

Reclassification of *Geobacillus pallidus* (Scholz *et al.* 1988) Banat *et al.* 2004 as *Aeribacillus pallidus* gen. nov., comb. nov.

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Although *Anoxybacillus* and *Geobacillus*, two genera of thermophilic bacteria close to the genus *Bacillus*, have only been described recently, the number of species in these genera has increased rapidly. Four thermophilic, lipolytic strains (DR01, DR02, DR03 and DR04) isolated from a hot spring in Veracruz (Mexico), which could not be identified phenotypically, were subjected to 16S rRNA gene sequence analysis. Three strains were identified as belonging to the genus *Anoxybacillus*, but strain DR03 was identified as *Geobacillus pallidus*. This result led us to perform a phylogenetic analysis of the genera *Anoxybacillus* and *Geobacillus* based on 16S rRNA gene sequences from all the type strains of these genera. Phylogenetic trees showed three major clusters, *Anoxybacillus*–*Geobacillus tepidamans*, *Geobacillus sensu stricto* and *Geobacillus pallidus*, while the 16S rRNA gene sequences of *G. pallidus* (DR03 and the type strain) showed low similarity to sequences of *Anoxybacillus* (92.5–95.1 %) and *Geobacillus* (92.8–94.5 %) species, as well as to *Bacillus subtilis* (92.2–92.4 %). In addition, *G. pallidus* could be differentiated from *Anoxybacillus* and *Geobacillus* on the basis of DNA G+C content and fatty acid and polar lipid profiles. From these results, it is proposed that *Geobacillus pallidus* should be classified in a novel genus, for which we propose the name *Aeribacillus*, as *Aeribacillus pallidus* gen. nov., comb. nov. The type strain of *Aeribacillus pallidus* is H12^T (=ATCC 51176^T =DSM 3670^T =LMG 19006^T).

The thermophilic bacterial genera *Anoxybacillus* and *Geobacillus* were described in the radiation from the Gram-positive genus *Bacillus* (Pikuta *et al.*, 2000; Nazina *et al.*, 2001). The genus *Geobacillus* was described for six species belonging to the aerobic thermophilic *Bacillus* group 5, including *Geobacillus stearothermophilus* as the type species, and two novel species (*Geobacillus subterraneus* and *Geobacillus uzonensis*), based on physiological characteristics, fatty acid composition, DNA–DNA hybridization studies and 16S rRNA gene sequence analysis (Nazina *et al.*, 2001). Members of this genus are Gram-positive, spore-forming rods, neutrophilic, moderately thermophilic and aerobic or facultatively anaerobic. They contain iso-C_{15:0},

iso-C_{16:0} and iso-C_{17:0} as the major cellular fatty acids. The G+C content of the genomic DNA is 48.2–58 mol% and 16S rRNA gene sequence analysis of these species results in a very compact phylogenetic cluster, exhibiting internal levels of sequence similarity higher than 96.5 %.

To date, 18 *Geobacillus* species have been described, including ‘*Geobacillus zalihae*’ (Rahman *et al.*, 2007), the name of which has not been validly published. This continuing description of novel species has introduced some discrepancies with respect to the original characteristics of the genus. For some species, the G+C content of the DNA is below 48 mol% and/or the fatty acid profile (when determined) is different from that described for the genus. Moreover, low pairwise 16S rRNA sequence similarity values have been described among *Geobacillus* species, as in the case of *Geobacillus debilis* with respect to *G. stearothermophilus* (93 %) and *Geobacillus pallidus* (91 %) (Banat *et al.*, 2004).

The genus *Anoxybacillus* includes Gram-positive, spore-forming rods, alkaliphilic or alkalitolerant, thermophilic

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains DR01, DR02, DR03 and DR04 are respectively EU621359–EU621362.

A 16S rRNA gene sequence similarity matrix and maximum-likelihood tree, polar lipid profiles and a comparison of G+C content and fatty acid profiles of *Anoxybacillus* and *Geobacillus* species are available with the online version of this paper.

and aerotolerant or facultative anaerobes. The main fatty acid is iso-C_{15:0} and the DNA G+C content is 42–57 mol%. Sequences of the 16S rRNA gene showed at least 96% similarity between the three *Anoxybacillus* species that were included in the emended description of the genus (Pikuta *et al.*, 2003). At the time of writing, the genus *Anoxybacillus* comprises a total of 10 species, including the slightly acidophilic species *Anoxybacillus amylolyticus* (Poli *et al.*, 2006) and the strict aerobe *Anoxybacillus rupiensis* (Derekova *et al.*, 2007), two species whose type strains show high 16S rRNA gene sequence similarity to that of *Geobacillus tepidamans* (97.5 and 96.5%, respectively).

Recently, we isolated four thermophilic, lipolytic strains (DR01, DR02, DR03 and DR04) from a hot spring in Veracruz (Mexico), and these were preliminarily identified as belonging to the genus *Anoxybacillus* (Pinzón *et al.*, 2007a, b). The authors reported the first characterization of thermostable lipases from *Anoxybacillus* strains, adding to some *Bacillus* and *Geobacillus* strains that have been described previously as thermostable-lipase producers by other authors (Schmidt-Dannert *et al.*, 1994; Castro-Ochoa *et al.*, 2005; Rahman *et al.*, 2007).

In the present study, 16S rRNA gene sequences of all four strains were determined as described previously (Miñana-Galbis *et al.*, 2007) in order to confirm their identity. Sequences were about 1500 bp long. Sequences of strains DR01, DR02 and DR04 showed only 1–2 nucleotide differences, as well as a high level of similarity to sequences of *Anoxybacillus ayderensis*, *Anoxybacillus gonensis*, *Anoxybacillus flavithermus* and *Anoxybacillus kamchatkensis* (>99%; Supplementary Table S1, available in IJSEM Online). In contrast, strain DR03 could not be assigned to the genus *Anoxybacillus* because its 16S rRNA gene sequence was almost identical to that of *G. pallidus* DSM 3670^T (2 nucleotide differences). This unexpected result was confirmed by DNA–DNA hybridization between strain DR03 and *G. pallidus* LMG 19006^T, performed by the BCCM/LMG (Belgian Coordinated Collections of Micro-organisms/Laboratorium voor Microbiologie from Universiteit Gent) Identification Service, as described previously (Miñana-Galbis *et al.*, 2007). This analysis revealed 82% DNA relatedness, and we therefore concluded that strain DR03 belongs to *G. pallidus* (Wayne *et al.*, 1987).

This result led us to perform a phylogenetic analysis of the genera *Anoxybacillus* and *Geobacillus* based on 16S rRNA gene sequences from all the type strains of these genera (taken from GenBank) and from our strains. Sequence alignment and phylogenetic analysis were conducted using MEGA version 4.0 (Kumar *et al.*, 2004; Tamura *et al.*, 2007) except in the case of the maximum-likelihood method, which was performed using PhyML version 3.0 (Guindon & Gascuel, 2003). Phylogenetic trees with complete- and pairwise-deletion options were reconstructed by maximum-parsimony and neighbour-joining methods. The latter method was performed using different distance

estimation models (Jukes–Cantor, Kimura two-parameter and Tamura–Nei). The topological robustness of the phylogenetic trees was evaluated by a bootstrap analysis through 1000 replicates. All the obtained dendrograms showed almost identical topologies. The neighbour-joining tree with the Jukes–Cantor model and the pairwise-deletion option is shown in Fig. 1, while pairwise 16S rRNA gene sequence similarities are presented in Supplementary Table S1.

Three major clusters can be observed in Fig. 1. The cluster of the genus *Anoxybacillus* grouped all *Anoxybacillus* species, strains DR01, DR02 and DR04 and *G. tepidamans* with a bootstrap value of 80%. Within this cluster, *G. tepidamans* was grouped in the same phylogenetic branch as *A. rupiensis*, although this phylogenetic relationship was not confirmed by the maximum-likelihood method (Supplementary Fig. S1). 16S rRNA gene sequence similarities between *G. tepidamans* and *Anoxybacillus* species and other *Geobacillus* species ranged from 94.0 to 98.0% (29–80 nucleotide differences) and 91.7 to 96.0% (52–106 nucleotide differences), respectively (Supplementary Table S1). Nevertheless, *A. rupiensis* and *G. tepidamans* shared higher sequence similarities with type strains of their respective genera than with those from the other genus (Supplementary Table S1). Further analyses such as housekeeping gene sequencing (Zeigler, 2005) are needed for clarification of the taxonomic and phylogenetic positions of *A. rupiensis* and *G. tepidamans*.

In a second cluster, all species of the genus *Geobacillus*, except for *G. tepidamans* and *G. pallidus*, were grouped with a bootstrap value of 92%. *G. pallidus* strains (the type strain and DR03) were grouped in a third cluster (100% bootstrap), constituting an independent phylogenetic line. The 16S rRNA gene sequence similarity between *G. pallidus* (DR03 and the type strain) and species of the *Anoxybacillus* and *Geobacillus* clusters ranged from 92.5 to 95.1% (72–103 nucleotide differences) and 92.8 to 94.5% (74–108 nucleotide differences), respectively; the range was 92.2–92.4% with respect to the 16S rRNA gene sequence of the *Bacillus subtilis* type strain, which was included in the phylogenetic analysis as an outgroup (Supplementary Table S1). This result was confirmed by the maximum-likelihood method (Supplementary Fig. S1), suggesting that *G. pallidus* could represent a new genus in the family *Bacillaceae*. Although there are no clear-cut recommendations for genus delineation (Gillis *et al.*, 2001), these similarity levels were significantly lower than 96.5%, the value suggested by Nazina *et al.* (2001) for members of the genus *Geobacillus*.

Additionally, comparison of DNA G+C content and fatty acid profiles between species belonging to the genera *Anoxybacillus* and *Geobacillus* was performed (Supplementary Table S2). The G+C content ranged from 42 to 50 mol% in the genus *Anoxybacillus* and from 44 to 58 mol% in the genus *Geobacillus*, except in the case of *G. pallidus* (39–41 mol%) and *G. tepidamans* (42–43 mol%).

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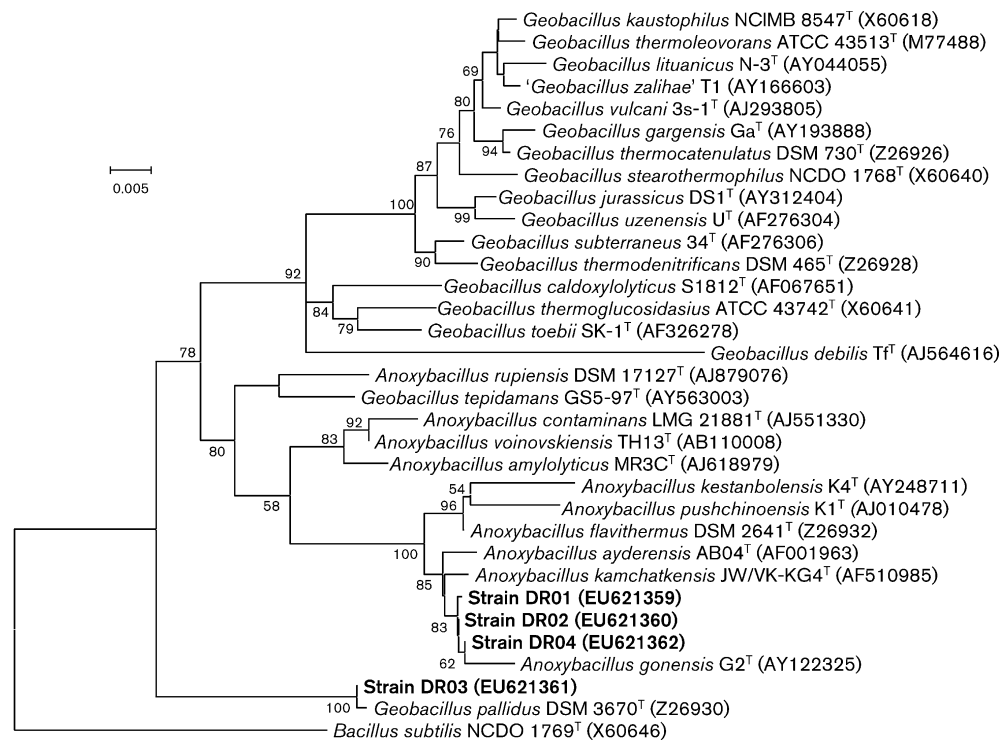


Fig. 1. Consensus neighbour-joining phylogenetic tree (Jukes–Cantor model and pairwise-deletion option) obtained from 33 sequences of the 16S rRNA gene, encompassing all *Anoxybacillus* and *Geobacillus* species. GenBank accession numbers are indicated in parentheses. Bar, distance of 0.005 substitutions per nucleotide position as calculated by MEGA. Bootstrap values (>50%) after 1000 replicates are shown.

The main fatty acid in *Anoxybacillus* species is iso-C_{15:0} (>38%), whereas the major cellular fatty acids in *Geobacillus* are iso-C_{15:0}, iso-C_{16:0} and iso-C_{17:0} (>60%), except in the case of *Geobacillus vulcani* (52.2%) and *G. pallidus* (19.5%), where the main fatty acid is C_{16:0} (50%). The fatty acid profile of *G. pallidus* LMG 19006^T determined in this study comprised C_{16:0} (50.0%), iso-C_{16:0} (9.3%), C_{14:0} (8.5%), anteiso-C_{17:0} (6.5%), iso-C_{15:0} (6.2%), anteiso-C_{15:0} (4.9%), iso-C_{17:0} (4.0%), C_{18:0} (2.1%), anteiso-C_{14:0} (1.6%), iso-C_{14:0} (1.6%) and C_{15:0} (1.2%).

Polar lipid profiles of *Anoxybacillus* and *Geobacillus* species could not be compared because they have not been reported. Because of that, polar lipids of *Anoxybacillus pushchinoensis* DSM 12423^T, *G. pallidus* LMG 19006^T and *G. stearothermophilus* CECT 43^T were extracted and analysed as reported previously (Tindall, 1990; Kämpfer *et al.* 2006; Tindall *et al.* 2007). Results were compared with *Bacillus subtilis* ATCC 6051^T and standards diposphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Sigma-Aldrich). The polar lipid profile of the type strain of the type species of the genus *Anoxybacillus* exhibited DPG, PE, PG and two unknown polar lipids (aminolipid AL1 and polar lipid L1). In contrast, *G. pallidus* LMG 19006^T produced large to moderate amounts of DPG, PG, L1 and four unknown

polar lipids (glycolipids GL1 and GL2, glycophospholipid GPL1 and polar lipid L2) and small amounts of three other polar lipids (glycolipids GL3 and GL4 and phospholipid PL1) and lacked PE. The polar lipid composition of the type strain of the type species of the genus *Geobacillus* was also different from those of the other two type strains, since this strain exhibited large to moderate amounts of DPG, PE, AL1 and an unknown aminophospholipid (APL1) and small amounts of L1 and an unknown aminophospholipid (APL2) and lacked PG (Supplementary Fig. S2).

Therefore, *G. pallidus* can also be distinguished from *Anoxybacillus* species and other *Geobacillus* species by the DNA G+C content and the fatty acid and polar lipid profiles (Table 1).

On the basis of 16S rRNA gene sequence similarities, phylogenetic analyses, DNA G+C contents and cellular fatty acid and polar lipid profiles, we propose that *Geobacillus pallidus* (Banat *et al.*, 2004) is reclassified as *Aeribacillus pallidus* gen. nov., comb. nov.

Description of *Aeribacillus* gen. nov.

Aeribacillus (Ae.ri.ba.cil'lus. L. n. *aer*, *aeris* air; L. masc. n. *bacillus* a small rod; N.L. masc. n. *Aeribacillus* aerobic small rod).

Table 1. Characteristics that distinguish the genus *Aeribacillus* gen. nov. from the genera *Anoxybacillus* and *Geobacillus*

Data were taken from the following studies: *Aeribacillus* gen. nov., this study and Scholz *et al.* (1987); *Anoxybacillus*, this study and Pikuta *et al.* (2000, 2003); *Geobacillus*, this study and Nazina *et al.* (2001). Members of all three genera are thermophilic and have MK-7 as the main isoprenoid quinone. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unknown aminolipid; APL, unknown aminophospholipid; GPL, unknown glycolipid; GL, unknown glycolipid; L, unknown polar lipid.

Characteristic	<i>Aeribacillus</i>	<i>Anoxybacillus</i>	<i>Geobacillus</i>
Relation to:			
O ₂	Aerobe	Aerotolerant or facultative anaerobe	Aerobe or facultative anaerobe
pH	Alkalitolerant	Alkaliphilic or alkalitolerant	Neutrophilic
Catalase activity	+	+/-	+
Oxidase activity	+	+/-	+/-
Major fatty acid(s)	C _{16:0}	iso-C _{15:0}	iso-C _{15:0} , iso-C _{16:0} , iso-C _{17:0}
Major polar lipids*	DPG, PG, 2GL, GPL, 2L	DPG, PE, PG, AL, L	DPG, PE, AL, APL
DNA G + C content (mol%)	39–41	42–57	48–58

*The same aminolipid (AL1) was produced by *Anoxybacillus* and *Geobacillus* species. One of the unknown polar lipids (L1) was produced by *Aeribacillus*, *Anoxybacillus* and *Geobacillus*, but the latter genus exhibited only minor amounts.

Cells are aerobic, thermophilic, alkalitolerant, motile, Gram-positive rods (0.8–0.9 × 2–5 µm) that occur singly, in pairs or in chains. Ellipsoidal to cylindrical endospores are formed centrally to terminally within slightly swollen sporangia. Catalase- and oxidase-positive. The main menaquinone is MK-7. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids, an unknown glycolipid and two unknown polar lipids. In addition, two other unknown glycolipids and an unknown phospholipid may be present. The main fatty acid is C_{16:0} and the fatty acids C_{14:0}, C_{15:0}, C_{18:0}, anteiso-C_{14:0}, anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{14:0}, iso-C_{15:0}, iso-C_{16:0} and iso-C_{17:0} may be present in amounts less than 10%. The DNA G + C content ranges from 39 to 41 mol%. The type species is *Aeribacillus pallidus*.

Description of *Aeribacillus pallidus* comb. nov.

Aeribacillus pallidus (pal'li.dus. L. masc. adj. *pallidus* pale, referring to the pale colony colour).

Basonym: *Bacillus pallidus* Scholz *et al.* 1988.

Homotypic synonym: *Geobacillus pallidus* (Scholz *et al.* 1988) Banat *et al.* 2004.

In addition to the description given for *Bacillus pallidus* by Scholz *et al.* (1987), the species exhibits the properties listed in the genus description above. The type strain is H12^T (=ATCC 51176^T =DSM 3670^T =LMG 19006^T).

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