A halophilic thermophilic bacterium isolated from a coastal hot spring in Lutao, Taiwan

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A heterotrophic, moderately thermophilic bacterial strain, designated strain T501, was isolated from a hot spring on the coast of Lutao, Taiwan. This bacterial strain was a strictly aerobic, Gram-negative rod, normally 0.6–0.7 µm in diameter and 4–10 µm in length, and motility was achieved by one to several lateral flagella. Its minimum and maximum growth temperatures were approximately 30 and 60 °C, respectively, with an optimal temperature at about 50 °C. The G + C ratio of its DNA was 38.1 mol %. Strain T501 tolerated a pH range from 6 to 9. It was halophilic, growing optimally in about 2% (w/v) salt, and Na⁺ was indispensable unless Mg²⁺ or Ca²⁺ were available. The addition of either cation to Na⁺-sufficient media significantly enhanced growth. Halophilic heterotrophic bacteria capable of aerobic growth over a temperature range of 40–55 °C have not been reported previously.

Introduction

Micro-organisms growing optimally at temperatures higher than 45 °C are considered to be thermophiles, the majority of which are members of either Archaea or Bacteria (Brock, 1985; Stetter *et al.*, 1990). Those growing optimally at temperatures of more than 80 °C are called hyperthermophiles, and are usually unable to grow below 60 °C. The most extremely thermophilic species grow at temperatures between 80 and 110 °C (Stetter *et al.*, 1990) and are strictly anaerobic archaea. Salt-dependent hyperthermophilic archaea of various genera have been isolated from shallow-water or deep-sea submarine hydrothermal areas (Fiala *et al.*, 1986; Kurr *et al.*, 1991; Miroschnichenko *et al.*, 1989; Neuner *et al.*, 1990; Pledger & Baross, 1989, 1991; Pley *et al.*, 1991). The genus *Thermotoga* includes the most thermophilic members of Bacteria, which may grow in environments up to 90 °C (Stetter *et al.*, 1990). Salt is absolutely essential for three species of this genus (Huber *et al.*, 1986; Windberger *et al.*, 1989). A variety of aerobic and anaerobic thermophilic bacteria, mainly heterotrophic species, have been described besides *Thermotoga* spp. (Kristjansson, 1992). All of them, however, only grow below 80–85 °C. *Rhodothermus marinus*, found in shallow-water submarine hot springs, is presently the

only aerobic halophilic heterotroph among them (Alfredsson *et al.*, 1988). It requires approximately 2% (w/v) salt and a temperature of 65 °C for optimal growth. Many thermophilic strains of *Thermus* spp. with an optimal growth temperature of 70–75 °C and a minimum growth temperature of about 55 °C will grow in the presence of salt at 3% or higher (Kristjansson *et al.*, 1986), but they are halotolerant rather than halophilic since their growth is not stimulated by salt. Several halotolerant thermophilic species of *Bacillus* grow between 40 and 65 °C (Claus & Berkeley, 1986). They do not require salt for growth but tolerate a salt concentration of 2–7%.

A halophilic, strictly aerobic, thermophilic heterotroph has been isolated in this laboratory in a study of thermophilic bacteria in the coastal hot springs of Lutao, Taiwan. This strain differs from all known species of halophilic thermophilic bacteria. The isolation and characterization of this micro-organism are described here.

Methods

Sample collection. Lutao is a small island (ca 16 km²) in the Pacific Ocean, 19 miles south-east of Taiwan. Several hot springs on the south-eastern coast and the intertidal zone around this island are slightly acidic and saline due to the mixture of underground and/or surface seawater. The temperature in the hot-water ducts of different springs ranges from 50 to 90 °C. A water sample, which had an original

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temperature of 59 °C, was collected from the opening of a hot spring on the coast at a depth of about 10 cm.

Media. The peptone-yeast (PY) broth medium used for enrichment consisted of 4 g Bacto-peptone (Difco), 2 g Bacto-yeast extract (Difco), 25 g NaCl and 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 1 litre deionized water and adjusted to pH 7.0. PY broth supplemented with CaCl_2 (10 mg l^{-1}) and MnCl_2 was used to detect endospore formation. PY agar was prepared by adding 20 g agar (Difco) to 1 litre PY broth. This medium was supplemented with Tween 80 (0.001%) and CaCl_2 (0.1 g l^{-1}) for the lipase test. Four modified PY agar media containing casein (4 g l^{-1}), starch (4 g l^{-1}), gelatin (4 g l^{-1}) and DNA (2 g l^{-1}), respectively, were used to test the hydrolysis of these substrates.

The peptone-yeast-carbohydrate (PYC) broth media were prepared from two stock solutions: (1) 4 g Bacto-peptone, 2 g Bacto-yeast extract, 25 g NaCl, 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 4.5 g MOPSO (Sigma) dissolved in 900 ml deionized water and adjusted to pH 7.0; and (2) 5 g of glucose, galactose, saccharose, cellobiose, xylose, mannose, trehalose, lactose, mannitol or dulcitol dissolved in 100 ml deionized water. The two solutions were autoclaved separately and mixed at room temperature. The modified PYC broth media used for carbohydrate fermentation tests were prepared from two stock solutions: (1) 4 g Bacto-peptone, 2 g Bacto-yeast extract, 0.03 g bromothymol blue, 25 g NaCl, 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.6 g Tris (*ca* 5 mmol) dissolved in 900 ml deionized water and adjusted to pH 7.4; and (2) 5 g of one of the above carbohydrates (see composition of PYC broth media) was dissolved in 100 ml deionized water.

Mineral media containing NH_4Cl (0.1 or 0.5 g l^{-1}) as the sole nitrogen source and various carbohydrates (5 g l^{-1}) as the sole carbon sources were modified from Shieh *et al.* (1988). MOPSO instead of Tris was added to the mineral media at 20 mM, and the pH of these media was adjusted to 7.0. Three media used for the tests of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase were modified from Falco lysine decarboxylase broth (MacFaddin, 1980). They consisted of 4 g Bacto-peptone (Difco), 2 g yeast extract, 0.5 g glucose, 25 g NaCl, 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.02 g bromocresol purple, and 5 g of L-arginine, L-lysine or L-ornithine, respectively, in 1 litre deionized water and adjusted to pH 6.8. All pH adjustments were made with HCl (1.0 M), KOH (1.0 M) or both.

Isolation of thermophilic bacteria. The water sample was serially diluted with a sterile NaCl-MOPSO buffer (25 g NaCl and 0.45 g MOPSO in 1 litre deionized water, pH 7.0). The cultures for enrichment of thermophilic bacteria were prepared by inoculating portions (1 ml) of 10^0 – 10^5 dilutions of the water sample into tubes containing PY broth (5 ml). They were incubated at 50 °C in the dark for 2–5 d under aerobic conditions. The cultures that developed an OD_{600} of greater than 0.2 were used (10 μl) to inoculate fresh PY broth (5 ml) for a secondary enrichment cultivation, streaked (one loopful) on PY agar plates and incubated aerobically at 50 °C in the dark for 2–3 d. Various types of colonies appearing on the plates were picked off and purified by successive streaking on PY agar. The cultures were maintained in both PY agar and PY broth and stored at 50 °C. One strain, designated as T501 was used in this study.

Characterization of thermophilic isolate. One loopful of culture was inoculated into 5 ml of each of the carbohydrate fermentation media, and the air in each culture tube was replaced by Ar after sealing with a rubber stopper. The cultures were examined daily for colour changes for up to 7 d. The cultures for the tests of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase were also incubated anaerobically under Ar and were examined for colour changes after incubation for 7 d. Cells cultivated at 50 or 55 °C in the modified PY broth medium were observed daily for endospores with a phase-contrast microscope for 7 d by spore staining (Smibert & Krieg, 1981). Luminescence, pigmentation, swarming and agarase (Agbo & Moss,

1979) were determined after growth for 24–48 h on PY agar plates. Luminescence was recorded after the plates had been in the dark for 5 min. Casein hydrolysis, amylase, gelatin hydrolysis, lipase and DNAase were tested by methods similar to those of Smibert & Krieg (1981). Tests for oxidase, catalase and Gram reaction were performed on cells grown for 24–48 h on PY agar plates (Shieh *et al.*, 1988). Cells grown for 24–48 h in PY broth were observed for motility by phase-contrast microscopy and fixed with 3% (v/v) glutaraldehyde and negatively stained with potassium phosphotungstate (1%, w/v, pH 7.0) for electron microscopy. All test cultures were incubated at 50 °C in the dark for 5 d under aerobic conditions unless otherwise stated.

Determination of antibiotic sensitivity. Sensitivity to the following antibiotics was determined with discs (Difco): ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), penicillin G (10 units), polymyxin B (300 units), streptomycin (10 μg), tetracycline (30 μg). Discs were placed on PY agar plates which had been spread with 0.1 ml culture of strain T501. Inhibition zones around the discs were measured after overnight incubation at 50 °C under aerobic conditions.

Test for diaminopimelic acid. The presence or absence of diaminopimelic acid in the cell hydrolysates (4 M-HCl, 16 h, 100 °C) of strain T501 were determined by paper chromatography (Rhuland *et al.*, 1955).

DNA base composition. The G+C content of bacterial DNA was determined by HPLC of nucleosides formed by P1 hydrolysis (Shieh *et al.*, 1987).

Determination of the effects of various factors on growth. Strain T501 was routinely precultivated in PY broth. One loopful of early stationary-phase preculture was inoculated into a tube containing 5 ml PY broth or its modifications, to determine the effects of temperature, pH, Na^+ , Mg^{2+} and Ca^{2+} on growth. The mineral media were used for the determination of growth on single carbon sources. The effects of various carbohydrates on growth were determined in PYC broth media. Bacterial growth was monitored by measuring culture optical density using a Spectronic 20 spectrophotometer (Milton Roy Company) at 600 nm. All growth experiments were carried out at 50 °C in the dark under aerobic conditions unless otherwise stated.

Results

Characterization of thermophilic micro-organism

Strain T501 was a rod-shaped, motile, Gram-negative bacterium, 0.6–0.7 μm in diameter and 4–10 μm in length. Motility was achieved by one to several lateral flagella. Endospore formation was suspected since it did not grow above 65 °C, but survived at 80 °C for several h to 1 d. Endospore-like structures were observed with a low frequency (< 10%) at the centre of the unstained cells but sporulation was not confirmed by spore staining. Cells grown on PY agar for 1 d developed circular, slightly umbonate, non-pigmented colonies with an entire margin. None of the test carbohydrates (glucose, saccharose, galactose, mannose, lactose, cellobiose, trehalose, xylose, mannitol dulcitol) were fermented. Additional physiological and biochemical characteristics are listed in Table 1.

Diaminopimelic acid was detected in the cell hydrolysates of strain T501, it was sensitive to all 10 of the test

Table 1. Characteristics of strain T501

Characteristic	Reaction*
Gram stain	—
Anaerobic growth	—
Halophilism	+
Motility	+
Growth at:	
25 °C	—
40–55 °C	+
65 °C	—
Luminescence	—
Pigmentation	—
Swarming	—
Casein hydrolysis	+
Agarase	—
Oxidase	+
Catalase	w
Lipase	—
Gelatinase	—
Deoxyribonuclease	w
Amylase	—
Arginine dihydrolase	—
Lysine decarboxylase	—
Ornithine decarboxylase	—

* +, Positive; —, negative; w, weakly positive.

Table 2. Sensitivity of strain T501 to various antibiotics

Antibiotic	Inhibition zone width (mm)*
Ampicillin	19
Chloramphenicol	12
Erythromycin	12
Gentamicin	12
Kanamycin	8
Nalidixic acid	10
Penicillin G	20
Polymyxin B	5
Streptomycin	3
Tetracycline	18

* From the edge of the discs to the edge of bacterial growth. All data represent mean values of duplicate experiments.

antibiotics (Table 2) and had a G+C content of 38.1 mol%.

Effects of temperature, pH and carbon sources on growth

Strain T501 grew significantly in PY broth over a range of 40–55 °C, and rapidly at about 50 °C, to an OD₆₀₀ of more than 0.2 within 1 d. The growth rate was significantly lowered at 30 to 35 °C and the OD₆₀₀ of the cultures was only about 0.1 even after incubation for 2 d. Strain T501 grew weakly (OD₆₀₀ < 0.1) or not at all at 60 °C after up to 7 d incubation, but some replicate cultures exhibited significant growth. No growth oc-

Fig. 1. Influence of salt concentration on growth of strain T501 in modified PY broth media supplemented with 2.5 mM-Mg²⁺ (a), 2.5 mM-Ca²⁺ (b) or neither of the two bivalent cations (c). OD₆₀₀ was measured at 24 (▽), 48 (□), 72 (△) and 96 h (○) as shown. Each point represents the mean of five cultures.

curred at 25 or 65 °C. Strain T501 tolerated a variation of pH from 6.0 to 9.0, but growth did not occur at pH 5.0 or 10.0. No growth occurred in any of the mineral media aerobically or anaerobically, nor in PY or any of the PYC broth media under anaerobic (100% Ar) conditions.

Effects of Na⁺, Mg²⁺ and Ca²⁺ on growth

Strain T501 grew in 0–8% salt (NaCl) in media containing 2.5 mM-Mg²⁺, with the most rapid and maximal growth at 2–4% salt (Fig. 1a). Growth did not occur at higher than 6% salt if Ca²⁺ (2.5 mM) instead of

Mg²⁺ was added to the medium (Fig. 1*b*). The NaCl tolerance range was more narrow in media deficient in Mg²⁺ and Ca²⁺. Without these cations, significant growth (OD₆₀₀ > 0.1) occurred only in about 2% salt; growth was weak in 0.5–1% salt and even negligible outside the salt concentration range between 0.5 and 3% (Fig. 1*c*). Substitution of KCl (1–4%) for NaCl did not support bacterial growth if Mg²⁺ and Ca²⁺ were not added (not shown). The presence of 0.025–0.25 mM-Mg²⁺ or Ca²⁺ could not support the growth of bacteria under salt-deficient conditions (not shown). All concentrations of the cations Na⁺, Mg²⁺, Ca²⁺ and K⁺ quoted here represent the minimal concentrations of these ions due to their presence in complex media used in these experiments.

Discussion

Moderately thermophilic bacteria with temperature optima of 40 to 60 °C and unable to grow above 70 °C had been isolated prior to the 1960s (Brock, 1984; Kristjansson, 1992). None of them were halophilic, although a few moderately thermophilic species of the genus *Bacillus* exhibited halotolerant characteristics (Claus & Berkeley, 1986). The moderately thermophilic strain T501 isolated in this study could be considered as moderately halophilic since its optimal growth occurred at about 2% NaCl. Strain T501 required Na⁺ but not Cl⁻ for growth under conditions deficient in Mg²⁺ and Ca²⁺. The requirement for Na⁺ could not be satisfied by K⁺, indicating that the requirement was not an osmotic function. The presence of Mg²⁺ or Ca²⁺ at a concentration of 2.5 mM allowed strain T501 to grow under Na⁺-deficient conditions, indicating that either cation could substitute for the Na⁺ requirement of this bacterium. Na⁺ was not an efficient substitute of Mg²⁺ and Ca²⁺ since 2.5 mM-Na⁺ was not nearly as effective as either of the two divalent cations at the same concentration (Fig. 1). Moderately halophilic bacteria (mesophiles or psychrophiles) in the marine environment generally require 70 to 700 mM-Na⁺ for optimal growth and yield (Reichelt & Baumann, 1974; Baumann & Baumann, 1977; Baumann *et al.*, 1983, 1984*a, b*). This requirement for Na⁺ can be reduced but not replaced by the addition of a certain concentration of Mg²⁺ and Ca²⁺ (Reichelt & Baumann, 1974). Strain T501 is thus the first halophilic bacterium exhibiting a requirement for Na⁺ that can be replaced by Mg²⁺ or Ca²⁺, although Na⁺ with either Mg²⁺ and Ca²⁺ was necessary for the most rapid and maximal growth.

Na⁺ is required in mesophilic halophilic bacteria for a number of complex and essential cellular functions. This includes permease systems involved in the uptake of exogenous substrates (Drapeau *et al.*, 1966; Forsberg *et*

al., 1970; Thompson *et al.*, 1970; Thompson & MacLeod, 1974; Fein & MacLeod, 1975), the retention of solutes within the cells (Wong *et al.*, 1969) and the maintenance of cell wall integrity (Forsberg *et al.*, 1970). Na⁺ has also been found to stimulate the activities of amylase (Onishi, 1972) and cytochrome oxidase (Kushwaha *et al.*, 1977) in mesophilic halophilic bacteria. Whether Na⁺ has any of the above functions in cells of strain T501 remains to be investigated.

Strain T501 is a strict aerobe that did not ferment any of the carbohydrates tested, nor did it grow anaerobically in PY or any of the PYC broth media. None of the carbohydrates tested could be utilized as the sole carbon source.

The majority of Gram-negative bacteria as well as most species of the genus *Bacillus* contain a peptidoglycan of the directly cross-linked diaminopimelic acid type (Claus & Berkeley, 1986; Schleifer & Kandler, 1972). The cell wall peptidoglycan of strain T501 may also belong to this type since diaminopimelic acid has been detected from the cell hydrolysates of the strain.

Strain T501 has been excluded from the genus *Bacillus* (Claus & Berkeley, 1986) on the basis of its halophilic and Gram-negative properties, and its lateral flagellation. The characterization that strain T501 is a motile, non-pigmented halophile, which is unable to grow at temperatures above 70 °C shows it is not a species of the genus *Thermus* (Brock, 1984). Halophilic heterotrophic eubacteria are almost all mesophilic or psychrophilic, but few are thermophilic. They include species of the genera *Rhodothermus* (Alfredsson *et al.*, 1988), *Thermotoga* (Huber *et al.*, 1986; Jannasch *et al.*, 1988; Windberger *et al.*, 1989), *Thermosiphon* (Huber *et al.*, 1989) and *Spirochaeta* (Aksenova *et al.*, 1992). Strain T501 cannot be placed in any of the latter three genera since none of them include strict aerobes. *Rhodothermus marinus*, the only aerobic species of halophilic thermophilic heterotrophs reported, has a minimum growth temperature at about 55 °C. Strain T501 is thus the first halophilic heterotrophic bacterium capable of aerobic growth at temperatures between 40 and 55 °C. It has a G+C content of 38.1 mol%, far lower than *Rhodothermus marinus*. This, together with other data (Table 3), may be sufficient to propose a new genus for the strain, but a formal proposal will not be made until the sequence data of 16S and/or 5S rRNA have been obtained.

The author has isolated more than 40 bacterial strains of aerobic, halophilic, thermophilic heterotrophs from the coastal hot springs of Lutao, a few of which, like strain T501, can grow under Na⁺-deficient conditions in the presence of 2.5 mM-Mg²⁺ or -Ca²⁺ (unpublished data). They are, however, somewhat more thermophilic than strain T501; their optimum and maximum growth temperatures are approximately 55 to 60 °C and 65

Table 3. Characteristics distinguishing strain T501 from *Rhodothermus marinus*

Characteristic	Strain T501	<i>Rhodothermus marinus</i> *
Growth at:		
40–50 °C	+	–
65–70 °C	–	+
Motility	+	–
Pigmentation	–	+
Oxidase	+	–
Casein hydrolysis	+	–
Sensitivity to:		
Streptomycin	+	–
Kanamycin	+	–
Gentamicin	+	–
G+C content (mol %)	38.1	64

* Data from Alfredsson *et al.* (1988).

to 70 °C, respectively (unpublished data). Halophilic thermophilic bacteria closely related to strain T501, as well as these isolates, may be confined to a very narrow zone near the openings of coastal or submarine hot springs due to the requirement for oxygen, organic matter, a moderately high temperature and the metal cations Na⁺, Mg²⁺ and Ca²⁺.

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