Extremophiles (2006) 10:347–356
DOI 10.1007/s00792-006-0505-4

ORIGINAL PAPER

Lei Wang · Yun Tang · Shuo Wang · Ru-Lin Liu
Mu-Zhi Liu · Yan Zhang · Feng-Lai Liang · Lu Feng

Isolation and characterization of a novel thermophilic *Bacillus* strain degrading long-chain *n*-alkanes

Received: 18 August 2005 / Accepted: 12 December 2005 / Published online: 8 April 2006

Abstract A thermophilic *Bacillus* strain NG80-2 growing within the temperature range of 45–73°C (optimum at 65°C) was isolated from a deep subterranean oil-reservoir in northern China. The strain was able to utilize crude oil and liquid paraffin as the sole carbon sources for growth, and the growth with crude oil was accompanied by the production of an unknown emulsifying agent. Further examination showed that NG80-2 degraded and utilized only long-chain (C15–C36) *n*-alkanes, but not short-chain (C8–C14) *n*-alkanes and those longer than C40. Based on phenotypic and phylogenic analyses, NG80-2 was identified as *Geobacillus thermodenitrificans*. The strain NG80-2 may be potentially used for oily-waste treatment at elevated temperature, a condition which greatly accelerates the biodegradation rate, and for microbial enhancing oil recovery process.

Communicated by G. Antranikian

Lei Wang, Yun Tang and Shuo Wang contributed equally to this study.

L. Wang · Y. Tang · M.-Z. Liu · L. Feng (✉)
TEDA School of Biological Sciences and Biotechnology,
Nankai University, 23# Hong Da Street, TEDA,
300457 Tianjin, People's Republic of China
E-mail: fenglu63@nankai.edu.cn
Tel.: +86-22-66229592
Fax: +86-22-66229596

L. Wang · L. Feng
Tianjin Key Centre for Functional Genomics and
Biochip Research, TEDA College, Nankai University,
23# Hong Da Street, TEDA, 300457 Tianjin,
People's Republic of China

S. Wang · Y. Zhang
Tianjin Key Laboratory of Food Nutrition and Safety,
Faculty of Food Engineering and Biotechnology,
Tianjin University of Science and Technology,
300222 Tianjin, People's Republic of China

R.-L. Liu · F.-L. Liang
College of Life Sciences, Nankai University,
300071 Tianjin, People's Republic of China

Keywords Long-chain *n*-alkanes · Degradation · *Geobacillus thermodenitrificans* · Thermophile · Characterization

Introduction

The ability to utilize saturated hydrocarbons (*n*-alkanes) is widely distributed among diverse microbial populations (Atlas 1981; Leahy and Colwell 1990). As *n*-alkanes are major constituents of most crude oils, these microbes play important roles in bioremediation of crude oil contamination. Several biological technologies using either natural or specialized microorganisms have been developed for the cleanup of oily sludges and oil-contaminated soils (van Hamme et al. 2003).

n-Alkane-degrading bacteria are readily isolated from oil-contaminated sites, and most reported are mesophilic strains belonging to different bacterial species such as *Acinetobacter* sp. M1 (Sakai et al. 1994), *Rhodococcus* sp. (van Hamme and Ward 2001), *Nocardia* sp. CF8 (Hamamura and Arp 2000), *Planococcus alkanoclasticus* (Engelhardt et al. 2001), and *Pseudomonas putida* (formerly *P. oleovorans*) (van Beilen et al. 1994). These bacterial strains show the ability to degrade *n*-alkanes of various chain lengths from C2 to C44. Only a limited number of thermophilic *n*-alkane-degrading bacterial strains have been reported, including a strain of *Bacillus* (*Geobacillus*) *stearothermophilus* isolated from oil-contaminated Kuwaiti desert capable of growing on C15–C17 (Sorkhoh et al. 1993), and two strains of *G. jurassicus* isolated from a high-temperature petroleum reservoir capable of growing on C6–C16 (Nazina et al. 2005). A *Bacillus thermoleovorans* strain isolated from deep subterranean petroleum reservoirs was shown to be able to degrade *n*-alkanes up to C23 at 70°C (Kato et al. 2001), and a *Thermus brockii* strain was found to degrade hexadecane at 70°C (Feitkenhauer et al. 2003). The most common pathway for *n*-alkane

degradation is via terminal oxidation, in which *n*-alkanes are first oxidized to the corresponding primary alcohol, which is further oxidized by alcohol and aldehyde dehydrogenases, respectively (Berthe-Corti and Fetzner 2002). The terminal oxidation pathway for *n*-alkane degradation is encoded by the OCT plasmid in *P. putida* Gpo1 (van Beilen et al. 1994), and a membrane-bound *n*-alkane hydroxylase system containing an integral membrane monooxygenase encoded by *alkB*, a rubredoxin and a rubredoxin reductase, was responsible for the first step of the pathway (van Beilen et al. 2001). The *alkB* homologs have been reported in many *n*-alkane-degrading bacterial strains, and some have been functionally examined (van Beilen et al. 2003).

One of the major factors limiting the degradation of hydrocarbons such as *n*-alkanes is their low availability to the cells. Microorganisms employ several strategies to enhance availability of those hydrophobic pollutants, such as biofilm formation and biosurfactant production (Bognolo 1999; Christofi and Ivshina 2002; Johnsen et al. 2005). On the other hand, bioavailability may also be enhanced by elevating the temperature, a condition favorable for the growth of thermophilic microorganisms, during hydrocarbon degradation (Margesin and Schinner 2001). Feitkenhauer et al. (2003) summarized several advantages by using thermophilic microorganisms for bioremediation of hydrocarbons over mesophilic organisms. Briefly, elevated temperature can increase the solubility of hydrophobic pollutants, decrease their viscosity, enhance their diffusion, and transfer long-chain *n*-alkanes from solid phase to liquid phase.

In this study, we report the isolation of a thermophilic bacterial strain NG80-2 from the deep subterranean oil-reservoir of Dagang oil field in northern China, which was able to grow on crude oil as the sole carbon source at 73°C. Further studies showed that the isolate grew on and degraded *n*-alkanes ranging from C15 to C36, but not those less than C14. Based on phenotypic and phylogenic analysis, the isolate was identified as *Bacillus thermodenitrificans*. To our knowledge, this is the first report showing a thermophilic bacterial strain degrading long-chain *n*-alkanes up to at least C36 and utilizing them as the sole carbon sources.

Materials and methods

Chemicals

Crude oil was obtained from Dagang oil field, 69-8, in northern China. Liquid paraffin was purchased from Tianjin Science and Technology Co. Ltd. The detergent Plysurf A-210G was kindly provided by Daiichi Kogyo Seiyagu Co. in Japan. All chemicals used in this study were of analytical grade. *n*-Alkanes with different chain lengths, pristane and squalane, were purchased from Sigma or Fluka.

Media and growth conditions

Luria Bertani (LB) medium was used for the isolation of single colonies from samples and optimal growth tests of NG80-2. The mineral medium containing 0.6 g Na_2HPO_4, 0.2 g KH_2PO_4, 4 g $NaNO_3$, 0.01 g $CaCl_2$, 0.01 g $FeSO_4$, 0.3 g $MgSO_4$, 0.1 g yeast extract per liter, pH 7.2, and minimal medium (medium A) (Mutzel et al. 1996) with and without the addition of yeast extract (0.01% w/v) were used in other experiments. Crude oil, liquid paraffin, and *n*-alkanes were added into the media as indicated. NG80-2 was cultivated with shaking at 180 rpm on an orbital shaker (Thermo Electron Co.) at 60°C–73°C as indicated.

Isolation of bacteria

Samples were taken from formation water of the deep subterranean oil-reservoir, Guan 69-8, in Dagang oil field, northern China. The temperature of the wells was 73°C and the depth was estimated to be 2,000 m. The samples were incubated at 73°C for 10 days and were used to streak LB agar plates for single colonies. Pure cultures from single colonies were first cultivated in LB broth overnight and 5-ml of the fully-grown culture was then transferred into 100-ml of the mineral medium supplemented with liquid paraffin (2%, w/v) in a sealed flask. After 7 days' incubation at 73°C, 5% of the culture was transferred into the same medium supplemented with crude oil (2%, w/v) and incubated for 28 days. Emulsification activity of the culture was examined visually for reduced surface adherence and formation of homologous solution after shaking. Degradation of crude oil was estimated by measuring absorbance at A_{250} using a DU 640 spectrophotometer (Beckman) after extraction of residual oil in individual flasks with 100-ml of hexane. This method is based on the presence of UV-absorbing compounds such as aromatics in crude oil, and a decrease of A_{250} indicates the degradation of crude oil.

Phenotypic characterization of strain NG80-2

The following characteristics were determined according to the "Bergey's Manual of Determinative Bacteriology, 9th edition" (Holt et al. 1994): the Gram stain, motility, starch, casein and gelatin hydrolysis, indole, catalase and urase production, and reduction of nitrate. Growth and acidification of carbohydrates tests were performed using API 50 CHB/E test strips (BioMérieux, Marcyl' étoile, France) according to the manufacturer's instruction. Temperature range for growth was tested by incubation of cells at 37–80°C in LB for 3 days; samples were taken at different intervals and decimal dilutions were made to obtain a number of colony forming units (CFU), and growth rate was assessed by calculating doubling time based on the viable

counts. Salinity tolerance was tested by incubation of bacterial cells in LB supplemented with 0–10% (w/v) NaCl, and growth was monitored by measuring absorbance at A_{600}.

Phylogenic characterization of strain NG80-2

Genomic DNA was extracted from LB cultures using the method described previously (Bastin and Reeves 1995) except that double amount of lysozyme was used and sequenced using a conventional whole genome shotgun strategy (Fleischmann et al. 1995) with an ABI 3730 automated DNA sequencer (ABI, Foster City, USA). Genome annotation was performed by comparing protein sequences with those in the NCBI protein database (http://www.ncbi.nlm.nih.gov). Sequences of 16s rRNA, *araA* (coding for arabinose isomerase), *mdh* (coding for malate dehydrogenase), and *recZ* (coding for DNA repair and genetic recombination protein) genes were retrieved from the genome sequence database of NG80-2 (separate study). Phylogenic trees were constructed by alignment of 16s rDNA, *araA*, *mdh,* and *recZ* with sequences of related genes from GenBank, respectively, using the program MEGA3 (Kumar et al. 2004).

Degradation experiments

To test the ability of NG80-2 to grow on crude oil and liquid paraffin as the sole carbon sources, cells from 10-ml overnight LB culture were washed twice with sterile saline and were used to inoculate 100-ml of medium A without yeast extract and supplemented with 2% (w/v) crude oil or liquid paraffin as the sole carbon sources in 300-ml Erlenmeyer flasks. The flasks were sealed with rubber stoppers to minimize evaporation and incubated at 60°C for 22 days. Individual flasks were taken at different intervals and decimal dilutions were used to obtain number counts of CFU on LB agar plates.

To test the ability of NG80-2 to degrade long-chain *n*-alkanes, 10-ml of overnight LB culture was inoculated into 100-ml of the mineral medium supplemented with 0.007% (w/v) each of *n*-pentacosane (C25), *n*-pentatriacontane (C35), and *n*-hexatetracontane (C46) in 300-ml Erlenmeyer flasks. The flasks were sealed with rubber stoppers and incubated at 65°C for 21 days. A set of uninoculated flasks served as control and was incubated in parallel. Individual flasks were taken at different intervals and residual *n*-alkanes were analyzed by gas chromatography (GC).

To test the range of *n*-alkanes degraded by NG80-2, cells from overnight LB culture were washed twice with sterile saline and were used to inoculate 30-ml of medium A without yeast extract (for growth test) or with yeast extract (for degradation test) in 100-ml culture bottles supplemented with 1% (w/v) individual *n*-alkanes (ranging from C8 to C44; see Table 2 for the full list) as the sole carbon source to an initial OD_{600} of 0.05. For *n*-alkanes ranging from C22 to C44, 0.001% (w/v) of plysurf A-210G was added into the medium as dispersant. For the degradation test, an additional set of uninoculated bottles served as controls and was incubated in parallel. The bottles were sealed with rubber stoppers and were incubated at 60°C for 10 days. Growth of cells was assessed by measuring absorbance at A_{600} after the removal of residual *n*-alkanes from the medium, which otherwise interfere with the reading, by precipitation method as described (Witholt 1972). Residual *n*-alkanes in individual bottles were analyzed by GC.

GC analysis

Residual *n*-alkanes were extracted using a continuous liquid–liquid extraction technique with a self-designed extractor made by Tianjin Glassware Service Factory. The total contents of the culture flask or culture bottle were transferred to the liquid–liquid extractor. The interior of the flask was rinsed with hexane and the washings poured into the central well of the extractor allowing the solvent to rise slowly up through the sample and overflow into the round bottom reflux flask. The sample was extracted for 24 h and the extract was adjusted to the same volume as that of the original culture, supplemented with 0.006% (w/v) of squalane as internal standard. Finally, it was analyzed by GC for *n*-alkanes less than C36 and high-temperature capillary gas chromatography (HTGC) for *n*-alkanes longer than C40. Extraction recovery rate was calculated by comparing the concentration of an alkane before and after extraction, and was $92.2 \pm 4.22\%$ for C25, $93.7 \pm 3.12\%$ for C35, and $83.5 \pm 4.01\%$ for C46.

GC analysis was performed using an Agilent Technologies 6820N gas chromatograph equipped with an on-column injection, FID detector, and SPB^{TM}-5 capillary column (30 m×0.53 mm i.d., 1.5 μm thickness) (Supelco). Nitrogen was used as a carrier and set at a constant flow rate of 35 ml/min. Oven temperature was set at 150°C for 5 min and then programmed from 150 to 280°C at 15°C/min. Injector and detector temperature was 280 and 350°C, respectively. HTGC was performed using an Agilent Technologies 6890N gas chromatograph equipped with an on-column injection, FID detector, and CP-SimDist Ultimental column (10 m×0.53 mm i.d., 0.17 μm film thickness) (Varian). Nitrogen was used as a carrier, set at a constant flow rate of 2 ml/min, and make-up flow rate was 30 ml/min. Oven temperature was first programmed from 130 to 250°C at 5°C/min, further rising to 380°C with a programmed rate of 4°C/min; injector temperature was from 150 to 390°C within 24 min, and detector temperature was 390°C.

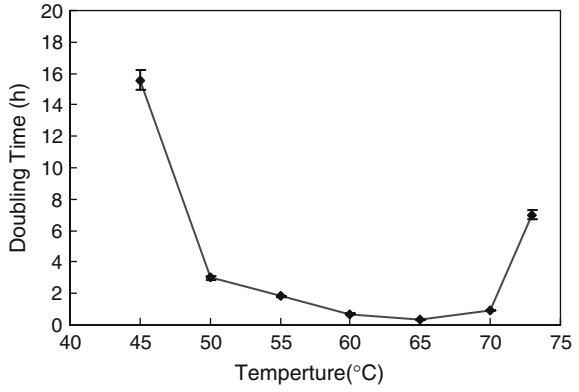

Fig. 1 Growth rates of NG80-2 at different temperatures. Cells were grown in 100-ml of LB in 500-ml Erlenmeyer flasks for 3 days on a rotary shaker (180 rpm) at the temperature indicated. Samples were taken from different intervals, and growth rate was indicated by doubling time calculated based on viable cell counts. Data are the means of duplicate samples

Results

Isolation of thermophilic *n*-alkane-degrading strain NG80-2

A total of 36 strains were isolated from the formation water of deep subterranean oil-reservoirs in Dagang oil field of north China, 14 of which showed good ability to emulsify crude oil by visual observation for reduced surface adherence and formation of homologous solution after shaking. Three strains including NG80-2, which showed highest emulsifying activities, were further examined for their ability to degrade crude oil. After incubation in the mineral medium supplemented with 2% crude oil at 73°C for 7 days, NG80-2 appeared to be the best degrader and degraded 42% of the crude oil as estimated by measuring absorbance at A_{250} spectrometrically. Strain NG80-2 was further characterized and assessed for its ability to degrade *n*-alkanes.

Characterization of strain NG80-2

Cells of strain NG80-2 stained Gram-positive and presented as straight rods, with peripheral flagellum, motile, and 0.6–1.0×3.1–5.5 μm in size. An ellipsoidal spore was located terminally or subterminally within a swollen sporulating cell. It did not require NaCl for growth, and the growth was inhibited by 5% (w/v) NaCl. NG80-2 showed noticeable growth in the temperature range between 45 and 73°C, and the optimum growth was obtained at 65°C (Fig. 1). Growth and acidification of carbohydrates by NG80-2 as determined by using API 50 CHB/E test strips showed that NG80-2 exhibited all the biochemical characteristics of *Geobacillus thermodenitrificans* strains described by Manachini et al. (2000) (Table 1). The colonies of strain NG80-2 were flat with fimbriate margin and had rather dry appearance, which were traits shared by *G. thermodenitrificans* strains (Manachini et al. 2000).

Phylogenic analysis of 16S rDNA, and the genes *araA*, *mdh,* and *recN* were carried out. Phylogenic analysis of the 16S rDNA placed strain NG80-2 firmly into the species of *G. thermodenitrificans* (Fig. 2a). When compared with 16S rDNA sequences of 40 *G. thermodenitrificans* strains available from the Genbank, the 16S rDNA of strain NG80-2 was found to share 99.931, 99.862, 99.794, 99.725, 99.656, and 99.587% identity with 7, 10, 9, 6, 4, and 1 of the *G. thermodenitrificans* strains, respectively. Only 3 of 40 *G. thermodenitrificans* strains shared with NG80-2 identities the same as or lower than NG80-2 shared with *G. uzenensis* BGSC92A2, *G. kaustophilus* BGSC W9A78 and *G. subterraneus* BGSC91A2 (99.312%), the next nearest relatives of NG80-2 in the phylogenic tree. Comparison of genes *araA*, *recN,* and *mdh* also placed strain NG80-2 into species of *G. thermodenitrificans* (Fig. 2b, c, d). The sequences of 16S rDNA, *araA*, *mdh,* and *recN* have been submitted to GenBank under accession numbers DQ243788, DQ243786, DQ243785, and DQ243787, respectively.

Table 1 Selected phenotypic characteristics of NG80-2 and reference strains

Characteristics	*G. thermodenitrificans* DSM 465[a]	NG80-2	*G. stearothermophilus* ATCC 12980[a]	*G. thermoleovorans* ATCC 43513[a]
Growth at 45°C	+	+	w	–
Growth at 70°C	+	+	–	+
NaCl (3%)	+	+	+	–
Denitrification	w	+	–	–
Utilization of				
Rhamnose	–	–	–	–
Lactose	+	w	–	–
Cellobiose	+	+	–	+
Galactose	+	w	–	+
Xylose	+	+	–	+
Ribose	+	+	–	+
Arabinose	+	+	–	–
Citrate	–	–	–	w

Geobacillus stearothermophilus and *G. thermoleovorans* are nearest phenotypic neighbors of *G. thermodinitrificans*
+ positive, – negative, *w* weak reaction
[a]Type strains of representative bacterial genus. Data were adapted from Manachini et al. (2000)

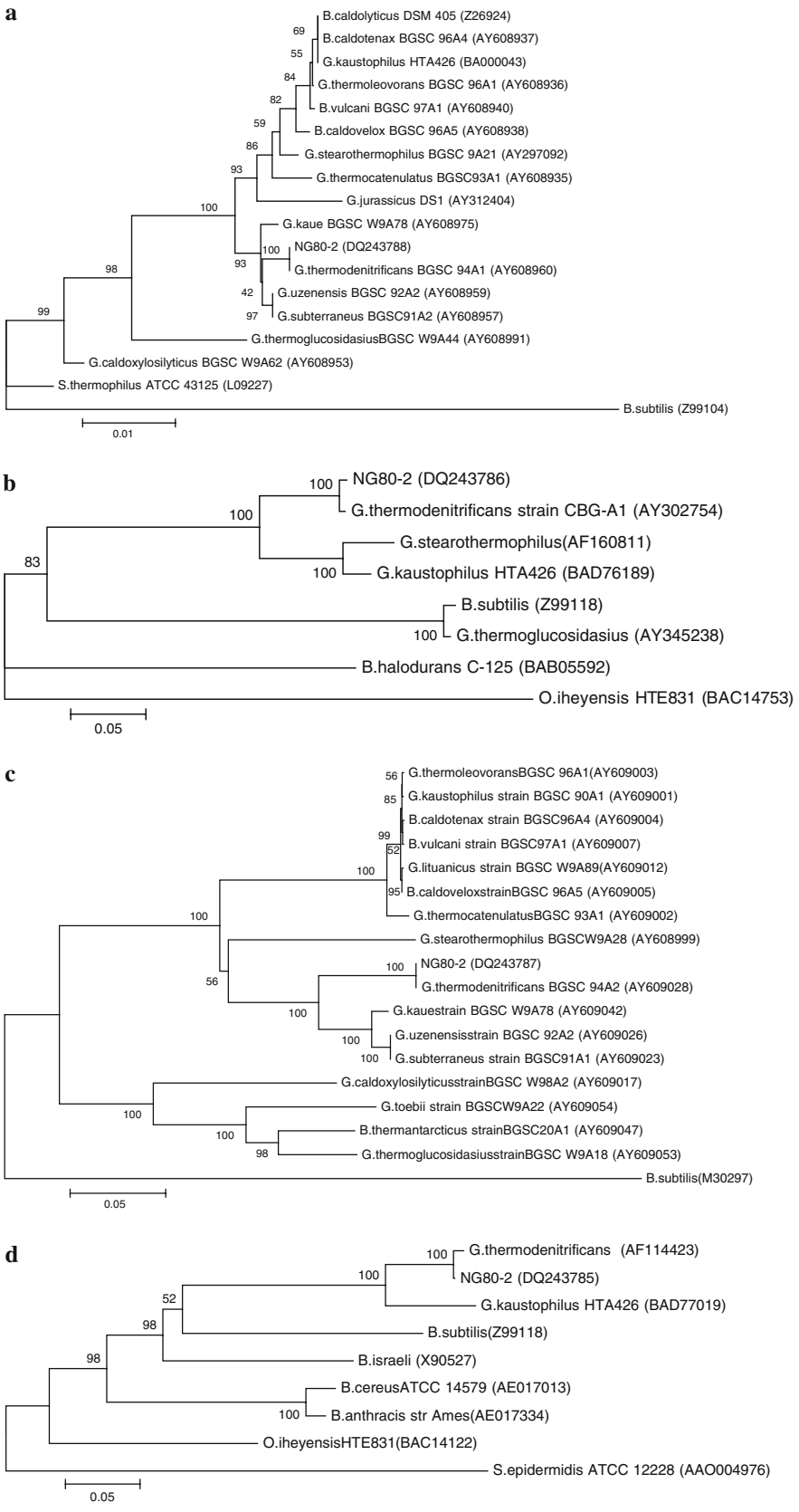

Fig. 2 Phylogenic trees showing the evolutionary distances of strain NG80-2 within the radiation of the genus *Geobacillus* and related taxa based on DNA sequences of 16S rDNA (**a**), *araA* (**b**), *recN* (**c**), and *mdh* (**d**). Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are shown at branching points. Bar 1 (**a**) and 5 (**b**–**d**) substitution per 100 nt; *G Geobacillus*; *B Bacillus*

On the basis of phenotypic and phylogenic analysis, NG80-2 was confidently identified as a strain of *G. thermodenitrificans*.

Utilization of crude oil and liquid paraffin as the sole carbon source by NG80-2

When washed cells of strain NG80-2 were inoculated into the minimal medium (medium A) without the addition of yeast extract and supplemented with 2% (w/v) crude oil or liquid paraffin as the sole carbon source, a decline of CFU numbers was observed in the first 2 days' incubation in both media (Fig. 3). This was apparently due to the shock encountered by the cells during washing procedure and changed to a less favorable environment. Growth with liquid paraffin started from day 3, while growth with crude oil started from day 8 (Fig. 3). During the lag period, crude oil-containing medium became emulsified (data not shown), indicating production of emulsifying agent by NG80-2. There was about threefold increase of growth as indicated by CFU after 15 and 17 days' incubation in liquid paraffin- and crude oil-containing media, respectively. The growth started to decline rapidly afterward, indicating that the nutrients became limited. Therefore, strain NG80-2 was able to use crude oil and liquid paraffin as the sole carbon source for growth, and the growth of NG80-2 with crude oil was accompanied by the emulsification of the medium. As liquid paraffin consists of *n*-alkanes ranging from C12 to C18 and the *n*-alkanes in crude oil have a much broader range, the ability to grow on crude oil and liquid paraffin indicates that *n*-alkanes, at least within the range of liquid paraffin, were degraded by NG80-2.

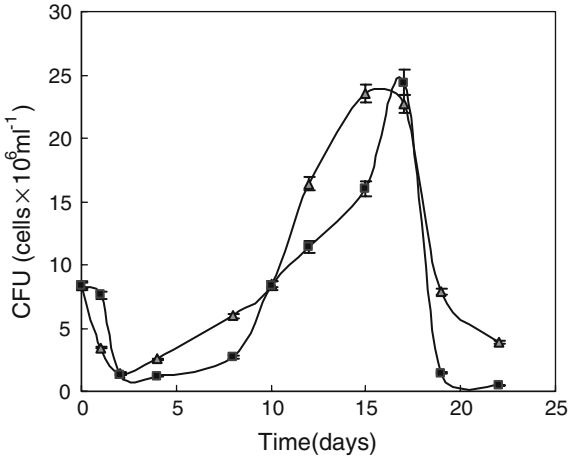

Fig. 3 Growth of NG80-2 in minimal medium (medium A) supplemented with 2% (w/v) crude oil (*filled square*) and liquid paraffin (*filled triangle*) as the sole carbon source. Cells were grown at 60°C on a rotary shaker (180 rpm), and residues were analyzed by GC. Data are the means of duplicate flasks

Degradation of long-chain *n*-alkanes by NG80-2

The ability to degrade long-chain *n*-alkanes by NG80-2 was further investigated by culturing the bacteria in the mineral medium supplemented with *n*-pentacosane (C25), *n*-pentatriacontane (C35), and *n*-hexatetracontane (C46). Analysis of the residues by high-temperature GC showed significant degradation of C25 and C35, but not C46 by NG80-2, as compared to the control samples without addition of any bacteria, which were cultured in parallel (Fig. 4). Degradation trends of C25, C35, and C46 without addition of bacteria and with addition of NG80-2 are summarized in Fig. 5. Both C25 and C35 were degraded most rapidly by NG80-2 in the first 3 days, and the rate slowed down afterward. The control samples, which had no bacteria added, also showed to a certain extent losses of the *n*-alkanes. By the end of 21 days' incubation, 90.01 and 93.80% of C25 and C35 were degraded in the samples with NG80-2, while 59.8 and 43.6% C25 and C35 were lost in the controls without bacteria, respectively. Taking out the consideration losses caused by non-bacterial effects, significant degradation of C25 and C35 by the action of NG80-2 was clearly demonstrated.

In the case of C46, the degradation trend curves of the sample with NG80-2 added and the control without bacteria were almost parallel. This indicates that NG80-2 was unable to degrade C46. There was around 40% of degradation at the end of day 21 for both samples, and this was apparently due to non-bacterial effects.

Test for the chain-length specificity of *n*-alkane degradation by NG80-2

When washed cells of strain NG80-2 were inoculated into the minimal medium (medium A) without yeast extract and supplemented with 1% (w/v) individual *n*-alkanes ranging from C8 to C44 as the sole carbon sources, growth was obtained in the cultures containing *n*-alkanes ranging from C15 to C36 after 10 days' incubation as estimated by measuring A_{600}, but there was no growth when *n*-alkanes less than C14 and greater than C40 were used (Table 2). The best growth was observed for *n*-alkanes ranging from C15 to C28. Whether or not the growth was related to the degradation of the *n*-alkanes was tested using C28. The results showed that the cell number increased by 10.7-fold and 22.8% of C28 was degraded in 13 days, and by the end of 35 days, 34.1% of C28 was degraded (Fig. 6). Degradation of the individual *n*-alkanes of the same range by NG80-2 was also tested in the same medium but supplemented with yeast extract. As assessed by GC analysis, it was also shown that NG80-2 degraded *n*-alkanes ranging from C15 to C36 only, while *n*-alkanes less than C14 and greater than C40 were not degraded (Fig. 7). The highest degradation rate was obtained with C16, and up to 55.6% of the compound was degraded after 10 days' incubation.

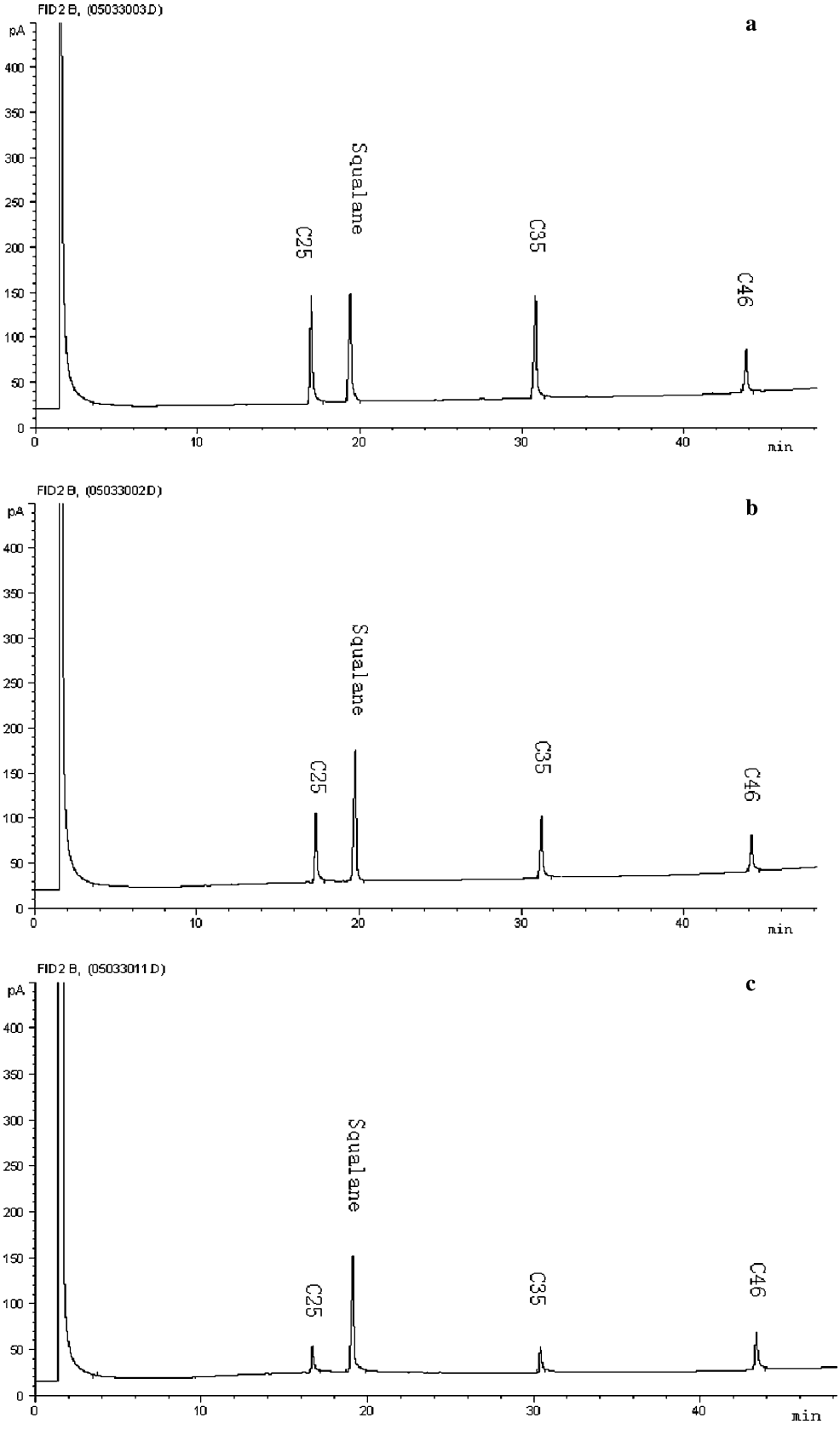

Fig. 4 High-temperature gas chromatograms of C25, C35, and C46. The cells were grown in 100-ml of the mineral medium containing 0.05% yeast extract and supplemented with 0.007% (w/v) each of C25, and C35 and C46 in 300-ml Erlenmeyer flasks at 65°C on a rotary shaker (180 rpm). **a** Uninoculated control at day 0; **b** Uninoculated control after 14 day's incubation; **c** Inoculated with NG80-2 after 14 days' incubation

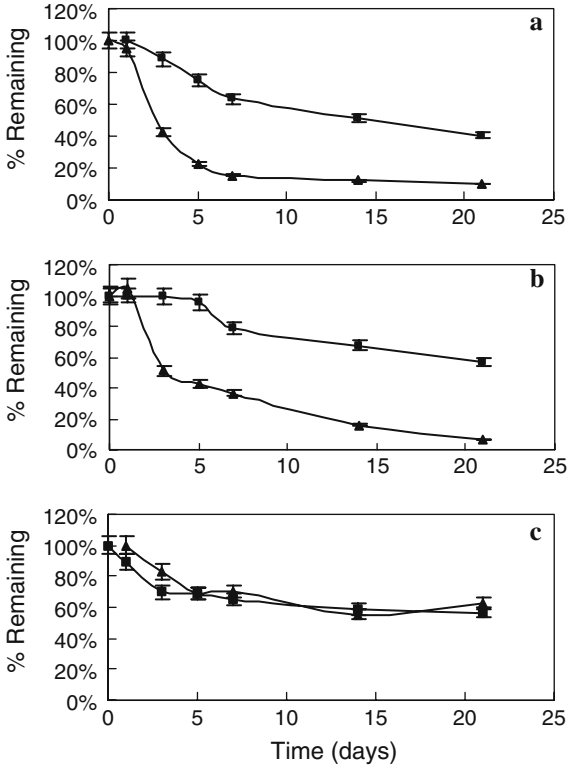

Fig. 5 Degradation curves of C25 (**a**), C35 (**b**), and C46 (**c**). The percentages of the *n*-alkanes remaining with addition of NG80-2 (*filled triangle*) and without addition of any bacteria (*filled square*) are shown. The cells were grown in 100-ml of the mineral medium containing 0.05% yeast extract and supplemented with 0.007% (w/v) each of C25, and C35 and C46 in 300-ml Erlenmeyer flasks at 65°C on a rotary shaker (180 rpm) for 21 days and individual flasks were taken at different intervals. The residual *n*-alkanes were extracted and analyzed by GC. Data are the means of duplicate flasks

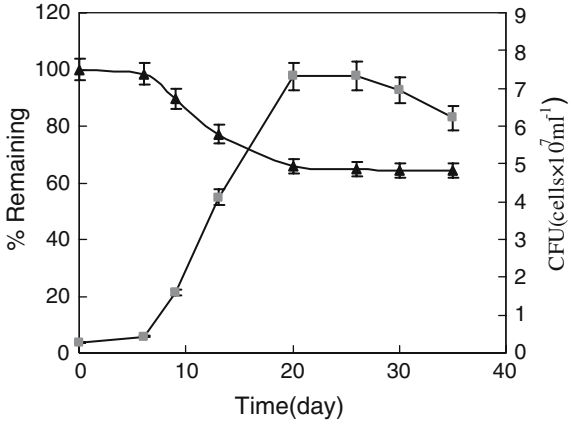

Fig. 6 Time courses of C28 utilization by NG80-2. Cells were grown in 30-ml of minimal medium (medium A) supplemented with 1% (w/v) of C28 as the sole carbon source in 100-ml culture bottles at 60°C on a rotary shaker (180 rpm). Individual bottles were taken at different intervals for viable counts and residue analysis. The residual *n*-alkanes were extracted and analyzed by GC. *Filled square* growth of cells, *filled triangle* degradation of C28. Data are the means of duplicate culture bottles

Discussion

Comparing the composition of the liquid paraffin and crude oil, the former contains *n*-alkanes ranging from C12 to C18 and is more easily dispersed in water. In contrast, crude oil is a complex mixture mainly containing insoluble compounds, including long-chain *n*-alkanes (up to C40 and above) that are not easily dispersed in water. Therefore, crude oil has much lower availability to the cells than liquid paraffin. The lag period observed when NG80-2 was growing on crude oil was apparently due to the lower availability of crude oil to the cells. When the medium became emulsified, the solubility of crude oil was increased and therefore the growth of cells was enhanced. Production of emulsifying agent by NG80-2 was expected as most of *n*-alkane-degrading bacteria produce emulsifying agents when they grow on crude oil-containing media. At this stage, the nature of emulsifying agent produced by NG80-2 is unknown.

When yeast extract (0.01%) was included in the minimal medium (medium A), even without supplementary carbon sources, the growth of NG80-2 was still compatible to the level as in the same medium without yeast extract but supplemented with crude oil or liquid paraffin (2%) (data not shown). Therefore, in carbon utilization experiments, yeast extract was not added into the medium. Despite the fact that NG80-2 was selected as the best crude oil degrader based on decreased A_{250}, which indicated decreased level of aromatic compounds in crude oils, NG80-2 was unable to utilize carbozole as the sole carbon and nitrogen sources, and dibenzothiophene (DBT) and benzothiophene (BT) as the sole carbon and sulfur sources for growth (data not shown). Whether or not NG80-2 degrades these and other

Table 2 Growth of NG80-2 on selected *n*-alkanes as the sole carbon sources

n-Alkane	OD_{600}
Octane (C8)	0.059
Dodecane (C12)	0.094
Tridecane (C13)	0.093
Tetradecane (C14)	0.088
Pentadecane (C15)	0.227
Hexadecane (C16)	0.207
Octadecane (C18)	0.269
Docosane (C22)	0.326
Tetracosane (C24)	0.256
Octacosane (C28)	0.226
Dotriacontane (C32)	0.170
Hexatriacontane (C36)	0.132
Tetracotane (C40)	0.062
Tetratetracotane (C44)	0.014

NG80-2 was cultured in minimal medium (medium A) supplemented with 1% (w/v) of individual *n*-alkane as indicated and incubated at 60°C for 10 days. The cells from fully-grown LB culture were washed twice with sterile saline before inoculation. The initial OD_{600} was 0.05. The data are the means of duplicate culture bottles

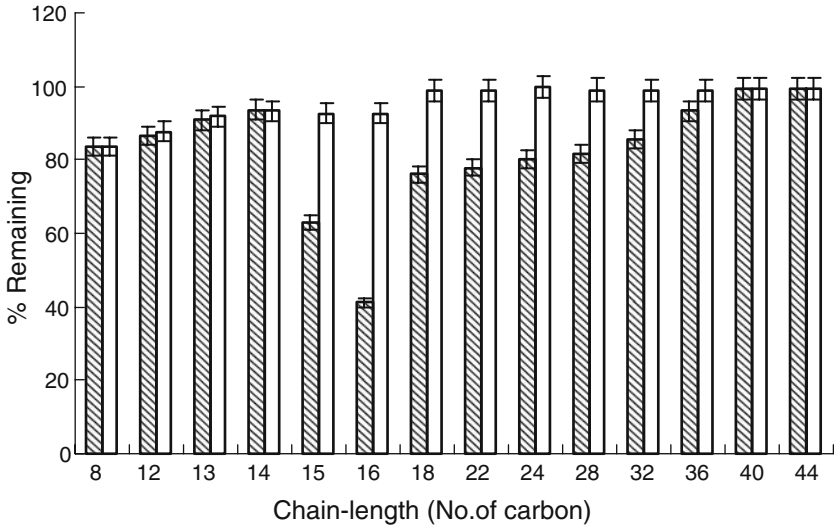

Fig. 7 Degradation of individual n-alkanes by NG80-2. The percentages of *n*-alkanes remaining with addition of NG80-2 (*hatched bars*) and without addition of any bacteria (*open bars*) are shown. The cells were grown in 30-ml of the minimal medium (medium A) supplemented with 0.01% yeast extract and 1% (w/v) of individual *n*-alkanes as indicated in 100-ml culture bottles at 60°C on a rotary shaker (180 rpm) for 10 days. Individual bottles were taken at different intervals, and the residual *n*-alkanes were extracted and analyzed by GC. Data are the means of duplicate culture bottles

aromatic compounds present in crude oil without utilizing them as the sole carbon, nitrogen or sulfur source, still needs to be investigated.

The ability to degrade *n*-alkanes ranging from C15 to C36, but not those less than C14 indicates that NG80-2 has only evolved as a mechanism for degradation of long-chain *n*-alkanes, but not short chains. The thermophilic nature of NG80-2, which grows between 45 and 73°C, and the volatility property of light fraction *n*-alkanes, which evaporates rapidly at elevated temperatures, may explain the lack of a mechanism for short-chain *n*-alkanes degradation in NG80-2. The inability to degrade *n*-alkanes longer than C40 may be due to the extremely insoluble nature of those compounds even after the use of dispersant. However, we cannot rule out the possibility of requirement for a different system for the degradation of those extremely long-chain *n*-alkanes, which is absent in NG80-2 at this stage.

Losses of *n*-alkanes observed in the control flasks without bacteria may be due to the effects of evaporation and chemical decomposition caused by high temperature and high rotation, or adsorption to rubber stoppers. Although long-chain alkanes are less easily evaporated and decomposed than short chains, a certain extent of losses may be expected. Regarding adsorption, even though the stoppers were flushed through out before the extraction, it was also possible that traces of *n*-alkanes, especially very long-chain *n*-alkanes such as C46, still remained. When the concentration of *n*-alkanes is very low as in the case shown in Figs. 4 and 5 (0.021%), even slight evaporation, decomposition, or adsorption can be significant as judged by percentages. However, when the concentration of *n*-alkanes is high as in the case shown in Fig. 7 (1%), losses are much less significant, especially with long-chain *n*-alkanes. Therefore, the experimental conditions used in this study are more suitable when relatively high concentrations of *n*-alkanes are used. Experimental conditions for testing degradation of alkanes with lower concentration still need to be investigated in future studies.

Most of the reported *n*-alkane-degrading bacteria are mesophiles, mainly degrading *n*-alkanes ranging from C6 to C16, and only a couple of *Rhodococcus* strains degrade *n*-alkanes up to C36 (van Beilen et al. 2003). *Acinetobacter* sp. M1 was the only other strain reported to degrade long-chain *n*-alkanes (C13–C44) (Sakai et al. 1994). However, reports on *n*-alkane-degrading thermophilic bacteria are limited. A *Bacillus thermoleovorans* strain degraded *n*-alkanes up to C23 at 70°C, but was unable to utilize *n*-alkanes as the sole carbon source for growth (Kato et al. 2001). To our knowledge, this was the first report on the degradation of long-chain *n*-alkanes up to at least C36 by a thermophilic bacterial strain and utilization of those compounds for growth as the sole carbon source.

In almost all of the *n*-alkane-degrading strains, homologs to the membrane-bound *n*-alkane hydroxylases AlkB have been identified, and some of them have been functionally examined (van Beilen et al. 2003). However, there were no *alkB* homologs found in NG80-2 (separate study). As the AlkB system acts mainly on *n*-alkanes ranging from C6 to C16 (van Beilen et al. 2003), the lack of an *alkB* homolog in NG80-2 also indicates that the thermophile employs different hydroxylase systems that act on long-chain *n*-alkanes. In *Acinetobacter* sp. M1, a copper-dependent *n*-alkane dioxygenase was found to be involved in the degradation of long-chain *n*-alkanes (Maeng et al. 1996), but the corresponding genes have not been identified. With the completion of NG80-2 genome sequencing in our separate study, the follow-up studies will include identification of genes responsible for the degradation of *n*-alkanes in NG80-2, which may lead to the discovery of novel hydroxylase systems specifically used for the degradation of long-chain *n*-alkanes.

The ability to degrade long-chain *n*-alkanes (C15 to at least C36) and at the same time produce an emulsifying agent makes NG80-2 an attractive candidate strain for oily-waste treatment, as the process can be largely accelerated under elevated temperatures. On the other hand, the temperature is usually high for oily-waste

discharge; there are obviously advantages on cost-savings and efficiency of the treatment by using thermophiles over mesophiles. NG80-2 also has a potential to be used as an inoculum for microbially enhanced oil recovery (MEOR) process, as the strain is able to proliferate under high-temperature conditions of oil-reservoirs, reduce the viscosity of crude oils, and decrease the ratio of light/heavy fraction of crude oils by degrading long-chain *n*-alkanes. Both aspects of application are worth investigating in the future studies.

Acknowledgements This research was supported by the National 863 Program (2002AA226011) and funding from Tianjin Science and Technology Committee (033105811), China. We thank David Bastin for critical reading of the manuscript.

References

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 45:180–209

Bastin DA, Reeves PR (1995) Sequence and analysis of the O antigen gene (*rfb*) cluster of *Escherichia coli* O111. Gene 164:17–23

van Beilen JB, Wubbolts MG, Witholt B (1994) Genetics of *n*-alkane oxidation by *Pseudomonas oleovorans*. Biodegradation 5:161–174

van Beilen JB, Panke S, Lucchini S, Franchini AG, Rothlisberger M, Witholt B (2001) Analysis of *Pseudomonas putida n*-alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. Microbiology 147:1621–1630

van Beilen JB, Li Z, Duetz WA, Smits THM, Witholt B (2003) Diversity of *n*-alkane hydroxylase systems in the environment. Oil Gas Sci Technol 58:427–440

Berthe-Corti L, Fetzner S (2002) Bacterial metabolism of *n*-alkanes and ammonia under oxic, suboxic and anoxic conditions. Acta Biotechnol 22:299–336

Bognolo G (1999) Biosurfactants as emulsifying agents for hydrocarbons. Colloids Surf 152:41–52

Christofi N, Ivshina IB (2002) Microbial surfactants and their use in field studies of soil remediation. J Appl Microbiol 93:915–929

Engelhardt MA, Daly K, Swannell RP, Head IM (2001) Isolation and characterization of a novel hydrocarbon-degrading, Gram-positive bacterium, isolated from intertidal beach sediment, and description of *Planococcus alkanoclasticus* sp.nov. J Appl Microbiol 90:237–247

Feitkenhauer H, Muller R, Markl H (2003) Degradation of polycyclic aromatic hydro-carbons and long chain *n*-alkanes at 60–70°C by *Thermus* and *Bacillus* spp. Biodegradation 14:367–372

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM et al (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269(5223):496–512

Hamamura N, Arp DJ (2000) Isolation and characterization of n-alkane-utilizing *Nocardioides* sp. strain CF8. FEMS Microbiol Lett 186:21–26

van Hamme JD, Ward OP (2001) Physical and metabolic interactions of *Pseudomonas* sp. strain JA5-B45 and *Rhodococcus* sp. strain F9-D79 during growth on crude oil and effect of a chemical surfactant on them. Appl Environ Microbiol 67:4874–4879

van Hamme JD, Singh A,Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549

Holt JG, Krieg NR, Sneath PHA, Staley JT, Willams ST (1994) Bergey's manual of determinative bacteriology. In: Holt JG, Schleifer KH,Tully JG, Ursing J, Bryant M, Krieg NR, Liston J,Moulder JW, Murray RGE, Niven CF, Pfenning N (eds) Group 18: Endospore-forming Gram-positive rods and cocci, 9th edn. Williams & Wilkins, Baltimore, pp 559–564

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71–84

Kato T, Haruki M, Iamanaka T (2001) Isolation and characterization of long-chain *n*-alkane degrading *Bacillus thermoleovorans* from deep subterranean petroleum reservoirs. J Biosci Bioeng 91:64–70

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315

Maeng JH, Sakai Y, Tani Y, Kato N (1996) Isolation and characterization of a novel oxygenase that catalyzes the first step of *n*-alkane oxidation in *Acinetobacter* sp. strain M-1. J Bacteriol 178:3695–3700

Manachini PL, Mora D, Nicastro G, Parini C, Stackebrandt E, Pukall R, Fortina MG (2000) *Bacillus thermodenitrificans* sp. nov., nom. rev. Int J Syst Evol Microbiol 50:1331–1337

Margesin R, Schinner F (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments (review). Appl Microbiol Biotechnol 56:650–663

Mutzel A, Reinscheid UM, Antranikian G, Müller R (1996) Isolation and characterization of a thermophilic *bacillus* strain that degrades phenol and cresols as sole carbon source at 70°C. Appl Biochem Biotechnol 46:593–596

Nazina TN, Sokolova DSh, Grigoryan AA, Shestakova NM, Mikhailova EM, Poltaraus AB, Tourova TP, Lysenko AM, Osipov GA, Belyaev SS (2005) *Geobacillus jurassicus* sp. nov., a new thermophilic bacterium isolated from a high-temperature petroleum reservoir, and the validation of the *Geobacillus* species. Syst Appl Microbiol 28:43–53

Sakai Y, Maeng JH, Tani Y, Kato N (1994) Use of long-chain n-alkanes by an isolate, *Acinetobacer* sp. M-1. Biosci Biotechnol Biochem 58:2128–2130

Sorkhoh NA; Ibrahim AS; Ghannoum MA; Radwan SS (1993) High-temperature hydrocarbon degradation by *Bacillus stearothermophilus* from oil-polluted Kuwaiti desert. App. Microbiol Biotechnol 39:123–126

Witholt B (1972) Method for isolating mutants overproducing NAD and its precursors. J Bacteriol 109:350–364