Assignment of Facultatively Alkaliphilic *Bacillus* sp. Strain C-125 to *Bacillus lentus* Group 3

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Facultatively alkaliphilic *Bacillus* sp. strain C-125 grew at temperatures of more than 50°C, did not reduce nitrate to nitrite, and did not deaminate phenylalanine. The guanine-plus-cytosine content of the chromosomal DNA of this organism was 42 mol%. These characteristics indicate that strain C-125 is related to *Bacillus lentus* group 3.

A large number of alkaliphilic *Bacillus* spp. strains have been isolated. Most of these strains have been surveyed for production of extracellular enzymes that are stable over a broad pH range and active at alkaline pH values ranging from 10 to 12. Because of their stability and suitability for industrial applications, enzymes produced by alkaliphiles have interested applied enzymologists (20, 21). Alkaliphiles are extremophiles that can grow at pH 10 or higher. A few *Bacillus* strains have been investigated as model systems for understanding the mechanisms of alkaliphily and alkali tolerance. One such organism is *Bacillus* sp. strain C-125, a facultative alkaliphilic strain. This organism is assigned to the genus *Bacillus* because of its gram-positive staining characteristics and possible aerobic sporulation (22). The cell wall structures of this organism have been studied extensively (1, 2, 12). Strain C-125 might adapt to alkaline environments by altering its cell surface (4, 6, 7, 10). It is very important that gene manipulation and cell fusion systems be established in this organism (3, 7, 8). The bioenergetics of the alkaliphilic organisms *Bacillus firmus* RAB and OF4 has been studied previously (17, 18). Determining the taxonomic position of strain C-125 is essential for comparing data obtained with other alkaliphilic *Bacillus* strains.

Strain C-125 was grown primarily at 37°C in an alkaline complex medium (pH 10) containing 0.1 M Na₂CO₃ (4), unless specified otherwise. The same medium containing 0.2 M NaCl instead of Na₂CO₃ was adjusted to pH 7.2 (4). This medium was adjusted to a variety of pH values with Na₂CO₃ and NaOH (9) for observing the morphological characteristics of strain C-125 cells. If necessary, the media were solidified with 1.5% (wt/vol) agar.

Morphological characteristics of strain C-125 were determined by conventional methods (13) by using cells grown at different constant pH values in pH-stat cultures. The morphology of strain C-125 varied with the culture pH, the cultivation temperature, and the growth phase. The cells grew singly and in pairs at 37°C and pH 7.5 to 10 (Fig. 1B and C). A few cells formed long chains during the exponential phase of growth. The organism grew in filaments at pH 6.8 and 10.8 (Fig. 1A and D). These pH values were close to the lower and upper pH limits for growth. Most filaments were coiled at pH 6.8 and straight at pH 10.8 (Fig. 1A and D); this coiling was more pronounced at 30°C than at 37°C (data not shown). Most cells grown at pH 7.0 to 7.5 formed coiled filaments at 30°C. Old cultures contained many long chains and filaments; extremely long filaments were formed at pH 10.8 (Fig. 1E to H). The rate of cross-linking among peptide moieties of peptidoglycans de-

pends on the culture pH (11); low cross-linking rates might be one explanation for coil formation at pH 7. The cell dimensions depended on the culture pH. Cells grown at lower pH values were shorter and wider than cells grown at higher pH values. Cells grown at pH values greater than 8 were straight rods with round ends, and cells grown at pH 6.8 appeared to be coccobacillary (Table 1).

The motility of cells was determined by the hanging drop method. Flagella were observed microscopically by using cells stained with ammoniacal AgNO₃ after treatment with tannic acid (9) and electron microscopically by using cells that had been negatively stained. The cells had numerous peritrichous flagella and were actively motile when they were grown at an alkaline pH. Flagellin synthesis depends on the culture pH (9). Most cells grown at pH values below 7 had no flagella or a few short flagella. Sporulation frequency was somewhat dependent on pH. Strain C-125 sporulated abundantly at pH 7 to 9, but less effectively at pH values above 10.

Strain C-125 formed circular, convex, entire, smooth, cream-white colonies on the neutral (pH 7.2) agar. Initially the colonies were circular, raised, entire, rough, and cream-white on the alkaline (pH 10) agar; these colonies gradually became irregular and undulate. The colonies were butyrous on each type of agar. No water-soluble pigment was formed around the colonies. Thus, colonial appearance varied depending on the pH of the agar medium. The variable appearance was probably due to differences in the amounts of cell wall components at different culture pH values (1, 4).

Strain C-125 grew at relatively high temperatures (up to 53°C at pH 7 and up to 57°C at pH 10) (Table 1). Interestingly, this organism did not grow as well at pH 7 as it did at pH 10 at low temperatures; the minimum temperatures for growth were 20°C at pH 10 and 30°C at pH 7 when growth was observed after incubation for 5 days. The effect of pH on growth was determined in pH-stat cultures by using the complex medium and a variety of pH values at 37°C. The doubling times of the organism grown in pH-stat cultures were about 1, 0.5, and 2 h at pH 6.8, 9.0, and 10.8, respectively. Strain C-125 grew in the presence of 10% (wt/vol) NaCl in the neutral medium containing 0.1% KH₂PO₄ (initial pH, 8).

Vitamin requirement, nitrate assimilation, and carbon compound utilization tests were performed by using an alkaline synthetic basal medium. The alkaline synthetic medium (pH 10) used previously (4, 10) was modified so that it contained (per liter of deionized water) 13.7 g of K₂HPO₄, 5.9 g of KH₂PO₄, 10.6 g of Na₂CO₃, 0.05 g of nitrilotriacetic acid, 0.05 g of MgSO₄ · 7H₂O, 5 g of glucose, 1 g of (NH₄)₂SO₄, and 1 g of KNO₃. The carbon and nitrogen sources were varied in each test. Other cultural characteristics were determined by stan-

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FIG. 1. Cellular morphology of strain C-125. Alkaliphilic *Bacillus* sp. strain C-125 was grown aerobically in the complex medium at 37°C, and cellular morphology was observed at the late exponential phase (A to D) or stationary phase (30 h) (E to H) of growth. The culture pH was kept maintained at pH 6.8 (A and E), pH 7.5 (B and F), pH 9.0 (C and G), or pH 10.8 (D and H). The same magnification was used for all micrographs. Bar = 10 μ m.

TABLE 1. Characteristics of alkaliphilic *Bacillus* sp. strain C-125^a

Characteristic	Strain C-125
Morphological characteristics	
Cell shape	Rods, short rods, coccobacilli
Cell size at pH 7.5 to 10 (µm)	0.4–0.7 × 1.9–3.4
Gram stain	+
Motility at pH 7	–
Motility at pH 10	+
Flagella at pH 7	None or few
Flagella at pH 10	Peritrichous
Sporulation	+
Spore shape	Ellipsoidal
Spore size (µm)	0.5–0.7 × 1.0–1.2
Sporangium shape	Swollen
Position of sporangia	Central to subterminal
Cultural characteristics	
Reaction to oxygen	Strictly aerobic
Oxidation-fermentation test	Oxidative
Growth temp range at pH 7 (°C)	30–53
Growth temp range at pH 10 (°C)	20–57
Growth pH range at 37°C	6.8–10.8
NaCl tolerance (%)	≤10
Assimilation of nitrate	+
Reduction of nitrate to nitrite	–
Indole production	–
Levan formation	–
Splitting of MUG	+
Hydrolysis of gelatin	+
Hydrolysis of casein	+
Hydrolysis of starch	+
Hydrolysis of pullulan	+
Hydrolysis of Tween 60	+
Hydrolysis of Tween 80	–
Hydrolysis of hippurate	+
Vitamin requirement	–
Biochemical characteristics	
Oxidase activity	+
Catalase activity	+
Arginine dihydrolase activity	–
Phenylalanine deaminase activity	–
Lecithinase activity	–
Urease activity	–
G+C content of DNA (mol%)	42.2
Menaquinones ^b	MQ-7, MQ-6, MQ-5
mDAP in peptidoglycan	+

^a +, positive; –, negative; MUG, 4-methyl-umbelliferyl-β-D-glucuronide; MQ, menaquinone; mDAP, meso-diaminopimelic acid.

^b The percentages of the menaquinones are as follows: menaquinone 7, 68%; menaquinone 6, 25%; menaquinone 5, 7%.

dard methods (25). Strain C-125 grew by assimilating nitrate instead of ammonium ions, but did not exhibit anaerobic denitrification. This organism was strictly aerobic and positive for oxidase and catalase activities. The enzyme activities listed in Table 1 were determined by standard methods (25). The carbon sources assimilated by the organism included several pentoses, hexoses, hexitol, and oligosaccharides (Table 2).

The chromosomal DNA of strain C-125 was digested with nuclease P1 (26). The resulting nucleotides were separated by high-performance liquid chromatography by using a Cosmosil 5C₁₈ column (4.6 by 150 mm; Nakalai Tesque, Kyoto, Japan) that was eluted with 10 mM KH₂PO₄–NaOH buffer (pH 7.0) at a flow rate of 1 ml/min. The G+C content of the chromosomal DNA was 42 mol%.

Cells grown in the alkaline complex medium were extracted twice with methanol-chloroform (1:2, vol/vol). The resulting extracts were washed with water and concentrated to dryness in a rotary evaporator. The acetone-soluble apolar lipids were obtained from the residue and resolved on a silica gel thin layer in *n*-hexane–diethylether (4:1, vol/vol). Yellow compounds with an *R_f* value corresponding to the *R_f* value of menaquinone prepared from *Bacillus subtilis* GSY1026 (4) were recovered and applied to a reverse-phase column (ODS-H-1251; 4.6 by 250 mm; Senshu Science, Tokyo, Japan), and the column was eluted with an acetonitrile-isopropanol mixture (23). The isoprenoid quinones were detected by measuring *A*₂₇₀. The quinone was measured by using menadione as a standard. The menaquinone system of the organism was similar to that found in *B. subtilis*. No ubiquinone-like pigment was found in the lipid fraction. The isoprene side chains of the quinone ranged from five to seven isoprene units long. The predominant quinone was menaquinone 7. The quinone composition depended to some extent on the culture pH (data not shown).

Peptidoglycan was prepared from strain C-125 as described previously (5). The isomerism of the diaminopimelic acid was determined by thin-layer chromatography (19) of peptidoglycan hydrolysates (4 M HCl, 105°C, 15 h). The peptidoglycan type of strain C-125 was type A1γ. The γ-carboxyl group of L-glutamic acid or the ω-carboxyl group of meso-diaminopimelic acid was not amidated. The glycan chain was composed of *N*-acetylglucosamine and *N*-acetylmuramic acid (4). This subtype of type A1γ peptidoglycan is found in the cell

TABLE 2. Comparison of the properties of strain C-125 and *B. firmus*–*B. lentus* intermediates

Taxon	Carbohydrate utilization ^a														Decomposition of:		Production of urase	Reduction of nitrate to nitrite	Deamination of phenylalanine	Hydrolysis of hippurate	Growth at 50°C	Sensitivity to <i>B. firmus</i> ATCC 5475 bacteriophage ^b
	Ara	Rib	Xyl	Glc	Fru	Gal	Man	Mal	Mel	Lac	Suc	Tre	Raf	Sor	Casein	Gelatin						
<i>B. firmus</i>	– ^c	NR	–	+	NR	NR	–	NR	–	NR	+	NR	–	–	+	±	–	±	+	–	–	+
Intermediates	±	NR	±	+	NR	NR	±	NR	±	NR	±	NR	±	±	±	±	±	±	±	±	±	±
<i>B. lentus</i> group 1	+	NR	+	+	NR	NR	+	NR	+	NR	+	NR	+	±	±	±	–	±	–	–	–	–
<i>B. lentus</i> group 2	+	NR	+	+	NR	NR	+	NR	+	NR	+	NR	+	±	±	±	–	±	–	–	–	–
<i>B. lentus</i> group 3	+	NR	+	+	NR	NR	+	NR	+	NR	+	NR	+	–	±	±	–	±	–	–	–	–
Strain C-125	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	NT

^a Abbreviations: Ara, L-arabinose; Rib, D-ribose; Xyl, D-xylitol; Glc, D-glucose; Man, D-mannose; Mal, maltose; Mel, melibiose; Lac, lactose; Suc, sucrose; Tre, trehalose; Raf, raffinose; Sor, sorbitol. The utilization of carbohydrates by strain C-125 was determined in this study; the utilization of carbohydrates by other strains was determined by examining acid production from carbohydrates in another study (15).

^b See reference 15.

^c +, strains are positive; –, strains are negative; ±, some strains are positive and some strains are negative; NR, not reported; NT, not tested.

TABLE 3. Comparison of the properties of strain C-125 and the six groups classified by Fritze et al.^a

Taxon	G+C content (mol%)	Colony color	Growth at 50°C	Deamination of phenylalanine	Reduction of nitrate
Group 1	38.2–40.8	Bright yellow	–	+	–
Group 2	38.2–40.8	Dull yellow	–	–	–
Group 3	42.1–43.9	Cream-white	+	–	+
Group 4	42.1–43.9	Cream-white	+	–	–
Group 5	34.0–37.5	Cream-white	–	–	–
Group 6	34.0–37.5	Cream-white	–	–	+
Strain C-125	42.2	Cream-white	+	–	–

^a The properties of strain C-125 were determined in this study, and the data for the other taxa were obtained from reference 14.

walls of *B. firmus*, *Bacillus lentus*, and *Bacillus megaterium*, but not in the cell walls of *Bacillus licheniformis* or *B. subtilis* (24).

Several other alkaliphilic *Bacillus* spp. strains also grow at pH 7 (4). Gordon and Hyde investigated variants capable of growth at pH 7.0 that were obtained from 174 alkaliphilic *Bacillus* strains (15). These organisms were assigned to five subgroups of the *B. firmus*-*B. lentus* complex on the basis of their physiological characteristics. The characteristics of neutrophilic strains of *B. firmus* and *B. lentus* are very diverse (16). The characteristics of the pH 7.0 variants obtained from the alkaliphilic strains are also very diverse. The variants designated *B. lentus* were further subdivided into three groups on the basis of growth at 50°C, sensitivity to some bacteriophages, and reduction of nitrate to nitrite, as shown in Table 2.

Alkaliphilic *Bacillus* sp. strain C-125 grew at 57°C on the alkaline agar and at 53°C on the neutral agar. Nitrate was not reduced to nitrite by strain C-125. These properties are consistent with the properties of *B. lentus* group 3. Strain C-125 is not related to *B. firmus*. The cellular morphology of *B. lentus* group 3 as described by Gordon and Hyde was similar to the morphology of strain C-125 grown at 37°C and pH 7.5 to 10. The spore and sporangium characteristics of *B. lentus* group 3 were also the same as those of strain C-125 at pH 7 to 9. The other properties of *B. lentus* group 3 were also consistent with those of strain C-125, as shown in Table 2. Therefore, strain C-125 is most similar to *B. lentus* group 3.

The DNA base compositions of several alkaliphilic *Bacillus* spp. strains have been determined by the buoyant density centrifugation method (14) or by chemical analysis of nucleosides released enzymatically from DNA (26). A wide range of DNA base compositions has been found in the alkaliphiles. The alkaliphilic *Bacillus* strains have been arbitrarily divided into three groups on the basis of their DNA G+C contents as follows: group A, 34.0 to 37.5 mol%; group B, 38.2 to 40.8 mol%; and group C, 42.1 to 43.9 mol% (14). Each group has been further subdivided into two phenotypic groups, as shown in Table 3. Table 3 also shows the properties that were used to classify the six phenotypic groups. The G+C content of strain C-125 is the same as the G+C contents of members of groups 3 and 4 proposed by Fritze et al. (14). The colony color and other phenotypic characteristics of strain C-125 indicated that this organism belongs to group 4. This group contains all but one strain of *B. lentus* group 3 of Gordon and Hyde (14, 15). My observations indicated that strain C-125 is related to *B. lentus* group 3 of Gordon and Hyde.

This work was supported in part by a grant for the "Biodesign Research Program" from RIKEN to R. Aono.

I thank M. Yamamoto, M. Ogino, and H. Kaneko for assistance, and I am indebted to K. Horikoshi for earnest discussions.

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