International Journal of Systematic Bacteriology (1998), 48, 1373–1382 Printed in Great Britain

Bacillus weihenstephanensis sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group

Sabine Lechner,[1] Ralf Mayr,[1] Kevin P. Francis,[1] Birgit M. Prüß,[1] Thomas Kaplan,[1] Elke Wießner-Gunkel,[1] Gordon S. A. B. Stewart[2] and Siegfried Scherer[1]

Author for correspondence: Siegfried Scherer. Tel: +49 8161 713516. Fax: +49 8161 7114512. e-mail: 100424.2340@compuserve.com

[1] Institut für Mikrobiologie, Forschungszentrum für Milch und Lebensmittel Weihenstephan, Technische Universität München, Vöttinger Str. 45, D-85354 Freising, Germany

[2] Department of Applied Biochemistry and Food Science, University of Nottingham, Sutton Bonington Campus, Nottingham LE12 5RD, UK

The *Bacillus cereus* group comprises the four valid species *Bacillus cereus*, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus anthracis*. Some isolates of *B. cereus* are known to be psychrotolerant (growth at 7 °C or below). Here, specific sequence differences are described between the 16S rDNA, the 23S rDNA, the 16S–23S rDNA spacer region and the genes of the major cold-shock protein homologue *cspA* in a variety of psychrotolerant and mesophilic *B. cereus* and *B. mycoides* strains. Randomly amplified polymorphic DNA analysis using three different primers clearly separated psychrotolerant strains of both species from the rest of the *B. cereus* group, as did inverse PCR patterns of the rDNA operons. These data strongly support a hitherto unrecognized fifth sub-group within the *B. cereus* species group comprising psychrotolerant, but not mesophilic, *B. cereus* strains. Despite the latter finding, the DNA sequences investigated exhibited a high degree of sequence similarity indicating a close relationship between the species of the *B. cereus* group. Considering the unusual importance of *B. cereus* in both food poisoning and food spoilage and to avoid merging all species of the group, a new species, *Bacillus weihenstephanensis* sp. nov., comprising psychrotolerant '*cereus*' strains, is proposed. Isolates of the new species grow at 4–7 °C but not at 43 °C and can be identified rapidly using rDNA or *cspA* targeted PCR. The type strain is *B. weihenstephanensis* WSBC 10204^T (= DSM 11821^T).

Keywords: *Bacillus cereus* group, *Bacillus mycoides*, taxonomy, *Bacillus weihenstephanensis* sp. nov.

INTRODUCTION

Few clear physiological characteristics are available to distinguish the four valid species of the *Bacillus cereus* group, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides* and *Bacillus anthracis* (20, 40, 48). All four species share a significant degree of genetic similarity as demonstrated by DNA–DNA hybridization studies (44, 26) and comparison of bacterial rRNA or rDNA at the 16S, 23S (1, 2) and 16S–23S spacer regions (5). It is accepted that the 16S–23S spacer region in particular provides a good marker for comparing both closely and distantly related organisms, since the selective pressure influencing this area of the genome is different from that for the rDNA structural genes (21, 28). Comparison of the genomic structure and organization between different strains of *B. cereus* and *B. thuringiensis* indicates that the diversity within these species is as large as the diversity between them (8) with some strains of *B. cereus* being more similar to strains of *B. thuringiensis* than to other *B. cereus* strains. Interestingly, the intraspecific genomic variability of *B. cereus* is considerable when compared to *Bacillus licheniformis* (14).

This paper is dedicated to Martin Busse, Freising, who initiated our studies on psychrotolerant *Bacillus cereus*.

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The EMBL accession numbers for the sequences reported in this paper are Z84575–Z84594.

Using different molecular and chemical identification methods such as arbitrary PCR patterns (24), multilocus enzyme electrophoresis (8), ribotyping (9), carbohydrate profiles (55) and IS231-derived sequences (23), it is possible to discriminate *B. anthracis* from the remaining three species, which are difficult to distinguish. Therefore, the debate continues as to whether the members of the *B. cereus* group should be regarded as a single species.

In addition to the four different sub-groups, psychrotolerant and mesophilic strains of the *B. cereus* group are known. Like their mesophilic counterparts, psychrotolerant strains have an optimal growth temperature at approximately 25–35 °C (35). They can be distinguished from the mesophilic strains by their ability to grow at temperatures of 7 °C and below. At low temperatures, psychrotolerant strains have slower metabolic rates and higher catalytic efficiencies than mesophiles (25). In contrast to psychrophilic microorganisms, these strains are characteristic of habitats where the temperature fluctuates diurnally and seasonally as they are 'adaptable' and can grow over a wide temperature range (41). Such strains are ecologically segregated and are able to co-exist with mesophilic strains.

So far, little attention has been directed towards a systematic investigation of psychrotolerant strains of the *B. cereus* group. Therefore, several psychrotolerant and mesophilic strains of the *B. cereus* group were characterized by DNA homology studies, 16S rDNA, 23S rDNA and spacer region sequence analysis, and rDNA operon patterns as well as by the nucleotide sequence of the major cold shock protein, CspA (32), and randomly amplified polymorphic DNA (RAPD) analysis.

METHODS

Organisms and culture conditions. Strains included in this study are listed in Table 1. Strains designated by the prefix WSBC were isolated from milk (33). Many milk samples were taken from three different South German dairies immediately after pasteurization over a period of 2 years, incubated at $7 \pm 0{\cdot}5$ °C for 10–16 d and plated on Plate Count (PC) agar. To confirm psychrotolerance, purified colonies were transferred to liquid culture (PC medium, see below) and incubated at $7 \pm 0{\cdot}5$ °C under agitation until growth was visible. Isolates were identified by morphological and biochemical characteristics (API 50CH and API 50CHB; bioMérieux). PC medium contained (l^{-1}) 5·0 g peptone, 2·5 g yeast extract, and 1·0 g glucose, with the pH adjusted to 7·0. Trypticase soy (TS) medium contained (l^{-1}) 17 g tryptone, 3 g phytone, 5 g NaCl, 2·5 g K_2HPO_4, 2·5 g glucose, and 6 g yeast extract, with the pH adjusted to 7·3. The bacteria were generally cultured overnight at 30 °C with shaking.

DNA similarity studies. For analysis of DNA homologies between different strains, bacteria were grown overnight in 500 ml medium to the stationary phase and harvested by centrifugation (3000 *g* for 10 min), resulting in 2–3 g fresh weight. DNA was isolated and purified by hydroxyapatite chromatography according to Cashion *et al.* (11). DNA–DNA hybridization was performed at the DSMZ, Braunschweig, Germany according to De Ley *et al.* (15) with a Gilford System 2000 spectrophotometer and a Gilford 2527-R thermoprogrammer and plotter.

Small-scale isolation of bacterial DNA. For PCR amplification, genomic DNA of the bacterial strains was isolated following a Miniprep protocol (53) with some modifications. After lysis (0·5 % SDS, 0·2 mg proteinase K ml^{-1}, 37 °C, 5 h), cell wall debris, denatured proteins, and polysaccharides were complexed to CTAB (hexadecyltrimethylammonium bromide) and removed by chloroform/phenol extraction (42). DNA was precipitated with 0·7 vol. 2-propanol, centrifuged, washed in 70 % ethanol, and air-dried. The DNA concentration was determined spectrometrically at 260 nm.

PCR amplification, cloning and sequencing. Primers and PCR conditions used for amplification are summarized in Table 2. Each amplification reaction started with a denaturation step at 94 °C for 5 min and ended with a final elongation step at 72 °C for 5 min. Reaction mixtures (100 µl) contained 50 pmol each primer, 200 µmol each dNTP (dATP, dTTP, dGTP, dCTP), 10 µl 10 × reaction buffer (100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl, 1 mg gelatin ml^{-1}, pH 8·3), 0·5 U Goldstar polymerase (Eurogentec) and 100 ng template DNA. The amplifications were carried out using a Perkin-Elmer thermal cycler. Results were checked on 1 % agarose gels.

For cloning of the 16S, 23S and the 16S–23S spacer, the *Bam*HI and *Sal*I restriction sites in the primers were used. After restriction digestion, the fragments were purified by agarose gel electrophoresis and ligated to a *Bam*HI/*Sal*I-cut, dephosphorylated pBS SK(−) vector (Stratagene). Ligation (T4 ligase; Boehringer) was carried out overnight at 18 °C. *Escherichia coli* JM101 cells were transformed by heat shock (42). Transformants were screened on LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, pH adjusted to 7·4) containing 80 µg ampicillin ml^{-1}. Amplified *cspA* gene fragments were directly sequenced as described below.

For DNA sequencing, plasmids were further purified following the Qiagen Midiprep protocol (Qiagen). DNA sequence determination (Sequiserve) was performed using a Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and reaction products were analysed using an Applied Biosystems model 373A automatic DNA sequencer according to the manufacturer's protocol. For each sequence, a single clone was analysed with internal primers to create overlapping fragments. To verify specific 16S rDNA signatures, PCR fragments amplified from internal primers were used directly in sequencing reactions. The fragments were purified by agarose gel electrophoresis and sequenced following the protocol of Casanova *et al.* (10). Sequences were aligned and analysed using the software package MacMolly Tetra (SoftGene). Corrections of the alignments were performed manually.

RAPD analysis. Three different primers were used (54): OPA7 (5′ GAAACGGGTG 3′), OPA18 (5′ AGGTGACCGT 3′) and OPB18 (5′ CCACAGCAGT 3′). PCR assays were performed with 200 ng total genomic DNA template in a 50 µl mixture containing 100 pmol primer, 200 µmol each dNTP, 5 µl 10 × reaction buffer and 0·5 U Goldstar polymerase. The reaction conditions were 94 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min (35 cycles) with an initial denaturation step at 94 °C for 7 min, and a final elongation step at 72 °C for 5 min. Amplification products were

Table 1. Bacterial strains and growth temperatures

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, MD, USA; HER, Centre de reference pour virus bacteriens, Felix d'Herelle Universite Laval, H.-W. Ackermann, Quebec, Canada; WS, Weihenstephan Culture Collection, Institute of Microbiology, FML Weihenstephan, Germany; WSBC, Weihenstephan *Bacillus* Collection, Institute of Microbiology, FML Weihenstephan, Germany.

Organism	Growth temperature (°C)				
	4	7	10	40	43
B. cereus (mesophilic)					
DSM 31$^{T\ meso.}$*	−	−	+	+	+
WSBC 10027†	−	−	+	+	+
WSBC 10028†	−	−	+	+	+
WSBC 10030†‡	−	−	+	+	+
WSBC 10039†	−	−	+	+	+
WSBC 10040†	−	−	+	+	+
WSBC 10041†	−	−	+	+	+
WSBC 10032†	−	−	+	+	+
WSBC 10033†	−	−	+	+	+
WSBC 10034†	−	−	+	+	+
WSBC 10035†	−	−	+	+	+
WSBC 10036†	−	−	+	+	+
WSBC 10037†‡	−	−	+	+	+
WSBC 10038†	−	−	+	+	+
WSBC 10042†	−	−	+	+	+
WSBC 10043†	−	−	+	+	+
WSBC 10044†	−	−	+	+	+
HER 1399†	−	−	+	+	+
HER 1414†	−	−	+	+	+
ATCC 7064†	−	−	+	+	+
ATCC 27877‡	−	−	+	+	+
B. cereus (psychrotolerant)§					
WSBC 10201†‡	+	+	+	−	−
WSBC 10202†	+	+	+	−	−
WSBC 10204$^{T\ psych.}$†‡	+	+	+	−	−
WSBC 10205†	+	+	+	−	−
WSBC 10206†‡	+	+	+	−	−
WSBC 10207	+	+	+	−	−
WSBC 10208†	+	+	+	−	−
WSBC 10209†	+	+	+	−	−
WSBC 10210†‡	+	+	+	−	−
WSBC 10211†	+	+	+	−	−
B. mycoides					
DSM 2048^{T}*	−	+	+	−	−
WSBC 10276†‡	−	+	+	−	−
WSBC 10277	−	+	+	−	−
WSBC 10278†‡	−	+	+	−	−
WSBC 10279†	−	+	+	−	−
B. thuringiensis					
DSM 2046^{T}*	−	−	+	+	+
HER 1211‡	−	−	+	+	+
HER 1230	−	−	+	+	+
HER 1231	−	−	+	+	+
HER 1232‡	−	−	+	+	+
HER 1233‡	−	−	+	+	+
HER 1236	−	−	+	+	+
HER 1357†	−	−	+	+	−
HER 1380‡	−	−	+	+	+
HER 1387‡	−	−	+	+	+
HER 1404‡	−	−	+	+	+
HER 1410†	−	−	+	+	+
HER 1418	−	−	+	+	+
WS 2632	−	−	+	+	+

* 16S rDNA sequence obtained from literature.

† *cspA* sequence determined in this study.

‡ 16S rDNA sequence determined in this study.

§ *Bacillus* '*cereus*' WSBC 10204^{T} is the type strain of the new species *Bacillus weihenstephanensis* but is listed in this table under the old nomenclature. The same is true for all psychrotolerant *Bacillus* '*cereus*' strains listed in this table.

visualized on 1·5% agarose gels and the results were documented with the VDS video documentation system (Pharmacia). The fragment patterns were analysed (Imagemaster 1D software; Pharmacia) and the combined data derived from the three primers were used to construct a similarity network with the neighbour-joining method (TREECON; 50).

Inverse PCR pattern of rDNA operons. Genomic DNA (200 ng) was completely digested with *Hha*I (cuts approximately 250 bp from the start of 16S rDNA). The enzyme was heat-inactivated at 85 °C for 30 min and the DNA fragments self-ligated with DNA ligase (Promega). PCR was performed with 200 µm dNTP, 2 mM $MgCl_2$, 50 pmol each primer and 8 ng ligated template (for details see Table 2). PCR products were visualized on a 1·2% agarose gel. To verify that rDNA was amplified, a 16S rDNA, digoxigenin-labelled universal probe consisting of a 194 bp fragment of 16S rDNA was synthesized by PCR. The probe was hybridized for 16 h at 50 °C to the DNA fragments blotted on nitrocellulose. Chemiluminescent detection of the bands was performed with an alkaline phosphatase-conjugated antibody directed against digoxigenin (Boehringer). Band patterns were analysed (Imagemaster 1D software; Pharmacia) and the data were used to construct a similarity network (neighbour-joining method, TREECON; 50).

RESULTS

Growth range

For characterization of individual growth temperature ranges, isolates were incubated as liquid cultures up to 14 d under agitation at 4, 7, 10, 40 and 43 ± 0·5 °C. Three thermal groups emerged (see Table 1). Psychro-

Table 2. Primers and PCR conditions used in this study

*Bam*HI and *Sal*I restriction sites are included in the 5′ end of the primers. PCR was run for 30 cycles except for the inverse PCR of rDNA operons which was run for 35 cycles.

Amplification target	Primer name	Primer sequence*	PCR conditions
16S rDNA gene	9-25†	5′ CGCGGGATCC^{BamHI}GAGTTTGATCCTGGCTC 3′	94 °C, 30 s
	1514-1492†	5′ GGCCGTCGAC^{SalI}ACGG(A/C)TACCTTGTTACGACTT 3′	60 °C, 30 s
			72 °C, 90 s
16S–23S spacer	16Sf-R2‡	5′ CGCGGGATCC^{BamHI}TTGTACACACCGCCCGTC 3′	94 °C, 30 s
23S fragment	23Sr-R10‡	5′ GGCCGTCGAC^{SalI}CCTTTCCCTCACGGTACTG 3′	60 °C, 30 s
			72 °C, 60 s
cspA gene§	BcF2§	5′ CGAATTTGATAATGTGTGGATTC 3′	95 °C, 15 s
	CSPU3‖	5′ CCCGGATCCGGTTACGTTA(G/C)C	50 °C, 30 s
		(A/T)GCT(T/G)(C/G)(A/C/T)GG(T/G/A)CC 3′	72 °C, 30 s
Inverse PCR pattern		5′ GGT GAG GTA ACG GCT CA 3′	95 °C, 15 s
of rDNA operons		5′ GCC GCC TTT CAA TTT CGA 3′	55 °C, 60 s
			72 °C, 180 s

* For nucleotides in parentheses, any one of the nucleotides given may be present.

† From Stackebrandt & Liesack (45), derived from *E. coli*.

‡ From Gürtler & Stanisich (21), derived from *E. coli*.

§ This study.

‖ From Francis & Stewart (17).

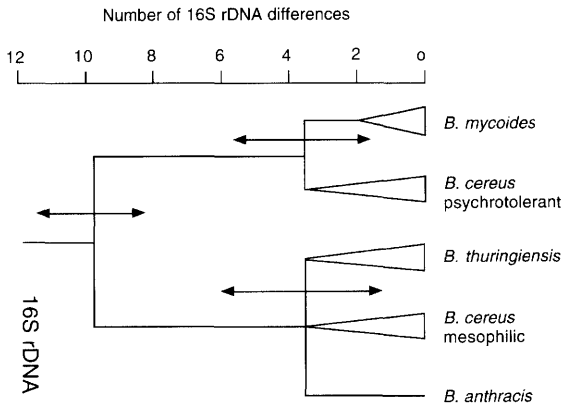

Fig. 1. Similarity between the species of the *B. cereus* group based on 16S rDNA sequence differences. The figure was constructed from a mean difference matrix which was based on four psychrotolerant *B. cereus*, three *B. mycoides*, six *B. thuringiensis*, four mesophilic *B. cereus* and the literature sequence of *B. anthracis*. Arrows at the nodes indicate the variation range in sequence differences. Due to considerable intra-species variation, *B. mycoides* and psychrotolerant *B. cereus* on the one hand and *B. thuringiensis*, *B. anthracis* and mesophilic *B. cereus* on the other cannot be differentiated. However, these two clusters are significantly different.

tolerant *B. cereus* grew at 4 °C but not at 43 °C, while mesophilic *B. cereus* and all but one *B. thuringiensis* grew at 43 °C but not at 7 °C. The five isolates of *B. mycoides* grew at 7 °C but not at 4 or 40 °C. It was important to test isolates for growth in agitated liquid culture since very slow growth of a few mesophilic *B. cereus* at 7 °C was observed on agar plates (data not shown).

Analysis of the 16S rDNA sequences

Almost complete 16S rDNA sequence data of seven *B. cereus*, two *B. mycoides* and six *B. thuringiensis* strains were determined. The sequences differ in only a few nucleotides and their comparison is shown in Fig. 1, including data of *B. cereus*, *B. mycoides* and *B. thuringiensis* available from databases (sequences taken from the literature, however, contain a certain number of undetermined nucleotides). The most significant sequence differences were observed between psychrotolerant strains of *B. cereus* and *B. mycoides* versus *B. thuringiensis*, *B. anthracis* and mesophilic strains of *B. cereus*, respectively. Significant differences between psychrotolerant *B. cereus*/*B. mycoides* on the one hand and all other strains of the *B. cereus* group on the other are evident and the variation is approximately three times greater than that observed within species. There is one region with typical base changes in two nucleotides (Table 3). This signature was confirmed by sequencing 20 mesophilic and 15 psychrotolerant strains (data not shown) and can be used to differentiate psychrotolerant from mesophilic *B. cereus* by PCR (51).

Table 3. Two signature sequences which differentiate between mesophilic and psychrotolerant strains of *Bacillus cereus*

Nucleotides printed in bold represent the signature nucleotides upon which differentiation of mesophilic and psychrotolerant isolates is based.

16S rDNA signature*	
Mesophilic	^{1008}CCTAGAGATAGG
Psychrotolerant	^{1008}**T**CTAGAGATAG**A**
cspA signature†	
Mesophilic	^{1}GCAGTA
Psychrotolerant	^{1}**A**CAGT**T**

* Nomenclature according to *E. coli*. Signatures are based on sequences of nine psychrotolerant and 11 mesophilic strains.

† The first base given is number 4 of the *cspA* gene. Signature is based on sequences of 20 mesophilic and 11 psychrotolerant strains.

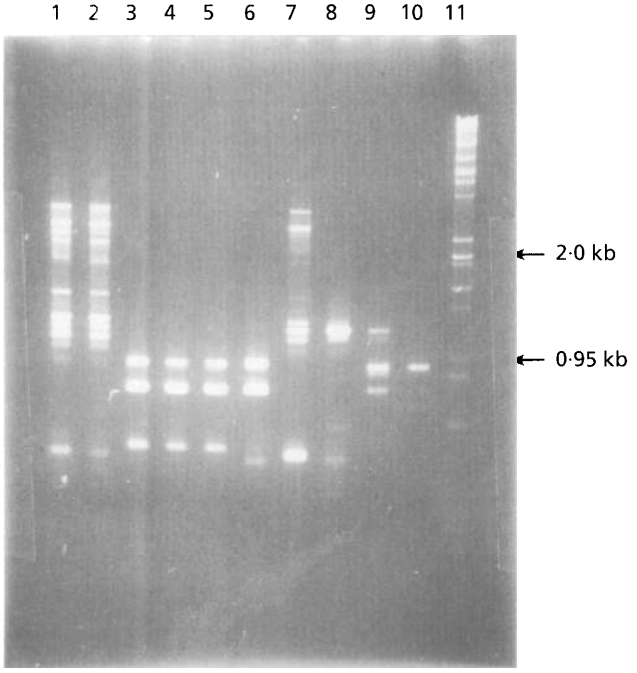

Fig. 2. Separation of fragments produced by RAPD/PCR amplification in a 1·5% agarose gel. Total genomic DNA of the following strains was used for amplification. Lanes: 1 and 2, psychrotolerant *B. mycoides* strains WSBC 10276 and WSBC 10279, respectively; 3–6, *B. thuringiensis* strains HER 1211, HER 1232, HER 1233 and HER 1404, respectively; 7, psychrotolerant *B. mycoides* type strain; 8, psychrotolerant *B. cereus* WSBC 10206; 9 and 10, mesophilic *B. cereus* WSBC 10032 and HER 1414, respectively; and 11, λ DNA (*Eco*RI- and *Hin*dIII-digested). For this experiment, primer OPB18 was used. See Table 1 for strain designation.

Analysis of the 16S–23S rDNA spacer region and partial 23S rDNA sequences

For further discrimination between the organisms of the *B. cereus* group, the spacer regions between the 16S and 23S rDNA genes and nucleotides 1–525 of the 23S rDNA gene were sequenced from three psychrotolerant strains (one *B. cereus* and two *B. mycoides*) and a mesophilic strain of *B. cereus* and *B. thuringiensis*, respectively. The spacer regions of these strains were 142 (*B. thuringiensis*) and 143 (*B. cereus* and *B. mycoides*) nucleotides and had 14 variable positions. No tRNA genes were detected within the spacer region. The 5′ region of the 23S rDNA includes seven sequence differences between the mesophilic and psychrotolerant strains. Both spacer regions and 23S rDNA sequences indicated a close relationship between psychrotolerant *B. cereus* and *B. mycoides* and between the mesophilic *B. cereus* strains and *B. thuringiensis*, thus corroborating results from 16S rDNA analysis.

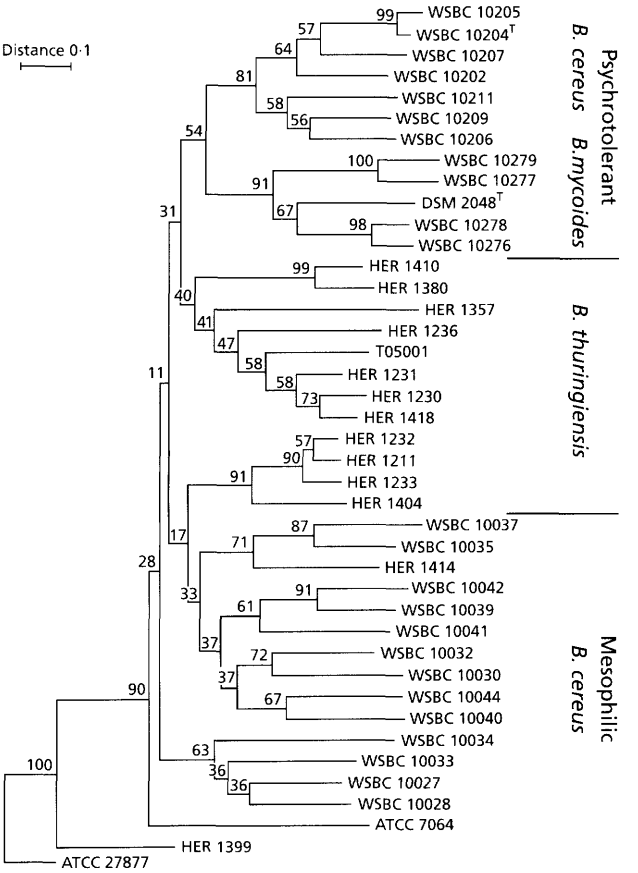

Fig. 3. Similarity pattern resulting from RAPD analysis of the different strains of the *B. cereus* group. Bootstrap analysis was performed with 100 repeats. The mesophilic *B. cereus* ATCC 27877 was used as an outgroup.

cspA sequences from psychrotolerant and mesophilic *B. cereus* strains

Analysis of the coding region for the major cold shock protein of 10 psychrotolerant and 20 mesophilic *B. cereus* strains comprising 171 nucleotides revealed differences in nine distinct positions. The nucleotide changes at positions 4 and 9 (Table 3) have signature

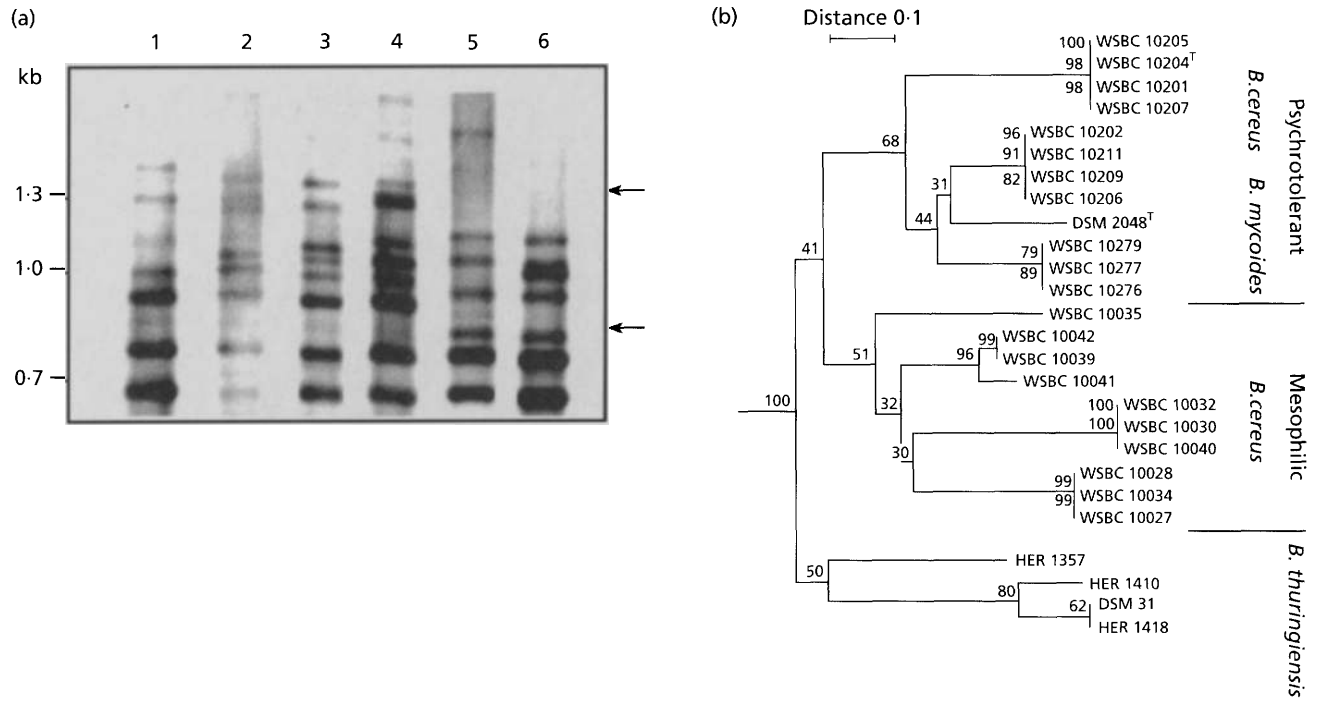

Fig. 4. (a) Separation of fragments produced by inverse PCR in a 1·2% agarose gel. Total genomic DNA of the following strains was used for the amplification. Lanes: 1–4, mesophilic *B. cereus* strains WSBC 10027, WSBC 10030, WSBC 10042 and WSBC 10035, respectively; and 5 and 6, psychrotolerant *B. cereus* strains WSBC 10206 and WSBC 10204^T, respectively. Arrows point to bands which are specific for mesophilic (upper arrow) and psychrotolerant (lower arrow) isolates. Molecular size standards are given on the left. (b) Similarity pattern resulting from inverse PCR of strains of the *B. cereus* group. Bootstrap analysis was performed with 100 repeats. For strain designations see Table 1.

character as they are specific for the differentiation of psychrotolerant strains from mesophilic strains of the *B. cereus* group (18). Further nucleotide changes were found at positions 78, 90, 111, 124, 135, 162 and 165 of the coding region. The high degree of similarity of the structural *cspA* genes (96–100%) also indicates a close phylogenetic relationship between all strains, but psychrotolerant *B. cereus* strains can be clearly differentiated from the mesophilic strains.

RAPD analysis

This is a powerful tool to characterize and distinguish closely related bacteria. Seven psychrotolerant and 17 mesophilic *B. cereus* strains, five psychrotolerant *B. mycoides* strains and 12 *B. thuringiensis* strains were analysed by RAPD/PCR, each with three different primers. All assays were repeated three times to verify the reproducibility of the fragment pattern. An example of a RAPD amplification is shown in Fig. 2. The fragment length patterns of amplifications resulting from three different primers were combined for each strain and data were analysed with the program TREECON using the neighbour-joining method for RAPD data (Fig. 3). The combination of the three primers each binding independently and randomly to genomic fragments clearly demonstrates that the psychrotolerant *B. cereus* and *B. mycoides* strains form a separate group. This group remains stable when individual RAPD patterns resulting from single primers were analysed. Other computer programs such as the maximum-likelihood method for restriction fragment analysis of the software package PHYLIP (version 3.75c, J. Felsenstein, Department of Genetics, University of Washington, Seattle, USA) were also used to analyse the data set and resulted in stable separation of psychrotolerant from mesophilic isolates (data not shown).

Inverse PCR pattern of rDNA operons

It is well-known that bacteria possess several rDNA operons (reviewed by 13). Since clear differences occurred between 16S rDNA sequences, it is speculated that the dispersal of the different operons over the genome and/or inter-operon sequences may vary. rDNA patterns were studied by inverse PCR (Fig. 4a) and reveal clear differences between strains. Two patterns were found for psychrotolerant *B. cereus* strains and two patterns were found for *B. mycoides*, while five and three patterns were observed for mesophilic *B. cereus* and *B. thuringiensis*, respectively. Specific bands for mesophilic isolates occurred around 1·3 kb, while at 800 bp, a band specific for all psychrotolerant strains was found. These patterns were used to construct a similarity network (Fig. 4b), thus dem-

Table 4. DNA–DNA hybridization (%) between mesophilic and psychrotolerant isolates of the *Bacillus cereus* group

1, *B. cereus* WSBC 10201; 2, *B. cereus* WSBC 10204$^{T\text{-psych.}}$; 3, *B. cereus* WSBC 10206; 4, *B. mycoides* DSM 2048^{T}; 5, *B. mycoides* WSBC 10278; 6, *B. mycoides* WSBC 10276; 7, *B. cereus* WSBC 10030; 8, *B. cereus* WSBC 10037; 9, *B. cereus* ATCC 27877.

	Psychrotolerant isolates						Mesophilic isolates		
	1	2	3	4	5	6	7	8	9
WSBC 10204^{T}	94·0								
WSBC 10206	80·1	80·1							
DSM 2048^{T}	88·0	86·9	84·7						
WSBC 10278	75·6	83·0	100	85·5					
WSBC 10276	66·8	68·1	62·9	60	73·4				
WSBC 10030	83·3	55·1	75·6	89·3	91·0	55			
WSBC 10037	67·2	63·1	62·1	61·2	59·9	69·8	70·8		
ATCC 27877	72·6	73·9	68·6	79·6	77·0	81·5	82·6	94·6	
B. thuringiensis WS 2632	54·3	69·3	48·0	63·8	54·4	71·5	53·6	82·7	94·6

onstrating that psychrotolerant *B. cereus* plus *B. mycoides* are separate from mesophilic *B. cereus* and *B. thuringiensis*. This result confirms the RAPD analysis (compare figure 3).

DNA–DNA hybridization

DNA–DNA hybridization data within the *B. cereus* group (Table 4) demonstrate large heterogeneity. The *B. cereus* and *B. mycoides* strains exhibited mean identity values of 75 and 73%, respectively. Between the *B. cereus* and *B. mycoides* strains, an identity of 77% was found. Neither the psychrotolerant nor the mesophilic *B. cereus* strains had significantly higher identity to the *B. mycoides* strains (80 and 74%, respectively). The only obvious difference was found between the psychrotolerant *B. cereus*/*B. mycoides* strains and *B. thuringiensis* WS 2632 (57 and 63%, respectively), whereas the mesophilic *B. cereus* strains exhibited 77% identity to WS 2632.

DISCUSSION

All species of the *B. cereus* group are closely related. Analysis of a variety of biochemical characters indicates the closeness of *B. cereus*, *B. mycoides*, *B. thuringiensis* and *B. anthracis* (20, 40, 48). Previous studies on a limited number of strains demonstrated the high degree of sequence similarity (> 99%) between strains of the *B. cereus* group for 16S rDNA (1), 23S rDNA (2) and the 16S–23S spacer region (5, 22). In general, these results were confirmed in this study.

There are physiological parameters which are considered to be unique for the different groups: production of an insecticidal toxin by *B. thuringiensis*, enterotoxin production by *B. cereus* and production of anthrax toxin of *B. anthracis*, as well as mycoidal growth in the sub-group *B. mycoides*. However, these characters may be disputed as taxonomic markers as it is possible to transfer plasmids of *B. thuringiensis* encoding insecticidal crystals to *B. cereus* and *B. anthracis*, making *B. cereus* and *B. anthracis* look like crystal-producing *B. thuringiensis* (3, 19). Some strains of *B. anthracis* lack the toxin and capsule virulence plasmids pXO1 and pXO2 (30). These strains look like *B. cereus* strains unless further tests such as bacteriophage susceptibility are performed (24). Enterotoxins are not only produced by *B. cereus*, but also by *B. thuringiensis* (7, 38) and *B. mycoides* (16). A physiological marker which is unequivocal and not easily transferable (29, 31) is the ability to grow at low temperatures, as observed in psychrotolerant *B. cereus* strains. Despite considerable efforts, mesophilic *B. cereus* could not adapt to grow at 7 °C in this study.

The limit for separation of species on the basis of DNA–DNA relatedness is suggested to be 70% (52). DNA–DNA hybridization studies for analysis of the relatedness of the organisms of the *B. cereus* group did not give clear results. The scatter of the data was too high to draw any convincing conclusions. DNA–DNA hybridization studies reported by Kaneko *et al.* (26) also demonstrated a large heterogeneity between the different strains of the *B. cereus* group which led them to suggest that *B. cereus*, *B. mycoides*, *B. thuringiensis* and *B. anthracis* form a single species. In contrast, Nakamura & Jackson (36) have shown, using DNA–DNA hybridization, that *B. cereus* and *B. thuringiensis* are closely related but are different species with levels of relatedness of 59–69%, while *B. cereus* strains clearly differed from the *B. mycoides* strains, with values of 22–44%. These results, which are also contradictory to molecular data, were not confirmed in this study. Stackebrandt & Goebel (46) demonstrated that 16S rDNA analysis and DNA–DNA hybridization may not correlate. Comparison of 16S and DNA–DNA hybridization data of the *B. cereus* group

led to a similar result (data not shown). DNA–DNA hybridization data varied unsystematically at 48–100% and, using strains from this study, did not correlate at all with 16S rDNA and RAPD.

Psychrotolerant strains of *B. cereus* and *B. mycoides* may form a separate sub-group. Psychrotolerant *B. cereus* strains have been reported widely in food microbiology studies (e.g. 27, 34). It was not possible to separate psychrotolerant and mesophilic strains of *B. cereus* in terms of their fermentation pattern or other biochemical parameters (data not shown). This study, for the first time, systematically compares psychrotolerant and mesophilic strains of the *B. cereus* group at the molecular level. 16S rDNA sequences display clear differences between psychrotolerant and mesophilic *B. cereus* group strains. Such differences were also found in the structural genes of the cold shock protein CspA. Interestingly, nucleotide changes in *cspA* occurred mainly at the wobble position and resulted in an amino acid change only at three positions: position 4, Thr or Ala; position 111, Asp or Glu; and position 162, Asp or Glu. The nucleotide substitutions are highly conserved. A function for these signatures in cold shock genes is not obvious at this stage.

The five *B. mycoides* strains studied were all psychrotolerant and their 16S rDNA sequences clustered clearly with the psychrotolerant *B. cereus* strains. Bell & Friedman (4) characterized populations of *B. mycoides* by standard metabolic tests, multilocus enzyme electrophoresis, RFLP and hybridization techniques. Their results indicated that the *B. mycoides* strains comprise two different groups which could be separated only with molecular assays but not by biochemical tests. It would be interesting to investigate whether these two groups represent psychrotolerant and mesophilic strains, respectively. However, the authors did not provide any information concerning the minimal growth temperature of the different strains.

RAPD analysis has been used to distinguish and characterize commercially available *B. thuringiensis* strains (6) as well as isolates of *B. cereus* (47). RAPD assays resulted in a clear separation of the psychrotolerant *B. cereus* strains (Fig. 3). Together with the psychrotolerant *B. mycoides* strains, they form a separate branch in the similarity network, thus confirming the results of rDNA and *cspA* analysis. *B. thuringiensis* did not form a clear group separated from, for example, mesophilic *B. cereus*. The result of RAPD analysis was confirmed by the pattern arising from inverse PCR of rDNA operons, except that in this case *B. thuringiensis* may form a separate group.

Schraft *et al.* (43) performed an epidemiological study of 62 *Bacillus cereus* strains isolated from food. They reported that fatty acid profiles clearly grouped 'psychrotrophic' and mesophilic *B. cereus* into different clusters, which is in good accordance with results reported here, although these authors define 'psychrotrophy' as growth at 8 °C within 100 h as measured in a Bioscreen C multiwell photometric plate reader. These data demonstrate clearly that psychrotolerant isolates of the two species *B. cereus* and *B. mycoides* (i) cluster closely and (ii) are more closely related to one another than to mesophilic *B. cereus* and *B. thuringiensis*. The taxonomy of the *B. cereus* group therefore needs revision.

Based on their high similarity, it has been repeatedly proposed that all species of the *B. cereus* group be merged into a single species (1, 2, 7, 26, 39, 40). It is suggested, however, that there are good reasons, in spite of the close relationship of the species, to maintain the taxonomy of the *B. cereus* group at the species level. This group comprises economically and medically important species which have been treated under their species names in a most diverse body of fundamental and applied literature (identification of food poisoning organisms and insecticidal toxin producers and separation of the extremely dangerous *B. anthracis*). Renaming all these species as *B. cereus* subspecies would create enormous confusion.

There are other examples such as the *Mycobacterium* species *Mycobacterium avium* and *Mycobacterium paratuberculosis* which are much more closely related than these *Bacillus* species, having only nine mismatches in the 16S rDNA genes, one mismatch in the 23S rDNA genes, and two mismatches in the 16S–23S spacer region (49). However, differences in growth rate and mycobactin dependence appear to be sufficient to separate these two species in view of their medical importance. Another example is the newly described, medically important species *Rickettsia peacockii* which has a 16S rDNA similarity to *Rickettsia rickettsii* or *Rickettsia slovaca* of 99·7% (37).

Sequences of 16S rDNA and CspA, as well as overall genomic organization resulting in different RAPD patterns and inverse PCR patterns of rDNA operons warrant the designation of a new, psychrotolerant species for which the name *Bacillus weihenstephanensis* sp. nov. is proposed. Although psychrotolerant *B. mycoides* formed a group that was separate from psychrotolerant '*cereus*' in RAPD analysis, such differences were not found at the 16S rDNA, 23S rDNA or *cspA* level. *B. mycoides* is more similar to the newly described species than to any other species of the *B. cereus* group. However, *B. cereus* as well as *B. weihenstephanensis* are known to cause food poisoning and, therefore, belong to hazard group 2. Because no case of food poisoning has been attributed to *B. mycoides*, it is recommended that *B. mycoides* is maintained as a separate species belonging to hazard group 1 to minimize confusion.

Description of *Bacillus weihenstephanensis* sp. nov.

Bacillus weihenstephanensis (we'ihen.ste'phan.en'sis. M.L. masc. adj. *weihenstephanensis* referring to Freising-Weihenstephan in southern Germany, the location where the type strain was isolated).

Gram-positive, facultatively anaerobic, spore-forming rods. Cell morphology and substrate utilization similar to *B. cereus* as described by Claus & Berkeley (12). Can be differentiated from *B. cereus* by its ability to grow aerobically at 7 °C in agitated liquid culture, absence of growth at 43 °C, by the presence of the 16S rDNA signature sequence ^{1003}TCTAGAGATAGA and the signature sequence ^{4}ACAGTT of the major cold shock gene *cspA*. Can be differentiated from *B. mycoides* by its non-mycoidal colony morphology. For differentiation from *B. thuringiensis* and *B. anthracis*, the same characters apply as have been described for differentiation of these species from *B. cereus* (12). Should be grouped within hazard group 2 due to its food poisoning potential. The type strain of *B. weihenstephanensis* is WSBC 10204^{T} and has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braundschweig, Germany, as DSM 11821^{T}.

ACKNOWLEDGEMENTS

R.M. was supported by grant 1116/92-5.1 from the Kommission der Europäischen Union.

REFERENCES

1. **Ash, C., Farrow, J. A. E., Dorsch, M., Stackebrandt, E. & Collins, M. D. (1991).** Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rDNA. *Int J Syst Bacteriol* **41**, 343–346.
2. **Ash, C. & Collins, M. D. (1992).** Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. *FEMS Microbiol Lett* **94**, 75–80.
3. **Battisti, L., Green, B. D. & Thorne, C. B. (1985).** Mating system for transfer of plasmids among *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. *J Bacteriol* **162**, 543–550.
4. **Bell, J. A. & Friedman, S. B. (1994).** Genetic structure and diversity within local population of *Bacillus mycoides*. *Evolution* **48**, 1698–1714.
5. **Bourque, S. N., Valero, J. R., Lavoie, M. C. & Levesque, R. C. (1995).** Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and of closely related species. *Appl Environ Microbiol* **61**, 1623–1626.
6. **Brousseau, R., Saint-Onge, A., Prefontaine, G., Masson, L. & Cabana, J. (1993).** Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. *Appl Environ Microbiol* **59**, 114–119.
7. **Carlson, C. R. & Kolstø, A.-B. (1993).** A complete physical map of a *Bacillus thuringiensis* chromosome. *J Bacteriol* **175**, 1053–1060.
8. **Carlson, C. R., Cougant, D. A. & Kolstø, A.-B. (1994).** Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl Environ Microbiol* **60**, 1719–1725.
9. **Carlson, C. R., Johansen, T. & Kolstø, A.-B. (1996).** The chromosome map of *Bacillus thuringiensis* subsp. *canadiensis* HD224 is highly similar to that of the *Bacillus cereus* type strain ATCC 14579. *FEMS Microbiol Lett* **141**, 163–167.
10. **Casanova, J.-L., Pannetier, C., Jaulin, C. & Kourilsky, P. (1990).** Optimal conditions for directly sequencing double-stranded PCR products with Sequenase. *Nucleic Acids Res* **18**, 4028.
11. **Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977).** A rapid method for the base ration determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
12. **Claus, D. & Berkeley, R. C. W. (1986).** Genus *Bacillus* Cohn 1872. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams and Wilkins.
13. **Condon, C., Squires, C. & Squires, C. L. (1995).** Control of rRNA transcription in *Escherichia coli*. *Microbiol Rev* **59**, 623–645.
14. **Daffonchio, D., Borin, S., Frova, G., Manachini, P. L. & Sorlini, C. (1998).** PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergeneric tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. *Int J Syst Bacteriol* **48**, 107–116.
15. **De Ley, J., Cattoir, H. & Reynaerts, A. (1970).** The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
16. **Dietrich, R. & Märtlbauer, E. (1997).** Personal communication.
17. **Francis, K. P. & Stewart, G. S. A. B. (1997).** Detection and speciation of bacteria through PCR using universal major cold shock protein primer oligomers. *J Ind Microbiol Biotechnol* **19**, 286–293.
18. **Francis, K. P., Mayr, R., von Stetten, F., Stewart, G. S. A. B. & Scherer, S.** Discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of major cold shock protein genes. *Appl Environ Microbiol* **64**, 3525–3529.
19. **Gonzales, J. M., Jr, Brown, B. J. & Carlton, B. C. (1982).** Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. *Proc Natl Acad Sci USA* **79**, 6951–6955.
20. **Gordon, R. E., Haynes, W. C. & Pang, C. H.-N. (1973).** *The Genus Bacillus*. Washington, DC: US Department of Agriculture.
21. **Gürtler, V. & Stanisich, V. A. (1996).** New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
22. **Harrell, L. J., Andersen, G. L. & Wilson, K. H. (1995).** Genetic variability of *Bacillus anthracis* and related species. *J Clin Microbiol* **33**, 1847–1850.
23. **Henderson, I., Dongzheng, Y. & Turnbull, P. C. B. (1995).** Differentiation *of Bacillus anthracis* and other '*Bacillus cereus* group' bacteria using IS231-derived sequences. *FEMS Microbiol Lett* **128**, 113–118.
24. **Henderson, I., Duggleby, C. J. & Turnbull, P. C. B. (1994).** Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. *Int J Syst Bacteriol* **44**, 99–105.
25. **Jay, J. M. (1987).** The tentative recognition of psychrotrophic Gram-negative bacteria in 48 h by their surface growth at 10 °C. *Int J Food Microbiol* **4**, 25–32.

26. **Kaneko, T., Nozaki, R. & Aizawa, K. (1978).** Deoxyribonucleic acid relatedness between *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. *Microbiol Immunol* **22**, 639–641.

27. **Larsen, H. D. & Joergensen, K. (1997).** The occurrence of *Bacillus cereus* in Danish pasteurized milk. *Int J Food Microbiol* **34**, 179–186.

28. **Leblond-Bourget, N., Philippe, H., Mangin, I. & Decaris, B. (1996).** 16S rDNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intra-specific *Bifidobacterium* phylogeny. *Int J Syst Bacteriol* **46**, 102–111.

29. **Lindsay, J. A. (1995).** Is thermophily a transferrable property in bacteria? *Crit Rev Microbiol* **21**, 165–174.

30. **Makino, S. I., Iinuma-Okada, Y., Maruyama, T., Ezaki, T., Sasakawa, C. & Yoshikawa, M. (1993).** Direct detection of *Bacillus anthracis* DNA in animals by polymerase chain reaction. *J Clin Microbiol* **31**, 547–551.

31. **Margesin, R. & Schinner, F. (1994).** Properties of cold-adapted micro-organisms and their potential role in biotechnology. *J Biotechnol* **33**, 1–14.

32. **Mayr, B., Kaplan, T., Lechner, S. & Scherer, S. (1996).** Identification of a family of dimeric major cold shock protein homologs from the psychrotrophic *Bacillus cereus* WSBC10201. *J Bacteriol* **178**, 2916–2925.

33. **Mayr, R. (1997).** *Kältetolerante Bacillusarten in pasteurisierter Trinkmilch: Flora, Verderbsanalysen und Phagentypisierung von Bacillus cereus*. PhD thesis, Technische Universität München.

34. **Meer, R. R., Baker, J., Bodyfelt, F. W. & Griffiths, M. W. (1991).** Psychrotrophic *Bacillus* spp. in fluid milk products: a review. *J Food Protein* **54**, 969–979.

35. **Morita, R. Y. (1975).** Psychrophilic bacteria. *Bacteriol Rev* **39**, 144–167.

36. **Nakamura, L. K. & Jackson, M. A. (1995).** Clarification of the taxonomy of *Bacillus mycoides*. *Int J Syst Bacteriol* **45**, 46–49.

37. **Niebylski, M. L., Schrumpf, M. E., Burgdorfer, W., Fischer, E. R., Gage, K. L. & Schwan, T. G. (1997).** *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in Western Montana. *Int J Syst Bacteriol* **47**, 446–452.

38. **Perani, M., Bishop, A. H. & Vaid, A. (1998).** Prevalence of β-exotoxin, diarrhoeal toxin and specific δ-endotoxin in natural isolates of *Bacillus thuringiensis*. *FEMS Microbiol Lett* **160**, 55–60.

39. **Priest, F. G. & Alexander, B. (1988).** A frequency matrix for probabilistic identification of some bacilli. *J Gen Microbiol* **134**, 3011–3018.

40. **Priest, F. G., Goodfellow, M. & Todd, C. (1988).** A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847–1882.

41. **Russel, N. J. (1990).** Cold adaptation of microorganisms. *Philos Trans R Soc Lond B Biol Sci* **326**, 595–611.

42. **Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

43. **Schraft, H., Stelle, M., McNab, B., Odumeru, J. & Griffiths, M. W. (1996).** Epidemiological typing of *Bacillus* spp. isolated from food. *Appl Environ Microbiol* **62**, 4229–4232.

44. **Somerville, H. J. & Jones, M. L. (1972).** DNA competition experiments within the *Bacillus cereus* group of bacilli. *J Gen Microbiol* **73**, 257–261.

45. **Stackebrandt, E. & Liesack, W. (1992).** The potential of rDNA in identification and diagnostics. In *Nonradioactive Labeling and Detection of Biomolecules*, pp. 232–239. Edited by C. Kessler. Berlin: Springer.

46. **Stackebrandt, E. & Goebel, B. M. (1994).** A place for DNA–DNA reassociation and 16S rDNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

47. **Stephan, R. (1996).** Randomly amplified polymorphic DNA (RAPD) assay for genomic fingerprinting of *Bacillus cereus* isolates. *Int J Food Microbiol* **31**, 311–316.

48. **Turnball, P. C. B. & Kramer, J. M. (1991).** Bacillus. In *Manual of Clinical Microbiology*, 5th edn, pp. 296–303. Edited by A. Balows, W. J. Hausler, Jr, K. L. Herrmann, H. D. Isenberg & H. J. Shadomy. Washington, DC: American Society for Microbiology.

49. **Van der Giessen, J. N. B., Haring, R. M. & van der Zeijst, B. A. M. (1994).** Comparison of the 23S ribosomal RNA genes and the spacer region between the 16S and 23S rDNA genes of the closely related *Mycobacterium avium* and *Mycobacterium paratuberculosis* and the fast-growing *Mycobacterium phlei*. *Microbiology* **140**, 1103–1108.

50. **Van de Peer, Y. & De Wachter, R. (1994).** TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Appl Biosci* **10**, 569–570

51. **von Stetten, F., Francis, K. P., Lechner, S., Neuhaus, K. & Scherer, S.** Rapid discrimination of psychrotolerant and mesophilic strains of the *Bacillus cereus* group by PCR targeting of 16S rDNA. *J Microbiol Methods* (in press).

52. **Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

53. **Wilson, K. H. (1987).** Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.2. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhls. New York: Wiley.

54. **Woodborn, M. A., Yousten, A. A. & Hilu, K. H. (1995).** Random amplified polymorphic DNA fingerprinting of mosquito-pathogenic and non-pathogenic strains of *Bacillus sphaericus*. *Int J Syst Bacteriol* **45**, 212–217.

55. **Wunschel, D., Fox, K. F., Black, G. E. & Fox, A. (1994).** Discrimination among the *Bacillus cereus* group, in comparison to *B. subtilis*, by structural carbohydrate profiles and ribosomal RNA spacer region PCR. *Syst Appl Microbiol* **17**, 625–635.