Extremophiles (1999) 3:293–296

ORIGINAL PAPER

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Reidentification of the keratinase-producing facultatively alkaliphilic *Bacillus* sp. AH-101 as *Bacillus halodurans*

Received: June 10, 1999 / Accepted: August 6, 1999

Abstract Alkaliphilic *Bacillus* sp. AH-101 was characterized in terms of physiological and biochemical characteristics, and 16S rDNA sequence homology and DNA–DNA hybridization analyses were performed. Phylogenetic analysis of strain AH-101 based on comparison of 16S rDNA sequences revealed that this strain is closely related to *Bacillus halodurans*. DNA–DNA hybridization of AH-101 and related *Bacillus* reference strains showed that the highest level of DNA–DNA relatedness (88%) was found between strain AH-101 and the *B. halodurans* type strain (DSM497). Our findings demonstrate that strain AH-101 is a member of the species *B. halodurans*.

Key words Alkaliphilic *Bacillus halodurans* AH-101 · DNA–DNA hybridization · Phylogenetic tree · Identification

Introduction

Since 1969, we have isolated a great number of alkaliphilic *Bacillus* strains from various environments and have purified many alkaline enzymes (Horikoshi 1996). Since the first report on the alkaline protease from alkaliphilic *Bacillus clausii* 221(DSM2512) was published, there have been extensive studies on alkaline proteases from other strains of alkaliphilic *Bacillus* such as YaB (Tsai et al. 1983), NKS-21 (Tsuchida et al. 1986), B-21-2 (Fujiwara and Yamamoto 1987), and AH-101 (Takami et al. 1990). The alkaliphilic bacterium strain AH-101 (JCM9161), isolated in 1989 (Takami et al. 1989), was identified as a member of the genus *Bacillus* capable of producing an extremely thermostable alkaline keratinase. This enzyme was found to be most active toward casein at pH 12–13 and was stable for 10 min at 60°C in the pH range 5–12. The temperature optimum for activity was about 80°C in the presence of 5 mM Ca^{2+}. This enzyme readily digests human hair and nail in alkaline buffer (pH 11–13) containing 1% thioglycolic acid (Takami et al. 1992) and offers great advantages for industrial processes such as leather tanning and wastewater treatment, and for domestic products such as toiletries for depilation. The unique properties of the thermostable alkaline keratinase produced by *Bacillus* sp. AH-101, as mentioned, have often been compared with those of alkaline proteolytic enzymes from other *Bacillus* strains (Fujiwara et al. 1993; Kobayashi et al. 1995; Horikoshi 1996; Ito et al. 1998). Thus, strain AH-101 is very important as a reference strain among alkaline protease producers and for industrial application.

Recently, alkaliphilic *Bacillus* strains were classified into 11 groups on the basis of 16S rDNA sequence data, and the major alkaliphilic *Bacillus* species were proposed to be the following: *B. pseudofirmus, B. agaradhaerens, B. clarkii, B. halodurans, B. clausii, B. cohnii, B. halmapalus, B. horikoshii, B. pseudoalcalophilus, and B. gibsonii* (Fritze et al. 1990; Nielsen et al. 1995). The taxonomic placement of *Bacillus* sp. AH-101 is still unclear, although this strain is similar to *Bacillus clausii* or *Bacillus halodurans* in some physiological properties.

Analysis of the genome of *Bacillus halodurans* C-125 (Takami and Horikoshi 1999) was initiated in 1998 as a standard model for facultatively alkaliphilic *Bacillus* strains, and the systematic sequencing of the whole genome of alkaliphilic *Bacillus halodurans* C-125 will be finished soon (Takami et al. 1999). Thus, we can expect that knowledge of the complete nucleotide sequence of the *Bacillus halodurans* genome will facilitate identification of the regulatory regions controlling enzyme production in alkaliphilic *Bacillus* strains.

In this article, we attempted to identify strain AH-101 not only based on conventional physiological and biochemical characteristics but also through phylogenetic analysis based on comparison of 16S rDNA sequences and through comparison of DNA–DNA hybridization patterns.

Communicated by G. Antranikian

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Materials and methods

Isoprenoid quinones

Isoprenoid quinones were extracted from dried cells with chloroform–methanol (2:1) and purified by thin-layer chromatography. The purified isoprenoid quinones were analyzed by reverse-phase high-performance liquid chromatography (Komagata and Suzuki 1987), and the absorbance was measured at 270 nm using menaquinone as a standard.

DNA studies

DNA was extracted by a previously described method (Saito and Miura 1963). The G + C content was determined by reverse-phase high-performance liquid chromatography (Tamaoka and Komagata 1984). For analysis of relatedness, DNA–DNA hybridization was carried out at 40°C for 3 h and measured fluorometrically by a previously described method (Ezaki et al. 1989).

16S rDNA sequencing and analysis

Polymerase chain reaction (PCR) amplification of the 16S rDNA was performed with a DNA thermal cycler (model 9600; Perkin Elmer, Norwalk, CT, USA) using 50-μl PCR reaction mixtures under the conditions recommended by the enzyme manufacturer (Takara, Otsu, Japan) according to the procedure reported previously (Takami et al. 1997). Prokaryote-specific primers (Takami et al. 1997) and the following *Bacillus halodurans* specific primers were used for gene amplification and sequencing: ABSF (5′-TTTATCGGAGAGTTTGATCCTGGCTC-3′), and ABSR (5′-AGAAAGGAGGTGATCCAGCCGCACC-3′). Sequencing of PCR-amplified fragments was performed with a DNA sequencer ABI PRISM 377 using a Taq Dye Terminator Cycle Sequencing Kit (Perkin Elmer). 16S rDNA sequences were aligned using the Clustal multiple-alignment program (Clustal W) (Thompson et al. 1994). Sites involving gaps were excluded from all analyses. A phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei 1987), using the DNADIST and NEIGHBOR programs in the PHYLIP package, version 3.57 (Felsenstein 1995). The nucleotide sequence data reported in this article have been submitted to DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number AB027713.

Results and discussion

Morphological, physiological, and biochemical properties

Morphological and physiological properties of *Bacillus* sp. AH-101 were investigated according to the methods described in *The Genus Bacillus* (Gordon et al. 1973) and *Bergey's Manual of Determinative Bacteriology* (Sneath et al. 1986). *Bacillus* sp. AH-101 was found to be aerobic, spore-forming, gram-positive, rod-shaped, 2.5–5 μm long and 0.8–0.9 μm wide, and motile. The cells were found to

Table 1. Characteristics of alkaliphilic *Bacillus* sp. strain AH-101

Cell shape	Rods
Cell size	0.8–0.9 × 2.5–5.0 μm
Spore shape	elipsoidal
Sporangium swollen	+ (w)
Anaerobic growth	+
VP reaction	−
Gram behavior	+
KOH test	−
Growth	
At 40°C	+
At 50°C	+
At 55°C	+
Growth	
In medium pH 7.5	+
In medium pH 9.7	+
Growth	
In NaCl 2%	+
In NaCl 5%	+ (w)
In NaCl 7%	−
Hydrolysis of	
Starch	+
Gelatin	+
Casein	+
Tween 60	−
Tween 40	+
Tween 20	n.g.
Use of citrate	+
NO_2 from NO_3	−
Phenylalanine deaminase	n.g.
Aminopeptidase	−
Catalase	+
Oxidase	+ (w)
Major isoprenoid quinone	7
G + C content	44.1

w, weak; n.g., no growth

Table 2. DNA–DNA hybridization between *Bacillus* sp. AH-101 and other related strains

Strain	DNA–DNA hybridization (%) with			
	Bacillus sp. AH-101 (JCM 9161)	*Bacillus halodurans* (DSM 497^T)	*Bacillus halodurans* C-125 (JCM 9153)	*Bacillus alcalophilis* (JCM 485^T)
Bacillus sp. AH-101(JCM 9161)	100	81	87	13
Bacillus halodurans (DSM 497^T)	88	100	91	17
Bacillus halodurans C-125 (JCM 9153)	97	92	100	15
Bacillus alcalophilis (JCM 485^T)	16	20	21	100

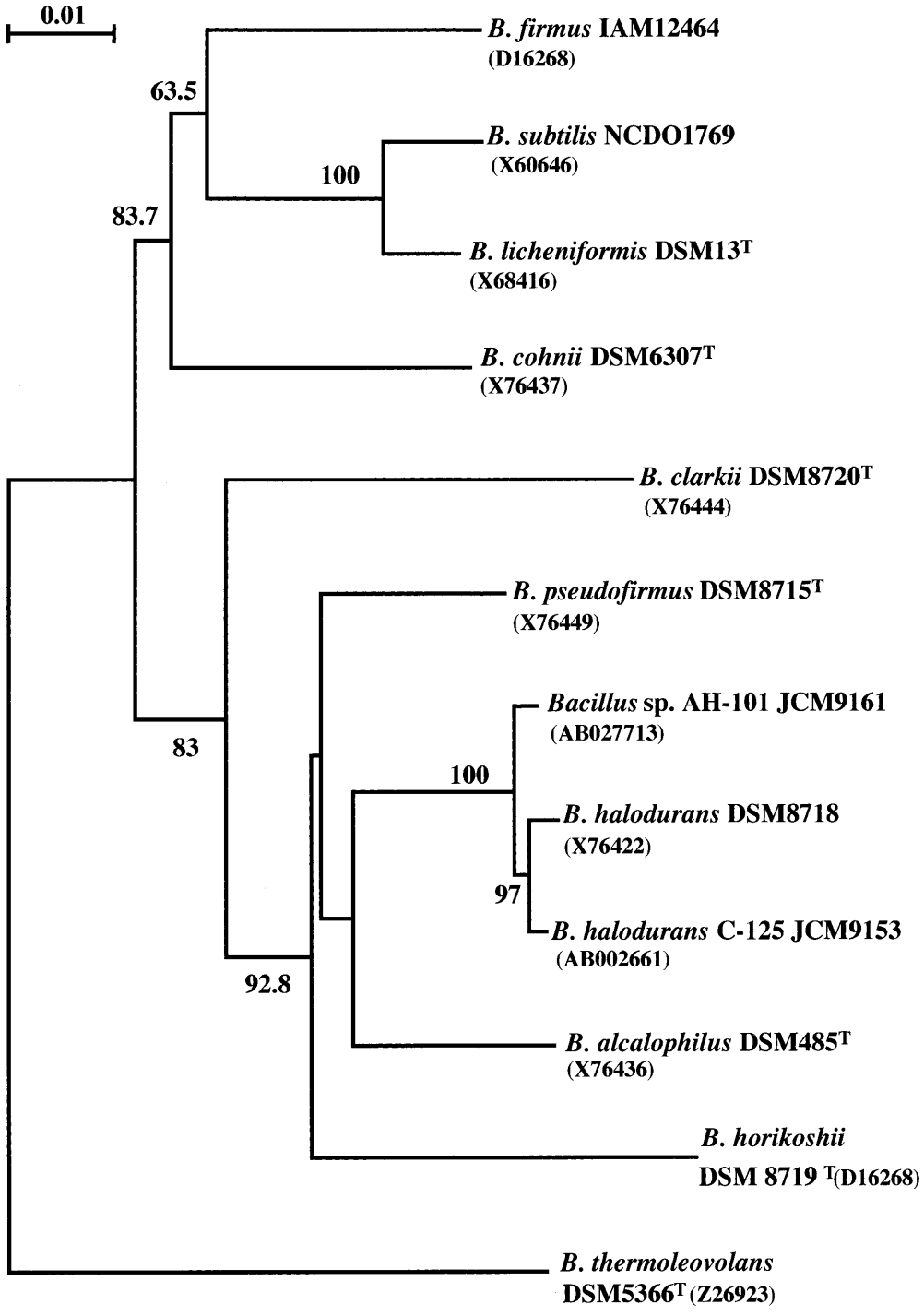

Fig. 1. Unrooted phylogenetic tree showing the relationship of strain AH-101 to other *Bacillus* strains. The *numbers* indicate the percentages of bootstrap samples, derived from 1000 samples, that supported the internal branches (Felsenstein 1985). Bootstrap probability values less than 50% were omitted from this figure. *Bar*, 0.01 Knuc unit

have numerous peritrichous flagella and were actively motile when grown at alkaline pH. The characteristics of alkaliphilic *Bacillus* sp. AH-101 are summarized in Table 1. Strain AH-101 was found to grow at relatively high temperatures (up to 55°C). Other physiological characteristics were essentially the same as described in previous reports (Takami et al. 1989). The major isoprenoid quinone in strain AH-101 was menaquinone-7 (MK7), which accounted for 93% of the total isoprenoid quinones, the same as in most alkaliphilic *Bacillus* strains. The G + C content of the DNA of strain AH-101 was found to be 44.1 mol%, a value quite similar to that of *B. clarkii* (44.8 mol%) and *B. halodurans* (43.7 mol%).

16S rDNA sequencing and analysis

In a previous study, a preliminary study of the properties of strain AH-101 was undertaken by examining conventional

physiological and biochemical characteristics (Takami et al. 1989), but the phylogenetic placement of this strain had not been established. For further characterization of strain AH-101, we constructed a phylogenetic tree based on comparison of the 16S rDNA sequence of this strain and those of type strains of *Bacillus* species. Homology values in the range of 90.8%–99.4% were obtained comparing the 16S rDNA sequence of strain AH-101 and those of 11 other *Bacillus* strains. The results of phylogenetic analysis using 16S rDNA sequence information indicate that *Bacillus* sp. AH-101 is phylogenetically distant from *B. clarkii* (DSM8718^T) although the physiological properties and the G + C content of the DNA of strain AH-101 are similar to those of *B. clarkii*. As shown in Fig. 1, strain AH-101 is closely related to *B. halodurans* strains DSM8718^T and C-125 (JCM9153).

DNA–DNA hybridization analysis

DNA–DNA hybridization analysis was carried out comparing strain AH-101 and other related strains (Table 2). The similarity between *Bacillus alcalophilus* type strain (DSM485^T) and strain AH-101 was quite low (13%–16%). The DNA–DNA relatedness between strain AH-101 and two strains of *B. halodurans*, DSM497^T and JCM9153 (strain C-125), was 81%–88% and 87%–97%, respectively, indicating that *Bacillus* sp. AH-101 should be classified as a member of the species *B. halodurans*.

Alkaliphilic *Bacillus halodurans* C-125 has been employed as a good model strain in basic and applied studies of alkaliphilic *Bacillus* isolates since 1977. Analysis of the C-125 genome has been initiated and the systematic sequencing of the whole genome will be finished soon. As we have correctly identified strain AH-101 as *B. halodurans* in this study, knowledge of the complete sequence of the C-125 genome will be useful in developing further industrial applications of strain AH-101, particularly its extremely thermostable alkaline keratinase. We have already identified a protease gene in the C-125 genome that shows very high identity (>90%) to the AH-101 keratinase gene (data not shown), although we were unable to detect thermostable keratinase activity in cultures of strain C-125.

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