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The triangular halophilic archaebacterium *Haloarcula japonica* strain TR-1

K. Horikoshi, R. Aono and S. Nakamura

Department of Bioengineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 227 (Japan)

Abstract. We have isolated a predominantly triangular disc-shaped halophilic archaebacterium, strain TR-1, from a Japanese saltern soil. The taxonomical characteristics of this strain led us to propose a new species *Haloarcula japonica*. The cell division of *Ha. japonica* strain TR-1 was analyzed by time lapse microscopic cinematography. Cell plates were laid down asymmetrically, generating triangular or rhombic daughter cells which then separated. We have demonstrated the occurrence of a glycoprotein with an apparent molecular mass of 170 kDa on the cell surface of *Ha. japonica*. The release of this cell surface glycoprotein (CSG), accompanied by a morphological change (triangular to spherical), was observed after lowering the magnesium concentration in the medium. Thus, it is likely that the CSG plays an important role in maintaining the characteristic shape of *Ha. japonica*.
Key words. Triangular bacterium; *Haloarcula japonica*; cell division; surface layer; cell surface glycoprotein; cell morphology.

Introduction

The extremely halophilic bacteria which belong to the archaebacteria[43] often exhibit unusual morphologies in liquid culture. Some of the cells may be ribbon-shaped, disc-shaped, or occasionally square or triangular[4–6]. Walsby[40] has observed square halophilic bacteria in naturally occuring brines, and Javor et al.[11] have isolated box-shaped halophilic bacteria from Californian salterns. However, the square morphology has not been convincingly demonstrated in the laboratory, and high proportions of triangular cells are not usually seen in cultures of halophilic bacteria.

The extremely halophilic archaebacteria are now divided into six genera: *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium*, and *Natronococcus*[2–6, 12, 18, 19, 39]. The genus *Haloarcula* contains a number of pleomorphic isolates[5]. Thus, the square bacteria described by Walsby[40] and Javor et al.[11] are likely to be *Haloarcula* spp.

In the course of screening a large number of samples from hypersaline environments for new isolates of halophilic bacteria, we have found a predominantly triangular disc-shaped halophilic bacterium strain TR-1. In this contribution, we describe the growth conditions, the taxonomic characteristics, and the mode of cell division responsible for the triangular morphology. The role of a cell surface glycoprotein is also discussed.

Isolation of a triangular bacterium

About 600 samples were collected from various hypersaline environments in Japan. Small amounts of material were suspended in CM medium (yeast extract 10 g; casamino acid 7.5 g; trisodium citrate 3.0 g; KCl 2.0 g; $MgSO_4 \cdot 7H_2O$ 20 g; $MnCl_2 \cdot 2H_2O$ 0.36 mg; $FeSO_4 \cdot 7H_2O$ 50 mg; and NaCl 200 g, in 1,000 ml of water, pH 7.2–7.8)[30] and then cultured at 37 °C for 6 days under aerobic conditions. The strain TR-1 was isolated from a saltern soil located at Noto Peninsula in Japan[27, 36]. The organism is motile by flagella (probably situated at one apex), non-spore forming, and has red flat cells that are predominantly triangular in shape although rhomboidal cells are also observed. The cells are typically 1–2 μm × 0.2–0.3 μm in size in liquid media. This typical triangular flat shape was clearly shown in differential interference microscopy (fig. 1A)[24].

The strain TR-1 is extremely halophilic and requires a high salt concentration in the medium[24, 27, 36]. The organism was cultured at 37 °C in a liquid CM medium in which the content of NaCl varied between 0 and 4.3 M [25% (w/v)] and of $MgSO_4$ between 0 and 650 mM [16% (w/v) of $MgSO_4 \cdot 7H_2O$]. Growth was observed between 1.7 and 4.3 M NaCl and maximal growth occurred at 2.6 to 4.3 M, depending on the $MgSO_4$ concentration. Mg^{2+} ion was required for the growth of strain TR-1, from 41 to 650 mM. The optimal concentration of $MgSO_4$ was about 160 mM. The Mg^{2+} concentration was critical for the formation of the triangular shape. At around 160 mM Mg^{2+}, typical triangular morphologies were observed. When grown in the presence of 3.4 M NaCl and low concentrations of Mg^{2+} (below 41 mM), strain TR-1 had spherical forms, while at higher concentrations of Mg^{2+} (above 320 mM), large, irregular cells became more frequent. The temperature range for growth was 24–45 °C, with an optimal temperature of 42 °C. Growth of the organism occurred within a pH range of 6.0–8.0, measured at 42 °C, with an optimal pH range of 7.0–7.5. No growth was observed above pH 8.5.

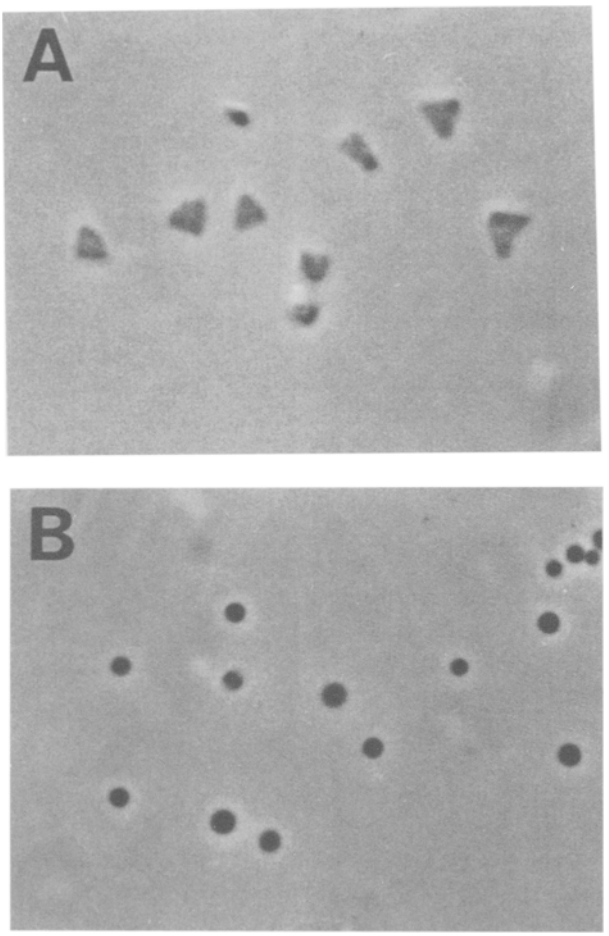

Figure. 1. Phase contrast photomicrographs of *Ha. japonica* cells before (*A*) and after (*B*) conversion to spheroplasts. (Modified from Nakamura et al.[24]).

Taxonomic characteristics

Biochemical characteristics of the strain TR-1 are as follows[27,36]: mono- and di-carbohydrates including galactose, arabinose, xylose, rhamnose, sucrose, glycerol, maltose, trehalose and cellobiose, and sugar alcohols such as mannitol and sorbitol, were utilized as sole carbon sources with acid production. No hydrolysis of casein, gelatin or starch was observed. Reduction of nitrates with gas, production of indole and H_2S were positive. No amino acid requirement was observed. Growth was inhibited by bacitracin, novobiocin and anisomycin.

Chemical composition of the cell envelope of strain TR-1[25,27] was almost the same as that of the cell envelope of *Haloarculas hispanica*[13]. Amino acid analysis of the bulk protein[27] indicated that the TR-1 bulk protein contained relatively higher concentrations of acidic amino acids such as aspartic acid and glutamic acid as compared with *Ha. hispanica*[13].

The strain TR-1 had C_{20}, C_{20} diether core lipids[27,36]. C_{25}, C_{20} (sesterterpanyl) diethers were not detected. The TR-1 cells contained C_{20}, C_{20} derivatives of phosphatidyl glycerophosphate, phosphatidyl glycerosulphate, phosphatidyl glycerol and triglycosyl diether (TGD-2) as the main polar lipids[27,36]. When compared with *Haloarcula* (formerly *Halobacterium*) *vallismortis* ATCC 29715, the presence of three unknown minor lipids was characteristic for strain TR-1.

Chromosomal DNA was extracted and purified from the TR-1 cells by the method of Tindall et al.[37] The molar ratio of guanine plus cytosine (G + C) was calculated to be 63.3 mol%[27,36]. DNA-DNA hybridization experiments[37] were carried out using ^{3}H-labeled chromosomal DNA preparations from reference strains. The DNA homology between the strain TR-1 and *Ha. vallismortis* ATCC 29715 was 20%, that between TR-1 and *Haloarcula* (formerly *Halobacterium*) *marismortui* ATCC 43049 was 32%, that between TR-1 and *Haloarcula californiae* ATCC 33799 was 30%, that between TR-1 and *Haloarcula sinaiiensis* ATCC 33800 was 24%, and that between TR-1 and *Ha. hispanica* ATCC 33960 was 35%, although that bewteen TR-1 and *Halobacterium salinarium* CCM 2148 was only 7%[27,36].

The 16S ribosomal RNA (rRNA) gene was cloned from the chromosomal DNA of TR-1 by using oligonucleotide probes corresponding to the 5′- and 3′-ends of the 16S rRNA gene of *Halococcus morrhuae*[21]. The sequence of the DNA fragment encoding the 16S rRNA was determined, and homology to 16S rRNA sequences of reference strains[7,10,21,26] was investigated. The strain TR-1 exhibited very high homology with *Ha. vallismortis* (99.3%) or *Ha. marismortui* (99.4%)[27]. Other extreme halophiles *Hb. salinarium*, *Haloferax* (formerly *Halobacterium*) *volcanii* and *Hc. morrhuae* exhibited lower homology with TR-1 (90.4%, 89.8% and 90.1%, respectively).

The genus *Haloarcula* currently comprises the type species *Ha. vallismortis and Ha. hispanica*[5,13]. However, a number of bacteria whose taxonomic status is uncertain because of a lack of detailed descriptive information. These include *Ha. marisomortui*, *Ha. californiae* and *Ha. sinaiiensis*[5,13]. Members of the genus are characterized by the possession of the triglycosyl diether lipid TGD-2, the lack of any requirement for amino acids in the growth medium, the ability to metabolize a wide range of sugars, and the ability to reduce nitrate with gas production[38]. Therefore, the isolated strain TR-1 is clearly a member of the genus on this basis. Furthermore, the mol% G + C content of DNA isolated from the TR-1 cells is within the range quoted for the genus *Haloarcula*[5]. The 16S rRNA sequence of the organism also indicates that strain TR-1 belongs to the genus *Haloarcula*.

The strain TR-1 differs from previously described organisms in the genus *Haloarcula* in being unable to utilize mannose and lactose and in requiring relatively high concentrations of Mg^{2+} ions. TR-1 also shows a very stable triangular morphology in liquid media. Of

the other *Haloarcula* spp. or related organisms, *Ha. vallismortis* and *Ha. hispanica* are normally rod-shaped and starch-degrading, whereas *Ha. marismortui*, although somewhat similar in morphology, is non-motile and indole negative. DNA-DNA hybridization experiments confirm that the homologies between the DNA of TR-1 and those of other *Haloarcula* strains are within the range considered to define a species[5, 29]. Accordingly we propose that strain TR-1 represents a new species as *Haloarcula japonica*[27, 36]. *Haloarculas japonica* is deposited as the strain TR-1 in JCM (Japan Collection of Microorganisms, The Riken Institute, Wako-shi, Saitama 351-01, Japan).

Cell division

The manner of cell division of *Ha. japonica* strain TR-1 attracted our attention because of its characteristic morphology. We analyzed the course of cell division by time-lapse microscopic cinematography[8, 27]. Cells of *Ha. japonica* were placed on a thin agar medium, mounted on a glass slide and incubated at 42 °C for 2 days. Cell division was recorded at appropriate intervals with a time-lapse cinematomicroscope equipped with a 16 mm movie camera in a plastic chamber maintained at 42 °C. The cell plate formed between the apex of a triangle and the middle of the opposite side, or between the middle of any two sides. In the former case, separation occurred roughly symmetrically to form two triangular cells. Rhomboid-shaped cells were generated by cell division in the latter, asymmetric manner. The second cell plate formed perpendicular to the first cell plate, approximately at the middle of the other two sides. In most of the rhomboid cells, cell plates formed between the middle of two opposite sides and two rhomboid-shaped daughter cells were thus generated after this division. In a few cases, the daughter cells separated between the opposite two apices. Figure 2 shows photographs of typical cell division processed by a color image analyzer[27].

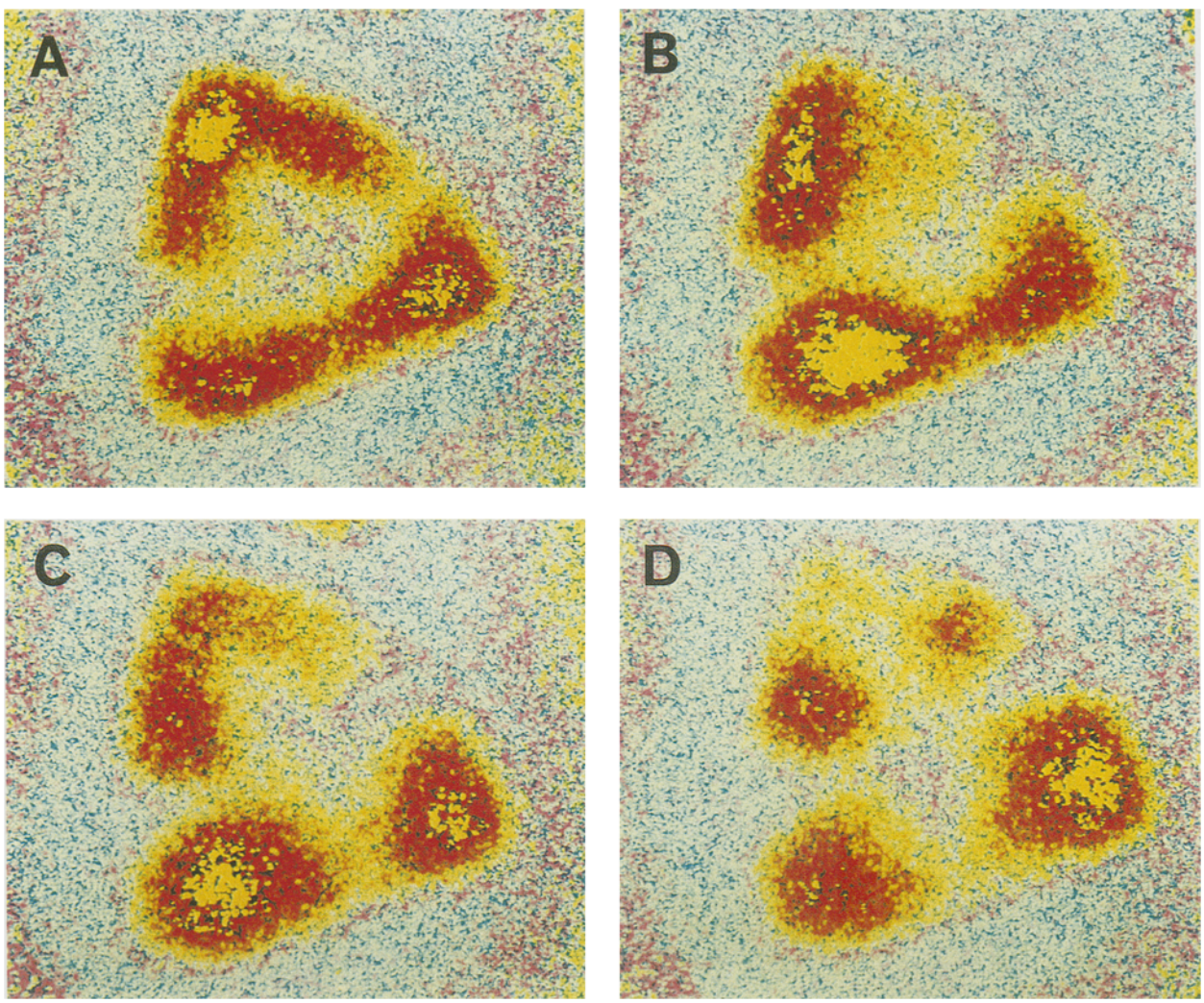

Figure. 2. Photographs of the cell division of *Ha. japonica*. Cell division was recorded with a time-lapse cinematomicroscope and then processed by a color image analyzer. The division occurred from *A* to *D*. (Modified from Otozai et al.[27]).

We initially expected that cell division must take place in a symmetrical manner. But as we observed, most of the divisions occurred asymmetrically and thus generated rhomboid-shaped cells. Some of these became triangular after division. The number of triangular cells in liquid medium is very high (more than 80% by light microscopy[24]) compared with cells on an agar medium. It is possible that the cells on agar in some way lose flexibility of cell shape so that the change to triangular proceeds more slowly than in a liquid medium, and in consequence cell plates form asymmetrically.

Ultrastructure of cell surface

The cell surfaces of many strains of halophilic archaebacteria are covered by a surface layer (S layer)[17,31–34,41]. The S layer of *Ha. japonica* cells was observed by electron microscopy of thin sections using the freeze-substitution method[25]. Higher magnification micrographs of the apex of a cell revealed that the S layer was 20–25 nm in thickness. A periodic arrangement of globular projections were seen in the outermost layer. Moreover, grazing sections along the S layer indicated hexagonal arrays of surface structures. The hexagonal surface patterns were confirmed by digital image processing techniques. The presence of similar hexagonal units has been reported in other halophilic archaebacteria[1,16,31,33,34].

Cell surface glycoprotein and cell morphology

Hb. salinarium, *Hb. halobium* and *Hf. volcanii* have glycoprotein on the cell surface[16,23,42]. The cell surface glycoprotein (CSG) is the major constituent of the S layer of *Hb. salinarium* and *Hb. halobium*[23,42]. Proteolytic degradation of the CSG on the cell surface and the shedding of the CSG by removal of Mg^{2+} ions from the culture medium caused morphological changes in *Hb. salinarium* and *Hf. volcanii*, respectively[22,35]. Thus, it seems that the CSG is the structural component responsible for maintenance of cell morphology in each strain. The genes coding for the CSG have been cloned from *Hb. halobium* and *Hf. volcanii*[20,35]. However, relatively few halobacteria have been examined and the analyses are confined to examples from the genera *Halobacterium* and *Haloferax*[9,16,19,20,22,23,31,35,41,42]. No study of the morphology of *Haloarcula* spp. has been done to date.

The cell envelope fraction of *Ha. japonica* strain TR-1 was prepared and examined by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. A high molecular mass protein was detected as a major band at 170 kDa by staining with Coomassie brilliant blue R-250 (CBB stain), although most of the envelope proteins had a molecular mass of 130 kDa or less[24]. This protein became visible as a clear zone after prolonged silver staining. A single band was observed with the same mobility as the above 170 kDa protein by the periodate-Schiff method (PAS stain). Comparison of CBB-, silver-, and PAS-stained gels indicated that this band stained poorly with CBB and silver compared with the lower molecular mass proteins. A similar phenomenon had been observed with the CSG of *Hb. salinarium*[23]. These results suggest that the cell envelope of *Ha. japonica* has a high molecular mass, carbohydrate-containing protein as the major envelope protein[24].

Ha. japonica has a predominantly triangular shape in liquid medium (fig. 1A). However, chelation of Mg^{2+} ions by adding an equimolar amount of ethylenediaminetetraacetic acid (EDTA) to the growth medium converted the cells to spherical forms (fig. 1B)[24]. The resulting spheroplasts were removed by centrifugation and the supernatant was analyzed by SDS-PAGE. A 170-kDa protein band, presumed to be the CGS, was detected by silver- and PAS-staining, although more than 95% of the 170-kDa protein molecules were retained on the cell surface. Thus, EDTA treatment simultaneously caused the release of CSG and the morphological change in the cells of *Ha. japonica*. The same morphological change and release of the CSG were also observed when the triangular cells were collected by centrifugation and suspended in medium without $MgSO_4$. So it is likely that the CSG is important for the maintenance of the characteristic shape of *Ha. japonica*[24].

We have demonstrated the occurrence of the glycoprotein on the cell surface of *Ha. japonica*. The release of less than 5% of the CSG caused a drastic morphological change. These findings led us to propose the 'arch model' (fig. 3) for the putative structure of the S layer of

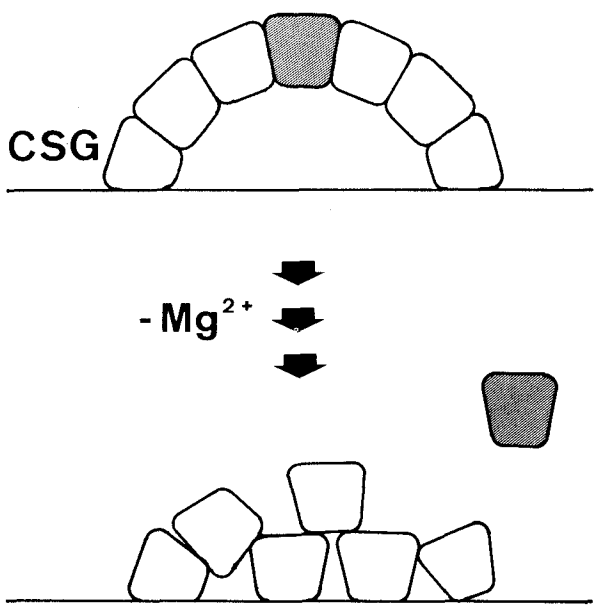

Figure. 3. Schematic illustration of the 'arch model'. The CSG molecules are arranged on the cell surface in an arch-like structure. Once a proportion of the CSG is released from the cell surface, the arch structure is no longer maintained and the triangular shape collapses.

Ha. japonica. In this model, the CSG molecules are arranged on the cell surface, just like an arch, repeating hexagonal symmetry to maintain the cell shape rigidly. Elimination of Mg^{2+} ions from culture media breaks the arch structure and thus causes the morphological change of *Ha. japonica* cells.

In halophilic archaebacteria examined to date, the hexagonal subunits are commonly composed of a CSG[15, 17, 32, 33]. However, halophilic archaebacteria showed different susceptibilities to proteolysis, suggesting that individual strains have CSGs with different moieties[14]. Further molecular studies combined with intrastructural investigations are necessary to clarify the contribution of the CSG to maintenance of the cell morphology of *Ha. japonica*.

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