Bacillus haloalkaliphilus sp. nov.

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Ten obligately alkaliphilic, extremely halotolerant *Bacillus* isolates were studied and compared with strain WN13^T (T = type strain), an earlier isolate provided by H. G. Trüper. All of these strains produced round, terminally located spores in swollen sporangia. DNA-DNA hybridization values (78 to 91%) and phenotypic similarity analyses revealed that 10 of the 11 strains formed a relatively homogeneous group, and although one strain (strain AH/6/1) could not be distinguished phenotypically, it exhibited hybridization values of only 46 to 47%. This group of strains is sufficiently different from all previously validly described *Bacillus* species in its morphological, physiological, and biochemical properties that a separate species is considered appropriate, for which the name *Bacillus haloalkaliphilus* sp. nov. is proposed.

Increased industrial interest in alkaliphilic organisms, especially members of the genus *Bacillus*, long after the original description of *Bacillus alcalophilus* (21), has resulted in the isolation of numerous strains. To date, a number of approaches have been used to define this heterogeneous group taxonomically (7, 9, 16). This has led to valid descriptions of additional alkaliphilic *Bacillus* species, including *Bacillus cohnii* (20) and *Bacillus agaradhaerens*, *Bacillus clarkii*, *Bacillus clausii*, *Bacillus gibsonii*, *Bacillus halmaphysus*, *Bacillus halodurans*, *Bacillus horikoshii*, *Bacillus pseudocaliphilus*, and *Bacillus pseudofirmus* (15). A more physiological and ecological approach to these organisms is the question of how life at pH values above 9 or even 10 or 11 is possible (10).

In 1985 isolation of obligately alkaliphilic, extremely halotolerant *Bacillus* sp. strain WN13^T (T = type strain) from the lakes of Wadi Natrun in Egypt was described, and the method of osmoregulation in this organism was discussed (14, 23). In a study of the taxonomy of 78 alkaliphilic *Bacillus* strains (6), Wadi Natrun strain WN13^T was the only isolate that produced round spores and exhibited extreme halotolerance. Differences in phenotypic properties and low levels of DNA-DNA hybridization with other alkaliphilic strains, as well as neutrophilic *Bacillus* species, suggested that this strain may represent a new species. After similar alkaliphilic, halophilic strains that form round spores were isolated, a more detailed study of the taxonomic position of this group of organisms was undertaken.

MATERIALS AND METHODS

Bacterial strains. The *Bacillus* strains used in this study and their sources are listed in Table 1. Strain WN13^T was provided by H. G. Trüper, Bonn, Germany. Additional haloalkaliphilic strains were isolated from brine and dried soil, mud, and dung samples which were collected in 1992 from various places in the Wadi Natrun by D. Claus. Type strains and other representative strains used for reference purposes were obtained from the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Enrichment and isolation. Portions (0.5 g) of the samples (Table 1) were soaked and dissolved in 5-ml portions of alkaline nutrient broth supplemented with 20% (final concentration) NaCl. The resulting preparations were incubated overnight at 40°C. Loopfuls from these enrichment cultures were streaked onto agar plates containing the same medium, and the plates were incubated at 40°C for 2 to 3 days until distinctive colonies were visible. Three or four colonial types developed, and whitish or yellowish colonies that were 1 to 2 mm in diameter and had smooth to matt surfaces were selected and examined with a microscope. These colonies usually consisted of slender cells that were approximately 0.4 by 7 µm. When these organisms were plated onto alkaline nutrient agar supple-

mented with 5% NaCl, the colonies that developed contained cells which produced round, terminally located spores in swollen sporangia.

Phenotypic characterization. Phenotypic characteristics were determined largely by the methods of Gordon et al. (8), as described previously (20), with the additional modification that test media were supplemented with 5% NaCl. Two media buffered with either phosphate or citrate were used to test for tolerance to pH 7.

Chemosystematic characterization. The following analyses were performed by using previously described methods: cell wall analysis (11), fatty acid analysis (17), and quinone analysis (12).

DNA isolation, base composition, and DNA-DNA hybridization. DNA was isolated and DNA hybridization experiments were performed as described by Spanka and Fritze (20). The G+C content of the DNA was determined by the buoyant density method (7), spectrophotometrically (20), or by high-performance liquid chromatography (HPLC) (13).

RESULTS

DNA base composition and DNA-DNA hybridization. G+C contents of some representative strains were as follows: strain WN13^T (= DSM 5271^T), 37.1 mol% as determined by the buoyant density method and 37.5 mol% as determined by HPLC; strain AH/1 (= DSM 9473), 37.9 mol% as determined by HPLC; and strain AH/6/1 (= DSM 9478), 39.0 mol% as determined by HPLC. A direct comparison of the melting curves of the DNAs of these strains revealed that their melting points differed by at most, 0.4°C, which reflected a difference in G+C contents of 1 mol%.

Of the 10 haloalkaliphilic isolates that formed round spores, 9 exhibited DNA-DNA reassociation values between 78 and 91% with reference strain WN13^T (= DSM 5271^T). One isolate, strain AH/6/1 (= DSM 9478), exhibited a level of hybridization with WN13^T of only 46 to 47%.

Cellular fatty acid composition, quinone system, and cell walls. Whole-cell fatty acid patterns were determined by using nonsporulated cells grown at 30°C on plates containing Trypticase soy broth (BBL) agar supplemented with 5 and 0.5% NaCl. The fatty acid patterns were only insignificantly different when the salt concentration was 0.5%. In the presence of 5% NaCl the major fatty acids in all of the strains were saturated branched-chain fatty acids (iso-C_{15:0}, 40 to 59% [mean, 48%]; anteiso-C_{15:0}, 6 to 15% [mean, 11%]; iso-C_{17:0}, 4 to 8% [mean, 6%]; anteiso-C_{17:0}, 6 to 15% [mean, 11%]). Low levels of unsaturated fatty acids were also present (C_{16:1} and C_{17:1} accounted for 15 to 24% of the fatty acids [mean, 19%]). When the cells were grown in the presence of an NaCl concentration of 0.5%, the amounts of iso-C_{15:0}, anteiso-C_{15:0}, and anteiso-C_{17:0} were slightly higher (4, 3, and 2% higher, respectively), whereas the amounts of iso-C_{17:0} and C_{17:1} were slightly lower (3 and 5% lower, respectively).

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TABLE 1. Haloalkaliphilic *Bacillus* strains examined in this study

Strain	Other designation	Source at Wadi Natrun ^a
DSM 5271 ^T	WN13 ^T	Brine and mud
DSM 9473	AH/1	Camel dung
DSM 9474	AH/2	Sandy loam
DSM 9475	AH/3	Solid loam
DSM 9476	AH/4	Solid loam
DSM 9477	AH/5	Loamy sand
DSM 9478	AH/6/1	Solid loam
DSM 9479	AH/7/2	Camel dung
DSM 9480	AH/8	Solid briny loam
DSM 9481	AH10/1	Briny sand
DSM 9482	AH/11	Salt crystal

^a Strain WN13^T was obtained from H. G. Trüper, Universität Bonn, Bonn, Germany; all of the other strains were isolated in this study.

The major quinone of strain WN13^T (= DSM 5271^T) was MK-7, which accounted for >90% of the total quinones. A small amount of MK-6 (about 8%) was also found.

The walls of vegetative cells of all 11 strains tested contained diaminopimelic acid.

Phenotypic characterization. The slender vegetative cells of the alkaliphilic, extremely halotolerant isolates were 0.3 to 0.5 μm wide and 3 to 8 μm long. The spores were round and were produced terminally, swelling the sporangia (Fig. 1). The colonies were cream white on alkaline nutrient agar supplemented with 5 to 15% NaCl. A slight yellowish color occurred on nutrient agar containing 20% NaCl. Most strains also formed more translucent colonies which contained nonsporulated or poorly sporulated cells. Sporulation was enhanced at NaCl concentrations close to 5% and was delayed at higher concentrations. Cell morphology and growth were strongly influenced by NaCl concentrations (Fig. 1). On media without additional NaCl or with only 0.5% NaCl, no growth or only very weak growth occurred, and under the microscope the cells appeared to be wrinkled, interwoven filaments.

The strains tested in this study were positive for oxidase and catalase reactions, growth at 15 and 40°C (at 45°C strains WN13^T, AH/3, and AH/10/1 did not grow and the growth of strain AH/11 was delayed), growth in the presence of 20% NaCl (in the presence of 25% NaCl strains AH/4 and AH/5 did not grow and the growth of strain AH/2 was variable), growth on defined medium at pH 8 and 9.7, hydrolysis of gelatin (most strains exhibited weak hydrolysis), weak hydrolysis of starch (very narrow [2- to 3-mm] clearing zones formed around colonies after 10 days; strains AH/6/1 and AH/10/1 were negative), hydrolysis of hippurate, and cleaving of 4-methyl-umbelliferone glucuronide.

The strains tested in this study were negative for the KOH and aminopeptidase reactions, growth at 5 and 10°C, growth in nutrient media not supplemented with NaCl (very weak, variable growth occurred in some cases), growth at pH 7, hydrolysis of casein (strains WN13^T and AH/8 were positive; strains AH/3 and AH/4 were weakly positive), hydrolysis of Tween 20 and Tween 80, hydrolysis of pullulan, the lecithinase reaction, reduction of nitrate to nitrite, and cleavage of urea.

DISCUSSION

After the extremely halotolerant, obligately alkaliphilic strain WN13^T was isolated from Wadi Natrun briny mud, it was used as a model organism to study the physiological strategies which allow an organism to survive in the presence of NaCl concentrations of 20% or more (23). Compatible solutes

FIG. 1. *B. haloalkaliphilus* cell morphology after growth for 3 days in nutrient broth (pH 9.7) containing NaCl at different concentrations. (A) Strain DSM 5271^T, 0.5% NaCl. (B) Strain DSM 5271^T, 5% NaCl. (C) Strain DSM 9473, 5% NaCl. (D) Strain DSM 5271^T, 10% NaCl. Bar = 10 μm .

help the organisms to resist osmotic stress, and it was shown that strain WN13^T accumulated different solutes when it was grown on complex media containing high salt concentrations, whereas it synthesized mainly ectoine when it was grown on mineral medium having a salt concentration of 10% (14). When betaine was offered as a compatible solute in the culture medium, this compound was taken up and accumulated rather than the strain synthesizing its own metabolites. Weisser and Trüper tried to classify strain WN13^T, largely on the basis of its

TABLE 2. Characteristics of *B. haloalkaliphilus* and physiologically or morphologically similar *Bacillus* species

Species	Spore shape	Sporangium shape	Growth at:		Growth in the presence of 20% NaCl	Deamination of phenylalanine	NO ₃ reduced to NO ₂	Hydrolysis of:		Presence of diaminopimelic acid	G+C content (mol%)
			pH 7	pH 9.7				Starch	MUG ^a		
<i>B. haloalkaliphilus</i> ^b	Round	Swollen	— ^c	+	+	—	—	+/-	+	+	37-38
<i>B. sphaericus</i>	Round ^d	Swollen ^d	+ ^d	— ^b	— ^b	— ^e	— ^d	— ^e	— ^b	— ^d	37.1 ^f
<i>B. pasteurii</i>	Round ^d	Swollen ^d	+ ^d	— ^b	ND	ND	+ ^e	— ^e	ND	— ^d	38.7 ^f
<i>B. schlegelii</i>	Round ^d	Swollen ^d	+ ^d	— ^g	— ^g	ND	+	— ^g	ND	ND	66.3 ^f
<i>B. halodenitrificans</i> ^h	—	—	+	+	+	—	+	—	ND	ND	38
<i>B. pantothenicus</i>	Ellipsoidal ^d	Swollen ^d	+ ^d	+ ^b	— ^b	— ^e	+ ^d	+ ^e	— ^b	+ ^d	36.8 ^f
<i>B. agaradhaerens</i> ^b	Ellipsoidal	Swollen	—	+	—	—	+	+	—	ND	39-40
<i>B. clarkii</i> ^b	Ellipsoidal	Swollen	—	+	—	—	+	—	—	ND	42-43
<i>B. halophilus</i> ⁱ	Ellipsoidal	Not swollen	+	—	+	—	—	—	ND	+	51.5
<i>B. pseudofirmus</i> ^b	Ellipsoidal	Not swollen	d	+	—	+	—	+	—	ND	39-41

^a MUG, 4-methyl-umbelliferone-glucuronide.^b Data from this study.^c —, negative or not present; +, positive or present; +/-, weakly positive; d, different results are obtained with different strains; ND, not determined.^d Data from previously published papers were confirmed in this study.^e Data from reference 2.^f Data from reference 5.^g Data from reference 18.^h Data from reference 4.ⁱ Data from reference 22.

alkaliphily, and found that it clearly differed from the only validly described alkaliphilic *Bacillus* species available at that time, *B. alcalophilus* (21), and another alkaliphilic *Bacillus* taxon that had not been validly described, "*B. alcalophilus* subsp. *halodurans*" (1). These findings were later confirmed (6).

The conspicuous properties of WN13^T include the shape and localization of the spores, as well as the shape of the sporangium; similar characteristics are found in only three other *Bacillus* species. However, in contrast to *Bacillus sphaericus* and *Bacillus pasteurii*, which also produce spherical spores that clearly distend the sporangium, the walls of strain WN13^T vegetative cells contain diaminopimelic acid. The cell wall composition of *Bacillus schlegelii*, an organism that can grow chemoautotrophically, is not known, but a number of properties, including its G+C content (66.3 mol%, as determined by the buoyant density method), clearly differentiate this organism from the strains used in this study. The presence of diaminopimelic acid in its cell walls also distinguishes WN13^T from *Bacillus globisporus*, *Bacillus insolitus*, *Bacillus marinus*, and *Bacillus psychrophilus*; in addition, the spores of these species are not wider (or not clearly wider) than the vegetative cells.

Few *Bacillus* species that grow in the presence of NaCl concentrations of 20% or more and/or are obligately alkaliphilic have been described. It has been reported that *Bacillus halophilus* (22) tolerates up to 30% total salts, grows at pH 6 to 8, and has an optimum pH of around 7. *Bacillus halodenitrificans* (4), which was described as an organism that does not form spores, grows at pH 5.8 to >9.6 and has an optimum pH of 7.4; NaCl concentrations up to 25% are tolerated by this organism. *Bacillus pantothenicus* grows at pH 9.7 but not in the presence of an NaCl concentration of 20% (only up to 10% NaCl reported as positive). The highest NaCl concentration at which three recently described alkaliphilic *Bacillus* species (16) definitely grow is 16%, and a few strains tolerate up to 18% NaCl. Two of these organisms, *B. agaradhaerens* and *B. clarkii*, are obligately alkaliphilic organisms whose optimum pH is around 10. *B. pseudofirmus* has an optimum pH of around 9, and a few strains grow at pH 7. None of these organisms

produces round spores. Table 2 shows the relevant properties of morphologically or physiologically similar *Bacillus* species.

In previous studies (6, 7) a number of DNA-DNA hybridization experiments were performed with strain WN13^T and the type strains or representative strains of morphologically or physiologically similar *Bacillus* species that have similar G+C compositions. None of the hybridization values obtained indicated that strain WN13^T belongs to a previously described species. The levels of binding (3) with strain WN13^T DNA were as follows: *B. alcalophilus* DSM 485^T, 29%; *B. lentus* DSM 9^T, 32%; *B. cohnii* DSM 2528, 25%; *B. cohnii* DSM 6362, 24%; *B. marinus* DSM 1297^T, 15%; *B. sphaericus* DSM 28^T, 23%; *B. pasteurii* DSM 33^T, 22%; and *B. globisporus* DSM 4^T, 28%.

Considerable advances have been made in the taxonomy of the genus *Bacillus* as a result of modern methods. However, the results obtained with these methods have also underscored the significance of features such as spore shape and position and shape of the sporangium, as well as physiological and biochemical properties. Despite the fact that certain morphological and physiological types may not consistently be monophyletic, the data presented in this study show that the new isolates which I studied represent a novel species belonging to the genus *Bacillus* as it is currently defined.

It has been recommended that a species description should be based on at least 10 isolates to show the variation within a taxon (19). Therefore, the group of strains studied here is considered to be genetically and phenotypically distinct, and the name *Bacillus haloalkaliphilus* sp. nov. is proposed below for this group of organisms.

Description of *Bacillus haloalkaliphilus* sp. nov. *Bacillus haloalkaliphilus* (hal.o.al.ka.li'phi.lus. Gr. n. *hals*, salt; Arabic n. *al qaliy*, soda ash; Gr. adj. *philos*, loving; M.L. adj. *haloalkaliphilus*, loving briny and alkaline media). Cells are gram positive (as determined by the KOH and aminopeptidase tests). The cells are 0.3 to 0.5 µm wide and 3 to 8 µm long. Motile. Spores are round and located terminally. Sporangia are swollen. On alkaline nutrient agar supplemented with 5 to 10% NaCl the colonies are creme white. In the presence of 20% NaCl the colonies are slightly yellowish.

The major cellular fatty acids are the saturated branched-chain fatty acids iso-C_{15:0} (48%), anteiso-C_{15:0} (11%), anteiso-C_{17:0} (11%), and iso-C_{17:0} (6%). A small amount (19%) of unsaturated fatty acids is also present. MK-7 is the major menaquinone (>90% of the total quinones). Ubiquinones are not present. The peptidoglycan side chains are directly linked via *m*-diaminopimelic acid.

Chemoorganotrophic. Does not grow (or grows only very poorly) in nutrient broth or on nutrient agar without NaCl. Catalase positive. Oxidase positive. Mesophilic. Three strains do not grow at 45°C; no growth occurs at 50°C. Obligately alkaliphilic. No growth occurs at pH 7, and good growth occurs at pH 9.7. Halophilic and extremely halotolerant. Growth occurs in the presence of up to 25% NaCl (two strains are negative under these conditions); no growth or only very weak growth occurs without added NaCl. Hydrolysis of starch is weak, and pullulan is not hydrolyzed. Gelatin is hydrolyzed. No hydrolysis or only weak hydrolysis of casein occurs. Hippurate is hydrolyzed. Tween 20 and Tween 80 are not hydrolyzed. Nitrate is not reduced to nitrite. Egg yolk lecithinase negative. 4-Methyl-umbelliferone-glucuronide is hydrolyzed. Urea is not cleaved.

The DNA base composition of type strain WN13 (= DSM 5271) is 37 mol% (as determined by the buoyant density method) to 38 mol% (as determined by HPLC). Isolated from alkaline, highly saline mud from Wadi Natrun, Egypt.

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