Taxonomic Characteristics of a Thermophilic Acidophilic Strain of *Bacillus* Producing Thermophilic Acidophilic Amylase and Thermostable Xylanase

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Taxonomic characteristics of a strain of thermophilic acidophilic bacillus, *Bacillus* sp. 11-1S, which had the ability to produce thermophilic acidophilic amylase and thermostable xylanase were examined. Cells of the organism were aerobic, heterotrophic, Gram-positive, spore-forming rods. It grew at temperatures between 45 and 70°C (optimum 65°C) in media of pHs ranging from 2.0 to 5.0 (optimum 3.5~4.0). Physiological and biochemical characteristics were identical with those of *Bacillus acidocaldarius*, and % GC of DNA (59%) was close to that of the latter (61~62%). From these results it was concluded that the organism belongs to *B. acidocaldarius* Darland and Brock.

In previous works, we have shown that a strain of thermophilic acidophilic bacillus, *Bacillus* sp. 11-1S, is capable of production of a thermophilic, unusually acidophilic amylase¹⁾ and a thermostable xylanase,²⁾ and their enzymatic properties have been characterized. It was of interest to determine the taxonomic position of the bacterium. In the present paper we report the taxonomic characteristics of the organism. It grows at temperatures between 45 and 70°C in media of pHs ranging from 2 to 5, and is an aerobic, heterotrophic, Gram-positive and spore-forming rod. Its characteristics are identical with those of *Bacillus acidocaldarius* Darland and Brock.^{3,4,11)}

MATERIALS AND METHODS

Bacterial strain. *Bacillus* sp. 11-1S is one of the bacteria isolated by us from hot-spring waters in the Hokkaido area of Japan. The isolation procedures were similar to those described previously⁵⁾ except that the enrichment was performed at 65°C using the following medium with a pH of 3.5. The basal salt medium, to which 0.5% of glucose and 0.1% of yeast extract (Difco) were added, was used for the enrichment. The organism had been maintained on an agar slope of the same composition.

Basal salt medium. The basal salt medium consisted of KH_2PO_4 2 g, NaCl 1 g, NH_4Cl 2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g,

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 25 mg, MnSO_4 25 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g, and distilled water to 1 liter.

Growth experiments. For growth studies, a carbon source (usually glucose, 0.5%) was supplemented to the basal salt medium and the medium was adjusted to desired pHs with 3N HCl or 2N NaOH. To determine specific growth rate, L-shaped tubes (Monod-type, 100 × 80 × 18 mm) containing 6 ml of medium were shaken (70 strokes per min) in a constant temperature water bath. In the case of stationary cultures, test tubes (180 × 18 mm) containing 5 ml of medium were used. Growth was measured turbidimetrically with an ADS electrophotometer (Fuji Kogyo Co.) with a No. 660 filter. Specific growth rate was calculated from the data obtained during the exponential phase of growth.

Morphological studies. Morphological properties were examined by conventional methods.⁶⁾

Utilization of carbon compounds. Carbohydrates and other carbon compounds were tested for utilization as the sole source of carbon and energy. The basal salt medium (pH 4.0) supplemented with 0.5% of the compounds was incubated for 4 days at 65°C without shaking.

Physiological and biochemical reactions. Starch hydrolysis was detected by flooding 0.02N I_2 -KI solution over the agar plate of the basal salt medium (pH 4.2) containing starch (0.5%) and peptone (Difco, 0.5%) after incubation for 1 and 2 days at 65°C.

The Voges-Proskauer reaction and indole production were tested according to the methods described⁶⁾ except

that the media of a pH of 4.5 were incubated at 65°C.

The test for nitrate as a source of sole nitrogen was carried out at 65°C using the basal salt medium with glucose by replacing NH_4Cl with KNO_3 or NaNO_3 .

Gelatin hydrolysis was detected by examination at 0°C of liquefaction of the basal salt medium (pH 4.4), to which 0.5% glucose and 15% gelatin were added, after incubation for 2 days at 65°C.

For testing anaerobic growth, test tubes containing the basal salt medium supplemented with 0.5% glucose and 0.1% yeast extract, in the presence or absence of 0.1% KNO_3 , were evacuated and flushed with N_2 gas after inoculation. The tubes were incubated for 6 days at 65°C.

%GC of DNA. DNA was extracted by the methods of Saito and Miura⁷⁾ and further purified by hydroxyapatite chromatography.⁸⁾ The guanine-plus-cytosine content (mol%) was estimated chromatographically⁹⁾ after hydrolysis with Nuclease P_1 .¹⁰⁾

The other physiological and biochemical tests were performed by conventional methods.⁶⁾

Materials. RNase I and RNase T_1 were purchased from Worthington Biochemical Corp., Freehold, N.J., USA; hydroxyapatite (DNA-grade Bio-Gel HTP) and ion-exchange resin AG 50W-X4 (-400 mesh) were from Bio-Rad Laboratories, Richmond, CA, USA; Nuclease P_1 was from Yamasa Shoyu Co., Choshi, Japan; and 5'-deoxymononucleotides were from Sigma Chemical Co., St. Louis, MO, USA. The other chemicals were of analytical grade commercially available.

RESULTS

Morphology

The cells of *Bacillus* sp. 11-1S were Gram-positive rods (0.6~0.7 × 1.5~2.5 μm), occur-

FIG. 1. Photomicrograph of *Bacillus* sp. 11-1S.

Cells grown in glucose-yeast extract-basal salt medium for 12 hr at 65°C without shaking, stained with crystal violet. The scale bar indicates 10 μm .

red singly or in pairs (rarely filamentous), and formed refractile endospores (oval form) located terminally to subterminally. The sporangia were slightly swollen. Heat resistance of the spores was demonstrated by heating for 3 min in boiling water.

On an agar plate of glucose-yeast extract-basal salts after 1 day incubation at 60°C, the unpigmented colonies were irregular with an undulate margin, convex elevation, smooth surface, translucent and butyrous.

In unaerated broth cultures, membranous surface growth was noted.

Temperature and pH relationships

The organism grew in the range from 45 to 70°C and at pHs from 2.0 to 5.0, but no growth was observed at temperatures of 40°C or lower, nor at pH 1.5 or 5.5. The optimal temperature and pH were found to be 65°C and 3.5~4.0, respectively. In shake cultures, the specific growth rates at 65°C and 45°C were 1.12 and 0.263 (hr^{-1}), respectively.

Physiological and biochemical reactions

1) *Relation to O_2 .* The organism was aerobic and failed to grow anaerobically in the presence or absence of nitrate.

2) *Nitrate reduction.* It failed to reduce nitrate to nitrite.

3) *Voges-Proskauer reaction.* The test was negative.

4) *Production of indole.* The organism was negative.

5) *Hydrolysis of starch.* The organism hydrolyzed it considerably.

6) *Hydrolysis of gelatin.* Liquefaction was observed.

7) *Growth-factor requirements.* It did not require any growth factors and grew in a medium of basal plus glucose or galactose.

8) *Nitrate as nitrogen source.* It was unable to utilize nitrate as a sole source of nitrogen.

9) *Utilization of carbon compounds.* Table I shows that the organism utilized 8 kinds of compounds tested as a source of carbon and energy. Organic acids such as

TABLE I. UTILIZATION OF CARBON COMPOUNDS AS SOLE SOURCES OF CARBON AND ENERGY

Bacillus sp. 11-1S was grown in basal salt medium (pH 4.0) supplemented with the indicated carbon source (0.5%) for 4 days at 65°C.

Compound	11-1S	<i>B. acidocaldarius</i> ⁴⁾
Glucose	+	+
Galactose	+	+
Casamino acids **	+	+
Starch	+	+
Glycerol	+	+
Sucrose	+	+
Gluconate	+	+
Inositol	—	v
Ribose	—	v
Rhamnose	+	v
Lactose	—	v
Ethanol	—	—
Sorbitol	—	—
Acetate	—	—
Succinate	—	—
Citrate	—	—

* +, positive; —, negative; v, variable between strains.

** Bacto-Casamino acids (Difco).

acetate, succinate and citrate were not utilized for growth.

%GC of DNA

The guanine-plus-cytosine mol% of the DNA was estimated to be 59.2.

DISCUSSION

The fact that the cells of the bacterium were aerobic, Gram-positive and spore-forming rods indicates that the organism should be classified in the genus *Bacillus*.

Limits of temperature and of pH for growth were 45~70°C and 2.0~5.0, respectively, and

it grew with glucose, galactose, starch, glycerol and Casamino acids as the sole source of carbon and energy, but not with ethanol, sorbitol, acetate, succinate or citrate. Growth occurred with ammonium salt but not with nitrate as the nitrogen source. No growth factors were required for growth of the organism. These results were identical with those of *B. acidocaldarius*,⁴⁾ and the GC mol% of DNA (59.2) was close to that of the latter (61~62).

Based on the bacterial properties mentioned above, *Bacillus* sp. 11-1S can be identified as *Bacillus acidocaldarius* Darland and Brock.^{3,4,11)}

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