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Direct proof for the presence and expression of two 5% different 16S rRNA genes in individual cells of *Haloarcula marismortui*

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Abstract A single-cell-derived pure culture of *Haloarcula marismortui* was obtained using the optical tweezers technique. Analysis of the 16S rRNA genes unambiguously demonstrated two 5% different 16S rRNA sequences. In addition, expression of both operons in the same cell was shown by fluorescence in situ hybridization with operon-specific probes.

Key words 16S rDNA heterogeneity · Archaea · *Haloarcula marismortui* · Fluorescence in situ hybridization

Introduction

Analysis of SSU rRNA sequences is widely used to obtain a first assessment of the prokaryotic diversity of microbial environments (Hugenholtz et al. 1998). This method is based on the assumption that every different prokaryote is characterized by a unique 16S rRNA gene. This gene may be present in multiple copies. For example, *Rhodobacter sphaeroides*, *Escherichia coli*, and *Clostridium paradoxum* harbor 3, 7, and 15 rRNA operons, respectively (Dreyden and Kaplan 1990; Carbon et al. 1979; Rainey et al. 1996). However, the sequence difference among operons was less than 2%, supporting the common opinion that the different SSU rDNA copies within a given genome were nearly identical (Clayton et al. 1995). During recent years, more and more reports have been published for the archaeon *Haloarcula* that claim pure cultures harboring two different 16S rRNA genes with more than 2.5% sequence differences.

Mylvaganam and Dennis (1992) were the first reporting two 16S rRNA genes (*rrn*A and *rrn*B) from the halophilic archaeon *Haloarcula marismortui* with sequence similarity of 95%. Generally, sequence differences of 2.5% are considered to represent at least different species (Stackebrandt and Goebel 1994). Within the genus *Haloarcula*, *H. vallismortis*, "*H. sinaiiensis*," and *H. quadrata* were reported to possess two heterogeneous 16S rRNA genes whereas *H. argentinensis* and *H. mukohataei* were found to contain only one single 16S rDNA (Kamekura 1998; Oren et al. 1999).

On the other hand, given the irregular shape of *Haloarcula* cells and the uncertainty of obtaining pure cultures by traditional purification methods, it remains unclear whether pure cultures had been examined (for discussion of culture purity, see Gottschal et al. 1992). To clarify this situation, we analyzed the presence and expression of two different 16S rRNA genes in the halophilic archaeon *H. marismortui* by two techniques that allow the direct characterization of prokaryotic populations: "optical tweezers" single-cell isolation under direct microscopic inspection followed by cultivation (Ashkin et al. 1987; Huber et al. 1995) and fluorescence in situ hybridization (FISH) (Amann et al. 1995).

Results

Single cells of *H. marismortui* DSM3752 were obtained by the optical tweezers technique and transferred into fresh culture medium as described by Huber et al. (1995). Within 2 weeks of incubation at 37°C, the cloned cells gave rise to pure cultures. This cloning procedure was repeated once. The resulting clone of *H. marismortui* (labeled as LL2B) was used for DNA extraction and subsequent 16S rDNA amplification with primers 8aF/1406uR as previously described (Eder et al. 1999). PCR products were cloned into

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pAMP1 vector (Life Technologies, Gaithersburg, MD, USA). The resulting ligation products were used to transform *Escherichia coli* DH5α cells. To distinguish between the two possible 16S rDNA sequences (Mylvaganam and Dennis 1992), the cloned 16S rDNAs were digested with *Nla*III (New England Biolabs, Beverly, MA, USA). One clone representative of each restriction pattern (designated as GA48 and GA50) (Fig. 1) was chosen and their 16S rDNA inserts were sequenced by an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). Computer analyses of sequences were performed with the ARB software package (Ludwig and Strunk 1997). Distance-matrix (Jukes–Cantor correction), and maximum-parsimony methods were applied for tree reconstruction as implemented in the ARB software package.

The sequences from the clones GA48 and GA50 were found to be identical to the *rrn*B and *rrn*A sequences described in Mylvaganam and Dennis (1992), as shown in Fig. 2. These results clearly indicate that single-cell-derived pure cultures of *H. marismortui* harbor two significantly different 16S rRNA genes. To estimate the expression of the two genes by fluorescence in situ hybridization, *H. marismortui* DSM3752 cultures were fixed and hybridized (Antón et al. 1999) with probes targeting each of the two 16S rRNA genes. A region of high heterogeneity between the two operons was chosen to design the operon-specific probes 668A (5′-TCC GTC TTC CTG AGG TGG-3′) and 668B (5′-TCC GGT CTC TCA ACG TGG-3′) targeting the region spanning the positions 668 to 685 (*E. coli* 16S rRNA numbering) (Brosius et al. 1981). The two probes were of similar GC content to avoid variation in hybridization signal intensities resulting from differences in affinity between the probes and their target regions.

Hybridization, DAPI staining, and microscopy were carried out as described previously (Snaidr et al. 1997). Hybridization buffers with different formamide concentrations (from 0% to 75%) were assayed to determine optimum hybridization conditions: 35% formamide was found

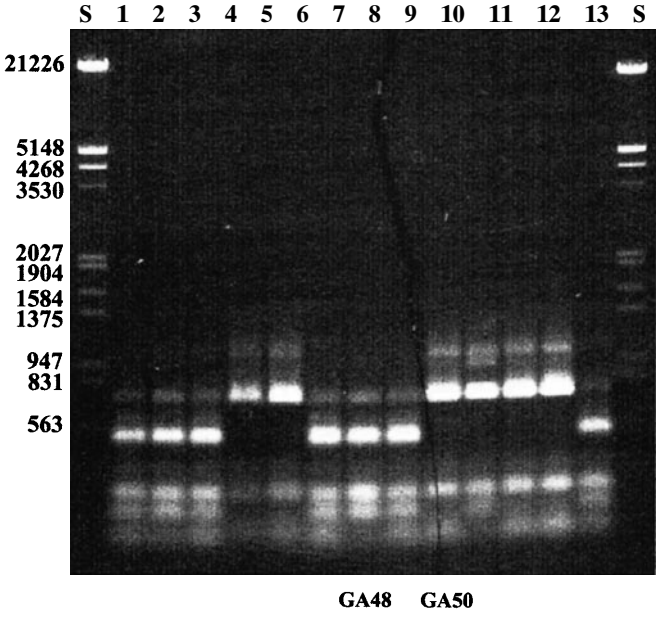

Fig. 1. *Lanes 1–3, 6–8, 13*, restriction digestion patterns of cloned rrnB of *Haloarcula marismortui* LL2B using *Nla*III (clone GA48, lane 7, was sequenced as representative for rrnB); *lanes 4, 5, 9–12*, restriction digestion patterns of cloned rrnA of *Haloarcula marismortui* LL2B using *Nla*III (clone GA50, lane 9, was sequenced as representative for rrnA); *lanes S*, size standard, lambda DNA digested by *Eco*RI/*Hind*III (bp)

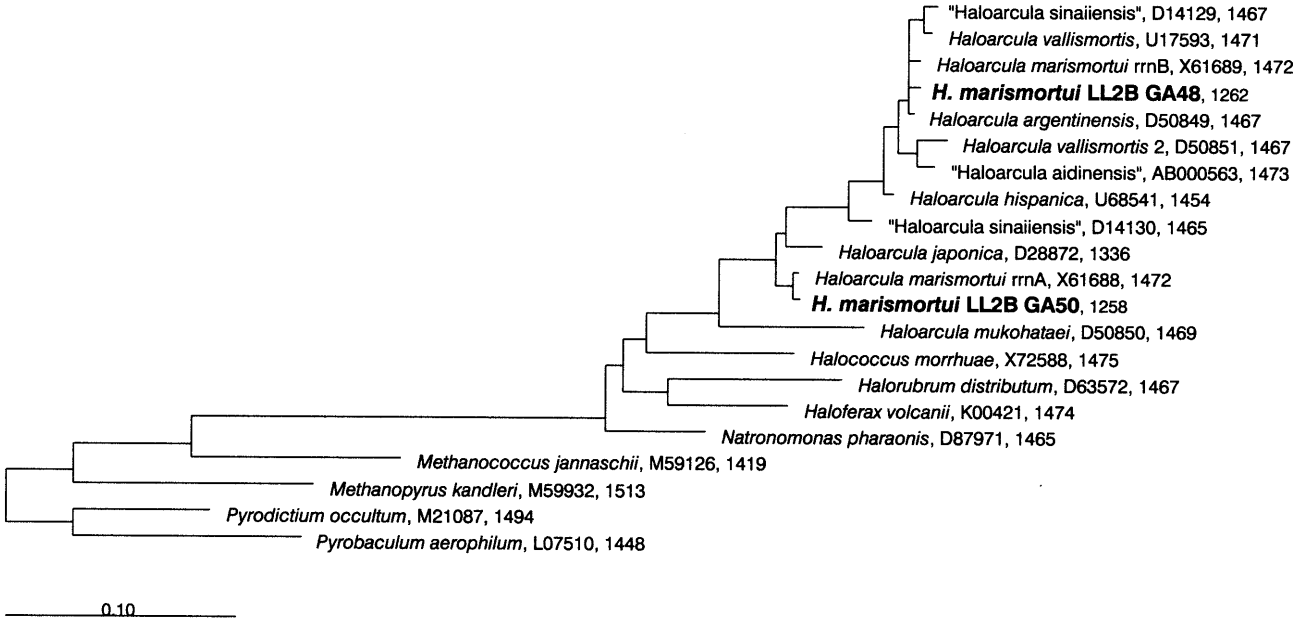

Fig. 2. 16S rRNA phylogenetic tree based on maximum-parsimony analysis including essentially the different *Haloarcula* species. GenBank accession numbers and number of sequenced nucleotides are indicated after the name of the species. *Bar* 10% estimated difference in nucleotide sequences

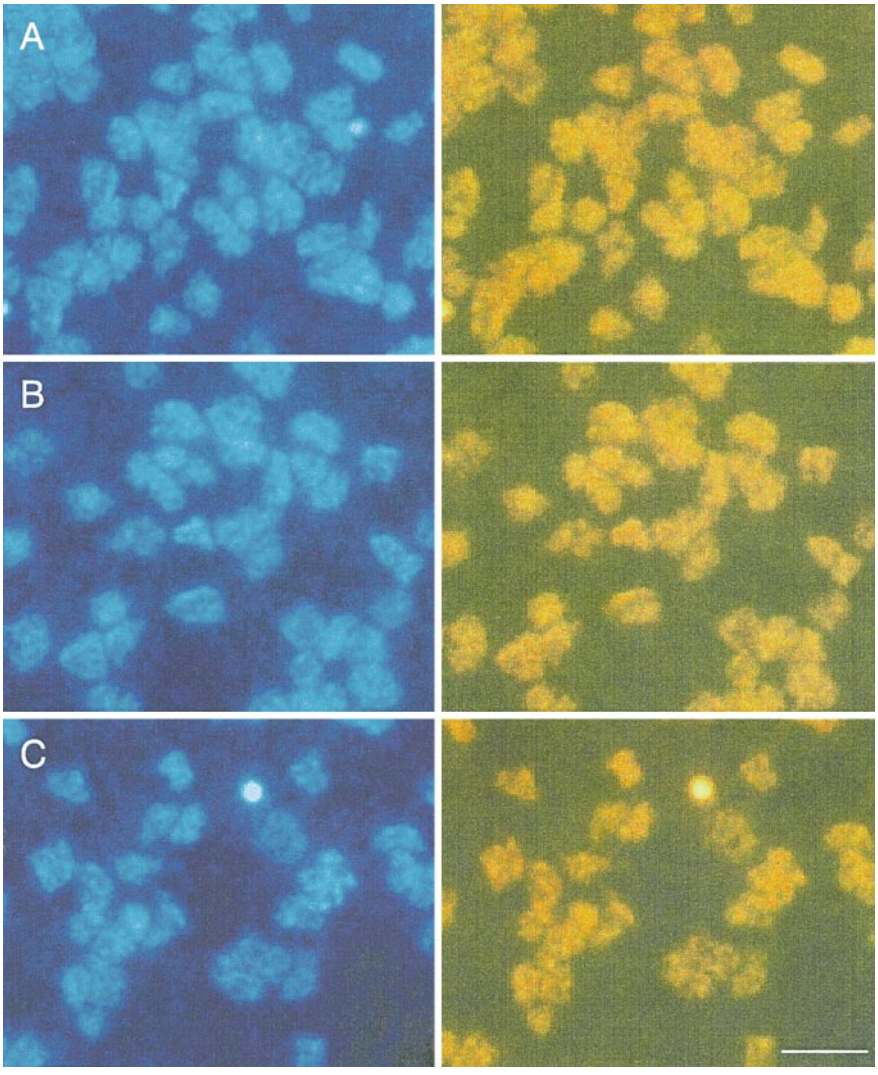

Fig. 3A–C. FISH analysis of *H. marismortui* cultures. The same microscopic field has been visualized with an epifluorescence microscope using filter sets specific for DAPI (*left*) and CY3 (*right*). Hybridization with CY3-labeled probes: **A** HRCU1502, specific for the genus *Haloarcula*, **B** 668A, specific for the *rrnA* gene of *H. marismortui*, and **C** 668B, specific for the *rrnB* gene of *H. marismortui. Bar* 5 μm

to ensure specificity for each probe and signals of high intensity, so mismatches were excluded and efficient hybridization was easily observed. The cultures of *H. marismortui* were also hybridized with probe HRCU1502 (Antón et al. 1999) specific for the genus *Haloarcula*, showing a homogeneous hybridization signal (Fig. 3) that indicated the consistent good condition of the culture.

As shown in Fig. 3, pure cultures of *H. marismortui* gave uniform hybridization signals with probes 668A and 668B. Thus, we conclude that both operons (*rrn*A and *rrn*B) are being simultaneously expressed in every cell of *H. marismortui*. We found that both probes hybridized uniformly along the growth curve of *H. marismortui*, indicating that both operons were expressed in all the growth phases analyzed (i.e., lag, exponential, and stationary phase), although in all the cases the hybridization signal with probe 668B was stronger than with 668A. This finding, however, cannot be used as a proof of different levels of expression for the two operons, because two different probes, even when targeting the same region and with the same GC content, do not necessarily yield the same hybridization signal.

Discussion

We have unambiguously demonstrated that single cells of *H. marismortui* harbor and simultaneously express two different 16S rRNA genes. Although 16S rDNA microheterogeneity seems to be a common phenomenon, to date it remains unclear if 16S rDNA macroheterogeneity is widespread in nature. Recently, significant interoperon heterogeneity has also been reported for members of the bacterial domain: *Thermobispora bispora* and *Thermomonospora chromogena* were found to comprise two types of rRNA operons with sequence similarities lower than 94% (Wang et al. 1997; Yap et al. 1999). Therefore, conclusions about a possible population diversity exclusively based on 16S rRNA genes retrieved from the environment should be

interpreted with more caution in future, keeping in mind that 16S rDNA macroheterogeneity exists. Based on our results, single-cell-derived cultures and in situ hybridization appear essential to avoid misinterpretation and overestimation of microbial diversity.

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