TITLE: Verification of Degradation of *n*-Alkanes in Diesel Oil by *Pseudomonas aeruginosa* Strain WatG in Soil Microcosms

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Abstract. Degradation of *n*-alkanes in diesel oil by *Pseudomonas aeruginosa* strain WatG (WatG) was verified in soil microcosms. The total petroleum hydrocarbon (TPH) degradation level in two bioaugmentation samples was 51 and 46% for 1 week in unsterilized and sterilized soil microcosms, respectively. The TPH degradation in the biostimulation was of control level (15%). The TPH degradation in aeration-limited samples was clearly reduced when compared with that in aeration-unlimited ones under both sterilized and unsterilized conditions. Addition of WatG into soil microcosms was accompanied by dirhamnolipid production only in the presence of diesel oil. These findings suggest that degradation of *n*-alkanes in diesel oil in soil microcosms would be facilitated by bioaugmentation of WatG, with production of dirhamnolipid, and also by participation of biostimulated indigenous soil bacteria.

Key words : *Pseudomonas aeruginosa* strain WatG; Soil microcosms; Bioaugmentation; *n*-Alkanes; Diesel oil; Rhamnolipids.

As industrialization is expanded, petroleum hydrocarbons are a potentially greater source of contaminants in water and soil environments [8]. Once the soil environment is contaminated with petroleum hydrocarbons, intrinsic bacteria are thought to degrade and utilize them as carbon sources [7]. However, if the contamination is so heavy that the intrinsic bacteria can no longer remove it effectively, some artificial decontamination processes are required. Biostimulation is a technique where the activity of the intrinsic bacteria is stimulated by adding nutrients or by aeration. While in bioaugmentation species or strains of microorganism that can degrade xenobiotics effectively are artificially added to the soil contaminated with petroleum.

Pseudomonas aeruginosa strain WatG is considered to be a good candidate for bioaugmentation of petroleum-contaminated soils, because it very efficiently degrades petroleum products, such as diesel oil, heavy oil and kerosene in a liquid medium containing mineral salts [13]. In this article, the ability of a single strain of *P. aeruginosa* to degrade petroleum in the soil environment has been examined and we discuss the practical utility of this strain in bioaugmentation in the soil environment.

Materials and Methods

Bacterial strains and culture media. Petroleum-degrading *P. aeruginosa* strain WatG (WatG) and *P. aeruginosa* strain type strain JCM5962^T as reference were described previously [13]. WatG was deposited to National Institute of Technology and Evaluation (NITE) Patent Microorganisms Depositary, Chiba, Japan (No. NITE AP-97). Soil characteristics were described previously [2].

Design and preparation of soil microcosms. Two grams of dried soil were put into 15-ml screw-capped or porous silicon-capped test tubes. When necessary, soil was autoclaved twice at 121˚C for 30 min to sterilize it completely. In this study Luria-Bertani (LB) medium was used as fertilizer to soil, because preliminary results showed that WatG exhibited a very low degradation activity of diesel oil in the soil supplemented with mineral salts medium (MSM) [13].

To change the inoculum size, 250 and 500 µl of the pre-culture of WatG were inoculated to 2 g of the soils. The former and latter microcosms were designated 12.5%-WatG-added and 25%-WatG-added groups, respectively. Filter-sterilized commercial diesel oil, NISSEKI ZOA (Nihon Sekiyu, Co. Ltd., Tokyo, Japan) was added to each soil microcosm to be at a final concentration of 1.0% (w/w). After mixing well with sterilized spatulas, each soil microcosm was left at 20˚C for 1 week.

Extraction and analysis of total petroleum hydrocarbons from soil microcosms. The procedure for extraction of total petroleum hydrocarbons (TPH) from soil microcosms was based on the Environmental Protection Agency method 3550B [12] with slight modifications. Briefly, an internal standard, *n*-dodecane (*n*C12) (Kishida Chemical Co. Ltd. Osaka, Japan), was added to each soil microcosm to give a final concentration of 0.3% (v/w) before extraction. An equal volume of solvent, consisting of dichloromethane (DCM) and ethanol (1:1, v/v), was added to the soil samples and mixed well with a vortex mixer. The samples were then sonicated at 30˚C for 1.5 h in an ultrasonic bath, followed by reciprocal shaking at 180 rpm at 20˚C for 2 days. All samples were centrifuged at 1,000 *g* for 10 min. The DCM-ethanol phase containing hydrocarbons was carefully removed from the lower soil phase and analyzed by gas chromatography and gas chromatography –

mass spectrometry [13].

For the calculation of the extraction efficiency of TPH, a solution of DCM and ethanol (1:1, v/v) containing 1% (w/v) diesel oil was regarded as the 100% extraction efficiency sample. The extraction efficiency of each sample was calculated by comparing the amount of TPH extracted with that of the 100% extraction efficiency sample.

Extraction and thin-layer chromatography analysis of biosurfactants. Twenty grams of soil supplemented with 12.5% (v/w) LB culture of WatG or control strain *P. aeruginosa* JCM5962^{T}, 12.5% (v/w) fresh LB medium, and 1% (w/w) diesel oil were put into 100-ml silicon-capped flasks and then incubated at 20°C for 1 week. Biosurfactants were extracted from the soil according to the methods of Hori et al. [6] and Sim et al. [10] with some modifications. The solvent system used was chloroform, methanol and acetic acid (65:15:2, v/v/v) [6]. Lipid spots on the plate were detected under UV after spraying with purimuline, and then glycolipids were visualized by the α-naphthol method [3].

Statistical data analyses. The group mean of extraction efficiency of TPH was analyzed using the Kruskal Wallis test at the $P < 0.05$ level of significance [1] and, if significant, the two sample t test was used to analyze for differences between the control and test groups at the $P < 0.05$ level of significance.

Results

Degradation of *n*-alkanes in diesel oil by WatG in soil microcosms. The recovery of TPH from 2 g of unsterilized soils containing 1.0% (w/w) diesel oil was 97% (Table 1). Spontaneous evaporation of diesel oil from the same amount of sterilized soils in the screw-capped (non-aerated) and porous silicon-capped (aerated) tubes was 7 ± 1.4% and 15 ± 5.0%, respectively (Table 1). The decrease of TPH in screw-capped test tubes would be attributed to the spontaneous evaporation of diesel oil to the head space of test tubes. The difference by approximately 4% between control and biostimulation samples of 25%-WatG-screw-capped sample would be attributed to the involvement of soil intrinsic bacteria that can grow under microaerobic condition.

The TPH degradation level of the 12.5%-WatG-silicon-capped biostimulation sample was the same as that of the control sample (Table 1). When the TPH degradation levels of two 12.5%-WatG-silicon-capped bioaugumentation samples were examined, they were 51% ± 16.9% and 46% ± 3.5% for those using unsterilized and sterilized microcosms, respectively. These results demonstrate that biostimulation is not effective in this system at least for 1 week and that bioaugmentation using unsterilized soil is more efficient than that using sterilized soil.

The inoculum size is another crucial point for bioaugumentation in soil microcosms. The results of 12.5%-WatG-silicon-capped samples were compared with those of 25%-WatG-silicon-capped samples. The TPH degradation levels of the former were approximately 20% higher than that of the latter under unsterilized conditions. Similarly under sterilized conditions the former was 30% higher than the latter (Table 1). These values were significant at the $P < 0.05$ level of significance when compared with the control and biostimulation samples (15 ± 5.0% and 15 ± 5.7%, respectively, Table 1).

To examine the effects of aeration conditions on diesel oil degradation, WatG was also inoculated to screw-capped test tubes to prevent spontaneous aeration as well as evaporation of diesel oil components. As shown in Table 1, TPH degradation by biostimulation and two bioaugumentation samples (non-aerated) in 25%-WatG screw-capped test tubes was obviously reduced, when compared with that in 25%-WatG porous silicon-capped samples (aerated). In particular, TPH degradation (24 ± 11.2 %) under non-aerated conditions was only 40% of that (41 ± 2.9 %) under aerated conditions in unsterilized soil.

Rhamnolipid production by WatG in soil microcosms. As shown in Fig. 1, two α-naphthol-positive lipids with R_f values of 0.26 and 0.20 were detected only in the WatG-added soil microcosms contaminated with diesel oil (lanes 5 and 6). The R_f value (0.26) of the former lipid was the same as that of dirhamnolipid from the WatG-grown liquid medium (Fig. 1, lane 1). Thus, the lipid was identified as dirhamnolipid. The production of dirhamnolipid in the soil microcosms containing WatG only in the presence of diesel oil implies that WatG would synthesize dirhamnolipid using diesel oil as inducer and secrete it into soil. To our knowledge, no reports on rhamnolipid production in soil microcosms have been presented. No obvious spot corresponding to monorhamnolipid was observed in any samples. The lipid with the R_f value of 0.20 would be probably a cellular lipid commonly derived from two *P. aeruginosa* strains (lanes 5 to 10) but not from soil microcosms (lanes 2 to 4), because of its occurrence only from all the cells-added microcosm samples (lanes 5 to 10).

Discussion

WatG could degrade diesel oil up to 51% in 1 week in soil microcosms at 20°C (Table 1). The finding that the diesel oil degradation capacity was much higher in 25%-WatG-silicon-capped culture than in 25%-WatG-screw-capped culture suggests that air (oxygen) would become a limiting factor during the cultivation of WatG in screw-capped test tubes. This ability of WatG to degrade *n*-alkanes in diesel oil in soil microcosms is comparable to that of a bacterial consortium consisting of *Nocardia nova* and *Rhodotorula glutinis var. dairensis* [11] and to that of active sewage sludge [5]. Thus it is considered that WatG can be adopted to a practical single degrader of petroleum products under aerobic conditions in the soil environment.

It should be emphasized that the percentage of TPH degradation was unexpectedly higher in the 12.5%-WatG-added groups than in the 25%-WatG-added groups (Table 1). The amendment of a fresh medium to soil microcosms would stimulate growth of WatG and indigenous soil bacteria, by which the consumption of diesel oil would be enhanced. Therefore, intermittent provision of fresh medium to soil microcosms may much more facilitate the efficiency of degradation of diesel oil in the soil environment than the provision of a larger inoculum in spent medium.

The finding that TPH degradation was clearly higher in unsterilized soil microcosms than in sterilized ones (Table 1) is reasonable, because soils may inherently include petroleum-degrading bacteria. Although no direct evidence has been provided, it is likely that indigenous soil bacteria work cooperatively with WatG to degrade diesel oil in soils. This cooperation may be mediated by rhamnolipids produced by WatG, because it is known that phenanthrene is degraded in soils co-inoculated with phenanthrene-degrading and biosurfactant-producing bacteria [4] and that the amendment of rhamnolipids to non-sterile soil enhances *n*-alkane degradation [9].

In conclusion 1) WatG exhibited a high diesel oil (mainly *n*-alkanes) degradation ability in soil provided with rich nutrients; 2) WatG produced dirhamnolipid in soil; 3) dirhamnolipid production by WatG in soil may facilitate its bioaugmentation ability by biostimulating indigenous soil bacteria.

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Figure legends

Fig. 1. The thin-layer chromatogram of crude rhamnolipids extracted from WatG-added soil microcosms. WatG was added to soil microcosms and then incubated for 1 week under conditions shown in the figure. *P. aeruginosa* strain JCM5962^{T} was used as a negative control. Lane 1, rhamnolipids of WatG cultivated in LB medium; lane 2, Bioaugmentation with WatG at 0 time; lane 3, Control; lane 4, Biostimulation; lanes 5 to 8, Bioaugmentation with WatG; lanes 9 and 10, Bioaugmentation with *P. aeruginosa* JCM5962^{T}. + : present; – : absent.

Table 1

TPH degradation in each soil microcosm

Experiment	Soil condition	TPH recovery [%][a]	Percentage of TPH degradation [%][a,b]		
			12.5% WatG silicon-capped	25% WatG silicon-capped	25% WatG screw-capped
100% extraction		100 ± 6.4			
0 time	unsterilized	97 ± 4.0			
Control [c]	sterilized		15 ± 5.0 [d]	15 ± 5.0 [d]	7 ± 1.4
Biostimulation	unsterilized		15 ± 5.7 [e]	15 ± 5.7 [e]	11 ± 2.5
Bioaugmentation	unsterilized		51 ± 16.9	41 ± 2.9	24 ± 11.2
Bioaugmentation	sterilized		46 ± 3.5	32 ± 5.1	20 ± 16.5

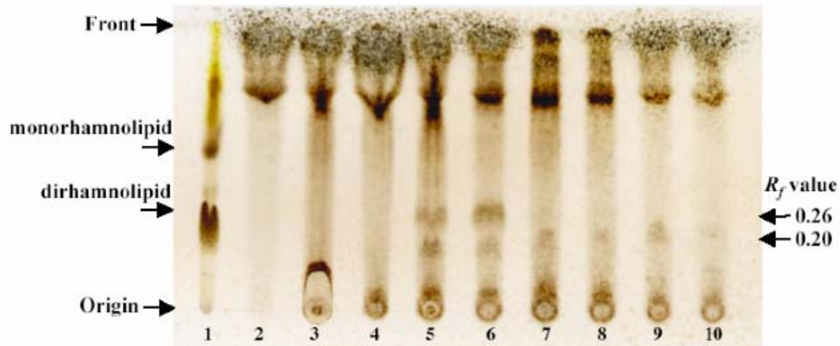
[a] Mean value ± standard deviation (*n*=3).

[b] Defined as the total degradation in which TPH recovery is taken into account.

[c] Defined as the disappearance of diesel compounds in the absence of biological activity (spontaneous evaporation).

[d] The value from the same experiments.

[e] The value from the same experiments.



	1	2	3	4	5	6	7	8	9	10
Sterilization of soil		-	+	-	-	+	-	+	-	+
Soil intrinsic bacteria		+	-	+	+	-	+	-	+	-
1% diesel oil		+	+	+	+	+	-	-	+	-
WatG		+	-	-	+	+	+	+	-	-
***P. aeruginosa* JCM5962^T**		-	-	-	-	-	-	-	+	+

Fig. 1.
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