Description of *Bacillus thermoaerophilus* sp. nov., To Include Sugar Beet Isolates and *Bacillus brevis* ATCC 12990†

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Isolates of thermophilic bacteria obtained from an Austrian beet sugar factory were screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and freeze-fracture electron microscopy for the presence of glycosylated crystalline cell surface layers (S-layers). On the basis of similarities in the protein band patterns on SDS-PAGE gels and the lattice geometry of the S-layers as revealed by electron micrographs, the 31 isolates which we studied were clustered into five groups (groups I to V) and several strains which exhibited no common characteristics (group 0). We found that the organisms belonging to groups I to III had glycosylated S-layer proteins, but the highest carbohydrate contents were observed in group III organisms. Partial sequencing of the 16S ribosomal DNAs of selected representative strains of each group revealed that the group I, II, IV, and V isolates and the few group 0 strains were different from the group III strains. The results of DNA-DNA hybridization experiments, SDS-PAGE, and an analysis of polar lipids demonstrated that group III isolates L419-91, L420-91^T (T = type strain), and L438-91 belong to the same species. We chose the group III organism *Bacillus* sp. strain L420-91^T for further analysis because of the high carbohydrate content of its S-layer protein. The taxonomic position of this isolate was determined by using a polyphasic approach. Phenotypic, chemotaxonomic, and genomic analyses revealed that strains L420-91^T, L419-91, and L438-91 represent a new *Bacillus* species. We observed high levels of similarity between these strains and *Bacillus brevis* ATCC 12990, which also had a glycosylated S-layer protein. Our results show that strains L420-91^T, L419-91, and L438-91 and *B. brevis* ATCC 12990 belong to the same species and that this species is a new *Bacillus* species, which we name *Bacillus thermoaerophilus*. The type strain of this species is strain L420-91 (= DSM 10154).

For many years the extraction plants of beet sugar factories have been recognized as sources for isolation of thermophilic bacterial strains (11, 15). Freeze-etched preparations have revealed that most of these isolates, which belong mainly to the species *Bacillus stearothermophilus*, *Thermoanaerobacter thermohydrosulfuricus* (*Clostridium thermohydrosulfuricum*), *Thermoanaerobacterium thermosaccharolyticum* (*Clostridium thermosaccharolyticum*), and *Desulfotomaculum nigrificans*, have a crystalline cell surface layer (S-layer) (for reviews see references 3, 29, 38, 39, and 40) that is composed of identical protein or glycoprotein species (16, 26, 27, 37). However, workers have observed remarkable strain-specific differences in the S-layer lattice type (oblique, p2; square, p4; hexagonal, p6 or p3 symmetry) and lattice dimensions, the molecular weight of the S-layer monomers, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the degree and type of glycosylation of the S-layer proteins (for reviews see references 26, 28, and 29).

In a systematic survey in which the goal was identification of glycosylated S-layer proteins in bacteria, we investigated 31 isolates which had been isolated during the 1991 and 1992 beet

sugar campaign from the extraction plant at the beet sugar factory in Leopoldsdorf, Austria. A preliminary characterization of these bacteria based on cell shape, spore formation, and several phenotypic features indicated that they are thermophilic organisms that belong to the genus *Bacillus*. These isolates were further characterized by electron microscopy, including freeze-etching and thin sectioning of whole cells, and SDS-PAGE of SDS extracts of the biomass (31). On the basis of the results of these studies, the isolates that produced similar band patterns on SDS gels and had similar lattice parameters on freeze-fracture micrographs were clustered into five groups. Several strains (group 0) had completely different parameters. Periodic acid-Schiff staining of SDS gels of whole bacteria confirmed that the group I to III isolates possessed glycosylated S-layer proteins. Since group III isolate L420-91^T (T = type strain) exhibited the strongest carbohydrate staining reaction, it was chosen for further analysis.

The results of a taxonomic precharacterization study (24) indicated that isolate L420-91^T is related to the recently reinvestigated species *Bacillus aneurinolyticus* (36). In order to determine the taxonomic affiliation of strain L420-91^T, this organism was compared with other isolates obtained from the 1991 to 1992 beet sugar campaign and reference strains, including *Bacillus brevis* ATCC 12990 (a thermophilic *B. brevis* strain [12] with a glycosylated S-layer protein). In this study high levels of similarity were observed between isolates L420-91^T, L419-91, L438-91, and *B. brevis* ATCC 12990. In this

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† Dedicated to Ruth E. Gordon.

TABLE 1. Bacterial strains used in this study

Strain	Source ^a
L400-91	New isolate
L407-91	New isolate
L415-91	New isolate
L417-91	New isolate
L418-91	New isolate
L419-91	New isolate
L420-91 ^T	New isolate
L426-91	New isolate
L435-91	New isolate
L438-91	New isolate
<i>Bacillus brevis</i> ATCC 12990	F. Hollaus from R. E. Gordon ^b
<i>Bacillus aneurinolyticus</i> DSM 5562 ^T	DSM
<i>Bacillus aneurinolyticus</i> NCIMB 10056	NCIMB
<i>Bacillus migulanus</i> DSM 2895 ^T	DSM
<i>Bacillus subtilis</i> CCM 2216 ^T	CCM
<i>Bacillus coagulans</i> DSM 1 ^T	DSM
<i>Bacillus methanolicus</i> NCIMB 13113 ^T	NCIMB
<i>Bacillus smithii</i> DSM 4216 ^T	DSM
<i>Bacillus sphaericus</i> DSM 28 ^T	DSM
<i>Paenibacillus (Bacillus) alvei</i> CCM 2051 ^T	CCM
<i>Paenibacillus (Bacillus) polymyxa</i> DSM 36 ^T	DSM
<i>Bacillus brevis</i> DSM 30 ^T	DSM
<i>Bacillus laterosporus</i> DSM 25 ^T	DSM
<i>Bacillus stearothermophilus</i> DSM 22 ^T	DSM
<i>Bacillus kaustophilus</i> NCIMB 8547 ^T	NCIMB
<i>Bacillus thermoglucosidasius</i> DSM 2542 ^T	DSM
<i>Saccharococcus thermophilus</i> ATCC 43125 ^T	ATCC
<i>Bacillus therronuber</i> DSM 7064 ^T	DSM
<i>Bacillus thiaminolyticus</i> DSM 5713	DSM
<i>Bacillus thiaminolyticus</i> DSM 5748	DSM

^a The new isolates were enriched from extraction juices obtained from the beet sugar factory in Leopoldsdorf, Austria. ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen, United Kingdom; CCM, Czechoslovak Collection of Microorganisms, Masaryk University, Brno, Czech Republic.

^b See reference 12.

paper we describe these strains as members of a new thermophilic species of the genus *Bacillus*.

MATERIALS AND METHODS

Isolation and bacterial strains. The strains which we used were isolated by incubating smears of sugar beet extraction juice at approximately 60°C under aerobic conditions on TYG agar (0.5% Bacto Tryptone [Difco Laboratories, Detroit, Mich.], 0.25% Bacto Yeast extract [Difco], 0.1% glucose, 2.2% Bacto Agar [Difco]). Single colonies were plated onto TYG agar, and the procedure was repeated until pure cultures were obtained.

The new isolates and reference strains used in this study are listed in Table 1. Working stocks were cultivated on nutrient broth (10) at 30 or 50°C and were stored at 4°C on nutrient agar slopes.

Morphology. Light microscopy was performed with a Polyvar light microscope (Reichert-Jung, Vienna, Austria). We used previously described methods to prepare organisms for freeze-fracturing and thin sectioning (27). Micrographs were taken with a model CM100 electron microscope (Philips, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV.

Phenotypic characterization. Unless indicated otherwise, the methods of Gordon et al. (12) were used to phenotypically characterize the strains investigated (see Table 3). All assays were performed in duplicate and repeated when inconsistent results were obtained. The carbohydrate fermentation tests included tests performed with the following compounds: glucose, sucrose, fructose, D-arabinose, L-arabinose, trehalose, xylose, mannitol, glycerol, galactose, maltose, and raffinose. Tween 80 hydrolysis and urease activity were determined as described by Lányi (23). For the urease test we used peptone-free Bacto Urea broth (catalog no. 0272-01-0; Difco). Thiamine decomposition was detected as described by Abe et al. (1). *Bacillus thiaminolyticus* DSM 5713 and DSM 5748 were used for control experiments. Hydrolysis of DNA was determined by using Bacto DNase test agar (Difco).

Electrophoresis of whole-cell proteins. After a 20-mg (wet weight) portion of biomass was boiled for 2 min in an SDS solution, it was analyzed by performing

SDS-PAGE as described by Laemmli (22) with 8 to 20% gradient gels as described by Podszus (32). The gel preparation procedure which we used was adapted for the Mini-Protein II electrophoresis apparatus (Bio-Rad, Hercules, Calif.). The gels were stained for proteins with Coomassie brilliant blue R350, and carbohydrates were detected after the gels were blotted onto nitrocellulose membranes as described previously (5). Densitometric scanning of the gels for molecular weight determinations was performed with a model Elscipit 400-AT densitometer (Hirschmann, Munich, Germany).

Polar lipid analysis. Lyophilized biomass was extracted with chloroform-methanol-0.3% sodium chloride (1:2:0.8) as described by Bligh and Dyer (4) and analyzed by one- and two-dimensional thin-layer chromatography (TLC) as described previously (30). Phosphate groups were detected with the molybdenum blue reagent, amino groups were detected with ninhydrin, glycolipids were detected with α -naphthol, and quaternary ammonium compounds were detected with the Dragendorff spray reagent.

Polyamine analysis. The growth temperatures on PYE medium (0.3% peptone from casein, 0.3% yeast extract; pH 7.2) that we used were 55°C for L420-91^T and *B. brevis* ATCC 12990 and 30°C for all other test strains. Extraction of polyamines and detection of polyamine patterns were performed as described previously (6). The high-performance liquid chromatography (HPLC) apparatus (Waters) that we used was equipped with two Waters model 510 pumps, a model U6K injector, and a Jasco model 821-FP spectrofluorimetric detector.

Cellular fatty acid and quinone compositions. Biomass grown on 3% Trypticase soy broth-1.5% agar medium was treated with sodium hydroxide and methanol for 30 min at 100°C in order to saponify the lipid material. Sodium salts of the free cellular fatty acids were converted to their methyl esters by heating the preparations for 10 min at 80°C with methanol and hydrochloric acid and were then extracted with *n*-hexane and *tert*-butylethylether. The extracts were analyzed by gas-liquid chromatography with a Hewlett-Packard model 5890 Series II chromatograph by using phenylmethyl silicone as the stationary phase, and the components were identified by flame ionization detection.

A quinone analysis was carried out as described by Tindall (44). After hexane-methanol (1:2, vol/vol) treatment of 100 mg of lyophilized biomass, the combined and concentrated hexane phases were analyzed by reversed-phase HPLC on an RP18 column (5 μ m; 250 by 4.6 mm; Shandon) by using methanol-1-chlorobutane (20:1, vol/vol) as the mobile phase and detection at 269 nm.

16S rDNA sequence analysis. Genomic DNA extraction, PCR-mediated amplification of the 16S ribosomal DNA (rDNA), and purification of PCR products were carried out as described previously (33, 35). Purified PCR products were sequenced by using a *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany) as directed by the manufacturer. Sequence reaction mixtures were electrophoresed by using an Applied Biosystems model 373A DNA sequencer. The 16S rDNA sequences were aligned manually with the sequences of representatives of the genus *Bacillus* and related taxa.

Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (19). The least-squares distance method of DeSoete (9) was used to construct a phylogenetic dendrogram from distance matrices.

DNA-DNA hybridization method. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion et al. (7). DNA-DNA hybridization was carried out as described by De Ley et al. (8), with the modifications described by Huss et al. (17), by using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (18).

Determination of G+C content of DNA. DNA was isolated as described by Cashion et al. (7). The G+C content of the DNA was determined by HPLC as described by Mesbah et al. (25).

Nucleotide sequence accession numbers. The 16S rDNA sequences of *B. aneurinolyticus* DSM 5562^T, *Bacillus migulanus* DSM 2895^T, isolate L420-91^T, and *B. brevis* ATCC 12990 have been deposited in the EMBL data library under accession numbers X94194, X94195, X94196, and X94197, respectively.

RESULTS

Isolation and precharacterization. During the 1991-1992 beet sugar campaign five different extraction juice samples were collected in the Leopoldsdorf beet sugar factory on different days to monitor the bacterial population of the extraction plant. Cultures were enriched in petri dishes on TYG medium at 60°C and assigned isolation numbers (Tables 1 and 2).

During our systematic survey for glycosylated S-layer proteins, the 31 isolates studied were clustered into groups I to V and group 0 on the basis of the presence of S-layers, the degree of glycosylation of the S-layer protein, the apparent molecular weight of the S-layer promoter as determined by SDS-PAGE, and the S-layer lattice type (31). An analysis of the five different extraction juice samples showed that in each sample there was a specific distribution of isolates that was not present in

TABLE 2. Comparison of selected sugar beet isolates used in partial 16S rDNA sequencing experiments

Isolate	Extraction juice sample	Group to which isolate was assigned ^a	S-layer		16S rDNA sequence determined	Closest relative ^d
			Occurrence ^b	Glycosylation ^c		
L419-91	V26A-19	III	+	+	Partial	L420-91 ^T
L420-91 ^T	V26A-19	III	+	++	Full	
L438-91	V26A-19	III	+	+	Partial	L420-91 ^T
L415-91	V26A-19	IV	—	NI ^e	Partial	<i>Bacillus licheniformis</i>
L418-91	V26A-19	IV	—	NI	NA ^f	
L417-91	V26A-19	0	—	NI	Partial	<i>B. subtilis</i>
L435-91	V26A-19	0	—	NI	Partial	<i>B. subtilis</i>
L407-91	V24-4	I	+	+	Partial	<i>B. stearothermophilus</i>
L400-91	V19A-2	II	+	+	Partial	<i>B. smithii</i>
L426-91	V32A-4	V	—	NI	Partial	NF ^g

^a Determined in the precharacterization study of Neuninger (31).^b +, S-layer is present; —, no S-layers are observed in freeze-fracture and ultrathin sectioning experiments (31).^c +, weak glycosylation; ++, strong glycosylation.^d Based on a partial 16S rDNA sequence comparison.^e NI, not investigated.^f NA, not analyzed.^g NF, not found.

any other sample (Table 2). Isolate L420-91^T, which was chosen in the precharacterization study (31) because of the high carbohydrate content of its S-layer protein, was found in only one sample (sample V26A-19). Table 2 shows the isolates obtained from sample V26A-19 and the strains which were selected for the comparative studies and the partial-sequence experiments.

The SDS-PAGE patterns of the SDS-soluble cellular proteins are shown in Fig. 1. The protein patterns of the different groups exhibited considerable heterogeneity. On the other

hand, the patterns of the group III strains were almost identical. Unfortunately, with the exception of the S-layers, most of the cellular proteins of the group III organisms did not stain very well with Coomassie brilliant blue. Such different staining

FIG. 1. SDS-PAGE gradient gel of whole-cell proteins of the isolates listed in Table 2. The positions of the S-layer proteins are indicated by arrowheads. Lanes M, marker proteins; lane 1, L407-91; lane 2, L400-91; lane 3, L419-91; lane 4, L420-91^T; lane 5, L438-91; lane 6, L415-91; lane 7, L418-91; lane 8, L417-91; lane 9, L435-91; lane 10, L426-91; lane 11, *B. brevis* ATCC 12990. For details see Table 2.

FIG. 2. One-dimensional TLC analysis of polar lipids of the isolates listed in Table 2. The lipids were stained with molybdenum blue. Lane 1, L407-91; lane 2, L400-91; lane 3, L419-91; lane 4, L420-91^T; lane 5, L438-91; lane 6, L415-91; lane 7, L418-91; lane 8, L417-91; lane 9, L426-91. For details see Table 2.

TABLE 3. Morphological characteristics of *Bacillus* strains

Strain	Cell length (μm) ^a	Cell diam (μm) ^a	Spore position	Flagella	S-layer lattice type	Center-to-center spacing (nm)	Molecular mass of S-layer protomer (kDa)	Glycosylated S-layer proteins
L420-91 ^T	4.5 $\pm$ 0.8	1.13 $\pm$ 0.07	Central	Peritrichous	Square (p4)	10.0	116	+
<i>B. brevis</i> ATCC 12990	4.8 $\pm$ 0.6	1.11 $\pm$ 0.07	Central	Peritrichous	Square (p4)	10.0	153	+
<i>B. aneurinolyticus</i> DSM 5562 ^T	3.0–5.0 ^b	0.7–0.9 ^b	Terminal ^b	Peritrichous ^b	Square (p4)	10.1	100	–
<i>B. migulanus</i> DSM 2895 ^T	2–6 ^c	0.5–1.0 ^c	ND ^d	Peritrichous ^c	Square (p4)	9.9	100	–

^a Based on 20 independent measurements.^b Data from reference 36.^c Data from reference 43.^d ND, not determined.

behaviors of whole-cell extracts, as well as differences in the apparent molecular weights of the S-layer protomers of even closely related strains, have been observed previously with other S-layer-containing bacteria (27). The results of SDS-PAGE (Fig. 1) and a one-dimensional polar lipid analysis (Fig. 2) of isolates L400-91, L407-91, L415-91, L417-91, L418-91, L419-91, L420-91^T, L426-91, L435-91, and L438-91 were confirmed by the results of the partial 16S rDNA sequencing experiments (Table 2). The sequence comparison showed that strain L438-91 exhibited the highest levels of similarity to strains L419-91 and L420-91^T. Strain L415-91 (group IV) exhibited the highest level of sequence similarity to *Bacillus licheniformis*, whereas the sequences of the two strains assigned to group 0 (L417-91 and L435-91) were similar to the sequences of members of the *Bacillus subtilis* group. Strains L407-91 and L400-91 grouped with the *B. stearothermophilus* group and *Bacillus smithii*, respectively. On the basis of the results obtained with group III isolates, only isolate L420-91^T and *B. brevis* ATCC 12990 (12) were selected for detailed characterization and determination of their taxonomic affiliation.

Morphology. Strain L420-91^T and *B. brevis* ATCC 12990 grew on nutrient agar plates and after overnight incubation formed irregular colonies with diameters of approximately 6 to 8 and 4 to 6 mm, respectively, frequently with swarming. After 24 h isolation of single colonies was often impossible because of extensive swarming on the agar surface. The morphological characteristics of selected strains are given in Table 3.

Figure 3 shows the cellular morphology of strain L420-91^T.

The cells of strains L420-91^T and ATCC 12990 were rods that had an average length of 3.7 to 5.4 μm and an average diameter of approximately 1.1 μm . The cells were peritrichous with several flagella (Fig. 3). Ultrathin sectioning of either organism revealed the cell envelope profile typical of gram-positive eubacteria (data not shown). L420-91^T and *B. brevis* ATCC 12990 produced central spores (data not shown). Like *B. aneurinolyticus* (36) and *B. migulanus* (43), both of these strains were completely covered with square S-layer lattices (Fig. 3b and Table 3) which consisted of glycosylated S-layer subunits in L420-91^T and *B. brevis* ATCC 12990. The glycan structures of the two organisms differed considerably (20, 21), as did the apparent molecular masses of the S-layer subunits (Table 3).

Phenotypic characterization. The growth temperatures of strains L420-91^T and ATCC 12990 on nutrient broth, which was the medium used for comparative purposes, were different from the growth temperatures of *B. aneurinolyticus* and *B. migulanus* (Table 4). Optimal growth of the latter two organisms was observed only in the mesophilic temperature range (36, 43). At 55°C *B. migulanus* showed only weak growth after 3 days (36) and the type strain of *B. aneurinolyticus* did not grow at all. This is in contrast to L420-91^T and the thermophilic organism *B. brevis* ATCC 12990 (12), which did not grow at 30 or 37°C and grew best at temperatures between 50 and 55°C. None of the organisms tested grew at 65°C. The temperatures used for the phenotypic tests were 30 and 55°C (Table 5). All of the strains tested were positive for growth in the presence of 3% sodium chloride, development of an alkaline pH (pH 8.0 to 8.5) in the Voges-Proskauer medium, and acid

FIG. 3. Electron micrographs of an intact cell of *B. thermoaerophilus* L420-91^T. (a) Negatively stained preparation showing the peritrichous flagella. Bar = 0.5 μm . (b) Freeze-etched and shadowed preparation showing the square S-layer lattice and the insertion sites of flagella (arrowheads). Bar = 100 nm.

TABLE 4. Growth of organisms on nutrient agar plates at different incubation temperatures

Strain	Growth at:							
	30°C	37°C	40°C	45°C	50°C	55°C	60°C	65°C
L420-91 ^T	– (5) ^a	– (5)	+	++ (2)	+++ (1)	+++ (1)	+	– (5)
<i>B. brevis</i> ATCC 12990	– (5)	– (5)	+	++ (2)	+++ (1)	+++ (1)	+	– (5)
<i>B. aneurinolyticus</i> DSM 5562 ^{Tb}	+++ (1)					– (5)	– (5)	– (5)
<i>B. migulanus</i> DSM 2895 ^{Tc}	+++ (1)					– (5)	– (5)	– (5)

^a –, no growth; +, weak growth; ++, medium growth; +++, strong growth. The numbers in parentheses are the numbers of days of incubation.

^b The temperature range for growth is 20 to 50°C, and the optimal growth temperature is 37°C (36).

^c The temperature range for growth is 20 to 50°C, and the optimal growth temperature is 37°C (43).

production from glycerol. They were negative for growth under anaerobic conditions, growth in the presence of 7% sodium chloride, the Voges-Proskauer reaction, production of indole, hydrolysis of starch and urea, and acid production from sucrose, L-arabinose, trehalose, xylose, mannitol, galactose, maltose, and raffinose. Some characteristics, such as hydrolysis of Tween 80, gelatin, and casein, acid production from glucose, and the thiamine hydrolase type 1 reaction, were positive in L420-91^T and *B. brevis* ATCC 12990 but negative in the type strains of the other two species. The type strains of *B. aneurinolyticus* and *B. migulanus* gave positive reactions in tests for reduction of nitrate, hydrolysis of DNA, and decomposition of thiamine by thiamine hydrolase type 2. In some assays only one of the strains was positive; e.g., only *B. brevis* ATCC 12990 was positive for the catalase reaction and growth in the presence of 0.02% (wt/vol) sodium azide, only *B. aneurinolyticus* DSM 5562^T was positive for growth in the presence of 5% (wt/vol) sodium chloride, and only *B. migulanus* DSM 2895^T was positive for acid production from fructose and D-arabinose. Only strain L420-91^T was negative for growth in the presence of 0.001% (vol/vol) lysozyme (Table 4). Since catalase activity is an important differentiating characteristic, we also tested the catalase reaction in the two other members of group III (Table 2), isolates L419-91 and L438-91. Both of these strains were positive for the catalase reaction.

Polar lipid analysis. Our comparison of the polar lipid patterns determined by one-dimensional TLC revealed high levels of homology among isolate L420-91^T, *B. brevis* ATCC 12990, *B. aneurinolyticus* DSM 5562^T and NCIMB 10056, and *B. migulanus* DSM 2895^T (Fig. 4). The *R_f* values of the lipid compounds detected revealed that these organisms differed from all other members of the genus *Bacillus*. On the basis of the TLC data, we concluded that L420-91^T and *B. brevis* ATCC 12990 could be distinguished from *B. brevis* DSM 30^T (Fig. 4) and *Bacillus laterosporus* DSM 25^T by the absence of an unidentified phospholipid (*R_f* 0.387) in the latter two organisms. Moreover, L420-91^T and *B. brevis* ATCC 12990 produced the same staining pattern on two-dimensional TLC (data not shown), which supports the hypothesis that these organisms are closely related. Treatment with the Dragendorff spray reagent for quaternary amines revealed no distinct staining reaction. Glycolipids were observed in *B. subtilis*, *Bacillus coagulans*, *B. smithii*, *Paenibacillus polymyxa*, *B. stearothermophilus*, *Bacillus thermoglucosidarius*, and *Saccharococcus thermophilus* but not in L420-91^T and *B. brevis* ATCC 12990 (Fig. 4).

Polyamine analysis. Our analysis of the polyamine patterns of the thermophilic organisms strain L420-91^T and *B. brevis* ATCC 12990 revealed that spermine was the predominant compound and that there were significant amounts of spermidine, while only trace amounts of other polyamines were detected (Table 6). In the mesophilic organisms *B. aneurinolyticus* DSM 5562^T and NCIMB 10056, *B. migulanus* DSM 2895^T,

B. laterosporus DSM 25^T, and *B. brevis* DSM 30^T the main component was spermidine, but minor amounts of other polyamines were present. The thermophilic organism *Bacillus thermoruber*, however, also contained spermidine as its major polyamine (Table 6). The ratio of spermidine to spermine in this organism was comparable to the ratio in *B. migulanus*, although the absolute amounts were significantly higher in *B. thermoruber*.

Cellular fatty acid and quinone compositions. The only group III strain whose cellular fatty acid and quinone compo-

TABLE 5. Differentiating phenotypic characteristics^a

Characteristic ^b	Strain L420-91 ^{Tb}	<i>B. brevis</i> ATCC 12990 ^b	<i>B. aneurinolyticus</i> DSM 5562 ^{Tc}	<i>B. migulanus</i> DSM 2895 ^{Tc}
Catalase activity	– ^d	+	–	–
Growth in the presence of:				
0.001% (vol/vol) Lysozyme	–	+	+	+
0.02% (wt/vol) Sodium azide	–	+	–	–
5% (wt/vol) Sodium chloride	–	–	+	–
Nitrate reduction	–	–	+	+
Hydrolysis of:				
Tween 80	+	+	–	–
Gelatin	+	+	–	–
Casein	+	+	–	–
DNA	–	–	+	+
Decomposition of thiamine by:				
Thiamine hydrolase type 1	w	w	–	–
Thiamine hydrolase type 2	–	–	+	+
Acid production from:				
Glucose	+	+	–	–
Fructose	–	–	–	+
D-Arabinose	–	–	–	+

^a All four strains did not grow under anaerobic conditions, grew in the presence of 3% (wt/vol) sodium chloride but not in the presence of 7% (wt/vol) sodium chloride, were Voges-Proskauer reaction negative, produced a pH of 8.0 to 8.5 in Voges-Proskauer medium, were indole negative, did not hydrolyze starch or urea, did not produce acid from sucrose, L-arabinose, trehalose, xylose, mannitol, galactose, maltose, or raffinose, and did produce acid from glycerol.

^b The temperature used in all tests was 55°C.

^c The temperature used in all tests was 30°C.

^d –, negative reaction; w, weak positive reaction; +, positive reaction.

FIG. 4. Schematic representation of the results of a one-dimensional TLC analysis of *Bacillus*, *Paenibacillus*, and *Saccharococcus* strains. Lane 1, *B. laterosporus* DSM 25^T; lane 2, *B. brevis* DSM 30^T; lane 3, L420-91^T; lane 4, *B. brevis* ATCC 12990; lane 5, *B. aneurinolyticus* DSM 5562^T; lane 6, *B. aneurinolyticus* NCIMB 10056; lane 7, *B. migulanus* DSM 2895^T; lane 8, *B. subtilis* CCM 2216^T; lane 9, *B. coagulans* DSM 1^T; lane 10, *Bacillus methanolicus* NCIMB 13113^T; lane 11, *B. smithii* DSM 4216^T; lane 12, *Bacillus sphaericus* DSM 28^T; lane 13, *Paenibacillus alvei* CCM 2051^T; lane 14, *P. polymyxa* DSM 36^T; lane 15, *B. stearothermophilus* DSM 22^T; lane 16, *Bacillus kaustophilus* NCIMB 8547^T; lane 17, *B. thermoglucosidasius* DSM 2542^T; lane 18, *Saccharococcus thermophilus* ATCC 43125^T. The organisms were placed in rRNA groups as described by Ash et al. (2). For each strain three different possible staining reactions are shown. Symbols: ●, positive molybdenum blue reaction; ■, positive ninhydrin reaction; ▼, positive naphthol reaction; ⊙, ⊠, and ▽, weak staining reactions. The area enclosed by a dashed-line box is the area of interest.

sitions were investigated was isolate L420-91^T (Table 2). The major cellular fatty acids found in strain L420-91^T and *B. brevis* ATCC 12990 were iso-C_{15:0} and iso-C_{17:0} (Table 7); however, the percentages of these fatty acids in *B. brevis* ATCC 12990 were higher than their percentages in L420-91^T (54 versus 48 and 33 versus 23%, respectively). Both strains could be clearly distinguished from the two *B. aneurinolyticus* strains by their higher levels of iso-C_{17:0} (23 to 33 versus 3%) (Table 7).

The major menaquinone in strain L420-91^T, *B. brevis* ATCC 12990, *B. aneurinolyticus*, and *B. migulanus* was menaquinone 7 (which accounted for more than 90% of the total), which is characteristic of members of the genus *Bacillus*.

16S rDNA sequence analysis. We determined the almost complete 16S rDNA sequences of *B. aneurinolyticus* DSM

5562^T, *B. migulanus* DSM 2895^T, strain L420-91^T (= DSM 10154^T), and *B. brevis* ATCC 12990 (= DSM 10155). A phylogenetic analysis revealed that these four strains represent a distinct lineage within the radiation of the genus *Bacillus* and related taxa (Fig. 5). The 16S rDNA similarity values (Table 8) show that strain L420-91^T and *B. brevis* ATCC 12990 are closely related (level of sequence similarity, 99.8%). *B. aneurinolyticus* and *B. migulanus* exhibit a level of sequence similarity of 99.7%. The range of similarity values for the four strains belonging to this cluster is 96.6 to 99.8%. The level of similarity or relatedness between *B. brevis* ATCC 12990 and *B. brevis* DSM 30^T was 90.9%, which was not significant.

DNA-DNA homology studies and G+C content. The level of DNA-DNA homology between strain L420-91^T and *B. brevis*

TABLE 6. Polyamine contents of *Bacillus* strains

Strain	Polyamine concn (μmol/g [dry wt])					
	1,3-Diamino-propane	Putrescine	Cadaverine	Spermidine	Norspermidine	Spermine
L420-91 ^T		0.74		2.18		4.90
<i>B. brevis</i> ATCC 12990	1.52			7.40	Tr ^a	14.11
<i>B. aneurinolyticus</i> DSM 5562 ^T	0.06	0.15		17.51	Tr	4.75
<i>B. aneurinolyticus</i> NCIMB 10056	0.09	0.15		10.63		1.90
<i>B. migulanus</i> DSM 2895 ^T	1.96	0.21		26.88	0.12	4.05
<i>B. laterosporus</i> DSM 25 ^T	0.15	0.12		6.63	Tr	1.21
<i>B. brevis</i> DSM 30 ^T		0.27		42.66		0.49
<i>B. thermoruber</i> DSM 7064 ^T		1.84	0.08	63.44		9.82

^a Tr, traces.

Strain	C _{11:0}	iso-C _{14:0}	C _{14:0}	iso-C _{15:1} F	iso-C _{15:0}	anteiso-C _{15:0}	C _{15:0}	iso-C _{16:0}	C _{16:1 ω11c}	C _{16:1 ω7c}	C _{16:0}	iso-C _{17:0}	anteiso-C _{17:0}	iso-C _{17:1 ω10c}	C _{17:0} cyclo	C _{17:0}	C _{18:0}	C _{20:0}
L420-91 ^T	0.1	0.2	0.2		54.3	0.6	0.6	2.3			3.5	32.8				0.2	0.3	4.4
<i>B. brevis</i> ATCC 12990	0.2	0.2	0.3		47.7	10.1	0.3	2.6			3.6	23.4						
<i>B. aneurinolyticus</i> DSM 5562 ^T	1.2	2.4	1.3	1.9	57.2	1.3		1.2			9.9	3.1						
<i>B. aneurinolyticus</i> NCIMB 10056	0.9	1.2	1.2	1.9	60.8	2.8		2.1	13.3	3.5	2.5	3.4	1.2		2.3	1.3		

TABLE 7. Analysis of cellular fatty acids

% of:

FIG. 5. Phylogenetic dendrogram based on a 16S rDNA sequence comparison, showing the position of *B. thermoaerophilus* within the radiation of the genus *Bacillus* and related taxa. Abbreviations: *B.*, *Bacillus*; *P.*, *Paenibacillus*; *S.*, *Saccharococcus*. Scale bar = five substitutions per 100 nucleotides.

ATCC 12990 was 94%. The levels of homology between the DNA of group III strain L419-91 and the DNAs of L420-91^T and *B. brevis* ATCC 12990 were 80.8 and 75.4%, respectively. The G+C contents of L420-91^T and *B. brevis* ATCC 12990 are 46.7 and 46.3 mol%, respectively.

DISCUSSION

In this paper we describe the isolation and characterization of a group of thermophilic aerobic bacteria, including strains L419-91, L420-91^T, and L438-91, obtained from the beet sugar factory in Leopoldsdorf, Austria. The isolates were collected on different days. Interestingly, strains which appeared in one sample (Table 2) were not found in other samples. Therefore, the appearance of a particular organism seemed to be strongly influenced by minor changes in the conditions of the continuous sugar beet extraction process, such as small changes in the temperature or pH value. However, we identified several isolates which belong to the same species (e.g., L419-91, L420-91^T, and L438-91) (Table 2) by partially sequencing 16S rDNA

TABLE 8. Levels of 16S rDNA similarity for strains L420-91^T and ATCC 12990 and related taxa within the radiation of the genus *Bacillus*

Organism ^a	% 16S rDNA similarity																		
Strain ATCC 12990	99.8	96.7	99.7	91.9	96.2	95.8	96.6	95.3	94.4	95.9	92.5	94.9	89.8	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus migulanus</i>	96.8	97.0	92.1	91.7	96.0	95.2	96.0	93.2	94.4	92.8	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus aneurinolyticus</i>	91.2	91.2	91.3	91.3	96.2	95.8	96.6	95.3	94.4	92.8	92.5	94.9	89.8	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus subtilis</i>	92.2	92.2	91.6	91.7	96.0	95.2	96.0	93.2	94.4	92.8	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus cohnii</i>	91.4	91.4	90.9	90.9	95.8	95.2	96.0	95.3	94.4	92.8	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus cereus</i>	92.2	92.2	91.6	91.6	96.6	95.5	96.6	95.3	94.4	92.8	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus methanolicus</i>	91.6	91.7	91.2	91.2	94.3	93.0	94.0	93.2	94.4	92.8	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus globisporus</i>	90.7	90.8	90.2	90.1	93.7	92.3	93.4	93.0	93.2	92.8	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus pasteurii</i>	90.0	90.0	90.2	90.2	94.0	94.1	94.6	93.4	93.8	92.2	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus alcalophilus</i>	90.6	90.6	91.3	91.1	94.1	94.0	94.0	93.3	93.4	92.2	92.2	94.9	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Bacillus sp. strain DSM 7821</i>	89.5	89.6	90.3	90.3	90.5	90.4	90.9	89.5	89.7	88.8	88.4	90.5	89.8	94.1	94.5	88.6	96.2	94.4	91.9
<i>Paenibacillus amylolyticus</i>	90.1	90.2	89.9	89.8	89.6	89.1	89.4	88.4	88.9	89.4	88.8	90.8	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Paenibacillus gordonae</i>	89.4	89.5	89.6	89.6	90.3	89.5	89.9	89.1	89.4	88.4	88.4	90.8	89.1	94.1	94.5	88.6	96.2	94.4	91.9
<i>Paenibacillus polymyxa</i>	90.9	90.9	91.5	91.7	90.4	90.6	90.7	90.1	90.6	89.0	89.1	90.5	90.2	88.8	89.1	89.1	96.2	94.4	91.9
<i>Bacillus brevis</i>	90.3	90.3	91.3	91.5	91.0	90.5	91.9	91.5	90.6	89.5	88.8	90.1	90.1	90.1	89.4	89.1	96.2	94.4	91.9
<i>Bacillus laterosporus</i>	91.6	91.7	91.6	91.7	91.7	90.5	90.9	93.2	94.4	92.3	92.2	91.9	92.6	89.0	88.8	88.7	90.3	90.3	91.9
<i>Bacillus thermoruber</i>	92.4	92.4	91.7	91.6	93.7	92.6	93.4	93.2	94.4	92.3	92.2	91.9	92.6	89.0	88.8	88.7	90.3	90.3	91.9
<i>Bacillus stearothermophilus</i>	92.2	92.2	91.9	91.6	94.6	93.6	94.1	94.0	95.3	93.2	92.8	92.5	92.9	89.5	89.0	89.1	90.6	90.6	91.9
<i>Bacillus thermoglucosidasius</i>	92.8	92.8	92.0	92.0	95.3	94.6	94.8	94.6	95.9	93.8	93.3	93.8	93.9	90.1	89.7	90.0	90.6	90.3	91.9
<i>Saccharococcus thermophilus</i>	91.6	91.8	90.7	90.7	94.7	93.4	94.5	93.6	94.9	94.1	93.5	93.2	91.9	89.1	89.3	89.5	90.5	90.6	91.9
" <i>Bacillus flavothermus</i> " ^a	91.2	91.2	90.9	90.9	93.4	92.6	92.8	92.6	93.0	92.0	92.0	91.2	91.2	88.4	89.4	88.8	89.6	89.7	91.1

^a For the strains used see Fig. 5.

preparations and by performing DNA-DNA hybridization experiments and chemotaxonomic analyses.

In this paper we also characterize *B. brevis* ATCC 12990 and compare it with the new isolates, in particular L420-91^T. Because of the high level of similarity between *B. brevis* ATCC 12990 and L420-91^T, the taxonomic status of strain ATCC 12990 as a *B. brevis* strain should be reconsidered. Strains L420-91^T and ATCC 12990 were compared by using chemotaxonomic characteristics and analyses at the molecular level with appropriate type strains (Table 1) in order to determine their taxonomic affiliations.

The high level of DNA relatedness (94%) between strain L420-91^T and *B. brevis* ATCC 12990 indicates that these organisms belong to the same species. This finding is supported by a high level of 16S rDNA sequence similarity (99.8%). It is interesting that although the type strains of *B. aneurinolyticus* and *B. migulanus* exhibit a level of 16S rDNA similarity of 99.7%, they have been shown by DNA-DNA homology studies to represent distinct species (36). These results highlight the need for DNA-DNA homology studies when high levels of 16S rDNA similarity are found between strains, as pointed out by Stackebrandt and Goebel (42).

The results of the 16S rDNA sequence analysis confirm the isolated position of *B. aneurinolyticus* described by Ash et al. (2) and show that *B. migulanus* is the closest phylogenetic neighbor of *B. aneurinolyticus*. These results also show that the thermophilic organisms strain L420-91^T and *B. brevis* ATCC 12990 are phylogenetically related to *B. aneurinolyticus* and *B. migulanus* and not related to the main group of thermophilic *Bacillus* species previously shown to group around *B. stearothermophilus* (2, 34).

We deduced from the growth temperature data that L420-91^T and *B. brevis* ATCC 12990 are thermophilic bacteria (Table 4), whereas the related organisms *B. aneurinolyticus* (36) and *B. migulanus* (43) are mesophilic bacteria. Morphologically, the strains described in this paper appeared to be quite similar. However, we found significant differences in the molecular masses of the S-layer protomers (Table 3) and the chemical compositions of the glycan chains of L420-91^T (20) and *B. brevis* ATCC 12990 (21). Since the glycan structures of S-layers do not have taxonomic significance (26, 27) and determination of these structures is complicated and time consuming, only strains L420-91^T and ATCC 12990 were investigated in detail. The S-layer proteins of *B. aneurinolyticus* and *B. migulanus* are not glycosylated (Table 3). Some of the phenotypic characteristics (Table 5) also reveal that the two groups of organisms are distinct. On the basis of the differences in specific cellular fatty acids (Table 7), we concluded that L420-91^T and *B. brevis* ATCC 12990 are different from *B. aneurinolyticus* and *B. migulanus*. This conclusion was supported by the results of the catalase test. While *B. aneurinolyticus* and *B. migulanus* had negative catalase reactions (36, 43), isolates L419-91 and L438-91 and *B. brevis* ATCC 12990 were positive in this test. However, the catalase reaction of L420-91^T was negative. A comparable situation with this differential characteristic has been described for *B. stearothermophilus* strains (41). The common polyamine pattern of L420-91^T and *B. brevis* ATCC 12990 (Table 6), in which spermine is the predominant compound, allowed us to differentiate these organisms from the closely related species *B. aneurinolyticus* and *B. migulanus*, in which spermidine is the major polyamine. So far, predominance of spermine has been detected only in other moderately thermophilic bacilli (13) and in members of the genus *Alicyclobacillus* (14). However, spermidine is the major polyamine in *B. thermoruber*. These data provide evidence that accumulation of spermine is not a useful characteristic for differenti-

ating between rod-shaped endospore-forming mesophilic and thermophilic members of the genus *Bacillus*.

The 16S rDNA sequence data indicate that new isolates L419-91, L420-91^T, and L438-91 and *B. brevis* ATCC 12990 fall within the radiation of the genus *Bacillus* and related taxa (34). However, it is clear from both the phylogenetic dendrogram and the 16S rDNA similarity values that these strains are not closely related to the type species of the genus *Bacillus*, *B. subtilis*. The 16S rDNA sequence analysis data indicate that these strains and perhaps *B. aneurinolyticus* and *B. migulanus* represent the core of a new genus. At the present time, there are not sufficient phenotypic and chemotaxonomic characteristics to differentiate this group of organisms and provide defining characteristics for such a new genus. Therefore, we propose that strains L419-91, L420-91^T, and L438-91 and *B. brevis* ATCC 12990 should be classified in a new *Bacillus* species, *Bacillus thermoaerophilus*. Future characterization of these and other strains may lead to reorganization of the genus *Bacillus* and creation of new genera for certain species currently placed in the genus *Bacillus*.

Description of *Bacillus thermoaerophilus* sp. nov. *Bacillus thermoaerophilus* (ther.mo.aer.o'phi.lus. Gr. adj. *thermos*, hot; Gr. masc. n. *aer*, air; Gr. adj. *philos*, loving; M. L. adj. *thermoaerophilus*, loving heat and air, i.e., thermophilic and strictly aerobic). Cells are rod shaped (length, 3.7 to 5.4 µm; diameter, 1.0 to 1.2 µm), gram positive, motile, and peritrichous with central spores in swollen sporangia. Colonies are flat on nutrient agar. Growth occurs at temperatures ranging from 40 to 60°C at pH 7 to 8. Positive for growth in the presence of 3% sodium chloride, development of an alkaline pH in the Voges-Proskauer reaction, hydrolysis of Tween 80, gelatin, and casein, acid production from glucose and glycerol, weak decomposition of thiamine by thiamine hydrolase type 1, and the presence of a specific glycosylated S-layer protein. Negative for anaerobic growth, the Voges-Proskauer reaction, growth in the presence of a sodium chloride concentration of 5% or higher, reduction of nitrate to nitrite, indole production, hydrolysis of starch and urea, degradation of DNA, acid production from sucrose, fructose, D- and L-arabinose, trehalose, xylose, mannitol, galactose, maltose, and raffinose, and decomposition of thiamine by thiamine hydrolase type 2. Variable for catalase production and growth in the presence of 0.001% (vol/vol) lysozyme and 0.02% (wt/vol) sodium azide. The major cellular fatty acids are iso-C_{15:0} and iso-C_{17:0}. The major quinone is menaquinone 7. The G+C contents of the strains examined are 46.7 mol% (L420-91^T) and 46.3 mol% (*B. brevis* ATCC 12990).

The type strain is strain L420-91. This strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 10154. *B. thermoaerophilus* ATCC 12990 (formerly *B. brevis* ATCC 12990, which is no longer available from the American Type Culture Collection) has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 10155.

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