In: The Marine Environment
Editor: Adam D. Nemeth, pp. 157-166
ISBN 978-1-61209-265-2

Chapter 6

POTENTIAL FOR PETROLEUM ALIPHATIC HYDROCARBON DEGRADATION OF THE KEY BACTERIA IN TEMPERATE SEAS

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ABSTRACT

The potential for petroleum aliphatic-hydrocarbon degradation of the key bacteria in temperate seas was evaluated. As the possible key bacteria, *Alcanivorax*, *Marinobacter* and three taxonomically novel strains (one showed 96.6% similarity in its 16S rRNA gene sequence to *Oleibacter marinus*; and the other two showed less than 90% respective similarity to *Teredinibacter turnerae* and *Acinetobacter johnsonii*) were isolated after enrichment on crude oil in a continuous supply of Japanese seawater. In contrast to our previous results with tropical seawater, all the *Alcanivorax* isolates were closely related to *Alcanivorax borkumensis*, suggesting *A. borkumensis* to be the key *Alcanivorax* species characterized for temperate seawater. *A. borkumensis* strains, especially two new isolates, showed the highest activity for both *n*-alkane and branched-alkane degradation, indicating that these two new *A. borkumensis* isolates would be useful for the bioaugmentation strategy. The *n*-alkane-degrading activities of the *Marinobacter* isolate and *Thalassolituus oleivorans* (the already known key species) MIL-1^T were the lowest, and their branched-alkane-degrading activities were not significant. These results, providing insights into petroleum biodegradation in temperate seas, will promote the rational bioremediation strategies.

Keywords: *Alcanivorax*, *Thalassolituus*, *Marinobacter*, petroleum aliphatic hydrocarbons, temperate marine environment

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INTRODUCTION

A wide variety of micro-organisms are known to degrade petroleum hydrocarbons (Head et al. 2006; Prince 2005). However, most studies have not quantitatively evaluated their hydrocarbon-degrading activities. Among the hydrocarbonoclastic bacteria, *Alcanivorax* (Yakimov et al. 1998; Kasai et al. 2001; Hara et al. 2003; Roling et al. 2004; Yakimov et al. 2005) and *Cycloclasticus* (Dyksterhouse et al. 1995; Kasai et al. 2002a; Maruyama et al. 2003) strains have been identified as key micro-organisms in the respective degradation of aliphatic and aromatic hydrocarbons in marine environments (Harayama et al. 2004). *Alcanivorax* strains, which are distributed throughout the world (Golyshin et al. 2005), show high *n*-alkane- and branched alkane-degrading activities (Teramoto et al. 2009); this is consistent with the observations that *Alcanivorax* strains predominate in crude oil-impacted temperate marine environments (Kasai et al. 2001; Kasai et al. 2002b; Roling et al. 2002; Hara et al. 2003; Roling et al. 2004; Yakimov et al. 2005; Cappello et al. 2007) and in branched alkane-contaminated temperate seawater microcosms (Hara et al. 2003; McKew et al. 2007). Aliphatic hydrocarbon-degrading *Thalassolituus* (Yakimov et al. 2004) strains have been shown to dominate in *n*-alkane-contaminated temperate seawater microcosms (Yakimov et al. 2005; McKew et al. 2007) and in crude oil-contaminated temperate estuarine seawater microcosms (McKew et al. 2007; Coulon et al. 2007), while their hydrocarbon-degrading activities remain to be quantitatively evaluated. In tropical marine environments, *Oleibacter* strains (Teramoto et al. 2010) are considered to be important for petroleum aliphatic hydrocarbon degradation, because the *Oleibacter* strains, showing high *n*-alkane-degrading activity comparable to that of *Alcanivorax*, have dominated in crude oil-impacted beach-simulating microcosms with Indonesian seawater (Teramoto et al. 2009).

In addition to these ‘professional’ hydrocarbonoclastic bacteria, many ‘non-professional’ hydrocarbonoclastic bacteria such as *Marinobacter hydrocarbonoclasticus* (Gauthier et al. 1992) have also been isolated. *Marinobacter* strains (Gauthier et al. 1992; Sproer et al. 1998; Huu et al. 1999; Hedlund et al. 2001) are of increasing interest as many marine hydrocarbonoclastic isolates from various marine environments, including pristine areas, have been suggested for classification into this genus (González and Whitman 2006).

The batch culture technique has most commonly been used to isolate bacteria that are capable of degrading hydrocarbons of interest. However, this method is highly selective, resulting in the enrichment of a few species with a selective growth advantage under laboratory conditions (Dunbar et al. 1996; Dunbar et al. 1997; Watanabe et al. 1998). On the other hand, the use of alternative methods such as continuous flow-through culture has enabled dominant bacterial populations in actual environments to successfully be isolated (Watanabe et al. 1998; Kasai et al. 2001; Kasai et al. 2002b; Stach and Burns 2002). Thus, the present study used the flow-through culture method with crude oil and Japanese seawater to isolate such key bacteria for petroleum hydrocarbon degradation in temperate seas. The petroleum hydrocarbon-degrading activities of the possible key degraders isolated from these cultures and of the already known key degraders in temperate seas were comparatively evaluated to better understand the petroleum biodegradation that occurs in temperate marine environments and to promote the rational bioremediation strategies.

MATERIALS AND METHODS

Seawater sample. The seawater samples used for isolating the petroleum hydrocarbon-degrading bacteria were collected in April and May 2006 off Katsuura city, Japan (35°8' N, 140°18' E). We called them Katsuura seawater.

Preparation of crude oil and its chocolate mousse. Arabian light crude oil treated at 214 ˚C for 10 h to remove the volatile fraction (30% in volume) was used in this study. 'Chocolate-mousse' crude oil was prepared by mixing the crude oil and fresh Katsuura seawater in a ratio of 1:5 (w/w), this being followed by vigorous and continuous shaking for one day. The resultant chocolate mousse was stable for several weeks.

Strains. *Alcanivorax borkumensis* $SK2^T$ (ATCC 700651; Yakimov et al. 1998) was obtained from ATCC, while *Thalassolituus oleivorans* MIL-1^T (DSM 14913; Yakimov et al. 2004) was from DSMZ. The strains isolated in this study were deposited at NBRC (NITE Biological Resource Center) under numbers NBRC 105770 to NBRC 105776.

Continuous-flow culture. The conditions used for continuous-flow culture with the chocolate-mousse crude oil were as described previously (Teramoto et al. 2009), except for using Katsuura seawater instead of Indonesian seawater. The continuous-flow culture was conducted in two different modes to isolate hydrocarbon-degrading bacteria of wider diversity. In the first mode (culture 1), non-sterilized SW medium [seawater supplemented (per litre) with 1 g NH_4NO_3, 0.2 g K_2HPO_4 and 12 mg $FeCl_3$] prepared with fresh seawater was supplied for 13 days. The surface of the chocolate-mousse crude oil and the aqueous phase of the culture were then spread on to a SW medium plate [1.5% (w/v) agar; 9 cm in diameter] covered with 30 µl crude oil. The plates were incubated at 18˚C, and the bacterial colonies appearing on the crude oil-covered SW medium plates were purified at around 25˚C on 1/5 MB plates composed (per litre) of 15 g agar, 0.2 l distilled water, 0.8 l seawater and 7.48 g Marine Broth 2216 (Difco). In the second mode of the continuous-flow culture (culture 2), non-sterilized SW medium prepared with fresh seawater was supplied for the first 3 days, and sterilized SW medium, prepared by autoclaving, was supplied for the next 13 days. The surface of the chocolate-mousse crude oil and the aqueous phase of the culture were then spread on to a 1/5 MB plate (9 cm in diameter) covered with 30 µl crude oil. These plates were incubated at 18˚C, and the bacterial colonies appearing on these crude oil-covered plates were purified at around 25˚C on 1/5 MB plates.

Analysis of 16S rRNA gene. The almost full-length 16S rRNA gene was sequenced as described previously (Teramoto et al. 2009). The sequences obtained were compared to those in the GenBank database using BLAST (Altschul et al. 1990). The sequences were aligned using CLUSTAL_X (version 1.83; Thompson et al. 1997). The distance matrices for the aligned sequences, including all gaps, were calculated, and a neighbor-joining tree (Saitou and Nei 1987) was constructed using the NJPlot software in the CLUSTAL_X program.

Other methods. Repetitive extragenic palindromic sequence PCR (rep-PCR) was performed and analyzed as described previously (Teramoto et al. 2009). The crude oil-degrading activity and emulsifying ability of each strain at 25˚C were evaluated as described previously (Teramoto et al. 2009). The dMB plate was comprised of (per litre) 15 g agar, 0.9 l seawater, 0.1 l distilled water and 3.74 g Marine Broth. Salinity of the seawater used for the physiological characterization (seawater collected from the Pacific Ocean, 300 km off the

coast of Tokyo, Japan) was measured by a hand-held ATC-S/Mill-E refractometer (Atago, Tokyo, Japan), and was 3.5%.

RESULTS AND DISCUSSION

Isolation of the possible key petroleum-hydrocarbon degraders in temperate seawater.

Petroleum hydrocarbon-degrading bacteria were enriched on chocolate-mousse crude oil in a flow-through system in which Japanese seawater supplemented with nitrogen, phosphorus, and iron nutrients was continuously supplied. After the cultivation, bacteria were isolated either from the surface of the chocolate-mousse crude oil or from the aqueous phase. Colonies of different morphologies were selected to isolate bacteria of wider diversity. Thirty isolates each were obtained from the surface of the chocolate-mousse crude oil and from the aqueous phase of culture 1, while 60 isolates each were obtained from the surface of the chocolate-mousse crude oil and from the aqueous phase of culture 2. The isolates were grown in SW medium supplemented with 0.1% (v/v) crude oil for GC-MS analysis to examine the degradation of petroleum hydrocarbons. Among the isolates, 37 isolates exhibiting significant *n*-alkane-degrading activity or both *n*-alkane-degrading and oil-emulsifying activities were chosen as the possible key petroleum-hydrocarbon degraders for further analysis. None of the isolates degraded aromatic hydrocarbons.

Taxonomy of the petroleum aliphatic hydrocarbon-degrading isolates.

The possible key 37 *n*-alkane degraders isolated were subjected to a rep-PCR analysis for strain typing, and the almost full-length 16S rRNA gene sequence of each of the strains exhibiting distinct rep-PCR patterns was determined (Table 1). Phylogenetic relationships of the alkane-degrading isolates and their phylogenetically closest bacterial species are shown in Figure 1 based on the 16S rRNA gene sequences. The 16S rRNA gene sequence of strain A*l*26 was 99.6% identical to that of *A. borkumensis* SK2, and thus all the *Alcanivorax* isolates obtained in this study were closely related to *A. borkumensis*. This is in contrast to our previous result that the *Alcanivorax* strains similarly selected from Indonesian seawater were of types other than *borkumensis* (Teramoto et al. 2009). In addition, *A. borkumensis* has dominated in the actual marine environment polluted by an oil-spill accident in the temperate zone (Kasai et al. 2001). These results may indicate that the key *Alcanivorax* species characterized for temperate seas is *A. borkumensis*.

On the other hand, three taxonomically novel strains were obtained: strain T13 showed 96.6% similarity in the almost full-length 16S rRNA gene sequence to the closest bacterial species, *Oleibacter marinus* (which had been similarly selected from Indonesian seawater in our previous study; Teramoto et al. 2009, 2010); strain 25 showed 89.2% similarity to *Teredinibacter turnerae*; strain *l*30 showed 89.8% similarity to *Acinetobacter johnsonii* (Table 1). Strain T13 grew much more slowly than the *O. marinus* strains on the dMB plate supplemented with 0.5% (w/v) pyruvate at 25˚C (data not shown), indicating that the strain T13 was not an *O. marinus* strain. These findings expand the genera that degrade petroleum hydrocarbons.

Petroleum aliphatic hydrocarbon-degrading activities of the possible key degraders (isolates) and the already known key degraders in temperate seas.

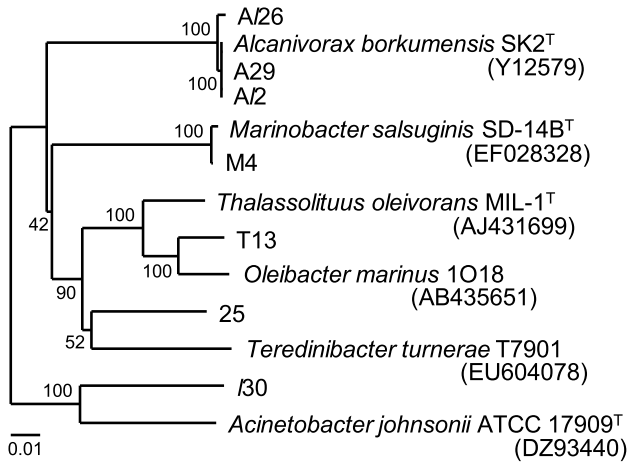

Figure 1. Phylogenetic relationships based on almost full-length 16S rRNA gene sequences of the alkane-degrading strains obtained in this study, their phylogenetically closest bacterial species, and the already-known key alkane-degrading species, *Thalassolituus oleivorans*. Bootstrap values are indicated at the nodes. The scale bar indicates 0.01 substitutions per site.

Table 1. Taxonomic affiliation of alkane-degrading strains obtained in this study, based on almost full-length 16S rRNA gene sequences

Isolate[a] (accession no.)	Closest GenBank relative[b] (accession no.)	Similarity (%)
A29, A*l*2 (AB435569)	*Alcanivorax borkumensis* SK2^T (Y12579)	100
A*l*26 (AB435570)	*Alcanivorax* sp. Wf-1 (AB055208)	100
M4 (AB435571)	*Marinobacter salsuginis* SD-14B^T (EF028328)	99.7
T13 (AB435572)	*Oleibacter marinus* 1O18 (AB435651)	96.6
25[c] (AB435573)	*Pseudomonadales* clone (AF102866)	89.7
*l*30[d] (AB435574)	Freshwater strain IMCC1704 (DQ664237)	95.7

[a] A29 and A*l*2 showed different rep-PCR patterns, although their 16S rRNA gene fragment sequences were 100% identical. The *l* in the names of isolates indicates that the isolate was obtained from the liquid phase, while the others were from the oil surface.

[b] All closest GenBank relatives belonged to *γ-Proteobacteria*.

[c, d] The validly described bacterial species with the highest 16S rRNA gene sequence similarity were *Teredinibacter turnerae* (T7901; EU604078; 89.2% similarity) for strain 25, and *Acinetobacter johnsonii* (ATCC 17909^T; Z93440; 89.8%) for strain *l*30.

Figure 2 compares the petroleum aliphatic hydrocarbon-degrading activities of the isolates (Table 1) and of type strains of the already known key species, *A. borkumensis* SK2^T (Yakimov et al. 1998; Kasai et al. 2001; Kasai et al. 2002b) and *T. oleivorans* MIL-1^T (Yakimov et al. 2004; Yakimov et al. 2005; McKew et al. 2007). When these strains were cultivated on crude oil, *Alcanivorax* strains SK2^T, A29 and A*l*2 and taxonomically novel strain *l*30 showed crude oil-emulsifying ability, but the other strains did not. *Alcanivorax* strains, especially strains A29 and A*l*2, showed the highest *n*-alkane- and branched alkane-degrading activities. Although *Alcanivorax* strain A*l*26 did not emulsify crude oil, this strain's degradation activity was comparable to those of the other *Alcanivorax* strains,

indicating that the emulsification is not indispensable for the efficient alkane biodegradation by this genus.

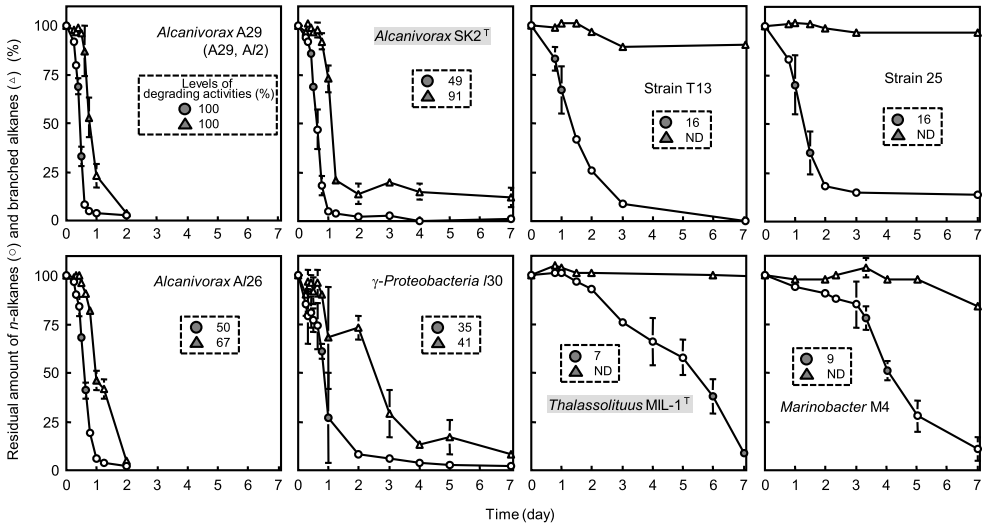

Figure 2. Degradation of *n*-alkanes and branched alkanes at 25˚C by the alkane-degrading strains and by type strains of the already-known key species, *A. borkumensis* SK2^T and *T. oleivorans* MIL-1^T. Colonies from each strain freshly grown at 25˚C on dMB plates supplemented with 0.5% (w/v) pyruvate were suspended in filter-sterile seawater until the OD_{600} was 0.5. Then 150 μl of this bacterial suspension was inoculated into 3 ml SW medium containing 3 μl crude oil and the culture was incubated for the indicated period at 25˚C. Non-inoculated sterile samples were similarly incubated and served as controls (100%). Oil was extracted from the cultures as described in Methods. The *n*-alkanes (C_{12-22}; circles) and branched alkanes (pristane and phytane; triangles) were quantified. Strains A29 and A*l*2 showed similar biodegradation profiles, and the profile of strain A29 is shown. Each value is the mean ± SE from two or more independent experiments. Inset data show levels of the degrading activities compared to those of strain A29 (100%). The values used for calculating the levels are indicated with filled symbols. ND, not determined.

Strain *l*30 degraded both *n*-alkanes and branched alkanes at slower rates than *Alcanivorax* strains. Other strains, T13, 25, MIL-1^T and M4, were unable to significantly degrade branched alkanes. Taxonomically novel strains T13 and 25 degraded *n*-alkanes at slower rates than strain *l*30. The *n*-alkane-degrading activities of *T. oleivorans* MIL-1^T and *Marinobacter* M4 were the lowest. The *n*-alkane-degrading activity of *Marinobacter* strains may be generally low, as another *Marinobacter* strain similarly selected from Indonesian seawater also shows low *n*-alkane-degrading activity (Teramoto et al. 2009).

T. oleivorans has been shown to dominate in *n*-alkane-contaminated temperate seawater microcosms (Yakimov et al. 2005; McKew et al. 2007) and in crude oil-contaminated temperate estuarine seawater microcosms (McKew et al. 2007; Coulon et al. 2007), and is thus recognized as the key degrader. However, the *n*-alkane-degrading activity of *T. oleivorans* MIL-1^T was low and its branched alkane-degrading activity was not significant (Figure 2). Therefore, the petroleum aliphatic hydrocarbon-degrading activity of *Thalassolituus* MIL-1^T, together with *Alcanivorax* SK2^T as a reference, was investigated in seawater diluted with water in order to reduce the salinity (to 2.7% from 3.5%), but was otherwise investigated in the same way as shown in Figure 2. The activity of strain MIL-1^T

was severely inhibited in the diluted seawater, and was much lower than that of *Alcanivorax* SK2^T (data not shown). It is possible that *Thalassolituus* strains will require some compounds produced by surrounding organisms in order to become dominant in an environment. This deserves further investigation.

A. borkumensis isolates A29 and A*l*2 expressed a high level of *n*-alkane- and branched alkane-degrading activities (Figure 2; Teramoto et al., 2009). Introducing these strains A29 and A*l*2 would be beneficial for accelerating the bioremediation of petroleum-contaminated marine environments. This bioaugmentation strategy with strains A29 and A*l*2 may also be advantageous in tropical marine environments especially for the branched alkane degradation. Tropical *Alcanivorax* isolates similarly selected as the possible key degraders from Indonesian seawater (all are types other than *borkumensis*) show lower branched alkane-degrading activity than control strain *A. borkumensis* SK2^T (levels of the branched alkane-degrading activity at 30˚C of the Indonesian *Alcanivorax* isolates are 26–58% compared to that of *A. borkumensis* SK2^T), and *Alcanivorax* strains have been indicated to be key bacteria for branched alkane degradation in tropical seas as well as in temperate seas (Teramoto et al. 2009). Information on the potential for petroleum aliphatic hydrocarbon degradation of the key bacteria in temperate seas that was provided in this study gives insights into the petroleum biodegradation that occurs in temperate marine environments and will promote rational bioremediation strategies.

CONCLUSION

A. borkumensis strains A29 and A*l*2, obtained by flow-through culture, showed the highest *n*-alkane- and branched alkane-degrading activities. Although *Marinobacter* and *Thalassolituus* strains are well-known petroleum aliphatic-hydrocarbon degraders and the *Thalassolituus* strains have dominated in petroleum-contaminated temperate seawater microcosms, their *n*-alkane-degrading activities were the lowest, and their branched-alkane-degrading activities were not significant.

A. borkumensis was suggested to be the key *Alcanivorax* species (the *Alcanivorax* species which dominates in actual environments) in the petroleum-polluted temperate marine environment, while other *Alcanivorax* species have been suggested to dominate in the petroleum-polluted tropical marine environment and show lower branched alkane-degrading activity than *A. borkumensis* (Teramoto et al. 2009). Introducing the *A. borkumensis* strains A29 and A*l*2 may be a useful bioremediation strategy for both tropical and temperate marine environments polluted by petroleum aliphatic hydrocarbons.

ACKNOWLEDGMENTS

We thank Kozue Anzai and Rieko Suzuki for the sequencer operation. This work was supported by a grant (P05032) from the New Energy and Industrial Technology Development Organization (NEDO).

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