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Identification of polyhydroxyalkanoates in *Halococcus* and other haloarchaeal species

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Abstract Polyhydroxyalkanoates (PHAs) are accumulated in many prokaryotes. Several members of the *Halobacteriaceae* produce poly-3-hydroxybutyrate (PHB), but it is not known if this is a general property of the family. We evaluated identification methods for PHAs with 20 haloarchaeal species, three of them isolates from Permian salt. Staining with Sudan Black B, Nile Blue A, or Nile Red was applied to screen for the presence of PHAs. Transmission electron microscopy and ^{1}H-nuclear magnetic resonance spectroscopy were used for visualization of PHB granules and chemical confirmation of PHAs in cell extracts, respectively. We report for the first time the production of PHAs by *Halococcus* sp. (*Halococcus morrhuae* DSM 1307^{T}, *Halococcus saccharolyticus* DSM 5350^{T}, *Halococcus salifodinae* DSM 8989^{T}, *Halococcus dombrowskii* DSM 14522^{T}, *Halococcus hamelinensis* JCM 12892^{T}, *Halococcus qingdaonensis* JCM 13587^{T}), *Halorubrum* sp. (*Hrr. coriense* DSM 10284^{T}, *Halorubrum chaoviator* DSM 19316^{T}, *Hrr. chaoviator* strains NaxosII and AUS-1), haloalkaliphiles (*Natronobacterium gregoryi* NCMB 2189^{T}, *Natronococcus occultus* DSM 3396^{T}) and *Halobacterium noricense* DSM 9758^{T}. No PHB was detected in *Halobacterium salinarum* NRC-1 ATCC 700922, *Hbt. salinarum* R1 and *Haloferax volcanii* DSM 3757^{T}. Most species synthesized PHAs when growing in synthetic as well as in complex medium. The polyesters were generally composed of PHB and poly-ß-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV). Available genomic data suggest the absence of PHA synthesis in some haloarchaea and in all other Euryarchaeota and Crenarchaeota. Homologies between haloarchaeal and bacterial PHA synthesizing enzymes had indicated to some authors probable horizontal gene transfer, which, considering the data obtained in this study, may have occurred already before Permian times.

Keywords Polyhydroxybutyrate · Haloarchaea · *Halococcus* · *Halobacterium* · Haloalkaliphile

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Introduction

Polyhydroxyalkanoates (PHAs) are produced and accumulated in prokaryotes as carbon and energy storage materials (Rehm 2003). They are water-insoluble polymers and stored in the cell cytoplasm as granules. Chemically, they consist mostly of poly-ß-hydroxybutyrate (PHB) and poly-ß-hydroxyvalerate or copolymers (poly-ß-hydroxybutyrate-*co*-3-hydroxyvalerate; PHBV). In recent years, PHAs have attracted increasing attention due to their biodegradable, biocompatible, and thermoplastic features; they could be substitutes for petrochemical-derived plastics and be used as packaging and biomedical materials (Madison and Huisman 1999).

The presence of PHA granules in haloarchaea was first reported in 1972 (Kirk and Ginzburg 1972). The strains were called at that time "*Halobacterium* sp. from the Dead Sea", but later identified as *Haloarcula marismortui* (Oren et al. 1990). Since then, strains of several other haloarchaeal genera, including *Haloferax*, *Halobiforma*, and *Haloquadratum*, have been found to accumulate PHAs, such as poly (3-hydroxybutyrate) or poly(3-hydroxybutyrate-*co*-hydroxyvalerate) (Fernandez-Castillo et al. 1986; Hezayen et al. 2002a; Burns et al. 2000). A recent review (Quillaguamán et al. 2010) lists PHA production by halophilic Archaea and Bacteria, with a focus on *Haloferax mediterranei*, which shows the highest potential of an archaeal source for industrial applications, and the characterization of enzymes involved in synthesis of PHA. No members of the genus *Halococcus* nor haloalkaliphiles, which grow optimally at pH values between 9 and 9.5 were yet reported to produce PHAs. In 2002, the first PHB synthase from an extremely halophilic archaeon was isolated from strain 56 (Hezayen et al. 2002b); this strain has been classified recently as *Halopiger aswanensis* (Hezayen et al. 2009).

In this work, species from several haloarchaeal genera, including members of the genus *Halococcus*, some haloalkaliphiles, and three isolates from Permo-Triassic rock salt (*Halococcus salifodinae* DSM 8989^T [Denner et al. 1994], *Halococcus dombrowskii* DSM 14522^T [Stan-Lotter et al. 2002] and *Halobacterium noricense* DSM 9758^T [Gruber et al. 2004]), were investigated for the production of PHAs to learn more about the distribution of this capacity, which might lead to the detection of novel producers, and to find rapid and simple methods of identification of the polyesters.

Material and methods

Archaeal and bacterial strains

The following strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7 B, 38124 Braunschweig, Germany): *Haloarcula hispanica* DSM 4426^T, *Hbt. noricense* DSM 9758^T, *Halococcus morrhuae* DSM 1307^T, *Hcc. dombrowskii* DSM 14522^T, *Halococcus saccharolyticus* DSM 5350^T, *Hcc. salifodinae* DSM 8989^T, *Hfx. mediterranei* DSM 1411^T, *Haloferax volcanii* DSM 3757^T, *Haloquadratum walsbyi* DSM 16790, *Halorubrum coriense* DSM 10284^T, *Natrinococcus occultus* DSM 3396^T, and *Bacillus megaterium* DSM 32^T. Strains *Halococcus hamelinensis* JCM 12892^T and *Halococcus qingdaonensis* JCM 13587^T were obtained from the Japan Collection of Microorganisms (RIKEN Bio-Resource Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan). *Halorubrum chaoviator* strains Halo-G* (DSM 19316^T), AUS-1 and Naxos II were isolates from Baja California, Western Australia and Naxos, Greece, respectively (see Mancinelli et al. 2009). *Halobacterium salinarum* NRC-1 (ATCC-700922) was purchased from LGC Teddington, UK. Cells of *Natronobacterium gregoryi* NCMB 2189^T and *Hbt. salinarum* R1 were gifts from Dr. Lawrence Hochstein, formerly at NASA Ames Research Center, USA.

Culture conditions

Strains were grown at 37 °C in side arm flasks in an incubator (Innova 4080) with a shaking platform (180 rpm). Growth in liquid culture was monitored spectrophotometrically at 520 nm (for synthetic media), or 600 nm (for complex media), respectively, using a Novaspec II photometer (Pharmacia). All strains were grown in both synthetic as well as in complex medium, unless indicated otherwise.

Synthetic media Basal synthetic medium was prepared similarly as described by Lillo and Rodriguez-Valera (1990); the pH was adjusted to 7.2 with NaOH. For growth of haloalkaliphiles, the NaCl content of the medium was increased to 200 g/l and $MgSO_4$ was reduced to 2 g/l; the pH was adjusted to 9.0 with NaOH.

Complex media *Halococcus* species and *Hbt. salinarum* R1 were cultured in M2 complex medium for neutrophilic halobacteria (Tomlinson and Hochstein 1976), except for *Hcc. hamelinensis* JCM 12892^T and *Hcc. qingdaonensis* JCM 13587^T, which were grown in DSM medium 372 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium372.pdf). *Hqr. walsbyi* (DSM 16790) was grown in JCM medium 457 (Bolhuis et al. 2004), *Hbt. salinarum* NRC-1 was grown in ATCC 2185 medium (Gruber et al. 2004); *Hbt. noricense* DSM 9758^T was grown in modified *Halobaculum gomorrense* medium DSM 823 (Gruber et al. 2004), *Nbt. gregoryi* NCMB 2189^T was grown in DSM medium 371 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium371.pdf), which had a final pH of 9.0-9.5. *B. megaterium* DSM 32^T was grown in nutrient broth, which contained (gram per liter): peptone 5; meat extract 3; the pH was adjusted to 7.0.

Staining procedures and microscopy

For staining, cells from early stationary growth phase were used; with few exceptions, cell density was high enough for direct microscopic observations. In the case of very low ODs (less than ca. 0.2) cells were concentrated by a factor of about 10 by centrifugation and resuspension.

Staining of cells with Sudan black B (Murray et al. 1994) Smears of cells deposited on a glass slide were heat-fixed and stained with a 3% (*w*/*v* in 70% ethanol) solution of Sudan Black B (Sigma) for 10 min, followed by immersion of the slide in xylene until it was completely decolorized. The sample was counterstained with safranin (Sigma; 5% *w*/*v* in deionized water) for 10 s, washed with water and dried. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined by phase contrast microscopy (Leica DM5000B).

Staining of cells with Nile Blue A (Ostle and Holt 1982) A 1% aqueous solution of Nile blue A (Sigma), was mildly heated and filtered before use (Ostle and Holt 1982). Heat-fixed smears of archaeal cells were stained with the Nile blue A solution at 55 °C in a water bath for 10 min or, in the case of *Halococcus* species, for 15 min. After staining, slides were washed with tap water followed by an 8% acetic acid solution for 1 min, washed again, and finally the stained smear was blotted dry with bibulous paper (remoistened with tap water) and covered with a glass cover slip. The samples were examined with a Leica DM5000B fluorescence microscope and observed with blue excitation wavelengths (filter BP 450-490).

Colony staining with Nile Red (Spiekermann et al. 1999) Nile Red (Sigma) was added from a stock solution of 25% (*v*/*v*) in dimethylsulfoxide to agar media at a final concentration of 0.5 μg/ml. Following growth, agar plates were exposed to UV light (365 nm) from a transilluminator (Type TFC-20 M; Vilber Lourmat, France) and photographed with a digital camera (Nikon COOLPIX S225).

Microscopes and acquisition of images For fluorescence and phase contrast microscopy, a Leica microscope type DM5000B with a mercury lamp (Hg100W; Leica Microsystems, Wetzlar, Germany), camera type DFC300FX (Leica Microsystems DI Cambridge) combined with the Leica Application Suite software 2.8.1 was used. Pictures were contrast-enhanced with Photoshop version 8.0.

Transmission electron microscopy (TEM) TEM was performed as described previously (Denner et al. 1994; Witte et al. 1990). Briefly, cells were fixed with 2% glutaraldehyde, stained with 3% lead citrate, and examined in a Siemens Elmiscop 101.

Chemical analysis of PHAs

Preparation of samples One hundred milliliters each of haloarchaeal cultures were obtained by growing cells of *Hfx. mediterranei* DSM 1411^T and *Har. hispanica* DSM 4426^T in the basal synthetic medium supplemented with 1% (*w*/*v*) glucose (see above). All other strains were grown in complex media as described in "Materials and methods" section. Cells were harvested by centrifugation at 7,000 rpm for 30 min at 20 °C in a SLA-3000 rotor in a Sorvall RC6 centrifuge. Cell pellets were washed with 30 ml sterile TN buffer (4 M NaCl and 50 mM Tris, pH 7.4) and centrifuged again. Pellets were transferred into sterile plastic tubes (1.5 ml volume each), frozen at −70 °C and subjected to lyophilisation on a laboratory freeze dryer Type Freezone 12 (Labconco). The dry weight of the freeze-dried samples was determined gravimetrically.

For extraction and enrichment of PHAs from haloarchaeal cells, two methods were used: (1) treatment with sodium hypochlorite which was performed similar as described by Lillo and Rodriguez-Valera (1990). Cells were incubated with 0.2% (weight per volume) sodium hypochlorite for 1.5-2 h at 37 °C and centrifuged; the pellets were washed with distilled water, acetone and finally 96% ethanol, followed by freeze-drying. (2) Repeated washing of haloarchaeal cells with water, which resulted in lysis of cells (Grant et al. 2001) and, following centrifugation, left an insoluble whitish residue, which contained PHAs. Following four to five washes with water, the material was freeze dried. In some cases, washes were done with aqueous 0.1% (*w*/*v*) sodium dodecyl sulfate solution instead of water, which improved yields slightly.

Nuclear magnetic resonance (NMR) spectroscopic analysis of PHB and PHBV in cell extracts To determine the amounts of PHB and PHBV, 20-70 mg of lyophilized cells were shaken with 1 ml of deuterochloroform ($CDCl_3$) for 10 min and sonicated for another 15 min to solubilize PHB and PHBV. After centrifugation, 20 mg ethyl sulfone was added to the supernatant as internal standard. One-dimensional ^1H NMR spectra with 128 scans were acquired on a Bruker AMX 360 MHz NMR spectrometer at 298 °K (Mukhopadhyay et al. 2005). Alternatively, spectra were obtained with a Varian Unity-300 spectrometer, operating at 299.94 MHz. For quantification, the signals of the methine protons at 3-position of PHB ($\delta(^1H)$=5.25 ppm) and PHBV ($\delta(^1H)$=5.17 ppm) were integrated and compared to the ethyl sulfone CH_2 signal ($\delta(^1H)$=3.00 ppm); in some experiments, quantification was done with benzene ($\delta(^1H)$=7.28 ppm). Lyophilized cell extracts, which were enriched for PHAs (see above), were treated similarly, except that the volumes of reagents were scaled down appropriately, since the dry weights were usually in the range of ≤5–10 mg, and the number of scans was increased to 1,024.

Results

Light and electron microscopical detection of PHA granules

The lipophilic stain Sudan Black B has long been regarded as a dye with particular high affinity for PHAs (Murray et al. 1994). Ostle and Holt (1982) demonstrated a higher specificity of the fluorescent dye Nile Blue A, a basic oxazine dye, for PHB. Both stains were applied here to cultures of haloarchaeal species. Following staining with Sudan Black B, stationary phase cells of haloarchaeal species contained prominent dark granules, as shown for *Hcc. morrhuae* DSM 1307^T in Fig. S1 (Online resource).

Staining with Nile Blue A was performed with cells of two strains, which are known producers of PHB, *Hfx. mediterranei* DSM 1411^T (Lillo and Rodriguez-Valera 1990) and *Hqr. walsbyi* DSM 16790 (Burns et al. 2007). Fluorescence microscopy at an excitation wavelength of 460 nm revealed numerous brightly fluorescent orange granules within the cells (Online resource, Fig. S2, A, C). Phase contrast micrographs of the same cells allowed already the detection of granules in *Hfx. mediterranei* DSM 1411^T and *Hqr. walsbyi* DSM 16790 as light refracting areas within the cytoplasm (Online resource, Fig. S2, B, D). Six species of the genus *Halococcus* (*Hcc. salifodinae* DSM 8989^T, *Hcc. hamelinensis* JCM 12892^T, *Hcc. dombrowskii* DSM 14522^T, *Hcc. qingdaonensis* JCM 13587^T, *Hcc. saccharolyticus* DSM 5350^T, *Hcc. morrhuae* DSM 1307^T) contained roundish granules of bright orange fluorescence within their cells, following staining with Nile Blue A (Fig. 1a-f). Six further haloarchaeal species are shown in Fig. S3 (Online resource): similar orange granules were detected in *Hbt. noricense* DSM 9758^T, *Ncc. occultus* DSM 3396^T, *Halorubrum* species (*Hrr. coriense* DSM 10284^T, *Hrr. chaoviator* DSM 19316^T, *Hrr. chaoviator* strain NaxosII), and *Har. hispanica* DSM 4426^T. In most cells, the granules were visible as distinct entities against a dark background; however, some cells showed also diffuse staining which delineated roughly the cell morphology. We attribute this faint color to a certain affinity of the lipophilic dye Nile Blue A to membranes or haloarchaeal cell envelopes. The number of granules in the cells differed to

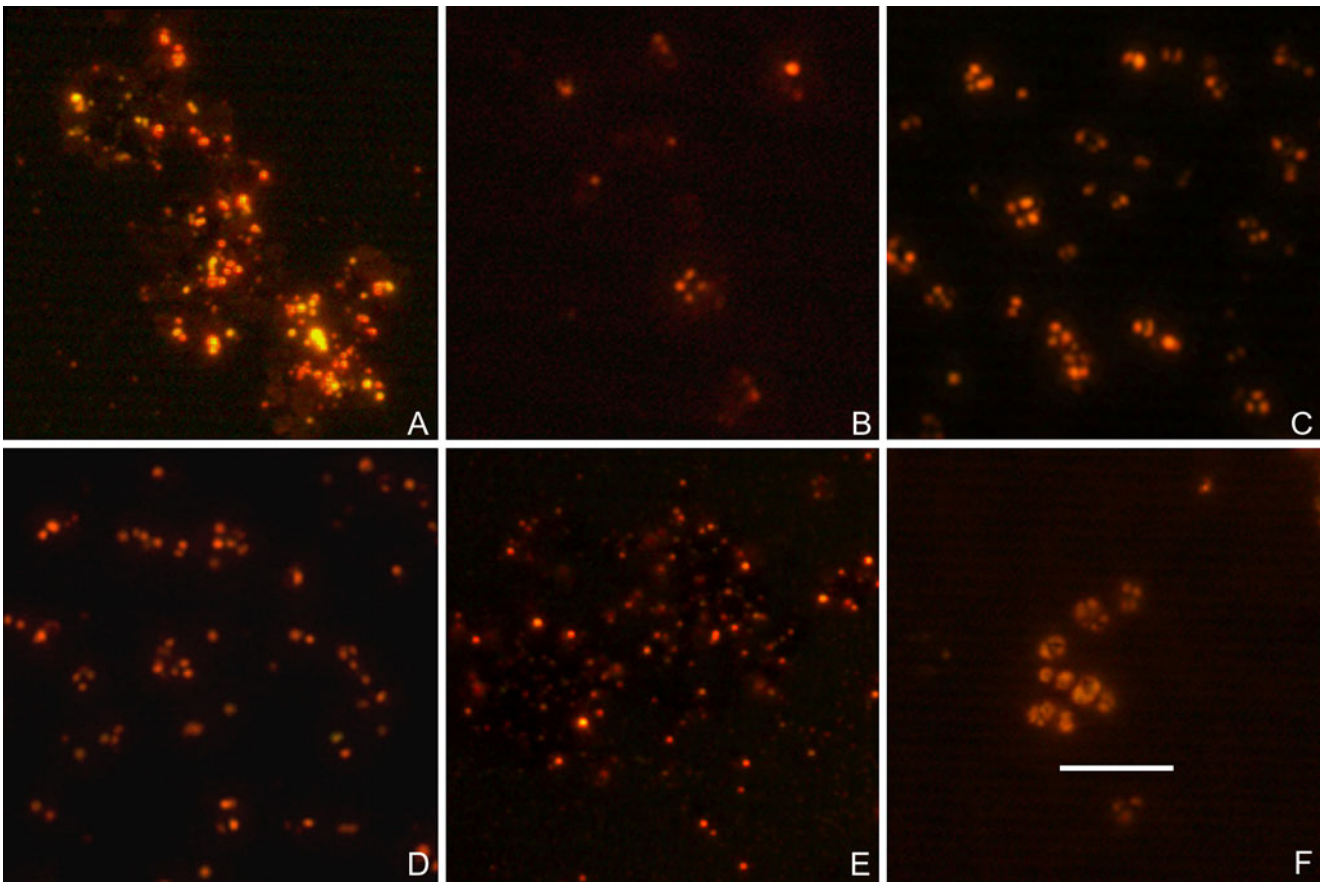

Fig. 1 Fluorescence microscopy of *Halococcus* species following staining with Nile Blue A. Growth media are indicated in brackets. First row: **a** *Hcc. salifodinae* DSM 8989^T (M2 medium); **b** *Hcc. hamelinensis* JCM 12892^T (DSM medium 372); **c** *Hcc. dombrowskii* DSM 14522^T (M2 medium). Second row: **d** *Hcc. qingdaonensis* JCM 13587^T (DSM medium 372); **e** *Hcc. saccharolyticus* DSM 5350^T (M2 medium); **f** *Hcc. morrhuae* DSM 1307^T (M2 medium). *Bar* 5 μm

some extent between the species; but in size and brightness, the orange fluorescent granules were similar as in the control organisms *Hfx. mediterranei* DSM 1411^T and *Hqr. walsbyi* DSM 16790 (see Online resource, Fig. S2).

In Table 1, staining results are summarized for growth in synthetic or complex medium, with "+" denoting the presence of orange granules due to Nile Blue A, or dark granules, due to Sudan Black B. *B. megaterium* was used as a negative control since this organisms possesses the capacity for production of PHAs, but does so only under limitation of nutrients (Findlay and White 1983) and not in complex medium.

Early TEM had revealed clear zones within haloarchaeal cells which, together with results from X-ray diffraction patterns, were identified as PHB granules (Kirk and Ginzburg 1972); a recent TEM image of PHBV granules in *Hfx. mediterranei* is displayed in Lu et al. (2008). Figure 2 shows a TEM photograph of *Hcc. morrhuae* DSM1307^T grown in M2 medium for 7 days. Each cell appeared to contain one or more PHB granules, which are visible as white inclusion bodies within the cytoplasm. The size of the PHB granules ranged from approximately 0.05-0.30 μm in diameter, as measured in the micrograph. While the structures in TEM pictures arise from random cuts through embedded material and therefore cannot be considered as an exact representation of the original material, the range of sizes was similar to the dimensions which were observed for bacterial PHB granules (Sudesh et al. 2000). Similar TEM pictures with PHB granules of comparable sizes were obtained with cells of *Hcc. salifodinae* DSM 8989^T (not shown).

Incorporation of Nile Red into colonies and cells

A selective solid medium was proposed by Spiekermann et al. (1999), containing the fluorescent dye Nile Red, which

Table 1 PHB accumulation in haloarchaeal species detected by staining with Nile Blue A or Sudan Black B. Cells were grown in synthetic medium + 1% glucose and/or complex medium (type indicated in brackets; see "Material and methods" sections for composition). The presence of stainable granules is indicated by+(Nile Blue A; Sudan Black B), or no staining (−)

Organism	Synthetic medium + 1% glucose		Complex medium	
	Nile Blue A	Sudan Black B	Nile Blue A	Sudan Black B
Hcc. dombrowskii DSM 14522^T	+	+	+ (M2)	ND
Hcc. salifodinae DSM 8989^T	+	+	+ (M2)	ND
Hcc. morrhuae DSM 1307^T	+	+	+ (M2)	+ (M2)
Hcc. saccharolyticus DSM 5350^T	+	+	+ (M2)	ND
Hcc. hamelinensis JCM 12892^T	ND	ND	+ (DSM372)	ND
Hcc. qingdaonensis JCM 13587^T	ND	ND	+ (DSM372)	ND
Ncc. occultus DSM 3396^T	+	+	ND	ND
Har. hispanica DSM 4426^T	+	+	+ (M2)	ND
Hfx. mediterranei DSM 1411^T	+	+	+ (M2)	ND
Hqr. walsbyi DSM 16790	ND	ND	+ (JCM 457)	+ (JCM457)
Hbt. noricense DSM 9758^T	+	+	+ (DSM823 mod.)	ND
Hrr. coriense DSM 10284^T	+	+	+ (M2)	ND
Hrr. chaoviator DSM 19316^T	+	+	+ (M2)	ND
Hrr. chaoviator strain Naxos II	+	+	+ (M2)	ND
Hrr. chaoviator strain AUS-1	ND	ND	+ (M2)	ND
Negative control: *B. megaterium* (DSM 32 ^{T})	ND	ND	– (nutrient broth)	– (nutrient broth)

ND not done

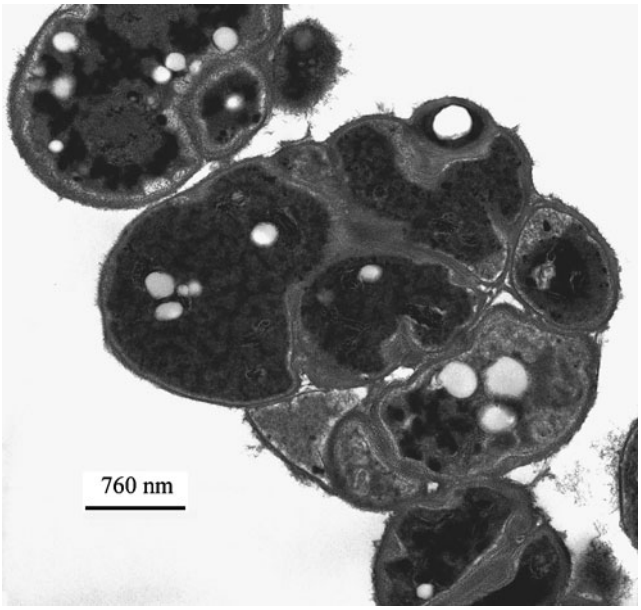

Fig. 2 Transmission electron micrograph of *Hcc. morrhuae* DSM 1307^T grown in complex medium (M2) for 7 days. Cells contain whitish poly-β-hydroxybutyrate inclusion bodies

enables discrimination between PHA accumulating and non-PHA-accumulating cells. Addition of Nile Red in low concentration (0.5 ug/ml) did not inhibit growth of haloarchaeal strains and caused intense fluorescence of colonies under UV light of 365 nm. *Hfx. mediterranei* DSM 1411^T and *Ncc. occultus* DSM 3396^T are shown in Fig. S4 (Online resource); other haloarchaeal strains, which were fluorescent on Nile Red containing agar plates, were *Har. hispanica* DSM 4426^T, *Hbt. noricense* DSM 9758^T, *Hrr. chaoviator* DSM 19316^T, and *Hrr. chaoviator* strain NaxosII (not shown). No or only weak fluorescence was detected with colonies of *Hbt. salinarum* NRC-1 (not shown). Cells from these plates were examined by fluorescence microscopy and exhibited orange-red staining, which confirmed the uptake of Nile Red (not shown).

A summary of the culturing conditions of haloarchaeal strains and the concomitant presence of stainable granules within cells, which suggested the production of PHAs, is given in Table 1. The cultures of the haloarchaeal strains were generally in their stationary growth phase when examined for PHAs. When synthetic medium was used, cell yields were in some cases low (OD≤0.2), as was to be expected from the known limited utilization of carbohydrates by some haloarchaea (Grant 2001a). Nevertheless, following concentration by centrifugation, enough biomass was obtained for successful staining experiments.

Chemical detection of PHAs

The content of PHAs was determined by proton NMR with chloroform extracts from freeze-dried cells and aqueous or hypochlorite extracts of cells. ^{1}H NMR spectra showed the characteristic peaks of PHB near 5.25 ppm and PHBV near 5.17 ppm (Jan et al. 1996; Mukhopadhyay et al. 2005), see Fig. S5 (Online resource). Table 2 shows the presence of PHAs synthesized by haloarchaeal species following analysis with ^{1}H NMR of extracts from freeze-dried cultures or fresh cells. The yield of PHAs was generally low and ranged from 0.06% to 0.25% of the cellular dry weight; however, it should be noted that no optimization of production of PHAs was attempted in this work. In addition, losses due to incomplete extraction of samples were likely occurring, as have been described previously for lipids and isoprenoids from haloarchaea (Kates and Kushwaha, 1995). Therefore, the numbers are to be considered the minimum amounts of PHAs in the respective strains. PHB was the main component produced by all investigated haloarchaeal species (Table 2); the copolymer PHBV was synthesized by several strains, but in much smaller amounts. Aqueous extracts yielded more enriched preparations from *Hfx. mediterranei* DSM 1411^T than was possible with chloroform treatment (about 69% PHB versus 0.2%; Table 2), and a somewhat improved preparation from *Hcc. dombrowskii* DSM 14522^T (0.4% PHB versus 0.15%; Table 2). Hypochlorite extracts (Lillo and Rodriguez-Valera 1990) increased the yield of PHB from *Nbt. gregoryi* NCMB 2189^T (Table 2). While the reproducibility of ^{1}H NMR spectra from the same sample was high (Table 2), variations between different preparations may be expected to be large, due to procedural influences as noted above.

Information from genomics

The classical route for the synthesis of PHA involves three enzymes (gene name in brackets): β-ketothiolase (*phaA*), acetoacetyl-CoA dehydrogenase (*phaB*), and poly(3-hydroxyalkanoate) synthase (*phaC*), although some additonal pathways have been identifed recently (Kalia et al. 2007). To date, the sequences of 12 genomes of the *Halobacteriaceae* are available, e. g., in the website resource of EMBL (http://www.ebi.ac.uk/embl/): *Har. marismortui* ATCC 43049, *Hbt. salinarum* NRC-1 ATCC700922, *Hbt. salinarum* R1, *Hfx. volcanii* DS2, *Halogeometricum borinquense* DSM 11551, *Halomicrobium mukohataei* DSM 12286, *Hqr. walsbyi* DSM 16790, *Halorhabdus utahensis* DSM 12940, *Halorubrum lacusprofundi* ATCC 49239, *Haloterrigena turkmenica* DSM 5511, *Natrialba magadii* ATCC 43099, *Natronomonas pharaonis* DSM 2160. In six of these, *Har. marismortui*, *Hgm. borinquense*, *Hmc. mukohataei*, *Hrd. utahensis*, *Hqr. walsbyi*, and *Htg. turkmenica*, the *phaC* gene is present. Recently highly homologous genes (*phaEC*) were identified in *Har. hispanica* (Han et al. 2007) and *Hfx. mediterranei* (Lu et al. 2008), which are coding for two subunits (E, C) of the PHA synthase. The genome of

Table 2 Contents of PHB and PHBV in haloarchaeal species, determined by proton NMR from cell extracts

Organism	Origin of material					
	Freeze-dried cells		Fresh cells, aqueous extract		Fresh cells, hypochlorite extract	
	PHB *w*/*w*% of cell dry weight	PHBV *w*/*w*% of cell dry weight	PHB *w*/*w*% of dried extract	PHBV *w*/*w*% of dried extract	PHB *w*/*w*% of dried extract	PHBV *w*/*w*% of dried extract
Hfx. mediterranei DSM 1411^T	0.19 0.20	0.03 0.05	69.6±0.43[a]	8.1 ± 0.3[a]	ND	ND
Hfx. volcanii DSM 3757^T	ND	ND	Not detectable	Not detectable	ND	ND
Hcc. dombrowskii DSM 14522^T	0.15	0.01	0.4±0[a]	0.07±0.05[a]	0.1	Not detectable
Hcc. salifodinae DSM 8989^T	0.05	0.01	ND	ND	ND	ND
Hcc. saccharolyticus DSM 5350^T	ND	ND	1.2	Not detectable	0.05	Not detectable
Har. hispanica DSM 4426^T	0.09	0.04	ND	ND	ND	ND
Hbt. salinarum R1	Not detectable	Not detectable	ND	ND	ND	ND
Hbt. salinarum NRC-1 ATCC 700922	ND	ND	Not detectable	Not detectable	Not detectable	Not detectable
Hbt. noricense DSM 9758^T	0.08	0.03	ND	ND	ND	ND
Hqr. walsbyi DSM 16790	ND	ND	0.1	Not detectable	ND	ND
Nbt. gregoryi NCMB 2189 ^{T}	0.1 0.44	0.03 0.18	0.1	Not detectable	0.4	Not detectable
Ncc. occultus DSM 3396 ^{T}	ND	ND	ND	ND	3.1	Not detectable

Freeze-dried cells were treated with $CDCl_3$ and sonicated prior to ^{1}H NMR analysis (see Methods); cell dry weights ranged from 33 to 170 mg. Water and hypochlorite extracts were prepared from fresh cells (see Methods for details) and ranged from 2 to 10 mg

ND not done

[a] Mean value ± standard deviation from three experiments each

Hbt. salinarum NRC-1 ATCC700922 contains homologues of genes *phaA* and *phaB*, but not of *pHaC* (Kalia et al. 2007). Using the HaloLex system (http://www.halolex.mpg.de/public/; see Pfeiffer et al. 2008), it is apparent that none of the available 43 genomes from Crenarchaeota and Euryarchaeota contain *phaE/C* genes.

Discussion

A useful screening method for the presence of PHAs is the incorporation of the dye Nile Red into colonies on agar plates and its detection by fluorescence (Spiekermann et al. 1999). The method has been used widely for the rapid identification of PHA-producing environmental bacteria (e.g., Berlanga et al. 2006), but not yet with archaea. In our experiments, correct predictions from the presence or absence of fluorescent colonies for the production of PHAs were obtained also for haloarchaeal strains. The method can thus be considered compatible with agar plates containing media of high ionic strength. Other methods which were used here included staining for cytoplasmic granules of PHB in cells, which is fast and generally informative. As noted earlier by Ostle and Holt (1982), Nile Blue A apparently has a greater affinity for PHB than Sudan Black B and, in connection with fluorescence microscopy, leads to superior images with high contrast. However, weak fluorescence indicative of unspecific binding to cell components, due to the lipophilic nature of Nile Blue A, may occasionally occur and lead to ambiguous results. Transmission electron microscopy is a superior method for visualizing PHB granules (see Fig. 2), but it is not a simple procedure. Chemical analysis is the preferable identification method, with the provision, that enough sample material must be used, especially when "weak PHA producers", such as most haloarchaea, are investigated, which are not stimulated by carbohydrates (Grant 2001a).

Strains of several haloarchaeal genera use carbohydrates as sources of carbon and energy, most notably *Hfx. mediterranei*, which has so far been identified as the best haloarchaeal PHA producer. PHA was detected in this work in strains from the genus *Halorubrum* (*Hrr. coriense*; three strains of *Hrr. chaoviator*), the genus *Haloarcula* (*Har. hispanica*) and the genus *Halococcus* (*Hcc. saccharolyticus*); since these strains are known to utilize various carbohydrates (McGenity and Grant 1995; Ventosa 2001; Grant 2001b), an investigation into the efficiency of their PHA production from different substrates may be warranted.

Haloalkaliphilic archaea, which require both alkaline conditions and high salt for growth (Kamekura 1998) were

shown here to include PHA producers (*Nbt. gregoryi* NCMB 2189^T, *Ncc. occultus* DSM 3396^T). The apparently high concentration of stainable granules in *Ncc. occultus* might indicate a rather profuse production of PHAs (see Online resource, Fig. S3).

The data presented here suggested that PHA granules were formed by many haloarchaeal strains during growth in both synthetic and complex media. It is likely that those haloarchaea constitutively produce the enzymes for synthesis of PHAs and accumulate the polymers continuously at low levels, independently of nutrient-rich or nutrient-limited conditions, similarly as shown for *Har. marismortui* (Han et al. 2007). The advantage for the cells would presumably be a fast response to changing environmental conditions with the commencement of production of storage materials.

Information from genome sequences about PHA synthases is still sparse. Of interest is the finding that high homologies exist to the bacterial set of enzymes as reported by several authors (Baliga et al. 2004; Bolhuis et al. 2006; Han et al. 2007; Lu et al. 2008; Quillaguamán et al. 2010), which suggests horizontal gene transfer (Kalia et al. 2007). *Hbt. salinarum* NRC-1 apparently acquired only two of the three enzymes, *pHaA* and *phaB,* but not *phaC*.

Six representatives of species from the genus *Halococcus* were shown here for the first time to produce PHA (Tables 1, 2; Fig. 1). Their geographical origins are global—Hamelin Pool, Shark Bay, Australia; Alpine salt, Austria; Qingdao beach, China; saltern in Cadiz, Southern Spain; salted fish from North American sea water. In addition, two of them (*Hcc. salifodinae* DSM 8989^T, *Hcc. dombrowskii* DSM 14522^T) are isolates from Permo-Triassic rock salt, as is the rod-shaped *Hbt. noricense* DSM 9758^T, which also contains PHAs (Tables 1, 2; Fig. S3, Online resource). It may be concluded that the transfer of all necessary enzymes has possibly occurred into these haloarchaea already some 250 million years ago.

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