Comparative phylogeny of *rrs* and *nifH* genes in the *Bacillaceae*

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The *rrs* (16S rDNA) gene sequences of nitrogen-fixing endospore-forming bacilli isolated from the rhizosphere of wheat and maize were determined in order to infer their phylogenetic position in the *Bacillaceae*. These rhizosphere strains form a monophyletic cluster with *Paenibacillus azotofixans*, *Paenibacillus polymyxa* and *Paenibacillus macerans*. Two of them (RSA19 and TOD45) had previously been identified as *Bacillus circulans* (group 2) by phenotypic characterization (API 50CH). Evidence for nitrogen fixation by *P. azotofixans*, *P. polymyxa*, *P. macerans* and putative *B. circulans* strains RSA19 and TOD45 was provided by acetylene-reduction activity, and confirmed by amplifying and sequencing a *nifH* fragment (370 nt). The phylogenetic tree of *nifH*-derived amino acid sequences was compared to the phylogenetic tree of *rrs* sequences. All *Paenibacillus nifH* sequences formed a coherent cluster distinct from that of related nitrogen-fixing anaerobic clostridia and Gram-positive high-G+C-content frankiae. The *nifH* gene was neither detected in the *B. circulans* type strain (ATCC 4513^T) nor in the type strains of *Bacillus subtilis*, *Bacillus cereus*, *Bacillus alcalophilus*, *Bacillus simplex*, *Brevibacillus brevis* and *Paenibacillus validus*. Accordingly, nitrogen fixation among aerobic endospore-forming *Firmicutes* seems to be restricted to a subset of species in the genus *Paenibacillus*.

Keywords: *Paenibacillus*, *Bacillus*, phylogeny, rDNA and *nifH* sequencing, nitrogen fixation

INTRODUCTION

Comparative *rrs* (16S rDNA) sequence analysis has demonstrated that the genus *Bacillus* consists of at least five monophyletic clusters (Ash *et al.*, 1991). The rRNA group 1 (*Bacillus sensu stricto*) of aerobic, endospore-forming bacilli includes type species *Bacillus subtilis*. The species *Bacillus pantothenicus* initially included in rRNA group 1 (Ash *et al.*, 1991) has been recently renamed *Virgibacillus pantothenicus* (Heyndrickx *et al.*, 1998). The rRNA group 3 (Ash *et al.*, 1991) which includes the species *Bacillus azotofixans*, *Bacillus polymyxa*, *Bacillus macerans*, *Bacillus amylolyticus*, *Bacillus macquariensis*, *Bacillus pabuli*, *Bacillus pulvifaciens*, *Bacillus larvae*, *Bacillus alvei* and *Bacillus gordonae* has been transferred to the new genus *Paenibacillus* (Ash *et al.*, 1993). Heyndrickx *et*

al. (1995) showed that the species *Paenibacillus gordonae* and *Paenibacillus validus* form a homogeneous group leading to the emended description of *P. validus*. *Paenibacillus larvae* and *Paenibacillus pulvifaciens*, have been combined into *P. larvae* as *Paenibacillus larvae* subsp. *larvae* and *Paenibacillus larvae* subsp. *pulvifaciens*, respectively (Heyndrickx *et al.*, 1996a). *Bacillus lautus* and *Bacillus peoriae* were reclassified as *Paenibacillus lautus* and *Paenibacillus peoriae*, respectively (Heyndrickx *et al.*, 1996b). Recently, further *Bacillus* species, *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis* and *Bacillus thiaminolyticus*, have also been transferred to the genus *Paenibacillus* (Shida *et al.*, 1997a, b).

Three of the former *Bacillus* species, *Paenibacillus azotofixans*, *Paenibacillus polymyxa* and *Paenibacillus macerans*, are dinitrogen-fixers, and they form a monophyletic cluster in the genus *Paenibacillus* (Ash *et al.*, 1993). *Paenibacillus durum*, which is also a nitrogen-fixing species, has recently been grouped into

Abbreviation: ARA, acetylene-reduction assay.

The EMBL accession numbers for the *rrs* and *nifH* sequences determined in this paper are AJ223987–AJ223997.

P. azotofixans (Rosado *et al.*, 1997). On the other hand, *Bacillus circulans* which includes some nitrogen-fixing strains (belonging to *B. circulans* group 2) (Priest *et al.*, 1981), remained in the genus *Bacillus* on the basis of the *rrs* sequence data obtained for the *B. circulans* type strain, ATCC 4513^T (Ash *et al.*, 1993).

In order to determine the taxonomic position of nitrogen-fixing strains which had been isolated from the rhizosphere of wheat (TOD45) and maize (RSA19) and identified as *B. circulans* (group 2) by phenotypic characterization (API 50CH) (Berge *et al.*, 1991), their *rrs* gene was sequenced. Since nitrogen fixation among aerobic endospore-forming bacilli is a physiological character of major importance both for taxonomic and phylogenetic purposes and for ecological assessment, the type strains of some representative species of the genera *Bacillus* and *Paenibacillus* were analysed for nitrogenase activity (acetylene-reduction assay, ARA) and for the presence of the *nifH* gene.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. Strains PMD230 (Mavingui *et*

al., 1992), CF43 (Heulin *et al.*, 1994) and TOD45 were isolated from the rhizosphere of wheat, and strain RSA19 was isolated from the rhizosphere of maize (Berge *et al.*, 1991). Strains were grown in LB medium (1⁻¹ distilled water: 10 g tryptone, 5 g yeast extract and 5 g NaCl) at 28 °C, for 48 h for DNA extraction, and up to an OD₅₉₀ of 0.5 for PCR amplification.

Phenotypic characters. All strains studied were checked microscopically and by colony morphology. Biochemical identification was carried out using both the API 50CH (bioMérieux; <http://www.biomerieux.fr/>) and Biolog systems (<http://www.biolog.com/>).

Amplification and sequencing of *rrs* genes. Ten microlitres of water-washed suspensions (OD₅₉₀ 0.5) of the cultures were used for PCR amplification using a pair of universal primers (FGP5-255 and FGPS1509'-153; Normand *et al.*, 1995) corresponding to positions 5–24 and 1524–1540 of the *Escherichia coli rrs* sequence (GenBank accession no. J01859). The PCR products were purified on a 1% (w/v) low-melting-point-agarose gel (Bio-Rad). They were sequenced directly according to Anderson *et al.* (1992) with the Genomix system (Beckman; <http://www.beckman.com/>), using primers described previously (Normand *et al.*, 1996).

Amplification and sequencing of *nifH* genes. A 370 bp

Table 1. Bacterial strains used, *rrs* and *nifH* sequence accession numbers and nitrogenase activities

EMBL accession numbers in bold are for sequences determined in this study.

Bacterial species	Strain	Sequence accession no.		Nitrogenase activity* (mean ± SD)
		<i>rrs</i>	<i>nifH</i>	
<i>Paenibacillus azotofixans</i>	ATCC 35681 ^T	X60608	U23649	9.5 ± 1.6
<i>Paenibacillus azotofixans</i>	LMG 14658 ^T		AJ223993	7.1 ± 1.3
<i>Paenibacillus macerans</i>	ATCC 8244 ^T	X57306	AJ223994	2.1 ± 1.0
<i>Paenibacillus sp.</i>	RSA19	AJ223987	AJ223995	1.3 ± 0.3
<i>Paenibacillus polymyxa</i>	PMD230	AJ223988	AJ223996	0.8 ± 0.3
<i>Paenibacillus polymyxa</i>	CF43	AJ223989		0.7 ± 0.1
<i>Paenibacillus larvae</i>	LMG 9820 ^T	X60619		Weak
<i>Paenibacillus amylolyticus</i>	LMG 11153 ^T	D85396		Weak
<i>Paenibacillus lautus</i>	LMG 11157 ^T	D78473		Weak
<i>Paenibacillus pabuli</i>	LMG 11158 ^T	X60625		Weak
<i>Paenibacillus polymyxa</i>	ATCC 842 ^T	X60632	AJ223997	Weak
<i>Paenibacillus polymyxa</i>	LMG 13294 ^T			Weak
<i>Paenibacillus peoriae</i>	LMG 14832 ^T	D78476		Weak
<i>Paenibacillus sp.</i>	TOD45	AJ223990	AJ223992	Weak
<i>Paenibacillus validus</i>	LMG 11161 ^T	D78320		Negative
<i>Paenibacillus apriarius</i>	LMG 17433 ^T	U49247		Negative
<i>Paenibacillus alvei</i>	LMG 13253 ^T	X60604		Negative
<i>Bacillus longisporus</i>	LMG 13275 ^T	AJ223991		Negative
<i>Bacillus alcalophilus</i>	LMG 7120 ^T			Negative
<i>Bacillus cereus</i>	LMG 6923 ^T	D16266		Negative
<i>Bacillus circulans</i>	ATCC 4513 ^T	X60613		Negative
<i>Bacillus subtilis</i>	LMG 7135 ^T	X60646		Negative
<i>Bacillus simplex</i>	LMG 11160 ^T			Negative
<i>Brevibacillus brevis</i>	LMG 7123 ^T	X60612		Negative

* ARA results in μmol C₂H₄ produced per tube measured after 4 d incubation under 2% C₂H₂; weak result, values ranging from 30 to 150 nmol C₂H₄ per tube; negative result, no difference compared with negative control.

fragment of the *nifH* gene corresponding to positions 111 and 483 of the *Bradyrhizobium japonicum nifH* sequence (GenBank accession no. E01169) was amplified using the following primers: forward 5'-GGAATTCTGTGATCCT-AAAGCTGA-3' and reverse 5'-AGCATACATTGCCAT-CATTTCACC-3' (modified from Zehr *et al.*, 1995). Four primers were used in the sequencing reaction of the 370 bp of *nifH* gene: the two primers used for amplification and two internal primers. The purification and sequencing steps were the same as described above for the *rrs* gene.

Acetylene-reduction tests. ARA was performed as described by Omar *et al.* (1989). Briefly, strains were grown for 48 h on solid Watanabe's modified medium (Omar *et al.*, 1989) containing 5 g l⁻¹ glucose, sucrose, starch and mannitol. They were incubated under 2% (v/v) acetylene/air for 4 d, and then analysed for ethylene production by GC (Omar *et al.*, 1989).

Data analysis. Sequences were aligned using CLUSTAL V (Higgins & Sharp, 1988). Indel-containing regions were excluded from the analysis. Matrix pairwise comparisons were corrected for multiple base substitutions by the two-parameter method of Kimura (1980). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and parsimony (Kluge & Farris, 1969) methods. A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein, 1985). Graphic representation of the resulting trees was made using the NJPlot software (Perrière & Gouy, 1996).

RESULTS AND DISCUSSION

Strains PMD230 and CF43 were identified as *P. polymyxa* and strains RSA19 and TOD45 as *B. circulans* by both API 50CH and Biolog systems.

The phenotypic identification of strains PMD230 and CF43 as *P. polymyxa* was confirmed by results from *rrs* sequencing (Fig. 1). However, the phenotypic identification of strains RSA19 and TOD45 as *B. circulans* (group 2) using the API 50CH and Biolog systems was not in accordance with the results from the comparison of the *rrs* sequences (Fig. 1). It can thus be inferred that these two strains constitute a new species yet to be described which will probably also include other strains formerly classified into *B. circulans* group 2.

These two rhizosphere strains were found to form a monophyletic cluster with *P. azotofixans*, *P. polymyxa* and *P. macerans* and with *P. peoriae* (Fig. 1). Emendation of *B. circulans* started recently with the description of *Paenibacillus illinoisensis* (Shida *et al.*, 1997b), another species for which nitrogen fixation capacity still has to be checked. On the basis of our data from *rrs* sequencing, '*Bacillus longisporus*' would also have to be transferred to the genus *Paenibacillus*. This last species is taxonomically closely related to *P. amylolyticus* and *Paenibacillus pabuli* (Fig. 1).

When this work was started, the only *nifH* gene sequence available in the genus *Paenibacillus* was that of *P. azotofixans* (Zehr *et al.*, 1995). In the present

study, a *nifH* amplicon of 370 nucleotides in the *P. polymyxa* and *P. macerans* type strains was detected, as well as in strains RSA19, CF43 and PMD230 (Table 1). For some other *Paenibacillus* strains, a weak amplicon was obtained using *nifH* primers (data not shown), but the sequences obtained were completely different from the expected *nifH* sequence. This illustrates that obtaining an amplicon with *nifH* primers is not enough to infer the presence of a *nif* gene, and confirmation by hybridization or sequencing is thus necessary.

Although a *nifH* gene was detected in the *P. polymyxa* type strain, ATCC 842^T (as well as LMG 13294^T), and in strain TOD45 (Fig. 2), only weak nitrogenase activity was measured (Table 1). Nitrogen fixation by the *P. polymyxa* type strain was determined by measuring nitrogen gains through micro-Kjeldahl analyses, while acetylene-reduction activity has never yielded positive results (Rhodes-Roberts, 1981). Rhodes-Roberts (1981) concluded that the *P. polymyxa* type strain is nitrogen-fixing, and that suitable conditions for demonstrating acetylene reduction remain to be found. The present study reinforces this conclusion.

Weak acetylene-reduction activity was detected for the type strains of *P. larvae*, *P. amylolyticus*, *P. lautus*, *Paenibacillus macquariensis* and *P. peoriae*, but no detectable *nifH* amplicon. A similar case was reported for *Streptomyces thermoautotrophicus* (Gadkari *et al.*, 1992). Conclusions on whether nitrogen fixation occurs in a bacterial strain should hence be drawn from both a positive nitrogenase activity test (acetylene-reduction assay, micro-Kjeldahl or ¹⁵N-uptake analyses), and by sequencing an amplicon from a conserved structural *nif* gene such as *nifH*.

All *Paenibacillus nifH* sequences formed a coherent cluster together with the sequence of *P. azotofixans* defined by Zehr *et al.* (1995), which was different from those of the closely related nitrogen-fixing anaerobic genus *Clostridium* and the Gram-positive high-G + C-content genus *Frankia*. Our data reinforce the assumption that *nifH* genes of aerobic genera (*Paenibacillus*, cyanobacteria, actinomycetes, proteobacteria) and anaerobic genera (*Clostridium*, *Desulfovibrio*, *Chromatium*), are deeply divergent, as previously claimed by Chien & Zinder (1994, 1996). These authors showed that the described *nifH* sequences could be distributed into four clusters (aerobes, anaerobes, alternative nitrogenase and archaea). The present study confirms that all the tested nitrogen-fixing members of the *Bacillaceae* belong to the group of aerobic nitrogen-fixers and are away from the phylogenetically related genus *Clostridium*. It highlights the fact that genes encoding non-ubiquitous and non-essential functions are poor phylogenetic markers because of the divergent selection pressures exerted on them (Woese, 1987).

The *nifH* phylogenetic tree (Fig. 2) was compared to

Fig. 1. Phylogeny of *rrs* genes of selected *Bacillaceae* species and strains analysed by the neighbour-joining method. The percentage of 1000 bootstrap replicates higher than 60% is indicated above nodes, and those supported by parsimony analysis are indicated by a 'P' below it. Almost complete sequences were used. Scale bar represents 0.01 substitutions per site.

Fig. 2. Phylogeny of a fragment of *nifH* amino acid deduced sequences analysed by the neighbour-joining method. The percentage of 1000 bootstrap replicates that support each topological element by more than 90% is indicated above nodes, and those supported by parsimony analysis are indicated by a 'P' below. Only partial sequences (99 aa) of the *nifH* gene of *Paenibacillus* species were determined in the present study. Scale bar represents 0.029 substitutions per site.

that of the *rrs* gene. Differences in the topology of both trees should not be regarded as evidence of lateral transfer events. Presumably they originate rather from the short length, and the high sequence conservation of the studied *nifH* gene fragment, which reduces the resolving power of the analysis. A longer fragment could not be obtained in the *nifH* gene since it has not yet been sequenced completely for a member of the *Bacillaceae*, and appropriate primers do not yet exist in the less conserved C-terminal part of the gene. A combination of *nifH* and *nifD* primers might be used. A similar strategy was performed in the high-resolution phylogenetic analysis of *Frankia* (Cournoyer *et al.*, 1993; Nalin *et al.*, 1995).

Neither in the *B. circulans* type strain, ATCC 4513^T, nor in the type strains of *B. subtilis*, *B. cereus*, *B. alvei*, *Bacillus alcalophilus*, *Bacillus simplex*, *Brevibacillus brevis* and *Paenibacillus validus* could a *nifH* amplicon be detected or ARA activity be demonstrated. This does not exclude the possibility that nitrogen-fixing *Bacillus* strains will not be isolated in the future, but nitrogen fixation should be assessed more systematically. This could allow more comprehensive conclusions to be drawn on the distribution of nitrogen fixation in the *Firmicutes*.

Even if our investigations were performed with only a part of the described *Bacillus* species, our results

suggest that nitrogen fixation among aerobic endospore-forming bacilli is restricted to the genus *Paenibacillus*. In contrast, in proteobacteria and cyanobacteria nitrogen-fixing taxa are interspersed with non-nitrogen-fixing ones. The situation in the *Bacillaceae* would be in accordance with that found in the other *Firmicutes* where nitrogen-fixers are rare and form the monophyletic taxa *Frankia* and *Clostridium*.

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