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ORIGINAL ARTICLE

Functional characterization of two alkane hydroxylases in a versatile *Pseudomonas aeruginosa* strain NY3

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Abstract *Pseudomonas aeruginosa* strain NY3 has an extraordinary capacity to utilize a wide range of substrates, including *n*–alkanes of lengths C_5 to C_{34}, aromatic compounds, phenols, diesel and crude oil, and it can produce a variety of small bioactive molecules, including rhamnolipids, which can enhance its metabolic capacity for hydrophobic organic pollutants. This capacity makes NY3 a good candidate for use in environmental pollution remediation. Alkane hydroxylases catalyze both the initial and rate-limiting step of the terminal oxidation of *n*–alkanes. To better understand the genetic mechanisms by which *P. aeruginosa* NY3 degrades such a wide range of *n*–alkanes, two putative coding genes of alkane hydroxylases were functionally characterized using a gene-knockout approach with three different degradation systems. The single *n*–alkane test indicated that the hydroxylase AlkB2 acted in the early growth phase and played a major role in the utilization of C_{12}–C_{18}. However, a double mutant showed a trend towards recovery when C_{20}–C_{24} were used as sole carbon source. This suggests that there are other enzymes capable of utilizing *n*–alkanes longer than C_{20}. Tests of both artificial *n*–alkanes mixture and crude oil-containing waste water showed similar results, suggesting that both AlkB1 and AlkB2 are involved in *n*–alkane degradation, and, moreover, that AlkB2 plays a major role. Finally, given the wider functional range of both AlkBs in the mixture of *n*–alkanes compared to that of single *n*–alkanes, these results hint at co-metabolism.

Keywords *Pseudomonas aeruginosa* · Alkane hydroxylase · *n*–alkanes · Crude oil-containing waste water · Degradation efficiency

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Introduction

Oil pollution resulting from environmental spillage or leakage of crude oil and fuels poses a serious threat to global ecosystems (Camilli et al. 2010). Although many technologies have been used for hydrocarbon removal, bioremediation has been considered one of the greenest and most economically, efficient methods for the elimination of these pollutants (Lu et al. 2012). Presently, approximately 60 genera of aerobic bacteria and 5 of anaerobic bacteria have been shown to degrade hydrocarbons (Liu et al. 2014). Among these, those from the species *Pseudomonas* have been increasingly recognized as ideal candidates for hydrocarbons biodegradation. This is due largely to their ability to degrade a wide range of organic compounds. Moreover, members of this species are also widely distributed in natural environments. To this end, *Pseudomonas aeruginosa* RR1 and DQ8 can degrade *n*–alkanes that range in length from C_{12} to C_{34} and C_{14} to C_{30}, respectively (Marín et al. 2003; Zhang et al. 2011). Similarly, *P. aeruginosa* MGP-1 can degrade *n*–alkanes of an even wider range—from C_{11} to C_{40} (Salgado-Brito et al. 2007). However, both the genetic characteristics of these bacteria and the mechanism(s) through which they degrade *n*–alkane are relatively unknown.

In recent years, *alkB*-related genes have been examined in several *P. aeruginosa* strains, including PAO1, DQ8 and SJTD-1 (Marín et al. 2003; Zhang et al. 2011; Liu et al.

2014). *P. aeruginosa* PAO1 has two *alkB* genes, which are involved in the degradation of C_{12}–C_{16} *n*–alkanes by heterologous expression (Marín et al. 2003). Additional research using a crude extract enzyme assay showed that three *alkB* genes of *P. aeruginosa* NCIMB 9571 were also involved in the degradation of *n*–alkanes (ranging from C_7 to C_{14}) (Vandecasteele et al. 1983). Finally, gene knockout methods used in *P. aeruginosa* SJTD-1 demonstrated that both *alkB1* and *alkB2* play a role in the degradation of C_{12}–C_{16} *n*–alkanes (Liu et al. 2014). All these strains harbored two or three *alk*B homologs, with the exception of DQ8, which contained only one *alk*B homolog (Zhang et al. 2011). Collectively, these works show that different alkane hydroxylases may have distinct properties. However, the differences among these distinct alkane hydroxylases are still not fully understood.

In this study, we sought to (1) genetically characterize the function of *alkB1* and *alkB2* in the oxidation of *n*–alkanes, and (2) understand their differences in the NY3 strain. NY3 was chosen because it has the capacity to utilize a wide range of *n*–alkanes, from pentane (C_5) to tetratriacontane (C_{34}), as well as aromatic compounds, phenols, diesel and crude oil (Nie et al. 2010, 2016). To answer these questions, two *alkB* genes were inactivated, and their single and double mutants were constructed. The differences among these three mutants, as well as wild-type strain NY3 were compared in three different degradation assays using a single *n*–alkane, an artificial *n*–alkanes mixture and crude oil-containing waste water. Our results provided data to better characterize *alkB1* and *alkB2* in strain NY3, in addition to providing a more convenient method for testing alkane hydroxylase function.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are described in Table 1.

Media and culture conditions

P. aeruginosa and *E. coli* were routinely grown in Luria-Bertani (LB) (Sambrook et al. 1989) broth or on LB agar, at 37 °C unless otherwise stated. Antibiotics were used at the following concentrations: for *P. aeruginosa*, gentamicin (Gm) at 15 μg mL^{-1} in LB or 150 μg mL^{-1} in *Pseudomonas* isolation agar (PIA), streptomycin (Sm) at 500 μg mL^{-1} in LB or 1500 μg mL^{-1} in PIA, carbenicillin (Cb) at 250 μg mL^{-1} in LB; For *E. coli*, kanamycin (Kan) at 50 μg mL^{-1}, ampicillin (Ap) at 100 μg mL^{-1}, spectinomycin (Spc) at 20 μg mL^{-1} and Gm at 15 μg mL^{-1} in LB.

The growth patterns of the *P. aeruginosa* strain NY3 with *n*-alkanes as well as their alkane-degrading ability were tested using Mineral Salt medium (MSM) (Nie et al. 2016) supplemented with either various *n*-alkanes or crude oil-containing waste water as the sole carbon source. The MSM liquid cultures were grown in 250 mL Erlenmeyer flasks and incubated at 30 °C on a rotary shaker at 150 rpm for 7 days. All *n*–alkanes of defined chain lengths were obtained from Sigma-Aldrich Shanghai Trading (Shanghai, China). For simplicity, *n*–alkanes of defined chain lengths will be referred to by the number of carbon atoms they contain, (e.g., C_{10} refers to decane, C_{11} refers to hendecane).

Cloning of alkane hydroxylase gene homologs from *P. aeruginosa* strain NY3

The *alkB1* and *alkB2* genes of *P. aeruginosa* strain NY3 were amplified by PCR using the following primers: B1EF2 (GTAGAATTCATGTTTGAAAATTTCTCTC), B1HR2 (GTGAAGCTTTCAGGAAGCTGCCGGCCGC), B2EF2 (GTAGAATTCGACGCTTTCCGCCAGCAG) and B2HR2 (GTGAAGCTTAGATGCGCTGGGTGTCGG). The underlined portions in the primer nucleotide sequences were restriction enzyme digestion sites. PCR was carried out using *Taq* polymerase (TaKaRa, Dalian, China) on an ABI PCR system. The amplified fragments were subsequently cloned into the plasmid pMD18-T (TaKaRa) and sequenced. Nucleotide sequences were compared with the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of *P. aeruginosa* NY3 *alkB* knockout mutants

A previously described SacB-based strategy was employed for the construction of gene knockout mutants (Hoang et al. 1998). Briefly, *alkB1* and *alkB2* PCR products were digested with *Eco*RI and *Hin*dIII restriction enzymes at the underlined sites in the primer sequences. PCR products were then cloned separately into plasmid pEX18Amp (Hoang et al. 1998). A DNA fragment containing the Gm^r-lacZ from pZ1918Gm (Schweizer 1993) was then inserted into the *alkB1* gene to construct the final plasmid pEXB1lacZ. For the *alkB2* mutant, 62 bp of the target gene was deleted by *Bam*HI digestion and then a DNA fragment containing the Sm^r/Spc^r from pHPΩ45 (Prentki and Krisch 1984) was inserted. The final plasmid was named pEXB2Sm. Finally, either pEXB1lacZ or pEXB2Sm was introduced into *P. aeruginosa* strain NY3 by triparental mating (Ditta et al. 1980) to give the final *alkB1* and *alkB2* mutants. The *alkB1/alkB2* double mutant was constructed by conjugating pEXB2Sm into the *alkB1* mutant using the same procedure outlined above. All resulting mutants were verified by PCR and their antibiotic resistance. The *alkB1* and *alkB2* single mutants were named NB1D and NB2D, respectively, and the *alkB1/alkB2* double mutant was named NB12DD.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Source or reference
Strains		
Pseudomonas aeruginosa NY3	Wild type, laboratory strain	This study
NB1D	*alk*B1 insertion mutant, GmR	This study
NB2D	*alk*B2 insertion mutant, SmR	This study
NB12DD	*alk*B1/*alk*B2 insertion mutant, GmR/ SmR	This study
Plasmids		
pEX18Amp	oriT$^+$sacB$^+$ gene replacement vector with multiple-cloning site from pUC18, AmpR	Hoang et al. 1998
pHpΩ45	Sm^r/Spc^r gene from the R100.1 plasmid, transcription-termination sequences from pMJK4–18 plasmid, SmR/SpcR	Prentki and Krisch 1984
pZ1918Gm	Source plasmid of Gm^r cassette, GmR	Schweizer 1993
pRK2013	Broad-host-range helper vector, KanR	Ditta et al. 1980
pEXB1	pEX18Amp containing a 1149 bp *alk*B1 fragment, AmpR	This study
pEXB2	pEX18Amp containing a 1433 bp *alk*B2 and upstream DNA fragments, AmpR	This study
pEXB1lacZ	pEXB1 containing Gm^r-lacZ fragment from pZ1918Gm insert in the *Sph*I site, AmpR/GmR	This study
pEXB2Sm	pEXB2 containing Sm^r/Spc^r fragment from pHpΩ45 insert in the *Bam*HI site, AmpR/SpcR	This study

[a] Antibiotic resistance markers: *AmpR* ampicillin, *GmR* gentamicin, *SmR* streptomycin, *SpcR* spectinomycin, *KanR* kanamycin

Test of *P. aeruginosa* NY3 and its *alkB* mutants in liquid medium supplemented with single *n*–alkane

P. aeruginosa NY3 and its *alkB* mutants were first grown in LB medium. The cultures were then inoculated at 5% (*v*/v) into 100 mL MSM medium supplemented with 500 mgL^{-1} *n*–alkanes ranging from C_{12} to C_{24}. Culture growth was measured at OD_{600} at regular intervals, whereby 100 mL of the cell cultures were collected, freeze-dried and weighed. OD_{600} values were converted to cell dry weight (mgL^{-1}) to plot resulting curves. All measurements were taken in triplicate.

Degradation efficiency of *n*–alkanes in both artificial *n*–alkanes mixture and crude oil-containing waste water by *P. aeruginosa* NY3 and its *alkB* mutants

The degradation efficiency of NY3 and its mutants in mixtures of *n*–alkanes was determined by measuring the loss of *n*–alkanes of different carbon lengths from each liquid culture. The inoculum was prepared as described above and supplemented with artificial *n*–alkanes mixture. This mixture was either made of 21 types of *n*–alkanes, with a total concentration is 33.6 g L^{-1} or of 2% (*v*/v) crude oil-containing waste water obtained from the Changqing oilfield. To exclude losses due to evaporation and extraction, two blank controls were also included. Residual cultures *n*–alkanes were extracted with three volumes of *n*–hexane, evaporated, and finally dissolved in the same volume of *n*–hexane. The concentration of each *n*–alkanes was determined by gas chromatography (GC). GC analysis was performed using an Agilent Technologies 6820 N gas chromatograph equipped with an on-column injection, FID detector and HP-5 MS silica capillary column (30 m × 320 μm × 0.25 μm). The carrier gas was Nitrogen (99.99%) at a flow rate of 30 mL min^{-1}. Undecane was the internal standard used to evaluate the rate of specific *n*–alkanes removal. The temperature program was run from 50 °C (7 min isotherm) to 100 °C at 20 °C/min, 1 min isotherm at 100 °C, and further increased to 290 °C at 5 °C/min with a hold temperature of 290 °C for 5 min. The injector and detector temperatures were 300 °C.

Results

Cloning of alkane hydroxylase gene homologs from *P. aeruginosa* NY3

Two *alkB* homologs were successfully amplified from the genomic DNA of *P. aeruginosa* NY3. The *alkB1* and *alkB2* sequence of strain NY3 had been deposited in GenBank under the accession numbers KM114054 and KM114055, respectively. The two *alkB* sequences of strain NY3 showed very high sequence identity with other *Pseudomonas alkBs* sequences. The *alkB1* and *alkB2* genetic sequences obtained from strain NY3 exhibited 69.6% sequence identity, and their derived proteins exhibited 66.2% amino acid sequence identity. The level of identity was conserved among *alkB*

orthologues from other *P. aeruginosa* strains (Smits et al. 2003; Wu et al. 2011; Liu et al. 2014).

Construction of *P. aeruginosa* NY3 *alkB* mutants

To test the *n*–alkanes degradation ability of AlkB1 and AlkB2 in *P. aeruginosa* NY3, we next knocked out the two genes separately and in combination. Two disruption cassettes were constructed for *alkB1* and *alkB2* as previously described in the Materials and methods. First, the *alkB1* gene of *P. aeruginosa* NY3 was disrupted by using the *alkB1* disruption plasmid pEXB1lacZ. Five gentamicin-resistant clones were analyzed by PCR. The *alkB1* region was correctly replaced by *alkB1*::Gm^r in all clones. One of these clones was named NB1D (*alkB1* mutant) and used for all subsequent experiments. Similarly, the *alkB2* gene was disrupted with the *alkB2* disruption plasmid (pEXB2Sm). Ten streptomycin-resistant clones were obtained and analyzed by PCR. One of the positive clones was named NB2D (*alkB2* mutant) and used for all subsequent experiments. The *alkB1/alkB2* double mutant was constructed by disrupting the intact *alkB2* gene in NB1D using the *alkB2* disruption plasmid (pEXB2Sm), and the three clones obtained from the selection plate were verified by PCR. One of these clones was named NB12DD (*alkB1/alkB2* double mutant) and used for all subsequent experiments. The resulting knockout mutants were verified by both PCR and their antibiotic resistance (Figs. 1, 2).

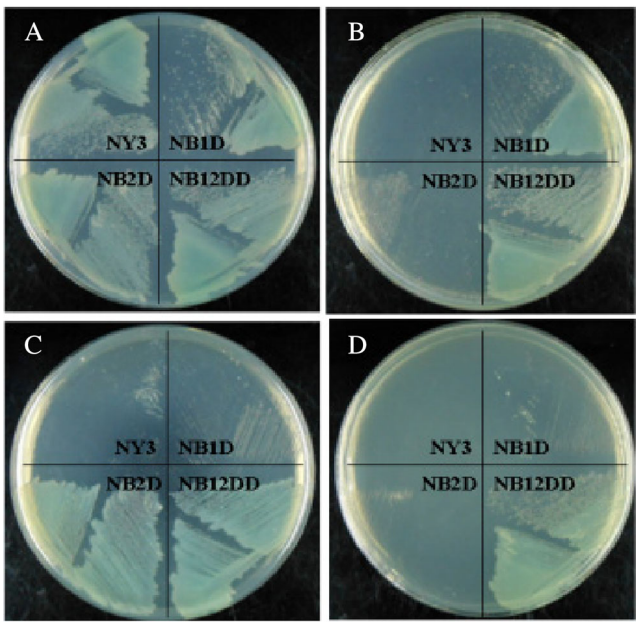

Fig. 1 Antibiotic resistance of NY3 and its mutants. The wide-type strain NY3 and all its three disruption mutants grew on *Pseudomonas* isolation agar (PIA) medium. Only NB1D and NB12DD grew on PIA medium containing gentamicin (Gm), and only NB2D and NB12DD grew on PIA medium containing streptomycin (Sm). NB12DD grew on PIA medium both containing Gm and Sm

Growth curves of NY3 and its *alkB* mutants in single *n*–alkanes

The ability of NY3 and its mutants to utilize *n*–alkanes was studied by monitoring their cell growth for 6 days in MSM medium containing a variety of *n*–alkanes ranging from C_{12} to C_{24}. As shown in Fig. 3, NB1D and NY3 started multiplying immediately after incubation, and reached the exponential growth phase within 24 h in all tested *n*–alkanes. Of all the *n*–alkanes, C_{16} resulted in the highest cell growth, followed by C_{18}. This result suggests that C_{16} is likely the best available carbon source for strain NY3. NB1D grew at a similar rate to the wild-type strain in all tested *n*–alkanes. Furthermore, it grew better than the NB2D mutant in the early growth phase, implying (1) that AlkB2 plays a major role in the early growth phase, and (2) that the initial step of *n*–alkanes utilization is performed predominantly by AlkB2. NB2D showed an obvious growth delay when compared with NB1D and NY3, suggesting that AlkB2 played a more important role in *n*–alkane utilization. NB12DD could not grow on medium containing only substrates of lengths C_{12}–C_{18} (Fig. 3a–d), further demonstrating that both AlkB1 and AlkB2 are responsible for the utilization of C_{12}–C_{18} in strain NY3. However, the addition of longer chain *n*–alkanes to NB2D and NB12DD cultures showed a recovery in their growth phenotype (Fig. 3e–g). This result suggests that there are other—yet unknown—genes involved in the utilization of longer chain-length *n*–alkanes in strain NY3.

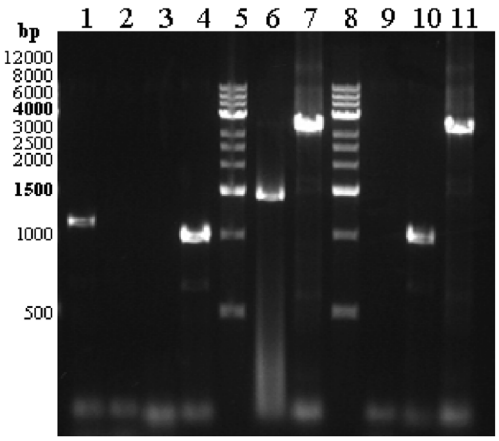

Fig. 2 PCR verification of *alkB* mutants. Lanes: *1* PCR product of NY3 strain using B1EF2/B1HR2 primer; *2* PCR product of NB1D using B1EF2/B1HR2 primer; *3* PCR product of NY3 strain using lacZ-F/B1HR2 primer; *4* PCR product of NB1D using lacZ-F/B1HR2 primer; *5*, *8* DNA ladder; *6* PCR product of NY3 strain using B2EF2/B2HR2 primer; *7* PCR product of NB2D using B2EF2/B2HR2 primer; *9* PCR product of NB12DD using B1EF2/B1HR2 primer; *10* PCR product of NB12DD using lacZ-F/B1HR2 primer; *11* PCR product of NB12DD using B2EF2/B2HR2

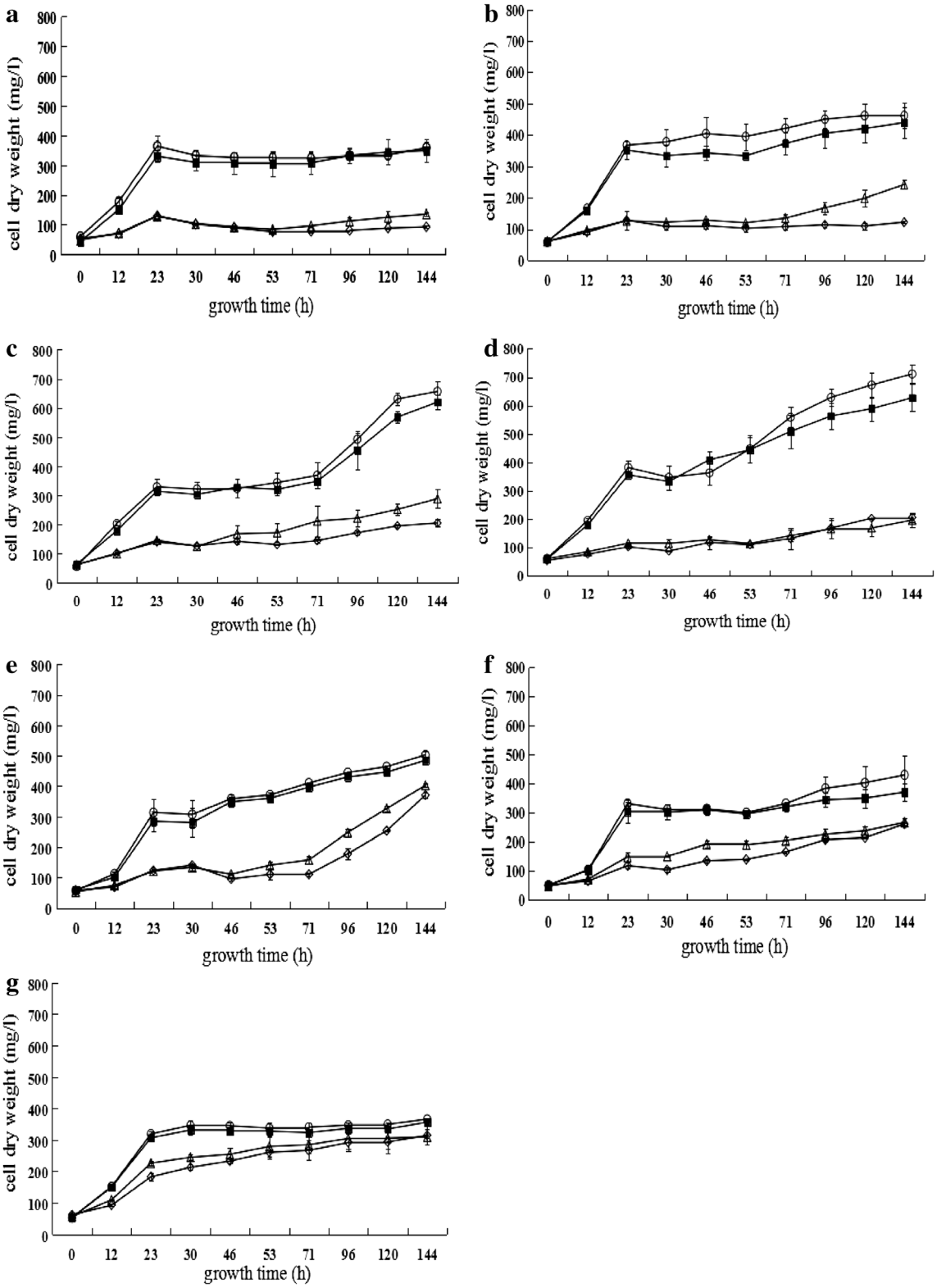

Fig. 3a–g Growth curves of *Pseudomonas aeruginosa* NY3 (O), NB1D (■), NB2D (Δ), and NB12DD (◊) in –alkanes. Strains were inoculated in MSM medium supplemented with 500 mg L^{-1} of the following n –alkanes: C_{12} (**a**), C_{14} (**b**), C_{16} (**c**), C_{18} (**d**), C_{20} (**e**), C_{22} (**f**), C_{24} (**g**) at 30 °C for 144 h. The values presented are the means of experiments run in triplicate

Characterization of the degradation ability of NY3 and its mutants in an artificial mixture of *n*–alkanes

The gas chromotogram presented in Fig. 4 was taken after 7 days of degradation, and revealed that the GC peak for residual *n*–alkanes was the lowest in NY3 and the highest in NB12DD. This indicates that the wild-type strain NY3 was the most efficient in degrading *n*–alkanes. According to the decrease area of each *n*–alkane GC peak, the degradation rates were subsequently calculated using the decreased area of each *n*–alkane GC peak and are shown in Fig. 5. For strain NY3, the degradation rate was between 65 ± 5.49% to 100% with all tested *n*–alkanes. NB1D showed a minor difference with NY3 for *n*–alkanes ranging from C_{10} to C_{20}, demonstrating a markedly lower degradative rate for C_{21} to C_{32} (11 ± 3.50% to 53 ± 2.74%). A similar tendency was seen in NB2D and NB12DD; however, the degradation rate was much lower. This was particularly true for NB12DD. Collectively, these results indicate that AlkB1 and AlkB2 were co-functional in the degradation of *n*–alkanes, with AlkB2 playing a major role in the degradation of middle-chain length *n*–alkanes.

Characterization of the degradation ability of NY3 and its mutants in crude oil-containing waste water

The GC peaks of the residual *n*–alkanes are shown in Fig. 6. These results were similar to those seen in the artificial *n*–alkanes mixture test. The degradation rate was highest in NY3, followed by NB1D and NB2D, with NB12DD being the lowest (Fig. 7). Taken together, this assay also showed that both AlkB1 and AlkB2 are involved in degradation of *n*–alkanes, with AlkB2 being of greater importance than AlkB1 in the degradation of *n*–alkanes.

Discussion

Members of the genus *Pseudomonas* have a rich metabolic diversity and are distributed widely in the ecological environment (Zhang et al. 2011), making them prime potential strains for use in environmental pollution remediation. Some members of the genus *Pseudomonas* have been reported to metabolize chemical pollutants in the environment, including a wide range of aliphatic hydrocarbons and aromatic hydrocarbons, as well as heterocyclic compounds (Kanaly and Harayama 2000; Nam et al. 2003; Onaca et al. 2007). When comparing the range of *n*–alkanes metabolized with that of other *P. aeruginosa* strains, NY3 showed it had a significant advantages in utilizing both short, middle, and long-chain *n*–alkanes. In comparison, other strains (e.g., PAO1, PG201) have been shown to utilize only mid-length *n*–alkanes (Smits et al. 2003; Hardegger et al. 1994), while others (e.g., RR1,DQ8, MGP-1, SJTD-1) utilize only mid- and/or long-chain *n*–alkanes (Marín et al. 2003; Zhang et al. 2011; Salgado-Brito et al. 2007; Liu et al. 2014). Still others (e.g., KSLA 473, NCIMB 8704, NCIMB 9571, ATCC 17423) use short and/or mid-chain *n*–alkanes (Van Beilen et al. 1994, 1998; Vandecasteele et al. 1983). In addition to its wider *n*–alkanes range, *P. aeruginosa* NY3 also produces structurally diverse rhamnolipids (Nie et al. 2010). These are important in that they could potentially enhance its metabolic capacity for hydrophobic organic pollutants. Thus *P. aeruginosa* NY3 was selected as a good candidate for handling crude oil-containing waste water (Nie et al. 2016).

However, the genetic characteristics of NY3 remain elusive, and little is known about the mechanisms through which it breaks down such a wide range of *n*–alkanes. Recent years have shown the importance of alkane hydroxylase enzymes, with *alkB*-related genes having been examined in several *P. aeruginosa* strains (e.g., PAO1,DQ8, SJTD-1) (Marín et al. 2003; Zhang et al. 2011; Liu et al. 2014). Only *P. aeruginosa* strain SJTD-1 has been analyzed using a straightforward gene knockout approach to characterize the function of AlkB (Liu et al. 2014). Other strains were either examined using heterologous expression or through crude extract enzyme assays (Marín et al. 2003; Zhang et al. 2011). To this end, this study inactivated two *alkB* homolog genes of *P. aeruginosa* strain NY3, leading to the construction of three gene knockout mutants. The differences among the three mutants, as well as in wild-type strain NY3, were compared using three different degradation assays.

In the single *n*–alkane test, *P. aeruginosa* strains NY3 showed a rapid multiple rate from *n*–alkanes ranging in length from C_{12} to C_{24}, the measured OD_{600} reached 0.5–0.7 within 24 h. In comparison, *P. aeruginosa* strain SJTD-1 reached 0.1–0.4 after 24 h (Liu et al. 2014). Moreover, *P. aeruginosa* strain MGP-1 reached 3.09×10^9 in 5 days, and the growth cycle was long at 26 days (Salgado-Brito et al. 2007). Three *P. aeruginosa* strains (UMI-82, UMI-88 and UMI-89) were isolated from polluted environmental zones, and entered exponential phase after nearly 3 days when 0.2% hexane and heptane was used as their sole carbon source (Abdelhaq et al. 2002). When the *alkB1* and *alkB2* genes were inactivated in strain NY3, mutant strains showed different growth tendencies. NY3 grew from 358 to 656 mg/L cell dry weight after 144 h, while NB12DD maintained 93 mg/L to 205 mg/L cell dry weight when C_{12}–C_{18} was the sole carbon source. This result indicated that both AlkB1 and AlkB2 functioned in the utilization of C_{12}–C_{18}. However, the dry cell weight of NB12DD was only reduced by 14%–26% when compared with NY3. This occurred when *n*–alkanes of lengths C_{20}–C_{24} were used as the sole carbon source, thereby implying that some unknown genes in NY3 are involved in utilization of these longer chain-length *n*–alkanes. In all the tested *n*–alkanes, NB1D grew at a similar rate to NY3, which was better than the NB2D mutant in the early growth phase. This finding implies that AlkB2 plays a

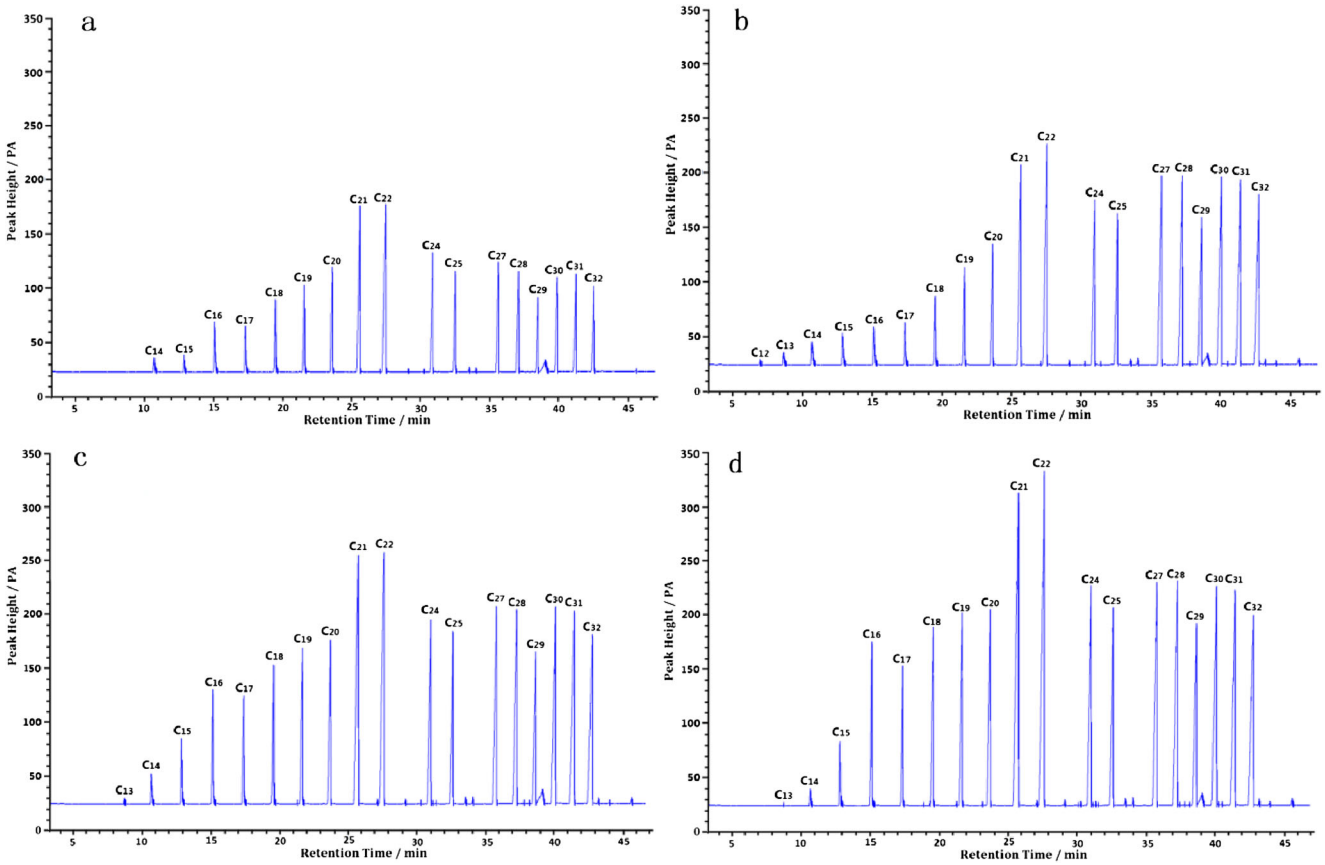

Fig. 4a–d Gas chromatograms of an artificial n- alkane mixture biodegraded by strain NY3 and its mutants after 168 h. Chromatograms are representative examples of experiments conducted in triplicate. Strains: **a** NY3, **b** NB1D, **c** NB2D, **d** NB12DD

major role in the early growth phase, and that the initial step of *n*–alkanes utilization is performed predominantly by AlkB2. This was further verified by Q-PCR, which showed that the *alkB2* gene was expressed during the entire growth phase, while *alkB1* was expressed only under the induction of *n*–alkanes (data not shown).

In the artificial *n*–alkanes mixture test, NY3 showed the highest degradation rate, followed by NB1D, then NB2D, with

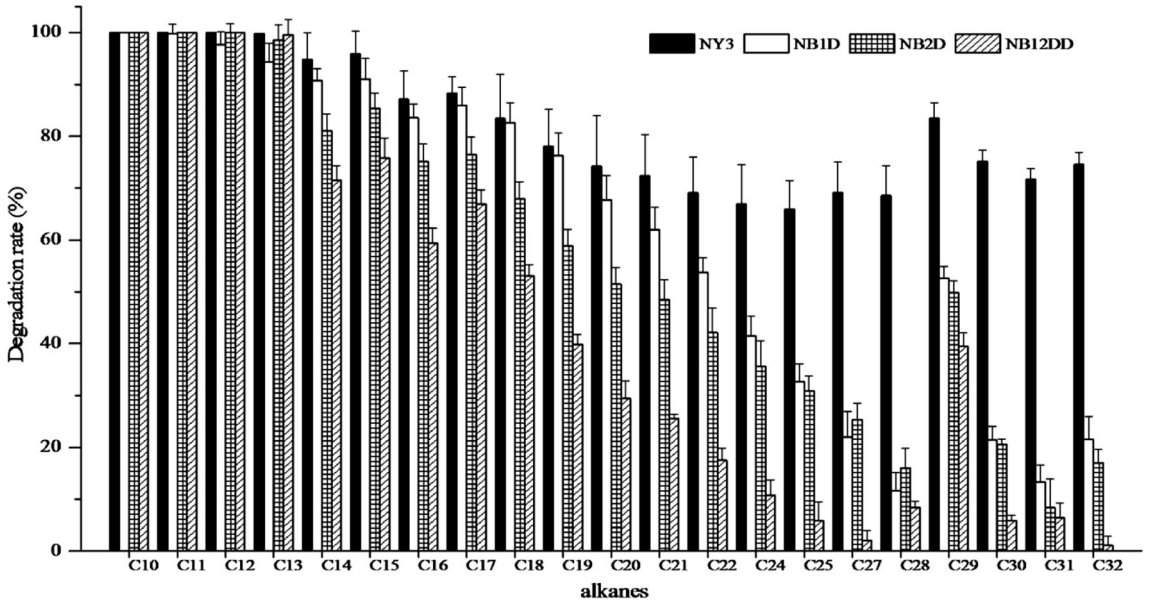

Fig. 5 Degradation rates of n –alkanes in an artificial n –alkane mixture after 168 h of biodegradation by NY3 and its mutants

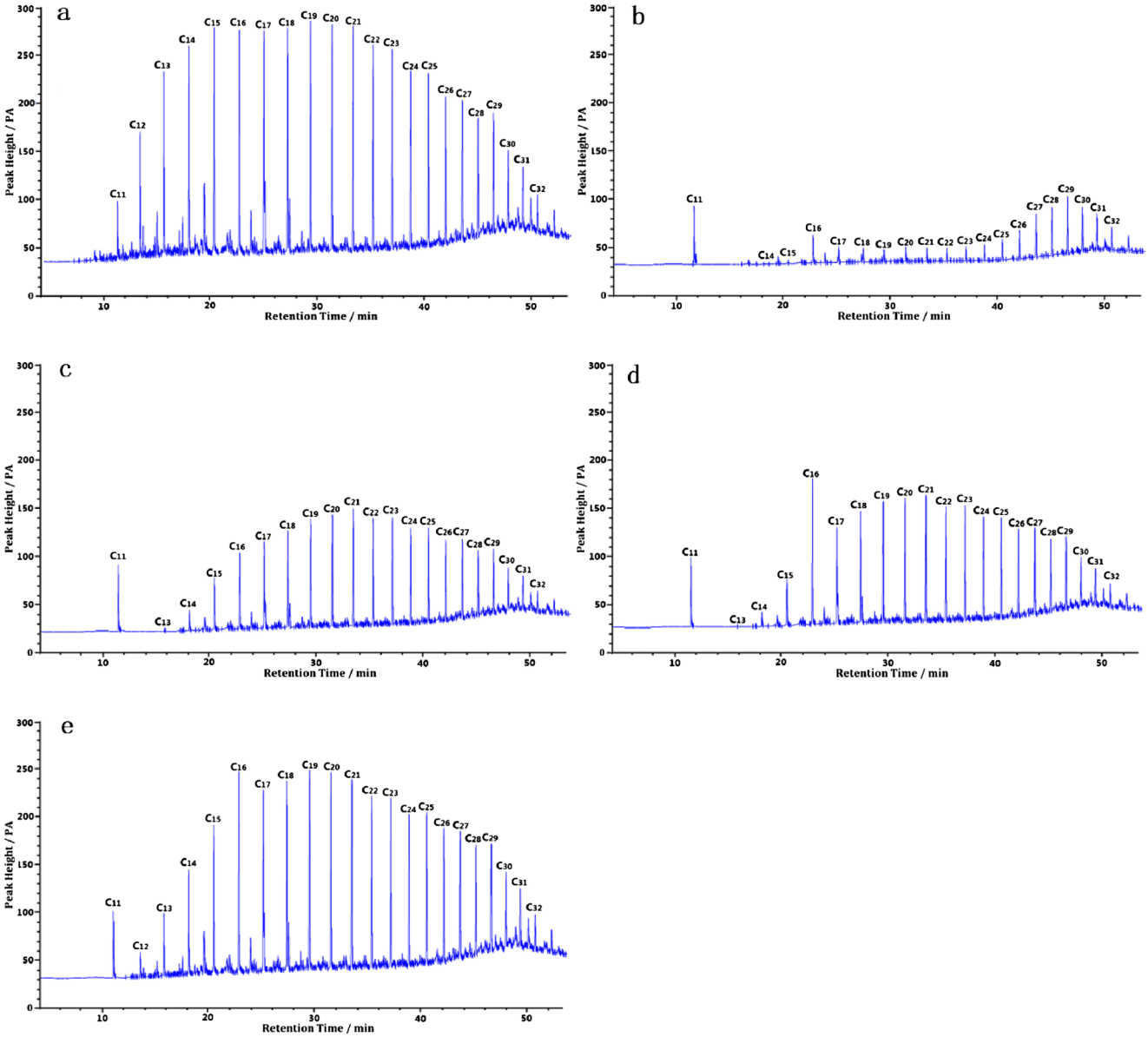

Fig. 6a-e Gas chromatogram of components in crude oil-containing waste water before (**a**) and after 168 h of biodegradation by strain NY3 (**b**), NB1D (**c**), NB2D (**d**) and NB12DD (**e**). Undecane was used as the internal standard. Experiments were performed in triplicate and chromatograms shown are representative of the three results

NB12DD being the lowest. These results were similar to those obtained from the single *n*–alkane test, showing that both AlkB1 and AlkB2 functioned in the degradation of *n*–alkanes, and moreover that AlkB2 played a major role. However, there were still some differences when compared with the single *n*–alkane test. First, NB12DD could not utilize C_{12}–C_{18}. In the artificial *n*–alkanes mixture, the degradation rate of C_{12} and C_{13} reached 100%, while the degradation rate for C_{14}–C_{18} reached 60% to 75% in NB12DD. The high degradation rate of C_{12} and C_{13} could be due partly to evaporation, while the high degradation rate for C_{14}–C_{18} hinted at the existence of the well-known phenomenon of co-metabolism. For instance, Bravo et al. (2015) reported that *P. citronellolis* UAM-Ps1 could oxidize methyl tert-butyl ether (MTBE) using co-metabolism in the presence of either *n*–pentane or *n*–octane. Similarly, Hasanuzzaman et al. (2007) found that *P. aeruginosa* strain WatG was unable to utilize either C_{36} or C_{40} as a sole carbon source; however, more than 25% of each *n*–alkane was degraded when crude oil was added. The mixture of *n*–alkanes may be responsible for the co-metabolism, but which carbon source caused the phenomenon will require further study. Second, the degradation rate of NB12DD for C_{19}–C_{32} was quite low, reaching no higher than 0.7%. We speculate that NB12DD prefers to use co-metabolism of carbon sources rather than initiate other AH systems for degradation. Until now, at least five alkane hydroxylases (AlkB1, AlkB2, P450-1, P450-2 and AlmA) have been found to be involved in

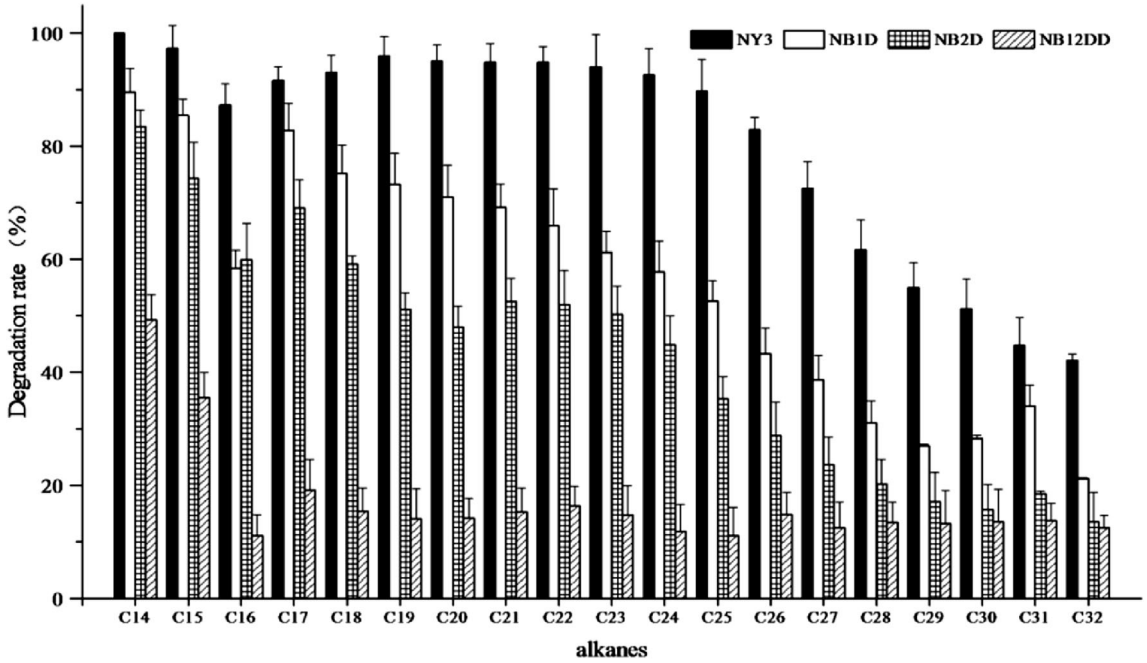

Fig. 7 Degradation rates of n- alkanes in crude oil-containing waste water after 168 h of biodegradation by NY3 and its mutants

the oxidization of *n*–alkanes in *Pseudomonas* (Smits et al. 2002; Funhoff et al. 2006; Throne-Holst et al. 2007). The other three alkane hydroxylases appeared to be silent when NB12DD was grown in the *n*–alkane mixture. In fact, *p450-1*, *p450-2*, and *almA* were acquired by PCR and verified by sequencing analysis in *P. aeruginosa* NY3 (data not shown). In the final test of crude oil-containing waste water, the tendencies of the three mutants in degrading *n*–alkanes were similar, although they did show a lower degradation rate than in the artificial *n*–alkanes mixture. This was particularly true for C_{14} to C_{21}. This may be due to the more complicated components found in the test crude oil (contains 90.66% saturated hydrocarbons, 5.35% aromatic hydrocarbons, 0.7% asphaltine, and 2.66% non-hydrocarbons), with some substances leading to inhibition of cell growth and AlkB activity.

To our knowledge, there has been no previous work testing the function of AlkBs in artificial mixture of *n*–alkanes. Our results also revealed that, in a mixture of *n*–alkanes, NB12DD preferred to use co-metabolism rather than initiate other AH systems. This interesting finding warrants further analysis. Since *P. aeruginosa* NY3 is a good candidate strain for the treatment of oil-contaminated environments, it will be necessary to study other AH systems in strain NY3 further to better understand their roles in *n*–alkanes degradation.

Conclusions

In this study, two homologous genes-*alkB1* and *alkB2*- to *alkBs* of *P. aeruginosa* strains were cloned, three *alkB* disruption mutants were constructed, and their function were tested under three different degradation systems. The single *n*–alkane test indicated that AlkB2 acts in the early growth phase and plays a major role in utilizing C_{12}-$C_{18.}$ This finding also suggested that there are other enzymes for utilization of *n*–alkanes with chain lengths over C_{20}. It is important to understand how strain NY3- in the absence of *alkBs*-still manages to survive on the long-chain *n*–alkanes. In the test of both artificial *n*–alkanes mixture and crude oil-containing waste water, the double mutant showed the lowest degradation rate with all tested *n*–alkanes, followed by the *alkB2* mutant then *alkB1* mutants. This suggests that both AlkB1 and AlkB2 are involved in *n*–alkanes degradation and that AlkB2 plays a significant role.

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