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Reidentification of facultatively alkaliphilic *Bacillus firmus* OF4 as *Bacillus pseudofirmus* OF4

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Abstract With a view toward verifying the original classification of alkaliphilic *Bacillus firmus* OF4, physiological and biochemical characteristics were more extensively catalogued than in original studies, and this catalog was supplemented with 16S rDNA sequence homology and more extensive DNA–DNA hybridization analyses. Phylogenetic analysis of this alkaliphile based on the comparison of multiple 16S rDNA sequences from *Bacillus* species indicated that this strain is most closely related to *Bacillus pseudofirmus*. Consistently, in the DNA–DNA hybridization analysis of the alkaliphile and *Bacillus* reference strains, the highest level of DNA–DNA relatedness (96%) was found between the alkaliphile and the *B. pseudofirmus* type strain (DSM 8715^T). The findings support the conclusion that this alkaliphile strain is more closely related to *B. pseudofirmus* than to *B. firmus*, and we propose the future use of the designation *B. pseudofirmus* OF4.

Key words Alkaliphilic *Bacillus pseudofirmus* OF4 · DNA–DNA hybridization · Phylogenetic tree · 16S rDNA

Introduction

Alkaliphilic *Bacillus* strains were recently, classified into 11 groups on the basis of 16S rDNA sequence data and the major alkaliphilic *Bacillus* species were proposed to be the following: *Bacillus pseudofirmus, Bacillus agaradhaerens, Bacillus clarkii, Bacillus halodurans, Bacillus clausii, Bacillus cohnii, Bacillus halmapalus, Bacillus horikoshii, Bacillus pseudoalcalophilus*, and *Bacillus gibsonii* (Fritze et al. 1990; Nielsen et al. 1995). The strain OF4, which is one of most intensely studied among the alkaliphilic *Bacillus* strains (Krulwich et al. 1998), had been identified as a *Bacillus firmus* based on limited DNA–DNA hybridization analysis in comparison with *Bacillus firmus* RAB, which in turn had a group of physiological properties in common with *B. firmus* (Guffanti et al. 1986). However, *Bacillus firmus* is not one of the species that have been more recently found among the alkaliphilic *Bacillus*-containing group. A more precise identification of the strain OF4 was therefore undertaken. At a time of rapidly developing genetic and molecular data on different alkaliphilic species, the distinctions are of particular importance because there are apparently some commonalities but also some differences among the key gene products that are required for alkaliphily in different species (Krulwich et al. 1997, 1998). Thus, we attempted to identify the strain OF4 not only based on an expanded array of 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 to the type strains of related species.

Materials and methods

Biolog test

The manufacturer's standard methodology (Release 3.50, version DE) is as follows (Biolog, Hayward, CA, USA): (1) grow bacteria on a nonselective agar medium; (2) prepare, within the standardized OD range, a uniform suspension of cells in sterile, normal (0.85% w/v) saline; (3) inoculate the cell suspension (150 µl per well) into the GP MicroPlate; (4) incubate the plate under appropriate conditions (37°C in this case); and (5) analyze the colorimetric changes by measurement of A_{590}.

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Isoprenoid quinones

Isoprenoid quinones were extracted from dried cells with chloroform: methanol (2:1 v/v) 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 of DNA 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). 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 AB029256.

Results and discussion

Physiological and biochemical properties

The physiological characteristics of strain OF4 and *B. pseudofirmus* DSM 8715^T were investigated by Biolog. Table 1 shows the specific carbon sources and nutrients showing positive action in the Biolog test. The strain OF4 assimilated maltotriose, acetic acid α-ketovaleric acid, L-malic acid, pyruvic acid, and L-asparagine, although some were negative in *B. pseudofirmus* DSM 8715^T. *B. pseudofirmus* assimilated three amino acids, D-alanine, L-alanine, and L-serine, in contrast to strain OF4 (Table 1). The assimilation pattern of substrates based on the Biolog database suggested that both alkaliphile strains OF4 and *B. pseudofirmus* DSM 8715^T were similar to *Bacillus brevis* with a similarity value from 0.64 to 0.81, although there are some differences in the assimilation patterns between strain OF4 and *B. pseudofirmus* DSM 8715^T, as just mentioned. Thus, *B. pseudofirmus* DSM 8715^T was incorrectly identified as *B. brevis* based on the assimilation pattern in the Biolog test, which was not useful for identification of *Bacillus* strain.

Table 1. Specific carbon sources and nutrients showing positive action in the Biolog test

Sources	Strain OF4	*Bacillus pseudofirmus* DSM 8715^T
Maltotriose	+	−
Acetic acid	+	+
α-Ketovaleric acid	+	+
L-malic acid	+	−
Methyl pyruvate	+	+
Pyruvic acid	+	+
L-asparagine	+	−
D-alanine	−	+
L-alanine	−	+
L-serine	−	+

The isoprenoid quinones of strain OF4 were menaquinone-7 (MK-7) and -6 (MK-6), which accounted for 74% and 26% of the total isoprenoid quinones, respectively. This menaquinone pattern is similar to those typically observed in *B. pseudofirmus* strains A-40-2 and 124-1 (DSM 516 and DSM 2517) or *B. clausii* strains 221 and Y-76 (DSM 2512 and DSM 2515) (Horikoshi 1991). The G + C content of the DNA of strain OF4 was found to be 40.4 mol%; this value is similar to that of *B. pseudofirmus* DSM 8715 (40.5 mol%).

16S rDNA sequencing and analysis

For further characterization of the alkaliphile strain, a phylogenetic tree was constructed 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% to 99.8% were obtained comparing the 16S rDNA sequence of *Bacillus* OF4 and those of 11 other *Bacillus* strains. The results of phylogenetic analysis using 16S rDNA sequence information indicate that the *Bacillus* OF4 is phylogenetically distant from *B. firmus* (IAM 12464), although the physiological properties of the strain OF4 were similar to those of *B. firmus*. As shown in Fig. 1, strain OF4 is closely related to *B. pseudofirmus* strain DSM 8715^T.

DNA–DNA hybridization analyses

DNA–DNA hybridization analyses were conducted to compare strain OF4 with various other alkaliphilic *Bacillus* strains and additional *Bacillus* type strains (Table 2). The hybridization values obtained in comparison of the *Bacillus alcalophilus* type strain (DSM 485^T) and *Bacillus* OF4 were

Table 2. DNA–DNA hybridization between strain OF4 and other related strains

Strain	DNA–DNA hybridization (%) with			
	OF4	*Bacillus pseudofirmus* (DSM8715^T)	*Bacillus halodurans* (DSM497^T)	*Bacillus alcalophilus* (DSM485^T)
OF4	100	**93**	22	18
Bacillus pseudofirmus (DSM8715^T)	**96**	100	39	45
Bacillus halodurans (DSM497^T)	49	49	100	53
Bacillus alcalophilus (DSM485^T)	3	1	28	100

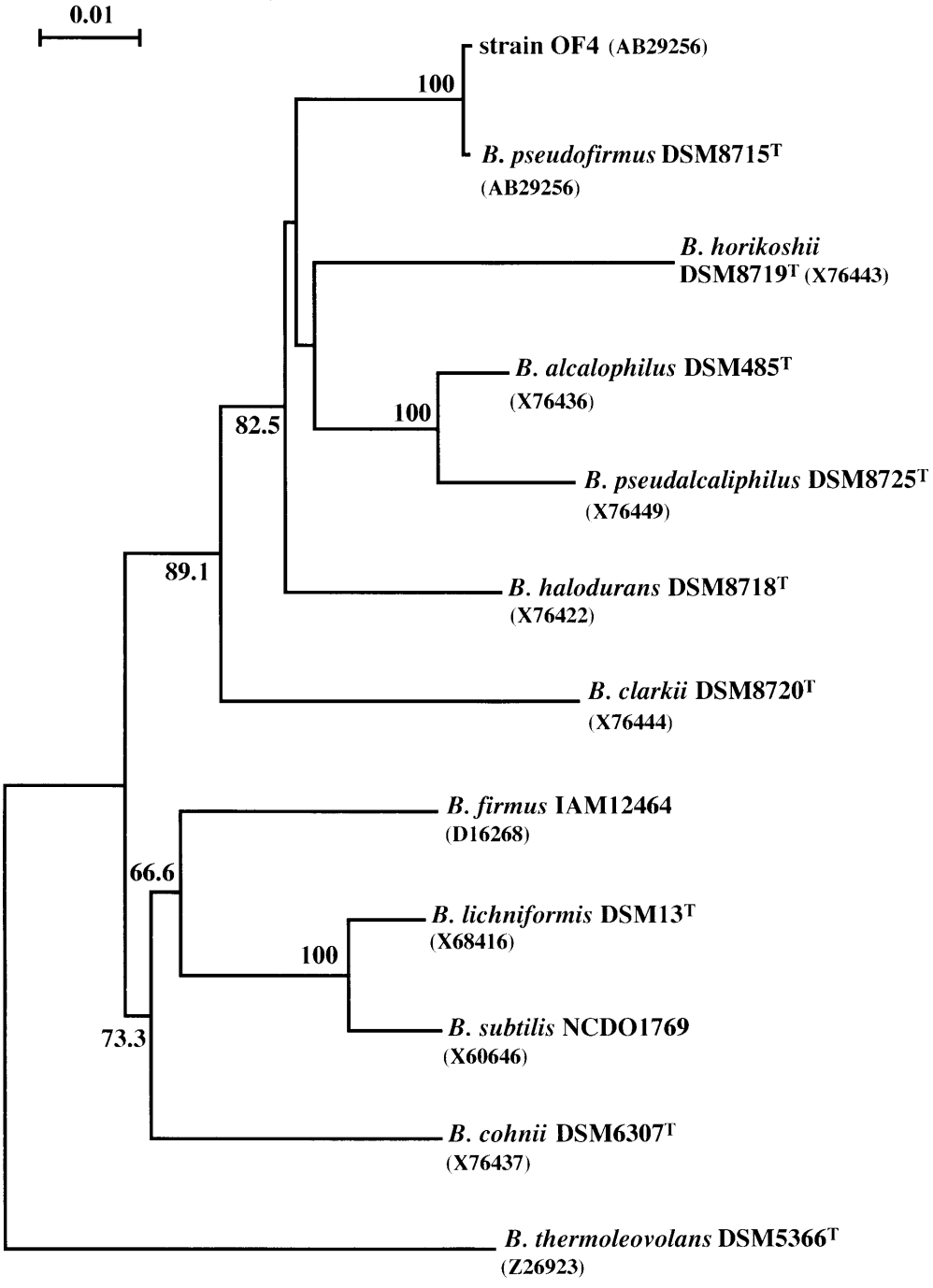

Fig. 1. Unrooted phylogenetic tree showing the relationship of the alkaliphile strain OF4 to other *Bacillus* strains. The numbers indicate the percentages of bootstrap samples, derived from 1000 samples, that supported the internal branches (Felsenstein 1985). Bootstrap probability values less than 50% were omitted from this figure. *Bar* 0.01 Knuc unit

quite low (3%–18%). The DNA–DNA relatedness between *Bacillus* OF4 and *B. halodurans*, DSM 497^T, and *B. pseudofirmus* DSM8715^T was 22%–49% and 93%–96%, respectively. These results led to the conclusion that *Bacillus* OF4 is not closely related to *B. halodurans* C125 (Takami and Horikoshi 1999) and should be classified as a member of the species *B. pseudofirmus*. In view of the findings of the present study, we will refer to this alkaliphile as *B. pseudofirmus* OF4 in future reports.

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