Two novel psychrotolerant species, *Bacillus psychrotolerans* sp. nov. and *Bacillus psychrodurans* sp. nov., which contain ornithine in their cell walls

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Eleven psychrotolerant *Bacillus* strains with ornithine as diamino acid in position 3 of the peptide side chain of the cell wall and a G+C range of 35.7–38.4 mol% were characterized taxonomically. DNA–DNA hybridization studies confirmed previously physiologically established groups. High DNA-binding values (>70%) were found within groups I A (consisting of the type strain of *Bacillus insolitus* DSM 5^T and *Bacillus insolitus* DSM 2272), I B (consisting of isolates 3H1^T, 71H1, 84E1, 87H2 and 4H2) and I C (consisting of isolates 68E3^T, 61E1, 4E3 and 67E1). Low DNA-binding values (<60%) were revealed between the three groups. Consequently, strains of groups I B and I C were considered as being representatives of new psychrotolerant species. For group I B strains the name *Bacillus psychrotolerans* sp. nov. is proposed with the type strain 3H1^T (= DSM 11706^T = NCIMB 13838^T) and for group I C strains the name *Bacillus psychrodurans* sp. nov. is proposed with the type strain 68E3^T (= DSM 11713^T = NCIMB 13837^T).

Keywords: psychrotolerant, *Bacillus*, ornithine

INTRODUCTION

Six psychrotolerant or psychrophilic *Bacillus* species have been described to date: *Bacillus globisporus* (Larkin & Stokes, 1967; Rüger & Richter, 1979; Nakamura, 1984), *Bacillus insolitus* (Larkin & Stokes, 1967), *Bacillus macquariensis* (now *Paenibacillus macquariensis*) (Ash *et al.*, 1993; Marshall & Ohye, 1966), *Bacillus marinus* (Rüger, 1983; Rüger *et al.*, 2000), *Bacillus psychrophilus* (Rüger & Richter, 1979; Larkin & Stokes, 1967; Nakamura, 1984) and *Bacillus*

psychrosaccharolyticus (Larkin & Stokes, 1967; Priest *et al.*, 1988).

To date, the taxonomy of psychrotolerant and psychrophilic *Bacillus* strains is somewhat uncertain as it is based on only a few strains and a few discriminating properties. However, studies on the 16S rRNA sequences as well as DNA–DNA hybridizations, including the type strains of these species (Stackebrandt *et al.*, 1987; Ash *et al.*, 1991; Fox *et al.*, 1992; Farrow *et al.*, 1994), confirmed the individual species position of *Bacillus globisporus*, *Bacillus insolitus*, *Bacillus macquariensis* (now *Paenibacillus macquariensis*), *Bacillus psychrophilus* and *Bacillus psychrosaccharolyticus*. Rüger *et al.* (2000) have provided an emended description of *Bacillus marinus*. *Bacillus marinus* has recently been reclassified as *Marinibacillus marinus* (Yoon *et al.*, 2001b), and *Bacillus globisporus*, *Bacillus psychrophilus* and *Bacillus pasteurii* have been transferred to *Sporosarcina* (Yoon *et al.*, 2001a).

In a previous study (Abd El-Rahman, 1988) a large number of psychrotolerant *Bacillus* isolates were sub-

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Abbreviation: CASO agar, casein-peptone soymeal-peptone agar.

The EMBL accession numbers for the 16S rDNA gene sequences of strains 3H1^T (DSM 11706^T) and 68E3^T (DSM 11713^T) are AJ277983 and AJ277984, respectively.

jected to various tests to reveal their morphological, physiological, biochemical and genetic properties. Fourteen reference strains of the species *Bacillus globisporus*, *Bacillus insolitus*, *Bacillus psychrophilus*, *Bacillus psychrosaccharolyticus* and *Paenibacillus macquariensis* were included in the study for comparison. By arrangement of the strains according to cell wall type, G+C content, and biochemical and physiological properties, distinct subgroups were established. Of these, group I A was representative for the species *Bacillus insolitus*, group II B for *Bacillus psychrosaccharolyticus*, group II F for *Bacillus macquariensis*, group III B for *Bacillus globisporus* and group III C for *Bacillus psychrophilus*. About a third of the psychrotolerant isolates could not be allocated to any of these groups, nor to any other species of the genus *Bacillus* or related aerobic spore-forming genera. They formed a number of separate clusters which obviously warrant separate species status.

For the present investigation, the 11 strains of group I (subgroup I A, including the type strain of *Bacillus insolitus*, DSM 5^T, and *Bacillus insolitus* DSM 2272; subgroup I B consisting of isolates 3H1^T, 71H1, 84E1, 87H2 and 4H2; and subgroup I C consisting of isolates 68E3^T, 61E1, 4E3 and 67E1) were subjected to a number of studies to reveal their taxonomic position.

METHODS

Bacterial strains. The *Bacillus* strains used in this study are listed in Table 1. The new isolates were obtained from samples collected in Egypt and Germany in 1985.

Phenotypic characterization. Phenotypic characterization was carried out according to Gordon *et al.* (1973) with the modification that casein-peptone soymeal-peptone agar (CASO agar; Merck 5458) was used instead of nutrient agar as standard.

Photography. For photomicrographs the cultures were prepared as described by Claus & Berkeley (1986). Photo-

micrographs were taken from cultures grown on CASO agar or Difco marine agar (Difco marine broth 2216 plus agar).

Chemotaxonomic characterization. Composition and structure of peptidoglycan was determined according to Schleifer & Kandler (1972) and Kandler & Weiss (1986).

DNA base composition and DNA-DNA hybridization. Determination of base composition was performed as described by Fritze *et al.* (1990) based on the methods reported by De Ley *et al.* (1970) and Gillis *et al.* (1970). DNA hybridizations were performed as described by Spanka & Fritze (1993).

16S rDNA sequence determination and analyses. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced by using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) as directed in the manufacturer's protocol. An Applied Biosystems 373A DNA sequencer was used for the electrophoresis of sequence reaction products. The ae2 editor (Maidak *et al.*, 1999) was used to align the 16S rDNA sequences determined in this study against the 16S rDNA sequences of representatives of the main bacterial lineages available from the public databases. Pairwise evolutionary distances were computed by using the correction of Jukes & Cantor (1969).

RESULTS

Cell walls

The diamino acid in position 3 of the peptide side chain of the peptidoglycan of all 11 strains had previously been determined to be ornithine. Accordingly, the strains had been grouped into cell wall group I (Abd El-Rahman, 1988). When these strains (including the type strain of *Bacillus insolitus*, DSM 5^T) were further analysed for the structure of their peptidoglycan, it was revealed that the peptide side chains were connected through an interpeptide bridge formed by glutamic acid, resulting in an L-Orn-D-Glu cell wall type (A4 β type, according to Schleifer & Kandler, 1972).

Table 1. List of strains investigated in this study

Name	G + C (mol %)	Physiological group	Source
<i>B. insolitus</i> DSM 5 ^T	35.8	I A	DSMZ (Larkin & Stokes, 1967)
<i>B. insolitus</i> DSM 2272	35.7	I A	DSMZ (Larkin & Stokes, 1967)
87H2	36.0	I B	Water, brook, Lutter, Göttingen, Germany
84E1	36.3	I B	Water, Weende, Göttingen, Germany
3H1 ^T (DSM 11706 ^T)	36.5	I B	Soil, field, Weende, Göttingen, Germany
4H2	36.6	I B	Soil, botanical garden, Göttingen, Germany
71H1	38.4	I B	Soil, field, Kafr-Hamsa, Kalyobia, Egypt
61E1	35.8	I C	Soil, field, Faculty of Agriculture, Shobra Khaima, Cairo, Egypt
4E3	36.3	I C	Soil, botanical garden, Göttingen, Germany
68E3 ^T (DSM 11713 ^T)	36.5	I C	Soil, garden, El-Kanater, Egypt
67E1	36.3	I C	Soil, field, Shobra Khaima, Cairo, Egypt

Table 2. DNA–DNA hybridization of psychrotolerant *Bacillus* strains of subgroups I A, I B and I C

Each value is the mean of at least two determinations.

Subgroup	Strain	Percentage DNA–DNA hybridization with:		
		DSM 5 ^T	87H2	68E3 ^T (DSM 11713 ^T)
I A	DSM 5 ^T	100	–	–
I A	DSM 2272	89	–	–
I B	87H2	44	100	–
I B	84E1	35	96	–
I B	3H1 ^T (DSM 11706 ^T)	42	100	44
I B	4H2	44	100	–
I B	71H1	48	71	–
I C	68E3 ^T (DSM 11713 ^T)	53	–	100
I C	4E3	10	–	76
I C	61E1	55	23	69
I C	67E1	57	53	86

DNA–DNA hybridization

The DNA of the 11 strains of group I were determined previously to have a G + C content of 35.7–38.4 mol% (Abd El-Rahman, 1988). When the DNAs of these strains were subjected to hybridization studies, three subgroups were found matching the previously established physiological groups I A, I B and I C. Within each group, hybridization values were higher than 70% (69% in one case) and between the groups hybridization values were below 60% (see Table 2). Group I A consisted of the type strain of *Bacillus insolitus* (DSM 5^T) and one further strain of this species, DSM 2272. Group I B consisted of the isolates 3H1^T, 71H1, 84E1, 87H2 and 4H2. Group I C contained the isolates 68E3^T, 61E1, 4E3 and 67E1.

16S rDNA sequence analysis

Of the strains investigated, six were selected for partial 16S rRNA gene sequence determination. Full sequences were determined for strains 3H1^T (DSM 11706^T) and 68E3^T (DSM 11713^T). Comparison of the sequences of these strains with reference sequences indicated that both species are members of rRNA group 2 (Ash *et al.*, 1991) in which they form a tight cluster (data not shown). The binary sequence similarity value is 99.8%. The closest relative within this cluster is represented by *Bacillus insolitus* showing a similarity value of 97.8 or 98.0%. All other species of *Bacillus* rRNA group 2 are more distantly related, having similarity values < 95%.

Phenotypic characterization

Nutrient agar or nutrient broth did not support or only weakly supported growth of the psychrotolerant strains. Therefore, all tests were performed on CASO

Fig. 1. (a) *Bacillus psychrotolerans* strain 3H1^T (= DSM 11706^T) grown for 3 days on CASO agar (Merck 5458) plus Mn²⁺ at 26 °C. (b) *Bacillus psychrodurans* strain 68E3^T (= DSM 11713^T) grown for 6 days on CASO agar (Merck 5458) plus Mn²⁺ at 22 °C. (c) *Bacillus psychrodurans* strain 68E3^T (= DSM 11713^T) grown for 3 days on marine agar (Difco 2216) at 20 °C. Bars, 10 µm.

Table 3. Phenotypic characters distinguishing between established groups

+, Positive; —, negative; ±, weak; v, variable among strains.

Character	Group IA <i>B. insolitus</i>	Group IB <i>B. psychrotolerans</i>	Group IC <i>B. psychrodurans</i>	<i>B. marinus</i> *	<i>B. globisporus</i>	<i>P. macquariensis</i>	<i>B. psychrophilus</i>	<i>B. psychrosaccharolyticus</i>
Sporangium swollen	—	+	+	—	+	+	+	+
Reduction of nitrate	—	—	+	v	—	—	+	+
Hydrolysis of starch	—	+	+	—	—	+	—	+
Hydrolysis of DNA	—	+	+	v	+	—	+	+
Anaerobic growth with KNO ₃ †	—	—	+	—	±	+	—	—
Growth at 3% NaCl	—	+	+	+	+	—	+	+
Growth at 5% NaCl	—	— (1+)	+	v	—	—	+	—
Cell wall	L-Orn-D-Glu‡	L-Orn-D-Glu	L-Orn-D-Glu	L-Lys-direct	L-Lys-D-Glu‡	DAP	L-Lys-D-Glu‡	DAP
rRNA group	2§	2	2	2	2§	3§	2§	1§

* Data for *B. marinus* from Rüger *et al.* (2000).† CASO medium with 0.1% KNO₃.‡ Data for cell wall composition from Stackebrandt *et al.* (1987).§ rRNA group allocation from Ash *et al.* (1991).

agar or in CASO broth (for further details, see Abd El-Rahman, 1988).

Spore formation could be induced only for strains DSM 2272, 68E3^T, 3H1^T, 71H1, 84E1 and 87H2. Round spores were observed with strains DSM 2272, 68E3^T, 3H1^T, 71H1 and 87H2. Strain 3H1^T also showed ellipsoid spores and strain 84E1 showed only ellipsoid spores. When strain 68E3^T was grown on Difco marine agar (instead of CASO agar) to enhance spore formation, mainly ellipsoid spores could be observed (Fig. 1). The spores of DSM 2272 did not distend the cell, whereas the spores of strains 3H1^T, 71H1, 87H2, 84E1 and 68E3^T did distend the cell.

The lower temperature limit for growth was tested down to –5 °C. For five strains (DSM 5^T, DSM 2272, 4H2, 3H1^T and 4E3) growth was still observed at –2 °C. Four strains (84E1, 61E1, 68E3^T and 67E1) were able to grow at 0 °C and two strains (71H1 and 87H2) had their lower growth limit at +1 °C.

The upper temperature limit for growth was determined to be 25 °C for one strain (DSM 5^T), 30 °C for five strains (DSM 2272, 3H1^T, 68E3^T, 87H2 and 61E1), 35 °C for four strains (67E1, 71H1, 4H2 and 4E3) and 40 °C for one strain (84E1).

Positive reactions for all tested strains (unless indicated otherwise) were found for catalase, oxidase (DSM 5^T negative), growth at +1 °C and hydrolysis of Tween 20, 40 and 60.

Negative reactions for all tested strains (unless indicated otherwise) were found for KOH (slightly positive for strains 61E1, 67E1 and 4H2) and aminopeptidase, growth at 40 °C (84E1 positive), growth in nutrient broth or on nutrient agar (or only very weak and variable growth), growth at pH 5.7, growth in the presence of 7% NaCl, utilization of citrate, hydrolysis of cellulose, hydrolysis of pullulan (71H1 positive), hydrolysis of pectin, hydrolysis of chitin, hydrolysis of aesculin in CASO medium, degradation of casein, acid

production from arabinose and xylose, the egg yolk lecithinase reaction, production of indole, production of dihydroxyacetone, Voges–Proskauer reaction (pH in Voges–Proskauer between 6.2 and 6.8), deamination of phenylalanine, tyrosine degradation, growth in the presence of lysozyme, cleavage of urea and hydrolysis of 4-methyl-umbelliferone glucuronide.

Characters variable within the established groups I A, I B and I C were hydrolysis of gelatin (negative for strains DSM 5^T, DSM 2272, 3H1^T, 4H2, 61E1), hydrolysis of Tween 80 (negative for strains 84E1, 71H1 and 68E3^T), acid from glucose (negative for strains DSM 5^T, DSM 2272 and 4H2, weakly positive for strains 4E3, 61E1, 3H1^T, 71H1, 84E1), production of acid from mannitol (weakly positive for strains 4E3, 61E1, 67E1, 68E3^T, 3H1^T and 84E1) and growth in the presence of 5% NaCl (positive for strains 84E1, 4E3, 68E3^T and 67E1).

Phenotypic characters varying between the established groups and helping to distinguish between them included the shape of the sporangium, reduction of nitrate, hydrolysis of starch and DNA, anaerobic growth with KNO₃ and growth in the presence of 3% NaCl (see Table 3).

DISCUSSION

In a previous evaluation of psychrotolerant *Bacillus* species and additional psychrotolerant isolates (Abd El-Rahman, 1988), high phenotypic similarity was found between all of these strains. These findings were in accordance with the original descriptions of the hitherto known psychrotolerant *Bacillus* species. Differentiation at the phenotypic level is especially complicated because of the physiological inactivity of these organisms (Rüger *et al.*, 2000).

Only through the application of a large range of physiological tests has it been possible to establish groups of organisms. A few characters have been

found to help distinguish among the newly established groups of psychrotolerant *Bacillus* isolates and between those and the known psychrotolerant *Bacillus* species. A detailed comparison of phenotypes of groups of psychrotolerant *Bacillus* is given in Abd El-Rahman (1988).

Group I, as defined by Abd El-Rahman (1988), was further evaluated in the present study. Group I A refers to *Bacillus insolitus*; groups I B and I C refer to two related groups of strains. Characteristic for all strains of group I are their unusual cell walls (L-Orn-D-Glu) which distinguish them from all other hitherto validly published aerobic spore-forming species (*Bacillus* spp. and relatives).

Physiological and morphological characters that suggested separation of the three groups from each other were the shape of the sporangium, reduction of nitrate, hydrolysis of starch and DNA, anaerobic growth with KNO₃ and growth in the presence of 3% NaCl.

The additional application of DNA–DNA hybridizations for the 11 strains of cell wall group I confirmed the separation of the three groups.

Full 16S rDNA gene sequence analysis of one representative strain of each of groups I B and I C suggested allocation of the organisms to *Bacillus* rRNA group 2 as established by Ash *et al.* (1991) and further underlined the novelty of groups I B and I C.

Obviously, *Bacillus insolitus* and its two neighbouring groups have to be considered as rare species, as, out of a set of 52 isolates and 14 reference strains, only two strains were determined to belong to the species *Bacillus insolitus*, and five and four strains, respectively, formed the two adjacent groups.

Following the results of the present study it is suggested that the strains of groups I B and I C be recognized as new psychrotolerant species of the genus *Bacillus* for which the names *Bacillus psychrotolerans* sp. nov. and *Bacillus psychrodurans* sp. nov. are proposed, respectively.

The two new species formed by groups I B and I C are easily distinguishable from *Bacillus insolitus* (group I A) and other psychrophilic aerobic spore-forming organisms by a number of characters (see Table 3). The two new species may be distinguished physiologically from each other by their abilities to reduce nitrate, to grow in the presence of 5% NaCl and to grow anaerobically in CASO medium with KNO₃.

The fact that the genus *Bacillus* is a large heterogeneous group in need of taxonomic rearrangement has been discussed extensively over the past decades. Recent work has led to the description of more than 20 genera of aerobic endospore-forming bacteria.

It is tempting to follow the trend and designate a new genus for the two new species described in the present

study. However, although the cell wall seems to differentiate both species strongly enough from other similar organisms, such single-parameter-based taxa are prone to be unstable and we therefore consider the description of a separate genus as premature. In future studies these two species, together with other organisms of *Bacillus* rRNA group 2, need to be subjected to a comprehensive polyphasic study to reveal 'good' borderlines between existing genera and potential new ones.

Species descriptions are given below for the strains of groups I B (*Bacillus psychrotolerans* sp. nov.) and I C (*Bacillus psychrodurans* sp. nov.).

Description of *Bacillus psychrotolerans* sp. nov.

Bacillus psychrotolerans (psy.chro.to'l'er.ans. Gr. adj. *psychros* cold; L. pres. part. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* cold-tolerating).

Cells are Gram-positive (according to KOH and aminopeptidase tests). Cell width, 0.4–1 µm; cell length, 2–7 µm. Spore formation rare. Spores predominantly round on CASO agar containing Mn²⁺, located terminally (one of the strains tested did not sporulate). Sporangium swollen. Peptidoglycan type L-Orn-D-Glu (A4β). The diamino acid in position 3 of the peptidoglycan side chain is ornithine, and glutamic acid forms an interpeptide bridge. Chemo-organotrophic. Does not grow (or only very poorly) in/on nutrient broth or nutrient agar. Good growth in/on CASO broth or agar. Catalase-positive. Oxidase-positive. No anaerobic growth with or without KNO₃. Psychrotolerant. Lower temperature limit for growth: –2 °C (type strain) up to 1 °C. Upper temperature limit for growth: 30 °C (type strain) up to 40 °C. No growth in the presence of lysozyme. No growth at pH 5.7. Growth at 3% NaCl; normally no growth at 5% NaCl (one of the five strains tested positive); no growth at 7% NaCl. No tyrosine degradation. DNA degradation positive. No or only weak acid production from D-glucose, L-arabinose, D-xylose and D-mannitol. Hydrolysis of starch. Usually no hydrolysis of pullulan (one of the strains tested positive). No hydrolysis of cellulose. No hydrolysis of pectin. Usually no hydrolysis of gelatin (one of the strains tested positive). No hydrolysis of casein. No hydrolysis of chitin. No hydrolysis of aesculin. Hydrolysis of Tween 20, 40, 60 and usually also of Tween 80 (two of the strains tested negative). No hydrolysis of urea. No hydrolysis of 4-methyl-umbelliferone glucuronide. No citrate hydrolysis. Nitrate not reduced to nitrite. Egg-yolk lecithinase-negative. Phenylalanine not deaminated. Voges–Proskauer reaction negative. pH in Voges–Proskauer > 6. Indole-negative. No production of dihydroxyacetone. Type strain is 3H1^T (= DSM 11706^T = NCIMB 13838^T). Isolated from field soil, Göttingen-Weende, Germany. G + C content of the DNA is 36–38% (Bd) with 36.5% for the type strain.

Description of *Bacillus psychrodurans* sp. nov.

Bacillus psychrodurans (psy.chro.dur'ans. Gr. adj. *psychros* cold; L. pres. part. *durans* enduring; N.L. part. adj. *psychrodurans* cold-enduring).

Cells are Gram-positive (according to KOH and aminopeptidase test). Cell width, 0.5–0.6 µm; cell length, 2–5 µm. Spore formation rare. Spores predominantly round on CASO agar and predominantly ellipsoid on Difco marine agar; located terminally (spores observed only with type strain). Sporangium swollen. Peptidoglycan type L-Orn-D-Glu (A4β). The diamino acid in position 3 of the peptidoglycan side chain is ornithine, and glutamic acid forms an interpeptide bridge. Chemo-organotrophic. Does not grow (or only very poorly) in/on nutrient broth or nutrient agar. Good growth in/on CASO broth or agar. Catalase-positive. Oxidase-positive. No anaerobic growth. With KNO₃ anaerobic growth positive. Psychrotolerant. Lower temperature limit for growth: –2 °C up to 0 °C (type strain). Upper temperature limit for growth: 30 °C (type strain) up to 35 °C. No growth in the presence of lysozyme. No growth at pH 5–7. Growth at 3 % NaCl and usually at 5 % NaCl positive (with 5 % NaCl one of the four strains tested negative); no growth at 7 % NaCl. No tyrosine degradation. DNA degradation positive. Acid production from D-glucose, L-arabinose, D-xylose and D-mannitol usually negative or only weak (two of the four strains tested, including the type strain, positive with D-glucose and D-mannitol). Hydrolysis of starch. No hydrolysis of pullulan. No hydrolysis of cellulose. No hydrolysis of pectin. Usually hydrolysis of gelatin (one of the four strains tested negative). No hydrolysis of casein. No hydrolysis of chitin. No hydrolysis of aesculin. Hydrolysis of Tween 20, 40, 60 and usually of Tween 80 positive (with Tween 80 type strain negative). No hydrolysis of urea. No hydrolysis of 4-methylumbelliferone glucuronide. No citrate hydrolysis. Nitrate is reduced to nitrite. Egg-yolk lecithinase-negative. Phenylalanine not deaminated. Voges-Proskauer reaction negative. pH in Voges-Proskauer > 6. Indole-negative. No production of dihydroxyacetone. Type strain is 68E3^T (= DSM 11713^T = NCIMB 13837^T). Isolated from garden soil, El-Kanater, Egypt. G+C content of the DNA is 36–37 mol % (Bd) with 36.3 % for the type strain.

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