Alkane Utilization by *Rhodococcus* Strain NTU-1 Alone and in Its Natural Association with *Bacillus fusiformis* L-1 and *Ochrobactrum* sp.

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Linear (*n*-hexadecane) and branched (pristane) alkanes were degraded by a mixed culture isolated from an oil-contaminated field. The degradation was accompanied by formation of bioflocculles. The culture was composed of *Rhodococcus* strain NTU-1, *Bacillus fusiformis* L-1, and *Ochrobactrum* sp. *Rhodococcus* strain NTU-1 carried out the degradation of the alkane via a hydroxylase. *Bacillus fusiformis* L-1 and *Ochrobactrum* sp. did not degrade the alkanes but aided the flocculation by forming more rigid bacterial aggregates that enhanced the trapping of alkanes. In batch cultures, transformation and removal of the linear and branched alkanes was achieved within 66 h with more than 95% efficiency.

Introduction

Petroleum hydrocarbons are widely used in everyday life and as a result often contaminate soil, groundwater, and marine environments. The problem of environmental contamination with crude oil is exacerbated by the branched alkanes that normally accompany it. Branched alkanes are generally more recalcitrant to biodegradation than linear alkanes (*1*−*3*). Possible strategies for the bioremediation of these polluted environments are the use of hydrocarbon-degrading microorganisms (*4*−*6*). Microbial biodegradation of linear alkanes can start via a hydroxylase by terminal or subterminal oxidation (*7*, *8*). The resulting primary or secondary alcohols then proceed to be metabolized toward the aldehyde, the acid, and the β-oxidation pathway. Microbial degradation of branched isoprenoid alkanes, such as pristane, also goes on to form an acid but before proceeding toward β-oxidation, and the methyl branching point is esterified with coenzyme A. The involvement of racemaces to help the oxidation of the resulting methyl-branched stereoisomers of pristane has been described (*9*).

Bioremediation of sites contaminated with mixed alkanes is often slowed by the low water solubility of the branched hydrocarbons (*10*). Attempts to shorten the time of bioremediation by adding surfactants that increase the solubility of the branched alkanes have been made but yielded mixed results (*11*, *12*). Still, there are microorganisms capable of the uptake and degradation of highly hydrophobic alkanes in reasonable lengths of time. Examples of Gram-positive microorganisms able to biodegrade pristane, a representative branched alkane, are *Brevibacterium* sp., *Corynobacterium* sp., *Nocardia globerula* 432, *Mycobacterium fortuitum*, *Mycobacterium* sp. strain P101, and *Rhodococcus* (*9*). Microorganisms such as *Rhodococcus* sp. produce bioflocculants while they utilize the alkane (*13*, *14*), suggesting a physiological adaptation. For example, *Rhodococcus* sp. strain Q15 utilized *n*-hexadecane more efficiently at low temperatures through the production of biosurfactant or bioflocculant. The bioflocculant consists of assemblies of polypeptides and lipids, especially mycolate-containing glycolipids (*15*, *16*). The bioflocculant apparently allowed the bacteria to access the substrate more easily (*17*). The production of bioflocculles during the utilization of long-chain alkane seems to be a common property of bacteria such as *Rhodococcus equi* that do not produce biosurfactants (*13*).

From a practical viewpoint, the formation of bioflocculles has two advantages for the bioremediation of the environment: (1) it promotes interfacial contact that facilitates the degradation of the alkane by the microorganism, and (2) the entrapping of the alkanes in the bioflocculle makes possible the physical removal of the pollutant for further treatment. In this study, we investigated alkane biodegradation by *Rhodococcus* strain NTU-1 alone and when in its natural association with *Bacillus fusiformis* L-1 and *Ochrobactrum* sp. We also report the effects of the bacterial floccules morphology in the removal of the alkane in batch cultures.

Materials and Methods

Cultures and Growth Conditions. The mixed culture (TN-4) was enriched from soil contaminated with crude oil from Tao-Yuan, Taiwan. The enriched culture was a consortium that formed cell aggregates containing consistently the three strains in the study (see Culture Composition). The bacterial stains were cultivated on mineral salt (MS) medium containing per liter 1.0 g NaCl, 1.0 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1 mL salt solution. The salt solution contained per liter 1.0 g $FeSO_4 \cdot 7H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, and 1.0 g $ZnSO_4 7H_2O$. The pH before sterilization was adjusted to 6.5. The alkane (*n*-hexadecane (C16) or pristane (C19)) was added to MS medium as the sole source of carbon and energy for growth. For the preparation of inocula, the bacterial stocks were maintained at 4 °C in mineral agar supplemented with pristane as the only carbon source and transferred to fresh agar medium monthly. For the degradation experiments, colonies were first picked up from a stock dish and cultivated in nutrient broth medium (0.8%, w/v). Cells were then harvested by centrifugation at 6000 rpm for 10 min after 18 h of cultivation, washed twice with MS medium, and suspended in an equal volume of MS medium to 1.2 OD_{600}. Aliquots were taken to inoculate the biodegradation

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tests (see next section). All chemicals in this study were of analytical grade (≥98% purity). The *n*-hexadecane (C16) and pristane (C19) were obtained from Sigma-Aldrich (U.S.A.). NaCl, K_2HPO_4, and Nutrient Agar were purchased from Merck (Germany), and Nutrient Broth was purchased from Difco (France). All other chemicals were purchased from Riedel-de Haën (Germany). A collection of organic acids/fatty acids were used as carbon and energy sources to test for growth. Erlenmeyer flasks (250 mL) with 100 mL of sterile MS medium were inoculated with 3 mL of culture (1.2 OD_{600}) per flask similarly as for the biodegradation tests (see next section). Concentrations of these acids were between 200 and 500 ppm. The cultures were sampled at 78 h of cultivation, and their optical density (OD_{600}) and pH values determined. The cultures in these conditions did not aggregate.

Biodegradation Test. Erlenmeyer flasks (250 mL) with 100 mL of sterile MS medium were inoculated with 3 mL of culture (1.2 OD_{600}) per flask. To these, 1000 ppmv (volume-to-volume ratio) of each alkane was added as appropriate. The flasks were then incubated at 30 °C on a water bath shaker at 100 rpm. Samples were taken at 20, 30, 42, 54, 66, and 78 h after inoculation.

The cell growth was directly measured by cell dry weight (*5*). The cells and cell aggregates were collected by filtration on a 0.3 μm glass-fiber filter, washed with 30 mL of ethyl acetate, and dried at 110 °C for 24 h. The method was adopted because determination of cell growth by measuring OD_{600} was inaccurate as a result of the formation of cell aggregates. Residual hydrocarbon after the incubation in each flask was recovered by extracting the culture medium with 30 mL of ethyl acetate. To determine the efficiency of the extractions, *n*-dodecane (100 μL) was added to each flask as an internal standard. Bacterial aggregates, when present, were separated through a stainless steel sieve (60 mesh) and allowed to collect nearly all bacterial pellets from the culture medium. The medium and cell aggregates were extracted separately with ethyl acetate. The ethyl acetate phase was collected after 2 h of extraction. Residual hydrocarbon was determined in a Perkin-Elmer autosystem gas chromatograph equipped with a flame ionization detector (*17*). A 30 m fused silica capillary column (i.d. 0.53 mm, film thickness 0.25 μm) with a temperature program of 200−230 °C at 10 °C·min^{-1} was used. The alkanes were identified and quantified by comparison to standards.

PCR Reactions, Northern Hybridizations, and 16S rDNA Analysis. Nucleic acid manipulations and Northern analyses were performed using standard protocols (*18*). Polymerase chain reactions (PCR) were performed on a Cycler system (BIO-RAD Hercules, CA) following the protocols and software of the manufacturer. The nucleotide sequence of the 16S rDNA gene was determined directly in the PCR amplified products obtained from the mixed culture and from the isolated strains at the Food Industry Research and Development Institute (Taiwan). PCR primers for the 16S rRNA gene were 8f and 1492R (*19*). Degenerate primers for the hydroxylase gene of alkane degraders were Ts2S and Deg1RE (*20*) or LK3F and ALK-3R (*21*). The former set of primers amplifies most hydroxylase genes, and the later is specific for the alkane hydroxylase genes of *Rhodococcus* sp. 1BN. Either of the two amplified DNA fragment were used to test for the expression of the *alkB* using Northern hybridizations. The partial nucleotide sequence of the *alkB* in *Rhodococcus* strain NTU-1 has the GenBank accession number DQ173199.

Results

Culture Composition. The isolated culture (TN-4) from crude-oil-contaminated fields was able to grow on plates and liquid batch cultures using the branched alkane pristane as its sole source of carbon and energy. Because of the recalcitrant nature of branched alkanes to biodegradation, we decided to further characterize this culture. Spreading culture TN-4 on nutrient-agar plates revealed at least three cell morphologies, suggesting more than one microorganism in TN-4. We proceeded to sequence the PCR amplified 16S rDNA genes of the microorganisms in culture TN-4 to determine their identity. Database comparisons (using BLAST) of the 16S-rDNA amplified sequences revealed that culture TN-4 was composed of three microorganisms similar to *Rhodococcus* sp. (99% sequence similarity), *Bacillus fusiformis* L-1 (98% similarity), and *Ochrobactrum* sp. (98% similarity). *Rhodococcus* sp. is described as an aerobic Gram-positive, nonmotile, rod-shaped bacterium possessing mycolic acid in its cell wall. *Bacillus fusiformis* L-1 is described as an aerobic Gram-positive, motile, oxidase-positive, and endospore-forming bacterium. *Ochrobactrum* is described as an aerobic Gram-negative bacterium that possesses flagellum and is oxidase-positive. Attempts made to enrich *Rhodococcus* strain NTU-1 in the mixed culture TN-4 using pristane for selection were not successful. Apparently the microorganisms derive some benefits in the TN-4 mixed culture that favors their association.

The 16S rDNA sequence of the *Rhodococcus* strain matched that of *Rhodococcus erythropolis*. *Rhodococcus* isolates are known to be capable of oxidizing alkanes using alkane-1-hydroxylase (*17*, *22*−*24*). The *Rhodococcus* strain of this study was able to metabolize C14 (tetradecane), C16 (hexadecane), and C18 (octadecane) *n*-alkanes and the branched alkane pristane (only C16 and pristane are reported here). The *B. fusiformis* L-1 and *Ochrobactrum* sp. strains were not capable of utilizing the alkanes for growth. PCR amplification using genomic DNA from the *Rhodococcus* strain of this work and degenerate primers for the loci of *alkB* genes in other alkane-degrading organisms yielded a DNA product. The nucleotide sequence of the amplified product was 99% similar to the gene of alkane-1-hydroxylase of *Pseudomonas frederiksbergensis*, *R. erythropolis* 35-O, and *Rhodococcus* sp. Q15. Because the partial sequence of the gene for *alkB* was not identical to the *alkB* genes in other bacteria and because of the similarity of its 16S rDNA nucleotide sequence to other *Rhodococcus*, the isolate in this study was named *Rhodococcus* strain NTU-1.

To ensure that the degradation of the alkane took place by the *alkB* in *Rhodococcus* strain NTU-1, we tested for its expression using Northern hybridizations and the probe for *alkB* (Figure 1). Only cultures of *Rhodococcus* NTU-1growing on the alkanes expressed *alkB*, demonstrating that its alkane hydroxylase carries out the degradation of the hydrocarbons in the mixed culture. Cells of *Bacillus fusiformis* and *Ochrobactrum* did not grow on the alkanes, suggesting that they did not possess an alkane hydroxylase. However, as reported below, the two accompanying strains influence the utilization of the alkane.

Alkane Utilization. To determine whether there was interdependence among the strains in culture TN-4, the utilization of alkanes in *Rhodococcus* strain NTU-1 alone and in association with *B. fusiformis* L-1 and *Ochrobactrum* sp. (culture TN-4) was monitored in batch cultures. The linear alkane hexadecane C16 (Figure 2) and branched alkane pristane (Figure 3) were used as representative for the alkane substrates. Because the biofloccules entrapped a significant amount of hydrocarbon, the

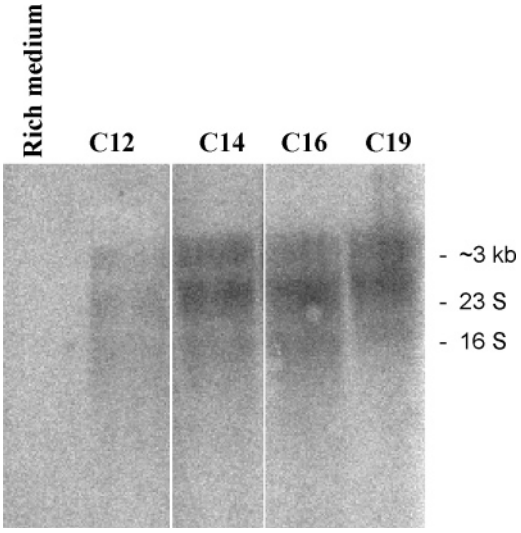

Figure 1. Expression of the hydroxylase of *Rhodococcus* strain NTU-1 when grown on rich medium (Luria Bertani broth), C12 (dodecane), C14 (tetradecane), C16 (hexadecane), and C19 (pristane). The probe used was the PCR amplified product of *alkB* generated with the primers described in the text and was hybridized to total RNA extracted from cells at stationary phase.

reduction of hydrocarbon in the solution should attribute to both biodegradation and entrapment in the pellets. (Figures 2B, 3B and Table 1) When grown on C16, *Rhodococcus* strain NTU-1 entered the stationary growth phase in about 40 h of incubation, and culture TN-4 entered the stationary phase in about 50 h (Figure 2A). Bioflocculation, or aggregation, occurred between 30 and 50 h for TN-4 culture and *Rhodococcus* strain NTU-1, respectively. After the appearance of biofloccules, progressive biodegradation was noted as trapping of alkane continued (see Table 1). At 66 h, the remaining hydrocarbon in solution was approximately 5% (e.g., pristane by NTU-1) or less (other cases). That is, more than 95% of 1000 ppmv (volume-to-volume ratio) were removed by biodegradation and entrapment in a relative short period (Table 1). Biodegradation was always accompanied with a significant drop in pH (Figures 2A and 3A), suggesting an accumulation of acid(s) in the medium. The pH of *Rhodococcus* strain NTU-1 medium dropped from 6.5 to 4.3 while the pH of the TN-4 culture fell from 6.5 to 5.4. The more marked drop of pH in the pure culture (NTU-1) suggested that *Bacillus fusiformis* L-1 and *Ochrobactrum* utilized the byproducts (acids) from the alkane degradation in the TN-4 mixed culture. The higher cell density in TN-4 culture can be attributed to the additional growth of *B. fusiformis* L-1 and *Ochrobactrum* on the byproducts. Table 2 summarized the growth on organic acids/fatty acids by these three organisms. *Ochrobactrum* sp. grew on some organic acid/fatty acids (Table 2) and caused pH change during the cultivation (Figure 4), which suggests the utilization of byproducts of the alkane degradation in the mixed culture TN-4. *B. fusiformi* L-1 seemed not to utilize these acids tested. It is likely that some other byproducts (from NTU-1 or *Ochrobactrum* sp.) supported the growth of *Bacillus*.

Aggregation and Appearance of Cell Floccules. When degrading alkanes, *Rhodococcus* strain NTU-1 alone and when mixed in culture TN-4 tended to bioflocculate and form cell aggregates. The flocculation phenomenon trapped the *n*-alkanes and pristane (Figure 5). *Rhodococcus* strain NTU-1 formed aggregates after approximately 50 h with hexadecane and after 63 h with pristane. When in the mixed culture TN-4, the bacterial aggregates were formed after 30 and at 40 h, respectively (Figures 2 and 3). The appearance of the aggregates (Figure 5) in both pure and mixed cultures was roughly similar at low alkane concentrations. The aggregation was in the form of yellowish rod-shaped compact clumps. At higher alkane concentration, the appearance changed and differed for the pure culture and the mixed culture. Aggregates formed by NTU-1 were white and loose. Aggregates formed by culture TN-4 were cream in color, round (0.1−2 cm in diameter), and relatively firmer and contained roughly 26−47% *Rhodococcus* strain NTU-1, 3−5% of *Bacillus fusiformis* L-1, and 50−69% of *Ochrobactrum* in terms of cell numbers (by plate count).

Alkane Removal. The occurrence of bacterial aggregates during cultivation facilitated the physical removal of residual alkane in the medium (Figures 2 and 3). The aggregates were separated from the medium, and the amounts of trapped alkane were determined (Table 1). Up to 95% of the alkane was removed (Table 1). The two nonflocculating bacteria, *B. fusiformis* L-1 and *Ochrobactrum* sp., contributed to bacterial pellet formation via co-aggregation with the flocculating *Rhodococcus* strain NTU-1. Hence, culture TN-4 tends to perform better in the removal of the alkanes (biodegraded and trapped) than *Rhodococcus* strain NTU-1 alone.

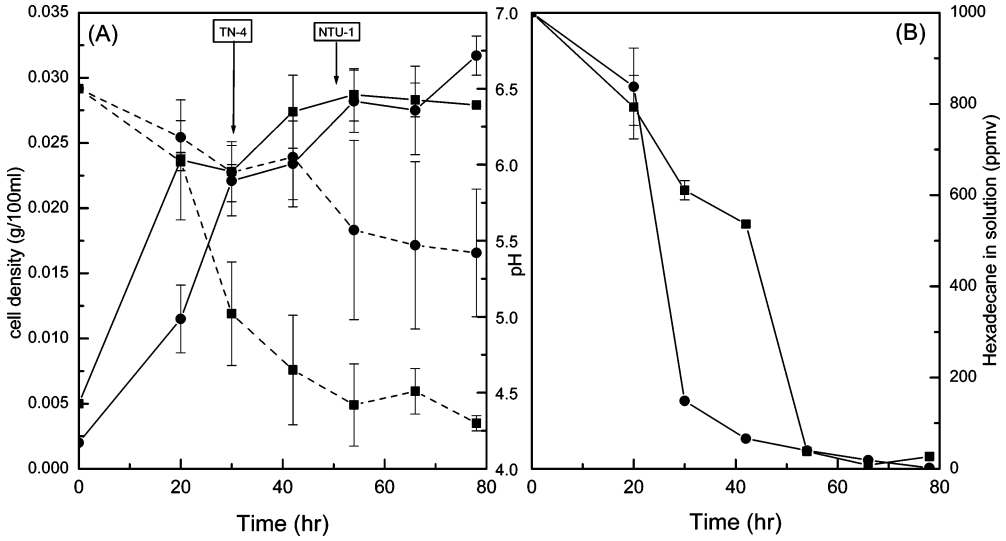

Figure 2. (A) Growth (—) and pH (- - -); (B) *n*-hexadecane (excluding the trapped amount when biofloccules formed) in cultures of *Rhodococcus* strain NTU-1 (■) and culture TN-4 (●). Initial concentration of *n*-hexadecane was 1000 ppmv with 3−6 duplicate experiments. Boxes with arrows (A) indicate when the formation of bacterial floccule occurred. Initial pH 6.5, 30 °C, 100 rpm.

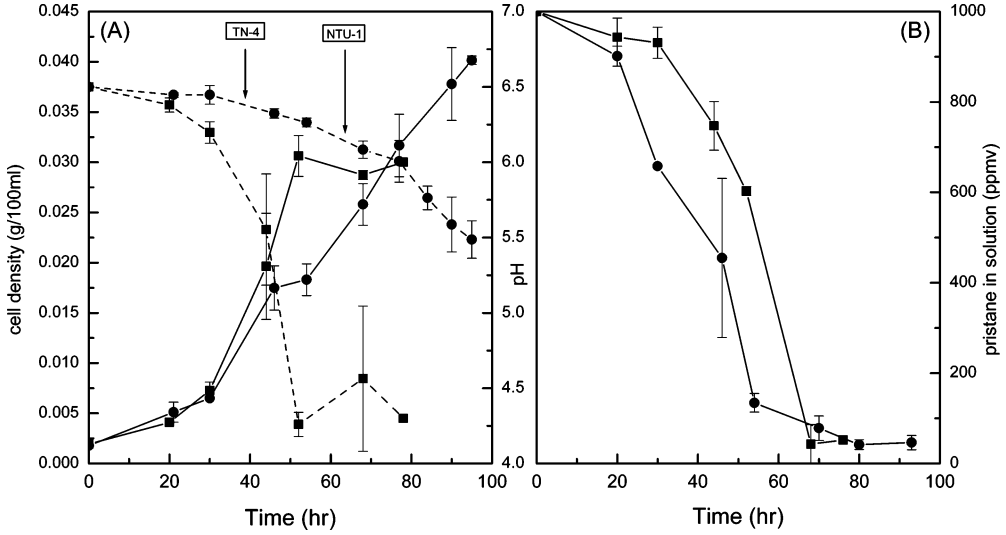

Figure 3. (A) Growth (—) and pH (- - -); (B) pristane (excluding the trapped amount when biofloccules formed) in cultures of *Rhodococcus* strain NTU-1(■) and culture TN-4(●). Initial concentration of pristane was 1000 ppmv with 3−6 duplicate experiments. Boxes with arrows (A) indicate when the formation of bacterial floccules occurred. Initial pH 6.5, 30 °C, 100 rpm.

Table 1. Alkane Distribution in Cultures of *Rhodococcus* Strain NTU-1 Alone and in the TN-4 Mixed Culture[a]

	hexadecane (ppmv)			pristane (ppmv)		
time (h)	in solution	bio-degraded	trapped	in solution	bio-degraded	trapped
			NTU-1			
0	1000			1000		
20	793	207		943	57	
30	611	389		931	69	
42	536	464		747	253	
54	**38**	**530**	**432**	603	397	
66	**8**	**570**	**422**	**43**	**418**	**539**
78	**27**	**620**	**353**	**52**	**419**	**530**
			TN-4			
0	1000			1000		
20	838	162		932	68	
30	**149**	**340**	**511**	**658**	**95**	**247**
42	**66**	**410**	**524**	**108**	**370**	**522**
54	**40**	**450**	**510**	**105**	**462**	**433**
66	**19**	**492**	**489**	**24**	**551**	**425**
78	**2**	**629**	**370**	**22**	**687**	**291**

[a] Bold numbers indicate the cells are flocculated. Initial pH 6.5, 1000 ppmv, 30 °C, 100 rpm.

Table 2. Growth of NTU-1, *Bacillus fusiformis* L-1, and *Ochrobactrum* sp. on Selected Organic Acids/Fatty Acids[a]

	NTU-1	*B. fusiformis* L-1	*Ochrobactrum*
formic acid			
acetic acid	0.204		
propionic acid	0.260		
butyric acid	0.369		
valeric acid	0.416		
hexanoic acid	0.446		
heptanoic acid			
octanoic acid			
nonanoic acid			
dodecanoic acid	0.130		0.413
tridecanoic acid	0.136		0.650
myristic acid	0.313		0.20
palmitic acid	0.188		0.136
oxalic acid			
L-(+)-tartaric acid			
succinic acid	0.199		0.211
citric acid	0.204		0.230

[a] Blank entries indicate that there was no obvious growth. Initial OD ≒ 0.076 (blank).

Discussion

Rhodococcus strain NTU-1, when in association with *Bacillus fusiformis* L-1 and *Ochrobactrum* sp., performed better than when in pure culture for the removal of linear and branched alkanes (Table 1). There are reports of mixed cultures of indigenous uncharacterized hydrocarbon-degrading bacteria being used to accelerate the bioremediation of oil-contaminated sites (*25*−*27*). However, in those studies the cultures were derived from consortia and it was not clear whether the accompanying bacteria that were incapable of alkane utilization played a role in the enhancing of the utilization of the alkanes. The interaction between two alkane-degrading microorganisms, *Pseudomonas aeruginosa* K1 and *Rhodococcus equi* P1, has been documented as well (*28*). Seeding endogenous soil populations with *Rhodococcus equi* P1 improved the biodegradation of hydrocarbons in contaminated soil. On the other hand, seeding with *Pseudomonas aeruginosa* K1 delayed the bioremediation of gasoline-contaminated soil (*28*). However, when used together and when hexadecane was sufficient, the strains were able to co-oxidize other linear and complex hydrocarbons (*28*). Although the amounts of alkane degraded changed by seeding these strains in contaminated soil, it was not evident whether there was a role for the endogenous soil bacteria incapable of degrading alkanes. There have been few evaluations of the effects of seeding alkane-degrading bacteria from a pure culture and from a mixed culture with bacteria incapable of degrading hydrocarbons. Our work is an example of an association between hydrocarbon-degrading bacterium with bacteria incapable of degrading hydrocarbons that can be used efficiently for the removal of alkanes.

Bacterial flocculation in oil-contaminated fields could be used to help remediate a spill and, perhaps, to facilitate the physical removal of the hydrocarbon for further treatment. *Rhodococcus equi* tended to flocculate while metabolizing long-chain *n*-alkanes, probably an adaptation for the utilization of hydrophobic substrates (*13*). Not surprisingly, *Rhodococcus* strain NTU-1 was capable of producing floccules when grown in *n*-alkanes and branched alkanes. Environmental factors such as substrate gradients, chemical or physical stress, and predation are known to trigger bacterial aggregation (*29*, *30*). Moreover, specific cell−cell interactions that involved protein interaction and cell-surface hydrophobicity also contributed to bioflocculation (*8*). For example, *Saccharomyces cerevisiae* had a higher

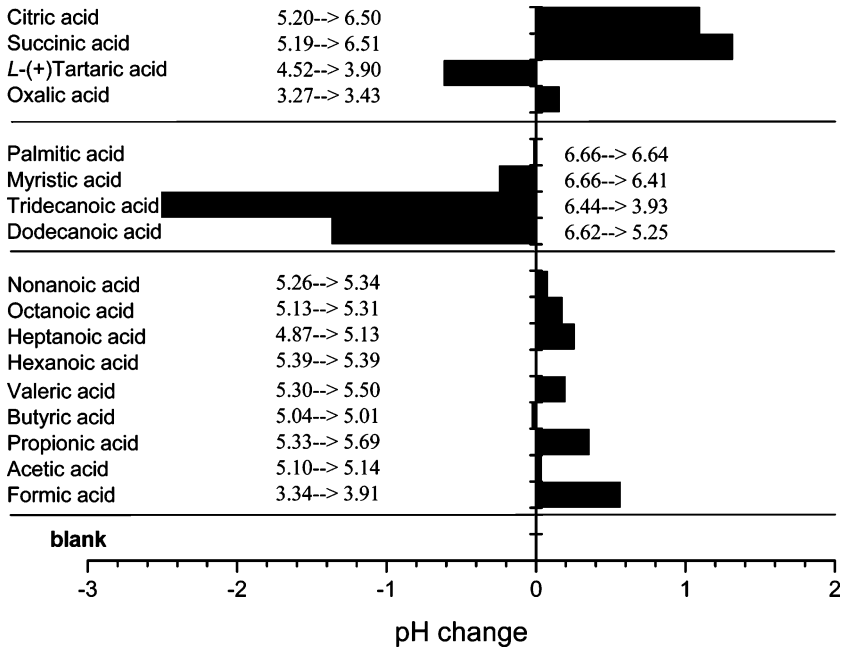

Figure 4. Change in pH of the *Ochrobactrum* sp. spent medium with the carbon source indicated.

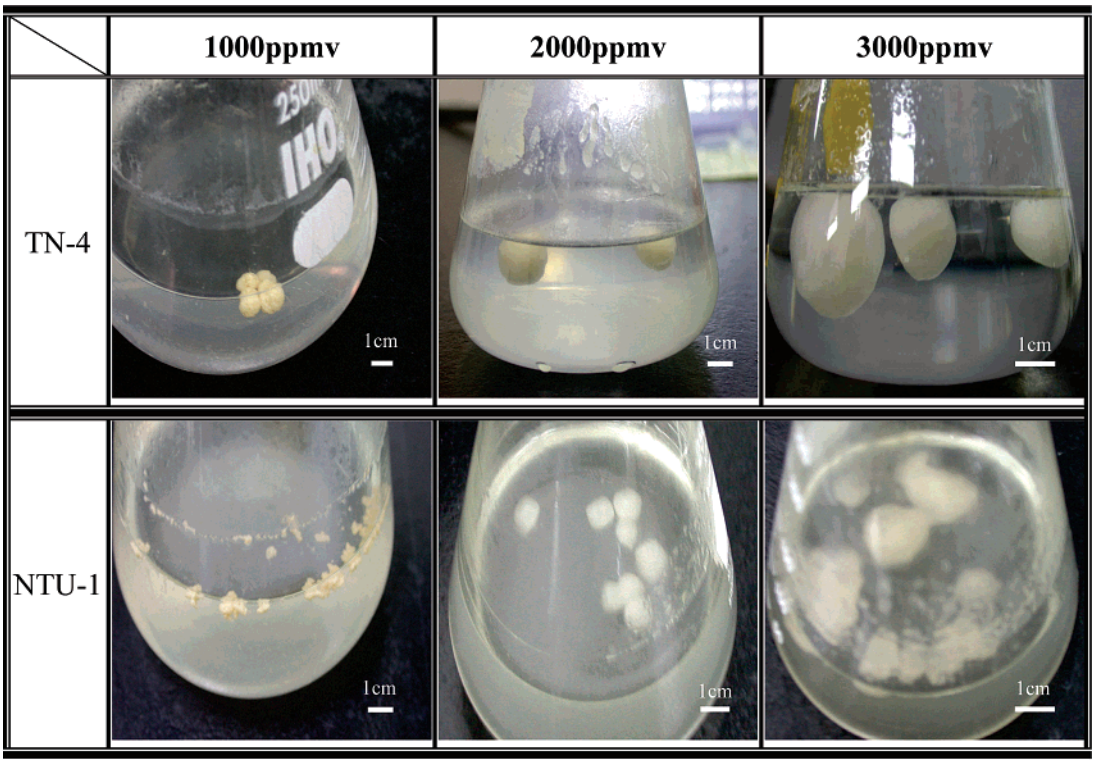

Figure 5. Appearance of floccules in cultures of NTU-1 and TN-4 at three concentrations of pristane. Initial pH 6.5, 30 °C, 100 rpm.

flocculation tendency during the stationary phase than during exponential phase and death phase (*31*). In our study, once bioflocculation of NTU-1 and TN-4 was observed, the cell mass and remnant alkane were separated with relative ease by filtration or centrifugation. This separation was more efficient in the aggregates of the mixed culture TN-4 than in the pure culture of *Rhodococcus* strain NTU-1 facilitated by the larger size of the floccules (Figure 5). The alkane degradation may continue in the aggregates since there is evidence that cells are capable of alkane degradation in the interface of the floccules (*13*). The bacteria that associate with these cultures and are incapable of alkane utilization may derive a benefit by utilizing the byproducts of the degradation. In our study, after incubation for 78 h, the culture medium of NTU-1 was rather acidic (close to pH 4; Figures 2A and 3A). A less marked decrease in the pH in the mixed culture TN-4 was observed. This suggests that *Bacillus fusiformis* L-1 and *Ochrobactrum* possibly consumed the acidic products produced during the biodegradation process. However, the growth of *Rhodococcus* strain NTU-1 was hindered at this low pH in the batch cultures because the remaining substrate in the medium was not utilized. Given that efficiencies of up to 95% removal were achieved in the batch cultures, transferring the floccules to fresh medium may remove the remaining 5% of the alkane. This approach offers a promising bioremediation strategy.

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