Cohnella thermotolerans gen. nov., sp. nov., and classification of '*Paenibacillus hongkongensis*' as *Cohnella hongkongensis* sp. nov.

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A Gram-positive, rod-shaped, endospore-forming organism, strain CCUG 47242^T, was isolated from a sample of industrial starch production in Sweden. 16S rRNA gene sequence analysis demonstrated that this isolate was moderately related to species of the genus *Paenibacillus*, with <94.4% sequence similarity to all other hitherto described *Paenibacillus* species. Strain CCUG 47242^T showed the greatest sequence similarity (96.5%) to '*Paenibacillus hongkongensis*' HKU3, a strain with a name that has not yet been validly published. Chemotaxonomic data [major menaquinone, MK-7 (98%); major polar lipids, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, lysyl-phosphatidylglycerol, two unknown phospholipids, four unknown aminophospholipids; major fatty acids, iso-C16:0 and anteiso-C15:0] showed some significant differences when compared with the type species of the genus *Paenibacillus*, *Paenibacillus polymyxa*. Physiological and biochemical tests allowed clear phenotypic differentiation of strain CCUG 47242^T from strain HKU3. On the basis of 16S rRNA gene sequence analysis, in combination with chemotaxonomic data, strains CCUG 47242^T and HKU3 represent two novel species of a new genus of endospore-forming bacteria for which we propose the names *Cohnella thermotolerans* gen. nov., sp. nov. (type strain CCUG 47242^T = CIP 108492^T = DSM 17683^T) and *Cohnella hongkongensis* sp. nov. (type strain HKU3^T = CCUG 49571^T = CIP 107898^T = DSM 17642^T).

The genus *Paenibacillus* was originally proposed by Ash *et al.* (1993, 1994) for rRNA group 3 bacilli, mainly on the basis of 16S rRNA gene sequence data and the fact that this group was distinct from other groups which they defined within the bacilli. The genus description was emended by Shida *et al.* (1997) and, to date, the genus comprises more than 60 species, most of which were listed by Aguilera *et al.* (2001), Berge *et al.* (2002), Bosshard *et al.* (2002), Elo *et al.* (2001), Genersch *et al.* (2006), Iida *et al.* (2005), Kim *et al.* (2004),

Meehan *et al.* (2001), Montes *et al.* (2004), Rivas *et al.* (2005a, b), Rodríguez-Díaz *et al.* (2005), Saha *et al.* (2005), Sánchez *et al.* (2005), Šmerda *et al.* (2005), Takeda *et al.* (2005), von der Weid *et al.* (2002) and Yoon *et al.* (2003, 2005). The taxonomic dissection of the genus *Bacillus* (initiated by Ash *et al.*, 1991), based largely on a 16S rRNA gene sequence perspective, has been summarized by Stackebrandt & Swiderski (2002).

Strain CCUG 47242^T was isolated on blood agar at 37 °C during hygiene control checks at a starch-producing company in Sweden. Subcultivation was done on tryptone soy agar (TS agar; Oxoid) at 30 °C for 24 h. On this agar, strain CCUG 47242^T was able to grow at 20–55 °C, but not at 10 or 60 °C. Growth at 30 °C was also observed on nutrient agar and R2A agar, but not on *Salmonella*–*Shigella* agar (SS agar;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of CCUG 47242^T is AJ971483.

Phylogenetic trees constructed using the neighbour-joining and maximum-parsimony methods are available as supplementary figures in IJSEM Online.

Fig. 1. Polar lipid profiles after separation by two-dimensional thin layer chromatography. (a) Strain CCUG 47242^T; (b) HKU3; (c) *Bacillus subtilis* DSM 10^T; (d) *Paenibacillus polymyxa* DSM 36^T. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL1–5, unknown phospholipids; LPG, lysyl-phosphatidylglycerol; PN2–4, unknown aminophospholipids; GL1, β -gentiobiosyldiacylglycerol; GL2 and GL3, unknown glycolipids.

all media from Oxoid). Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under a Zeiss light microscope at $\times 1000$, using cells that had been grown for 24 h at 30 °C on TS agar. Details of cell morphology are given in the species description. The 16S rRNA gene sequence was analysed as described previously (Kämpfer *et al.*, 2003). Analysis of the sequence data was performed by using MEGA version 2.1 (Kumar *et al.*, 2001), after multiple alignment of sequences by CLUSTAL_X (Thompson *et al.*, 1997). A distance-matrix method (distance options according to the Kimura two-parameter model) that used clustering with the neighbour-joining method (see Supplementary Fig. S1 in IJSEM Online), as well as a discrete character-based maximum-parsimony method (Supplementary Fig. S2 in IJSEM Online), were performed. In each case, bootstrap values were calculated based on 1000 replications. The 16S rRNA gene sequence of strain CCUG 47242^T was a continuous stretch of 1486 bp. Sequence similarity calculations indicated that strain CCUG 47242^T showed the greatest degree of similarity to '*Paenibacillus hongkongensis*' HKU3 (GenBank accession no. AF433165; 96.58%), which was described by Teng *et al.* (2003) (this name has not been validly published to date). Significantly lower sequence similarities (<94.5%) were found with all other recognized species of the genus *Paenibacillus*.

Chemotaxonomic analyses were performed as follows: respiratory quinones (Tindall, 1990), polar lipids (Tindall, 1990) and fatty acids (Kämpfer & Kroppenstedt, 1996).

Although respiratory quinones have low resolution within the bacilli, the presence of MK-7 supports the affiliation of strains CCUG 47242^T and HKU3 to the genus *Paenibacillus*, where all species investigated to date have MK-7 as the major quinone. MK-7 represented 98% of the quinones of strains CCUG 47242^T and HKU3; in addition, small amounts of MK-6 (1%) and MK-8 (1%) were detected.

Strains CCUG 47242^T and HKU3 displayed a complex polar lipid profile, which consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, lysyl-phosphatidylglycerol, two unknown phospholipids and four unknown aminophospholipids (Fig. 1).

The fatty acid profiles of strains CCUG 47242^T and HKU3 (given in the species description) were similar to those of other *Paenibacillus* species; however, it was striking that the amounts of iso-C16:0 (45.5% for strain CCUG 47242^T) or C16:0 (25.3% for strain HKU3) were very large. Such large amounts of iso-C16:0 have not been reported for *Paenibacillus* species before. Members of the genus *Thermobacillus*, described by Touzel *et al.* (2000), showed a similar fatty acid content to strain CCUG 47242^T with respect to C16:0 and C17:0, but not for the other fatty acids. Kämpfer (2002) compiled fatty acid data for *paenibacilli* and found that the major fatty acids of the genus *Paenibacillus* (with ranges as percentages of total given in parentheses) are anteiso-C15:0 (36–80%), iso-C16:0 (0.5–6%), iso-C15:0 (1–12%) and anteiso-C17:0 (6.7%).

Although some progress has been made in the reclassification of the bacilli, there is still some question as to what constitutes the genus *Bacillus* and whether other genera created over the years should be split further or combined with other genera. Redefining the genus *Bacillus* or the genus *Paenibacillus* would need extensive comparative work that would at present hinder the creation of novel taxa that may be closely associated with the genera *Bacillus* or *Paenibacillus*. This problem is clearly evident in a recent publication by Heyrman *et al.* (2005), where novel species within *Bacillus* group 2 are, by default, also placed in the genus *Bacillus*, although this group also contains members of the genera *Kurthia*, *Caryophanon* and *Sporosarcina*. The alternative is to define clearly the properties of the type species of the genera *Bacillus* and *Paenibacillus* and to create novel genera where clear differences can be found without having first to wait for extensive taxonomic rearrangements. This approach can be most effectively undertaken using a combination of 16S rRNA gene sequence data and chemical composition analysis. Such an approach leads to a rather different perspective of the potential taxonomic groupings than that based on 16S rRNA gene sequence data alone. It should be noted that features such as physiology, biochemistry and morphology may also be correlated with these data and all such data should be used to evaluate the taxonomic significance once groupings have been established. A clear distinction has to be made between groupings and genera. In the future, this may be expanded to include more detailed information from total genome analysis, although it is essential that such information should be coupled to other aspects such as biochemical pathways and structural proteins etc. (i.e. the epigenetic level).

It is well documented that the chemical composition of members of the bacilli is heterogeneous (Minnikin & Goodfellow, 1981; O'Leary & Wilkinson, 1988). However, in recent years, much emphasis has been put on the use of fatty acids. While some differentiation is possible, they appear to be of limited value in further differentiating within the bacilli. In the case of respiratory lipoquinones, menaquinones dominate, with isoprenoid chain lengths from 6 to 9. The most commonly encountered menaquinone is MK-7. Closer examination of polar lipids indicates a greater degree of variation, although this method has not been widely applied in recent years. In the case of members of *Bacillus* group 1 (Ash *et al.*, 1991), there is chemical heterogeneity within the group (Minnikin & Goodfellow, 1981; O'Leary & Wilkinson, 1988). However, *Bacillus subtilis*, the type species of the genus *Bacillus*, produces diphosphatidylglycerol, phosphatidylglycerol, diphosphatidylethanolamine, an aminophosphatidylglycerol and a glycolipid, which has been identified as a β -gentiobiosyldiacylglycerol (Minnikin & Goodfellow, 1981). Species sharing this polar lipid pattern, together with MK-7 and the fatty acid pattern listed in Table 1, should, in the future, constitute the 'core' of the genus *Bacillus*. The polar lipid composition of the type species of the genus *Paenibacillus*, *Paenibacillus polymyxa*, is different. The single glycolipid present does not have the

Table 1. Fatty acid content (%) of *Bacillus subtilis*, *Paenibacillus polymyxa*, *Cohnella thermotolerans* CCUG 47242^T and *Cohnella hongkongensis* HKU3^T

The data for *B. subtilis* and *P. polymyxa* are taken from Kämpfer (2002) and were compiled from several species descriptions. 1, *B. subtilis*; 2, *P. polymyxa*; 3, *C. thermotolerans* CCUG 47242^T; 4, *C. hongkongensis* HKU3^T.

Fatty acid	1	2	3	4
ai-13:0				0.8
i-14:0	1		2.1	2.3
14:0			1	5
i-15:0	27	1	3.2	8.1
ai-15:0	39	63	28.4	31.2
15:0			1.4	8
i-16:0	1	6	45.5	11.9
i-16:1	1			
16:0	1	9	6.6	25.3
16:1 ω 11c				0.9
i-17:0	8	2		
ai-17:0	1	17	6.7	2.6
i-17:1	3		1.1	1.9
17:0				1.2
ai-17:1	10			
17:1 ω 6c			1	
Other	8			
18:1 ω 7c			4	

same R_f value as β -gentiobiosyldiacylglycerol. While the major phospholipids present are diphosphatidylglycerol, phosphatidylglycerol and diphosphatidylethanolamine, they only serve (together with the presence of menaquinones and iso/ anteiso fatty acids) to confirm that this species belongs to the bacilli. The presence of a number of unidentified phospholipids serves to differentiate this species. Preliminary work on the polar lipid composition of the genus *Paenibacillus* indicates that this group is also heterogeneous (S. Kirschner and B. J. Tindall, unpublished; Minnikin & Goodfellow, 1981; O'Leary & Wilkinson, 1988); only those species sharing a similar chemical composition to the type species should be retained in the genus *Paenibacillus* in the future.

In the case of the two strains studied here, their chemical composition also centres around the presence of menaquinones (MK-7), the dominance of iso and anteiso fatty acids, together with diphosphatidylglycerol, phosphatidylglycerol and diphosphatidylethanolamine as the predominant phospholipids. However, among the polar lipids, the two strains share the same property of having a number of phospholipids and aminophospholipids that are not present in the type species of the genera *Bacillus* and *Paenibacillus*.

In particular, the lipid labelled LPG had the same R_f value as authentic lysyl-phosphatidylglycerol, which has been identified in *Staphylococcus* (Nahaie *et al.*, 1984) and *Listeria* (Fischer & Leopold, 1999) species. The biosynthesis of this lipid is unusual in that lysine tRNA serves as the source of

lysine (Gould & Lennarz, 1967, 1970; Lennarz *et al.*, 1966). A specific enzyme has been identified that is responsible for the incorporation of lysine into the parent phospholipids (Oku *et al.*, 2004). This lipid, and other amino acid derivatives of phospholipids, have also been found in other bacilli (Minnikin & Goodfellow, 1981; O'Leary & Wilkinson, 1988), as well as in members of the genera *Enterococcus*, *Lactobacillus*, *Listeria*, *Vagococcus* and *Staphylococcus* (Fischer & Leopold, 1999; Fischer & Arneith-Seifert, 1998; Nahaie *et al.*, 1984; O'Leary & Wilkinson, 1988). The ability to synthesize such lipids (including other amino acid derivatives) must also be seen in a taxonomic and evolutionary context, being particularly prevalent in this branch of the Gram-positive bacteria. This study, and previous work, clearly indicates the value of chemotaxonomy within the bacilli and further supports the need for the inclusion of such analyses in all future taxonomic work on this group. This is also consistent with the often forgotten remarks of the ad hoc committee (Wayne *et al.*, 1987), which emphasized the need to carry out more chemotaxonomic work.

The results of physiological characterization, using methods that have been described previously (Kämpfer *et al.*, 1991), are given in the species descriptions. Strain CCUG 47242^T was able to utilize many carbohydrates, but organic acids and amino acids were not utilized. In the case of HKU3, no positive results for carbon source utilization tests were reported (Teng *et al.*, 2003). On the basis of the results presented, we propose that strains CCUG 47242^T and HKU3 represent separate novel species of a new genus, *Cohnella* gen. nov.

Description of *Cohnella* gen. nov.

Cohnella (Coh.nel'la. N.L. fem. dim. n. *Cohnella* named after Ferdinand Cohn, the German microbiologist who first described the bacterial genus *Bacillus* in 1872).

Cells are Gram-positive, spore-forming, aerobic, non-motile, rod-shaped and thermotolerant. Good growth occurs after 24 h incubation on TS and nutrient agars at 25–30 °C; good growth also occurs at 55 °C. Main menaquinone is MK-7. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lysyl-phosphatidylglycerol. In addition, two unknown phospholipids and four unknown aminophospholipids are present. The major fatty acids are iso C16:0, anteiso C15:0 and C16:0. The type species is *Cohnella thermotolerans*.

Description of *Cohnella thermotolerans* sp. nov.

Cohnella thermotolerans (ther.mo.tol'er.ans. Gr. n. *therme* heat; L. pres. part. *tolerans* tolerating; N.L. part. adj. *thermotolerans* able to tolerate high temperatures).

Displays the following properties in addition to those given in the genus description. Oxidase-positive and shows an oxidative metabolism. The fatty acid profile of the type strain comprises iso C16:0 (45.5%), anteiso C15:0 (28.4%), anteiso C17:0 (6.7%), C16:0 (6.6%),

C18:1 ω 7c (4.0%), iso C15:0 (3.2%), iso C14:0 (2.1%), C15:0 (1.4%), iso C17:0 (1.1%), C14:0 (1.0%) and C17:1 ω 6c (1.0%). Aesculin and p-nitrophenol (pNP) β -D-glucopyranoside are hydrolysed. Arbutin, L-arabinose (weakly), D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, α -D-melibiose, L-rhamnose (weakly) and D-ribose are utilized as sole sources of carbon. No acid production from glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, sorbitol, L-arabinose, raffinose, rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl D-glucoside, erythritol, melibiose, D-arabitol or D-mannose. No hydrolysis of o-NP β -D-galactopyranoside, pNP β -D-glucuronide, pNP α -D-glucopyranoside, pNP β -D-xylopyranoside, bis-pNP phosphate, pNP phenylphosphonate, pNP phosphorylcholine, 2-deoxythymidine-5'-pNP phosphate, L-alanine p-nitroanilide (pNA), L-glutamate- γ -3-carboxy pNA or L-proline pNA. The following carbon sources are not utilized: N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, sucrose, salicin, D-trehalose, D-xylose, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, acetate, propionate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, L-malate, mesaconate, oxoglutarate, pyruvate, suberate, L-alanine, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The DNA G + C content is 59 mol%.

The type strain, CCUG 47242^T (=DSM 17683^T=CIP 108492^T), was isolated from a sample of industrial starch production in Sweden.

Description of *Cohnella hongkongensis* sp. nov.

Cohnella hongkongensis (hong.kong.en'sis. N.L. fem. adj. *hongkongensis* pertaining to Hong Kong).

The name '*Paenibacillus hongkongensis*' has been used for this organism, but it has not been validly published and cannot be formally cited as a synonym.

In addition to the description given by Teng *et al.* (2003), the type strain utilizes several sole carbon sources after 7 days of incubation: N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, arbutin, L-arabinose (weakly), D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-maltose, D-mannose, α -D-melibiose, L-rhamnose and D-ribose. The following carbon sources are not utilized: sucrose, salicin, D-trehalose, D-xylose, adonitol, i-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, acetate, propionate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, L-malate, mesaconate, oxoglutarate, pyruvate, suberate, L-alanine, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The fatty acid profile of the type strain contains iso C16:0 (11.9%), anteiso C15:0 (31.2%),

anteiso C17:0 (2.6%), C16:0 (25.3%), iso C15:0 (8.1%), anteiso C13:0 (0.8%), iso C14:0 (2.3%), C15:0 (8.0%), C16:1 ω 11c (0.9%), iso C17:0 (1.9%), C14:0 (5.0%) and C17:0 (1.2%). The DNA G+C content is 60.9 mol% (method according to Peña *et al.*, 2005).

The type strain is HKU3^T (= DSM 17642^T = CIP 107898^T = CCUG 49571^T).

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