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Revision of the taxonomic position of the xylanolytic *Bacillus* sp. MIR32 reidentified as *Bacillus halodurans* and plasmid-mediated transformation of *B. halodurans*

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Abstract *Bacillus* sp. MIR32 has been isolated using xylan as the only carbon source, and one of its xylanolytic enzymes has been extensively studied. Biochemical analysis first related this strain to *Bacillus amyloliquefaciens*, but further studies based on a comparison of 16S rDNA sequences, G + C content, and DNA–DNA hybridization showed that strain MIR32 should be classified as a member of the species *Bacillus halodurans*. This change is also supported by the typical phenotype observed and by the results of PCR amplification directed toward spacers in rDNA and tDNA genes, which were assayed and compared with those of *B. halodurans* DSM 497^T. Although among alkaliphilic bacilli competence development has not been experimentally demonstrated, in this work both *B. halodurans* MIR32 and DSM 497^T were transformed according to a simple procedure developed in our laboratory, reaching 10^2–10^3 stable transformants per microgram of plasmid DNA.

Key words *B. halodurans* MIR32 · Molecular characterization · Transformation · 16S rDNA sequence analysis · DNA–DNA hybridization · 16S–23S Intergenic spacer region

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Introduction

The genus *Bacillus* is one of the most utilized sources for industrial processes because species of this genus are capable of producing large amounts of enzymes and other products (Priest and Sharp 1989). In our laboratory, *Bacillus* sp. MIR32 was isolated from soil samples as a good xylan-degrading bacterium by using bagasse xylan and potassium nitrate as sole carbon and nitrogen sources, respectively (Breccia et al. 1995). Then an endo-1,4-β-D-xylan-xylanohydrolase with an optimum pH of 6.8–7.0, an isoelectric point of 10.1 and showing high stability in alkaline environments at 50°C was purified and characterized (Breccia et al. 1998a, b). Isolation of the strain and xylanase characterization were performed under neutral conditions, and *Bacillus* sp. MIR32 was reported as *B. amyloliquefaciens* on the basis of the biochemical traits observed by using API standardized methods (BioMérieux, Marcy l'Etoile, France). Nevertheless, further studies revealed that the strain was able to grow under both neutral and alkaline conditions. This property has been previously described for several well-studied alkaliphilic and alkalitolerant *Bacillus* strains (Nielsen et al. 1995; Takami and Horikoshi 2000).

With the objective of verifying the taxonomic position of *Bacillus* sp. MIR32, we propose here a revision on the basis of specific phenotypic characteristics and a molecular characterization of the strain, including 16S rDNA sequence analysis, G + C content, DNA–DNA hybridization, and PCR fingerprinting methods, which sample intergenic spacers in rDNA and tDNA genes. Assessment of length polymorphism of such regions is now more frequently used for microbial typing and also for evolutionary and diversity studies (Gürtler and Mayall 2001; Senesi et al. 2001).

Recently, the entire genome of *B. halodurans* C-125 was completely sequenced and compared with the taxonomically related *B. subtilis*. Among the conclusions of the whole genome analysis of *B. halodurans* C-125, it was demonstrated that this strain lacks some of the necessary genes for competence (Takami et al. 2000). As we reported previously, *Bacillus* sp. strain MIR32 could be easily transformed with plasmid DNA by using a procedure that has been

shown to be useful for transforming various *Bacillus* species (Martínez et al. 1999). In this work, we confirmed those results, including the plasmid-mediated transformation of the *B. halodurans* DSM 497^T type strain.

Materials and methods

Bacterial strains and media

For routine growth and storage, *B. halodurans* MIR32 was grown in a xylan medium (XM), consisting of (w/v) 1% bagasse xylan (Breccia et al. 1995), 0.1% casamino acids, 0.1% yeast extract, 2% NaCl, and 0.1% K_2HPO_4, at pH 7 and at pH 10. For DNA extractions, the xylan was replaced by 0.5% glucose. The review of some phenotypic characteristics was performed according to Nielsen et al. (1995). For transformation assays, strains were grown on nutrient broth plates (NB, Merck, Darmstadt, Germany) at pH 7 and at pH 10. After autoclaving, the pH of the media was adjusted to approximately 10 by the addition of 100 mM sodium sesquicarbonate buffer. *B. halodurans* DSM 497^T was grown according to Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) instructions (http://www.dsmz.de). *B. subtilis* 1 A1 and *B. amyloliquefaciens* 10 A1 reference strains were kindly provided by Dan Zeigler from the *Bacillus* Genetic Stock Center (BGSC, http://bacillus.biosci.ohio-state.edu); their growth and maintenance is described in the BGSC catalog.

DNA extraction and studies

To obtain genomic DNA, 1.5 ml of each overnight culture was centrifuged at 13,000 *g* for 3 min. The pellets were resuspended in 1 ml of 10 mM Tris HCl, 10 mM EDTA, 100 mM NaCl, 2% (v/v) SDS, and 400 µg ml^{-1} proteinase K; the mixture was incubated at 56°C for 30 min. After phenolization and chloroform purification steps, DNA was precipitated with 1 volume of 2-propanol, washed twice with 70% (v/v) ethanol, and finally resuspended in 200 µl of sterile water.

G + C content was determined by scanning calorimetry according to the method of Klump and Herzog (1984), by using a VP-DSC MicroCalorimeter (MicroCal, Northamton, MA, USA). The DNA–DNA hybridization analysis was carried out as described by De Ley et al. (1970) with modifications described by Huss et al. (1983) and Escara and Hutton (1980).

16S rDNA sequencing and analysis

Universal primers were used to amplify 16S rDNA (Weisburg et al. 1991): 8–27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR products were recovered from 0.8% (w/v) agarose gels using a Prep-A-Gene DNA purification system (Bio-Rad, Hercules, CA, USA). DNA sequencing of both strands was performed by using the dideoxy chain termination method with an ABI prism 3100 DNA Analyzer, and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The sequence was registered in the GenBank Data Library under accession number AY017348. Sequences belonging to the same or closely related species available through the public databases were aligned, and a similarity matrix was calculated by using Similarity Matrix version 1.1 software (Maidak et al. 2000). Only unambiguously aligned positions from all sequences were used to calculate the matrix, and gaps were not included in the match/mismatch count.

PCR fingerprinting of 16S–23S rDNA and tDNA intergenic spacers

Both the 16S–23S rDNA and tDNA intergenic regions were amplified according to Daffonchio et al. (1998) in a Gene Amp PCR 9700 system (Applied Biosystems) by using primers proven to be useful for *Bacillus* species. For intergenic spacer region amplification (ISR-PCR), primers were S-D-Bact-1494-a-S-20 (5′-GTCGTAACAAGGTAGCCGTA-3′) and L-D-Bact-0035-a-A-15 (5′-CAAGGCATCCACCGT-3′), while for tDNA-PCR, the primers were T3A (5′-AGTCCGGTGCTCTAACCAACTGAG-3′) and T5B (5′-AATGCTCTACCAACTGAACT-3′).

Transformation of alkaliphilic *Bacillus*

Both *B. halodurans* MIR32 and DSM 497^T strains lack natural plasmids and were transformed with plasmid vectors extensively used in the *Bacillus* genus: pUB110 (Km^r), pBC16 (Tc^r), and pHV1432 (Cm^r) (BGSC). Plasmid DNA was obtained from *B. subtilis* cultures by using the boiling method described by Bron (1990). Transformation was performed as described previously (Martínez et al. 1999), by initially growing strains on NB agar plates at pH 7 and 10 at 37°C for 12–14 h, resuspending scraped cells in the transformation solution (per liter: 0.75 g yeast extract; 8 g glucose; 1.0 mM $CaCl_2$, and 10 mM $MgCl_2$; pH 7), and then adding 10 µl of crude extract containing 100–300 ng of plasmid DNA. Transformants were selected on NB pH 7 agar plates plus 10 µg ml^{-1} of the corresponding antibiotics. As a positive control, *B. subtilis* 1 A1 was also transformed.

Results and discussion

Characterization of *Bacillus* sp. MIR32

This strain was isolated during a screening program for xylan-degrading bacteria (Breccia et al. 1995). Studies were then focused on an endo-1,4-β-D-xylan-xylanohydrolase with potential industrial uses in chemical pulp bleaching (Breccia et al. 1998a). *Bacillus* sp. MIR32 was identified as *B. amyloliquefaciens*, a member of the *B. subtilis* group, on the basis of morphological and biochemical assays by using API standardized tests. Takami et al. (2000) reported that *B.*

subtilis is taxonomically related to *B. halodurans* C-125, except for the alkaliphilic phenotype. Although API systems have been successfully used for classification and identification of *Bacillus* isolates, several alkaliphilic *Bacillus* were unable to produce a detectable reaction. Even though the system could be modified for use at high pH, or the organisms could be first adapted to growth at pH 7, Nielsen et al. (1995) reported that such an artificial approach could lead to misleading physiological reactions and erroneous results. Recent observations of phenotypic characteristics and ISR-PCR fingerprints of *Bacillus* sp. MIR32 strain led us to review our previous results and attempt to reidentify the isolate.

Cells of *Bacillus* sp. MIR32 were Gram-positive, spore-forming, motile rods. Optimal growth occurred aerobically at 45°C and pH 10 on NB and XM agar, developing cream white-colored colonies with a slightly filamentous margin. Table 1 shows some morphological, physiological, and biochemical characteristics of the studied strain, which exhibits good growth in 2%, 5%, and 7% NaCl, and even in 10% NaCl when this test is performed at pH 10 and 45°C. The G + C content of strain MIR32 was found to be 43 mol%, a value comparable to that found in *B. halodurans* (42.1–43.9 mol% G + C; Nielsen et al. 1995).

Table 1. Morphological, physiological, and biochemical characteristics of *Bacillus* sp. MIR32

Morphology	Rod-shaped cells occurring singly or in short chains, with subterminal and ellipsoidal spores showing a weakly swollen sporangium
Gram staining	+
Growth at pH 7	+
Growth at pH 10	+
Growth at	
15°C	–
30°C	+
37°C	+
45°C	+
55°C	+
Growth in NaCl	
5%	+
7%	+
10%	+[a]
G + C content	43 mol%
Catalase	+
Nitrate reduction	–
Use of citrate	+
Hydrolysis of	
Starch, pullulan, and dextrin	+
Gelatin and casein	+
Tween 20	+
Tween 40	+
Tween 60	+

[a] This test was positive only at pH 10 and 45°C

DNA–DNA hybridization analysis

In order to confirm our findings, DNA reassociation reactions were performed that compared our native strain with *B. halodurans* DSM 497^T and *B. amyloliquefaciens* 10 A1. Results, shown in Table 2, indicated that strain MIR32 is genomically unrelated to *B. amyloliquefaciens* 10 A1, while reassociation values of 88%–91% were obtained with the *B. halodurans* DSM 497^T type strain, indicating that *Bacillus* sp. MIR32 should be classified as a member of species *B. halodurans*.

16S rDNA sequence analysis

The *Bacillus* sp. MIR32 16S rDNA sequence (AY017348) was compared with sequences from related taxa available in public databases, and a similarity matrix was calculated (Table 3). The 16S rDNA sequence analysis showed that this strain was closely linked to members of alkaliphilic *Bacillus* species, sharing more than 0.99 sequence similarity with other *B. halodurans* strains. The most similar sequences, a 16S rDNA sequence similarity value (*S*) of 0.998, unequivocally related our native strain to *B. halodurans* DSM 497^T and C-125 (JCM9153). Lower similarity levels were found with other alkaliphilic *Bacillus* strains, particularly *B. gibsonii* DSM 8722^T and *B. clausii* DSM 8716^T (S = 0.970 in both cases). These results allowed us to conclude that *Bacillus* sp. MIR32 is highly related to *B. halodurans* and not closely related to *B. amyloliquefaciens*.

ISR-PCR and tDNA-PCR fingerprints

Evaluation of intergenic length polymorphism of hypervariable parts of conserved genomic regions revealed typically

Table 2. DNA–DNA reassociation values between *Bacillus* sp. MIR32, *B. halodurans* DSM 497^T, and *B. amyloliquefaciens* 10 A1

Strain	DNA–DNA hybridization (%) with		
	Bacillus sp. MIR32	*B. halodurans* DSM 497^T	*B. amyloliquefaciens* 10 A1
Bacillus sp. MIR32	100	91	25
B. halodurans DSM 497^T	88	100	37
B. amyloliquefaciens 10 A1	21	32	100

Table 3. 16S rDNA sequence similarity matrix

16S rDNA sequence similarity (*S*)													
Strain		1	2	3	4	5	6	7	8	9	10	11	12	13
1	MIR32	1												
2	C-125	.998												
3	DSM 497^T	.998	1											
4	DSM 485^T	.966	.966	.966										
5	DSM 8715^T	.966	.966	.966	.967									
6	DSM 8725^T	.964	.964	.964	.986	.970								
7	DSM 8719^T	.947	.947	.947	.953	.942	.952	.957						
8	DSM 8723^T	.955	.955	.955	.956	.943	.955	.962	.990					
9	DSM 8722^T	.970	.970	.970	.948	.953	.943	.952	.934	.940				
10	DSM 6307^T	.938	.938	.938	.952	.948	.948	.950	.980	.980	.934			
11	DSM 8716^T	.970	.970	.970	.946	.954	.945	.950	.931	.934	.945	.923		
12	DSM 8720^T	.949	.949	.949	.946	.942	.945	.950	.936	.939	.934	.934	.920	
13	ATCC23350^T	.942	.943	.944	.933	.941	.932	.943	.942	.918	.942	.928	.923	

GenBank accession numbers are in brackets. 1, *Bacillus* sp. MIR32 [AY017348] and related taxa; 2, *B. halodurans* C-125 (JCM9153) [AB002661]; 3, *B. halodurans* DSM 497^T [AJ302709]; 4, *B. alcalophilus* DSM 485^T [X76436]; 5, *B. pseudofirmus* DSM 8715^T [X76439]; 6, *B. pseudalcalophilus* DSM 8725^T [X76449]; 7, *B. horikoshii* DSM 8719^T [AB043865]; 8, *B. halmapalus* DSM 8723^T [X76447]; 9, *B. gibsonii* DSM 8722^T [X76446]; 10, *B. cohnii* DSM 6307^T [X76437]; 11, *B. clausii* DSM 8716^T [X76440]; 12, *B. clarkii* DSM 8720^T [X76444]; 13, *B. amyloliquefaciens* ATCC 23350^T [X60605]

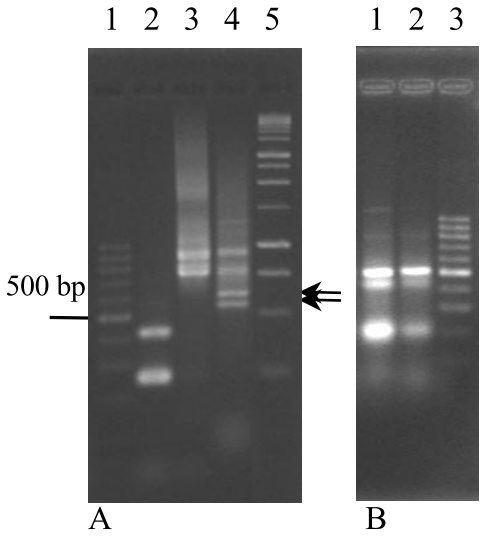

Fig. 1. A Two-percent agarose gels of intergenic spacer region (ISR) PCR patterns. *Lanes: 1*, 100 bp DNA ladder; *2*, *B. subtilis* 1 A1; *3*, *Bacillus* sp. MIR32 with bands ranging from 750 to 1,400 bp; *4*, *B. halodurans* DSM 497^T, bands ranging from 650 to 1,000 bp; *5*, 1 kb DNA ladder (Promega). The *arrows* indicate the absence of two major bands between 600 and 700 bp in *Bacillus* sp. MIR32. **B** Two-percent agarose gels of tDNA-PCR patterns. *Lanes: 1*, *Bacillus* sp. MIR32; *2*, *B. halodurans* DSM 497^T; *3*, 100-bp DNA ladder (Promega)

large 16S–23S intergenic regions in both alkaliphilic *Bacillus* compared with those described for other *Bacillus* species (Wunschel et al. 1994; Daffonchio et al. 1998). Although this analysis revealed the existence of a polymorphism affecting the size and number of ISR (Fig. 1A), a common feature was the large size of the spacers, which is consistent with observations of Nakasone et al. (2000) and which has been observed in other alkaliphilic *Bacillus* isolates in our laboratory (data not shown).

Amplification of tDNA intergenic spacers generally generates species-specific band patterns that have successfully been used to discriminate species of the same genus and even strain clusters in several bacteria, including in alkaliphilic bacilli (De Gheldre et al. 1999; Senesi et al. 2001). Fingerprints obtained showed complete identity between *Bacillus* sp. MIR32 and *B. halodurans* DSM 497^T with amplicon lengths ranging from 200 to 1,200 bp (Fig. 1B).

Transformation of alkaliphilic *Bacillus* by plasmid DNA

We previously showed that *Bacillus* sp. MIR32 could be transformed by using a previously described method (Martínez et al. 1999). We decided to confirm our earlier data with the newly reclassified *B. halodurans* MIR32 and also to test the transformation of the *B. halodurans* DSM 497^T type strain. Transformations were confirmed by isolation and restriction characterization of plasmids from transforming colonies, and transformation frequencies were estimated as the number of stable transformants per microgram of plasmid DNA in three independent experiments at both initial growing conditions, pH 7 and 10 (Table 4). In our experience, plasmid concentrations of unpurified plasmid preparations are critical to obtain good transformation efficiencies, because it is sometimes useful to concentrate transformed cells before plating them onto selection media.

Concluding remarks

Bacillus sp. MIR32 is a facultative alkaliphilic organism that has been reclassified as a member of *B. halodurans* on the

Table 4. Transformation of *B. halodurans* species with different plasmids

Strain	Transformants (μg plasmid DNA^{-1})		
	pUB110	pBC16	pHV1432
B. subtilis 1A1	4×10^5	2×10^5	1.7×10^3
B. halodurans MIR32	2×10^3	4×10^2	0.5×10^2
B. halodurans DSM 497^T	1×10^3	1×10^3	0.3×10^2

basis of data from the present study. By using this correct identification and the accumulated knowledge on this species, we have already cloned and overexpressed genes from this strain involved in xylan-degrading activity (unpublished data).

Finally, the completely sequenced *B. halodurans* C-125 strain lacks some genes involved in competence development (Takami et al. 2000). Considering that *B. halodurans* MIR32 and DSM 497^T are closely linked to that strain, the results obtained here support the utility of the procedure previously reported for plasmid-mediated transformation of different *Bacillus* species, even those in which genetic competence has not been experimentally demonstrated.

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