International Journal of Systematic Bacteriology (1999), 49, 531–540 Printed in Great Britain

Transfer of *Bacillus lentimorbus* and *Bacillus popilliae* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus lentimorbus* comb. nov. and *Paenibacillus popilliae* comb. nov.

Bertil Pettersson,[1] Karen E. Rippere,[2] Allan A. Yousten[2] and Fergus G. Priest[3]

Author for correspondence: Fergus G. Priest. Tel: +44 131 451 3464. Fax: +44 131 451 3009. e-mail: f.g.priest@hw.ac.uk

[1] Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden

[2] Biology Department, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

[3] Department of Biological Sciences, Heriot Watt University, Edinburgh EH14 4AS, UK

Almost complete 16S rRNA gene sequences were generated for the type strains of the obligate insect pathogens *Bacillus lentimorbus* and *Bacillus popilliae* and a second strain of *Bacillus popilliae* (NRRL B-4081) received as '*Bacillus popilliae* var. *melolonthae*'. A phylogenetic tree was constructed which grouped these strains into a well defined subcluster within the genus *Paenibacillus*. *Bacillus popilliae* NRRL B-4081 occupied an intermediate position between the type strains of *Bacillus lentimorbus* and *Bacillus popilliae* but with a marked clustering to the latter. The phylogenetic assignment of these strains to *Paenibacillus* is in contrast to earlier studies which placed these bacteria in the genus *Bacillus*, close to *Bacillus subtilis*. Indeed, the rRNA sequences generated in this study share less than 88% similarity to the deposited sequences for *Bacillus popilliae* ATCC 14706^T and *Bacillus lentimorbus* ATCC 14707^T. The results obtained by using different tree algorithms, bootstrap analysis, branch lengths and verification by signature nucleotide analysis supported the reclassification of these species in the genus *Paenibacillus* as *Paenibacillus lentimorbus* comb. nov. and *Paenibacillus popilliae* comb. nov.

Keywords: *Paenibacillus lentimorbus* comb. nov., *Paenibacillus popilliae* comb. nov., insect pathogens, 16S rRNA

INTRODUCTION

Bacillus lentimorbus and *Bacillus popilliae* were first described by Dutky (1940) as the causative agents of milky disease in Japanese beetle and related scarab larvae (Klein & Kaya, 1995). Although there have been suggestions in the past that *Bacillus popilliae* and *Bacillus lentimorbus* may be varieties of a single species, DNA hybridization and RAPD analysis have shown that the two taxa are closely related but distinct and appropriately placed in separate species (Rippere *et al.*, 1998). Physiologically, differences between the species are few, but growth in the presence of vancomycin or 2% NaCl is generally associated with strains of *Bacillus popilliae* (Rippere *et al.*, 1998). The two species are responsible for slightly different forms of milky disease. *Bacillus popilliae* causes type A milky disease and *Bacillus lentimorbus* is associated with type B milky disease (Dutky, 1940). The latter is characterized by the appearance of brown clots which block the circulation of haemolymph in the larva and lead to gangrenous conditions in the affected parts (Stahly *et al.*, 1992).

In a comparative analysis of 16S RNA sequences of 51 species of the genus *Bacillus*, Ash *et al.* (1991) described five principal groups of strains of which two, rRNA groups 3 and 4, have since been afforded generic status as *Paenibacillus* (Ash *et al.*, 1993) and *Brevibacillus* (Shida *et al.*, 1996), respectively. In this original study, the type strains of *Bacillus popilliae* and *Bacillus*

The GenBank accession numbers for the 16S rRNA gene sequences of *Bacillus lentimorbus* ATCC 14707^T, and *Bacillus popilliae* ATCC 14706^T and NRRL B-4081 are AF071861, AF071859 and AF071860, respectively.

lentimorbus were recovered in rRNA group 1, a large group containing the type species of the genus, *Bacillus subtilis* (Ash *et al.*, 1991). However, *Bacillus popilliae* and *Bacillus lentimorbus* have numerous physiological and ecological traits in common with various species of *Paenibacillus*, including poor sporulation *in vitro*, swollen sporangia, a requirement for complex media, variable Gram stain reaction and lack of catalase (in common with *Paenibacillus larvae* subsp. *larvae*). Indeed, in a numerical analysis of phenotypic features *Bacillus popilliae* and *Bacillus lentimorbus* were recovered in a cluster with other paenibacilli associated with insects, including *Paenibacillus larvae* subsp. *larvae* and *Paenibacillus larvae* subsp. *pulvifaciens* (Priest *et al.*, 1981). These physiological and ecological observations suggested to us that *Bacillus popilliae* and *Bacillus lentimorbus* may be misclassified in rRNA group 1 and are actually more closely related to the bacteria of the genus *Paenibacillus*. Here we report a phylogenetic analysis based on 16S rRNA sequences of the type strains of *Bacillus popilliae* (ATCC 14706^T) and *Bacillus lentimorbus* (ATCC 14707^T) and present data consistent with the transfer of these species to the genus *Paenibacillus*.

METHODS

Strains and growth conditions. The strains used are listed in Table 1. Although strain NRRL B-4081 has been designated '*Bacillus popilliae* var. *melolonthae*', given its uncertain varietal status (Stahly *et al.*, 1992; Rippere *et al.*, 1998) we shall refer to it simply by its species name. *Bacillus lentimorbus* and *Bacillus popilliae* strains were grown in MYPGP agar and broth (Dingman & Stahly, 1983) at 30 °C and stored as described previously (Rippere *et al.*, 1998). All other strains were grown on nutrient agar and broth at 37 °C.

Fatty acid analysis. Fatty acids were extracted from strains ATCC 14706^T and ATCC 14707^T by a single tube method as described by Mayberry & Lane (1993). Following esterification, fatty acids were analysed by GC on a Hewlett Packard 5890 using a flame-ionization detector. Microbial Identification System software (Microbial ID) was used for analysis.

DNA preparation and amplification for identification. DNA was prepared from 1 litre MYPGP broth as described previously (Rippere *et al.*, 1998). The specific primers PAEN515F (Shida *et al.*, 1997) and PAEN862F were used in conjunction with a reverse primer (RNA1484R) to the distal end of the 16S rRNA gene (Table 2) in PCR amplifications for presumptive identification of strains of the genus *Paenibacillus*. As a control, the forward primer (RNA31F), which recognizes the proximal end of the 16S rRNA gene, was used. Amplification conditions for both reactions were denaturation at 94 °C for 5 min followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1·5 min and extension at 72 °C for 1·5 min followed by a final extension at 72 °C for 5 min. Products were separated in 1·0% agarose gels in TAE buffer.

***In vitro* amplification of the 16S rRNA gene.** The 16S rRNA gene from genomic DNA preparations of *Bacillus lentimorbus* ATCC 14707^T and *Bacillus popilliae* strains ATCC 14706^T and NRRL B-4081 was amplified with a pair of primers complementary to the regions close to the 5′ and the 3′ termini of the gene. Genomic DNA of the respective strain (10 ng) was used for amplification with the primers RIT604 and RIT621B (Table 2). PCR products (1 µl) were subjected to two different semi-nested amplifications. The primers RIT604 and RIT390B were used in one of the amplifications and RIT388 and RIT621B (Table 2) were used in the second semi-nested reaction. Biotinylated PCR products, suitable for solid-phase DNA sequencing, were generated with 10 pmol of each primer and the following thermocycling profile was used: denaturation at 96 °C for 15 s and a combined primer annealing/extension step at 70 °C for 90 s was repeated 30 times. A final extension at 72 °C for 10 min was also used.

Direct solid-phase 16S rDNA sequencing. Immobilization of the biotinylated PCR products followed by strand separation and template preparation were performed with superparamagnetic beads (Dynabeads M-280 Streptavidin; Dynal). The nucleotide sequences from both strands were determined by direct solid-phase DNA sequencing (Hultman *et al.*, 1991, 1989) with ALFexpress (Amersham Pharmacia Biotech) as described previously (Pettersson *et al.*, 1996a, b, c). The sequencing primers are listed in Table 2.

Comparison of 16S rRNA genes. The 16S rDNA sequences from the *Bacillus popilliae* and *Bacillus lentimorbus* strains determined in this study were checked against deposited 16S rDNA sequences in non-redundant GenBank, EMBL and

Table 1. Bacterial strains used in the study

Strain	rRNA group*	Reaction with *Paenibacillus*-specific PCR	Source/description
Bacillus subtilis NCIMB 3610^T	1	−	Priest *et al.* (1988)
Bacillus sphaericus 2362	2	−	Alexander & Priest (1990)
Paenibacillus alvei DSM 29^T	3	+	Priest *et al.* (1988)
Paenibacillus chibensis NRRL B-142^T	3	+	Shida *et al.* (1997)
Bacillus lentimorbus ATCC 14707^T	3	+	Rippere *et al.* (1998)
Bacillus popilliae ATCC 14706^T	3	+	Rippere *et al.* (1998)
'*Bacillus popilliae* var. *melolonthae*' NRRL B-4081	3	+	Rippere *et al.* (1998)
Brevibacillus borstelensis HRS 23	4	−	De Silva *et al.* (1998)

* rRNA groups as defined by Ash *et al.* (1991).

Table 2. Primers used for PCR and DNA sequencing

Primer*	Position†	Sequence (5′–3′)‡	Application
PAEN515F	470–494	GCTCGGAGAGTGACGGTACCTGAGA	*Paenibacillus*-specific
PAEN862F	842–862	TCGATACCCTTGGTGCCGAAGT	*Paenibacillus*-specific
RNA31F	8–31	gcgcaagcttAGAGTTTGATCCTGGCTCAGGACG	PCR
RNA1484R	1507–1484	gcggatccTACCTTGTTACGACTTCACCCCA	PCR
604	11–35	USP-GTTYGATCCTRGCTCAGGAYIAACG	PCR
388	334–356	USP-CCARACTCCTACGGRAGGCAGC	PCR
390B	939–917	CTTGTGCGGGYYCCCGTCAATTC	PCR
620B	1542–1520	RSP-GAAAGGAGGTRWTCCAYCCSCAC	PCR
USP		CGTTGTAAAACGACGGCCAG	Sequencing
631C	534–519	ATTACCGCGGCKGCTG	Sequencing
538C	800–818	GTAGTCCACGCCGTAAACG	Sequencing
390C	939–917	CTTGTGCGGGYYCCCGTCAATTC	Sequencing
623C	1184–1200	GGGGATGATGTCAARTC	Sequencing
624C	1237–1220	GTAGYAYGTGTGWAGCCC	Sequencing
RSP		CACAGGAAACAGCTATGACC	Sequencing

* B and C indicate that the actual oligonucleotide was labelled with a biotin and an indodicarbocyanine (Cy5), respectively.

† *E. coli* numbering (Brosius *et al.*, 1978).

‡ USP and RSP indicate that the PCR primer has a handle at the 5′ end with the sequence of the universal sequencing primer and the reverse sequencing primer, respectively. Lower case letters at the 5′ ends of RNA31F and RNA1484R indicate two flanking residues and six residues constituting endonuclease restriction sites previously used for cloning. K, R, S, W and Y denote degenerate positions according to the IUB letter code. The 'I' in primer 604 denotes the presence of an inosine residue.

DDBJ databases by using the gapped BLAST option (Altschul *et al.*, 1997), implemented as a sequence search tool at the home page of The National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov). Close relatives were retrieved and aligned with other relatives maintained at the Ribosomal Database Project (Maidak *et al.*, 1996). A secondary structure of the 16S rRNA of *Bacillus subtilis* (Maidak *et al.*, 1996) was used for proper alignment of stems and loops. The alignment was performed manually using GDE (Genetic Data Environment) software (Smith, 1992). The phylogenies were inferred by using programs contained in the Phylogenetic Inference Package, PHYLIP 3.51c (Felsenstein, 1993). Evolutionary distances were calculated by DNADIST, correcting for multiple base changes at single locations by the method of Jukes & Cantor (1969). User trees based on the distance matrix were computed by the neighbour-joining method (Saitou & Nei, 1987) using the NEIGHBOR program. Parsimony analysis was performed by using DNAPARS. The trees obtained from neighbour-joining and parsimony were analysed for stability by performing bootstrap analysis by using SEQBOOT in sets of 500 resamplings. Majority-rule consensus trees were computed from the user trees by using the CONSENSE program.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rRNA sequences of the reference strains used for comparison in this study are as follows: *Alicyclobacillus acidocaldarius* ATCC 27009^T, X60742; *Alicyclobacillus cycloheptanicus* DSM 4006^T, X51928; *Aneurinibacillus aneurinilyticus* ATCC 12856^T, X60645; *Aneurinibacillus migulanus* ATCC 9999^T, D78462; *Bacillus circulans* ATCC 4513^T, X60613; *Bacillus megaterium* IAM 13418^T, D16273; *Bacillus methanolicus* NCIMB 13114, X64465; *Bacillus* sp., X60622 (formerly *Bacillus lentimorbus* ATCC 14707^T); *Bacillus* sp., X60633 (formerly *Bacillus popilliae* ATCC 14706^T); *Bacillus stearothermophilus* ATCC 12980^T, X60640; *Bacillus subtilis* ATCC 6051^T, X60646; '*Bacillus tipchiralis*' C-type, AF039408; *Brevibacillus borstelensis* NRRL NRS-818^T, D78456; *Brevibacillus brevis* ATCC 8246^T, X60612; *Paenibacillus alginolyticus* DSM 5050^T, D78465; *Paenibacillus alvei* ATCC 6344^T, X57304; *Paenibacillus amylolyticus* NRRL B-377^T, D85396; *Paenibacillus apiarius* NRRL NRS-1438^T, U49247; *Paenibacillus azotofixans* NRRL B14372^T, D78318; *Paenibacillus chibensis* NRRL NRS-142^T, D85395; *Paenibacillus chondroitinus* DSM 5051^T, D82064; *Paenibacillus curdlanolyticus* IFO 15724^T, D78466; *Paenibacillus glucanolyticus* DSM 5162^T, D78470; *Paenibacillus gordonae* ATCC 29948^T, X60617; *Paenibacillus illinoisensis* NRRL NRS-1356^T, D85397; *Paenibacillus kobensis* IFO 15729^T, D78471; *Paenibacillus larvae* subsp. *larvae* ATCC 9545^T, X60619; *Paenibacillus lautus* NRRL NRS-666^T, D78473; *Paenibacillus macerans* DSM 24^T, X60624; *Paenibacillus macquariensis* NCTC 10419^T, X60625; *Paenibacillus pabuli* DSM 3036^T, X60630; *Paenibacillus peoriae* IFO 15541^T, D78476; *Paenibacillus polymyxa* IAM 13419^T, D16276; '*Paenibacillus*' strain C-168, Y16129; '*Paenibacillus*' strain T-168, Y16128; *Paenibacillus thiaminolyticus* JCM 8360^T, D78475; *Paenibacillus thiaminolyticus* JCM 7540, D88513; *Paenibacillus validus* DSM 3037^T, D78320.

RESULTS AND DISCUSSION

Presumptive allocation of *Bacillus lentimorbus* and *Bacillus popilliae* to the genus *Paenibacillus*

Two diagnostic oligonucleotide primers targeted to unique regions of the 16S rRNA gene of paenibacilli (Ash *et al.*, 1993; Shida *et al.*, 1996) were used in PCR reactions with template DNA from *Bacillus lenti-*

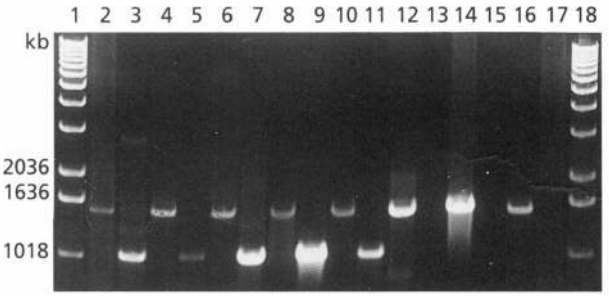

Fig. 1. Agarose gel electrophoresis of PCR products generated with control primers (even lanes) and a *Paenibacillus*-specific primer (odd lanes) for various endospore-forming bacteria. Lanes: 1, size markers (1 kb ladder); 2 and 3, *Paenibacillus alvei* DSM 29^T; 4 and 5, *Paenibacillus chibensis* NRRL B-142^T; 6 and 7, *Bacillus popilliae* ATCC 14706^T; 8 and 9, *Bacillus lentimorbus* ATCC 14707^T; 10 and 11, *Bacillus popilliae* NRRL B-408; 12 and 13, *Bacillus subtilis* NCIMB 3610^T; 14 and 15, *Bacillus sphaericus* 2362; 16 and 17, *Brevibacillus borstelensis* HRS 23; 18, size markers.

morbus ATCC 14707^T, *Bacillus popilliae* ATCC 14706^T and NRRL B-4081, and representative strains of *Bacillus*, *Brevibacillus* and *Paenibacillus* (Fig. 1). The control reaction resulted in the predicted band of 1·5 kb in agarose gels of PCR products from all strains. However, when the specific primer PAEN515F was used, only the DNA from the *Paenibacillus* strains and from *Bacillus lentimorbus* and *Bacillus popilliae* acted as template, resulting in the predicted product of around 1 kb (Fig. 1). DNA was not amplified from *Bacillus subtilis* (rRNA group 1), *Bacillus sphaericus* (rRNA group 2) and *Brevibacillus borstelensis* (rRNA group 4) with this primer. This suggests that *Bacillus lentimorbus* and *Bacillus popilliae* are indeed members of the genus *Paenibacillus* and confirms the utility of this oligonucleotide as a diagnostic tool for strains of the genus *Paenibacillus*. Similar results (not shown) were obtained with the primer PAEN862F (Ash *et al.*, 1993).

16S rRNA gene sequences of *Bacillus lentimorbus* and *Bacillus popilliae*

Direct solid-phase 16S rDNA sequencing resulted in virtually complete (> 95%) sequences of the 16S rRNA genes of *Bacillus popilliae* ATCC 14706^T (1470 nt) and NRRL B-4081 (1470 nt), and *Bacillus lentimorbus* ATCC 14707^T (1479 nt). By using this procedure, the nucleotide sequence data of the 16S rRNA genes belonging to different rRNA operons is determined simultaneously. In all cases both strands were sequenced and all positions were unambiguously determined. Interestingly, microheterogeneities (polymorphisms) in which two nucleotide residues share the same nucleotide position in different alleles, as described for *Bacillus sporothermodurans*, were detected for *Bacillus popilliae* ATCC 14706^T and *Bacillus lentimorbus* ATCC 14707^T. These polymorphisms were situated in or close to the hypervariable regions V1 and V2 (Gray *et al.*, 1984) of the 16S rRNA molecule. This has also been found to be the case for other aerobic, endospore-forming bacteria, e.g. *Bacillus sporothermodurans* (Pettersson *et al.*, 1996b) and *Paenibacillus alginolyticus* DSM 5050^T (B. Pettersson, unpublished). In contrast, polymorphisms in the 16S rRNA genes of mycoplasmas have been found to be positioned in different parts of the 16S rRNA molecule with no tendency for being concentrated in the V1 and V2 regions (Pettersson *et al.*, 1996a, c, 1998).

The different nucleotides in a polymorphic position were present in roughly equal ratios (i.e. 1:1). The *Bacillus popilliae* ATCC 14706^T rRNA genes contained three polymorphic sites 145 (C/T), 173 (A/T) and 258 (G/A) (unless otherwise stated *Escherichia coli* numbering is used; Brosius *et al.*, 1978). Only one heterogeneous position was found in the 16S rRNA gene of *Bacillus lentimorbus* ATCC 14707^T, namely in the first nucleotide position of the tetra-loop starting at nt 208. Instead of the G residue at this position, as commonly found in other *Paenibacillus* strains, the *Bacillus lentimorbus* ATCC 14707^T rRNA gene contained an A and a T. Moreover, the stem of the actual helix was extended by five extra base pairs in *Bacillus lentimorbus* ATCC 14707^T. This higher order structural attribute was not found among any of the other members of the genus *Paenibacillus* for which 16S rRNA gene sequence data could be retrieved, or indeed any other bacilli. Moreover, elongations of this region are present in only some of the other (eu)bacterial phyla (e.g. some *Actinomyces* and *Actinopolyspora* species, members of the *Desulfotomaculum* group and the β-, γ- and δ-subclasses of the *Proteobacteria*), however, without substantial similarity of primary structure. Therefore, this extension facilitates the identification of *Bacillus lentimorbus* by using a PCR-based strategy with one of the primers targeting the region between positions 202 and 215 with the sequence **GTTCC**GA(A/T)TAA**TCGGGGC** (underlined residues are those involved in base pairing and bold type denotes the additional nucleotides explicitly found in the 16S rRNA molecule of *Bacillus lentimorbus* ATCC 14707^T). It is likely that this elongation is present in the matured 16S rRNA molecule because it forms a stable stem structure containing canonical and non-canonical base pairs of the common U·G type (reviewed by Gutell *et al.*, 1994) and because slight variations in stem lengths of the other helices in the V2 region of the molecule are known to exist in other taxa (Maidak *et al.*, 1996). However, it remains to be shown by sequence analysis of more *Bacillus lentimorbus* strains if this extension is idiosyncratic for this species.

Previous studies have shown that the bidirectional solid-phase 16S rDNA sequencing technique results in highly accurate sequence data (e.g. Pettersson *et al.*, 1994, 1996a, b, c). Therefore, the microheterogeneities discussed above are due to differences between the various rRNA genes rather than the result of sequencing artefacts. It has been claimed that 16S

rRNA gene sequences from different operons should be used for accurate phylogenetic inferences since different operon nucleotide sequences may result in altered tree topologies when closely related taxa are studied (Clayton *et al.*, 1995; Pettersson *et al.*, 1996b, 1998). However, because *Bacillus popilliae* ATCC 14706^T and *Bacillus lentimorbus* ATCC 14707^T only showed 3 and 1 polymorphic sites, respectively, the 16S rDNA sequences from individual operons will not significantly alter the evolutionary relationships presented in Fig. 2.

Phylogenetic analysis of *Bacillus lentimorbus* ATCC 14707^T, and *Bacillus popilliae* ATCC 14706^T and NRRL B-4081

The phylogenetic tree shown in Fig. 2 is based on distance matrix analysis of aligned nucleotide positions ranging from nt 45 to 1430 of the 16S rRNA genes from the type strains and selected members of the genera *Aneurinibacillus*, *Bacillus*, *Brevibacillus* and *Paenibacillus* with *Alicyclobacillus acidocaldarius* ATCC 27009^T as outgroup. Neither gaps nor ambiguously determined nucleotides were removed prior to computation of phylogenetic relationships for reasons to be detailed below. For comparison, trees were also constructed from differently corrected alignments by removing gapped positions and/or by omitting ambiguously aligned positions. These trees revealed no substantial changes in their overall topology from that shown in Fig. 2 which we judge to be representative with regard to phylogenetic stability. Moreover, topologies obtained by parsimony analysis strongly resembled those generated by neighbour-joining algorithms (not shown). The bootstrap percentage values included at the nodes of the tree attest to the stability of the main branches and it is reassuring that the tree is in overall agreement with a recently published dendrogram by Shida and co-workers (Shida *et al.*, 1997).

All trees computed in this study revealed that *Bacillus lentimorbus* ATCC 14707^T, and *Bacillus popilliae* ATCC 14706^T and NRRL B-4081 are members of a monophyletic group comprising the genus *Paenibacillus*. While this is in full agreement with phenetic studies (Gordon *et al.*, 1973; de Barjac, 1981; Priest *et al.*, 1981; Priest, 1994), it contrasts with earlier 16S rRNA sequence analyses (Ash *et al.*, 1991) which placed the type strains of *Bacillus lentimorbus* and *Bacillus popilliae* (under the GenBank accession numbers X60633 and X60622, respectively) in rRNA group 1 close to *Bacillus subtilis*. Pairwise sequence comparisons indicated a distant relationship between the different sequences of the respective species, i.e. < 88% (not shown), which is reflected in the phylogenetic positions shown in bold type in Fig. 2. Thus, while our data suggest the reclassification of *Bacillus popilliae* ATCC 14706^T and *Bacillus lentimorbus* ATCC 14707^T as members of the genus *Paenibacillus*, rather than belonging to the *Bacillus subtilis* group, confusing results are likely to occur if appropriate 16S rRNA gene sequences are not used for phylogenetic inferences. Since we have carefully checked our strains and they have been subjected to both phenotypic and molecular characterization (Rippere *et al.*, 1998) we recommend that accession numbers AF071861 and AF071859 should be used for future studies of *Bacillus lentimorbus* and *Bacillus popilliae*, respectively, and that accession numbers X60633 and X60622 should represent two separate *Bacillus* strains.

Nucleotide motifs (signatures) in the 16S rRNA genes to assist identification of different groups within the family *Bacillaceae* were published by Ash *et al.* (1993). All signatures except nt position 845 (which we here correct to 844) continue to define the genus *Paenibacillus* in the light of the current study. While the vast majority of the *Paenibacillus* sequences have a G residue at position 844, members belonging to the two branches *Paenibacillus apiarius/alvei* and *Paenibacillus curdlanolyticus/kobensis*, all possessed an A at this site. Moreover, we have corrected one more position, namely the base pair 126·236 (A·U) which should be 126·235 (A·U). Therefore, the signature nucleotide positions in the 16S rRNA molecule useful for the identification of *Paenibacillus* from other *Bacillus* rRNA groups and *Alicyclobacillus* involve the following positions: 126·235 (A·U), 157·164 (C·G), 458 (G), 493 (G), 494 (A), 606 (U), 607 (U), 631 (U), 632 (C), 852 (G), 867 (A), 941·1342 (A·U), 948·1233 (U·A), 1252 (C), 1335 (U) and 1423 (A). The sequence data of the 16S rRNA genes of *Bacillus lentimorbus* ATCC 14707^T and *Bacillus popilliae* strains ATCC 14706^T and NRRL B-4081 presented in this study harboured these motifs, supporting their association with the paenibacilli. These motifs were not present in the previously deposited nucleotide sequences for the type strains of these species.

The signatures are useful for the design of genus-specific oligonucleotides as shown in Fig. 1. We found both PAEN515F (Shida *et al.*, 1997) and PAEN862F (Ash *et al.*, 1993) valuable for identification of paenibacilli, although the latter sometimes gave rise to non-specific bands with some *Bacillus* species.

The *Paenibacillus lentimorbus/popilliae* phylogenetic subcluster

Bacillus lentimorbus ATCC 14707^T, and *Bacillus popilliae* ATCC 14706^T and NRRL B-4081 were recovered in a subcluster of the *Paenibacillus* cluster consisting of eight taxa. This subgroup was obtained in all of the computed trees (represented by Fig. 2) and is regarded to be a stable phylogenetic entity as judged from bootstrap values and branch lengths. Hereafter, we will refer to this cluster as the *Paenibacillus lentimorbus/popilliae* cluster, since these species were the first described species belonging to this cluster.

The *Paenibacillus lentimorbus/popilliae* subcluster encompasses the parent species, and *Paenibacillus*

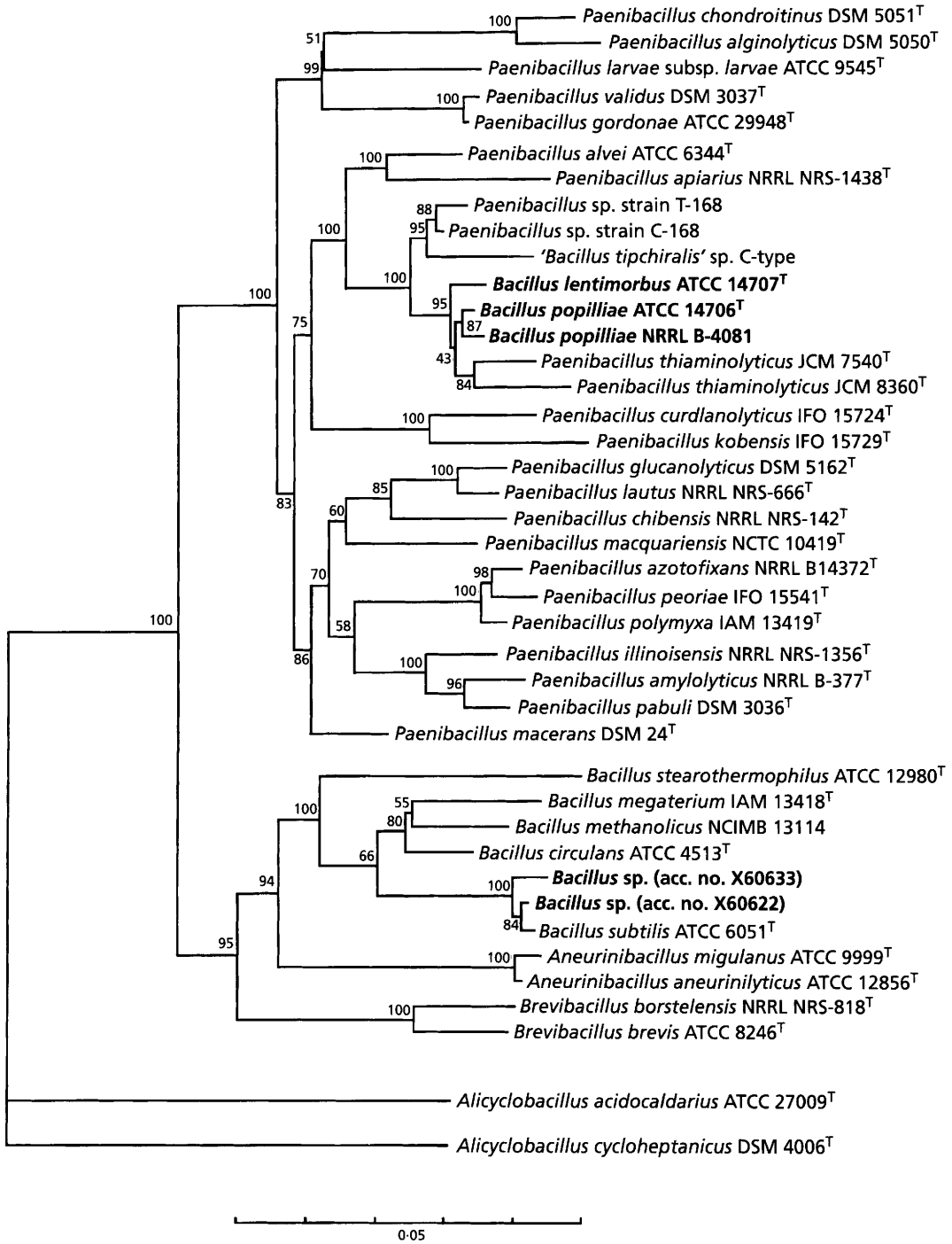

Fig. 2. Phylogenetic tree, based on neighbour-joining (Saitou & Nei, 1987), derived from an alignment comprising 16S rRNA gene sequences, ranging from position 45 to 1430 (*E. coli* numbering; Brosius *et al.*, 1978) from *Paenibacillus* species and selected members of closely related genera. *Alicyclobacillus acidocaldarius* ATCC 27009^T served as outgroup. *Bacillus lentimorbus* ATCC 14707^T, and *Bacillus popilliae* ATCC 14706^T and NRRL B-4081 are shown in bold type as are the previous phylogenetic positions obtained when using sequences deposited under the accession numbers X60622 and X60633 for *Bacillus lentimorbus* ATCC 14707^T and *Bacillus popilliae* ATCC 14706^T, respectively. The data set was resampled 500 times by using the bootstrap option and the percentage values are given at the nodes, showing the frequency at which the taxa to the right of the actual node cluster together. The scale bar indicates the number of substitutions per nucleotide position.

Table 3. Unique nucleotide positions in the 16S rRNA molecule of the *Paenibacillus lentimorbus/popilliae* subcluster

Lower case letters indicate that the actual nucleotide residue was found in one of the 16S rRNA sequences of a strain or species of that group. Positions are given according to *E. coli* numbering (Brosius *et al.*, 1978).

Position of base or pair	*P. lentimorbus/ popilliae* subcluster	*P. apiarius/ P. alvei*	*P. curdlanolyticus/ P. kobensis*	Other paenibacilli
121	U	C	C	C
155	C	C	U	A/U
156	A	A/C	U	C/G/U
167	G	A	U	A/g
217	C	U	U	U/c
236	A	G	G	G
471	U/c	C	C	C/u
593	G/u	G	G	U

thiaminolyticus JCM 8360^T and JCM 7540 (Shida *et al.*, 1997) which have no history of involvement with insects. Three other strains in this subcluster are '*Paenibacillus*' C-168 and T-168 (GenBank Y16129 and Y16128, respectively) and '*Bacillus tipchiralis*' (Rudner *et al.*, 1998). The genus of the latter should be investigated since it conflicts with the monophyletic origin of the paenibacilli (e.g. Ash *et al.*, 1993; Shida *et al.*, 1997; Fig. 2) and the phylogenetic evidence points to it being a member of *Paenibacillus*. None of these bacteria are known to have close associations with insects (Rudner *et al.*, 1998).

Signature nucleotide positions (compiled in Table 3) were determined for the subcluster which were characterized by nucleotide residues found only in the 16S rRNA genes of the taxa of this subcluster to the exclusion of all other paenibacilli. However, most of these nucleotide motifs were present in the highly variable V1 and V2 regions (Gray *et al.*, 1984) of the 16S rRNA gene. The reason for this remains unclear, but might reflect that the members of the *Paenibacillus lentimorbus/popilliae* subcluster represent a recently evolved group of organisms.

Most closely related to the *Paenibacillus lentimorbus/ popilliae* subcluster are members of the *Paenibacillus apiarius/alvei* branch and the *Paenibacillus curdlanolyticus/kobensis* branch. While *Paenibacillus alvei* (Cheshire & Cheyne, 1885) and *Paenibacillus apiarius* (Katznelson, 1955) were both isolated from dead honeybee larvae, neither is actually responsible for larval pathogenicity and *Paenibacillus curdlanolyticus* and *Paenibacillus kobensis* have no history of involvement with insects. It therefore seems that the only members of this part of the tree with entomopathogenic properties are *Paenibacillus lentimorbus* and *Paenibacillus popilliae*.

Paenibacillus larvae subsp. *larvae* is phenetically similar to *Paenibacillus lentimorbus* and *Paenibacillus popilliae* in being catalase-negative, unable to grow in nutrient broth and sporulating poorly, if at all, *in vitro* (Gordon *et al.*, 1973;de Barjac, 1981; Stahly *et al.*, 1992). This bacterium is responsible for 'American foulbrood' of honeybee larvae, a disease which is similar to the milky diseases of scarabaeid larvae and results from massive growth and sporulation of the bacteria in the larval haemolymph. It is therefore reassuring that the three endospore-forming, obligate entomopathogens should all be classified in *Paenibacillus*. The different phylogenetic placements (Fig. 2) may possibly reflect the evolutionary divergence of the insect hosts and the considerable time period in which the pathogens have been evolving separately.

Phylogenetic relationships of *Paenibacillus lentimorbus* and *Paenibacillus popilliae*

The fine relationships between *Paenibacillus lentimorbus* and *Paenibacillus popilliae* were determined from trees constructed from sequence alignments which were corrected by removing gaps and unsolved positions and from those in which such positions were included. The former grouped *Paenibacillus popilliae* NRRL B-8041 together with *Paenibacillus lentimorbus* ATCC 14707^T rather than with the type strain of *Paenibacillus popilliae* ATCC 14706^T, while the latter showed the *Paenibacillus popilliae* strains with the closest similarity (Fig. 2). This indicated a certain instability in this part of the tree, the reason for which was further examined by carefully scrutinizing the alignment. The branch swapping was found to occur because some of the phylogenetically informative positions within the 16S rRNA gene sequences were deleted by gap removal to create the final data set to be used for subsequent phylogenetic calculations. The main problem was due to lack of compositional information in the primary structures of the actual positions in some of the operational taxonomic units. By comparing the 16S rDNA sequences of *Paenibacillus popilliae* ATCC 14706^T and NRRL B-4081,

Table 4. Distinctive phenotypic properties of *Paenibacillus lentimorbus, Paenibacillus popilliae* and related paenibacilli

Data from Gordon *et al.* (1973), Priest *et al.* (1988), Shida *et al.* (1997) and Rippere *et al.* (1998). +, > 90 % strains positive; −, < 10% strains positive; v, 11–89% strains positive; NT, not tested.

Character*	*P. alvei*	*P. apiarius*	*P. curdlanolyticus*	*P. kobensis*	*P. larvae* subsp. *larvae*	*P. larvae* subsp. *pulvifaciens*	*P. lentimorbus*	*P. popilliae*	*P. thiaminolyticus*
Spore shape	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval
Swollen sporangia	+	+	+	+	+	+	+	+	+
Parasporal crystal	−	−	NT	NT	−	−	v	+	−
Anaerobic growth	+	+	−	−	+	+	+	+	+
Catalase	+	+	+	+	−	−	−	−	+
Oxidase*	+	−	−	−	NT	−	−	−	+
Nitrate reduction	−	+	+	+	v	v	−	−	+
Acid from:									
Arabinose	−	−	+	+	−	−	−	−	v
Glucose	+	+	+	+	+	+	+	+	+
Mannitol	−	−	−	−	−	+	−	−	−
Xylose	−	−	+	+	−	−	−	−	−
Production of:									
Acetylmethylcarbinol	+	−	−	−	v	v	−	−	−
Indole	+	−	−	−	+	+	−	−	+
Decomposition of:									
Tyrosine	v	+	NT	NT	−	−	−	−	+
Hydrolysis of:									
Casein	+	+	−	−	+	+	−	−	+
Starch	+	+	+	+	−	−	−	−	+
Use of citrate:	−	+	−	−	−	−	−	−	+
Growth in:									
Nutrient broth	+	+	+	+	−	+	−	−	+
2% NaCl	+	+	NT	NT	+	+	+	−	+
5% NaCl	v	+	−	−	−	−	NT	NT	v
0·001% Lysozyme	+	+	+	+	+	+	+	+	−
Growth at:									
50 °C	−	−	−	−	−	−	−	−	−
pH 5·6	−	+	NT	NT	−	−	−	−	−

* Based on reactions of type strains only for *Paenibacillus lentimorbus* and *Paenibacillus popilliae*.

and *Paenibacillus lentimorbus* ATCC 14707^T with those from the other members of the *Paenibacillus lentimorbus popilliae* subcluster and the species of the neighbouring branches, a more detailed picture of the nucleotide similarity percentage values was thus obtained. The non-corrected similarity percentage values (not shown) obtained from this comparison indicated that *Paenibacillus popilliae* ATCC 14706^T and NRRL B-4081 are indeed more closely related to each other than to *Paenibacillus lentimorbus*, revealing a 16S rRNA gene similarity value of 99·2% but showing 98·6 and 98·8%, respectively, to *Paenibacillus lentimorbus* ATCC 14707^T. Therefore, the tree in Fig. 2 is most likely to be representative regarding the branching order between these strains. This conclusion is congruent with recent DNA–DNA reassociation experiments where *Paenibacillus popilliae* NRRL B-4081 showed 73–77% similarity to strains of *Paenibacillus popilliae* but only 59–61% to strains of *Paenibacillus lentimorbus* (Rippere *et al.*, 1998) and supports the varietal status for NRRL B-4081. However, further strains of this taxon must be isolated and characterized before any firm conclusions about subspecific classification can be entertained.

In conclusion, the results of this study revealed that the species *Bacillus popilliae*, represented by strains ATCC 14706^T and NRRL B-4081, and *Bacillus lentimorbus* ATCC 14707^T cluster tightly together in a highly defined subgroup within the genus *Paenibacillus* in accordance with their phenetic properties and we therefore propose transfer of these species to the genus *Paenibacillus* as *Paenibacillus popilliae* comb. nov. and *Paenibacillus lentimorbus* comb. nov. The phenotypic distinction of these species from phylogenetically and phenetically related taxa is given in Table 4.

Emended description of *Paenibacillus lentimorbus* (Dutky 1940) comb. nov.

Paenibacillus lentimorbus (len.ti.mor'bus. L. adj. *lentus* slow; L. n. *morbus* disease; M.L. n. *lentimorbus* the slow disease).

This description is based on this work and the studies of Dutky (1940), Gordon *et al.* (1973) and Rippere *et al.* (1998). Cells are rods, 0·5–0·7 μm by 1·8–7 μm which stain Gram-variable or -negative in exponential-phase cultures. Ellipsoidal spores are located centrally or terminally in swollen sporangia and may or may not be accompanied by a refractile parasporal body visible by phase-contrast microscopy. The type strain does not produce a parasporal body. May be isolated from haemolymph of infected larvae of the Japanese beetle (*Popillia japonica* Newman) and related larvae. Nutritionally fastidious, requires special media such as J-broth (Gordon *et al.*, 1973) or MYPGP (Costilow & Coulter, 1971). On these media forms colonies which are cream-coloured and less than 1 mm in diameter.

Sporulation in laboratory media has not been reported. Spores are produced during *in vivo* growth in susceptible larvae. Maximum growth temperature is 35 °C, minimum is 20 °C. Facultative anaerobe, produces slight acidity (pH 5·9–6·5) in Voges–Proskauer broth but negative reaction for acetoin. Acid is produced from fermentation of galactose, glucose, maltose, mannose and trehalose but not arabinose, mannitol or xylose. Grows in J-broth in presence of 0·001 % lysozyme. The organism is negative for catalase and nitrate reduction reactions and fails to grow in the presence of 2% NaCl (most strains) or 1 μg vancomycin ml^{-1}. Casein, gelatin and starch are not hydrolysed, indole is not produced and phenylalanine not deaminated. Predominant fatty acids of the type strain are: $C_{15:0}$, 2·7 %; iso-$C_{15:0}$, 6·6 %; anteiso-$C_{15:0}$, 37 %; C_{16}, 33 % (results of duplicate assays). One strain that did not produce a paraspore had 90 % DNA similarity to the type strain, whereas seven paraspore-forming strains had 73–78 % similarity to the type strain (Rippere *et al.*, 1998). The type strain is ATCC 14707^T.

Emended description of *Paenibacillus popilliae* (Dutky 1940) comb. nov.

Paenibacillus popilliae (po.pil'li.ae. M.L. n. *Popillia* generic name of the Japanese beetle; M.L. gen. n. *popilliae* of Popillia).

This description is based on this work and the studies of Dutky (1940), Gordon *et al.* (1973) and Rippere *et al.* (1998). Cells are rods, 0·5–0·8 μm by 1·3–5·2 μm which stain Gram-variable or -negative in exponential-phase cultures. Sporulating cells stain Gram-positive. Ellipsoidal spores are located centrally or terminally in swollen sporangia. The spore is often accompanied by a refractile parasporal body visible by phase-contrast microscopy. Isolated from haemolymph of infected larvae of the Japanese beetle (*Popilliae japonica* Newman) and related larvae such as the northern masked chafer (*Cyclocephala borealis*, Arrow). Nutritionally fastidious, requires special media such as MYPGP (Costilow & Coulter, 1971) or J-broth (Gordon *et al.*, 1973). On these media forms colonies which are cream-coloured and less than 1 mm in diameter. Sporulation in laboratory media is poor. Spores are produced during *in vivo* growth in susceptible larvae. Maximum growth temperature is 31 °C, minimum is 20 °C. Facultative anaerobe, produces acid in Voges–Proskauer broth (pH 5·7–6·2) and grows in presence of 0·001 % lysozyme in semi-solid J agar. Negative for catalase and Voges–Proskauer reactions, does not grow in 5 % NaCl or nutrient broth. No acid from arabinose, mannitol or xylose. Casein, gelatin and starch are not hydrolysed. No production of indole or deamination of phenylalanine. Nitrate is not reduced. Most strains grow in the presence of 2 % NaCl or 150 μg vancomycin ml^{-1}. Acid is produced from glucose and trehalose. Predominant fatty acids of the type strain are: $C_{15:0}$, 6·8 %; iso-$C_{15:0}$, 3·2 %; anteiso-$C_{15:0}$, 32 %; C_{16}, 31 % (results of duplicate assays). DNA reassociation: 24 strains showed greater than 73 % similarity to the type strain (Rippere *et al.*, 1998). The type strain is ATCC 14706^T.

ACKNOWLEDGEMENTS

We are grateful to Raja Mazumder for analysis of fatty acids.

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