Bacillus kochii sp. nov., isolated from foods and a pharmaceuticals manufacturing site

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Three Gram-staining-positive, strictly aerobic, motile, catalase-positive, endospore-forming rods, designated WCC 4582^T, WCC 4581 and WCC 4583, were isolated from two different food sources and a pharmaceuticals production site. The three isolates were highly similar in their 16S rRNA gene sequences (100 % similarity) and *groEL* sequences (99.2–100 % similarity), Fourier-transform infrared spectroscopic fingerprints and other features tested. The isolates were most closely related to *Bacillus horneckiae*; the isolates and the type strain of *B. horneckiae* shared 97.6 % and 89.6 % 16S rRNA gene and *groEL* sequence similarities, respectively. The organisms grew optimally at 30 °C, at pH 7 and in the presence of 0.5 % (w/v) NaCl. The cell-wall peptidoglycan of WCC 4582^T contained *meso*-diaminopimelic acid (A1_γ) and the genomic DNA G + C content was 36.4 mol%. DNA–DNA relatedness between strain WCC 4582^T and *B. horneckiae* NRRL B-59162^T was 17 %. The three isolates are considered to represent a novel species of the genus *Bacillus*, for which the name *Bacillus kochii* sp. nov. is proposed. The type strain is WCC 4582^T (=DSM 23667^T=CCUG 59877^T=LMG 25855^T).

Microbial spoilage in food and contamination of pharmaceutical preparations pose a health risk to consumers and cause considerable economic losses in the industry. In particular, spore-forming bacteria are of importance, as they are heat resistant in food and endure desiccation and treatment with inorganic disinfectants, organic solvents and UV light in clean rooms. Formerly, the identification of spoilage micro-organisms was very costly and burdensome. Several research projects, therefore, developed an identification technology based on Fourier-transform infrared (FT-IR) spectroscopy, which fulfilled the following criteria: ease of use, fast and reliable results, high sample throughput and low costs as no consumables are required. During the course of many studies in our laboratory, thousands of micro-organisms have been isolated from industrial environments and identified by this method and novel species have been discovered (Bleicher *et al.*, 2010; Rieser *et al.*, 2012; Schmidt *et al.*, 2009, 2012).

Strains WCC 4582^T and WCC 4583 were isolated from products of two dairies in south Germany and strain WCC

4581 was isolated from a clean room in a pharmaceuticals manufacturing site in north Germany. Isolation from the dairy products was carried out using tryptone soy agar (TSA; Roth) supplemented with 0.1 % glucose (TSGA) or tryptone soy broth (TSB; Roth) solidified with 15 g agar l⁻¹ (TSBA); isolation from pharmaceutical clean room samples was carried out using TSA. For identification purposes, the FT-IR spectra of endospore-forming isolates were recorded as described by Kümmerle *et al.* (1998), Oberreuter *et al.* (2002) and Wenning *et al.* (2008) using an IFS-28B spectrometer and OPUS version 5.5 (Bruker Optics). The sample spectra were compared with a reference database for aerobic, mesophilic, spore-forming bacteria, including 770 spectra from 108 species and 12 genera. The three isolates described here displayed highly similar spectra but did not match any reference spectrum. The isolates were maintained as glycerol suspensions at –80 °C and as Weihenstephan culture collection (WCC) lyophilized cells.

Almost-complete 16S rRNA gene sequences of the isolates (each 1511 bp) were obtained according to Schmidt *et al.* (2009). The identification of phylogenetic neighbours was initially performed using BLAST, MEGABLAST and FASTA (Altschul *et al.*, 1997; Pearson & Lipman, 1988; Zhang *et al.*, 2000). Searches of public databases indicated that members of a presumably hitherto-unknown species of the

Abbreviation: FT-IR, Fourier-transform infrared.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains WCC 4582^T, WCC 4581 and WCC 4583 are FN995265, FR845720 and FR845721, respectively.

Three supplementary figures are available with the online version of this paper.

genus *Bacillus* had been isolated. Only *Bacillus horneckiae* 1P01SC^T (Vaishampayan *et al.*, 2010) and non-type strains of *Bacillus flexus*, '*Bacillus granadensis*', '*Bacillus pichinoty*' and *Bacillus bataviensis* exhibited >97 % 16S rRNA gene sequence similarity. Additionally, sequences of 58 type strains with the highest sequence similarity scores were selected for the calculation of pairwise sequence similarity using the global alignment algorithm, which was implemented at the EzTaxon server (Chun *et al.*, 2007). This comparison (Fig. S1, available in IJSEM Online) revealed that strains WCC 4582^T, WCC 4581 and WCC 4583 formed a distinct cluster (100 % 16S rRNA gene sequence similarity) within the genus *Bacillus* and were most closely related to *B. horneckiae* 1P01SC^T (97.6 % sequence similarity). The type strains of *Bacillus acidicola*, *B. pocheonensis*, *B. firmus*, *B. bataviensis*, *B. flexus*, *B. marisflavi*, *B. simplex*, *B. frigoritolerans*, *B. niacini*, *B. nealsonii*, *B. drentensis*, *B. shackletonii* and *B. circulans* were more distantly related (<96.8 % sequence similarity). Phylogenetic and molecular evolutionary analyses were also conducted using MEGA (Tamura *et al.*, 2007), CLUSTAL X (Thompson *et al.*, 1997), TREECON (Van de Peer & De Wachter, 1997) and PhyloWin (Gouy *et al.*, 2010). The topologies of trees calculated using neighbour-joining, maximum-parsimony and maximum-likelihood were basically all the same. Fig. 1 shows the position of the isolates among their nearest phylogenetic neighbours.

Partial *groEL* sequencing (530 bp) was performed in a 50 µl reaction mix containing 1 U Thermo-Start *Taq* DNA polymerase, 5 µl Thermo-Start PCR buffer, 1.5 mM MgCl₂, 200 µM each dNTP (all Thermo Scientific), 0.5 µM primer H279, 0.5 µM primer H280 (Goh *et al.*, 1996) and 1 µl lysate. Cycling conditions comprised an initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 40 °C for 45 s and extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. Pairwise comparison of the *groEL* sequences confirmed that the three isolates were highly similar (99.2–100 % sequence similarity) and that *B. horneckiae* NRRL B-59162^T was the most closely

related type strain (89.6 % *groEL* sequence similarity each). The neighbour-joining phylogenetic tree is shown in Fig. 2.

To further investigate the relationship between the isolates and their closest phylogenetic neighbour, a DNA–DNA reassociation study was performed by the DSMZ identification service (Braunschweig, Germany) based on spectroscopic hybridization. Bacterial cells grown in TSB (2 days, 30 °C, shaking culture) were disrupted using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). DNA–DNA relatedness between strain WCC 4582^T and *B. horneckiae* NRRL B-59162^T was 17 %. This value is significantly below the 70 % cut-off for the delineation of bacterial species suggested by Wayne *et al.* (1987).

Strains WCC 4582^T, WCC 4581 and WCC 4583 grew well on common media used for aerobic bacteria such as TSA, TSGA, TSBA, plate count agar (PCA; Merck) and PCA with powdered milk (PCMA; Roth) and in TSB aerated by shaking. The isolates produced ellipsoidal endospores in unswollen or sometimes only slightly swollen sporangia (Fig. S2, available in IJSEM Online). The biochemical and physiological characteristics of the isolates and the type strains of closely related species were determined (Logan *et al.*, 2009). Conditions for growth were assessed in duplicate in TSB for up to 14 days; temperature for growth was also assessed on TSA. Growth at pH 4.0–11.0 (at intervals of 0.5 pH units), using 1 M NaOH/HCl for pH adjustment and filtration for subsequent sterilization, and with 0–12 % (w/v) NaCl (at intervals of 1 %) were performed at 30 °C. The temperatures determined for growth were 5, 8, 10, 12, 15, 18, 25, 27, 30, 34, 37, 40, 45, 50

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic positions of *Bacillus kochii* sp. nov. and type strains of closely related species. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. *Bacillus subtilis* NCIB 3610^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

Fig. 2. Neighbour-joining tree based on partial *groEL* sequences determined in this study showing the phylogenetic positions of *Bacillus kochii* sp. nov. and type strains of closely related *Bacillus* species. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Bar, 0.02 substitutions per nucleotide position.

and 55 °C. Additionally, the isolates were characterized using the API 50 CH, API 20 NE, API Staph, API 20 E, API 20 Strep, API Coryne, ID 32 Staph, rapid ID 32 E, rapid ID 32 Strep, ID 32 GN and API ZYM systems (bioMérieux), according to the manufacturer's instructions except that the API 50 CH wells were covered with paraffin, the strips were incubated for up to several days at 30 °C in a humid chamber and API ZYM was analysed after 7 h at 30 °C. If different results were obtained from different systems, the API system was given priority over the ID system. Positive reactions were considered as positive after an incubation time according to the instructions of the supplier and as weakly positive after a prolonged incubation time. Resistance to lysozyme, anaerobic growth, hydrolysis of starch, production of dihydroxyacetone, reduction of nitrate, egg-yolk reaction and decomposition of casein, gelatin, xanthine, hypoxanthine and tyrosine were examined as described by Claus & Berkeley (1986) and Logan & De Vos (2009). Hydrolysis of Tweens 20, 40, 60 and 80 and tributyrin was determined as described by Harrigan & McCance (1976). Columbia blood agar with sheep blood (Oxoid), DNase activity agar medium (Merck), ONPG (β -galactosidase) strips (Oxoid) and Oxidase strips (Merck) were used as commercially available materials. The phenotypic characteristics are given in the species description and Table 1.

Analysis of the G+C content of the genomic DNA, the examination of quinones, polar lipids and the peptidoglycan and the determination of the cellular fatty acid compositions were carried out by the DSMZ. For G+C content, bacterial cells were obtained and DNA was purified as described for the DNA–DNA reassociation study. DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analysed by HPLC (Tamaoka & Komagata, 1984). DNA of strain WCC 4582^T was hydrolysed twice and the two resulting hydrolysates were injected three times into the HPLC system, which resulted in six independent measurements. The G+C content of strain WCC 4582^T was 36.4 ± 0.5 mol%. Cell mass for analysis of quinones and polar lipids was obtained from TSB shaken for 2 days at 30 °C. Quinones were examined as specified by Altenburger *et al.* (1996). The menaquinones of strain WCC 4582^T were MK-7 (97 %) and MK-6 (2 %), which is

in line with phylogenetically related taxa. Polar lipids were examined as described by Tindall (1990a, b). Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and aminophospholipid were the major polar lipids of strain WCC 4582^T. For cell-wall peptidoglycan and cellular fatty acid analysis, cell mass of strains was harvested from TSBA after cultivation for 24 h at 28 °C. The cell-wall peptidoglycan was isolated after disruption of the cells by shaking with glass beads and subsequent total hydrolysis (4 M HCl, 100 °C, 16 h). The amino acids and peptides in the hydrolysate were analysed by two-dimensional ascending TLC on cellulose plates using previously described solvent systems (Rhuland *et al.*, 1955; Schleifer, 1985). Strain WCC 4582^T contained the cell-wall peptidoglycan *meso*-diaminopimelic acid (A1 γ). Fatty acids were extracted and analysed by GLC according to the instructions of the Microbial ID system (MIDI; TSBA 40 version 4.10; Verbarg *et al.*, 2008). The major fatty acids of strains WCC 4582^T, WCC 4581 and WCC 4583 were iso-C_{15:0} (33–62 %), anteiso-C_{15:0} (11–25 %), iso-C_{14:0} (5–10 %) and iso-C_{16:0} (3–11 %). Some of the reference strains exhibited a similar pattern of major fatty acids, as shown in detail in Table 2. An FT-IR-based dendrogram of the isolates and their closest phylogenetic neighbours was calculated (Fig. S3, available in IJSEM Online). The distances of repeated measurements of each strain demonstrated the spectral variance within and between the species tested. This cluster analysis confirmed the distinct position of the isolates that was observed with the 16S rRNA gene and *groEL* sequence analyses.

Three isolates found at different geographical locations are described in this paper: strain WCC 4582^T from dairy A, strain WCC 4583 from dairy B and strain WCC 4581 from a pharmaceuticals production site. A variety of phenotypic characters (Table 1) were identical among the isolates. However, the isolates were not clonal, as demonstrated by the following differences: strain WCC 4581 and strain WCC 4583 had an identical 16S rRNA sequence whereas strain WCC 4582^T differed at position 183 (according to *Escherichia coli* numbering) where it showed an A/T polymorphism in the 16S rRNA operons. Strain WCC 4582^T and strain WCC 4583 could not be differentiated on the basis of partial *groEL* sequences (Fig. 2) or FT-IR fingerprints (Fig. S3), but were clearly different in these

Table 1. Characteristics that differentiate *Bacillus kochii* sp. nov. from type strains of closely related species

Taxa: 1, *Bacillus kochii* sp. nov. (WCC 4582^T, WCC 4581 and WCC 4583); 2, *B. circulans* DSM 11^T; 3, *B. firmus* DSM 12^T; 4, *B. horneckiae* NRRL B-59162^T; 5, *B. infantis* DSM 19098^T; 6, *B. nealsonii* DSM 15077^T; 7, *B. siralis* DSM 13140^T. All data were taken from this study unless otherwise indicated. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Cell length (µm)	3–13	2–7	1–5	4–20	3–10	4–9	2–5
pH for growth	6–10.5	6–9.5	6–10	5.7–10.5	ND	6–10	6.5–10
Nitrate reduction	–	–	+	+	–	–	+
Hippurate hydrolysis (ID 32 Strep)	–	ND	ND	+	ND	ND	–
Acid production from (API 50 CH):							
Glycerol	+	+	+	–	+	+	+
D-Ribose	+	+	+	–	+	–	–
Growth on (ID 32 GN):							
2-Ketogluconate	–	+	–	+	–	+	+
3-Hydroxybenzoate	+	–	–	–	–	–	–
Maltose	–	+	+	+	+	+	–
Sucrose	–	+	+	+	+	+	–
Lactate	–	+	+	+	+	–	+
L-Histidine	–	–	w	+	w	–	–
L-Proline	w	–	w	+	w	–	w
Phenylacetate	+	–	–	–	–	–	–
Propionate	w	–	–	+	–	–	–
DNA G + C content (mol%)	36.4 ± 0.5*	35.7–36.2†	40.7–41.4†	35.6–36.1†	40.8†	ND	38.1 ± 0.5

*DNA G + C content was determined only for strain WCC 4582^T.

†Data taken from Logan & De Vos (2009).

characters from strain WCC 4581. On the other hand, strain WCC 4581 and strain WCC 4583 were quite similar in their fatty acid compositions but different from strain WCC 4582^T (Table 2).

While the three isolates and the type strain of *B. horneckiae* showed clear similarities, a considerable number of phenotypic and chemotaxonomic differences were observed. These, taken together with the results of 16S rRNA gene and *groEL* sequence analyses, DNA–DNA relatedness and FT-IR typing, demonstrate that the isolates represent a novel species of the genus *Bacillus*, for which the name *Bacillus kochii* sp. nov. is proposed.

Description of *Bacillus kochii* sp. nov.

Bacillus kochii (ko.chi'i. N.L. gen. masc. n. *kochii* of Robert Koch, named in honour of the famous microbiologist with great merit in *Bacillus anthracis* research, to commemorate the centenary of his death on 27 May 1910).

Vegetative cells are motile rods (0.5–0.8 × 3–13 µm). Short chains are formed sporadically. Parasporal crystals are not produced. Ellipsoidal endospores lie paracentrally or subterminally and may slightly stretch sporangia sometimes. Colonies are cream, flat to low convex, spreading and translucent with irregular to filamentous margins. After 24 h on TSA at 30 °C, colony diameter is 3–5 mm; after 72 h, 15–20 mm. An off odour is produced. Growth occurs at 10–40 °C (optimum 30 °C), at pH 6.0–10.5

(optimum pH 7) and with 0–10 % (w/v) NaCl (optimum 0.5 % NaCl). Grows in the presence of 100 U lysozyme ml^{–1}, but not with 5 µg novobiocin ml^{–1}. Positive for Gram-staining, catalase and citrate utilization; negative for oxidase activity, anaerobic growth, Voges–Proskauer reaction and reduction of nitrate to nitrite. Does not produce dihydroxyacetone, gas from glucose, indole or hydrogen sulfide. Hydrolyses casein, DNA, gelatin and Tweens 20, 40, 60 and 80, but not aesculin, hippurate, lecithin, starch, tributyrin, tyrosine, urea, xanthine or hypoxanthine. On blood agar, β-haemolysis occurs. Weakly produces acid from glycerol and D-ribose; does not produce acid from α-cyclodextrin, D-adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, cellobiose, colistin, coumarate, dulcitol, erythritol, D-fructose, D- or L-fucose, D-galactose, gentiobiose, D-gluconate, D-glucose, D-glucuronate, glycerol, glycogen, inulin, itaconate, 2-keto-D-gluconate, 5-keto-D-gluconate, lactose, D-lyxose, maltose, D-mannitol, D-mannose, melezitose, melibiose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, *myo*-inositol, *N*-acetyl-D-glucosamine, palatinose, pullulan, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, starch, sucrose, D-tagatose, trehalose, turanose, xylitol or D- or L-xylose. As sole carbon sources for energy and growth, utilizes acetate, adipinate, L-alanine, citrate, D-gluconate, 3-hydroxybenzoate, 3-hydroxybutyrate, itaconate, malate, phenylacetate, L-proline, propionate, D-ribose, L-serine, suberate and valerate, but not L-arabinose, caprate, L-fucose, D-glucose,

Table 2. Cellular fatty acid compositions of *Bacillus kochii* sp. nov. and the type strains of closely related species

Strains: 1, *Bacillus kochii* sp. nov. WCC 4582^T; 2, *Bacillus kochii* sp. nov. WCC 4581; 3, *Bacillus kochii* sp. nov. WCC 4583; 4, *B. siralis* DSM 13140^T; 5, *B. infantis* DSM 19098^T; 6, *B. horneckiae* NRRL B-59162^T. Some rare trace derivatives were not given in the table. Data were taken from this study using cells harvested from TSBA after incubation for 24 h at 28 °C.

Fatty acid (%)	1	2	3	4	5	6*
Straight chain						
C _{14:0}	1.0	0.9	1.2	3.2	2.4	0.9 (1.1)
C _{15:0}	—	—	—	0.3	—	—
C _{16:0}	0.8	0.5	0.6	5.8	1.8	0.5 (0.5)
Branched						
iso-C _{13:0}	—	0.2	0.2	0.2	0.2	0.2 (0.2)
anteiso-C _{13:0}	—	—	—	—	0.1	—
iso-C _{14:0}	10.1	5.9	5.3	3.3	1.1	4.3 (4.0)
iso-C _{15:0}	33.4	62.1	59.5	46.0	44.5	57.4 (54.3)
anteiso-C _{15:0}	25.4	10.7	14.1	19.0	31.5	14.2 (19.9)
iso-C _{16:0}	10.7	3.4	2.8	3.0	1.6	3.3 (2.5)
iso-C _{17:0}	1.2	1.2	1.4	3.0	1.8	1.9 (1.3)
anteiso-C _{17:0}	6.1	1.5	2.0	3.6	8.0	2.1 (2.3)
iso-C _{17:1} ω10c	0.5	2.4	2.2	2.3	1.1	2.1 (2.3)
Unsaturated						
C _{16:1} ω7c alcohol	7.6	7.8	7.0	3.0	1.0	8.3 (6.8)
C _{16:1} ω11c	1.4	1.6	1.7	6.0	1.6	1.8 (2.0)
Summed feature 4†	1.8	1.8	2.1	1.2	2.8	2.8 (2.9)

*Data in parentheses were taken from Vaishampayan *et al.* (2010) using cells harvested from TSA after incubation for 24 h at 32 °C.

†Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 4 consisted of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

glycogen, L-histidine, 4-hydroxybenzoate, 2-keto-D-glucuronate, 5-keto-D-glucuronate, lactate, malonate, maltose, D-mannitol, D-mannose, melibiose, *myo*-inositol, *N*-acetyl-D-glucosamine, L-rhamnose, salicin, D-sorbitol or sucrose. With API, positive for alkaline phosphatase, glycyl-tryptophan arylamidase, leucine arylamidase, pyrazinamidase, pyroglutamate arylamidase and α -chymotrypsin; weakly positive for acid phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), leucine aminopeptidase, trypsin, tryptophan deaminase and valine arylamidase; negative for alanyl-phenylalanyl-proline arylamidase, arginine dihydrolase, α -fucosidase, α - and β -galactosidase, α - and β -glucosidase, β -glucuronidase, indoxyl phosphatase, lipase (C14), lysine decarboxylase, α - and β -mannosidase, *N*-acetyl-D-glucosaminidase, naphthol-AS-BI-phosphohydrolase, ornithine decarboxylase, phenylalanine deaminase and tetrathionate reductase. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0}. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and amino-phospholipid.

The type strain is WCC 4582^T (=DSM 23667^T=CCUG 59877^T=LMG 25855^T). Strain WCC 4582^T and reference strain (WCC 4583) were isolated from different German dairy products. Another reference strain, WCC 4581 (=DSM 24673), was isolated from a pharmaceuticals production line in Germany. The DNA G+C content of the type strain is 36.4±0.5 mol% (HPLC).

Acknowledgements

We thank J.L. Swezey (ARS Culture and Patent Culture Collection, Peoria, USA) for kindly providing *B. horneckiae* NRRL B-59162^T.

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