

Isolation and characterization of two new halophilic species of *Bacillus* from Saudi Arabia

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ABSTRACT

Two moderately halophilic bacterial isolates (BST and BSF) were obtained from Al-Kaseem Natural Salt Basin, Saudi Arabia. Isolate BST consists of short thick rods ($1.2-1.5 \times 2-3 \mu\text{m}$), with central spherical spores, large distinctive circular white colonies and a DNA-base composition of 45 mol %. Isolate BSF consists of long thin rods ($0.4-0.8 \times 3-5 \mu\text{m}$), with terminal spherical spores, small white-glossy circular colonies and a DNA-base composition of 38 mol %. Both isolates are Gram positive, heterotrophs, mesophiles, motile and require MgSO_4 and NaCl for their growth. The optimum NaCl concentration is 10–22%. KCl is not required for growth and it does not substitute NaCl. These isolates were classified as two new species of the genus *Bacillus* on the basis of their unique characteristics as compared to each other and with the species described in Bergey's Manual of Systematic Bacteriology.

INTRODUCTION

Salt and solute-tolerant organisms are widely distributed among bacteria, fungi, yeasts, algae and viruses (Hipkiss *et al.* 1980). There are a number of environments that are good sources of the above microbial populations, e.g. sea, natural or man-made lakes, solar salt, the Dead Sea, salted foods and natural salt deserts (Hipkiss *et al.* 1980; Brown *et al.* 1965).

Moderate halophiles were first described by Baxter & Gibbons (1956). They grow in the range of 3 to 20% and can withstand 20–30% NaCl (Forsyth *et al.* 1971). Lowering NaCl concentration than the optimum will lead to a change in cell morphology and a disintegration of the outer layers (Grant & Tindall 1980; Vareeland *et al.* 1984). This change cannot be prevented by KCl or by non-ionic solutes (Novitsky & Kushner 1975). KCl is less effective than NaCl in maintaining cellular integrity. Some halophiles, however, have a very high K^+ concentration, and hence, this ion may be growth limiting (Gochnauer & Kushner 1971). Of the divalent ions, Mg^{2+} is required for the stability of the ribosomes and for the stability and the activity of the enzymes of many halophilic bacteria. Therefore, almost all of the halo-media contain this ion (Grant & Tindall 1980; Shindler *et al.* 1977). Most of the halophilic cocci and rods have vitamin and amino acid requirements (Boffi 1969; Drapeau *et*

Table 1. Physiological characteristics of the two isolates

Test	Isolate	
	BST	BSF
Acid and/or gas from glucose, arabinose, xylose and mannitol ^a	—	—
Extracellular protein crystals	—	—
Acetoin ^a	—	—
Indole ^b	—	—
Hydrolysis of: ^b		
starch (halo-medium)	+	—
hippurate (halo-medium)	+	+
gelatin (halo-medium)	+	—
urea	+	—
casein	+	—
Nitrate reduction ^b	+	+
Growth in the presence of:		
0.001% lysozyme (halo-medium)	+	+
2% sodium azide (halo-medium)	+	+
Alkali on citrate salts agar ^b	+	+
Catalase (halo-medium) ^b	+	+
Egg yolk reaction ^b	—	—
Decomposition of tyrosine ^b	—	—
Deamination of phenylalanine ^b	—	—
Oxidase (halo-medium) ^b	—	—
Litmus milk ^b	—	—
Growth on:		
nutrient agar	+	+(weak)
blood agar base	+	+
trypticase soy agar	+	+
starch agar	+(weak)	+(weak)
brain heart infusion agar	+	+
Sabouraud dextrose broth and/or agar	—	—
Higuchi & Carolin medium without added sugar ^c	—	—
Higuchi & Carolin medium with the following sugars added: ^c		
glucose	+	—
mannitol	+	—
xylose	+	—
Mineral salts medium (autotrophically) ^d	—	—
Mineral salts medium + glucose ^d	—	—

^a Gibson & Gordon (1986); ^b Smibert & Krieg (1981); ^c Higuchi & Carolin (1957); ^d Krieg (1981).

al. 1966). Therefore, these requirements should be taken into consideration when isolating such microbes from their environments.

In this study, we report on two new halophilic bacterial species from Saudi Arabian soils.

MATERIAL AND METHODS

Sampling. Soil samples were taken from Al-Kaseem Natural Salt Basin, Saudi Arabia. This basin has a pH of 7.8 and a temperature of 0–24°C in winter and 32–45°C in summer. The salt content was not determined, but the basin is being used by many

people as a source of natural salt. The top layer, 2–3 cm, was removed and samples were collected in sterile bags.

Isolation of bacteria. Five grams soil were suspended in 100 ml of the halophile medium of Sehgal & Gibbons (1960). The flasks were shaken (100 rpm) at 30°C for 3 days, then a 10% transfer was made to a fresh medium and shaking was continued for another 23 h. A suitable dilution was seeded on agar plates of the same medium. The plates were incubated at 30°C and examined for growth after 48 h. The isolates were purified by streaking and were maintained at 4°C and subcultured monthly.

Characterization. The colonies were observed on halophile medium agar plates after an incubation period of 48 h at 30°C. The spore stain was made following the method of Schaeffer–Fulton. The Gram stain was made using 14 h cultures by the method of Hucker. The two methods were as described by Doetsch (1981). Cells were photographed by a photomicroscope III (Carl Zeiss).

Growth under anaerobic conditions was observed using halo-medium plates with pyrogallol and NaOH as an oxygen scavenger. The effect of temperature on growth was studied using halo-medium agar plates at 3, 15, 24, 30, 33, 37, 40, 45 and 50°C. Halo-medium broth with pH values of 6.5, 7.5, 8.5, 9.0, 9.5, 10.0 and 10.5 was used for the determination of the effect of pH. Other physiological studies were performed using a halo-medium (when indicated) with 15% NaCl concentration or using the methods and media described in the given references. The concentrations of NaCl and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in the media were adjusted throughout at 15% and 2%, respectively.

Deoxyribonucleic acid (DNA) base composition. The DNA base composition of the two isolates was determined by the thermal denaturation method (T_m) as described by Johnson (1981).

Effect of sodium chloride on growth. Cells were grown in a halo-medium broth containing 10% NaCl. Cell suspensions were diluted and plated on triplicate halo-medium agar plates containing 0–28% (w/v) of NaCl. The colonies were counted and the average number for each NaCl concentration was plotted against the concentration. The growth of the two isolates was also studied in halo-medium lacking MgSO_4 , halo-medium lacking KCl, halo-medium with 5–20% KCl and 1% NaCl, halo-medium with 1% NaCl and 2–15% sucrose, and halo-medium lacking NaCl but containing 2–15% sucrose.

RESULTS

Morphological characteristics. Two bacterial isolates were obtained on the medium containing 25% NaCl. Isolate BST has large distinctive circular white colonies, whereas isolate BSF has small white-glossy circular colonies (Fig. 1). They are both composed of Gram-positive spore-forming rods (Figs 2A, B, C and D). Isolate BST consists of short thick rods ($1.2\text{--}1.5\ \mu\text{m} \times 2\text{--}3\ \mu\text{m}$) with central nearly spherical spores, whereas isolate BSF consists of long thin rods ($0.4\text{--}0.8\ \mu\text{m} \times 3\text{--}5\ \mu\text{m}$) with terminal spherical spores. Chains are not formed and the cells are motile.

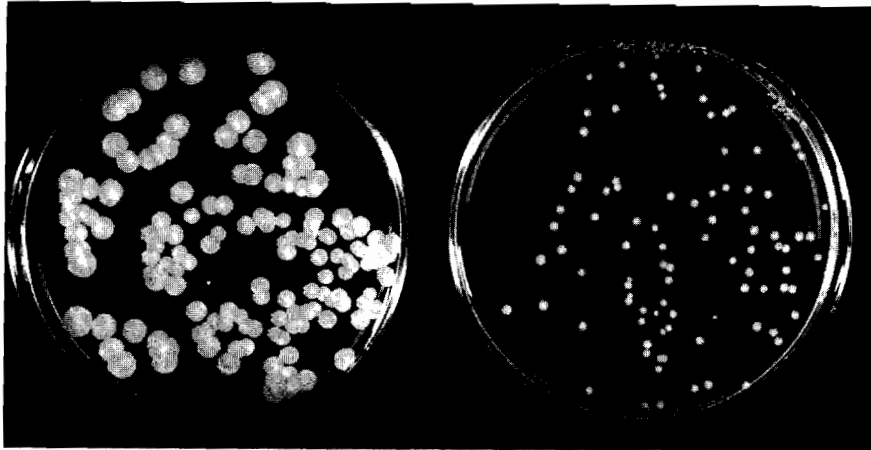


Fig. 1. Colonies of isolate BST (left) and isolate BSF (right), grown on halo-medium for 48 h at 30°C.

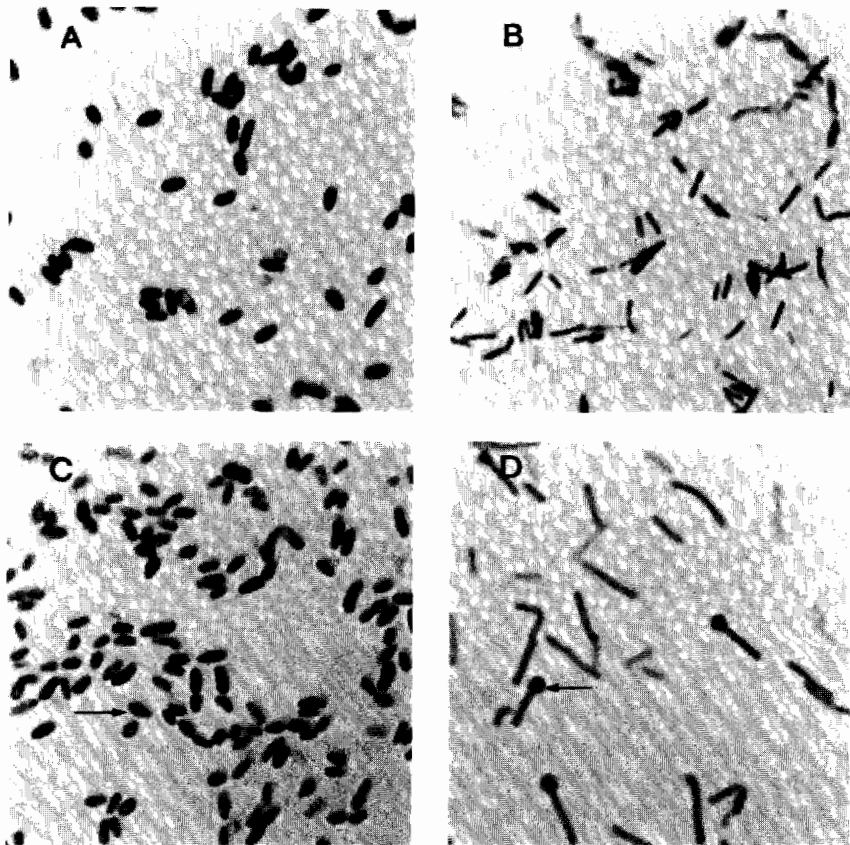


Fig. 2. Gram (A and B) and spore (C and D) stains of isolate BST (A and C) and isolate BSF (B and D). The arrows which are shown on C and D are pointing to an endospore-forming cell. The total magnification for both is 3500 \times .

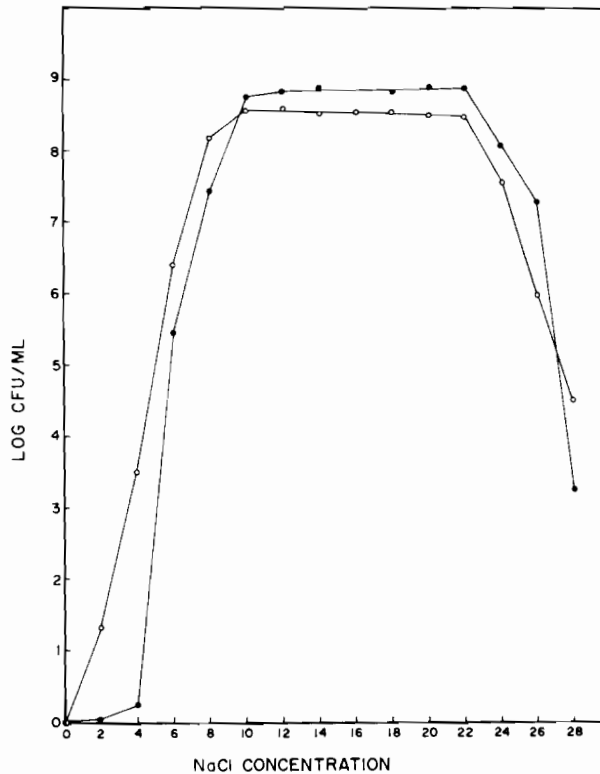


Fig. 3. Relationship between NaCl concentration (percentage) and log number of colony-forming units per ml. Open circles represent isolate BST, whereas closed circles represent isolate BSF.

Physiological characteristics. The maximum temperature for growth is between 40 and 45°C, whereas the minimum is around 20°C. So, both isolates are mesophilic bacteria. The isolates grow between a pH of 6.5 and 9.5 but not at 10 or 10.5. Their growth at pH 9.5, however, is weak. The optimum temperature is 33°C and the optimum pH is 7.5–8. Isolate BST grows aerobically and anaerobically, whereas isolate BSF grows only aerobically.

Deoxyribonucleic acid (DNA) base composition. The G + C content of DNA is 38 and 45 mol % (Tm) for the BSF and BST isolates, respectively.

Effect of sodium chloride on growth. Fig. 3 shows the effect of NaCl concentration on the growth of the two isolates. Both isolates require salt for growth. However, isolate BSF requires higher concentrations than isolate BST. The optimum salt concentration for both isolates is between 10 and 22%. The range of sodium chloride concentration which supports the growth of isolate BST is wider than that for isolate BSF.

The two isolates do not grow in a halo-medium lacking MgSO₄, halo-medium having 5–20% KCl and 1% NaCl, halo-medium having 1% NaCl and 2–15% sucrose, and halo-medium lacking NaCl and having 2–15% sucrose. The two isolates, however, were able to grow in a halo-medium lacking KCl.

DISCUSSION

Al-Kaseem Natural Salt Basin has been formed by evaporative concentration of a natural ground leaking water. The pH of the basin is 7.8. The salt composition was not determined, but the salt is used directly for human consumption. It is interesting to find two *Bacillus* species living in the presence of such high salt concentration. The two isolates belong to the genus *Bacillus* because they are Gram-positive spore-forming rods, aerobic (BSF) or facultative aerobic (BST) and catalase positive. They are two different species because they vary in shape, location of the endospore, oxygen relation and DNA-base composition.

To identify the two isolates to the species level, their morphological and physiological characteristics as well as their DNA-base composition were compared with those of individual *Bacillus* spp. listed in Gibson & Gordon (1986). Isolate BST has nearly spherical central endospores which distend the sporangium distinctly. It does not produce acid and/or gas from glucose nor does it produce acetoin or grow at 3°C. This isolate, however, hydrolyses starch, grows anaerobically and has a DNA-base composition of 45 mol % (Tm). Therefore, it does not resemble any of the *Bacillus* species described by Gibson & Gordon (1986). Isolate BSF has terminal spherical endospores which distend the sporangium distinctly. It does not produce acid and/or gas from glucose nor does it produce acetoin. In these characteristics it resembles *Bacillus sphaericus* and *Bacillus pasteurii*. *Bacillus sphaericus* reportedly deaminates phenylalanine, proteolyzes nutrient gelatin and some of its strains decompose casein and grow in Sabouraud dextrose broth and/or agar, whereas isolate BSF does not. Isolate BSF, however, reduces nitrate, whereas *Bacillus sphaericus* strains do not. Therefore, BSF is not a *Bacillus sphaericus* strain. Some of the *Bacillus pasteurii* strains digest gelatin and casein slowly and all strains are urease positive and have a pH optimum of 9, whereas isolate BSF does not. Therefore, isolate BSF is not a *Bacillus pasteurii* strain either. The above results along with the DNA-base composition of 38 mol % (Tm) confirm that BSF is a new species of the genus *Bacillus*.

The optimum growth at 10–22% NaCl is apparently not due to osmotic stabilization, since the two isolates were not able to grow at 5–20% KCl and 1% NaCl or at 2–15% sucrose and 1% KCl. It appears that NaCl is either a part of, or it is required for, the synthesis of integral part(s) of the cell.

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عزل نوعين بكتيريين جديدين محيين
للملوحة من المملكة العربية السعودية
وتصنيفها تحت جنس البكتريا بسلس

علي عبدالله سلامة

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خلاصة

تم عزل سلالتين بكتيريتين (BSF, BST) من مستنقع طبيعي مالح في منطقة القصيم بالمملكة العربية السعودية . السلالة BST تتكون من عصيات قصيرة وسميكة (عرضها ١,٢-١,٥ ميكرومتر وطولها ٢-٣ ميكرومتر) ، ولها أبواغ مركزية كروية تقريبا ، ومستعمرات كبيرة دائرية بيضاء متميزة ، والمحتوى القاعدي لحمضها النووي يساوي ٤٥ مول٪ . أما السلالة BSF فتتكون من عصيات طويلة رفيعة (عرضها ٠,٤-٠,٨ ميكرومتر وطولها ٣-٥ ميكرومتر) ، ولها أبواغ كروية طرفية ومستعمرات صغيرة دائرية بيضاء لامعة ، والمحتوى القاعدي لحمضها النووي يساوي ٣٨ مول٪ . كلا السلالتين موجبة لصبغة جرام ، متغايرة التغذية الكيميائية ، متوسطة المدى الحراري ، متحركة ، ويلزم لنموها كبريتات المغنيسيوم وكلوريد الصوديوم . التركيز المثالي لكلوريد الصوديوم يساوي ١٠-٢٢٪ . كلوريد البوتاسيوم ليس متطلبا للنمو ولم يحل كليا أو جزئيا محل كلوريد الصوديوم .

لقد تم تصنيف هاتين العزلتين على أنهما نوعان جديدان يتبعان جنس بسلس وذلك بناء على مقارنة صفاتها الخاصة بعضها البعض ، ومع السلالات التي وصفت في الطبعة التاسعة لكتاب بيرجى الخاص بوصف البكتيريا وتصنيفها .