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ORIGINAL PAPER

Gene cloning and characterization of an aldehyde dehydrogenase from long-chain alkane-degrading *Geobacillus thermoleovorans* B23

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Abstract *Geobacillus thermoleovorans* B23 is capable of degrading long-chain alkanes at 70°C. *Bt-aldh*, an aldehyde dehydrogenase gene in B23, was located in the upstream region of *p21* whose expression level was dramatically increased when alkane degradation was started (Kato et al. 2009, BMC Microbiol 9:60). Like *p21*, transcription level of *Bt-aldh* was also increased upon alkane degradation. Bt-Aldh (497 aa, MW = 53,886) was overproduced in *Escherichia coli*, purified, and characterized biochemically. Bt-Aldh acted as an octamer, required NAD^+ as a coenzyme, and showed high activity against aliphatic long-chain aldehydes such as tetradecanal. The optimum condition for activity was 50–55°C and pH 10.0. The activity was elevated to two- to threefold in the presence of 2 mM Ba^{2+}, Ca^{2+}, or Sr^{2+}, while Mg^{2+} and Zn^{2+} inhibited the enzyme activity. Bt-Aldh represents thermophilic aldehyde dehydrogenases responsible for degradation of long-chain alkanes.

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Introduction

Despite their chemical stability and inertness, alkanes are degradable by a wide range of bacteria, including genera *Pseudomonas*, *Acinetobacter*, and *Rhodococcus* that are well studied for their degradation mechanisms of alkanes (Geissdörfer et al. 1999; Ishige et al. 2000; van Beilen et al. 1994). *Pseudomonas putida* GPo1 (previously, *Pseudomonas oleovorans*) possesses OCT plasmid that is responsible for degrading C5-C12 alkanes. The plasmid contains two genetic loci, *alkB/F/G/H/J/K/L* and *alkS/T*, that encode all the proteins necessary to convert alkanes into corresponding primary alcohol, aldehyde, fatty acid, and acyl-CoA (van Beilen et al. 1994). *Acinetobacter* sp. M1 contains two alkane hydroxylases, one of which mainly oxidizes C12 to C16 alkanes, and the other showed a higher activity against C20 or larger alkanes (Maeng et al. 1996). Gram-positive *Rhodococcus* is a bacterial group which harbors multiple alkane hydroxylase gene (*alkB*) systems in the cells (Whyte et al. 2002; Takei et al. 2008). *Rhodococcus* sp. TMP2 is capable of degrading straight and branched chain alkanes and maintains at least five *alkB* genes (*alkB1-B5*, Takei et al. 2008). On the other hand, little information is yet available for alkane degradation genes and enzymes from thermophilic bacteria (Poelarends et al. 2000; Rueter et al.

1994; Zarilla and Perry 1987). Recently, the complete sequence of a cryptic plasmid pLW1071 (57,693 bp) of a long-chain alkane-degrading *Geobacillus thermodenitrificans* NG80-2 was analyzed and a new stand-alone dimeric alkane monooxygenase, LadA (ABO68832), has been characterized (Feng et al. 2007; Li et al. 2008). No other related enzymes for long-chain alcohol, aldehyde, and fatty acid metabolisms have remained unknown.

We had previously isolated *Geobacillus thermoleovorans* B23 from a deep petroleum reservoir in Niigata, Japan. B23 is capable of degrading long-chain alkanes with carbon chain length up to 30 at 70°C (Kato et al. 2001). Recently, it has been found that when the B23 cells are grown in the presence of alkane, production levels of two function-unknown membrane proteins P16 and P21, and several cytoplasmic proteins, such as superoxide disumutase (P24), catalase, and acyl-CoA oxidase, are significantly increased (Kato et al. 2009). We have analyzed 6.1 kb gene fragment which includes P21 gene and found that there are six possible genes in the franking region of *p21* and one of them encoded a putative aldehyde dehydrogenase. Aldehyde dehydrogenase is recognized as one of the essential enzymes for degradation process of various hydrocarbon compounds, including alkane and distributed in a wide range of living organisms. In order to get knowledge of aldehyde dehydrogenase from an extremely thermophilic and long-chain alkane-degrading bacterium *Geobacillus thermoleovorans* B23, Bt-Aldh was overproduced in *E. coli*, purified, and characterized biochemically. Recombinant Bt-Aldh demonstrated its unique preference for metal ions as co-factors and long-chain aldehydes as substrates. Differences of alkane degradation enzymes among related *Geobacillus* strains will be also discussed.

Materials and methods

Bacterial strains and plasmids

An extremely thermophilic alkane-degrading *G. thermoleovorans* B23 was isolated from a deep petroleum reservoir in Minami-aga oil field (Niigata, Japan) (Kato et al. 2001). *E. coli* DH5α [F^-, *φ*80, *lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_k^- m_k^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169, λ^-] (Sambrook et al. 1989) and plasmid vector pCR2.1 (Invitrogen Corp., San Diego, CA) or pUC18 were used for gene cloning experiments. *E. coli* BL21(DE3)pLysS [F^-, *ompT*, *hsdS*$_B$(r_B^- m_B^-), *gal*(*λcI857*, *ind1*, *Sam7*, *nin5*, *lacUV5-T7gene1*), *dcm*(DE3), pLysS (Cm^r)] (Studier et al. 1990) and plasmid pET-25b (Novagen, Madison, WI) were used for gene expression and over production of proteins.

Culture media

Strain B23 was cultivated at 70°C in L broth, which contained per liter 5 g of yeast extract (Difco, Detroit, MI), 10 g of Bacto tryptone (Difco), 5 g of NaCl, and pH was adjusted to 7.2 by 1 N NaOH. Standard gas oil (Tokyo Chemical Industry) was added (0.1% v/v) to the media. NZCYM medium containing per liter 10 g of NZ amine, 5 g of NaCl, 5 g of yeast extract, 1 g of casamino acids, 2 g of $MgSO_4$-$7H_2O$ was used for protein over production experiments in *E. coli*. All the recombinant *E. coli* cells were grown at 37°C in L broth or NZCYM medium containing 50 mg/l ampicillin.

Analysis of transcription level for p21 and Bt-aldh

Cells were collected by centrifugation and frozen on dry ice. Total RNA was isolated by the method as described by Reddy (1995). RT–PCR method was adopted to analyze the transcriptional level of the genes. QIAGEN OneStep RT–PCR Kit (QIAGEN, Valencia, CA) was used in this case (Privitera et al. 1999). RT–PCR primers were constructed to amplify a part (ca. 510 bp) of *p21* and *Bt-aldh* genes. Initial synthesis of cDNA was carried out at 50°C for 30 min using 0.1 μg total RNA as template. Temperature program for amplification reaction was; 95°C for 15 min in the first denaturation step, 35 cycles of 94°C for 60 s—55°C for 60 s—72°C for 60 s, and 72°C for 10 min in the last completion step. The PCR amplified products were analyzed by electrophoresis on 1% agarose gel and ethidium bromide staining.

Overproduction and purification of Bt-Aldh

Bt-aldh was amplified by PCR with a combination of forward (5′-TTTTTCAT*ATG*ACAATGATTTCGGCG-3′) and reverse (5′-TTTTTGAATTCTCCCTCCACGAGCG CCTT-3′) primers. Underlines represent *Nde*I and *Eco*RI sites, respectively, and the initiation codon is shown in italics. The resulting 1.5-kb DNA fragment was digested with *Nde*I and *Eco*RI, and ligated into the *Nde*I-*Eco*RI gap of pET-25b vector to create an overexpression plasmid pET-*Bt-aldh*. In this plasmid, transcription of *Bt-aldh* gene was under the control of T7 promoter. In order to overproduce Bt-Aldh, *E. coli* BL21(DE3)pLysS cells maintaining pET-*Bt-aldh* were grown at 37°C in NZCYM broth containing 50 mg/l ampicillin. When optical density at 660 nm of the culture reached 0.3, isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added at a final concentration 1 mM. Cultivation was continued for additional 3 h. Cells were then harvested by centrifugation at 8,000×*g* for 10 min at 4°C and subjected to the following purification steps.

All the purification procedures were carried out at 4°C. Cells were washed with 50 mM Tris–HCl buffer (pH 7.0). After centrifugation at 8,000×*g* for 10 min, the cell pellet was suspended with the same buffer. Cells were disrupted by a sonifier model 450 (Branson Ultrasonic Corp., Danbury, CT), and centrifuged at 15,000×*g* for 30 min. The supernatant was heated at 50°C for 30 min to precipitate host proteins. After removal of host proteins by centrifuge the resultant supernatant was adjusted to 40% $(NH_4)_2SO_4$ saturation and centrifuged at 15,000×*g* for 30 min at 4°C. The precipitate was suspended in the 50 mM Tris–HCl buffer (pH 7.0) and then dialyzed against excess of the same buffer. This crude enzyme solution was applied to HiTrapQ anionic exchange column fitted to a FPLC system (GE Healthcare, Waukesha, WI). The enzyme was eluted by linearly increasing the NaCl concentration from 0 to 1.0 M in 50 mM Tris–HCl buffer (pH 7.0). Purity of the enzyme was analyzed by SDS–PAGE (Laemmli 1970).

Enzyme activity

Aldehyde dehydrogenase activity was generally determined at 37°C by measuring the initial rate of NAD^+ reduction (NADH production) in an increase of absorption at 340 nm (Black 1951). The assay mixture (1 ml) contained 50 mM tricine buffer (pH 8.5), 1 mM NAD^+, 2% Triton X-100, 1 mM substrate, and an appropriate amount of the enzyme, unless specifically stated otherwise. The enzymatic activity against all-*trans* retinal was determined in 50 mM tricine buffer (pH 8.5) containing 1 mM NAD^+, 2% Triton X-100 and 1 mM all-*trans* retinal (Zhao et al. 1996). One unit (U) of activity is defined as the amount of enzyme catalyzing production of 1 μmol NADH/min.

To determine the enzyme activity against insoluble aldehydes whose carbon numbers are longer than eight, octanal (C8), the product fatty acid was extracted with an equal volume of ethyl acetate and the amount was directly quantified by gas chromatography (GC/FID). GC/FID analysis was performed on an HP6980 instrument (Hewlett–Packard, Palo Alto, CA) equipped with a 30-m non-polar capillary column (HP-1, Hewlett–Packard: internal diameter, 0.25 mm; coated with 0.25-μm film thickness cross-linked methyl siloxane) and a flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 25 ml/min. The temperature program was 80°C (5 min isothermal), 80–300°C (10°C/min), and 350°C (10 min isothermal). The sample (1 μl) was injected by split mode at a ratio of 50:1 at 250°C. Aldehydes with carbon chain length longer than C14, tetradecanal, were not tested because of their commercial unavailability.

Results

Aldehyde dehydrogenase gene from *G. thermoleovorans* B23

An aldehyde dehydrogenase gene (*Bt-aldh*) was located approximately 1.2 kb upstream of an alkane-inducible gene, *p21* (Fig. 1, AB047106, Kato et al. 2009). Bt-Aldh is composed of 497 amino acid residues and its calculated molecular weight is 53,886 Da. Bt-Aldh showed significant amino acid sequence identities of 99.3% to BAD77057 from *G. kaustophilus* HTA426 and 91.5% to ZP_03557706 from *Geobacillus* sp. Y412MC61, and moderate identities of 50.7% to YP_1940491 from extremely acidophilic candidates "Methylacidiphilum infernorum" V4, 49.5% to *p*-cumic aldehyde dehydrogenase, CymC, from *Pseudomonas putida* (AAB62298, Eaton 1997), 48.5% to a putative aldehyde dehydrogenase, DhaS, from *Bacillus subtilis* (CAB13823, Kunst et al. 1997), and 47.3% to retinal dehydrogenase, Raldh2, from *Mus musculus* (CAA67666, Zhao et al. 1996). Phylogenetic tree indicates that Bt-Aldh represents a new group of Aldhs (Fig. 2). Possible consensus sequence of the NAD^+ binding site, G-X-T-X-X-G at 238-243 (Liu et al. 1997) and the catalytic amino acid residues Glu260 (Abriola et al. 1987) and Cys294 (von Bahr-Lindström et al. 1985) are completely conserved in Bt-Aldh.

Transcriptional induction of p21 and Bt-aldh by alkane

Total RNA was prepared from B23 cells grown for 0, 4, and 10 days in the cultures containing alkane. In order to compare transcription level of the genes, RT–PCR was applied for each RNA sample. Although this method does not directly quantify the amount of mRNA, it is more sensitive than Northern blot analysis method and the amount of RT–PCR product roughly correlates to the transcription level for each gene (Privitera et al. 1999). It was demonstrated that the bands for both *p21* and *Bt-aldh* similarly and dramatically increased after 10-day cultivation, exactly when the alkane degradation started (Fig. 3, Kato et al. 2001). Although *p21* had previously shown to be solely transcribed from the result of Northern-Blot analysis (Kato et al. 2009), both *p21* and *Bt-aldh* are suggested to participate in alkane degradation pathway of *G. thermoleovorans* B23.

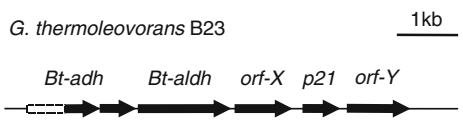

Fig. 1 Physical map of the genes encoding Bt-Adh, Bt-Aldh, ORF-X, P21, and ORF-Y (AB047106). *Arrows* indicate the direction of transcription. P21 is a membrane protein whose production level is specifically induced upon alkane degradation (Kato et al. 2009)

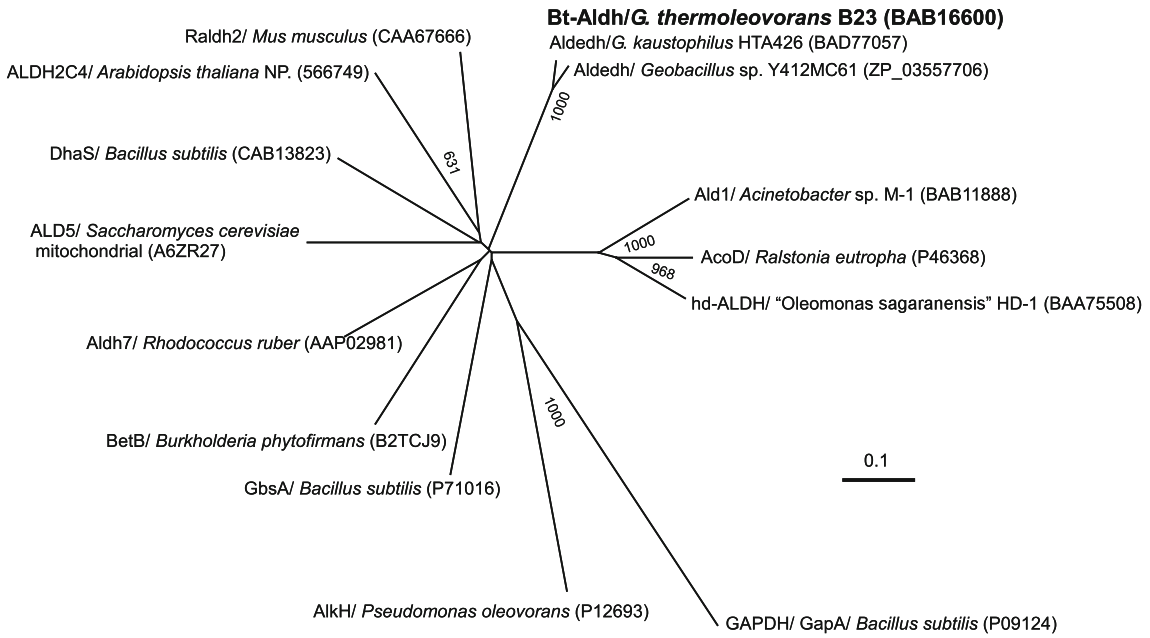

Fig. 2 Phylogenetic tree derived from amino acid sequence of several Aldhs containing Bt-Aldh. GAPDH, glyceraldehyde-3-P dehydrogenase was used as an out group. Bt-Aldh (BAB16600; *Geobacillus thermoleovorans* B23), Aldh (ZP_03557706; *Geobacillus* sp. Y412MC61), DhaS (CAB13823; *B. subtilis*), ALD5 (A6ZR27; *Saccharomyces cerevisiae* mitochondrial), Raldh2 (CAA67666; *Mus musculus*), ALDH2C4 (NP_566749; *Arabidopsis thaliana*), Aldh7 (AAP02981; *Rhodococcus ruber*), BetB (B2TCJ9; *Burkholderia phytofirmans* PsJN), GbsA (P71016; *B. subtilis*), AlkH (P12693; *Pseudomonas oleovorans*), Ald1 (BAB11888; *Acinetobacter* sp. M-1), AcoD (P46368; *Ralstonia eutropha*), hd-ALDH (BAA75508; "Oleomonas sagaranensis" HD-1), GAPDH (GapA, P09124; *B. subtilis*)

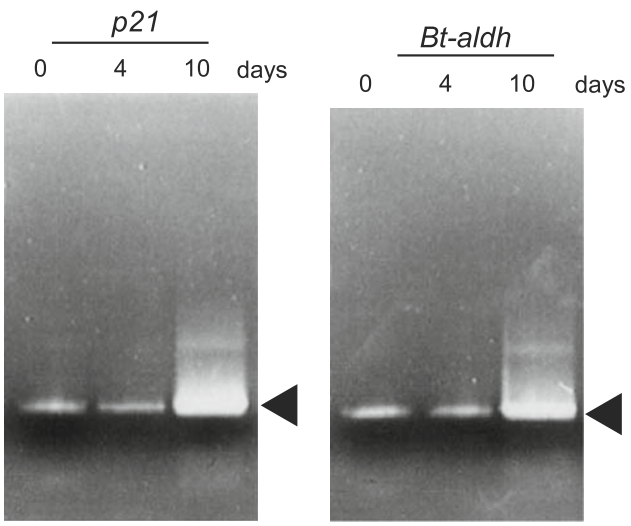

Fig. 3 Transcription levels of *p21* and *Bt-aldh* upon induction by alkane. Relative amounts of mRNA of *p21* and *Bt-aldh* are compared by RT–PCR method. RT–PCR analysis was performed using total RNA isolated from B23 cultures before (0 day) and after induction by alkanes for 4 and 10 days. RT–PCR products were loaded on an 1% agarose gel and stained by ethidium bromide. In every RT–PCR 0.1 μg of RNA was used as template. Positive signals were detected at expected position ca. 510 bp

Overproduction and purification of Bt-Aldh

Bt-Aldh was produced in the cells as a major protein upon induction of the gene by 1 mM IPTG (Fig. 4, lane 2). Its production level was roughly estimated to be 10 mg/l culture, judging from the intensity of the band visualized by Coomassie Brilliant Blue staining after SDS–PAGE. Most of the Bt-Aldh was recovered in a soluble fraction after sonication. After the purification steps, Bt-Aldh gave nearly a single band on SDS–PAGE gel (Fig. 4, lane 7). Finally 6.4 mg of Bt-Aldh was obtained from 2-l culture. The molecular weight was estimated at 55,000 Da by SDS–PAGE and 410,000 Da by gel permeation chromatography on Superdex-200, respectively. These results suggest that Bt-Aldh acts as an octameric form. Although it also eluted at a position equivalent to hexadecameric form, its specific activity was only about 5% of octameric enzyme. Although retinal dehydrogenase (Raldh2) has been reported to act as a tetramer (Zhao et al. 1996), no tetrameric form was observed for Bt-Aldh.

Properties of Bt-Aldh

The activity of Bt-Aldh was doubled in the presence of NAD^+ than $NADP^+$. This result demonstrates that Bt-Aldh is indeed a member of NAD^+-dependent aldehyde dehydrogenases. It was also found that the enzyme prefers alkaline conditions and optimum pH was around 10.0. Bt-Aldh was fully stable up to 55°C but its activity decreased to 12.7% after a heat treatment at 70°C for 30 min. The enzyme activity of Bt-Aldh was increased by

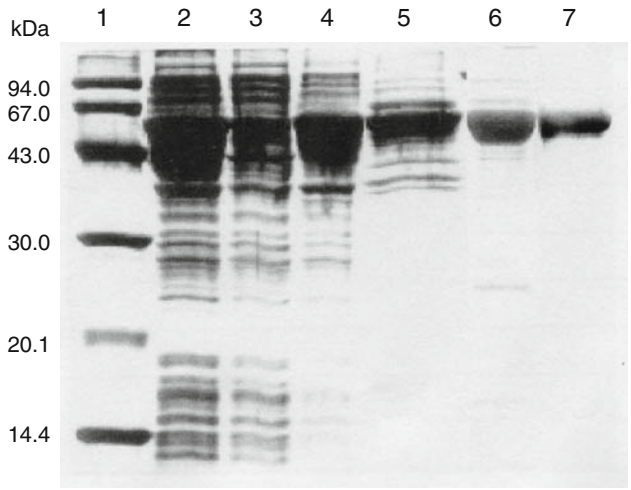

Fig. 4 Overproduction and purification of the recombinant Bt-Aldh. Samples were subjected to electrophoresis on 10% polyacrylamide gel in the presence of SDS. Lane 1, molecular weight markers; phosphorylase b, 94.0 kDa; bovine serum albumin, 67.0 kDa; ovalbumin, 43.0 kDa; carbonic anhydrase, 30.0 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa. Lane 2, whole cell extracts. Lane 3, insoluble fractions after sonication of the cells. Lane 4, soluble fractions after sonication of the cells. Lane 5, soluble fractions after heat treatment at 50°C for 30 min. Lane 6, proteins after 40% $(NH_4)_2SO_4$ precipitation. Lane 7, Bt-Aldh purified by anionic exchange column HiTrapQ

Table 1 Effects of divalent metal ions and EDTA on Bt-Aldh activity

Metal ions	Relative activity
None	1.0
EDTA	0.63
$BaCl_2$	2.1
$CaCl_2$	1.9
$CoCl_2$	1.2
$CuCl_2$	0.87
$MgCl_2$	0.14
$MnCl_2$	1.1
$NiCl_2$	0.97
$SrCl_2$	3.4
$ZnCl_2$	<0.01

The enzyme was previously treated with 2 mM EDTA and dialyzed against 50 mM Tris–HCl buffer (pH 7.0) before measuring its enzymatic activity. The enzyme activity was determined at 37°C in 50 mM glycine buffer (pH 10.0) containing 1 mM hexanal, 1 mM NAD^+, 2% Triton X-100, and 2 mM EDTA or 2 mM each metal ion

two- to threefold in the presence of 2 mM $BaCl_2$, $CaCl_2$, and $SrCl_2$, and inhibited by 2 mM $MgCl_2$ and $ZnCl_2$ (Table 1). $SrCl_2$ was the most effective co-factor for the activity. Although the effect of metal ions has not been examined for related Aldhs, activities of NADPH-dependent ubiquinone reductase from rat liver (Studier et al. 1990) and methanol dehydrogenase from *Paracoccus denitrificans* (Harris and Davidson 1994) have been reported to be enhanced by Sr^{2+} as well as Mg^{2+} or Ca^{2+}, respectively.

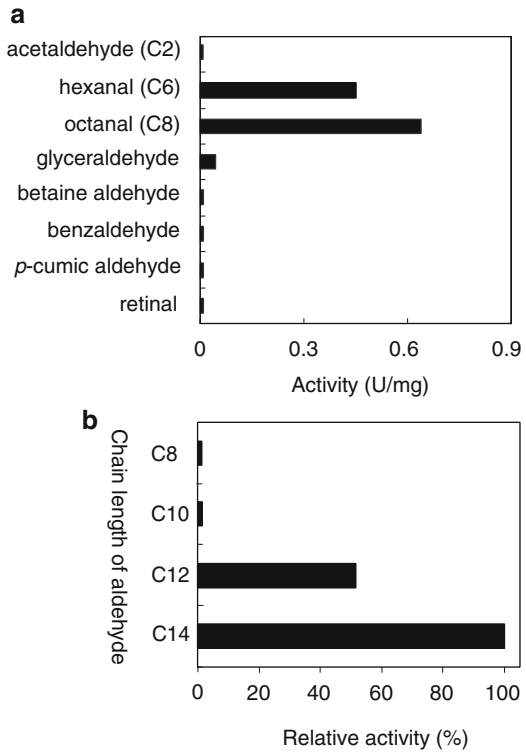

Fig. 5 **a** Substrate specificity of Bt-Aldh. Enzymatic activity was determined colorimetrically at 37°C for 10 min in 50 mM glycine buffer (pH 10.0) containing 2 mM $SrCl_2$, 1 mM NAD^+ and 2% Triton X-100. **b** Enzyme activity against aliphatic aldehydes with carbon chain longer than 8 (C8) was determined by directly measuring the amount of the product, fatty acid, by the GC/FID. Reaction temperature and time were 55°C and 10 min, respectively, in this case

It was obvious that Bt-Aldh is active against long-chain aldehydes. Among tested soluble substrates, Bt-Aldh showed the highest activity against octanal (C8) at a specific activity of 0.63 U/mg (Fig. 5a). The enzyme activities against insoluble long-chain aldehydes were determined by directly measuring the amount of reaction product (fatty acid) by GC/FID (Fig. 5b). In the range of substrate aldehydes with carbon numbers C2 to C14, Bt-Aldh was the most active against C14, tetradecanal at 55°C. According to this method, the activity against tetradecanal was seventy-three times higher than that against octanal.

Discussion

The genes encoding long-chain aldehyde dehydrogenase, *ald1* and *hd-aldh*, have already been cloned from *Acinetobacter* sp. M-1 and "Oleomonas sagaranensis" HD-1,

respectively (Ishige et al. 2000; Okibe et al. 1999; Kanamori et al. 2002). hd-Aldh showed ten times higher kcat/Km value for decanal than acetaldehyde (Okibe et al. 1999). Despite their substrate preference for long-chain alkyl aldehyde, Ald1 and hd-Aldh showed significant amino acid sequence similarity with another group of NAD^+-dependent Aldhs, including AcoD by 69 and 76% identities, respectively. AcoD is an Aldh involved in acetoin and ethanol catabolisms in *Ralstonia eutropha* (P46368; Priefert et al. 1992). Substrate specificity of the enzyme may be governed by a very limited number of amino acid residues.

The structure of retinal dehydrogenase, Raldh2 has been elucidated at 2.7Å (Lamb and Newcomer 1999). The structure was carefully compared with that of mitochondrial ALDH2 which is active against short-chain acetaldehyde. Preference of Raldh2 for the substrate with long alkyl chain has been explained by a characteristic disordered loop structure at C-terminal domain. Replacement of F459/G460 in ALDH2 with L459/N460 (non-Gly residue) in Raldh2 was shown to be crucial for the structural distortion to accept bulky substrates (Bordelon et al. 2004). Because respective amino acid residues were L453/S454 in Bt-Aldh, it could have a similar disordered loop structure like Raldh2. Further structure analysis and mutation works would contribute to identify the factors important for activity of thermophilic long-chain aldehyde dehydrogenase.

Bt-*aldh* was found in the upstream region of alkane-inducible *p21* (Fig. 1). Another gene encoding putative ORF-X (302 aa) was also found 196 bp upstream of *p21*. ORF-X has significant sequence identity (42%) with a probable ribonucleotide reductase small chain of *Mycobacterium tuberculosis* (3EE4_A; Andersson and Högbom 2009). Ribonucleotide reductase has been reported to form class I (large helixbundle proteins) diiron-carboxylate protein family (Fox et al. 1994) together with alkane hydroxylases and methane monooxygenases. ORF-X could be an alkane hydroxylase/monooxygenase in *G. thermoleovorans* B23. Another possible open reading frame, ORF-Y, was found at 143 bp downstream of *p21* but ORF-Y shares no significant sequence similarity with any other proteins in database.

Strain B23 showed significant identity of 16SrRNA gene sequence, 99.4%, with *Geobacillus kaustophilus* HTA426, which was isolated from the deep-sea sediments of the Mariana Trench (Takami et al. 2004a) and whose genome sequence has been determined (Takami et al. 2004b). We found that all the genes corresponding to *Bt-adh*, *Bt-aldh*, *orf-X*, *p21*, and *orf-Y* were completely conserved in *G. kaustophilus* HTA426 (BAD77059, BAD77057, BAD77056, BAD77055, BAD77054, respectively). Identity of the total nucleotide sequences of B23 and HTA426 in these gene regions was 99.3% (6087/6127 bases). It should be noted that these genes are missing in the genome of another long-chain alkane-degrading *G. thermodenitrificans* NG80-2. Moreover, HTA426 genome contains no orthologous gene of a plasmid-born alkane monooxygenase, LadA (ABO68832) from NG80-2. Long-chain alkane degradation systems in *G. thremoleovorans* B23 and *G. kaustophilus* HTA426 may be similar to each other, but they are different from *G. thermodenitrificans* NG80-2 (Feng et al. 2007).

Conclusion

A gene encoding a thermostable aldehyde dehydrogenase, *Bt-aldh*, from *G. thermoleovorans* B23 was cloned and overexpressed in *E. coli*. Special characteristics of the recombinant enzyme Bt-Aldh were the preference for long-chain aldehyde substrates, alkaline pH conditions, and Ba^{2+}, Ca^{2+}, Sr^{2+} ions. Expression level of *Bt-aldh* and *p21* were similarly increased at the time when alkane degradation started, suggesting their contribution to long-chain alkane degradation in B23 cells. Long-chain alkane degradation systems in *G. thremoleovorans* B23 and *G. kaustophilus* HTA426 may be similar to each other, but they are different from *G. thermodenitrificans* NG80-2.

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