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Maria S. Muntyan · Tatiana P. Tourova
Anatolij M. Lysenko · Tatiana V. Kolganova
Dagmar Fritze · Vladimir P. Skulachev

Molecular identification of alkaliphilic and halotolerant strain *Bacillus* sp. FTU as *Bacillus pseudofirmus* FTU

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Abstract The systematic position of the alkaliphilic and halotolerant strain *Bacillus* sp. FTU was refined in view of the comprehensive taxonomic revision of the group of alkaliphilic and alkalitolerant *Bacillus* strains. Sequence analysis of almost the entire 16S rRNA gene of *Bacillus* sp. FTU revealed 99.8% homology with two *Bacillus pseudofirmus* strains. Subsequent DNA–DNA hybridization analysis confirmed the close relationship of *Bacillus* sp. FTU with the type strain of *B. pseudofirmus* (the level of homology reached 86%). Results of physiological and biochemical characterizations relevant for the group clearly underlined the positioning of strain FTU within this species. It is therefore concluded that *Bacillus* sp. FTU represents a strain of the alkaliphilic species *B. pseudofirmus* and is to be renamed as *B. pseudofirmus* FTU. The phylogeny of different *Bacillus* species is discussed using N-terminal sequence homologies of some caa_3-type oxidase subunits.

Key words Alkaliphilic and halotolerant *Bacillus pseudofirmus* FTU · 16S rDNA · DNA–DNA hybridization · caa_3-type cytochrome *c* oxidase

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M.S. Muntyan · V.P. Skulachev (✉)
Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia
Tel. +7-095-9395530; Fax +7-095-9390338
e-mail: skulach@genebee.msu.su

T.P. Tourova · A.M. Lysenko · T.V. Kolganova
Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia

D. Fritze
Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

Introduction

The past decade marked a full revision of alkaliphilic *Bacillus* classification according to their phylogenetic and phenetic diversity (Fritze et al. 1990; Nielsen et al. 1994), and nine new alkaliphilic *Bacillus* species were proposed, *B. agaradhaerens*, *B. clarkii*, *B. clausii*, *B. gibsonii*, *B. halmapalus*, *B. halodurans*, *B. horikoshii*, *B. pseudalcaliphilus*, *B. pseudofirmus*, in addition to the pair of previously known species, *B. cohnii* and *B. alcalophilus* (Nielsen et al. 1995). The tapping of a sodium ion electrochemical gradient on membranes of some alkaliphilic bacilli to perform certain energetic functions (Skulachev 1991) has received much attention from bioenergeticists and has stimulated a search for molecular mechanisms responsible for $\Delta\bar{\mu}Na^+$ production in membranes of alkaliphilic bacilli. The alkaliphilic strain *Bacillus* sp. FTU, which was isolated on a marine synthetic medium, is an example of this type of bacillus. Its energy-conserving mechanisms are studied thoroughly inasmuch as the strain is spontaneously resistant to protonophore uncouplers and is capable of growing both under alkaline conditions and high NaCl concentrations (Semeykina et al. 1989). On the basis of physiological and biochemical tests and DNA–DNA hybridization analysis, the alkaliphilic and halotolerant strain *Bacillus* sp. FTU had previously been defined as *B. halodurans* (Grinkevich et al. 1997). The high halotolerance of the strain (up to 15% NaCl) in comparison to other alkaliphilic *Bacillus* representatives and the relatively high level of DNA homology with *B. halodurans* DSM 2513 (66%) and *B. halodurans* DSM 497 (58%) were decisive for this determination. Recently, doubts arose concerning these taxonomic conclusions. We therefore performed a series of extensive physiological and biochemical tests for the strain *Bacillus* sp. FTU as described for the group of organisms. In addition, we applied 16S rRNA gene sequencing and DNA–DNA hybridization analysis with the nearest phylogenetic relatives among alkaliphilic bacilli to find out the relationships of this strain in the context of the new classification of the alkaliphilic *Bacillus* group as suggested recently (Nielsen et al. 1995).

Methods

The bacterial strains used in the study are listed in the section "DNA–DNA hybridization analyses" (Results and discussion). *Bacillus* sp. FTU DSM 6716 was isolated and maintained at the A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, and deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Other *Bacillus* strains used were received from the DSMZ.

Physiological and morphological characteristics of the strain

Phenotypic tests were based on the methods described earlier (Gordon et al. 1973) and performed according to later modifications (Fritze et al. 1990). Growth and reactions were usually observed after 2 or 3 days. Growth at NaCl concentrations of 18% and 20% was recorded for up to 10 days. Hydrolysis of starch and pullulan was observed for up to 8 days and hydrolysis of Tweens and hippurate for 14 days. Reduction of NO_3^- was recorded for up to 11 days. Indole reaction was tested after 7 days. Vegetative cells and sporangia were observed under a phase contrast microscope (Univar, Reichert, Vienna, Austria) using the ×40 and the ×100 magnification. To keep cells in focus, agar coated slides were used.

16S rRNA sequencing

The 16S rRNA gene was amplified and sequenced using primers universal in most prokaryotes (Edwards et al. 1989). The medium for amplification contained: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.001% gelatin. The reaction mixtures of 100 µl each contained the standard concentrations of deoxynucleoside (5′-)triphosphate (dNTP) and equimolar quantities of pA and pH′ primers. Thirty amplification cycles of the following temperature profile were carried out: DNA denaturation at 94°C, 0.5 min; primer annealing at 40°C, 1 min; and elongation at 72°C, 2.5 min. After purification on low-melting agarose and on Wizard columns (Promega, Madison, WI, USA), the 16S rRNA sequencing was performed in both directions using forward and reverse universal primers and Sequenase (Biochemicals, Cleveland, OH, USA).

16S rRNA sequence analysis

Primary analysis of 16S rRNA gene nucleotide sequence similarity of the studied strains was carried out using the BLASTA server. The sequences were aligned against the corresponding 16S rRNA sequences of the related bacterial strains using the CLUSTAL.X program (Thompson et al. 1994). An unrooted phylogenetic tree of the studied bacteria was constructed using methods available in the TREECONW program package (Van de Peer and De Wachter 1994).

DNA–DNA hybridization

DNA for hybridizations was prepared from 300-ml cultures in tryptone-salt medium adjusted to pH 9, containing (in g l^{-1}): tryptone (Difco, Detroit, MI, USA) 1, NaCl 30, KCl 0.75, $(NH_4)_2SO_4$ 1.98, $MgSO_4 \cdot 7H_2O$ 1.23, Tris 6.1, ethylenediaminetetraacetic acid (EDTA) 0.003, $FeSO_4 \cdot 7H_2O$ 0.003, succinate-NaOH 3.54, KH_2PO_4 0.27 (added after autoclaving). DNA was isolated according to the method of Marmur (Marmur 1961). The G + C content of the DNA was determined using thermal denaturation curves recorded by a Pye Unicam SP 1800 spectrophotometer (Birmingham, UK) at a heating rate of 0.5°C min^{-1}. Melting was performed in 0.1 SSC buffer, containing 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0. The nucleotide composition was calculated according to the following equation: GC mol% = $T_{mlt} - 106.4$ (Owen et al. 1969). DNA reassociation studies were carried out as described previously (De Ley et al. 1970). DNA samples dissolved in 0.1 SSC were sonicated using an ultrasonic disintegrator (UZDN-2T, own-made; 0.4 mA, 2.5 min), denatured, and placed into spectrophotometer cuvettes heated up to the optimum reassociation temperature. Calculations were done according to the following equation:

$$D = \frac{44V_m - V_a - V_b}{2V_a \cdot V_b} 100\%,$$

where the symbols mean the following: D, DNA homology %; V_a, sample A reassociation rate; V_b, sample B reassociation rate; V_m, reassociation of two DNA samples in mixture in equimolar concentrations.

Amino acid sequence analysis

Searching of amino acid sequences of the terminal oxidases and analysis of their similarity were carried out using the BLASTA server.

Nucleotide sequence accession numbers

The 16S rDNA sequence of strain Z-9801^T has been deposited in GenBank under accession number AF406790.

Results and discussion

Morphological, physiological, and biochemical properties

Among 11 phena proposed as new and newly revised alkaliphilic *Bacillus* species (Nielsen et al. 1995), only members of phenon 1 (*B. pseudofirmus*) are able to deaminate phenylalanine. According to this single test, the strain *Bacillus* sp. FTU might be related to *B. pseudofirmus* (Table 1). In

Table 1. Physiological and biochemical properties of *Bacillus* sp. FTU

Shape of sporangium	Not swollen in young cells, swollen in old cells
Position of spores in sporangium	Subterminal
Shape of spores	Ellipsoid
Catalase	+
Oxidase	+
Motility	+
Temperature range for growth:	
5°C	–
10°C	+
40°C	+
45°C	–
Anaerobic growth	–
Growth in the presence of:	
10% NaCl	+
15% NaCl	+
17% NaCl	+
18% NaCl	+
20% NaCl	–
pH tolerance:	
6.0	–
7.2	+
8.1	+
9.1	+
10	+
Hydrolysis of:	
Starch	–/+
Casein	+
Gelatin	+
Pullulan	–
Tween 80	–
Tween 60	+/–
Tween 40	+/–
Tween 20	–
MUG	–
Hippurate	–
Deamination of phenylalanine	+
Production of $NO_3^- \rightarrow NO_2^-$	–
Indole	–
Urease	–

MUG, 4-methylumbelliferyl β-D-glucuronide

addition, phenon 1 is one of three phena (*B. pseudofirmus, B. agaradhaerens, B. clarkii*), of the 11 mentioned, exhibiting the highest NaCl tolerance (up to 17%–18%) and a high degree of alkaliphily (with growth at pH 10, the maximum being at pH 9 or 10), both features being inherent of *Bacillus* sp. FTU. Interestingly, *Bacillus* sp. FTU was unable to grow in the absence of sodium ions, similar to representatives of the phena *B. agaradhaerens* and *B. clarkii*, which had been described as a unique feature among all the taxa studied (Nielsen et al. 1995). It seems possible, however, that other alkaliphiles (e.g., from less saline environments) still require Na^+ but utilize its lower concentrations. We have also found that in *Bacillus* sp. FTU, sporangia appeared to be nonswollen by young spores but the old spores make the sporangium bulge (Fig. 1). In *B. halodurans*, sporangia are slightly swollen (Nielsen et al. 1995), while in *B. pseudofirmus* this feature is variable:

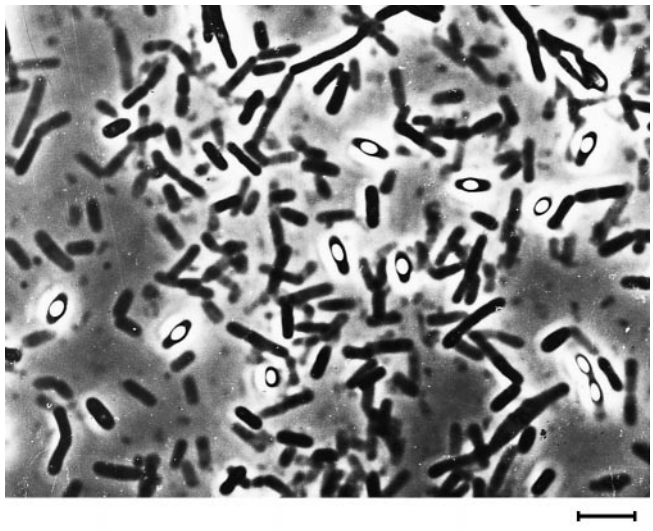

Fig. 1. Spores in *Bacillus* sp. FTU cells (phase contrast microscopy). *Bar* 5 μm

sporangia are nonswollen in the type strain (Nielsen et al. 1995) and are swollen in *B. pseudofirmus* OF4 (H. Takami, personal communication). Summarizing these and other physiological and biochemical tests (presented in Table 1), in particular the inability to reduce nitrate and to hydrolyze hippurate, MUG, and Tween 20, it is assumed that *Bacillus* sp. FTU falls into the *B. pseudofirmus* phenon.

16S rDNA sequencing and analysis

For further characterization of the alkaliphile strain FTU, a phylogenetic analysis was performed based on 16S rDNA sequencing. We determined an almost complete 16S rDNA sequence (1,524 nucleotides) for strain FTU, corresponding to positions 7–1,521 in *Escherichia coli* numbering. According to an initial phylogenetic analysis of GenBank by BLASTA, the highest score was found with *Bacillus* species of phylogenetic group 6 (Nielsen et al. 1994) which comprises 8 of the 11 alkaliphilic *Bacillus* species: *B. pseudofirmus*, *B. agaradhaerens*, *B. clarkii*, *B. halodurans*, *B. pseudalcaliphilus*, *B. alcalophilus*, *B. gibsonii*, *B. clausii*, and two noncharacterized species. The phylogenetic tree (Fig. 2) was constructed based on comparison of the 16S rDNA sequence of this strain and those of all strains (including type strains of alkaliphilic *Bacillus* species) of this group. *Bacillus* sp. FTU formed a tight cluster with two strains (including the type strain) of *B. pseudofirmus* with the maximum bootstrap index (100) and high level of sequence similarity (99.8%). The results of phylogenetic analysis using 16S rDNA sequence information also indicate that the *Bacillus* FTU is phylogenetically more distantly related to *B. halodurans* and *B. alcalophilus* (96.2% sequence similarity). Homology values in the range of 91.4%–94.1% were obtained comparing the 16S rDNA sequence of *Bacillus* sp. FTU and those of other reference alkaliphilic *Bacillus* strains. The obtained level of sequence similarity between strain FTU and members of *B. pseudofirmus* corresponds to

the intraspecies level as defined previously (Stackebrandt and Göbel 1994).

DNA–DNA hybridization analyses

The G + C contents of DNA are almost identical for *Bacillus* sp. FTU and *B. pseudofirmus* DSM 497^T and are slightly different from two strains (including type strain DSM8715^T) of *B. halodurans* (Table 2). DNA–DNA hybridization analyses were conducted to compare *Bacillus* sp. FTU strain with some additional alkaliphilic *Bacillus* strains (Table 2). The DNA–DNA relatedness between *Bacillus* sp. FTU and *B. pseudofirmus* DSM 497^T (86%) was at an intraspecies level (Wayne et al. 1987). The level of DNA–DNA relatedness between *Bacillus* sp. FTU and two strains (including type strain DSM 8715^T) of *B. halodurans* was lower and appeared to be an interspecies one. The interspecies level of DNA–DNA relatedness between the type strains of *B. pseudofirmus* and *B. halodurans* (51%–53%) is close to that obtained by Takami and Krulwich (49%) (Takami and Krulwich 2000). The hybridization values obtained earlier (Grinkevich et al. 1997) between the *B. alcalophilus* type strain (DSM 485 T) and *Bacillus* sp. FTU were quite low (no more than 13%). Thus the species *B. alcalophilus* is distantly related to the species *B. pseudofirmus* and *B. halodurans*.

Homology of N-terminal sequences of some *caa3*-type oxidase subunits

In the context of the phylogenetic relationships of *Bacillus* sp. FTU, we analyzed the sequence similarity of N-terminal amino acid fragments of the caa_3-type oxidase subunits in several representatives of the genus *Bacillus*. Sequencing of the full genome of *B. halodurans* C-125 (Takami et al. 2000) presented data on the previously unknown gene sequences of terminal oxidases in this bacterium. It was found that the sequence similarity of the analyzed amino acid fragments was significantly higher among alkaliphilic *Bacillus* representatives than between alkaliphilic bacilli and bacilli from other phylogenetic groups (Fig. 3). However, within the alkaliphilic organisms, appreciable differences were revealed between *Bacillus* sp. FTU and *B. halodurans* C-125, whereas nearly full similarity of the N-terminal fragments of the caa_3-type oxidase in *Bacillus* sp. FTU and *B. pseudofirmus* OF4 was found. This again evidenced that both strains are representatives of the same species. These

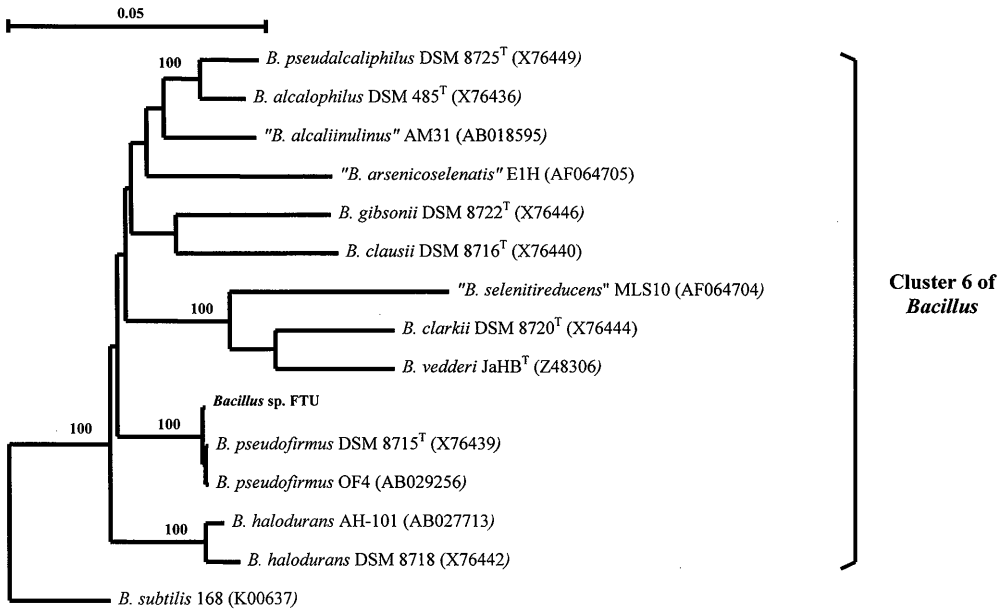

Fig. 2. The phylogenetic tree based on 16S rDNA sequence analysis showing the position of the strain *Bacillus* sp. FTU among the members of the *Bacillus* group 6. Bootstrap values (expressed as percentage of 100 replications) are shown at branch points; values greater than 95 were considered as significant. *Bar* indicates the distance corresponding to 5 nucleotides substitutions per 100 nucleotides (from Jukes and Cantor 1969)

Table 2. DNA–DNA hybridization of several *Bacillus* representatives

Strain	G + C mol%	Homology (%)		
		Bacillus FTU	*B. halodurans* 2513	*B. halodurans* 497
Bacillus FTU	41.3	100		
B. halodurans 2513	43.4	63	100	
B. halodurans 497	44.5	61	84	100
B. pseudofirmus	41.3	83	54	51
Escherichia coli K12 (standard)	51.7			

Bacterial srains	Amino acid sequences	Score (bits)
Subunit I		
B.pseudofirmus FTU	-ATQKQEK-SVIWDWLTTVDXKKIAIMYLXAG-	
B.pseudofirmus OF4	M.......-..........H........I..-	60.1
B.halodurans C-125	M....K..-..L........H..LG....I..-	55.5
B.subtilis (c)	MTE.RTRG.ML..Y.....H.....L..V..-	41.2
B.anthracis	MA....Y.....H.....L..I..-	41.2
Bacillus sp. YN2000	MAQK.GFG-ATV..Y.....H........I..-	41.2
B.stearothermophilus	M.RK.G-VGA.L..Y.....H....HL..I..-	38.9
Bacillus PS3	M.RK.G-VGA.L..Y.....H....HL..IS.-	37.7
B.subtilis (q)	LTYF.WK.-WL.SE.I....H..LG...IISA-	29.6
Subunit II		
B.pseudofirmus FTU	-CLGEENLTALDPKGPQAQWIYDNMILSIIXM-	
B.pseudofirmus OF4	MKLWKTASRFLPLSFLTLFLTG..........V.-	64.8
B.halodurans C-125	MKLWKTALRFLPLSLVVLFLAG.M..T.......Q....E.LF...L..LYV.-	54.7
Bacillus PS3	MNKGLCNWRLFSLF---GMMALLLAG.-.KPF.ST.Q.A.EV.DMQ.SL.L..TSI.-	<30
B.subtilis (c)	MVKHWRLI---LLLALVPLLLSG.-.KPF.ST.K.A.EV.DKQ..LTV..TLI.-	<30
B.subtilis (q)	MLGG.---S.ASV......V.EQQS.LIL...GF.-	<30

Fig. 3. Alignment of N-terminal amino acid sequences of caa_3-type oxidase major subunits of several *Bacillus* representatives. *B. pseudofirmus* FTU amino acid sequence was obtained directly from protein sequencing; *X* indicates unidentified amino acids. Other amino acid sequences were the predictions on the basis of nucleotide sequences obtained using the BLASTA server. In *B. subtilis, c* cytochrome-*c*-oxidase of caa_3-type, *q* quinol oxidase of aa_3-type. Leader peptides of subunits II are *boxed* (*dashed line*)

findings show that even limited information on amino acid sequences of constitutive proteins might be useful as a source of additional data when the relationships of alkaliphilic bacilli are evaluated. These results confirm the conclusion that *Bacillus* sp. FTU is not closely related to *B. halodurans* 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* FTU in future reports.

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