Bacillus alcalophilus subsp. *halodurans* subsp. nov.: an Alkaline-Amylase-Producing, Alkalophilic Organism

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An alkaline-amylase-producing, alkalophilic bacillus, NRRL B-3881, was characterized and compared with *Bacillus* sp. ATCC 21591 and *Bacillus alcalophilus* Vedder strain NCTC 4553 (=ATCC 27647), which is here designated as the type strain of *B. alcalophilus*. All three strains contained motile, gram-positive rods with rounded ends and swollen, clavate sporangia with oval, terminal to subterminal endospores. All three strains grew in soybean broth; were facultatively anaerobic; hydrolyzed starch, gelatin, and casein; reduced methylene blue; and fermented the following carbohydrates without gas production: sucrose, D-glucose, lactose, maltose, D-mannitol, D-xylose, L-arabinose, glycerol, sorbitol, and salicin. None produced acetylmethylcarbinol, indole, urease, or crystalline dextrans. *Bacillus* sp. ATCC 21591 and *Bacillus* sp. NRRL B-3881, but not NCTC 4553, reduced nitrate to nitrite, utilized citrate, and grew well in 12% NaCl and slowly in 15% NaCl. *B. alcalophilus* NCTC 4553 did not grow in 5% NaCl. In our opinion, these differences are sufficient to justify the establishment of a separate subspecies for ATCC 21591, NRRL B-3881, and similar strains. We propose the name *B. alcalophilus* subsp. *halodurans* as the name for this new subspecies. The name of the type subspecies, which contains the type strain, NCTC 4553, is *B. alcalophilus* subsp. *alcalophilus* Vedder. NRRL B-3881 is designated as the type strain of *B. alcalophilus* subsp. *halodurans* and is available from the Northern Regional Research Laboratory. It has also been deposited in the American Type Culture Collection under the number 27557.

There have been several papers in the past few years describing alkalophilic *Bacillus* species that produce extracellular alkaline protease (4), amylases (1, 5, 12), and pectinase (6). Also, alkaline-amylase-producing, alkalophilic bacilli have been described in a patent (Rikagaku Kenkyusho, Netherlands Pat. Appl. 70-13,396, 1970), as have alkaline-protease-producing, alkalophilic bacilli (K. Aunstrup et al., Great Britain Patent 1,243,784, 1971). *Bacillus alcalophilus* Vedder 1934 (11) produces an alkaline protease (K. Aunstrup et al., Great Britain Patent 1,205,403, 1970). The taxonomic positions of these alkalophilic bacilli that produce extracellular alkaline enzymes have not been ascertained.

Vedder (11) isolated 16 strains of *Bacillus alcalophilus* from human feces. He deposited one strain of *B. alcalophilus* with the National Collection of Type Cultures, London, under the number 4553. In 1956, the same strain was

deposited with the National Collection of Industrial Bacteria, Edinburgh, under the number 8772, where the strain is erroneously labeled *Bacillus subtilis* (K. Aunstrup et al., Great Britain Patent 1,205,403, 1970). This species grew only above pH 7, the pH range for optimal growth being 8.6 to 10. *B. alcalophilus* was listed in an appendix in the 6th edition of *Bergey's Manual of Determinative Bacteriology* (8), but it was not mentioned in the 7th edition (9); it was also listed in *Index Bergeyana* (2). In this paper, *B. alcalophilus* Vedder strain NCTC 4553 is compared with two other alkaline-amylase-producing, alkalophilic bacilli, namely, *Bacillus* sp. NRRL B-3881 (1) and *Bacillus* sp. ATCC 21591 (Rikagaku Kenkyusho, Netherlands Pat. Appl. 70-13,396, 1970).

This paper was presented in part at the 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., 23 to 28 April 1972.

MATERIALS AND METHODS

Bacterial strains. *Bacillus* sp. NRRL B-3881 (Northern Regional Research Laboratory, Peoria, Ill.) was isolated from dried sewage sludge by Boyer and Ingle (1); it has been deposited in the American Type Culture Collection, Rockville, Md., under the number 27557. *B. alcalophilus* Vedder strain NCTC 4553 (=ATCC 27647) was obtained from the National Collection of Type Cultures, London. *Bacillus* sp. ATCC 21591 was obtained from the American Type Culture Collection.

Media and methods. The media and tests used were described previously by Smith et al. (10), except that it was necessary to adjust the pH to the range required by these alkalophilic bacteria. A 10% solution of either NaHCO_3 or $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ was sterilized by autoclaving, and the amount necessary to provide a final alkali concentration of 1% was aseptically added to the test media. The pH values of the test media were 9.8 with NaHCO_3 or 8.8 with $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$. Sodium pyrophosphate buffer was substituted for sodium bicarbonate buffer in the casein and gelatin agar plates because when plates buffered with sodium bicarbonate were flooded with acid HgCl_2 precipitant, bubbles formed on the agar and masked the protease hydrolysis zones.

All tests were incubated at 37 C instead of 28 C except where noted otherwise. In the carbohydrate fermentation tests, ammonium phosphate was the nitrogen source, and the change in pH was measured with a glass electrode.

It was necessary to use baffled shake flasks (Bellco, no. 599) with pad closures to obtain sporulation in the alkaline amylase production medium described by Boyer and Ingle (1). The minimal medium used to ascertain the nutritional requirements was described by Ingle and Boyer (7).

Motile cells of strains ATCC 21591 and NCTC 4553 were obtained when grown in a medium containing 0.5% tryptone (Difco), 0.5% yeast extract (Difco), 0.1% K_2HPO_4 , 0.7098% Na_2HPO_4 , and 1% NaHCO_3 (TY medium) in unbaffled shake flasks. Motile cells of NRRL B-3881 were obtained when grown in a medium containing nutrient broth (Difco), 1% NaHCO_3 , and 0.1 mM MgCl_2 (NB medium) in unshaken culture tubes (R. L. Gherna, personal communication).

The effect on the growth of NRRL B-3881 of adding 5% NaCl to a medium at an acid pH was determined by inoculating (1% vol/vol) 50 ml of test medium in 250-ml triple baffled shake flasks (Bellco, no. 599) with pad closures. A gyratory cabinet shaker (1-inch circular orbit; 300 rpm) was used at 39 C for 18 h.

RESULTS

All three strains studied here contained gram-positive rods with rounded ends; the sporangia were swollen and clavate, and the spores were oval and terminal to subterminal (Fig. 1). All three strains were motile. When

suspensions of motile cells were stained according to Leifson's method, *Bacillus* sp. ATCC 21591 (Fig. 2), *B. alcalophilus* NCTC 4553 (Fig. 3), and *Bacillus* sp. NRRL B-3881 (Fig. 4) were found to be peritrichously flagellated.

FIG. 1. Sporulated cells of *Bacillus alcalophilus* subsp. *halodurans* NRRL B-3881. Gram-stain, $\times 1,200$. Phase contrast.

FIG. 2. Peritrichous cells of *Bacillus alcalophilus* subsp. *halodurans* ATCC 21591 from a 24-h-old TY broth culture incubated at 37 C. Stained by Leifson's method, $\times 1,200$. Phase contrast.

FIG. 3. Peritrichous cells of *Bacillus alcalophilus* NCTC 4553 from a 24-h-old TY broth culture incubated at 37 C. Stained by Leifson's method, $\times 1,200$. Phase contrast.

None of the strains produced acetylmethylcarbinol, indole, urease, or crystalline dextrins. All three strains grew in soybean broth; were aerobic and facultatively anaerobic; hydrolyzed starch, gelatin, and casein; reduced methylene blue; and fermented (without gas production) sucrose, D-glucose, lactose, maltose, D-mannitol, D-xylose, L-arabinose, glycerol, sorbitol, and salicin.

FIG. 4. Peritrichous cells of *Bacillus alcalophilus* subsp. *halodurans* NRRL B-3881 from a 24-h-old NB culture incubated at 37 C. Stained by Leifson's method. $\times 1,200$. Phase contrast.

NRRL B-3881 and ATCC 21591 grew only as pinpoint colonies, and NCTC 4553 did not grow when inoculated on plate count agar (Difco) incubated for 48 h at 37 C. However, the colony morphology of all three strains was similar on plate count agar containing 1% NaHCO_3 . After incubation for 48 h at 37 C, the colony diameters were 1 to 1.5 mm (NCTC 4553), 2.3 mm (NRRL B-3881), and 1.5 mm (ATCC 21591). The colonies were white, smooth, shiny, and convex with entire margins.

ATCC 21591 and NRRL B-3881 utilized citrate, reduced nitrate to nitrite, grew well in 12% NaCl, and grew slowly in 15% NaCl. NRRL B-3881 tolerated 9, 12, and 15% NaCl better than did ATCC 21591 (Table 1). *B. alcalophilus* did not grow in 5% NaCl. Only ATCC 21591 formed a pellicle on broth.

The differences between the three strains studied are summarized in Table 1.

A description of NRRL B-3881, designated below as the type strain of a new subspecies, follows. Vegetative cells are gram-positive, encapsulated, straight rods (0.9 to 1 μm by 3 to 4 μm) with rounded ends, occurring in small chains of two to six cells (Fig. 1). Chains were not produced in the growth on soybean agar. The cells were small, twisted, and bent when

TABLE 1. Differentiation of NRRL B-3881, ATCC 21591, and NCTC 4553^a

Characteristic	NRRL B-3881	ATCC 21591	NCTC 4553
Maximum temperature for growth in broth with aeration	>53 <54	>54 <57	>44 <46
Optimal growth temperature in broth with aeration	48	50.5	33-35
Doubling time at optimal temp with aeration	9 Min	8.5 Min	ND
Growth in NaCl ^b 0%	4+	4+, Pellicle	4+
5%	4+	4+, Pellicle	—
7%	4+	4+, Pellicle	—
9%	4+	3+, Pellicle	—
12%	4+	2+	—
15%	2+	1+, Very little growth	—
Hydrolysis of gelatin ^c	+, Good growth	+, Good growth	+, Little growth
Hydrolysis of casein ^c	+, Good growth	SI +, Good growth	SI +, Scant growth
Anaerobic growth	1+	2+	Scant growth
Reduction of methylene blue	+	++	SI +
Nitrate reduction	+	++	—
Growth in soybean broth	1+, Uniformly turbid	2+, Pellicle	1+, Uniformly turbid
Hydrolysis of starch ^c	+	+	+
Production of crystalline dextrins	—	—	—
Voges-Proskauer	—	—	—
Production of indole	—	—	—
Production of urease ^c	—	—	—
Utilization of citrate ^c	+	+	—

^a Symbols: ND, not done; SI +, slight reaction; +, positive; ++, more positive; —, negative.

^b Grown without shaking for 48 h at 37 C, pH 9.8.

^c 1% $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10 \text{H}_2\text{O}$ buffer (pH 8.8) instead of 1% NaHCO_3 buffer (pH 9.8) was used.

grown in media with no added NaCl at pH 7 to 8. The sporangia were swollen and clavate (Fig. 1). The spores were oval (0.5 to 0.8 μm by 1 to 1.5 μm), terminal to subterminal, and refractile (Fig. 1). Sporulation in the alkaline-amylase production medium at 37 C began at 12 h and was nearly complete after 48 h. The maximum temperature for growth in broth with aeration was between 53 and 54 C; the optimal temperature with aeration was 48 C. Without aeration, slow growth occurred at 26 C; a higher growth rate was obtained at temperatures above 40 C than at those below 33 C. Slow growth resulting in small colonies was obtained on TYG agar at pH 7.0; in nutrient broth (Difco) at pH 9, the growth was flocculent with a slimy sediment; there was growth but no sporulation on alkaline nutrient agar (Difco) slants and on alkaline soybean agar slants. The initial pH of glucose broth was 10, and the final pH was 8.98 after 18 h at 37 C. Growth was produced in a basal salts medium containing sodium citrate, magnesium sulfate, sodium phosphate, calcium chloride, sodium sesquicarbonate, ammonium sulfate, and carbohydrate at pH 9.3 (10). The addition of 5% NaCl to the basal medium at pH 6.6 resulted in growth, as measured by the change in optical density at 525 nm (ΔOD_{525}), equal to that obtained with a medium at pH 8.8 with no NaCl added (Table 2).

DISCUSSION

NRRL B-3881 is similar to ATCC 21591, but both are different from *B. alcalophilus* NCTC 4553. Both reduced nitrate to nitrite, utilized citrate, and grew well in 12% NaCl and slowly in 15% NaCl. However, *B. alcalophilus* NCTC 4553 did not utilize citrate, reduce nitrate to nitrite, or grow in 5% NaCl. There were some qualitative differences between NRRL B-3881 and ATCC 21591. However, in the genus *Bacillus* variations in characters frequently occur among strains within a species (9).

TABLE 2. Effect of NaCl and pH on *B. alcalophilus* subsp. *halodurans* NRRL B-3881

Medium	Initial pH	Final pH	ΔOD_{525}
Basal ^a	6.59	6.59	0
Alkaline basal ^b	8.79	8.54	0.36
Basal + 5% NaCl	6.59	6.86	0.36

^a Basal medium: 0.1% $(\text{NH}_4)_2\text{HPO}_4$, 0.02% KCl, 0.02% MgSO_4 , 0.02% yeast extract (Difco), and 1% lactose; pH 6.59.

^b Alkaline basal medium: basal medium plus 1% $\text{Na}_2\text{P}_2\text{O}_7 \cdot 10 \text{H}_2\text{O}$, pH 8.79.

ATCC 21591 and NRRL B-3881 can therefore be differentiated from *B. alcalophilus* on the basis of citrate utilization, nitrate reduction, and growth in salt solutions. In our opinion these differences are sufficient to justify the classification of NRRL B-3881 and ATCC 21591 in a new subspecies, for which we propose the name *Bacillus alcalophilus* subsp. *halodurans* (Gr. noun *hals*, *halis* salt; L. part. adj. *durans* hardening, resisting; M.L. part. adj. *halodurans* salt-resisting). NRRL B-3881 is designated as the type strain; a description of this strain is given above.

A composite description of *B. alcalophilus* subsp. *halodurans* subsp. nov. follows (the pH of the medium must be alkaline [preferably between pH 8 and 10] for normal growth and morphology; however, there is some growth at pH 7): gram-positive (rarely gram-variable), straight, motile rods with rounded ends and peritrichous flagella; sporangia are swollen and clavate; spores are oval, terminal and subterminal, and refractile; acetylmethylcarbinol, indole, urease, and crystalline dextrins are not produced; growth occurs in soybean broth; aerobic, facultatively anaerobic; starch, gelatin, and casein are hydrolyzed; methylene blue is reduced; sucrose, D-glucose, lactose, maltose, D-mannitol, D-xylose, L-arabinose, glycerol, sorbitol, and salicin are fermented without gas production; citrate is utilized; nitrate reduced to nitrite; good growth in 7% (one strain in 12%) NaCl; slow growth in 15% NaCl; maximum temperature for growth in broth with aeration, 53 to 55 C; optimal growth temperature in broth with aeration, 48 to 50 C; slow growth in broth without aeration at 26 C; colonies grown on plate count agar (Difco) containing 1% NaHCO_3 at 37 C for 48 h are white, smooth, shiny, and convex with entire margins.

A comparison of the characters reported here for *B. alcalophilus* NCTC 4553, one of Vedder's strains, with those originally described for 16 strains of *B. alcalophilus* by Vedder (11) shows that NCTC 4553 still has the characters of *B. alcalophilus* as originally described by Vedder. To our knowledge, a type strain has not previously been designated for *B. alcalophilus*; therefore, NCTC 4553 (=ATCC 27647) is here designated as the type strain of this species.

B. alcalophilus belongs to group II (9) of the genus *Bacillus*. Morphologically and physiologically it is related most closely to *Bacillus circulans*. However, it is not closely related to the strain of *B. circulans* described by Chislett and Kushner (3), which is capable of growing under highly alkaline conditions.

It is worthy of note that NRRL B-3881, the

type strain of *B. alcalophilus* subsp. *halodurans*, ordinarily does not grow in media below pH 7.0. However, if 5% NaCl is added to a medium at pH 6.6, it grows as well as it does in a medium at pH 8.8 without NaCl added. We cannot explain how the presence of 5% NaCl enables NRRL B-3881 to grow at pH 6.6. Further study on this phenomenon is warranted.

ACKNOWLEDGMENT

The technical assistance of Donald D. Stante is gratefully acknowledged.

REPRINT REQUESTS

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