Bacillus thermoruber sp. nov., nom. rev., a Red-Pigmented Thermophilic Bacterium

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The morphological, cultural, and physiological properties of a new species of *Bacillus* are reviewed. This microorganism, for which we propose the name *Bacillus thermoruber*, is motile, produces terminal or subterminal oval endospores in swollen sporangia, and shows optimal growth at 45 to 48°C. It is characterized by a high deoxyribonucleic acid guanine-plus-cytosine content (57 ± 0.8 mol%) and a red, endocellular, nondiffusible pigment. The type strain is strain BT2, which has been deposited in the collection of the Cattedra di Microbiologia Industriale, Università degli Studi di Milano (Italy) as strain MIM 30.8.38.

In the course of a study on thermophilic microfloras of the compost used for the production of mushrooms (*Agaricus campestris* var. *bispora*), we isolated a rod-shaped, sporeforming, aerobic, thermophilic bacterium belonging to the genus *Bacillus*. This microorganism produced a characteristic red, nondiffusible pigment. According to its morphological, cultural, and physiological properties, this strain could not be identified as a member of any species described previously. Therefore, we believe that this bacterium represents a new species of the genus *Bacillus* (2, 10).

Although this species has been described previously (2, 10) as "*Bacillus thermoruber*," this name did not appear on the Approved Lists of Bacterial Names (22). Therefore, a formal description which includes new data to support designation of a new species is given in this paper.

MATERIALS AND METHODS

Microorganism. Strain BT2^T (T = type strain) was grown on TER/1 agar slants (4) and was maintained at 4°C after incubation for 48 to 72 h at 45°C. TER/1 medium contained 20 g of crude maltose, 8 g of soya meal, 3 g of yeast extract (Difco Laboratories, Detroit, Mich.), and 20 g of agar in 1,000 ml of tap water (pH 7); sterilization was done at 121°C for 30 min.

Morphological characteristics. Morphological properties were determined by using 8-h- to 3-day-old cultures grown on TER/1 agar slants at 45°C. Gram and spore staining was done as described by Salle (19).

Electron microscopy. Cells grown in broth culture were washed twice with a sterile physiological solution. One drop of the washed cell suspension was placed on a polyvinyl-Formvar-coated 300-mesh copper grid. Excess liquid was removed, and 1 drop of 2% phosphotungstic acid was added. The grid was allowed to air dry. Specimens were examined with a Siemens Elmiskop model 1A electron microscope; photographs were taken on Afga Scientia plates.

Cultural and physiological characteristics. The media and procedures used for cultural and physiological characterizations were those described by Smith et al. (23), with some modifications. Unless otherwise indicated, the carbon sources used in the media were dissolved in distilled water and sterilized separately at 112°C for 30 min, and all tests were performed at 45°C for different times (1 to 5 days). Growth was also assessed on malt agar, malt broth, potato-glucose-yeast extract agar and broth (3), skim milk, TER/1

agar, TER/1 broth (at pH 5, 6, 7, and 8), and GYE agar and broth (containing 15 g of glucose, 5 g of yeast extract [Difco], and 0.2 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1,000 ml of tap water, pH 7). The temperature range and optimum temperature for growth were determined on GYE agar and broth and on potato-glucose-yeast extract agar by observing growth at different temperatures in a variable-temperature incubator (Politermomatic-Rossi, Piacenza, Italy) (10). Basic nutrient requirements were determined by using the following media: SM medium [containing 3 g of $(\text{NH}_4)_2\text{SO}_4$, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g of NaCl, and 0.1 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1,000 ml of distilled water, pH 7], SM medium supplemented with 0.1 g of yeast extract per liter, and SM medium supplemented with vitamins (100 µg of biotin per liter, 400 µg of calcium pantothenate per liter, 100 µg of folic acid per liter, 400 µg of niacin per liter, 300 µg of *p*-aminobenzoic acid per liter, 400 µg of pyridoxine hydrochloride per liter, 300 µg of riboflavin per liter, and 400 µg of thiamine hydrochloride per liter). Vitamin requirements were determined by the single-deletion technique (24).

Utilization of compounds as sole sources of carbon was determined by using SM medium lacking glucose but containing 0.1 g of yeast extract per liter. The test was performed by using sugars at concentrations of 0.5%, alcohols or organic acids at concentrations of 0.2%, and 0.01% phenol red as the indicator. The following substrates were used: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, D-melibiose, L-rhamnose, D-ribose, sorbitol, sucrose, trehalose, D-xylose, acetate, citrate, DL-lactate, L-malate, succinate, pyruvate, L-aspartate, and L-glutamate. The utilization of compounds as sole nitrogen sources was tested by using SM medium without $(\text{NH}_4)_2\text{SO}_4$ but containing 0.1 g of yeast extract per liter. The following nitrogen sources were tested: $(\text{NH}_4)_2\text{SO}_4$ (3 g/liter), NaNO_3 (3 g/liter), urea (3 g/liter), tryptone (Difco) (1 g/liter), Neopeptone (Difco) (1 g/liter), and soytone (Difco) (1 g/liter). Inoculated minimal media without added carbohydrates or N sources were used as controls. All tests were performed in 100-ml Erlenmeyer flasks containing 10 ml of medium. Each flask was inoculated with 0.1 ml of inoculum grown at 45°C for 12 h, incubated at 45°C in an alternating shaker (100 4-cm strokes per min), and examined after 24, 48, and 72 h. Cell density was measured at 650 nm by using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) immediately after inoculation and after incubation for different lengths of time. A wavelength of 650 nm was chosen because in the carbo-

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FIG. 1. Electron micrograph of *Bacillus thermoruber* strain BT2^T. Bar = 1 μ m.

hydrate test phenol red was used as the indicator. All tests were concluded after two subcultures on each medium.

Catalase production was determined by using cultures grown on TER/1 agar slants. The heat resistance of the spores was assessed by using a spore suspension in sterile 50 mM tris(hydroxymethyl) aminomethane (pH 7) containing about 10^8 spores per ml. After 20 min of incubation at different temperatures (80, 90, 100, 105, 110, 112, 114, and 116°C), 1 ml of the spore suspension was smeared on to TER/1 agar. Susceptibility to antibiotics was examined by using TER/1 agar and penicillin, tetracycline, vulcamycin, or chloramphenicol added at different concentrations. The results were recorded after 48 h of incubation at 40°C.

Pigment extraction. The red pigment was extracted and characterized by the method of Guicciardi et al. (10), with a minor modification. The cells (30 g, wet weight) were suspended in 50 ml of distilled water containing 30 mg of lysozyme (Boehringer, Mannheim, Federal Republic of Germany) and incubated at 37°C for 1 h. The mixture was centrifuged at $48,000 \times g$ for 30 min at 4°C, and the aqueous phase was removed. The pellet was washed with acetone, and the pigment was extracted several times with ethyl ether-ethanol (1:1, vol/vol) containing 1% gaseous HCl. Extracts were collected and evaporated, and the residue was dissolved in water and extracted with *n*-butanol. Crude pigment was purified by column chromatography on type MN300 cellulose (Macherei, Nagel & Co., Düren, Federal Republic of Germany) as previously described (10).

Cellular fatty acid composition. Fatty acids analysis was performed by using the procedure of Aragazzini et al. (1). Strain BT2^T was grown for 4 days at 45°C on an alternating shaker (100 strokes per min) in medium containing 20 g of glucose, 2 g of yeast extract, and 0.5 g of K_2HPO_4 in 1,000 ml of distilled water (pH 7). The cells were hydrolyzed by refluxing them under nitrogen for 4 h with 10% KOH in 50% aqueous methanol; the fatty acids were extracted with hexane, and methyl esters were prepared by treatment with diazomethane. Fatty acid composition was determined by gas-liquid chromatography (Fractovap G₁; Carlo Erba, Milan, Italy), using the following conditions: flame ionization detector; glass column (4 mm by 2.0 m) containing 20% ethylene glycolsuccinate or 10% Apiezon L on Chromosorb W-LA (80-100 mesh) and kept at 190°C; injector temperature, 270°C; carrier gas, N_2 at a flow rate of 40 ml/min. Unsaturated fatty acids were separated by the method of

Goldfine and Bloch (8). Fatty acids were identified by comparing their retention times on each column with the retention times of standard methyl esters (Applied Science Div., Alltech Associates, Inc., State College, Pa.).

Determination of DNA base composition. Deoxyribonucleic acid (DNA) was extracted by the method of Marmur (15), with the minor modifications described by Dellaglio et al. (6) and Manachini and Parini (13). The final isopropanol DNA precipitate was dissolved in $0.1 \times$ standard saline citrate at a concentration of 20 μ g/ml, and the DNA base composition was determined by the thermal denaturation method (16), using a Beckman model ACTA-C-III spectrophotometer fitted with a circulating temperature bath. The guanine-plus-cytosine (G+C) content of a DNA ($G+C_x$) was calculated from the following formula: $G+C_x = G+C_{STD} + 2.44(T_{m_x} - T_{m_{STD}})$ (14), where $G+C_{STD}$ and $T_{m_{STD}}$ were the G+C content and thermal denaturation midpoint temperature of the internal standard DNA, respectively, and T_{m_x} was the thermal denaturation midpoint temperature of the DNA being investigated. The DNA of *Escherichia coli* strain B (Sigma Chemical Co., St. Louis, Mo.), which has a G+C content of 50.9 mol%, was used as an internal standard.

RESULTS

Morphology. The cells of strain BT2^T were rod-shaped and 0.8 to 1.0 by 2.5 to 4.8 μ m and occurred singly and in short chains. The microorganism was gram positive; the endospores were oval (0.9 to 1.2 by 1.4 to 1.7 μ m) and usually distended the sporangium in a terminal or subterminal position. Strain BT2^T was motile, and lateral flagella were visible by electron microscopy (Fig. 1). The colonies were circular, entire, convex, red pigmented, and smooth and had glossy and mucilaginous surfaces.

Cultural and physiological characteristics. Strain BT2^T grows in skim milk with coagulation and peptonization. On TER/1 agar, GYE agar, and potato-glucose-yeast extract agar the growth is abundant, spreading, smooth, shiny, and red; in GYE broth strain BT2^T exhibits homogeneous turbid growth. The greatest intensity of pigmentation occurs during the stationary phase of growth, during the sporulation process. Growth is less intense on potato-glucose-yeast extract broth and glucose-asparagine agar. No growth is observed on SM medium; the addition of yeast extract (0.1 g/liter) to SM medium allows growth. The yeast extract can be replaced with a vitamin solution; in particular, strain BT2^T requires either biotin or thiamine.

Strain BT2^T does not grow under anaerobic conditions. The temperature range for growth is 34 to 58°C. Optimum growth occurs at 45 to 48°C; no growth is observed at 30 or 65°C.

Strain BT2^T does not synthesize acetylmethylcarbinol, indole, or hydrogen sulfide and does not produce catalase and tyrosine-degrading enzymes. No growth is produced in the presence of 5% NaCl; nitrate is not reduced to nitrite. Starch and casein are hydrolyzed, and gelatin is liquefied.

Strain BT2^T utilizes (without acid formation) L-arabinose, D-fructose, D-galactose, D-glucose, glycerol, inositol, maltose, mannitol, D-ribose, trehalose, and D-xylose. Citrate, L-malate, and pyruvate are not utilized. Utilization of acetate and succinate is weakly positive. Ammonium sulfate, urea, tryptone, soytone, and peptone are utilized as nitrogen sources, but $NaNO_3$ is not.

Spores resist heating at 100°C for 20 min, but do not survive at 112°C.

Strain BT2^T does not grow in the presence of 5 μ g of penicillin per ml and 5 μ g of tetracycline per ml; the

inhibitory concentration of chloramphenicol and vulcamycin is 50 µg/ml.

Red pigment. Strain BT2^T produces a red pigment on all media tested. The exact nature of this pigment has not been determined; a probable chinoid structure has been suggested (10). Elemental analysis gave the following results: 57.12% C, 6.28% H, and 3.40% N. The pigment is soluble in methanol, ethanol, *n*-butanol, ethyl acetate and chloroform and insoluble in petroleum ether. When it was dissolved in methanol, the pigment showed a λ_{max} at 405 nm ($E_{1\text{cm}}^{1\%} = 680$).

Cellular fatty acid composition. The total fatty acids obtained by alkaline hydrolysis represents 2 to 4% of the dry weight of strain BT2^T cells. A marked predominance of four branched-chain fatty acids is observed; iso-C₁₅, anteiso-C₁₅, iso-C₁₇, and anteiso-C₁₇ acids accounts for 80 to 90% of the total fatty acids. In particular, iso-C₁₅ acid is consistently the most abundant fatty acid (44 to 60%). The only unsaturated fatty acid present in very low amounts (0.1 to 3%) is *n*-C_{18:1} acid.

DNA base composition. The G+C content of the DNA of strain BT2^T is 57 ± 0.8 mol%.

DISCUSSION

The characteristics of strain BT2^T fit the description of the genus *Bacillus* as defined in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (7). Moreover, the fatty acid composition of strain BT2^T is in agreement with the compositions reported for several species of *Bacillus*; specifically, it is like that of some thermophiles, such as *Bacillus stearothermophilus*, *Bacillus caldolyticus*, and *Bacillus caldotenax* (12, 17).

The previously described species of the genus *Bacillus* differ considerably from strain BT2^T in morphological, cultural, and physiological characteristics; furthermore, strain BT2^T is easily distinguished from the other species by its peculiar red nondiffusible pigment. The base contents of the DNAs of members of the genus *Bacillus* are typically between 32 and 53 mol% G+C (7, 18); these values are lower than the value found for our isolate (57 mol% G+C). Higher G+C values have been found for the DNAs of some thermophilic species, including *Bacillus thermocatenulatus* (69 mol%) (9), *Bacillus schlegelii* (67 to 68 mol%) (20), *Bacillus caldovelox* (65 mol%) (11, 21), *Bacillus caldotenax* (64.8 mol%) (11, 21), and *Bacillus acidocaldarius* (61 to 62 mol%) (5). These differences suggest that strain BT2^T is not closely related to the previously described members of the genus *Bacillus*.

On the basis of the findings described above, we believe that strain BT2^T represents a new species of the genus *Bacillus*, for which we propose the name *Bacillus thermoruber*. A description of the new species is given below.

Bacillus thermoruber (ther'mo.ru.ber. Gr. n. *therme* heat; L. adj. *ruber* red; M.L. masc. adj. *thermoruber* heat loving and red pigment producing) cells are gram positive, straight rods 0.8 to 1.0 by 2.5 to 4.8 µm, with lateral flagellation; endospores are oval (0.9 to 1.2 by 1.4 to 1.7 µm) and terminal or subterminal and distinctly distend the sporangium. Colonies are red colored, circular, entire, and convex and have glossy and mucilaginous surfaces.

For growth, biotin or thiamine is required. L-Arabinose, D-fructose, D-galactose, D-glucose, glycerol, inositol, maltose, mannitol, D-ribose, trehalose, and D-xylose are utilized as sole carbon sources. Utilization of acetate and succinate is weakly positive. Ammonium sulfate, urea, tryptone, soytone, and peptone are utilized as nitrogen sources.

The temperature range for growth is 34 to 58°C, and the optimum temperature is 45 to 48°C. Positive physiological properties include gelatin liquefaction and starch and casein hydrolysis; negative properties include nitrate reduction, Voges-Proskauer reaction, indole production, tyrosine decomposition, hydrogen sulfide production, presence of catalase, and growth in the presence of 5% NaCl.

Iso-C₁₅, anteiso-C₁₅, iso-C₁₇, and anteiso-C₁₇ acids are the major cellular fatty acids.

The G+C content of the DNA is 57 ± 0.8 mol%.

Distribution: isolated from compost used for mushroom production.

The type strain is strain BT2, which has been deposited in the collection of the Cattedra di Microbiologia Industriale, Università di Milano, Milan, Italy, as strain MIM 30.8.38.

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