Extremophiles (2005) 9:427–435
DOI 10.1007/s00792-005-0459-y

ORIGINAL PAPER

Daqi Tu · Gregor Blaha · Peter B. Moore
Thomas A. Steitz

Gene replacement in *Haloarcula marismortui*: construction of a strain with two of its three chromosomal rRNA operons deleted

Received: 30 March 2005 / Accepted: 13 May 2005 / Published online: 22 June 2005

Abstract Site-directed mutagenesis were done in *Haloarcula marismortui* using the strategy that Khorana and coworkers devised for deleting the bacteriorhodopsin gene from *Halobacterium halobium* [Krebs et al. Proc Natl Acad Sci USA 90:1987–1991 (1993)]. Strains have been prepared from *H. marsimortui*, which normally has three rRNA operons, that are missing either its *rrnB* operon or both its *rrnB* and *rrnC* operons. In rich media, both strains grow at about the same rate as wild type. The G2099 in the *23S rRNA* gene of the single operon strain was changed to A, and a three amino acid deletion was introduced into the gene for ribosomal protein L22 of the wild-type organism. The structural consequences of these and other such mutations can be determined with unusual accuracy because crystals of the large ribosomal subunit of *H. marismortui* diffract to atomic resolution.

Keywords Haloarcula marismortui · Gene replacement · rRNA Operon · Ribosomal protein L22

Abbreviations *rrn*A: ribosomal RNA operon A · *rrn*B: ribosomal RNA operon B · *rrn*C: ribosomal RNA operon C · rRNA: ribosomal RNA · Mev^r: Mevinolin resistant

Communicated by J.N. Reeve

D. Tu · G. Blaha · P. B. Moore · T. A. Steitz (✉)
Department of Molecular Biophysics and Biochemistry,
Yale University, 266 Whitney Avenue, P.O. Box 208114,
New Haven, CT 06520-8114, USA
E-mail: eatherton@csb.yale.edu
Tel.: +1-203-4325617
Fax: +1-203-4323282

P. B. Moore (✉) · T. A. Steitz
Department of Chemistry, Yale University,
P.O. Box 208107, New Haven,
CT 06520-810, USA
E-mail: peter.moore@yale.edu
Tel.: +1-203-4323995
Fax: +1-203-4326144

T. A. Steitz
Howard Hughes Medical Institute,
New Haven, CT 06520-8114, USA

Introduction

The crystals that have been obtained of the 50S ribosomal subunits of the halophilic archaeon *Haloarcula marismortui* diffract to a higher resolution (2.2 Å) than any other ribosome or ribosomal subunit crystals so far reported. Thus, *H. marismortui* would be an attractive species in which to study the structural consequences of mutations in the large ribosomal subunit if site-directed mutagenesis of its rRNA and ribosomal protein genes could be done in that organism.

When the work reported here began, there were no reports of genetic manipulations done on *H. marismortui* of which we were aware. However, polyethylene-glycol-mediated transformation is very efficient in bacteria and yeast (Chang and Cohen 1979; Klebe et al. 1983), and Cline and Doolittle had shown its efficacy in halophilic archaea like *Halobacterium halobium* (Cline and Doolittle 1987), *Haloferax volcanii* (Charlebois et al. 1987), *Haloarcula hispanica* and *Haloarcula vallimortis* (Cline and Doolittle 1992). Futhermore, detailed protocols for transformating many halophilic archea are available on the web (Dyall-Smith 2003). Thus it seemed very likely that we would be able to obtain the strains of *H. marismortui* we needed in this way, and so it proved.

It is difficult to obtain homogeneous populations of ribosomes with mutated rRNAs from most prokaryotes, because their genomes contain several rRNA operons, and *H. marismortui* is typical in this respect. In 1989, Dennis and coworkers concluded that the genome of *H. marismortui* contains two rRNA operons: *rrnA* and *rrnB* (Mevarech et al. 1989; Dennis et al. 1998). While this manuscript was being prepared, the complete genome sequence of *H. marismortui* was published (Baliga et al. 2004). It shows that *H. marismortui* contains a third rRNA operon (*rrnC*), confirming what we had

already learned from the gene knockout experiments described below. In order to simplify the generation of ribosomes with mutant rRNAs, our first objective was to obtain a strain of *H. marismortui* that has a single rRNA operon.

A strain of *H. marismortui* containing a single rRNA operon was produced by deleting its *rrnB* and *rrnC* operons. Surprisingly, even though all three of these rRNA operons are expressed in the wild-type *H. marismortui*, the single rRNA operon strain obtained (*rrnA*, Δ*rrnB*, Δ*rrnC*) grows on rich media at about the wild-type rate. A point mutation G2099A has been introduced into the *23S rRNA* gene of the single rRNA operon strain, and another strain created carrying a three-amino-acid deletion in its *L22* ribosomal protein gene. Crystallographic studies done on large ribosomal subunits from *H. marismortui* that contain these non-lethal mutations confirm the utility of constructs of this kind (Tu et al. 2005).

Materials and methods

Strains and culture conditions

The *H. marismortui* strains used in this work are described in Table 1. The *H. marismortui* cells were grown in a slightly modified version of a medium described previously (Ban et al. 1998) that contains (per liter): 214 g of NaCl, 17 g of $MgSO_4 \cdot 7H_2O$, 3 g of trisodium citrate·2 H_2O, 1.7 g of KCl, 0.23 g of $CaCl_2 \cdot 2H_2O$, 4.3 g of yeast extract (Difco), 8.5 g of bacteriological peptone (Oxoid), 3.4 g of glucose, and 5.2 g of Tris, pH 7.2. After autoclaving and cooling, 1.7 ml/L was added of a mixture of trace elements (0.0218 g of $MnCl_2 \cdot 4H_2O$ and 0.486 g of $FeCl_3 \cdot 6H_2O$ per 100 ml) that had been sterilized by filtration. Cells were routinely grown at 42°C. When needed, solid and liquid media contained mevinolin that had been treated with NaOH to hydrolyze its lactone ring (Kita et al. 1980) at 4 and 1 μg/ml, respectively.

Transformation procedure

H. marismortui cells were transformed as previously described for *H. volcanii* (Cline et al. 1989) except for the following changes: cells were grown to an OD_{550} of 0.9–1.0; the spheroplasting solution contained 1 M NaCl; EDTA treatment to induce spheroplast formation was done at 37°C for 15 min; spheroplasts were mixed with PEG solution in 1.5-ml microcentrifuge tubes by 3–4 s of vigorous manual shaking; PEG treated cells were recovered by diluting them into 15 ml of *H. marismortui* medium, supplemented with 15% sucrose, and incubating at 37°C for 60 h before plating.

Genomic DNA and plasmid preparation from *H. marismortui*

H. marismortui genomic DNA was prepared with DNeasy Tissue Kit (Qiagen) as recommended by the manufacturer. Plasmid minipreps of *H. marismortui* cells were performed using the Wizard Plus Miniprep DNA purification System (Promega) according to manufacturer's instructions.

Plasmid construction

The plasmids used in this work are listed in Table 1, and the sequences of the PCR primers used are given in Table 2. Plasmids were subcloned and maintained in *Escherichia coli* using Efficiency DH5α cells (Invitrogen) and XL10-Gold Ultracompetent cells (Stratagene).

Table 1 *H. marismortui* strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
Wild type	*rrnA rrnB rrnC*	ATCC43049
DT29	Wild type/Δ*rrnB*	This study
DT38	DT29/Containing the G2099A mutation in *rrnA*	This study
DT41	DT38/Δ*rrnC*	This study
DT50	Wild type/Δ9bp in codons 122–124 of *L22* gene	This study
Plasmids		
pWL102	Shuttle vector containing halobacterial mevinolin resistance gene	Lam and Doolittle (1989)
pHC8	*rrnA* Subclone	Mevarech et al. (1989)
pHH10	*rrnB* Subclone	Mevarech et al. (1989)
pDT2	pWL102 Containing flanking sequences of *rrnB*	This study
pDT3	pWL102 Containing *rrnA* with G2099A mutation	This study
pDT4	pWL102 Containing S10-spc with Δ9bp in codons 122–124 of *L22* gene	This study
pDT5	pWL102 Containing flanking sequences of *rrnC*	This study

Table 2 Primers used in this work

Primer	Sequence (5–3′)[a]	Location
100G2A	GGCTTGCATGAATGGATTAACCA	2000–2022, 23S rRNA; fp[b]
2099RR	CACGGGCAGGATGGAGGG	2547–2564, 23S rRNA; rp[b]
BLA	GAATTCCAGACGATTATTGGCGAGTTTGGC	1 kb up-stream to *rrnB*; fp
BLB	GACGTCCGCGATTCAGAGGTGAGAATAATC	5′ end of *rrnB*; rp
BRA	GCGGCCGCATTCATACCTTTCATAGCCCACTC	3′ end of *rrnB*; fp
BRB	AAGCTTTGGCGGGGTTCATTGCGACTG	1 kb down-stream to *rrnB*; rp
DUTOP	CTC ATCGATTG AGATCTGC	Synthetic linker; plus strand
DUBOTM	GGCCGC AGATCTCA ATCGATGAGACGT	Synthetic linker; minus strand
HMAS10L	GAATTCCACAGTCTAGTGGCGCGACGTG	35nt up-stream to orf1 in S10-spc; fp
HMAS10R	AAGCTTGTCGACAGGTCGAACGCGAACG	75nt up-stream to S17; rp
BMF	AGATCTACGAGGATGTCGACGTCGAGG	334 nt up-stream to 3′ end of S3; fp
BMR	ACGCGTGAGCTGCTCGATGTCC	75 nt up-stream to L32e; rp
MHF	ACGCGTATCAACGACAAGGACGTGC	75 nt up-stream to L32e; fp
MHR	AAGCTTCGGTAGGAAATCCAGCCCTGCAA	232 nt down-stream to 3′ end of SecY; rp
CLA	GAATTCATTACGTTAACCAGTATTGAGTGATAATTGACACG	1.3 kb up-stream to *rrnC*; fp
CLB	GACGTCGTCCAGAAGACGGGGGGAACATATAA	5′ end of *rrnC*; rp
CRA	GCGGCCGCATTCATACCTTTCATAGCCCACTCAGGAGAGG	3′ end of *rrnC*; fp
CRB	AAGCTTGGCTCGACCGCGACGATGTC	1.3 kb down-stream to *rrnC*; rp
ACL1	ATGCCAGCTGGTGGATTGCT	11–30, 23S rRNA; forward primer
ACR1	CTCGCGTCTCGGTCATTCGT	562–581, 23S rRNA; reverse primer
HBKOLF	TACCGTCAGCATGAACTCAATCTCGATATTGC	1540 nt up-stream to 16S RNA/ *rrnB*; fp
HBSLR1	GAAATCAACAGTGAGGTGTTGCAAGTCGG	1142 nt up-stream to 16S RNA/ *rrnB*; rp
EXT23S4	TTTTCGAGCGCGTACCGCCC	2284–2303, 23S rRNA; rp

[a] Nucleotides *underlined* are adaptor restriction sites
[b] *fp* forward primer, *rp* reverse primer

pDT2

A 1 kb DNA fragment from *H. marismortui* genomic DNA that lies upstream of the *rrnB* transcription start site PB1 (Dennis et al. 1998) was amplified by PCR using primers BLA and BLB, which contain *Eco*RI and *Aat*II restriction sites, respectively. A 1 kb DNA fragment downstream of *rrnB 5S* gene was similarly PCR amplified using primers BRA and BRB, which contain *Not*I and *Hin*dIII sites, respectively. A short synthetic linker was made by annealing the primers DUTOP (19mer) and DUBOTM (27mer) to produce a duplex DNA that carries an *Aat*II site at one end and a *Not*I site on the other end. pDT2 was created (Fig. 1) by ligating the following four DNA fragments: plasmid pWL102 that had been digested with *Eco*RI and *Hin*dIII to remove its pHV2 portion, the two PCR fragments flanking *rrnB* described above that had been digested with *Eco*RI/ *Aat*II and *Not*I/ *Hin*dIII, respectively, and the synthetic duplex linker. The sequence information used for designing the DNA primers was obtained by sequencing the entire plasmid pHH10 (Mevarech et al. 1989), which includes the *rrnB* operon.

pDT3

The G at position 2099 of the *rrnA 23S rRNA* gene of plasmid pHC8 (Mevarech et al. 1989) was mutagenized to A using a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The mutagenized *rrnA* fragment that resulted was cut out of the pHC8 DNA using *Hin*dIII and *Cla*I, and ligated into pWL102 from which the pHV2 segment had been removed using the same restriction enzymes.

pDT4

A 14.9 kb fragment of the S10-spc operon lacking a promoter was obtained by PCR-amplifying three shorter consecutive fragments, each carrying restriction sites on both ends, from *H. marismortui* genomic DNA, and ligating them together into plasmid pUC19. The three fragments were amplified using primers pairs HMAS10L/HMAS10R (5.8 kb product), BMF/BMR (4.2 kb product), and MHF/MHR (5.1 kb product), respectively, using PfuTurbo DNA polymerase (Stratagene). These three PCR fragments were digested using the restriction enzymes *Eco*RI/ *Bgl*II, *Bgl*II/ *Mlu*I, and *Mlu*I/ *Hin*dIII, respectively, and then ligated into plasmid pUC19 that had been digested with *Eco*RI and *Hin*dIII. Sequence information for designing primers was taken from literature (Arndt 1990, 1992; Arndt et al. 1990; Arndt and Weigel 1990; Scholzen et al. 1991). In order to construct pDT4, a DNA fragment containing the *L22* gene from *H. marismortui* genomic DNA was PCR amplified using primers HMAS10L and HMAS10R, and cloned into a pUC19 plasmid that had been digested with *Eco*RI and *Hin*dIII to produce a smaller clone that was more suited for site-directed mutagenesis. This smaller construct was mutagenized to delete the nine bp of interest from the *L22* gene (QuickChange Site-Directed Mutagenesis kit, Stratagene), and digested with *Eco*RI/ *Bgl*II to release a fragment containing the mutagenized *L22* gene. The resulting mutant fragment was exchanged into the subclone of the full S10-spc operon described above. Finally, the mutagenized 14.9 kb S10-spc operon was cut out of the pUC19 plasmid using *Eco*RI and *Hin*dIII, and

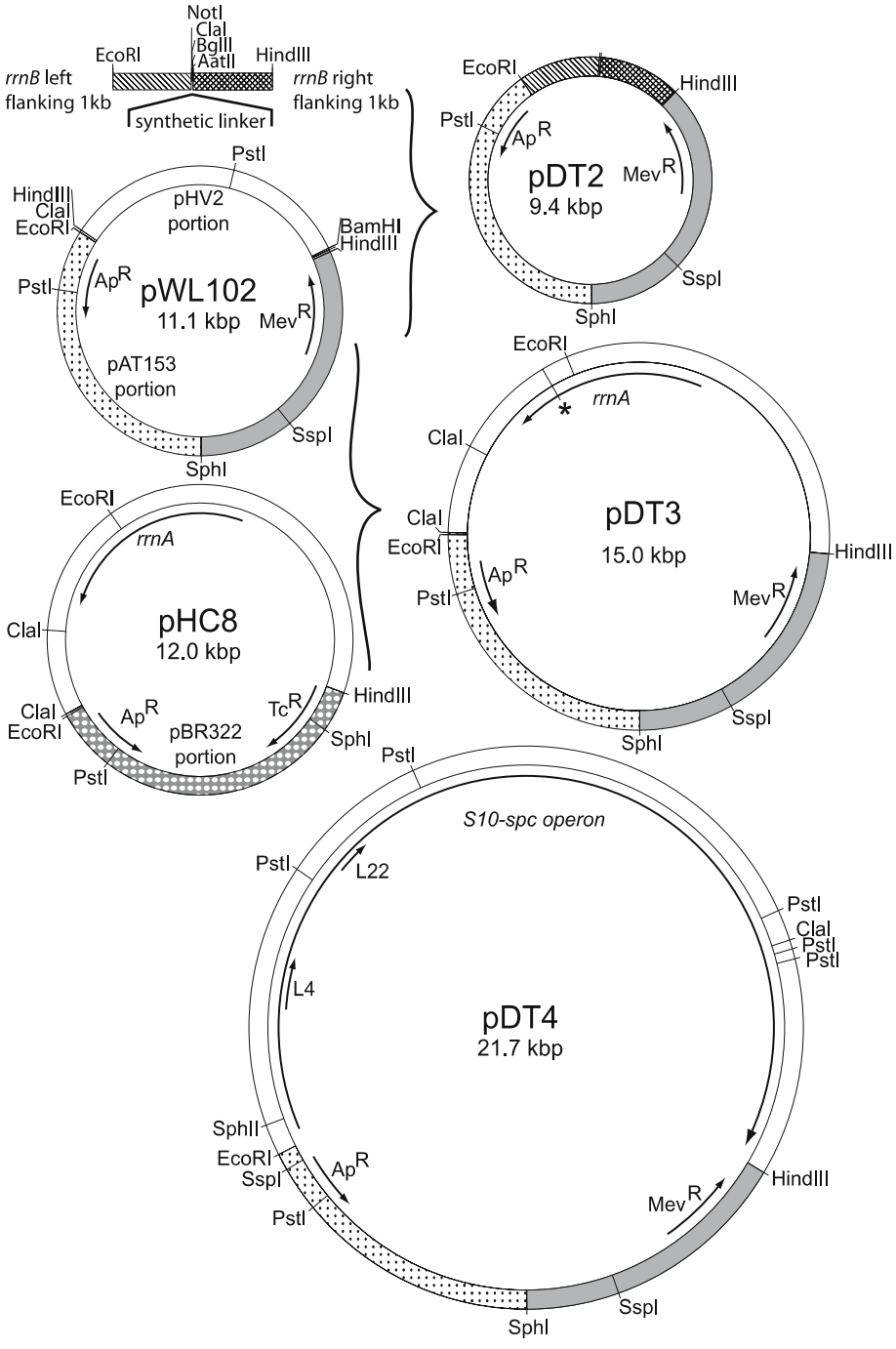

Fig. 1 Construction of plasmids pDT2, pDT3, and pDT4. The pHV2 portion of pWL102 was removed either by digestion with *Eco*RI and *Hin*dIII for pDT2 and pDT4, or by digestion with *Cla*I and *Hin*dIII for pDT3. In its place, a fragment containing a 1 kb left flanking sequence of the *rrnB* operon connected by a synthetic linker to a 1 kb right flanking sequence of the *rrnB* operon was inserted to create pDT2. pDT3 was made the same way using a fragment containing the entire *rrnA* operon with G2099A mutation in its *23S rRNA* gene, which was cut out of pHC8. The asterisk indicates the relative position of G2099A mutation in *rrnA*. pDT4 was constructed similarly using a fragment containing the entire S10-spc operon without promoters and with a 9-bp deletion in its *L22* gene (codons 122–124)

ligated into pWL102 that had been digested with the same restriction enzymes to remove the pHV2 portion, and to form pDT4.

pDT5

pDT5 was constructed the same way as pDT2. A 1.3 kb fragment that flanks the left side up-stream of the *rrnC* promoters, which was identified by its similarity to the *rrnB* promoters, and a 1.3 kb fragment that flanks the down-stream right side of the *rrnC 5S* gene were PCR-amplified from *H. marismortui* genomic DNA using the primer pairs CLA/CLB, and CRA/CRB, respectively. A synthetic duplex DNA linker was used to ligate these two fragments into pWL102 that had been digested to remove its pHV2 portion. Sequence information for designing primers was obtained from (http://zdna2.umbi.umd.edu, version 2).

Southern blot analysis

Genomic DNAs from *H. marismortui* strains were digested using restriction enzymes and transferred to GeneScreen Hybridization Transfer Membranes

(Perkin-Elmer) using the manufacturer's protocol. Radioactive probes were made using a Random Primed DNA Labeling Kit (Roche) and gel-purified (MinElute Gel Extraction Kit, Qiagen) PCR fragments as templates. The knockout of the *rrnB* operon was confirmed initially using a 400 bp template DNA that had been generated by PCR from a sequence 1.5 kb upstream of the *rrnB 16S rRNA* gene using the primer pair HBKOLF/HBSLR1. Later the *rrnB* and *rrnC* deletions were confirmed using a probe that covers a 565 bp sequence inside the *23S rRNA* gene extending from 2000 to 2564. The probe was based on a PCR fragment generated using the primer pair 100G2A/2099RR. Hybridization and washing were done according to the transfer membrane manufacturer's protocol except that washing was done at 65 °C. Membranes were exposed to Fuji phosphor imaging plates that were scanned using a Fuji BAS-2000 scanner, and digital images were analyzed with Image Gauge (Version 4.1; Science Lab).

Primer extension analysis

Sucrose gradient-purified large ribosomal subunits from wild-type *H. marismortui* (Ban et al. 1998) were phenol-chloroform extracted. The rRNA was ethanol precipitated and dissolved in DEPC-treated water. Primer extension was performed as described (Stern et al. 1988) with primer EXT23S4. The anode chamber buffer for electrophoresis included 1 M NaAc, pH 8.3, in addition to TBE. Dried gels were exposed to a phosphor imager plate overnight (Fuji imaging plate). The imager plates were scanned with Fuji BAS-2000 and the digital images were analyzed with Image Gauge (Version 4.1; Science Lab).

RT-PCR analysis

The RNA was isolated from *H. marismortui* strains with an RNeasy Mini kit (Qiagen) following the manufacturer's protocol with some minor modifications. Lysozyme was not necessary for cell lysis, but RNase-free DNase (DNase set, Qiagen) was used at the recommended point in the RNA isolation. The RT-PCR was performed with OneStep RT-PCR kit (Qiagen) using primers pair ACL1/ACR1 or 100G2A/2099RR (Table 2).

Results

Transformation of *H. marismortui*

All the plasmids used to transform *H. marismortui* are derivatives of pWL102, which was kindly provided by Ford Doolittle (Lam and Doolittle 1989). Both *H. vallismortis* and *H. hispanica* can be transformed using pWL102 DNA that has been passaged through *E. coli* (Cline and Doolittle 1992), but the transformation efficiency is very different for the two species, 500 transformants/μg have been reported for *H. vallismortis*, whereas 4×10^5 transformants/μg were obtained for *H. hispanica*. This difference reflects the presence of restriction enzymes in *H. vallismortis* that are absent from *H. hispanica*. The *H. marismortui* strain we used resembles *H. vallismortis* in this regard. Typically we obtained 100 transformants/μg of DNA using pWL102 DNA from *E. coli*, but the transformation efficiency increased to 1×10^5 transformants/μg after the plasmid has been passaged through *H. marismortui*. Passage of the plasmid through *E. coli* SCS110 (*dam*-, *dcm*-) did not improve transformation efficiency.

Construction of a *rrnB* deletion strain

The strategy used to obtain gene knockouts and replacements, which is outlined in Fig. 2, is based on the two-step procedure devised by Krebs et al. (1993) to delete the bacteriorhodopsin gene from *H. halobium*. To knock out the *rrnB* operon, a suicide vector named pDT2 (Fig. 1; Materials and methods) was transformed into *H. marismortui*, and the transformants were plated onto plates containing mevinolin (mev), which is toxic to halophilic archaea. The plasmid from which pDT2 was derived, pWL102, replicates in wild type *H. marismortui*, but pDT2 does not because it has no origin of replication that is active in halophiles, and its 3.5 kb Mev^r locus, which was isolated from *H. volcanii*, has no significant homology with the *H. marismortui* genome. Thus all the mev-resistant colonies that emerged should have had pDT2 DNA integrated into their genomes as a result of homologous recombinations involving the flanking regions of the *rrnB* operon (Fig. 2). Only three mev-resistant colonies were obtained this way, which corresponds to only 1 transformant/μg of pDT2 DNA, but all were shown to have pDT2 DNA integrated into their genomes by colony PCR, and the absence of free plasmid DNA from the transformed strains was confirmed by miniprep experiments (data not shown).

Cells with pDT2 integrated into their genome were subcultured three times in media lacking mevinolin to allow a second recombination to occur, and then grown on plates that lacked mevinolin. Twenty-five of the resulting colonies were picked, streaked onto mev-free plates, and then checked for the presence of the Mev^r gene by colony PCR. Five of the 25 lacked a Mev^r gene, indicating that a second recombinational event had taken place (Fig. 2), and Southern blot experiments showed that all of them were *rrnB* knockouts (data not shown). One of the strains obtained, DT29, was used for all subsequent experiments.

Mutation of G2099 in a Δ*rrnB* strain

Like all archaea, *H. marismortui* has a G at position 2099 in its 23S rRNA (2058 in *E. coli*), while in most

eubacteria the base at that position is an A. In eubacteria, A2058G (*E. coli*) mutations render cells resistant to MLS_B class antibiotics like erythromycin (Weisblum 1995), and not surprisingly, erythromycin binds so weakly to wild-type *H. marismortui* 50S ribosomal subunits that its interaction cannot be observed crystallographically (J.L. Hansen, P.B. Moore and T.A. Steitz, unpublished results).

Because of our interest in determining the structure of erythromycin bound to the large subunit, the first point mutation introduced into DT29, which at the time we thought was a strain containing a single rRNA operon, was a G2099A mutation in the 23S rRNA cistron of its *rrnA* operon. To this end, a vector called pDT3 was constructed from plasmid pWL102 that contains an entire *rrnA* operon, including its promoters and terminators and is G2099A in its 23S rRNA cistron (Fig. 1; Materials and methods). The pDT3 was transformed into DT29 using the strategy summarized in Fig. 2. The number of transformants obtained, about 5 μg of *E. coli* passaged pDT3 DNA, was five times higher than observed for the *rrnB* knockout vector pDT2, which is probably indicative of the difference in the length of the homologous DNA sequences available for recombination between the two vectors. Colonies lacking the Mev^r locus were obtained without incident following the second recombination step, and the 2099 region of the 23S rRNA cistrons in these colonies was amplified by PCR, and sequenced to determine the identity of residue 2099. To our surprise, all such strains showed both G and A at 2099. The experiments done to pursue this anomaly, which need not be described here, ultimately convinced us that *H. marismortui* must contain a third rrn operon, a result now confirmed by the genome sequence available for *H. marismortui* (Baliga et al. 2004). One of these two-operon strains, DT38, was selected for further study.

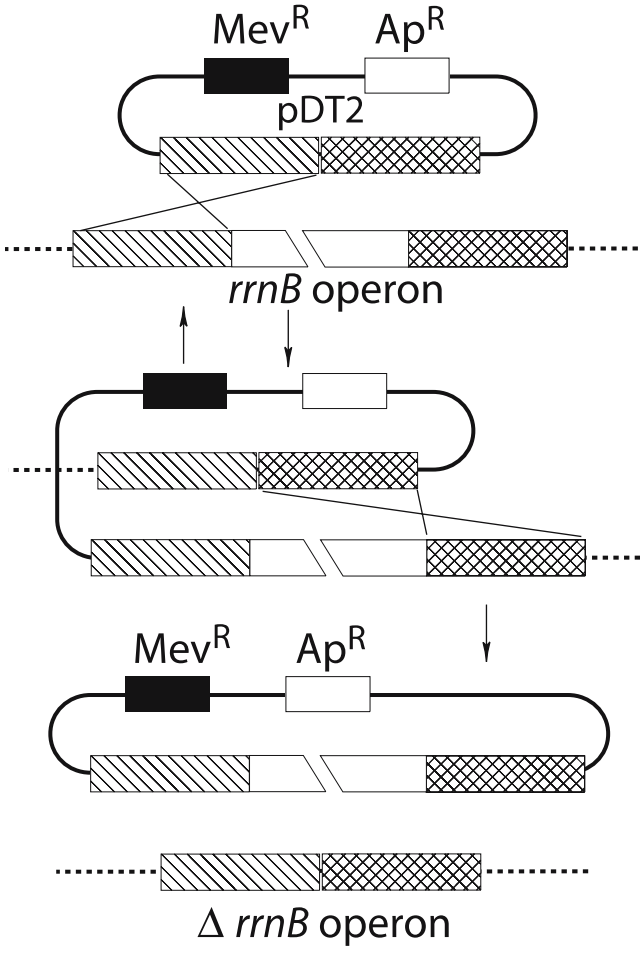

Fig. 2 Schematic diagram describing the deletion of the *rrnB* operon from *H. marismortui* genome. Plasmid pDT2 (described in Fig. 1 and in "Materials and methods" section) was integrated into the chromosome by homologous recombination between the hatched region in the plasmid and corresponding sequences in the chromosome, and mevinolin-resistant cells were selected. Following growth without selection, a second recombination event in the same region could lead either to loss of integrated plasmid in its original form and return to a wild-type chromosome, or to loss of the integrated plasmid carrying the *rrnB* operon, and deletion of *rrnB* sequence from the chromosome through recombination in the cross-hatched region

Deletion of the *rrnC* operon from a Δ*rrnB* strain

In the repository of partial *H. marismortui* genome sequences on the web (http://zdna2.umbi.umd.edu, Version 2), we found the complete sequence of the *rrnC* operon including its flanking region (within contig 146). Using this information pDT5 was constructed (Materials and methods) so that the *rrnC* operon could be deleted from DT38 in the usual way (Fig. 2). Several colonies emerged that were (Δ*rrnC*, Δ*rrnB*) and the one used for further study is designated DT41.

The success of the manipulations just described was confirmed by a Southern blot experiment, the results of which are shown in Fig. 3. Genomic DNA from wild-type *H. marismortui*, which has 3 rRNA operons, DT29, which has two (*rrnA* and *rrnC*), and DT41, which has only one (*rrnA*), was digested using three different restriction enzymes: *Xho*I, *Rsr*II, and *Pvu*I. Gels of these restriction digests were blotted and probed using a 565 bp, radioactively labeled, PCR fragment corresponding to bases 2000–2564 of 23S rRNA. As Fig. 3 shows, wild type DNA yields three restriction fragments bands that hybridize to the probe when digested with any one of the three restriction enzymes tested, DT29 yields 2, and DT41, only 1. The sizes of these fragments are consistent with the genomic DNA sequences available on the web (http://zdna2.umbi.umd.edu, Version 2).

Physiology of the single rRNA operon strain

Deletion of the *rrnB* and *rrnC* operons from *H. marismortui* has little effect on its growth rate. In our hands, there is a lot of variability in both the doubling time and the lag time of *H. marismortui* cultures grown under

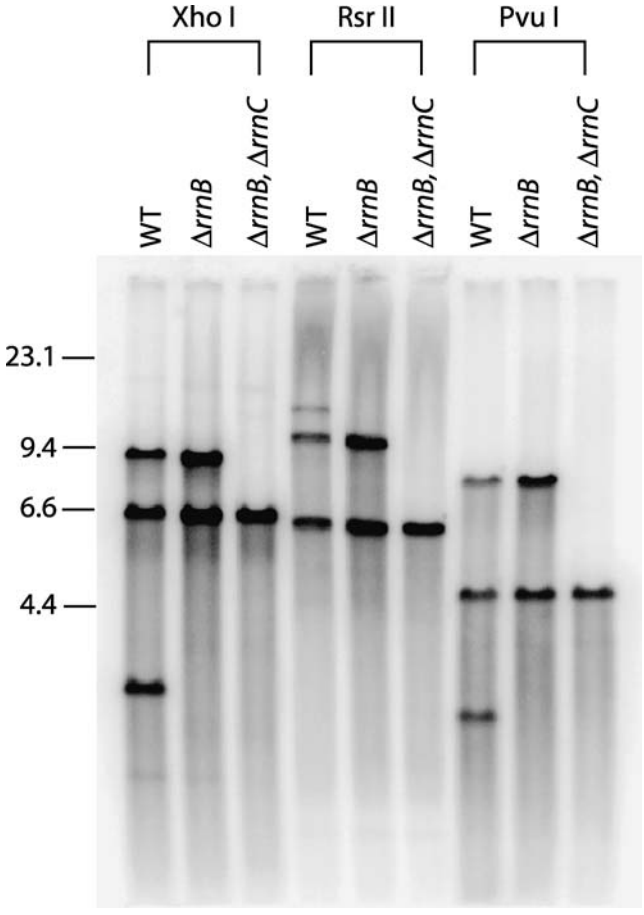

Fig. 3 Southern blot analysis of wild-type, the *rrnB* knockout, and the *rrnB*/*rrnC* double knockout strains. Genomic DNA was prepared from wild-type strain, the Δ*rrnB* strain, DT29, and the Δ*rrnB*/Δ*rrnC* strain, DT41. After digestion with *Xho*I, *Rsr*II or *Pvu*I, respectively, the DNA was hybridized with a probe that anneals to position 2000–2564 in *23S rRNA* gene. The genotypes of the strains are indicated above the slots. DNA size markers are from a λ DNA *Hin*dIII digest, and their sizes are indicated in kb

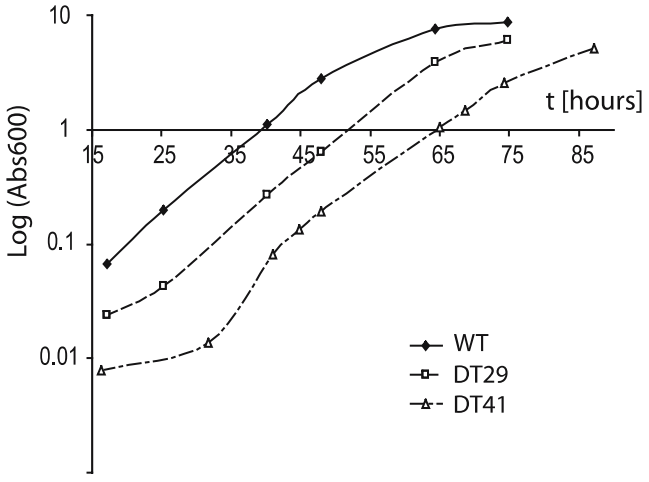

Fig. 4 Growth curve of wild-type, two operon (Δ*rrnB*) and single operon (Δ*rrnB*/Δ*rrnC*) strain. Cells were grown at 42 °C, as a 400 ml culture in a 2 L baffled flask on an orbital shaker (220 rpm). Generation time of WT is 5.4 h, DT29 (Δ*rrnB*) 6.3 h, and DT41 (Δ*rrnB*/Δ*rrnC*) 6.1 h

apparently identical conditions. The doubling times and lag times of the two-rRNA operon strain DT29 (Δ*rrnB*) and one-rRNA operon strain DT41 (Δ*rrnB* Δ*rrnC*) are indistinguishable from wild-type *H. marismortui* when grown on a rich medium (Fig. 4). One way this observation could be explained is if neither the *rrnB* nor the *rrnC* operons were expressed under these conditions.

To find out whether all of the rRNA operons are expressed in wild type *H. marismortui*, an RT-PCR experiment was done on the total cellular RNA from the wild-type strain, DT29 (Δ*rrnB*), and DT41 (Δ*rrnB*, Δ*rrnC*). The result is shown in Fig. 5. In the region of 23S rRNA around nucleotide 300, the sequences of the *rrnC* and *rrnA* operons differ at five positions, and the *rrnB* sequence is a combination of the two. It is clear that both the *rrnA* and *rrnC* variants are expressed in the wild-type organism, and that in the single rrn operon strain, DT41, only the *rrnA* version of the sequence is seen, as anticipated. In the regions of nucleotide 2170, *rrnA* and *rrnC* are identical, but *rrnB* differs from both at four positions. Clearly the *rrnB* variant is expressed in the wild type strain, but not in DT29, as expected. We note in passing that the plots obtained when genomic DNA is sequenced from these three strains are virtually identical to those shown in Fig. 5 (data not shown). However, DNase treated controls run in connection with this RT-PCR experiment show unequivocally that the sequence plots shown here derive from RNA not DNA. To further confirm these results, some regions of 23S rRNA isolated from wild type cells were sequenced by primer extension. At positions where the sequences of the three rrn operons diverge, the contribution of the divergent operon was clearly evident, and in many cases, the majority band was about twice as dark as the minority band (data not shown).

Gene replacement in the S10-spc operon

One of the ribosomal mutations known to cause resistance to macrolides in *E. coli* is a three-amino-acid deletion in ribosomal protein L22 (Chittum and Champney 1994), and it would be interesting to know what its structural consequences are because L22 is not a component of the macrolide binding site (Hansen et al. 2002). In archaea, ribosomal protein genes are clustered in operons just as they are in eubacteria (Ramìrez et al. 1993), and in *H. marismortui*, the gene for *L22* is a component of the S10/spectinomycin operon, which contains 25 open reading frames that encode 15 large ribosomal subunit proteins, seven small ribosomal subunit proteins, secY, and two unknown proteins (Arndt 1990, 1992; Arndt et al. 1990; Arndt and Weigel 1990; Scholzen et al. 1991). The synthesis of r-proteins is autogenously regulated at the level of translation in archaea, just as it is in eubacteria. When in excess, one of

the r-proteins in an r-protein operon binds to a regulatory site in its own, polycistronic mRNA, and thereby inhibits translation of all genes that mRNA encodes (Ramírez et al. 1993; Kraft et al. 1999).

In designing a gene replacement vector for *L22*, we decided to omit the promoter of the S10/spc operon so that problems caused by r-protein autoregulation would be avoided. However, because the promoter was to be omitted, the version of the S10/spc operon on the plasmid used for transformation had to be complete downstream of L22 because otherwise recombination between the plasmid and the chromosome would generate daughter chromosomes containing genes on the 3' side of the recombination site that could not be transcribed. The amino acids deleted in L22 were Gln-Gln-Gly corresponding to codons 122–124, positions that were identified by multiple sequence alignment of *H. marismortui* L22 protein with other bacterial and archaeal L22 proteins, and confirmed by structural alignment of the crystal structure of L22 from *Thermus thermophilus* (Unge et al. 1998) and that of L22 from *H. marismortui* as it exists in the structure of its large ribosomal subunit (Ban et al. 2000).

The pDT4 (methods section) was used to produce the mutation desired in wild-type *H. marismortui* using the strategy outlined in Fig. 2. Of the 12 colonies recovered after the second recombination step that had lost the Mev^r locus, only one had the desired 9 bp deletion in the gene for *L22* (data not shown). The low efficiency of the second step probably reflects the relatively skewed position of the 9 bp deletion, which is only about one-third of the way down the 14.9 kb S10/spc operon.

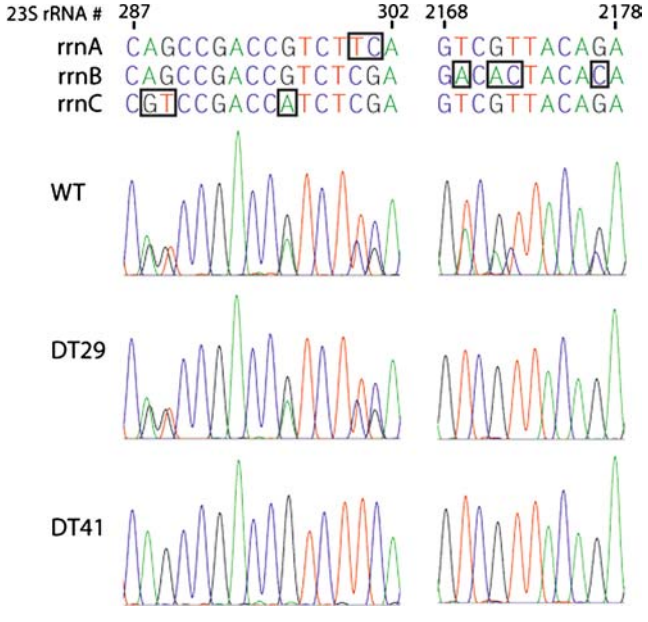

Fig. 5 RT-PCR sequencing of the total cellular RNA of three strains: wild type, DT29, and DT41. Total cellular RNA of wild-type strain, the Δ*rrnB* strain, DT29, and the Δ*rrnB*/Δ*rrnC*/mutant *rrnA* (G2099A) strain, DT41 was RT-PCR amplified in two regions using the primers pair ACL1/ACR1 or 100G2A/2099RR (Table 2). The PCR products were sequenced and the four-color sequencing plots are shown for all three cellular RNAs around 23S rRNA positions 300 and 2170

Discussion

It is clear from the data reported above that transcripts of all three rRNA operons are incorporated into 50S ribosomal subunits of wild-type *H. marismortui* at roughly equal levels. RT-PCR results showed that the 23S rRNA products of all three operons accumulate in cells, and primer extension results indicate that these 23S rRNAs are incorporated into ribosomes in a roughly unbaised way.

At first glance, the finding that *H. marismortui* strains carrying only one rRNA operon grow normally in rich media is not surprising. Archaea typically possess one to four rRNA operons, with 1.5 being the average (http://rrndb.cme.msu.edu, rrndb: the Ribosomal RNA Operon Copy Number Database, Version 2.5). Included among the archaea that get along with a single rRNA operon is *H. halobium*, a halophile closely related to *H. marismortui* that grows at about the same rate. The result becomes puzzling only when it is realized that in wild-type *H. marismortui*, all rRNA operons are expressed at roughly equal levels, as shown above. Why should an organism that appears to need only one rRNA operon retain three, and how does the regulatory system work that enables the single operon in DT41 to produce as much rRNA as three operons in the wild-type organism?

Whatever the explanation for this aspect of the physiology of *H. marismortui*. It is clear that the transformation system and the strains described above can be used to create strains of that organism that contain homogeneous populations of mutant ribosomes carrying mutant rRNA, provided that the mutations are not lethal. Strains of this kind have already been used to investigate issues related to antibiotic interactions (Tu et al. 2005). Their potential utility for high-resolution investigations of structure–function relationships is indicated by Fig. 6, which compares the electron density

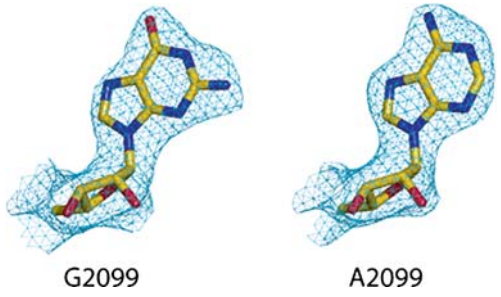

Fig. 6 Electron density for G2099 in the crystal structure of the 50S ribosomal subunit from wild type *H. marismortui* compared to the electron density for A2099 from the crystal structure of 50S ribosomal subunits from the strain DT41. In both cases, $2F_o$-F_c electron density map were calculated using amplitudes obtained from a crystal of the appropriate type, and model phases computed with the residue at position 2099 omitted

for the base at position 2099 obtained from crystals of the large ribosomal subunit prepared from DT41 (Fig. 6 right), and from wild-type *H. marismortui* (Fig. 6 left). It is obvious that the base at 2099 is an A in DT41, and G in the wild-type organism, as expected.

Acknowledgements We thank W. Ford Doolittle for providing pWL102, and Patrick P. Dennis for providing pHC8 and pHH10. Sequences from *H. marismortui* found at (http://zdna2.umbi.umd.edu, Version 2) were used in designing the *rrnC* knockout construct. This research was supported by National Institutes of Health grant GM022778 to T.A.S. and P.B.M., by a grant from the Agouron Institute to T.A.S. and P.B.M.

References

Arndt E (1990) Nucleotide sequence of four genes encoding ribosomal proteins from the 'S10 and spectinomycin' operon equivalent region in the archaebacterium *Halobacterium marismortui*. FEBS Lett 267:193–198

Arndt E (1992) The genes for ribosomal protein L15 and the protein equivalent to secY in the archaebacterium *Haloarcula (Halobacterium) marismortui*. Biochim Biophys Acta 1130: 113–116

Arndt E, Kromer W, Hatakeyama T (1990) Organization and nucleotide sequence of a gene cluster coding for eight ribosomal proteins in the archaebacterium *Halobacterium marismortui*. J Biol Chem 265:3034–3039

Arndt E, Weigel C (1990) Nucleotide sequence of the genes encoding the L11, L1, L10 and L12 equivalent ribosomal proteins from the archaebacterium *Halobacterium marismortui*. Nucleic Acids Res 18:1285

Baliga NS, Bonneau R, Facciotti MT, Pan M, Glusman G, Deutsch EW, Shannon P, Chiu Y, Weng RS, Gan RR, Hung P, Date SV, Marcotte E, Hood L, Ng WV (2004) Genome sequence of *Haloarcula marismortui*: a halophilic archaeon from the Dead Sea. Genome Res 14:2221–2234

Ban N, Freeborn B, Nissen P, Penczek P, Grassucci RA, Sweet R, Frank J, Moore PB, Steitz TA (1998) A 9 Å resolution X-ray crystallographic map of the large ribosomal subunit. Cell 93:1105–1115

Ban N, Nissen P, Hansen J, Moore PB, Steitz TA (2000) The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science 289:905–920

Chang S, Cohen SN (1979) High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol Gen Genet 168:111–115

Charlebois RL, Lam WL, Cline SW, Doolittle WF (1987) Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium. Proc Natl Acad Sci USA 84:8530–8534

Chittum HS, Champney WS (1994) Ribosomal protein gene sequence changes in erythromycin-resistant mutants of *Escherichia coli*. J Bacteriol 176:6192–6198

Cline SW, Doolittle WF (1987) Efficient transfection of the archaebacterium *Halobacterium halobium*. J Bacteriol 169:1341–1344

Cline SW, Doolittle WF (1992) Transformation of members of the genus *Haloarcula* with shuttle vectors based on *Halobacterium halobium* and *Haloferax volcanii* plasmid replicons. J Bacteriol 174:1076–1080

Cline SW, Lam WL, Charlebois RL, Schalkwyk LC, Doolittle WF (1989) Transformation methods for halophilic archaebacteria. Can J Microbiol 35:148–152

Dennis PP, Ziesche S, Mylvaganam S (1998) Transcription analysis of two disparate rRNA operons in the halophilic archaeon *Haloarcula marismortui*. J Bacteriol 180:4804–4813

Dyall-Smith M (2003) The Halohandbook: protocols for halobacterial genetics. http://www.microbiol.unimelb.edu.au/staff/mds/HaloHandbook/ (accessed March 2005)

Hansen JL, Ippolito JA, Ban N, Nissen P, Moore PB, Steitz TA (2002) The structures of four macrolide antibiotics bound to the large ribosomal subunit. Mol Cell 10:117–128

Kita T, Brown MS, Goldstein JL (1980) Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. J Clin Invest 66:1094–1100

Klebe RJ, Harriss JV, Sharp ZD, Douglas MG (1983) A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene 25:333–341

Kraft A, Lutz C, Lingenhel A, Grobner P, Piendl W (1999) Control of ribosomal protein L1 synthesis in mesophilic and thermophilic archaea. Genetics 152:1363–1372

Krebs MP, Mollaaghababa R, Khorana HG (1993) Gene replacement in *Halobacterium halobium* and expression of bacteriorhodopsin mutants. Proc Natl Acad Sci USA 90:1987–1991

Lam WL, Doolittle WF (1989) Shuttle vectors for the archaebacterium *Halobacterium volcanii*. Proc Natl Acad Sci USA 86:5478–5482

Mevarech M, Hirsch-Twizer S, Goldman S, Yakobson E, Eisenberg H, Dennis PP (1989) Isolation and characterization of the rRNA gene clusters of *Halobacterium marismortui*. J Bacteriol 171:3479–3485

Ramírez C, Köpke AKE, Yang D-C, Boeckh T, Matheson AT (1993) The structure, function and evolution of archaeal ribosomes. In: Kates M, Kushner DJ, Matheson AT (eds) The biochemistry of archaea (archaebacteria), vol 26. Elsevier Science Publishers BV, Amsterdam, The Netherlands, pp439–466

Scholzen T, Arndt E (1991) Organization and nucleotide sequence of ten ribosomal protein genes from the region equivalent to the spectinomycin operon in the archaebacterium *Halobacterium marismortui*. Mol Gen Genet 228:70–80

Stern S, Moazed D, Noller HF (1988) Structural analysis of RNA using chemical and enzymatic probing monitored by primer extension. In: Harry F, Noller J, Moldave K (eds) Methods in enzymology, vol 164. Academic Press Inc., San Diego, pp 481–489

Tu D, Blaha G, Moore PB, Steitz TA (2005) Structures of MLS_BK antibiotics bound to mutated large ribosomal subunits provide a structural explanation for resistance. Cell 121:257–270

Unge J, berg A, Al-Kharadaghi S, Nikulin A, Nikonov S, Davydova N, Nevskaya N, Garber M, Liljas A (1998) The crystal structure of ribosomal protein L22 from *Thermus thermophilus*: insights into the mechanism of erythromycin resistance. Structure 6:1577–1586

Weisblum B (1995) Erythromycin resistance by ribosome modification. Antimicrob Agents Chemother 39:577–585