG-rfp was observed under laser scanning confocal microscope (Figure 2D).

The stability of SG-rfp was tested in LB agar in absence of antibiotics and inducers by continuous passage cultivation for 30 generations. The results indicated that strain SG-rfp could steadily and constitutively express *rfp* gene in absence of antibiotics and inducers. Due to the deficiency of *amp* gene, strain SG-rfp is intolerant of ampicillin. In addition, SG-rfp is intolerant of kanamycin (equal or greater than 50 μg/mL), gentamicin (equal or greater than 10 μg/mL), and streptomycin (equal or greater than 50 μg/mL). Differ with the genes generally existing in petroleum reservoir or oil-polluted environments, such as, *srfA* gene, *licA* gene, *rhlR* gene and 16S rRNA gene, the inserted *rfp* gene makes it possible to efficiently distinguish the strain SG-rfp from the vast microorganisms around, or to be specifically quantified by PCR or qPCR. For the above advantages, strain SG-rfp shows great application potential for investigations of microbial migration in oil beating porous medium and MEOR process.

2.3 Growth, surfactant production, and oil recovery efficiency of SG-rfp

The cell density (OD_{600}) and surfactant production (surface tension) during SG and SG-rfp cultivation process were measured in shake flasks. As the time curves of cell growth show (Figure 3A),

the cell density increased with the extending of incubation time for both the SG and SG-rfp, and reached the stationary phase at 72 to 96 th. In the stationary phase, the cell density of SG was slightly higher than that of SG-rfp. The surface tension of culture liquid was decreased from approximate 65 mN/m to 30 mN/m in 24 h for both SG and SG-rfp (Figure 3A), indicating that chromosome integration of *rfp* gene in chromosome of *P. aeruginosa* SG did not obviously influence the production of biosurfactants. In addition, the fermentation broth of SG-rfp turned dark compared with that of SG, and the bubbles were smaller (Figure 3B). The phenomenon may be explained by the massively produced red fluorescence protein.

Consistent with strain SG, anaerobic cultivation performed in serum bottles indicated that SG-rfp is also capable of producing biosurfactants under anaerobic conditions. The surface tension of the culture could was decreased from 65 mN/m to approximate 30 mN/m. It has been confirmed that cell growth and biosurfactants production of *P. aeruginosa* in the absence of oxygen is feasible via anaerobic denitrification[18]. Biosurfactants produced by a wide variety of microorganisms consist of hydrophilic and lipophilic moieties, and consequently are able to partition at the oil-air or the oil-water interfaces and to lower surface or interfacial tension, respectively[1-2]. These properties make them ideal agents to recovery the entrapped or residual oil in small pores within rock matrix in petroleum reservoirs toward the end of the secondary stage of oil recovery. The oil recovery efficiency (ORE) of SG-rfp was measured via core-flooding test. As shown in Figure 4, the first round of water flooding recovered 48.86% crude oil from the control core and 51.91% from the experimental core. Compared with the control core (ORE=52.95%), more 9.6% of oil was recovered from the core injected SG-rfp fermentation broth in the second round of water flooding process.

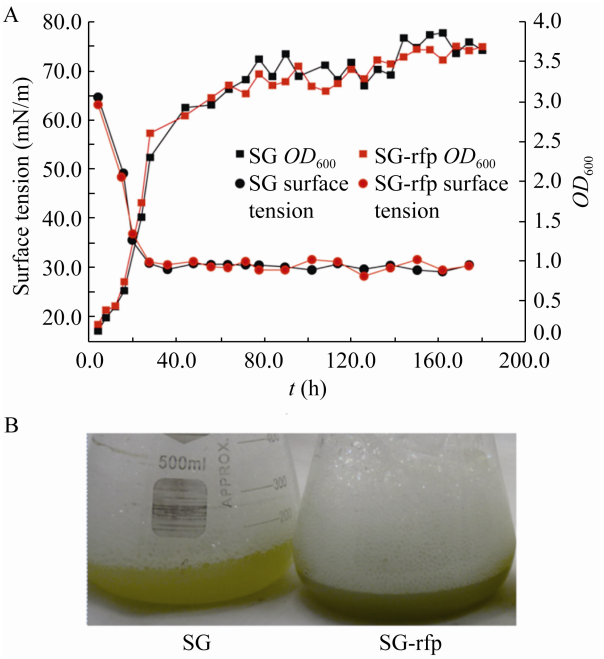

Figure 3 Time curves of cell growth and surface tension (A) and fermentation liquid (B) of *P. aeruginosa* SG and SG-rfp cultured in fermentation medium for 72 h

图 3 野生型菌株 SG 及荧光蛋白基因标记菌株 SG-rfp 生长与表面张力变化曲线(A)及其对应的 72 h 下发酵液状态(B)

2.4 Transfer of SG-rfp in oil-bearing porous medium

The transfer of microorganisms in oil-bearing strata is one of the most important issues that needed to be investigated, because it directly relates to swept volume of EMEOR[19], and may influences IMEOR process via influencing subsurface microbial community composition. Geographic isolation is supposed as an important habitat filter that influences

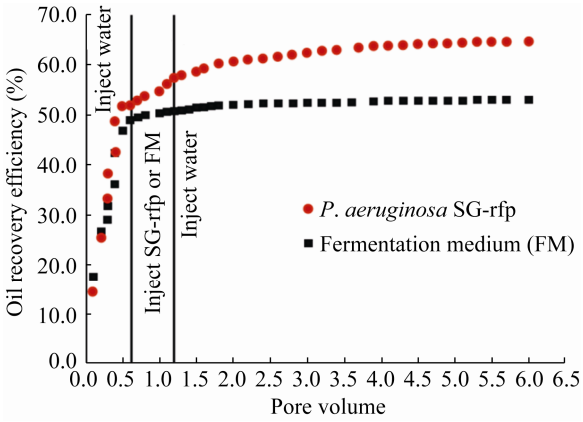

Figure 4 The curves of oil recovery efficiency with the increase of injected displacing liquid in core-flooding tests

图 4 岩心驱替实验下随驱替液注入量的变化原油采收率变化曲线

microbial community composition via preventing microbial dispersal[20]. For petroleum reservoir, the low permeability of oil-bearing strata will inevitably exert a significant influence on microbial dispersal. Ren et al. propose that the transportation of injected bacteria in oil-bearing strata was impacted by the varied permeability from injection well to each of the adjacent oil production wells[21]. Unfortunately, whether microorganisms could migrate or be brought into oil-bearing strata is still problematic, because it is hard to distinguish the injected microbial strain from the microorganisms around.

Using strain SG-rfp, microbial transfer along with displacing liquid in oil-bearing porous medium was investigated via core-flooding test. As shown in Figure 5, fluorescent signal was obviously observed in the effluent after 1 PV of displacing fluid containing SG-rfp was injected for the core with permeability of 1.373 μm^2. The fluorescent signal increased with the injection of displacing fluid, and reached up to maximum after 2 PV displacing fluid was injected. For the core with permeability of 0.203 μm^2, the fluorescent signal was observed in the effluent after 2 PV of displacing fluid was injected. The fluorescent signal increased slowly with the injection of displacing fluid, and reached up to maximum till 15 PV of displacing fluid was injected. It could be concluded that exogenous microorganisms could be transferred by injected displacing liquid in oil-bearing porous medium, and the transfer process is limited by formation permeability. However, a large number of detailed transfer mechanisms, such as, migration resulted by microbial flagella, microbial absorption in oil-bearing rocks, the dynamics of microbial migration, need to be further investigated. Although *P. aeruginosa* has been usually detected in petroleum reservoirs, and has been proven as an *in-situ* contributor in MEOR[11-13], there is still much to learn about the survival of SG-rfp and its impacts on subsurface microbial communities.

3 Conclusion

Strain SG-rfp that can constitutively express red fluorescent protein gene in absences of antibiotics selection was constructed by chromosomal integration of *rfp* gene in rhamnolipids-producer *P. aeruginosa* SG. Strain SG-rfp is sensitive to multiple antibiotics, satisfying the security requirements of application in petroleum reservoir. The strain SG-rfp shows promise as an effective tool for in-depth investigations of microbial transfer in petroleum reservoir, and sweep efficiency of MEOR process.

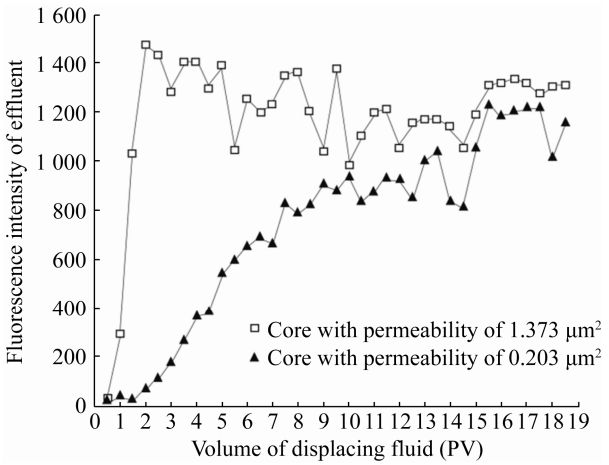

Figure 5 The fluorescent intensity of effluent from oil-bearing cores flooded by displacing liquid containing *P. aeruginosa* SG-rfp

图 5 荧光蛋白基因标记菌株 SG-rfp 岩心驱替实验中产出液荧光信号强度变化

REFERENCES

[1] Pannekens M, Kroll L, Müller H, et al. Oil reservoirs, an exceptional habitat for microorganisms[J]. New Biotechnology, 2019, 49: 1-9

[2] Youssef N, Elshahed MS, McInerney MJ. Microbial processes in oil fields: culprits, problems, and opportunities[J]. Advances in Applied Microbiology, 2009, 66: 141-251

[3] Wang WD, Lin JZ, Geng XL, et al. MEOR field test at block Luo801 of Shengli oil field in China[J]. Petroleum Science and Technology, 2014, 32(6): 673-679

[4] McInerney MJ, Maudgalya S, Nagle DP, et al. Critical assessment of the use of microorganisms for oil recovery[J]. Recent Research in Developmental Microbiology, 2002, 6: 269-284

[5] Ivanov MV, Belyaev SS, Borzenkov IA, et al. Additional oil production during field trials in Russia[J]. Developments in Petroleum Science, 1993, 39: 373-381

[6] Vigneron A, Alsop EB, Lomans BP, et al. Succession in the petroleum reservoir microbiome through an oil field production lifecycle[J]. The ISME Journal, 2017, 11(9): 2141-2154

[7] Wang XT, Li XZ, Yu L, et al. Characterizing the microbiome in petroleum reservoir flooded by different water sources[J]. Science of the Total Environment, 2019,

653: 872-885
[8] Kim DD, O'Farrell C, Toth CRA, et al. Microbial community analyses of produced waters from high-temperature oil reservoirs reveal unexpected similarity between geographically distant oil reservoirs[J]. Microbial Biotechnology, 2018, 11(4): 788-796
[9] Zhao F, Zhang J, Shi RJ, et al. Production of biosurfactant by a *Pseudomonas aeruginosa* isolate and its applicability to *in situ* microbial enhanced oil recovery under anoxic conditions[J]. RSC Advances, 2015, 5(45): 36044-36050
[10] Liu JF, Wu G, Yang SZ, et al. Structural characterization of rhamnolipid produced by *Pseudonomas aeruginosa* strain FIN2 isolated from oil reservoir water[J]. World Journal of Microbiology and Biotechnology, 2014, 30(5): 1473-1484
[11] Zhang F, She YH, Li HM, et al. Impact of an indigenous microbial enhanced oil recovery field trial on microbial community structure in a high pour-point oil reservoir[J]. Applied Microbiology and Biotechnology, 2012, 95(3): 811-821
[12] Zhao F, Zhou JD, Han SQ, et al. Medium factors on anaerobic production of rhamnolipids by *Pseudomonas aeruginosa* SG and a simplifying medium for *in situ* microbial enhanced oil recovery applications[J]. World Journal of Microbiology and Biotechnology, 2016, 32(4): 54
[13] Zhao F, Li P, Guo C, et al. Bioaugmentation of oil reservoir indigenous *Pseudomonas aeruginosa* to enhance oil recovery through *in-situ* biosurfactant production without air injection[J]. Bioresource Technology, 2018, 251: 295-302
[14] Hanahan D. Studies on transformation of *Escherichia coli* with plasmids[J]. Journal of Molecular Biology, 1983, 166(4): 557-580
[15] de Boer HA, Comstock LJ, Vasser M. The tac promoter: a functional hybrid derived from the trp and lac promoters[J]. Proceedings of the National Academy of Sciences of the United States of America, 1983, 80(1): 21-25
[16] Smits THM, Seeger MA, Witholt B, et al. New Alkane-responsive expression vectors for *Escherichia coli* and *Pseudomonas*[J]. Plasmid, 2001, 46(1): 16-24
[17] Lodge JM, Minchin SD, Piddock LJ, et al. Cloning, sequencing and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal *ampC* *β*-lactamase[J]. Biochemical Journal, 1990, 272(3): 627-631
[18] Chayabutra C, Wu J, Ju LK. Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: effects of limiting nutrients and carbon substrates[J]. Biotechnology and Bioengineering, 2001, 72(1): 25-33
[19] Youssef N, Simpson DR, McInerney MJ, et al. *In-situ* lipopeptide biosurfactant production by *Bacillus* strains correlates with improved oil recovery in two oil wells approaching their economic limit of production[J]. International Biodeterioration & Biodegradation, 2013, 81: 127-132
[20] Horner-Devine MC, Lage M, Hughes JB, et al. A taxa-area relationship for bacteria[J]. Nature, 2004, 432(7018): 750-753
[21] Ren HY, Xiong SZ, Gao GJ, et al. Bacteria in the injection water differently impacts the bacterial communities of production wells in high-temperature petroleum reservoirs[J]. Frontiers in Microbiology, 2015, 6: 505