

Distribution of Hydrocarbon-Degrading Bacteria in the Soil Environment and Their Contribution to Bioremediation

Yuki Fukuhara · Sachie Horii · Toshihide Matsuno ·
Yoshiki Matsumiya · Masaki Mukai · Motoki Kubo

Received: 17 January 2013 / Accepted: 28 February 2013 /
Published online: 22 March 2013
© Springer Science+Business Media New York 2013

Abstract A real-time PCR quantification method for indigenous hydrocarbon-degrading bacteria (HDB) carrying the *alkB* gene in the soil environment was developed to investigate their distribution in soil. The detection limit of indigenous HDB by the method was 1×10^6 cells/g-soil. The indigenous HDB were widely distributed throughout the soil environment and ranged from 3.7×10^7 to 5.0×10^8 cells/g-soil, and the ratio to total bacteria was 0.1–4.3 %. The dynamics of total bacteria, indigenous HDB, and *Rhodococcus erythropolis* NDKK6 (carrying *alkB* R2) during bioremediation were analyzed. During bioremediation with an inorganic nutrient treatment, the numbers of these bacteria were slightly increased. The numbers of HDB (both indigenous bacteria and strain NDKK6) were gradually decreased from the middle stage of bioremediation. Meanwhile, the numbers of these bacteria were highly increased and were maintained during bioremediation with an organic nutrient. The organic treatment led to activation of not only the soil bacteria but also the HDB, so an efficient bioremediation was carried out.

Keywords Biostimulation · Bioaugmentation · Indigenous hydrocarbon-degrading bacteria · Alkane hydroxylase gene · *Rhodococcus*

Introduction

Petroleum hydrocarbons are a major energy source. However, environmental contamination by petroleum hydrocarbons has become a serious problem all over the world. The leakage of petroleum hydrocarbons to nature causes the disruption of the natural ecosystem since petroleum hydrocarbons contain many kinds of toxic compounds [1]. To remediate hydrocarbon-contaminated sites, bioremediation systems have been developed [2]. In the bioremediation system, biostimulation and bioaugmentation procedures have been

Y. Fukuhara · S. Horii · T. Matsuno · Y. Matsumiya · M. Mukai · M. Kubo (✉)
Department of Bioscience and Biotechnology, Faculty of Life Science, Ritsumeikan University,
1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan
e-mail: kubo@sk.ritsume.ac.jp

investigated. Biostimulation using inorganic and organic nutrients has been developed for the activation of indigenous hydrocarbon-degrading bacteria (HDB) [1, 3]. For the construction of more efficient bioremediation systems, there have been attempts at isolating and characterizing many kinds of HDB for bioaugmentation [4–6].

Petroleum hydrocarbons include various types of compounds, such as normal alkanes (*n*-alkanes), cyclic alkanes (*c*-alkanes), and polyaromatic hydrocarbons (PAHs). Long-chain *n*-alkanes, long-chain *c*-alkanes, and PAHs are recalcitrant in the environment [7, 8]. The genera *Acinetobacter* and *Pseudomonas* can degrade long-chain *n*-alkanes, but *c*-alkane degradation by these genera requires the co-metabolism of *n*-alkanes [9]. The metabolic pathways for long-chain *n*-alkanes and related enzymes have been investigated in bacteria such as *Pseudomonas putida* GPO1 and *Acinetobacter baylyi* ADP1 [10, 11]. *n*-Alkanes are first converted to alcohol by alkane hydroxylase, this alcohol is further converted to aldehyde and fatty acid, and the resulting fatty acid is mineralized by β -oxidation to carbon dioxide [11–13].

On the other hand, several bacteria that can degrade cyclohexane and alkyl-cyclohexanes have been isolated, including *Alcanivorax* sp. MBIC4326, *Gordonia terrae* NDKY76A, and *Rhodococcus erythropolis* NDKK6 [9, 14, 15]. *G. terrae* NDKY76A and *R. erythropolis* NDKK6 can utilize *c*-alkanes without co-metabolism of *n*-alkanes, and their metabolic pathways for long-chain *c*-alkanes have been determined [15].

In a previous study by Hatayama et al. [16], an efficient bioremediation system was developed using specific HDB. To improve the efficiency of bioremediation, the activation of indigenous HDB and an analysis of their distribution in the soil environment will be important. In this study, the distribution of HDB in the soil environment and the dynamics of the indigenous HDB during bioremediation were investigated for construction of an efficient bioremediation system.

Materials and Methods

Bacterial Strains, Medium, and Soil Samples

Escherichia coli JM109 [17], *Pseudomonas aeruginosa* F721 [9], *R. erythropolis* NDKK6 [9], and *G. terrae* NDKY76A [15] were used. All bacteria were cultivated with Luria–Bertani medium (1 % peptone, 0.5 % yeast extract, and 0.5 % NaCl) at 30 °C. Soil samples were collected from different areas in Shiga Prefecture, Japan (23 samples: clay soil, 5 samples; silt soil, 14 samples; sand soil, 4 samples).

Analysis of Total Carbon and Hydrocarbon in Soil

Total carbon (TC) in the soil was analyzed using a total organic carbon analyzer (TOC-V CPH, Shimadzu Corp., Kyoto, Japan) with a solid sample module (SSM-5000A, Shimadzu Corp., Kyoto, Japan). Concentration and composition of hydrocarbons in the soil were analyzed by infrared spectroscopy (IR; Horiba oil content analyzer OCMA-355, Horiba Ltd., Kyoto, Japan) and gas chromatography (GC; GC-2010, Shimadzu Corp., Kyoto, Japan) with a capillary column (DB-1ht 30 m \times 0.25 mm \times 0.1 μ m, J&W Scientific, CA, USA), respectively. Hydrocarbon in the soil (2 g) was extracted with 10 ml of H-997 solvent (Horiba Ltd., Kyoto, Japan). The extract was quantified and analyzed by IR and GC. Concentration of hydrocarbon was calculated by the following equation: Concentration of hydrocarbon in soil (mg/kg)= $R \times D \times H$ (R , reading value of hydrocarbon from OCMA-355

(mg/kg); *D*, dilution factor; *H*, volume of H-997 (ml)). The conditions for GC analysis were as follows: injector temperature, 250 °C; oven temperature, 75 °C; detector temperature, 300 °C. The oven temperature was kept at 75 °C for 1 min and then increased to 300 °C at a rate of 6 °C/min, and the temperature was maintained for 20 min.

Primer Design for Quantification of HDB and *R. erythropolis* NDKK6

Primer sequences are shown in Table 1. The alkB-F/alkB-R degenerate primer set for quantification of HDB was designed based on conserved sequences of alkane hydroxylase genes (*alkB2* from *Gordonia* sp. TF6 (accession no. AB112870), *alkB* from *Gordonia* sp. SoCg (accession no. HQ026811), *alkB* from *R. jostii* RHA1 (accession no. CP000431.1), *alkB* from *R. opacus* B4 (accession no. NC_012522), *alkB2* from *Rhodococcus* sp. Q15 (accession no. AF388182), *alkB2* from *P. aeruginosa* PAO1 (accession no. NC_002516.2), *alkB* from *P. putida* GPO1 (accession no. AJ245436), *alkMa* from *Acinetobacter* sp. M-1 (accession no. AB049410.1), and *alkM* from *A. baylyi* ADP1 (accession no. AJ002316)). The size of PCR product is approximately 140 bp.

The alkB R2-F/alkB R2-R primer set for quantification of *R. erythropolis* NDKK6 was designed from *alkB* R2 sequence of *R. erythropolis* NDKK6 using a specific region of the gene. The size of PCR product is approximately 100 bp.

Quantification of the Total Number of Soil Bacteria

The total number of the soil bacteria was estimated from the quantity of environmental DNA (eDNA) extracted by the slow stirring method [18]. A standard curve for the estimation of the total bacteria based on the amount of eDNA was constructed using *R. erythropolis* NDKK6. The total number of the soil bacteria was calculated from the amount of eDNA using the following equation: Total number of soil bacteria (cells/g-soil)=Amount of eDNA (μg/g-soil)×4.0×10⁹.

Quantification of HDB and *R. erythropolis* NDKK6

The eDNA band was cut from agarose gel and placed into a bottom-punctured 600-μl centrifuge tube. The tube was then centrifuged at 14,000×*g* at 4 °C for 5 min. The resulting DNA solution was ethanol-precipitated, dried, and dissolved in 10 μl of water. Real-time PCR was carried out using a KAPA-SYBR qPCR kit (Kapa Biosystems Inc., MA, USA) and Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, CA, USA). The primers for the indigenous HDB and *R. erythropolis* NDKK6 are shown in Table 1. The cycling program for indigenous HDB was 3 min of initial preincubation at 95 °C followed by 40 cycles of 95 °C for 30 s and

Table 1 PCR primers for quantification of HDB and *R. erythropolis* NDKK6

Target	Primer	Sequence ^a
HDB	alkB-F	5'-AACTAYMTCGARCAYTAYGG-3'
	alkB-R	5'-TGRCTKSWRTGNCGYTGVARGTG-3'
<i>R. erythropolis</i> NDKK6	alkB R2-F	5'-CGGTTGTGTGCGCAGGAATC-3'
	alkB R2-R	5'-AACGACTGCGCCAGAGTGAT-3'

^a K=GT, M=AC, N=ATCG, R=AG, S=CG, V=ACG, W=AT, and Y=CT

60 °C for 35 s. The cycling program for strain NDKK6 was 10 min of initial preincubation at 95 °C followed by 30 cycles of 95 °C for 15 s and 60 °C for 30 s.

Bioremediation Experiment

The soil used in the bioremediation experiment (TC value was 10,000 mg/kg-soil without hydrocarbon) was prepared by mixing the sand soil (TC value was 200 mg/kg-soil) and silt soil (TC value was 39,000 mg/kg-soil). Hydrocarbon-contaminated soil (5,000 mg/kg-soil) was prepared by adding base oil (ENEOS type super oil T10, Shin Nihon Sekiyu Co., Ltd., Tokyo, Japan) into the soil. Bioremediation experiments were carried out using 2 kg of soil at 25 °C with the following conditions: (1) Inorganic condition of biostimulation was carried out by addition of four times concentrated modified SW (4×MSW) medium (1 % v/w; per liter: 4.84 g $[\text{NH}_4]_2\text{NO}_3$, 57.28 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 21.78 g KH_2PO_4 , 2 g NaCl, 0.986 g MgSO_4 , 11 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 59 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.801 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 mg $[\text{NH}_4]_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.08 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.16 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.06 mg $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 2 g polypeptone, and 1 g yeast extract). (2) Inorganic condition of bioaugmentation was carried out by addition of 4×MSW (1 % v/w) and *R. erythropolis* NDKK6 (final cell number was 1×10^8 cells/g-soil). (3) Organic condition of biostimulation was carried out by addition of chicken manure (5 % w/w; Hakkoukeifun, Agurienuwai, Hyogo, Japan), and TC value of the soil was adjusted to about 20,000 mg/kg-soil without hydrocarbon. The numbers of total bacteria and indigenous HDB in the manure were 4.3×10^{10} and 1.4×10^8 cells/g-sample, respectively. (4) Organic condition of bioaugmentation was carried out by addition of chicken manure (5 % w/w) and *R. erythropolis* NDKK6 (final cell number was 1×10^8 cells/g-soil). The numbers of total bacteria, indigenous HDB, strain NDKK6, and hydrocarbon concentration were analyzed after 1, 3, 7, 14, 21, and 28 days. Three independent experiments were carried out.

Results

Development of the Quantification Method for HDB in the Soil Environment

To quantify the number of indigenous HDB in the soil environment, a quantification method using the alkane hydroxylase gene (*alkB*) with real-time PCR was developed. In order to confirm *alkB* amplification, PCR was carried out using the primers with the total DNAs of *R. erythropolis* NDKK6, *G. terrae* NDKY76A, and *P. aeruginosa* F721, which carry *alkB* as a template. A DNA fragment (approximately 140 bp) was amplified from these strains, while no amplicon was observed when the total DNA from *E. coli* JM109 was used as a template (data not shown). This indicates that specific amplification of many kinds of *alkB* from HDB was amplified by the *alkB*-F/*alkB*-R primer set (Table 1).

For the quantification system of the number of HDB using the primers in the soil environment, a standard curve was constructed with real-time PCR using *R. erythropolis* NDKK6 (1×10^6 – 1×10^9 cells/g-soil). The standard curve between \log_{10} cell number and cycle threshold (C_t) value is shown in Fig. 1. The R^2 of the standard curve was 0.99, indicating that HDB can be quantified within the range of 1×10^6 – 1×10^9 cells/g-soil.

Quantification and Distribution of Indigenous HDB in the Soil Environment

To quantify the bacteria carrying *alkB* in the soil environment (indigenous HDB) and to investigate the distribution of these bacteria in the soil environment, various soil samples

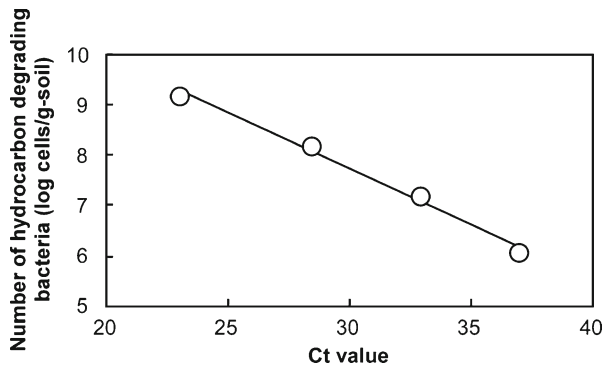


Fig. 1 Standard curve to quantify the indigenous HDB in soil samples by real-time PCR with *alkB*-F and *alkB*-R primers. The standard curve was produced from tenfold serial dilutions of *R. erythropolis* strain NDKK6 cells ranging from 1×10^6 to 1×10^9 cells/g-soil and by plotting the C_t versus the logarithm of the estimated cell number in the sample

(clay, silt, and sand soils) without hydrocarbon contamination were analyzed by the method. The total numbers of the soil bacteria and indigenous HDB are summarized in Table 2.

Table 2 Total number of bacteria and bacteria carrying *alkB* in various soil samples

Soil type	Soil sample	Total number of soil bacteria ($\times 10^9$ cells/g-soil)	Number of bacteria carrying <i>alkB</i> ($\times 10^7$ cells/g-soil)	Ratio of bacteria carrying <i>alkB</i> (%)
Clay	1	35	8.7	0.24
	2	54	21	0.36
	3	77	9.9	0.12
	4	29	13	0.42
	5	13	15	1.1
Silt	6	3.9	3.7	1.0
	7	4.7	8.4	1.8
	8	26	10	0.41
	9	43	50	1.1
	10	39	16	0.41
	11	85	13	0.15
	12	71	7.4	0.10
	13	37	8.5	0.23
	14	37	4.6	0.12
	15	19	20	1.1
	16	55	16	0.30
	17	32	14	0.45
	18	24	10	0.42
	19	30	4.8	0.16
Sand	20	4.2	7.3	1.7
	21	2.8	13	4.3
	22	4.5	4.2	0.86
	23	6.9	20	2.9

The total number of the soil bacteria in 23 soil samples tested in this study was 2.8×10^9 – 5.4×10^{10} cells/g-soil. Indigenous HDB were detected in all 23 soil samples. The number of indigenous HDB ranged from 3.7×10^7 to 5.0×10^8 cells/g-soil. The ratio of indigenous HDB was 0.1–4.3 % of the total soil bacteria. These results indicate that indigenous HDB were widely distributed in the soil environment.

Dynamics of HDB During Bioremediation Under Inorganic Environmental Conditions

In order to investigate the relationship between the number of HDB and hydrocarbon degradation during biostimulation and bioaugmentation under inorganic conditions, the total number of soil bacteria, the number of indigenous HDB, and the concentration of hydrocarbon in the soil were analyzed. In the bioaugmentation experiment, *R. erythropolis* NDKK6, which can degrade not only long-chain *n*-alkanes but also long-chain *c*-alkanes, was inoculated into hydrocarbon-contaminated soil.

In the case of biostimulation under inorganic condition, 30 % of hydrocarbon was degraded by the indigenous HDB in the soil over 28 days (Fig. 2). The total number of the soil bacteria tended to increase from 9.4×10^8 to 1.8×10^{10} cells/g-soil (Fig. 3). The number of indigenous HDB was also increased by about 88 times after 7 days and reached 11 % of total bacteria. After that, indigenous HDB gradually decreased (Fig. 4). GC analysis of hydrocarbons in the biostimulation experiment shows that the sharp peaks (*n*-alkanes) and the broad peak (*c*-alkanes) still remained in the soil (Fig. 5).

On the other hand, 36 % of hydrocarbon was degraded by strain NDKK6 and indigenous HDB in the soil over 28 days during bioaugmentation under inorganic condition (Fig. 2). The total number of the soil bacteria increased in the same way as in the biostimulation experiment (Fig. 3). The number of indigenous HDB gradually increased by about 22 times after 7 days to 16 % of total bacteria (Fig. 4), while the number of strain NDKK6 also increased after 3 days and decreased after 7 days (Fig. 6). GC analysis of hydrocarbons from bioaugmentation experiments showed a decrease in *n*-alkanes but a decline in *c*-alkanes during biostimulation (Fig. 5).

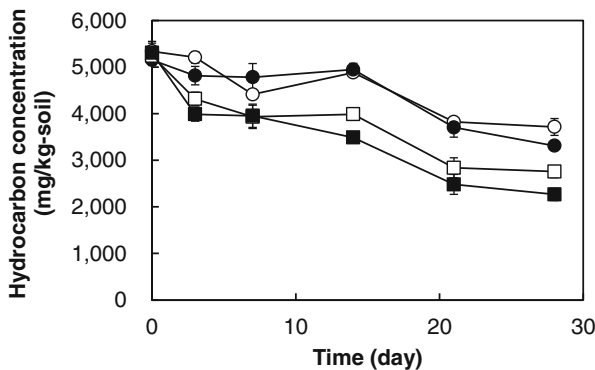


Fig. 2 Time course of hydrocarbon concentration during bioremediation. Petroleum hydrocarbons were extracted from the soil and analyzed by IR. *Empty circle*, biostimulation under inorganic conditions; *filled circle*, biostimulation and bioaugmentation under inorganic conditions; *empty square*, biostimulation under organic conditions; *filled square*, biostimulation and bioaugmentation under organic conditions. The data are the average \pm standard deviation of three experiments

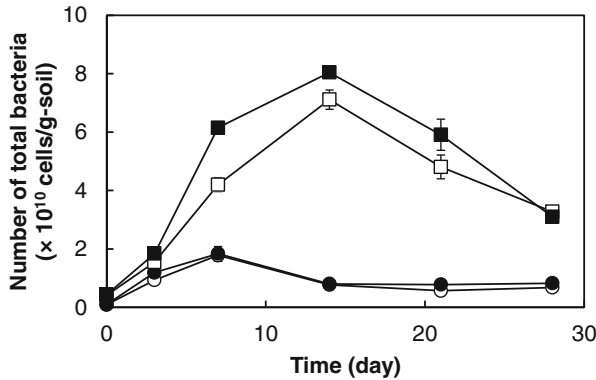


Fig. 3 Time course of the total number of soil bacteria. The total bacteria were quantified from eDNA in the soil sample. *Empty circle*, biostimulation under inorganic conditions; *filled circle*, biostimulation and bioaugmentation under inorganic conditions; *empty square*, biostimulation under organic conditions; *filled square*, biostimulation and bioaugmentation under organic conditions. The data are the average \pm standard deviation of three experiments

Dynamics of HDB During Bioremediation Under Organic Environmental Conditions

In order to construct a more efficient bioremediation system, chicken manure, which contains abundant nutrients, was added into the hydrocarbon-contaminated soil. The numbers of the total soil bacteria, indigenous HDB, strain NDKK6, and hydrocarbon concentration in the soil were analyzed.

During biostimulation under organic condition, 47 % of hydrocarbon was degraded by the indigenous HDB in the soil over 28 days (Fig. 2). The total number of bacteria increased during the experiment (Fig. 3). The number of indigenous HDB increased by about 19 times after 7 days to reach 9 % of total bacteria, and the number of bacteria was maintained compared with biostimulation under inorganic conditions (Fig. 4), suggesting that chicken manure assisted in maintaining the soil bacterial number. GC analysis of hydrocarbons in the biostimulation experiment shows that the sharp peaks (*n*-alkanes) mainly decreased (Fig. 7).

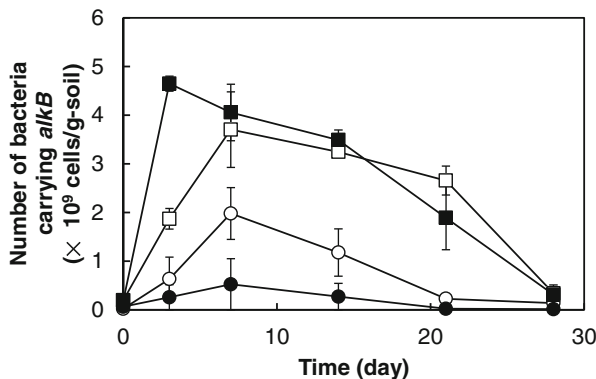


Fig. 4 Time course of the number of indigenous HDB during bioremediation. *Empty circle*, biostimulation under inorganic conditions; *filled circle*, biostimulation and bioaugmentation under inorganic conditions; *empty square*, biostimulation under organic conditions; *filled square*, biostimulation and bioaugmentation under organic conditions. The data are the average \pm standard deviation of three experiments

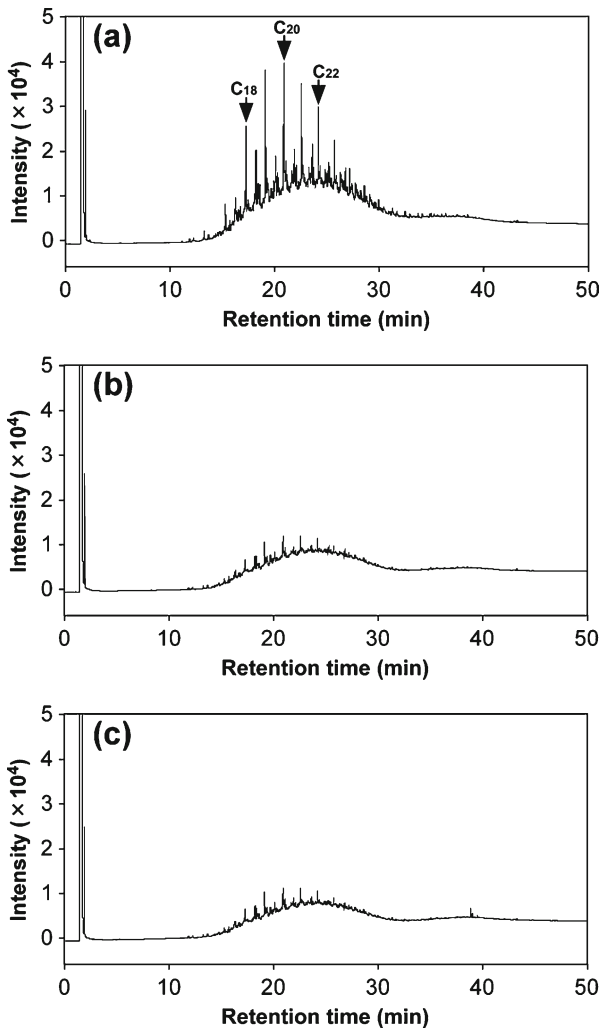


Fig. 5 Degradation of hydrocarbons by indigenous HDB and *R. erythropolis* strain NDKK6 during bioremediation under inorganic conditions. **a** Gas chromatogram of hydrocarbons from the soil at the start of the experiment. *Arrows* indicate the peaks for known *n*-alkanes and their carbon number. **b**, **c** Gas chromatograms of residual hydrocarbons from the soil of biostimulation and bioaugmentation, respectively, after 28 days of incubation

On the other hand, 57 % of hydrocarbon was degraded in the soil after 28 days in the bioaugmentation under organic condition (Fig. 2). The total number of the soil bacteria increased the same way as in the biostimulation experiment (Fig. 3). The number of indigenous HDB increased by 21 times after 7 days to 7 % of total bacteria (Fig. 4), and the number of strain NDKK6 increased by about 8.4 times after 14 days (Fig. 6). GC analysis of hydrocarbons shows that both the sharp peaks (*n*-alkanes) and the broad peak (*c*-alkanes) decreased; especially notable was the significant decrease of *c*-alkanes observed in this experiment (Fig. 7). These results indicate that the combination of biostimulation and bioaugmentation under organic conditions is effective for hydrocarbon degradation, and maintaining the bacterial number in the soil environment is important for bioremediation of hydrocarbon-contaminated soil.

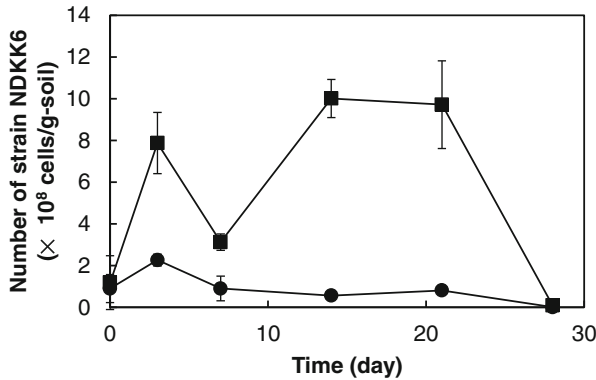


Fig. 6 Time course of the number of *R. erythropolis* NDKK6. Filled circle, biostimulation and bioaugmentation under inorganic conditions; filled square, biostimulation and bioaugmentation under organic conditions. The data are the average \pm vstandard deviation of three experiments

Discussion

HDB were isolated from various environments as described previously [9, 13, 19]. In the present study, distribution of indigenous HDB carrying the *alkB* gene in the soil environment was investigated. These bacteria were widely distributed in various soil environments even without the contamination of hydrocarbons.

The ratio of indigenous HDB was <4.3 %. Ammonia-oxidizing bacteria (carrying *amoA*) and nitrite-oxidizing bacteria (carrying *nirK*), which contribute to nitrogen circulation, are distributed in the soil environment at the ratio of 0.002–4.16 % [20–25]. Indigenous HDB seem to exist at almost the same ratio and so might have similar roles to ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. Various hydrocarbons exist in the soil environment, and there are several alkane hydroxylases which oxidize not only *n*-alkanes but also *c*-alkanes [16]. These bacteria may oxidize alkane like hydrocarbons such as plant wax and/or fatty acids in the soil environment [13, 26, 27] and take part in the carbon cycle.

Indigenous HDB are known to be increased at various hydrocarbon-contaminated sites [13, 19]. In the present study, the dynamics of HDB during bioremediation were analyzed. The bacteria increased accompanied with a decrease in hydrocarbon, especially of the short to the middle range of *n*-alkanes. Efficiency of biostimulation depended on the indigenous HDB. Activation of these bacteria will contribute to efficient biostimulation in addition to bioaugmentation. Analysis of the soil bacteria and indigenous HDB is important for efficient bioremediation.

The numbers of soil bacteria in hydrocarbon-contaminated soil are usually relatively low [18], and long-chain *n*-alkanes and *c*-alkanes tend to remain for a long time. Inorganic salts were used as nutrients to grow and maintain the indigenous HDB in a biostimulation system. Inorganic nitrogen (N) and phosphorous (P) were used for the adjustment of the C/N/P ratio against hydrocarbon based on the Redfield ratio [28]. Indigenous HDB were not always stimulated by the addition of inorganic salts [29].

In the present study, the total numbers of soil bacteria and HDB (strain NDKK6 and indigenous HDB) were maintained for a long time by addition of organic material and control of the TC. Several investigations have succeeded in biostimulation by addition of

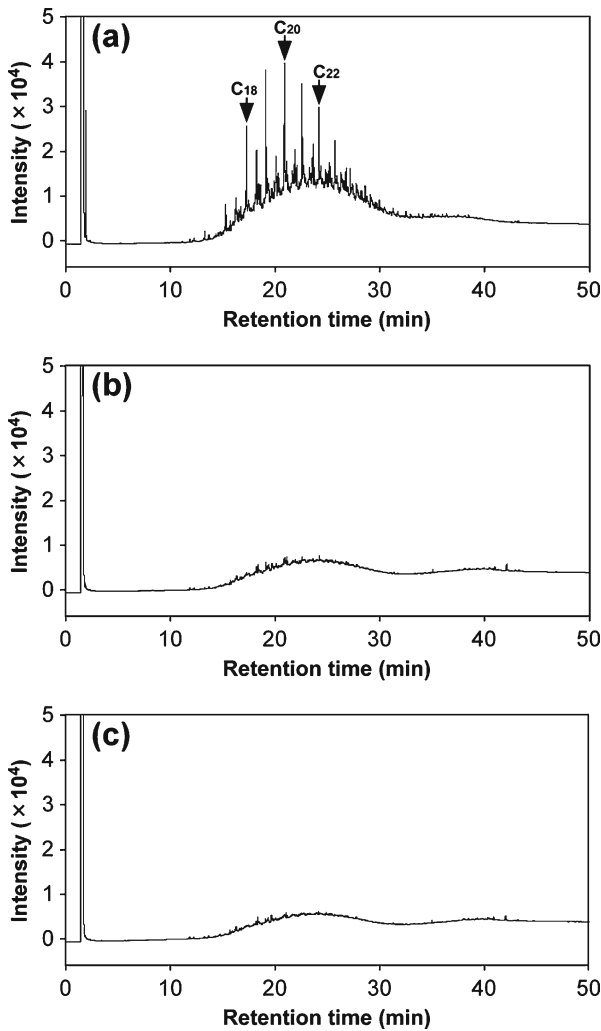


Fig. 7 Degradation of hydrocarbons by indigenous HDB and *R. erythropolis* strain NDKK6 during bioremediation under organic conditions. **a** Gas chromatogram of hydrocarbons from the soil at the start of the experiment. *Arrows* indicate the peaks for known *n*-alkanes and their carbon number. **b**, **c** Gas chromatograms of residual hydrocarbons from the soil of biostimulation and bioaugmentation, respectively, after 28 days of incubation

organic materials such as manure [29–31]. Total C and C/N ratio controls with organic materials will be useful for the growth of the soil bacteria and HDB.

Conclusion

The indigenous HDB were widely distributed throughout the soil environment. These bacteria were increased and maintained when chicken manure was used as a nutrient during bioremediation. The activation and maintenance of HDB by TC- and C/N-controlled environmental conditions contributed to efficient biodegradation.

References

1. Wuxing, L., Yongming, L., Ying, T., Zhengao, L., & Lena, Q. M. (2010). *Environmental Geochemistry and Health*, *32*, 23–29.
2. Hejazi, R. F., Husain, T., & Khan, F. I. (2003). *Journal of Hazardous Materials*, *99*, 287–302.
3. Margesin, R., & Schinner, F. (2001). *Applied and Environmental Microbiology*, *67*, 3127–3133.
4. Fujii, T., Narikawa, T., Takeda, K., & Kato, J. (2004). *Bioscience, Biotechnology, and Biochemistry*, *68*, 2171–2177.
5. Quatrini, P., Scaglione, G., Pasquale, C. D., Riela, S., & Puglia, A. M. (2007). *Journal of Applied Microbiology*, *104*, 251–259.
6. Piccolo, L. L., Pasquale, C. D., Fodale, R., Puglia, A. M., & Quatrini, P. (2011). *Applied and Environmental Microbiology*, *77*, 1204–1213.
7. Kirkwood, K. M., Chernik, P., Foght, J. M., & Gray, M. R. (2008). *Biodegradation*, *19*, 785–794.
8. Mao, D., Lookman, R., Diels, L., Weghe, H. V. D., Vanermen, G., Brucker, N. D., et al. (2009). *Journal of Chromatography. A*, *1216*, 1524–1527.
9. Kubota, K., Koma, D., Matsumiya, Y., Chung, S. Y., & Kubo, M. (2008). *Biodegradation*, *19*, 749–757.
10. Geissdörfer, W., Frosch, S. C., Haspel, G., Ehrh, S., & Hillen, W. (1995). *Microbiology*, *141*, 1425–1432.
11. van Beilen, J. B., Panke, S., Lucchini, S., Franchini, A. G., Röthlisberger, M., & Witholt, B. (2001). *Microbiology*, *147*, 1621–1630.
12. Wentzel, A., Ellingsen, T. E., Kotlar, H. K., Zotchev, S. B., & Throne-Holst, M. (2007). *Applied and Environmental Microbiology*, *76*, 1209–1221.
13. Rojo, F. (2009). *Environmental Microbiology*, *11*, 2477–2490.
14. Dutta, T. K., & Harayama, S. (2001). *Applied and Environmental Microbiology*, *67*, 1970–1974.
15. Koma, D., Sakashita, Y., Kubota, K., Fujii, Y., Hasumi, F., Chung, S. Y., et al. (2003). *Bioscience, Biotechnology, and Biochemistry*, *67*, 1590–1593.
16. Hatayama, K., Sakihama, Y., Matsumiya, Y., & Kubo, M. (2008). In J. B. Dominguez (Ed.) *Soil contamination research trends*. Nova Science, Hauppauge, 143–160.
17. Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., et al. (1997). *Science*, *277*, 1453–62.
18. Aoshima, H., Kimura, A., Shibutani, A., Okada, C., Matsumiya, Y., & Kubo, M. (2006). *Applied Microbiology and Biotechnology*, *71*, 875–880.
19. Harayama, S., Kasai, Y., & Hara, A. (2004). *Current Opinion in Biotechnology*, *15*, 205–214.
20. Okano, Y., Hristova, K. R., Leutenegger, C. M., Jackson, L. E., Denison, R. F., Gebreyesus, B., et al. (2004). *Applied and Environmental Microbiology*, *70*, 1008–1016.
21. Maeda, K., Toyoda, S., Shimojima, R., Osada, T., Hanajima, D., Morioka, R., et al. (2010). *Applied and Environmental Microbiology*, *76*, 1555–1562.
22. Long, X., Chen, C., Xu, Z., Oren, R., & He, J. (2012). *Soil Biology and Biochemistry*, *46*, 163–171.
23. Haase, S., Philippoth, L., Neumann, G., Marhana, S., & Kandelera, E. (2008). *Soil Biology and Biochemistry*, *40*, 1225–1234.
24. Chapuis-Lardy, L., Brauman, A., Bernard, L., Pablo, A. L., Toucet, J., Mano, M. J., et al. (2010). *Applied Soil Ecology*, *45*, 201–208.
25. Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., & Wei, W. (2012). *Microbial Ecology*, *63*, 446–459.
26. Post-Beittenmiller, D. (1996). *Annual Review of Plant Physiology and Plant Molecular Biology*, *47*, 405–430.
27. Schulz, S., Giebler, J., Chatzinotas, A., Wick, L. Y., Fetzter, I., Welzl, G., et al. (2012). *The ISME Journal*, *17*, 1–12.
28. Filler, D. M., Lindstrom, J. E., Braddock, J. F., Johnson, R. A., & Nickalaski, R. (2001). *Cold Regions Science and Technology*, *32*, 143–156.
29. Agarry, S. E., Owabor, C. N., & Yusuf, R. O. (2010). *Bioremediation Journal*, *14*, 189–195.
30. Hamdi, H., Benzarti, S., Manusadžianas, L., Aoyama, I., & Jedidi, N. (2007). *Soil Biology and Biochemistry*, *39*, 1926–1935.
31. Yakubu, B. M., Ma, H., & Zhang, C. (2009). *International Journal of Environmental Pollution*, *36*, 400–410.