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Degradation of long chain *n*-alkanes (C₃₆ and C₄₀) by *Pseudomonas aeruginosa* WatG

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Abstract

Pseudomonas aeruginosa strain WatG was unable to utilize either *n*-hexatriacontane (C₃₆) or *n*-tetracontane (C₄₀), which are both insoluble in a mineral salts medium (MSM), as sole carbon source. However, when C₃₆ and C₄₀ were added to MSM containing crude oil, more than 25% of each of the compounds was degraded by strain WatG after two weeks at 30°C. These results demonstrate that strain WatG has the ability to degrade long chain alkanes up to C₄₀, when they are solubilized by crude oil components.

1. Introduction

Petroleum hydrocarbons of crude and refined fossil fuel are the most widespread contaminants in the environment. Accidental spillage of petroleum often causes serious damage to the natural environment. Microbial degradation of spilled oil is one of the major routes of the natural removal of contaminants from the environment (Prince, 1993). Although *n*-alkanes are easily biodegradable, very long chain *n*-alkanes, branched-chain hydrocarbons, and polycyclic aromatic hydrocarbons are difficult to degrade.

Crude oil contains many different types of hydrocarbons and related compounds (Rosini, 1960). They are generally grouped into four major classes according to their differential solubility in organic solvents: saturates (including very long chain *n*-alkanes), aromatics, resins, and asphaltenes (Leahy and Colwell, 1990). Bacteria, yeasts, and fungi have been reported to degrade crude oil (Atlas, 1981).

Acinetobacter calcoaceticus S30 and *Alcaligenes odorans* P20 grew very well on *n*-alkanes up to C₃₃ (Lal and Khanna, 1996). Sakai et al. (1994) reported an *Acinetobacter* strain capable of degrading very long chain *n*-alkanes (up to C₄₄). However, it could grow on such hydrocarbons only in the presence of a chemical detergent. Most of the oil-degrading *P. aeruginosa* strains preferentially degrade middle to moderately long chain alkanes. Ko and Lebeault (1999) demonstrated that *P. aeruginosa* K1 and *Rhodococcus equi* P1 were able to degrade alkanes of chain lengths from C₇ to C₂₈. Although crude oil degradation by *P. aeruginosa* was reported (Abalos et al., 2004; Norman et al., 2002), the fate of the very long chain *n*-alkane components was not described. Wongsu et al. (2004) isolated a petroleum-degrading strain of *P. aeruginosa* that had the ability to degrade a broad range of hydrocarbons. Strain WatG almost completely degraded the hydrocarbons present in refined forms of petroleum, such as diesel oil or kerosene, that consisted of *n*-alkanes of middle to moderately

long carbon chains (up to C₂₄). However, the ability of strain WatG to degrade hydrocarbons longer than C₂₄ has never been tested.

In this study strain WatG was examined for its ability to degrade very long chain *n*-alkanes up to C₄₀ in a minimal liquid medium

2. Materials and methods

2.1. Petroleum hydrocarbons

The crude oil used throughout this study was kindly provided by Idemitsu Kosan Co. Ltd., Japan.

2.2. Culture media, bacterial growth conditions, and microbial count

Bacterial strains were grown in either Luria–Bertani (LB) medium or mineral salts medium (MSM) (Wongsa et al., 2004). Strain WatG was grown in LB medium at 30°C on a rotary shaker at 180 rpm until growth reached the stationary phase ($A_{600} = 5.8\text{--}6.0$). Cells grown on LB medium were transferred to 50 ml screw cap flasks containing 10 ml MSM supplemented with filter-sterilized crude oil (final concentration of 5.0 g L⁻¹) as the sole carbon source. Absorbance at 600 nm was adjusted to 0.1. A porous silicon plug was used for good aeration. Flasks were incubated at the appropriate temperature in a rotary shaker operating at 150 rpm. Three flasks were removed at each sampling time; two were used for the determination of crude oil degradation and one was used for a total viable cell count on LB plates at 30°C. Un-inoculated flasks containing an identical medium were used as controls in duplicate.

2.3. Extraction and GLC analyses of hydrocarbons

The residual petroleum hydrocarbons in culture media were extracted with an equal volume of dichloromethane. *n*-Dodecane was added to the cell culture as an internal standard before the extraction. The organic phase containing hydrocarbons was separated from the aqueous phase in a low-speed centrifuge. Anhydrous sodium sulfate was added to absorb residual water. The extracts were analyzed by a gas-liquid chromatograph (GLC; model GC 353B, GL Science, Japan) equipped with a flame ionization detector (FID) and a capillary column (TC-1, 30 m × 0.25 mm; film thickness = 0.1 μm). Injector and detector temperatures were 320°C and 340°C,

respectively. The sample was initially held 50°C for 5 min and then heated to 340 °C at a rate of 10°C min⁻¹ where it was held for 10 min. The percentage of total petroleum hydrocarbons degraded was calculated by the method of Wongsu et al. (2004).

*2.4. Degradation of *n*-hexatriacontane and *n*-tetracontane*

Solutions of *n*-hexatriacontane (C₃₆; Wako Chemical Co. Ltd., Japan) and *n*-tetracontane (C₄₀; Sigma-Aldrich) were prepared in chloroform at a concentration of 1 mg L⁻¹ and 0.5 mg L⁻¹, respectively. Amounts of C₃₆ (2 mg) and C₄₀ (1.5 mg) were each added to a flask containing 50 mg of crude oil. After complete vaporization of the chloroform, 10 ml of freshly prepared MSM was added. The degradation test was performed as described above.

3. Results and discussion

3.1. Growth of strain WatG and degradation of crude oil

A time course experiment was conducted to examine the growth of strain WatG and the degradation of crude oil (Fig. 1). At time zero, immediately after the oil was added, total viable cell numbers at both 20°C and 30°C were reduced to 50% of their value before the addition of oil, probably because of the presence of substance(s) in the oil that were toxic to strain WatG. The crude oil used in the experiment contained C₁₀ or shorter *n*-alkanes. *n*-Alkanes in the C₅–C₁₀ range have been shown to be inhibitory to many hydrocarbon-degrading bacteria at high concentrations as they disrupt the lipid membrane (Jeffrey, 1980). The viable cell count started increasing within 1 day and reached a maximum of 2.49×10^9 cells ml⁻¹ after 7 days of incubation at 30°C. At 20°C, a period approximately 3–4 times longer than that required at 30°C was necessary for cell numbers to recover from the initial reduction and for maximum viable cell numbers to be achieved. More than 85% and 75% of the crude oil was degraded by strain WatG at 30°C and 20°C, respectively.

*3.2. Degradation of individual *n*-alkanes*

Almost all of the *n*-alkanes (up to C₃₁, Fig. 2) were degraded. Approximately 15% of C₄₀ was degraded in one week at 30°C (data not shown). However, the extent of the degradation of

n-alkanes longer than C₃₆ by strain WatG was not always clear because of their extremely low concentration in crude oil. To evaluate their degradation by strain WatG, C₃₆ and C₄₀ were added as solids to MSM and MSM containing crude oil.

When either C₃₆ or C₄₀ was supplied as the sole carbon source in MSM, the *n*-alkanes remained insoluble and strain WatG could not utilize them. However, both C₃₆ and C₄₀ were degraded when they were supplied with crude oil, which most likely allowed the *n*-alkanes to become solubilized. Strain WatG degraded more than 17% of each of these *n*-alkanes after one week of incubation. Further incubation for another week resulted in 28% and 29% degradation for C₃₆ and C₄₀, respectively. The GLC profiles of C₃₆ and C₄₀ degradation are shown in Figure 3. The above results are summarized in Table 1.

Strains of *P. aeruginosa* are known to produce extracellular biosurfactant rhamnolipids in media containing petroleum hydrocarbons (Chayabutra and Ju, 2001; Jeong et al., 2004). However, it is not clear whether rhamnolipids are directly involved in the degradation of very long chain hydrocarbons. For strain WatG, externally added dirhamnolipid had no effect on the degradation of C₃₆ or C₄₀ in MSM (data not shown). Thus, it is considered that liquid components in crude oil might facilitate the solubilization of very long chain hydrocarbons such as C₃₆ and C₄₀. Similarly, C₂₄ *n*-alkane was degraded by strain WatG only when it was added to MSM together with liquid *n*-hexadecane (C₁₆; data not shown).

According to Sakai et al. (1994), *Acinetobacter* sp. strain M-1 is capable of degrading very long chain *n*-alkanes (up to C₄₄) only in the presence of an artificial detergent. In addition, two alkane hydroxylases encoded by *alkMa* and *alkMb* are induced by *n*-alkanes in different ways, that is, the former is induced by long chain alkanes (> C₂₂) in solid form and the latter is preferentially induced by liquid alkanes (C₁₆ to C₂₂; Tani et al., 2001). In *P. aeruginosa* strains, alkane hydroxylases that are responsible for the degradation of alkanes of different lengths (C₆–C₁₁ or C₁₂–C₁₆) have been identified (Smits et al., 2003). However, *P. aeruginosa* strains may have a third type of alkane hydroxylase specific to very long chain hydrocarbons such as C₃₆ or C₄₀.

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Table 1. Degradation of very long chain *n*-alkanes

<i>n</i> -Alkane type	Degradation (%)	
	7 days	14 days
Hexatriacontane (C ₃₆)	17.3 ± 0.4	28.3 ± 5.8
Tetracontane (C ₄₀)	16.7 ± 2.4	29.5 ± 8.0

Figure legends.

Fig. 1. Growth (○) and percentage of crude oil degradation (□) by *P. aeruginosa* strain WatG. Incubation temperatures were 20 °C and 30 °C. Bacterial growth was followed by measuring colony forming unit (cfu) per milliliter of cultures on LB medium.

Fig. 2. Degradation of selected *n*-alkanes in crude oil by *P. aeruginosa* strain WatG in MSM supplemented with crude oil. Strain WatG was incubated in MSM supplemented with 0.5% crude oil at 30 °C for 7 d with rotary shaking at 150 rpm.

Fig. 3. The Gas chromatograph profiles for very long chain *n*-alkane degradation. (A) and (B) are controls, (C) and (D) are inoculated with strain WatG; (A) and (C) are supplemented with C₃₆, (B) and (D) are supplemented with C₄₀.

Figure 1

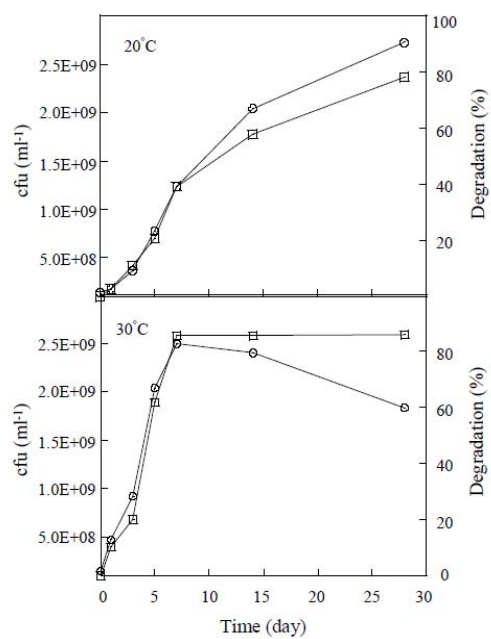


Figure 2.

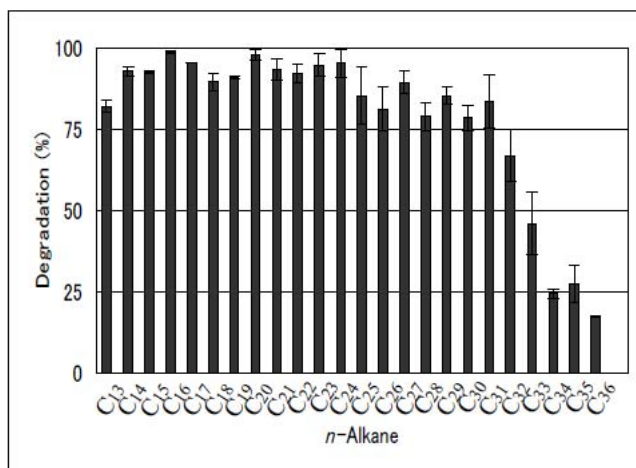


Figure 3.

