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# *Haloarcula* spp able to biosynthesize exo- and endopolymers

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**Three halobacteria strains (T5, T6, T7), growing optimally at a sodium chloride concentration of 3.5 M, were isolated from samples collected in the Tunisian marine saltern located close to Monastir. Although they were different in colony and cell morphology, the isolates were similar in most respects and all produced a homopolyester identified spectroscopically as polyhydroxybutyrate when grown on carbohydrates. Under the same conditions, the isolates formed acidic exopolysaccharides. Acid methanolysis of their complex lipids released archaeol (2,3-di-O-phytanyl-*sn*-glycerol). All the isolates had polar lipid patterns characteristic of representatives of the genus *Haloarcula*. Partial sequence analysis of 16S rRNA genes from strain T5 confirmed its assignment to the genus *Haloarcula*. Although strain T5 differed phenotypically from the species *Haloarcula japonica* in several respects, DNA–DNA hybridization indicated that the T5 isolate is a new strain of *H. japonica*.**

**Keywords:** *Haloarcula*; Tunisian marine saltern; polyhydroxybutyrate; exopolysaccharide; ether lipids

## Introduction

Polyhydroxyalkanoates (PHA), a heterogeneous family of polyesters, are synthesised exclusively by prokaryotes as a storage material and are accumulated by a number of microorganisms under restricted growth conditions [26,28]. The most prominent polyester biosynthesized by microorganisms is poly(*R*)-3-hydroxybutyrate (PHB), a linear, unbranched homopolymer built up of (*R*)-3-hydroxybutyric acid units, although copolyesters with a variety of compositions have been described [19,46]. Polyhydroxyalkanoates have advantages as biodegradable and biocompatible alternatives to thermoplastics, offering potential uses in pharmaceutical and clinical applications [44]. The presence of PHA in the cells has also been used as a chemotaxonomic marker to help in the identification of a new isolate. Several halotolerant prokaryotes belonging to the domain Bacteria and, notably, some haloarchaea (halobacteria) are able to produce these polyesters [37].

Microbial exopolysaccharides have also aroused interest in view of their widespread importance in microbial systems. Bacterial exopolysaccharides (EPS) provide important model systems for the study of molecular assembly and secretion, gene regulation, cell–cell interactions, symbiosis, and pathogenesis. EPSs enable free-living bacteria to adhere to and colonize solid surfaces forming biofilms where nutrients accumulate.

Moreover, increasing attention is being paid to these molecules because of their bioactive role and their wide range of commercial applications [42]. Thermophiles and methanogens are amongst organisms from extreme environments that produce exopolysaccharides [2,25,29,40]. A few halobacteria also produce exopolysaccharides, notably species of *Haloferax* and *Haloarcula* [44]. Recently, interest in the mass cultivation of microorganisms from hypersaline environments has grown considerably, because this represents an innovative low technology approach to biotechnological exploitation [44].

In a screening program to obtain polyhydroxyalkanoate and exopolysaccharide producers, we have isolated obligately halophilic microorganisms from a previously unexplored site in Tunisia. Hypersaline environments where salinities exceed 1.5 M are usually dominated by prokaryotes. Two main groups are to be found: the moderately halophilic bacteria are more abundant at intermediate salinities (1.5–2.5 M), whereas the halophilic archaea (the halobacteria) dominate at salinities greater than 2.5 M, often imparting spectacular red pigmentation to the environment due to high levels of carotenoids [13].

Representatives of the majority of archaeal genera are characteristic of neutral saline environments (*Halobacterium*, *Halorubrum*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halobaculum*, and *Natrialba* spp) [13,17,18,23,27,32] whereas alkaline saline environments harbour haloalkaliphilic halobacteria such as *Natronomonas* and *Natronobacterium* spp [18].

The application of molecular and biochemical techniques has indicated that specific successions of halobacteria occur in hypersaline waters as the waters become concentrated. Many neutral hypersaline environments at saturation point, harbour climax populations of halobacteria usually belonging to the genera *Halobacterium, Haloarcula* and *Halorubrum* [31]. Representatives of other genera are much less common [14,36], although detailed characterisation at the species level is seldom carried out.

In this paper we report the characterization of three halobacterial isolates from a saltern near Monastir in Tunisia, able to produce exo- and endopolymers, whose production and partial characterization are described.

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## Methods

### Media and strains

Culture collection strains of halobacteria *Haloarcula hispanica* DSM 4426 (Spanish saltern), *H. japonica* DSM 6131 (Japanese saltern soil), *H. marismortui* DSM 3752 (Dead Sea), *H. vallismortis* DSM 3756 (Death Valley USA), '*H. sinaiiensis*' ATCC 33800 (Sinai Sabka, Israel), '*H. californiae*' ATCC 33799 (California saltern) and Tunisian isolates were maintained on media containing (g $L^{-1}$): yeast extract 10; casamino acids 7.5; trisodium citrate 3.0; KCl 2.0; $MgSO_4 \cdot 7H_2O$ 20; NaCl 200; $MnCl_2 \cdot 4H_2O$ (mg $L^{-1}$) 0.36; $FeSO_4 \cdot 7H_2O$ 50; pH 7.2–7.8. The NaCl solution was made separately in half of the total volume of water and the rest of the components were made up in an equal volume of water, then autoclaved separately and allowed to cool before mixing them (standard complex medium). For solid media, agar at 1.8% final concentration was added. Cells were grown at 37°C in 25-ml flasks containing 10 ml of growth medium. Variations of this growth regime included incubation temperature, salt concentrations (NaCl was varied between 2 M and 5 M, and $Mg^{2+}$ between 0 M and 0.5 M) and the addition of other compounds to the medium as indicated. Growth was followed by measuring optical density at 540 nm. The ability to utilize different carbon sources was tested by reducing the yeast extract concentration to 1.0 (g $L^{-1}$), replaced by the compound tested at a concentration of 6.0 (g $L^{-1}$) in the saline solution described above (minimal medium).

### Source of organisms

Halobacteria were enriched from 50 samples collected at 1-m intervals in the last ponds of a marine saltern located close to Monastir in Tunisia. Enrichments were grown at 35°C, at different NaCl concentrations (2.0–5.0 M) and at different pHs ranging from 7.5 to 8.2 in the standard complex medium for 2 weeks. The cultures were purified by repeatedly streaking them on solid medium. Typically the isolates grew well after 4 days in the complex standard medium at 35°C, pH 7.5 with 3.5 M NaCl.

### Microscopy

Cellular morphology was determined by phase-contrast microscopy. Colony morphology was observed using a Leika M8 stereomicroscope. For scanning electron microscopy (SEM), cells were grown in liquid enrichment medium, harvested by mild centrifugation (38–153 × *g*, 10 min) and washed in several changes of 0.05 M phosphate buffer containing 2.0 M NaCl (buffer 1) at successive pHs of 5.0, 6.8 and 7.2. Cells were fixed in 1.5% glutaraldehyde in buffer 1 (pH 7.2) for 30 min at room temperature, washed in buffer 1 at decreasing pH and post-fixed in 1% $OsO_4$ at pH 6.8 for 30 min at 4°C. After fixation and ethanol dehydration, the samples were critical-point dried and then coated with carbon and gold in a sputter-coater. The microscopy observations were carried out with a Cambridge 250 Mark 3 SEM.

### Lipid analysis

Polar lipids were extracted from dried cells (1.0 g) [7]. The lipid extract was analysed by thin layer chromatography (TLC), using the solvent system $CHCl_3$:MeOH:$H_2O$ (65:25:4 v/v/v), and hydrolysed in 1 M methanolic HCl to cleave the polar head groups. The glycolipid and the core lipids were analysed by the procedure of Lanzotti *et al* [21].

### Biochemical tests

Gram stains were performed using both heat-fixed smears and smears fixed in acetic acid [10]. All biochemical tests were carried out at 37°C. Assays for production of indole (in tryptophan-enriched medium), nitrate reduction, catalase and oxidase tests were performed using standard techniques [12]. To examine starch hydrolysis, the organism was streaked on solid medium supplemented with 1% (w/v) soluble starch. After 10 days the plate was flooded with iodine solution. Clear zones around colonies were indicative of starch hydrolysis. Tween 60 (1% v/v) was incorporated into solid medium and plates were examined for opacity after 7 days. Gelatin liquefaction was carried out in standard medium containing 120 g $L^{-1}$ of gelatin. Urease formation was tested on solid medium without casamino acids and supplemented with (g $L^{-1}$) urea 20, glucose 6, and phenol red indicator 0.01.

### β-Polyhydroxyalkanoate (PHA) determination

PHA was extracted according to Sykes [43] and quantified either by the method of Law and Slepecky [22] or gravimetrically. The identification of polyester was performed by $^1H$ and $^{13}C$-NMR spectroscopy. The cells for PHA analysis were grown both in the complex standard medium and in the minimal media in the presence of glucose or starch (0.6%, w/v) or molasses (0.3%, v/v). Growth was stopped usually after 10 days. In order to follow PHA formation, samples of the cultures, in the medium containing starch, were withdrawn at 5, 8 and 10 days and the level of PHA was quantified.

### DNA extraction, polymerase chain reaction and sequencing 16S ribosomal DNA

Strains were grown in standard media [30] and incubated at 37°C. DNA was extracted using the guanidinium thiocyanate method of Pitcher *et al* [34] except that cells were not pre-treated with lysozyme, and sarkosyl was not included in the lysis reagent.

The gene encoding 16S rRNA was amplified using the PCR method of Embley [11] as modified by McGenity and Grant [27]. The forward amplification primer was 27F, TCCGGTTGATCCTGCCGGAG (positions 8–27), and the reverse amplification primer was 1525R, AAGGAGGTGATCCAGCC (positions 1541–1525).

The region between positions 415 and 1032 in the 16S rRNA gene was chosen for sequence comparisons as this covers the two variable regions where most of the species-specific sequences for *Haloarcula* spp are known to reside.

The double-stranded PCR product was sequenced directly in a linear PCR amplification [11] using the reverse primer (5′–3′) CGGGTCTCGCTCGTTG. Sequencing reactions were performed with the ABI PRISM™ Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit, with a Perkin Elmer Cetus thermal cycler (Norwalk, CT, USA), under the following conditions: 25 cycles of denaturation at 96°C for 30 s, annealing at 49°C for 15 s and extension at

60°C for 4 min. Reactions were analysed using an Applied Biosystems automated 373A DNA sequencer, a service provided by the Protein and Nucleic Acid Chemistry Laboratory of the University of Leicester.

The resulting sequence data from strain T5 were put into the alignment editor ac2 [24] and manually aligned. They were then compared with 16S rRNA gene sequences of representative organisms belonging to the genus *Haloarcula* [24]. The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment.

### *DNA/DNA hybridization*

Hybridizations were performed by the slot blot method of Seldin and Dubnau [38]. Hybridization was carried out in formamide at 62°C for 15 h, and the filters were washed at the hybridization temperature. DNA from strain T5 was labelled with [$\alpha^{35}$S]dCTP$\alpha$S (New England Biolabs, Beverly, MA, USA, NE Blot Kit) and used as the probe.

### *Extracellular polysaccharide (EPS) production*

The cells for EPS analysis were grown in minimal medium in the presence of glucose (0.6%, w/v). After growth for 10 days, 1 L of broth culture was centrifuged until the supernatant became clear (9800 × *g*, 20 min). Two volumes of cold ethanol were added to the supernatant drop by drop, with stirring. The alcoholic solution, kept at −18°C overnight, was centrifuged for 30 min at 15 300 × *g*. The pellet was then dissolved in hot tap water. The polysaccharide solution was dialysed against running tap water (72 h), followed by distilled water and dried by lyophilization. This EPS crude extract was analysed by $^1$H NMR. The polysaccharide solution was chromatographed on a column (2.5 × 40 cm i.d.) of Sephadex G-50, eluted with $H_2O$: pyridine: $CH_3COOH$ (250:5:2, v/v/v). The fractions (5 ml) were collected at a flow rate of 6 ml h$^{-1}$. The EPS fraction was re-chromatographed on a column (1.0 × 40 cm i.d.) of DEAE Sepharose CL6B eluted with 0.1 L of $H_2O$ and 1 L of a NaCl gradient from 0–1 M with a flow rate of 12 ml h$^{-1}$, the volume of each fraction was 10 ml. Total carbohydrate content of the EPS was determined according to the method of Dubois *et al* [9].

Acid hydrolysis of EPS: 1 ml of 0.5 M (for neutral sugars) or 2 M (for acid sugars) TFA was added to 1 mg of EPS. The solution was held for 16 h at 80°C. It was then dried under a stream of air. EPS sugar monomers were identified and quantified by high-pressure anion-exchange pulsed amperometric detection (HPAE-PAD, Dionex, Sunnyvale, CA, USA) with sugar standards for identification and calibration curves. The HPAE-PAD Dionex detector equipped with a Carbopac PA1 column was eluted isocratically with 15 mM NaOH for neutral sugars and 100 mM NaOH and 150 mM sodium acetate for acidic sugars [6]. Sulfate was identified by the method of Silvestri *et al* [39].

### *Methylation analysis*

The methylated material (0.5 mg) was hydrolysed with 2 M TFA at 120°C for 2 h and then transformed in partially methylated alditol acetates by reduction with $NaBH_4$, followed by acetylation with $Ac_2O$/pyridine (1:1, by vol) at 120°C for 3 h. Unambiguous identification of sugars was obtained by GLC and GC-MS using sugar standards. GLC runs were performed on a Hewlett-Packard (Palo Alto, CA, USA) 5890A instrument, fitted with a FID detector and equipped with a HP-5-V column and $N_2$ flux of 100 ml min$^{-1}$. The temperature program was: 170°C (1 min), from 170°C to 180°C at 1°C min$^{-1}$, 180°C (1 min), from 180°C to 210°C at 4°C min$^{-1}$. GC-MS was performed on a Hewlett-Packard 5890–5970 instrument equipped with a HP-5-MS column and with a $N_2$ flux of 50 ml min$^{-1}$; the temperature program was: 170°C (1 min), from 170°C to 250°C at 3°C min$^{-1}$.

Instrumental $^1$H and $^{13}$C NMR spectra were run on a Bruker (Karlsruhe, Germany) AMX 500 (500.13 MHz for $^1$H and 125.75 MHz for $^{13}$C) spectrometer. Chemical shifts were given in ppm ($\delta$) scale. The $CHCl_3$ signal was used as internal standard for PHB spectra ($\delta$ 7.26 $^1$H; 77.0 $^{13}$C). The $^1$H of EPS was run at 30°C and chemical shifts were reported relative to sodium 2,2,3,3,-$d_4$-(trimethylsilyl)propanoate. Prior to NMR spectroscopy, the EPS sample was exchanged twice in $D_2O$ with intermediate lyophilization and then dissolved in 500 $\mu$l $D_2O$ to a final concentration of 40 mg ml$^{-1}$.

## Results and discussion

### *Characterization of isolates*

The enrichment procedure we used selects mainly halobacteria [5]. Colonies from the enrichments that developed on solid media were about 1 mm in diameter, circular, entire and red-orange pigmented after 1 week of incubation at 37°C. The colonies were either smooth or rough, while the corresponding cells appeared as pleomorphic cocco-bacilli of different sizes or as spherical cells occurring in packets (sarcina-like), respectively. The cocco-bacillus morphology did not change over the growth period in liquid or solid medium (Figure 1) or at different concentrations of $Mg^{2+}$ and $Na^+$.

Strains T5 and T7 were cocco-bacilli and formed smooth colonies, whereas isolate T6 formed rough colonies and appeared sarcina-like. When EDTA was added in an equimolar amount to $Mg^{2+}$ to cultures of isolate T5, the cells were converted to regular spherical forms (figure not shown).

All isolates tested showed identical polar lipid compositions, namely archaeol derivatives of phosphatidylglycerol (PG), phosphatidylglycerophosphate (PGP), phosphatidylglycerosulphate (PGS) and non-sulphated triglycosyl glycolipid (TGD-2), typical for members of the genus *Haloarcula*.

Table 1 summarizes the biochemical characteristics of the Tunisian isolates. All isolates had similar features with respect to cardinal growth temperatures, pH and salt requirements, and all hydrolyzed starch and utilized a large variety of carbohydrates. Strain T5 was chosen for study in depth. It has the accession No. DSM 12772. Partial sequence analysis of 16S rRNA genes from strain T5 confirmed assignment to the genus *Haloarcula*: 99.5% similarity with *H. hispanica* and *H. japonica*, 98.8% similarity with *H. marismortui* and 96.8% similarity with *H. vallismortis*.

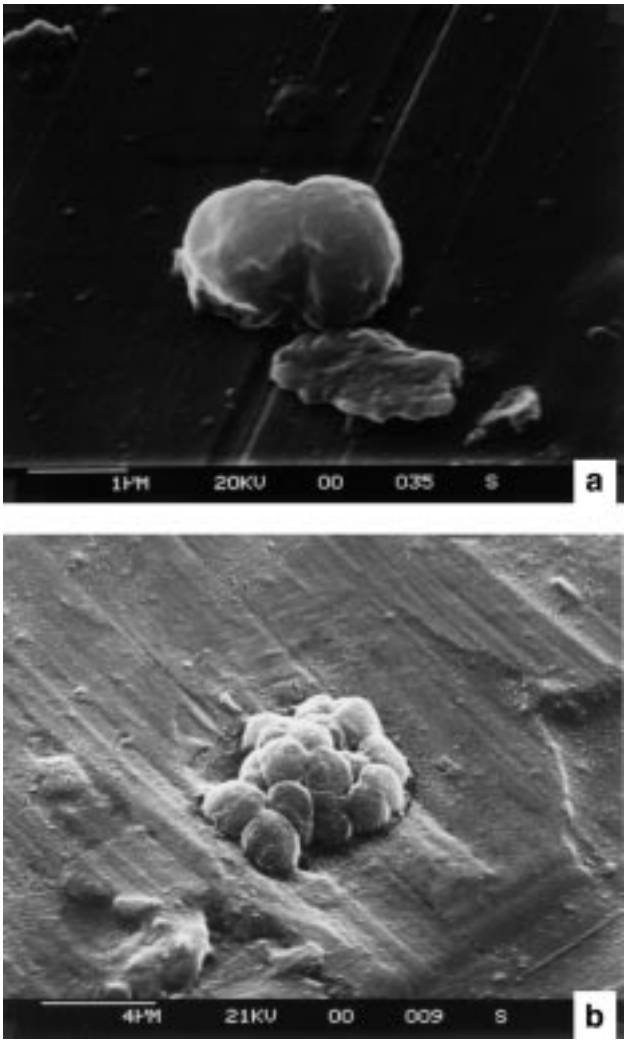


**Figure 1** Electronmicrograph of isolate T5 under SEM. (a) Cells grown on standard complex medium (pH 7.5) containing 80 mM $Mg^{2+}$, 3.5 M NaCl at 35°C. (b) Cells grown under conditions reported for (a), with the exception that $Mg^{2+}$ was 160 mM. The bar indicates the dimensions.

DNA/DNA hybridization experiments (Table 2) showed an intermediate value with *H. hispanica* (53%), high with *H. japonica* (85%), and low with all the other strains tested.

The differences in DNA/DNA homology between strain T5 and *H. japonica* and *H. hispanica* despite high 16S rDNA partial sequence homology is explainable since it is recognised that variable DNA/DNA homology values are often associated with organisms that have high but not complete sequence identity [45]. Additionally, we have not carried out a full sequence determination.

### *Accumulation of polyhydroxyalkanoate and NMR analysis of polyester*

A homopolyester of polyhydroxybutyrate (PHB) was identified by $^{1}H$ and $^{13}C$ NMR spectroscopy, in isolates grown in minimal medium containing glucose. $^{1}H$ and $^{13}C$ NMR spectra confirmed that in the three strains an isotactic homopolymer with a regular head-to-tail sequence of 3-hydroxybutyrate units was produced. In fact each peak of PHB in the $^{13}C$ NMR spectrum was very sharp and all resonances, both in $^{1}H$ and $^{13}C$ spectra (Figure 2), were typical of PHB [4,8]. PHB was not detected when the isolates were grown in the standard complex medium. The polyester concentration of strain T5 was 5 mg $g^{-1}$ of dried cells when the strain was grown in the minimal medium with either glucose or starch; the yield of the polymer reached its maximum in the presence of molasses (10 mg $g^{-1}$ of dried cells). The T5 PHB content was highest between 8 and 10 days of incubation, when the isolate was grown in the presence of starch. The synthesis of PHB has been reported for *H. marismortui* [20] and *H. vallismortis*, although PHB in *H. vallismortis* was produced only in small quantities using poorly aerated cultures [1]. For these

**Table 1** Biochemical characteristics of the strains

| Tests | Strain | | |
|---|---|---|---|
| | T5 | T6 | T7 |
| Gram stain | – | – | – |
| pH range for growth | 7.0–8.0 | 7.0–8.0 | 7.0–8.0 |
| T °C range for growth | 24–45 | 20–45 | 25–45 |
| Catalase | + | + | + |
| Oxidase | + | + | + |
| NaCl (M) range for growth | 2.5–5.0 | 2.5–5.0 | 2.5–5.0 |
| $MgSO_4$ (M) | | | |
| 0 | W | W | W |
| 0.005 | W | W | W |
| 0.1 | + | + | + |
| 0.5 | + | + | + |
| Starch hydrolysis | + | ++ | + |
| Nitrate reduction | + | + | + |
| Gelatin hydrolysis | – | – | – |
| Tween 60 hydrolysis | + | + | + |
| Urea hydrolysis | – | – | – |
| Indole production | + | + | + |
| Spore formation | – | – | – |
| Sensitive to bacitracin | + | + | + |
| *Utilization of carbon sources* | | | |
| Glucose | + | + | + |
| Fructose | + | + | W |
| Galactose | + | + | + |
| Arabinose | + | + | + |
| Raffinose | + | – | – |
| Xylose | + | + | + |
| Cellobiose | + | + | + |
| Sucrose | + | + | + |
| Rhamnose | + | – | + |
| Molasses | + | W | W |

+ Positive; – negative; W weak positive.

**Table 2** DNA/DNA hybridization among *Haloarcula* spp

| Organism | | % Hybridization with isolate T5 |
|---|---|---|
| *Haloarcula vallismortis* | DSM 3756 | 38 |
| *H. marismortui* | DSM 3752 | 25 |
| *H. hispanica* | DSM 4426 | 58 |
| *H. japonica* | DSM 6131 | 85 |
| Isolate T5 | | 100 |
| *'H. sinaiiensis'* | ATCC 33 800 | 39 |
| *'H. californiae'* | ATCC 33 799 | 19 |

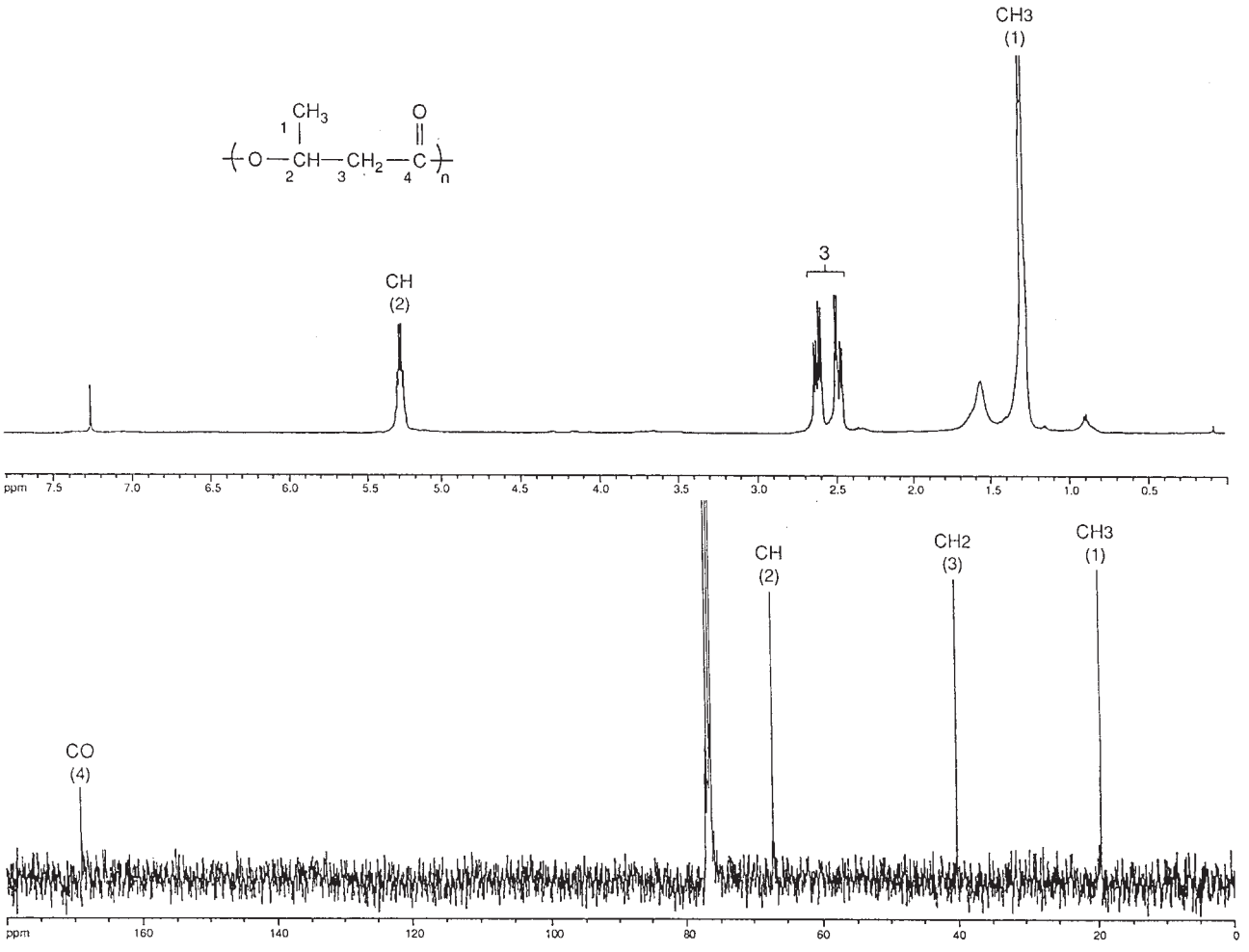


**Figure 2** 500-MHz $^{1}H$ and $^{13}C$ NMR spectra of polyhydroxybutyrate obtained from isolate T5.

last two species of *Haloarcula* there are no other detailed studies about the chemical identification of this polymer and its accumulation under various fermentation conditions.

### *Production and partial characterization of exopolysaccharide*

The isolates, grown on the minimal medium with glucose, were able to produce a sulfated extracellular polysaccharide (EPS), which was isolated from cell-free culture broth by precipitation with cold ethanol. The ethanolic precipitate, after dissolution in hot water, was centrifuged and the soluble fraction accounted for 80–90% of the total. This material was dialyzed, freeze-dried and weighed for each sample: 370 mg $L^{-1}$ (T5), 45 mg $L^{-1}$ (T6), 35 mg $L^{-1}$ (T7), respectively. A preliminary $^{1}H$ NMR spectrum of the crude EPS extract of isolate T5 in $D_2O$ showed the presence of five anomeric signals, two $\alpha$ and three $\beta$-linkages at $\delta$ 5.15, 5.01, 4.97, 4.95, and 4.90 (Figure 3).

The soluble fraction obtained from the T5 strain was desalted on Sephadex G-50 with a yield of 85%, and then chromatographed on DEAE-Sepharose CL-6B, with a yield of 80%. EPS fractions eluted at 0.5 M NaCl represented 50% of the total carbohydrate fractions. Hydrolysis of EPS with 2 M TFA yielded, as principal constituents: mannose, galactose and glucuronic acid in relative proportions of 2:1:3. Sugar analysis of crude EPSs of strains T6 and T7 yielded, as principal constituents: mannose, galactose and glucose in relative proportions of 1:0.2:0.2. Glucuronic acid was also detected. There have been reports that *Haloferax* spp produce EPS [2], and a report that *Haloarcula* spp also produce EPS [44]. However, only the *H. mediterranei* polymer has been partially characterized by Antòn *et al* [3], and fully characterized showing a unusual new structure by Parolis *et al* [33].

## Concluding remarks

Strain T5 differs from other *Haloarcula* spp (Table 3) in a number of respects. Morphologically, isolate T5 appears as pleomorphic coccobacilli, resembled *H. vallismortis* and *H. hispanica*; the latter species as well as strain T5 is also motile and able to hydrolyze starch, gelatin and Tween-60. Unlike *H. hispanica*, strain T5 was able to form PHB and was sensitive to bacitracin. The Tunisian isolate T5 shared this last property with the *Haloarcula* species: *H. japonica*, *H. marismortui* and *H. vallismortis*, but was different from these species in other features: indole production, tween and starch hydrolysis (Table 3).

Although the DNA–DNA hybridization experiments suggested that isolate T5 was closely related to *H. japonica*

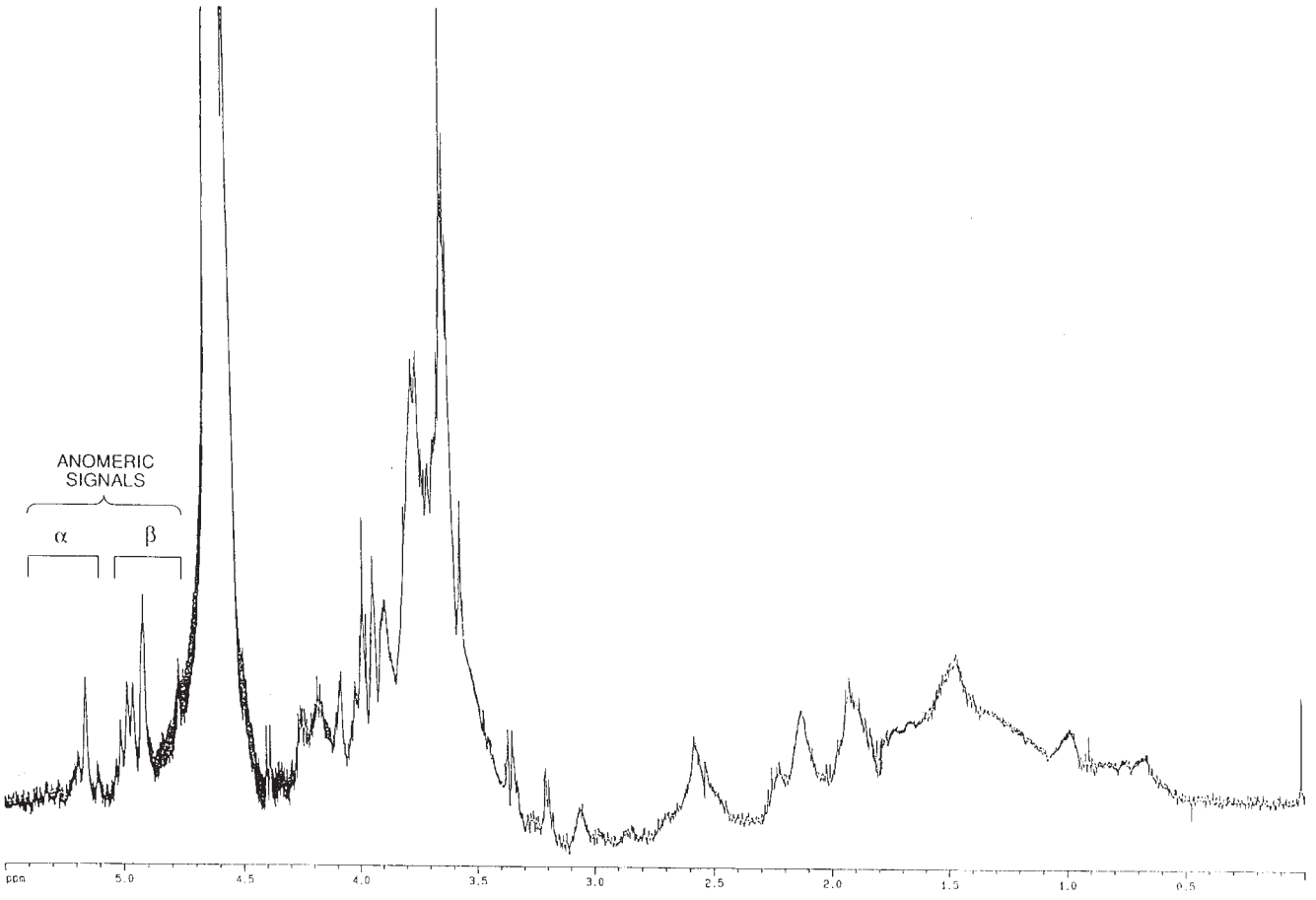


**Figure 3** 500-MHz $^{1}H$ spectrum of crude EPS from isolate T5.

**Table 3** Differential characteristics of the species of the genus *Haloarcula*

| Characteristic | Isolate T5 | *H. hispanica*[a] | *H. japonica*[a] | *H. marismortui*[a] | *H. vallismortis*[a] |
|---|---|---|---|---|---|
| Cell shape | Pleomorphic cocco-bacilli | Pleomorphic rods | Irregular flat triangles, rectangles | Irregular flat discs, rectangles | Pleomorphic rods |
| Cell dimension ($\mu$m) | 0.5–0.8 | 0.3–0.4 × 0.5–1.0 | 1.5–2.5 × 4.0–5.0 | 1.5–2.0 × 2.0–4.0 | 0.6–1.0 × 3.0–5.0 |
| Motile | + | + | + | – | – |
| Starch hydrolysis | + | + | – | W | W |
| Gelatin hydrolysis | – | + | – | – | – |
| Indole | – | [d] | + | – | + |
| Tween (60) hydrolysis | + | + | – | ND | – |
| Urea hydrolysis | – | – | ND | ND | + |
| Sensitive to bacitracin | + | – | + | + | + |
| PHB | +[b] | – | – | + | +[c] |

+ Positive; – negative; ND not determined; W weakly positive.
[a]Bergey's Manual of Systematic Bacteriology, vol 3, p 2225; Bergey's Manual of Systematic Bacteriology IX edition, p 744.
[b]Grown on glucose and on molasses.
[c]Grown in poorly aerated cultures.
[d]11–89% of strains are positive.

[41], the triangular morphology typical of this species [16] never appeared in our isolate over the growth period, even when the $Mg^{2+}$ concentration and/or the NaCl concentration were varied (Figure 1). It was reported that more than 80% of the cells of *H. japonica* were present as triangular forms in liquid medium, whilst in our case this characteristic was not observed either in liquid or on solid medium. On the basis of these considerations we conclude that the T5 isolate is a new strain of *H. japonica*.

Another feature that distinguished isolate T5 from *H. japonica* is the abilities to hydrolyze starch and to synthesize polyhydroxybutyrate.

Tunisian isolates had some interesting characteristics with respect to the related species, *H. japonica*, including PHB production, sulfated-exopolysaccharide formation and starch hydrolysis. It is of interest that the sulfated exopolysaccharide interferes with the adsorption and penetration of viruses into host cells and inhibited various retroviral reverse transcriptases [15,35]. Whereas the three Tunisian isolates biosynthesised the same polyester, they produced exopolysaccharides with different chemical structures.

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