Description of *Bacillus naganoensis* sp. nov.

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A new species, *Bacillus naganoensis*, is proposed for an obligately aerobic, moderately acidophilic, endospore-forming bacterium that produces a thermostable, acidic pullulanase (EC 3.2.1.41). The organism was isolated from soil by selection on solid, pullulan-containing medium at pH 4.0 and 30°C. The isolate required a medium pH of less than 6.5 for growth initiation. Fatty acid composition studies revealed that the major fatty acid of cells grown in nutrient broth supplemented with 1% starch was 14-methylpentadecanoic acid (iso-C_{16}) at 45 mol%. The guanine-plus-cytosine content of the DNA of this organism was 45 ± 2 mol%.

A type culture has been deposited with the American Type Culture Collection, Rockville, Md., as strain ATCC 53909.

Pullulanases (EC 3.2.1.41) are enzymes which specifically cleave the alpha-1,6-glycosidic linkages of pullulan, starch, glycogen, and amylopectin. Recently, these enzymes have found commercial utility in glucose- and maltose-manufacturing processes (10). Yeasts, higher plants, and microorganisms are known to be producers of pullulanase (17). Within the genus *Bacillus*, several species and strains have been reported to make pullulanase, including *Bacillus* sp. strain 202-1 (17), "*Bacillus acidopullulyticus*" (10), *Bacillus cereus* subsp. mycoides (23), *Bacillus macerans* (1), *Bacillus polymyxa* (9), and *Bacillus stearothermophilus* (13, 22). Of these, only the "*B. acidopullulyticus*" pullulanase has properties that allow its use in industrial saccharification processes.

In our search for a pullulanase that could operate under conventional glucose-manufacturing conditions (i.e., pH 4.3 and 60°C), we assumed that bacteria capable of growth at low pH values or high temperatures or both would produce acidic or thermostable enzymes. In this paper, we describe the isolation and characterization of a mesophilic, moderately acidophilic bacterium, designated *Bacillus naganoensis*, that makes a pullulanase with desirable acid and temperature stability characteristics.

**MATERIALS AND METHODS**

**Media and cultivation conditions.** Our initial screening medium (plate 1 medium) contained (per liter of distilled water) 1 g of yeast extract (Oxoid Ltd., Basingstoke, Hampshire, England), 2 g of tryptone (Difco Laboratories, Detroit, Mich.), 1 g of (NH_{4})_{2}SO_{4}, 0.3 g of KH_{2}PO_{4}, 0.2 g of MgSO_{4}·7H_{2}O, 10 mg of FeSO_{4}·7H_{2}O, 0.2 g of CaCl_{2}·2H_{2}O, 1 mg of MnCl_{2}·4H_{2}O, 20 g of agar, 10 g of soluble starch (Sigma Chemical Co., St. Louis, Mo.), 3 g of blue-colored soluble starch (20), and 7.5 g of red-colored pullulan. The pH was adjusted with 0.2 N sulfuric acid to pH 4.0. The color of the plates was dark violet. Incorporation of the two colored substrates into the plate medium allowed for the light purple-stained background of unhydrolyzed amylose to be distinguished from the dark blue zone, which is distinguishable from the light purple-stained background of unhydrolyzed amylopectin; amylose has a greater affinity for iodine than does amylopectin; thus, pullulanase activity can be detected by exposing plates to iodine vapors. A pullulanase-producing colony is surrounded by a dark blue zone, which is distinguishable from the light purple-stained background of unhydrolyzed amylopectin (G. C. Nielsen, I. V. Diers, H. Outtrup, and B. E. Norman, U.S. patent 4,560,651, December 1985). The pH values of both plate screening media were adjusted with 0.2 N sulfuric acid to pH 4.0.

**Growth characterization.** To determine the optimum temperature and pH for growth initiation, the organism was inoculated from a slant culture grown on plate 2 medium (pH 5.0) for 40 h into tryptic soy broth (Difco) containing 1% soluble starch or into the same medium amended with (per liter) 1 g of (NH_{4})_{2}SO_{4}, 0.3 g of KH_{2}PO_{4}, 0.2 g of MgSO_{4}·7H_{2}O, 0.2 g of CaCl_{2}·2H_{2}O, 10 mg of FeSO_{4}·7H_{2}O, and 1 mg of MnCl_{2}·4H_{2}O. The pH values of the media were adjusted with 4 N sulfuric acid. Growth was followed by determining values for optical density at 660 nm (OD_{660}) using a model DU-7 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Dilutions were made in phosphate-buffered saline (0.85%) to give OD_{660} readings ranging from 0.1 to 0.4.

**Biochemical characterization.** Biochemical tests with the isolate were performed by using the procedures of Gordon et al. (8), except that media were prepared at a pH of 5.5 (adjusted with 0.2 N sulfuric acid). Measurements of pH were used to assess acid production from sugars and organic acid utilization. To determine the fatty acid profile, the isolate was grown in nutrient broth supplemented with 1% starch (pH 5.5) at 33°C for 24 h. The fatty acids were analyzed by using the procedures of DeBoer and Sasser (4).
DNA base composition. High-molecular-weight DNA was prepared from lysozyme-treated cells by the method of Maniatis et al. (15). The guanine-plus-cytosine content was determined by thermal denaturation measurements (14).

Pathogenicity testing. The pathogenicity potential of the strain was tested in BALB/c mice as described by Reed and Coggin (19). Cells were grown in tryptic soy broth (pH 5.5) at 33°C for 24 h. Direct cell counts were obtained by using a Petroff-Hauser chamber, and 10-fold dilutions of the broth culture were made and introduced into the mice via oral or intraperitoneal routes (eight mice per group). Challenge doses as high as 10⁷ cells were administered. Animals were observed for 21 days and then examined postmortem.

RESULTS AND DISCUSSION

Isolation. A soil sample collected in the Kiso district of the Nagano Prefecture in Japan was suspended in sterile water (2 g of soil per 10 ml of water). A 0.1-ml portion of the suspension was spread onto plate 1 medium and incubated at 30°C. Discrete colonies which showed blue zones (i.e., were capable of hydrolyzing pullulan but not the straight-chain linkages of starch) were picked as presumptive pullulanase producers and transferred to plate 2 medium. Isolates which hydrolyzed the branched chains of amylopectin (i.e., showed blue zones surrounding the colonies on exposure to iodine vapors) were considered to be confirmed pullulanase producers. The pullulanase enzymes of these isolates were examined for temperature and pH stability (data not shown). One culture, designated D39, was found to produce a pullulanase of interest.

Morphology. Cells of strain D39 were rod shaped (0.5 to 1.0 by 2.1 to 10.0 μm) and occurred singly or in chains. The ends of the cells were rounded or square. The isolate was gram positive, as determined by conventional staining techniques. The endospores of this organism were oval and subterminally located and caused the sporangia to swell. No parasporal crystals were observed. Colonies were opaque, smooth, glistening, convex, and circular with entire margins. Moderate acido- phile; the pH range for growth is about 4.0 to 6.0. Growth occurs optimally at 30°C and does not occur at 20 or 45°C. Produces acid (after >14 days of incubation) from L-arabinose, D-xylose, D-glucose, D-mannitol, and lactose (weakly). No gas is produced from glucose. Starch hydrolysis, catalase, and growth in the presence of 5% NaCl are positive. Gelatin hydrolysis, casein hydrolysis, growth in the presence of 5% NaCl, phenylalanine deaminase, lecitinase, indole, Voges-Proskauer reactions, citrate utilization, and propionate utilization are negative. Does not decompose tyrosine or hippurate or produce dihydroxyacetone from glycerol. Reduction of methylene blue, reduction of nitrate to nitrite, and reduction of nitrite to N₂O are negative. The major fatty acid of the isolate grown in nutrient broth containing 1% starch is iso-C₁₆ (about 45 mol%). The guanine-plus-cytosine content is 45 ± 2 mol%. A subculture of B. naganoensis has been deposited in the American Type Culture Collection, Rockville, Md., as strain ATCC 53909; this is the type strain by monotypy.

Characteristics which distinguish B. naganoensis from related bacteria. At present, aerobic, endosporeforming, rod-shaped bacteria are classified in the genus Bacillus (3), which contains 33 species on the Approved Lists of Bacterial Names (21), as well as several recently described species (2, 5–7, 16, 18). Only five of these species are similar to B. naganoensis in that they are strict aerobes that produce oval spores which cause the sporangium to swell (Bacillus acidoterrestris, Bacillus cycloheptanicus, Bacillus globisporus, Bacillus schlegelli, and Bacillus sphaericus). B. cycloheptanicus and B. schlegelli are obligate thermophiles, while B. globisporus is a psychrophile; therefore, these organisms differ from B. naganoensis. Moreover, B. sphaericus, unlike B. naganoensis, does not produce acid from D-glucose, L-arabinose, D-xylose, or D-mannitol. B. acidoterrestris produces α-cyclohexane fatty acids and thus is distinguishable from B. naganoensis.

The fatty acid content of B. naganoensis appears to be unique. Kaneda (11) has divided the genus Bacillus into six groups based on the predominant fatty acids and the range of fatty acid chain lengths. B. naganoensis produces fatty acids...
with chains of 14 to 17 carbon atoms, a characteristic of four of the six groups; however, the predominant fatty acid of \( B.\ naganensis \) is iso-C\(_{16}\) (approximately 45 mol%). None of the groups of Kaneda have iso-C\(_{16}\) as the predominant fatty acid; thus, \( B.\ naganensis \) is novel in its fatty acid composition.

\( B.\ naganensis \) is different from the previously described pullulanase-producing \textit{Bacillus} strains. \textit{Bacillus} sp. strain 202-1 is an obligately alkalophilic organism (17); \( B.\ cereus \) is a Voges-Proskauer-positive, facultative anaerobe (3); \( B.\ macerans \) and \( B.\ polymyxa \) are facultative anaerobes that do not produce oval spores (3); and \( B.\ stearothermophilus \) is an obligate thermophile that does not produce oval spores and does not grow at a pH value less than 6 (3). \( B.\ naganensis \) and "\( B.\ acidopullulyticus \)" differ in their ability to cause sporangia to swell and in their ability to reduce nitrate (10). Furthermore, an analysis of the fatty acids from "\( B.\ acidopullulyticus \)" showed that this organism produces fatty acids with chains of 12 to 17 carbon atoms and that the predominant fatty acid is iso-C\(_{15}\) (data not shown). This places "\( B.\ acidopullulyticus \)" in group E of Kaneda (11).

**ACKNOWLEDGMENTS**

We thank American Type Culture Collection personnel for performing much of the biochemical testing, Microcheck, Inc. personnel for the fatty acid analyses, and J. C. Coggin, Jr. and Tin Cao of the University of South Alabama for determining the guanine-plus-cytosine content and for conducting the virulence potential studies.

**LITERATURE CITED**