J. Microbiol. Biotechnol. (2008), **18**(1), 63–66

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of Rhamnolipids on Degradation of Anthracene by Two Newly Isolated Strains, *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B

Cui, Chang-Zheng[1], Chi Zeng[1], Xia Wan[1], Dong Chen[1], Jia-Yao Zhang[2], and Ping Shen[1]*

[1]*College of Life Sciences, Wuhan University, Wuhan 430072, P. R. China*
[2]*College of Resource and Environmental Sciences, Wuhan University, Wuhan 430072, P. R. China*

Received: March 18, 2007 / Accepted: May 27, 2007

Anthracene is a PAH that is not readily degraded, plus its degradation mechanism is still not clear. Thus, two strains of anthracene-degrading bacteria were isolated from long-term petroleum-polluted soil and identified as *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B by a 16S rRNA sequence analysis. To further enhance the anthracene-degrading ability of the two strains, the biosurfactants produced by *Pseudomonas aeruginosa* W_3 were used, which were characterized as rhamnolipids. It was found that these rhamnolipids dramatically increased the solubility of anthracene, and a reverse-phase HPLC assay showed that the anthracene degradation percentage after 18 days with *Pseudomonas* sp. 12B was significantly enhanced from 34% to 52%. Interestingly, their effect on the degradation by *Sphingomonas* sp. 12A was much less, from 35% to 39%. Further study revealed that *Sphingomonas* sp. 12A also degraded the rhamnolipids, which may have hampered the effect of the rhamnolipids on the anthracene degradation.

Keywords: Anthracene, biosurfactant, rhamnolipids, biodegradation, *Sphingomonas* sp. 12A, *Pseudomonas* sp. 12B

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants [7]. Thus, the treatment of PAH-contaminated sites is of high concern owing to their toxic, carcinogenic, and mutagenic potentials [1]. Anthracene, a tricyclic PAH, is abundant in PAH-contaminated environments and represents a threat to the environment because of its toxicity to aquatic life, particularly *via* photo-induced higher toxicities [3]. In some cases, the effectiveness of degradation can be limited by the low water solubility and high hydrophobicity of anthracene. Therefore, for improved biodegradation, methods to enhance its solubility are required. This can be achieved by increasing the interfacial area of the aqueous phase and organic substrate or by adding surfactants [12].

The most important advantage of biosurfactants over synthetic surfactants is their ecological acceptance. Another advantage is that they can be modified by biotransformation to generate new products for specific requirements [2, 10]. Although not the strongest biosurfactant available, rhamnolipids, a glycolipid-type biosurfactant, are well suited to the bioremediation of pollutants because of their high emulsification activity and minor antibiotic effects. Accordingly, the objective of the present study was to investigate the effect of *Pseudomonas aeruginosa* W_3 rhamnolipids (RL_{W3}) at a critical micelle concentration (CMC) on the biodegradation of crystal anthracene.

Anthracene-degrading bacteria were isolated from a mixture of historically contaminated soil samples at the effluent treatment plant for the petrochemical industries and oil refinery in Wuhan, China. The soil samples (2 g) were suspended in 100 ml of MSM [5, 13], containing crystal anthracene at a concentration of 100 mg/l as the sole source of carbon. The medium containing the soil and crystal anthracene was then incubated at 30°C with orbital shaking (200 rpm) in the dark. Thereafter, the mixed culture was enriched and maintained by transferring 1 ml of the culture supernatant to fresh MSM every 10 days under the same conditions each time. After six transfers, 1 ml of the supernatant was diluted in an MSM solution, and then plated on a rich agar medium that contained (g/l): tryptone (10), yeast extract (5), NaCl (5), and 300 mg anthracene previously dissolved in *N,N*-dimethylformamide. The colonies displaying differences in colony shape and color were purified and tested for growth in liquid and solid MSM with anthracene as the sole source of carbon and energy [9]. On the basis of biochemical tests, two Gram-negative strains with the fastest growth were designated as strains 12A and 12B, and were found to be unable to produce biosurfactants.

*Corresponding author
Phone: 86-27-6875-4533; Fax: 86-27-8788-3833;
E-mail: ccz7808@yahoo.com.cn or pingshen@whu.edu.cn

The strains were identified using a 16S rRNA gene sequencing analysis according to standard protocol. The 16S rRNA gene was amplified from the genomic DNA by a PCR using the forward primer 5'-AGAGTTTGATCA-TGGCTCAG-3' and reverse primer 5'-TACGGTTACCT-TGTTACGACTT-3' [9]. The 16S rRNA gene sequence for 12A showed the highest similarity (99%) to that of *Sphingomonas* sp., whereas the 16S rRNA gene sequence for 12B was nearly identical (99%) to that of the type strain of *Pseudomonas* sp. The two strains were therefore designated as *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B, respectively.

For the production of biosurfactants, *Pseudomonas aeruginosa* W_3 was isolated from a mixture of historically petroleum-contaminated soil in Wuhan, China. This strain has been shown to possess a strong petroleum-degradation ability and to produce biosurfactants [15]. For the growth of *Pseudomonas aeruginosa* W_3 in a liquid culture, the basic salt medium [2] was supplemented with a high concentration (2%) of mannitol as the sole carbon source. The total rhamnolipid concentration in the sample was determined by measuring the concentration of hydrolysis-released rhamnose using the orcinol method after acid hydrolysis of the sample [4]. This measurement revealed that the rhamnolipid yield reached a maximum in 6 days, and therefore, the purified RL_{W3} was prepared as previously described [8], except the rhamnolipid-producing strain was cultured for only 6 days as opposed to 16 days.

To characterize the purified biosurfactants, liquid chromatography/mass spectrometry (LC/MS) analyses were performed using an equipped Agilent 1100 series LC/MSD trap. The electrospray ionization mass spectrum was run in the negative-ion mode, and the scanning mass range was from *m/z* 100 to 800. The samples (5 μl) were introduced by HPLC using an Agilent with a 2.1× 150 mm C18 reverse-phase column, and an acetonitrile-water gradient was used starting with 10% acetonitrile for 3 min, followed by increase to 90% acetonitrile over 30 min, which was then maintained for another 20 min. The HPLC flow rate was 0.25 ml/min. The structure and other parameters for the major components in RL_{W3} are listed in Table 1. As shown, the proportion of dirhamnolipid in RL_{W3} was rather high, possibly playing a major role in increasing the bioavailability of polycyclic aromatic hydrocarbons [14].

Table 1. Structure and relative abundance of rhamnolipids obtained from cultures with mannitol as the carbon source (only major components are shown).

Rhamnolipid	Retention time (min)	Pseudomolecular ion	Relative abundance (%)
Rha-Rha-C_8-C_{10}	25.5	621	15.5
Rha-Rha-C_{10}-C_{10}	28.6	649	57.4
Rha-Rha-C_{10}-$C_{12:1}$	30.5	675	7.6
Rha-C_{10}-C_{10}	315	503	9.3
Rha-Rha-C_{10}-C_{12}	32.0	677	8.4

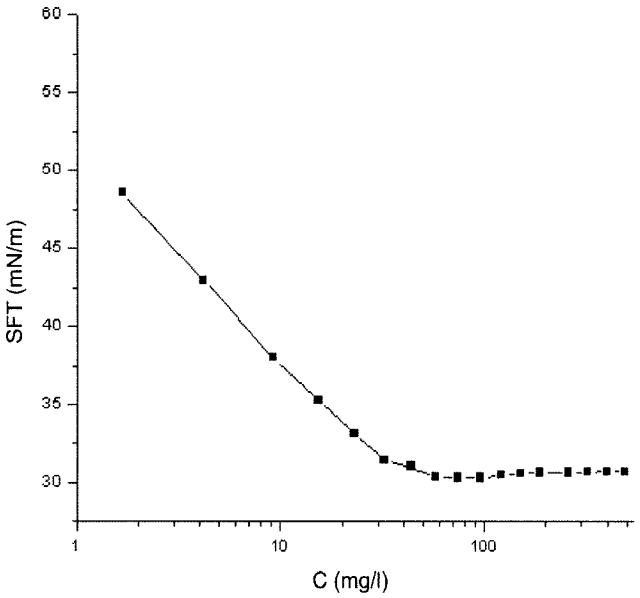

Fig. 1. Surface tension curve and CMC of rhamnolipid mixtures.

The surface tension measurements to evaluate the CMC of the RL_{w3} mixtures were measured at 30°C with a Krüss K100 (Germany) tension meter using the plate method. The rhamnolipids were dissolved at pH 7.0 with MSM. As illustrated in Fig. 1, the surface tension rapidly decreased from 71.4 mN/m to a minimum value of 30.5 mN/m as the rhamnolipid concentration was increased, and the CMC was determined as approximately 48 mg/l [4].

To measure the biodegradability and solubility of the anthracene, a quantitative volume of anthracene dissolved in dichloromethane was carefully added to the bottom of 50-ml micro-Erlenmeyer flasks to obtain crystal anthracene. The dichloromethane was then allowed to evaporate, and 15 ml of MSM with or without rhamnolipids was added to the flasks. These media were then inoculated with *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B from an anthracene MSM starter culture, respectively, to approximately 10^4 cells/ml. Two types of control were used: MSM containing only anthracene, and MSM containing both anthracene and rhamnolipids. All the cultures were incubated at 30°C with shaking at 200 rpm. The anthracene quantification was performed by reverse-phase HPLC using a Shimadzu LC-10ATVP liquid chromatograph (Shimadzu, Japan) with a Shimadzu SPD-10AVP (UV-Vis) detector at 254 nm. The separation column was a Shimadzu ODS-C18 4.6×250 mm. The mobile phase was methanol/water (80/20, v/v), and the flow rate was 0.5 ml/min.

The aqueous solubility of the anthracene in the absence of rhamnolipids was measured to be 50±3 μg/l. However,

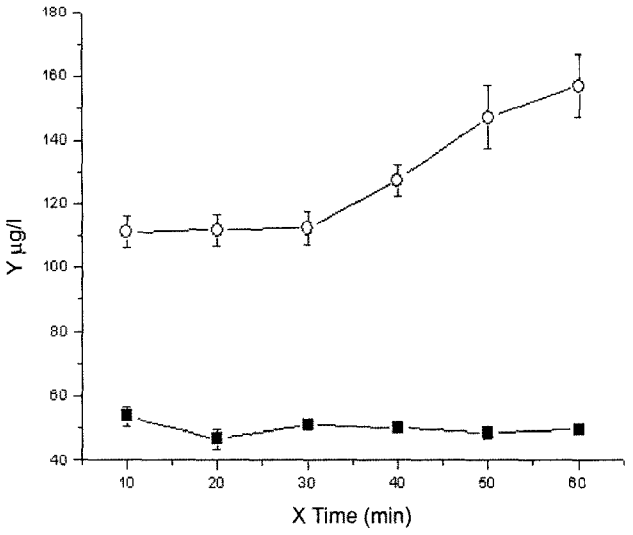

Fig. 2. Effect of rhamnolipid mixtures on dissolution of anthracene. Symbols are experimental data, and dotted lines are model fits. Symbols: (■) no rhamnolipids, (○) 48 mg/l rhamnolipids.

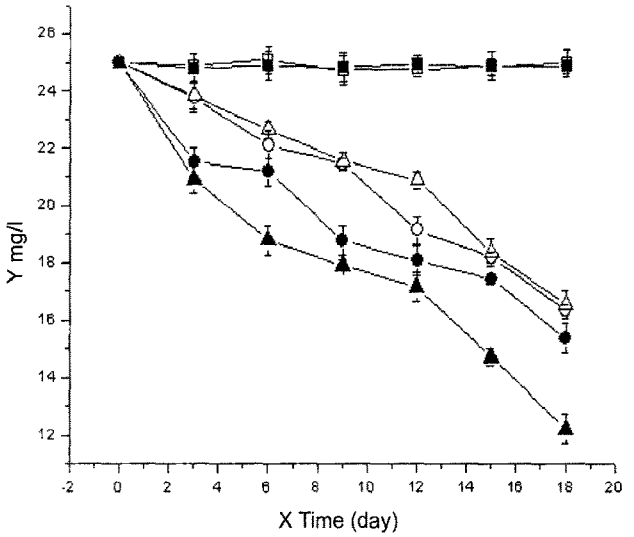

Fig. 3. Anthracene degradation in MSM amended with 48 mg/l rhamnolipids and no rhamnolipids.
Symbols are experimental data, and lines are model fit. Symbols: (▲) *Pseudomonas* sp. 12B+rhamnolipids, (●) *Sphingomonas* sp. 12A+ rhamnolipids, (△) only *Pseudomonas* sp. 12B, (○) only *Sphingomonas* sp. 12A. Controls included (■) no inoculum or rhamnolipids, (□) only rhamnolipids. (Note that the curves of ■ and □ are almost identical).

the solubility of the anthracene increased considerably, up to 157 µg/l, in the presence of the CMC of RL_{W3} (Fig. 2). It is widely believed that biosurfactants enhance the bioavailability of PAHs by increasing their solubility [1, 13]. However, the solubility of the anthracene remained level, even when the concentration of rhamnolipids was further increased to 10×CMC (data not shown).

Fig. 3 shows the effect of the rhamnolipids on the biodegradation of the anthracene. The controls showed almost no degradation, indicating that the anthracene did not degrade by itself in the dark, whereas the rhamnolipids alone did not enhance its degradation. The absence of the rhamnolipids resulted in a slow degradation of the anthracene, as *Pseudomonas* sp. 12B and *Sphingomonas* sp. 12A only degraded 34% and 35% of the anthracene, respectively, after 18 days, as would be expected from a substrate with a low water solubility and limited rate of mass transfer. When using RL_{W3} at the CMC (48 mg/l), the degradation rate with *Pseudomonas* sp. 12B was significantly enhanced to a degradation percentage (after 18 days) of 52%, further confirming the correlation between the extremely low dissolvability of anthracene and its limited degradation.

Interestingly, when using RL_{W3} at the CMC (48 mg/l) with *Sphingomonas* sp. 12A, there was only a slightly enhanced biodegradation of the anthracene (from 34% to 39%). Thus, to find the reason for this, it was investigated whether or not the two strains could also degrade rhamnolipids. One-hundred ml of MSM with 1×CMC rhamnolipids was added to 250-ml micro-Erlenmeyer flasks. These media were then inoculated with *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B, respectively. Two types of controls were used: only MSM, and MSM containing only rhamnolipids. The results revealed that *Sphingomonas* sp. 12A could degrade rhamnolipids, increasing the media surface tension from 30.5 mN/m to 50.9 mN/m in 9 days; however, the surface tension of the media inoculated with *Pseudomonas* sp. 12B and the media with only 1×CMC rhamnolipids remained at 30.5 mN/m, meaning that *Pseudomonas* sp. 12B could not degrade rhamnolipids. Since *Sphingomonas* sp. do not attach well to PAH-coated surfaces to form biofilms, the degradation ability of these strains would seem to depend on the solubility of the PAHs in the bulk liquid [6]. Therefore, it was extrapolated that the actual RL_{W3} concentration became lower than 1×CMC when 1×CMC was added, due to the consumption by *Sphingomonas* sp. 12A, thereby hampering any increase in its degradation of the anthracene.

In conclusion, the *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B isolated in this study, as well as the rhamnolipids produced by *Pseudomonas aeruginosa* W_3, would appear to be valuable for further research into the biodegradation of anthracene and its mechanism. In addition, this study highlights that when studying the effects of biosurfactants on the biodegradation of organic pollutants with a low water solubility, it is also important to determine whether the bacteria can consume or degrade the biosurfactant.

Acknowledgments

The authors are grateful to Wang Zhonghua and Shi Zhiguo for their skillful and diligent technical help. This work was supported by National Basic Research Program

of China (973 program) (2004CB719603) and grant from the National Natural Science Foundation of China (20277029).

References

1. Cameotra, S. S. and J. M. Bollag. 2003. Biosurfactant-enhanced bioremediation of polycyclic aromatic hydrocarbons. *Crit. Rev. Environ. Sci. Technol.* **30:** 111–126.
2. Costa, S. G. V. A. O., M. Nitschke, R. Haddad, M. N. Eberlin, and J. Contiero. 2006. Production of *Pseudomonas aeruginosa* LBI rhamnolipids following growth on Brazilian native oils. *Process Biochem.* **41:** 483–488.
3. Dean-Ross, D., J. D. Moody, J. P. Freeman, D. R. Doerge, and C. E. Cerniglia. 2001. Metabolism of anthracene by a *Rhodococcus* species. *FEMS Microbiol Lett.* **204:** 205–211.
4. Déziel, E., G. Paquette, R. Villemur, F. Lepine, and J. Bisaillon. 1996. Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. *Appl. Environ. Microbiol.* **62:** 1908–1912.
5. Fedorak, P. M. and D. Grbic-Galic. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. *Appl. Environ. Microbiol.* **57:** 932–940.
6. Johnsen, A. R. and U. Karlson. 2004. Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. *Appl. Microbiol. Biotechnol.* **63:** 452–459.
7. Johnsen, A. R., L. Y. Wick, and H. Harms. 2005. Principles of microbial PAH-degradation in soil. *Environ. Pollut.* **133:** 71–84.
8. Kang, Y. S., Y. J. Kim, C. O. Jeon, and W. Park. 2006. Characterization of naphthalene-degrading *Pseudomonas* species isolated from pollutant-contaminated sites: Oxidative stress during their growth on naphthalene. *J. Microbiol. Biotechnol.* **16:** 1819–1825.
9. Kiyohara, H., K. Nagao, and K. Yana. 1982. Rapid screening of bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl. Environ. Microbiol.* **43:** 454–457.
10. Laha, S. and R. G. Luthy. 1991. Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ. Sci. Technol.* **25:** 1920–1930.
11. Mata-Sandoval, J. C., J. Karns, and A. Torrents. 1999. High-performance liquid chromatography method for the characterization of rhamnolipid mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil. *J. Chromatogr. A* **864:** 211–220.
12. Tiehm, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. *Appl. Environ. Microbiol.* **60:** 258–263.
13. Tongpim, S. and M. A. Pickard. 1996. Growth of *Rhodococcus* S1 on anthracene. *Can. J. Microbiol.* **42:** 289–294.
14. Zhang, Y. M., J. M. Walter, and R. M. Maier. 1997. Effect of rhamnolipids on the dissolution, bioavailability, and biodegradation of phenanthrene. *Environ. Sci. Technol.* **31:** 2211–2217.
15. Zheng, J. X., J. Y. Zhang, Q. Zhao, L. Zhao, and C. T. Fu. 2006. Bacteria strains for petroleum degrading: Screening culture and UV-induced mutation. *Chinese Environ. Sci. Technol.* **29:** 1–2.