Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6535–6539, July 1992
Biochemistry

Introducing mutations into the single-copy chromosomal 23S rRNA gene of the archaeon *Halobacterium halobium* by using an rRNA operon-based transformation system

(archaebacteria/halobacteria/ribosome/thiostrepton/anisomycin)

A. S. MANKIN*†, I. M. ZYRIANOVA*, V. K. KAGRAMANOVA*, AND R. A. GARRETT‡

*Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, Russia; and ‡Institute of Biological Chemistry B, University of Copenhagen, Sølvgade 83, DK-1307 Copenhagen K, Denmark

Communicated by Carl R. Woese, April 3, 1992

ABSTRACT **A vector-transformation system is described that permits replacement of a portion of the single rRNA operon of the archaeon *Halobacterium halobium* with a homologous fragment from a vector-borne gene. The vector construct contains three functional sections: (*i*) an entire *H. halobium* rRNA operon with two selective mutations in the 23S rRNA gene, the substitutions of A → G at position 1159 conferring resistance to thiostrepton and C → U at position 2471 conferring resistance to anisomycin; (*ii*) the complete pHSB1 plasmid from *Halobacterium* sp. SB3, which interferes with vector maintenance in the transformed halobacterial cells; and (*iii*) a segment of the pBR322 plasmid that permits vector replication in *Escherichia coli*. Transformation of *H. halobium* with the vector plasmid generates cells resistant to both anisomycin and thiostrepton that can be selected for, and discriminated from spontaneous mutants, by a two-step selection procedure. After transformation, the plasmid recombines homologously with the chromosome so that the plasmid-borne rDNA segment with resistance markers substitutes for the corresponding region of the chromosomal rRNA operon, and the transforming plasmid is lost. Eventually, this leads to a homogeneous population of the mutant ribosomes in the cell. Other mutations that are engineered in the vector-borne rRNA sequences can be transferred to the chromosomal rRNA operon concomitantly with the selective markers. The system has considerable potential for ribosomal engineering.**

An important development in ribosomal studies over the past decade has been the growing recognition that ribosomal RNA plays an important functional role in translation and the consequent revival of the early concept that the original machinery of translation was composed primarily of RNA (1). Many of the modern RNA-oriented studies of the function and evolution of the ribosome require manipulations with the primary structures of rRNAs or their genes (see refs. 2 and 31 for review). Genes encoding rRNAs are clustered in operons and reiterated in genomes of most free-living organisms. Bacteria generally have several gene copies per chromosome, while hundreds or thousands of rRNA gene copies are usually present in eukaryotic genomes (3). This gene multiplicity renders application of the powerful tool of mutational analysis difficult, since a mutation occurring in one operon will generally be suppressed phenotypically by wild-type rRNAs transcribed from nonmutated genes. Thus, most of the natural rRNA mutations that have been characterized occur in the single-rRNA operons of mitochondria and chloroplasts (4). Another problem is that when an rRNA mutation is introduced into a cell on a self-replicating vector plasmid it will, with rare exceptions (5), yield a mixed population of chromosome-encoded wild-type and plasmid-encoded mutant ribosomes, which complicates functional analyses.

Organisms within the domain Archaea (formerly called "archaebacteria") (6) differ in many ways from both bacteria and eukaryotes (7). One of their characteristics is the low copy number of their rRNA genes, which in some halophiles, and most (if not all) thermophiles, is one set per genome (see ref. 8 for review). Consequently, these organisms are potentially excellent model systems in which to select for, and introduce, mutations into rRNA genes. Moreover, halophiles merit special attention because they can be grown easily in liquid culture, they readily produce colonies on agar plates and, furthermore, transformation of halobacteria with plasmid DNA has been demonstrated (9–11).

The rRNA gene cluster of the extreme halophile *Halobacterium halobium* has been cloned and sequenced, and its expression has been examined (12–17). The 16S, 23S, and 5S rRNA genes are organized in a single operon together with a small protein gene and two tRNA genes. The operon is transcribed from seven external promoters and one internal promoter, and processed rRNAs are incorporated in the ribosome. Spontaneous mutations in the rRNA genes that can confer resistance to the ribosomal drugs anisomycin, thiostrepton, and chloramphenicol (18–20) have been characterized for this organism. Here we describe a plasmid transformation system that can be used to introduce designed mutations into the single-copy rRNA operon of *H. halobium*. This provides intriguing possibilities for exploiting this organism as a model for studying the structure, function, and evolution of the rRNAs.

MATERIALS AND METHODS

Genetic Engineering Techniques. Cloning in *Escherichia coli*, colony hybridization screening, and Southern analysis were performed essentially as described in ref. 21. DNA probes for colony or Southern hybridization were labeled by using [α-^{32}P]dTTP (Amersham) and a random primer labeling kit (Boehringer Mannheim). Restriction enzyme hydrolyses were performed under standard conditions (21).

Growing of *H. halobium* Cells. *H. halobium* R_1 cells (22) were grown in complex liquid medium (23). Anisomycin or thiostrepton (both from Sigma) was added when necessary to concentrations of 10 μg/ml and 3 μg/ml, respectively. Agar plates were prepared by mixing 4 parts medium with 1 part water and gelling by adding 1.5% agar.

Selection for an Anisomycin- and Thiostrepton-Resistant Double Mutant of *H. halobium*. *H. halobium* cells were grown

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Ani^r, anisomycin-resistant; Ths^r, thiostrepton-resistant.

†To whom reprint requests should be addressed at: Sinsheimer Laboratories, University of California at Santa Cruz, CA 95064.

to a culture density of $OD_{550} \approx 1.2$ and 10^9 cells [assuming that an OD_{550} of $1 \approx 0.8 \times 10^9$ viable cells per ml (23)] were plated onto agar plates containing anisomycin at 10 μg/ml. Individual colonies appearing after 10 days were grown in the liquid medium in the presence of anisomycin at 10 μg/ml, and about 10^9 cells were plated onto agar medium containing anisomycin at 10 μg/ml and thiostrepton at 3 μg/ml. Anisomycin-resistant (Ani^r) thiostrepton-resistant (Ths^r) colonies appeared after about 1 week of incubation at 37°C and were further purified by streaking onto plates containing both antibiotics.

Isolation of Total RNA and DNA from Halobacteria. Total RNA from halobacterial cells was isolated as described previously (20). Total DNA was prepared as follows: Cells from a 5-ml stationary-phase culture were pelleted and resuspended in 0.5 ml TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA), and RNase A was added to a final concentration of 20 μg/ml. After incubation for 15 min at 37°C the water phase was extracted with TE-saturated phenol, phenol/chloroform, and chloroform. DNA was precipitated by adding an equal volume of ethanol, centrifuging, and washing with 70% ethanol. It was dissolved in sterile water and stored at 4°C.

Construction of the pHRZH Vector. A 5-μg sample of total DNA from the Ani^r Ths^r *H. halobium* double mutant was hydrolyzed with *Hin*dIII and *Bgl* II restriction enzymes, and the fragments were separated on a 0.7% agarose gel. DNA fragments ranging in size from 10 to 12 kilobases (kb) were eluted from the gel and ligated into pBR322 cleaved with *Hin*dIII and *Bam*HI. *E. coli* XL1 cells were transformed with the ligation mixture and ampicillin-resistant colonies were screened by hybridizing with uniformly labeled halobacterial rRNA as described earlier (13). Plasmid DNA was isolated from the hybridizing clones and the presence of the complete rRNA operon of *H. halobium* was confirmed by restriction site analysis and Southern hybridization. The resulting recombinant plasmid was designated pHRZ.

A pUC19 derivative containing pHSB1, a 1736-base-pair (bp)-long plasmid from *Halobacterium* sp. SB3 (24–26), cloned at its unique *Hin*dIII site (27), was used as a source of pHSB1. pHSB1 was excised from the pHSB1/pUC19 clone by *Hin*dIII digestion, isolated from the agarose gel, and ligated into pHRZ that had been cut at its *Hin*dIII site and dephosphorylated with calf intestine alkaline phosphatase. After transformation of *E. coli*, ampicillin-resistant clones were screened by hybridizing with the labeled pHSB1 DNA. The presence of pHSB1 was confirmed by *Hin*dIII hydrolysis of the plasmid DNA from the resulting clones, and its orientation was determined by subsequent restriction enzyme analysis.

Transformation of *H. halobium* with pHRZH. The polyethylene glycol procedure (23) was used for transformation of *H. halobium*. Transformation efficiency was monitored by using phage φH DNA (23) and was usually in the range of 10^4–10^5 plaques per μg of DNA. About 1 μg of the pHRZH DNA was applied in a typical transformation experiment. Transformed spheroplasts were diluted with 10 ml of a complex medium supplemented with 15% sucrose and were incubated overnight at 37°C. Cells were pelleted for 10 min at 7000 rpm and 20°C in a Sorvall SS 34 rotor and spread on the surface of agar plates containing anisomycin at 10 μg/ml. Alternatively (28), transformed spheroplasts were diluted with 2 ml of complex medium containing 15% sucrose, incubated 5 min at room temperature, centrifuged 3 min at 6500 rpm in an Eppendorf microcentrifuge, resuspended in another 2 ml of sucrose-containing medium, and incubated overnight at 37°C. Cells were pelleted for 5 min at 6500 rpm and applied to anisomycin-containing agar plates. Plates were sealed and incubated at 37°C for 10–14 days. Large and small colonies that appeared on plates were transferred in a replica fashion to an anisomycin (10 μg/ml)-containing plate and a thiostrepton (3 μg/ml)-containing plate. DNA from the transformants that were able to grow on both plates was subjected to further analysis.

Sequencing of the rRNA and the Plasmid DNA. Sequencing of the 23S rRNA of *H. halobium* was performed according to ref. 29, using about 8 μg of total RNA and 0.5 pM 5′-^{32}P-labeled deoxyoligonucleotide primers. Primers used were CCCGACTAACGCTGCTACTATGG, GTGTTTCATGTGTGACTC, and CGGTCGATATGTGCTCTTGCGAG, complementary to positions 1352–1374, 2187–2204, and 2482–2504 of the 23S rRNA (13).

Plasmid DNA was sequenced from the same primers by using a Sequenase kit (United States Biochemical).

RESULTS

Selection for an Ani^r and Ths^r Double Mutant of *H. halobium*. An rRNA operon from the double mutant of *H. halobium* that was resistant to ribosomal antibiotics anisomycin and thiostrepton provided the selective marker for the vector construct. The double mutant donor strain was generated by a sequential selection, first of Ani^r clones and then of clones that were both Ani^r and Ths^r.

About 10^9 wild-type halobacterial cells were initially plated onto solid medium containing anisomycin at 10 μg/ml. Several Ani^r spontaneous mutants appeared with a frequency of 10^{-7}–10^{-8} after 2 weeks of incubation. Sequencing of the peptidyl transfer region within domain V of the 23S rRNA (13) from several colonies revealed the presence of C → U transition at position 2471, which coincided with the previously described Ani^r mutations in *H. halobium* (18).

One of the Ani^r colonies was grown in a liquid culture in the presence of anisomycin, and 10^9 Ani^r cells were plated onto agar medium containing anisomycin at 10 μg/ml and thiostrepton at 3 μg/ml. Ani^r Ths^r double-resistant colonies appeared on the plate with the same frequency, 10^{-7} to 10^{-8}. Sequencing showed that these mutants acquired a second mutation, an A → G transition at position 1159, which has been shown previously to confer thiostrepton resistance (19). The Ani^r Ths^r mutant was named R1CUAG on the basis of the nucleotide changes in the 23S rRNA gene.

No colonies appeared when up to 10^{11} antibiotic-sensitive parental cells were spread directly onto plates containing both anisomycin and thiostrepton. This indicates that two spontaneous mutations arise in one cell at a very low rate.

Construction of the rRNA Operon-Based Vector pHRZH for *H. halobium*. From both the sequencing data and the restriction nuclease analyses (14, 15) it was known that the rRNA operon lies on a 10.4-kb *Hin*dIII–*Bgl* II fragment in the chromosome of *H. halobium*. This DNA fragment, isolated from the R1CUAG mutant, was inserted into the *E. coli* vector pBR322, which was cleaved with *Hin*dIII and *Bam*HI. The resulting plasmid, pHRZ, contained a complete rRNA operon with a 4-kb DNA segment upstream and a 1.3-kb DNA segment downstream from the rRNA genes. The presence of both Ani^r and Ths^r mutations, characterized previously in the R1CUAG mutant at the rRNA level, was confirmed by sequencing the corresponding regions of the pHRZ plasmid, employing the same primers that were used for rRNA sequencing.

Plasmid pHRZ can replicate in halobacterial cells probably due to the presence of an autonomously replicating sequence located upstream from the rRNA operon (A.S.M., N. I. Derckacheva, and V.K.K., unpublished data). To promote selection of cells for which recombination had occurred between plasmid and chromosome, it was necessary to inhibit plasmid replication. To achieve that a 1736-bp-long plasmid from the halobacterial strain SB3 (24–26) was inserted into pHRZ. The resulting plasmid, designated pHRZH

(Fig. 1), constituted 14.4 kbp and, in contrast to pHRZ, was not stably maintained in the transformed cells (see below).

Transformation of Halobacteria with pHRZH Plasmid. PEG 600-mediated transformation (23) was used to introduce pHRZH DNA into *H. halobium* cells. Transformed cells were plated onto anisomycin-containing plates. Colonies that formed within 2 weeks were of two types: those appearing at a frequency of 10^{-7} to 10^{-8} (thus yielding 10–100 colonies per plate) were large, about 3 mm in diameter; the others were smaller, about 1 mm in diameter, and their number varied from 1 to 40, correlating with the transformation competence of the cells. Both large and small colonies were challenged (in the liquid medium or by replica plating) for their ability to grow in the presence of thiostrepton at 3 μg/ml. About 80% of the smaller colonies turned out to be Ths^r, while all the larger colonies were sensitive to the drug. Formation of the small Ani^r Ths^r colonies was strictly pHRZH dependent: only Ani^r Ths^s "large" colonies were observed when no DNA was added during transformation. It could be inferred from these results, and from the observation that the rate of generation of spontaneous double mutants from the parental strain was very low (see above), that while formation of Ani^r Ths^s colonies was mostly due to spontaneous Ani^r mutations, the Ani^r Ths^r colonies originated from the cells transformed with pHRZH.

The fate of the pHRZH plasmid in the Ani^r Ths^r transformants was analyzed by Southern hybridization of total cellular DNA. As shown in Fig. 2, hybridization patterns for untransformed cells and Ani^r Ths^r transformants were indistinguishable for the restriction enzymes tested. This indicates that (*i*) the transforming plasmid was absent from the transformed cells; (*ii*) transformed cells retain a single copy of the rRNA operon; and (*iii*) no integration of either pBR322 or pHSB1 sequences occurred in the *H. halobium* chromosome as a consequence of transformation. Exactly the same results were obtained for DNA from several independent Ani^r Ths^r transformants. Thus, upon transforming *H. halobium* cells with pHRZH DNA, Ani^r and Ths^r mutations are probably transferred to the chromosomal copy of the rRNA operon and pHRZH plasmid is eventually lost.

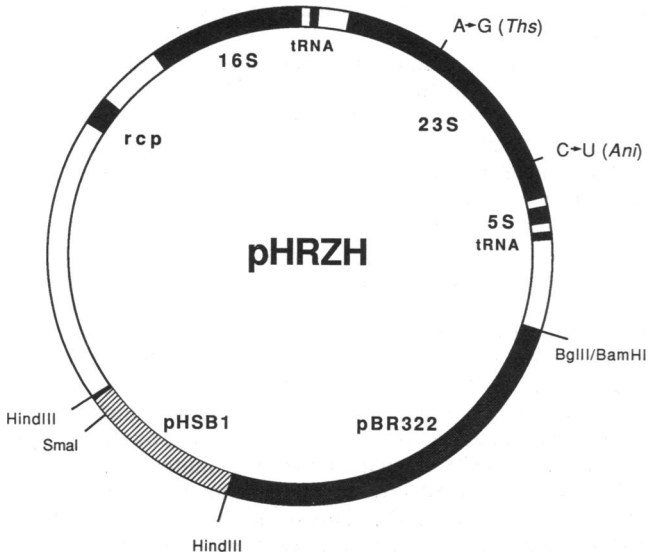

FIG. 1. Schematic map of the pHRZH plasmid. The pBR322 sequence is shown by a stippled bar and the pHSB1 sequence is represented by hatched bar. Location of the genes coexpressed in the rRNA operon is shown, including 16S, 23S, and 5S rRNA, rcp protein, and two tRNA genes. Cloning sites used for the pHRZH assembly are indicated alongside, with the *Sma* I site in pHSB1 indicative of its orientation in pHRZH.

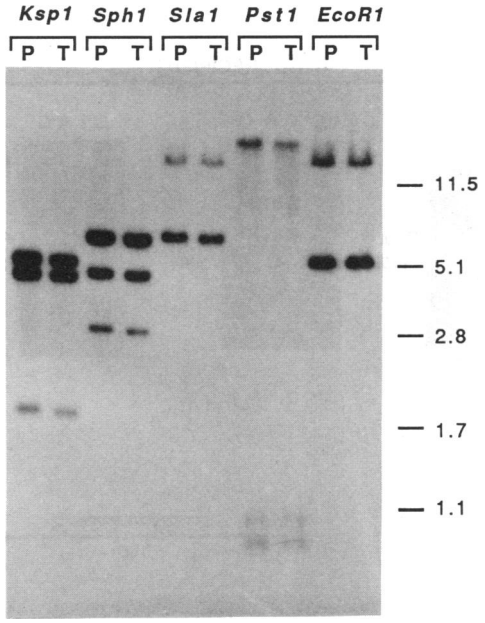

FIG. 2. Southern analysis of the total cellular DNA from *H. halobium* parental cells (lanes P) and cells transformed with pHRZH plasmid (lanes T). Randomly labeled pHRZH DNA was used as a probe. Restriction enzymes used for DNA digestion are indicated above the slots. Lengths of DNA size markers are indicated in kb.

Transformation of the R1AC *H. halobium* Mutant with pHRZH DNA. To gain insight into what happens with resistance markers during the transformation, use was made of a recently isolated chloramphenicol-resistant mutant of *H. halobium*, R1AC, which has an A → C substitution at position 2088 of the 23S rRNA gene (20), located between the *ths* and *ani* loci (Fig. 3*B*).

R1AC cells were used as recipients of the pHRZH plasmid in a transformation experiment and Ani^r Ths^r transformants were selected. Southern analyses confirmed the retention of a single rRNA operon in the transformants (not shown); total RNA was isolated from these clones and three sites in the 23S rRNA were sequenced, corresponding to the *ani* and *ths* loci and the position 2088. Fig. 3*A* demonstrates that acquisition of Ani^r and Ths^r mutations by transformants was accompanied by a loss of the A → C mutation originally present in the R1AC cells. Since no selection was made either for or against this mutation in these experiments, its loss most probably reflects replacement of part of the chromosomal rRNA operon with the corresponding region of the transforming pHRZH plasmid, spanning all three altered loci, as shown on the scheme in Fig. 3*B*.

DISCUSSION

We have described a transformation/recombination system that can be used to introduce desired mutations into the single-copy rRNA genes of the archaeon *H. halobium*. The approach is based on transformation of halobacteria with a plasmid that contains an rRNA operon with antibiotic-resistance mutations in it and has an impaired replication function. Analyses show that the only cells that survive under the selective growth conditions are those for which a mutated region in the plasmid-borne rRNA operon substitutes for the corresponding region of the chromosomal rRNA operon. This plasmid-to-chromosome marker transfer is accompanied by loss of plasmid and results in the transformed cells having a single copy of the rRNA operon with the selective mutations. Since other mutations engineered in the vector plasmid can, in principle, be transferred to chromosomal

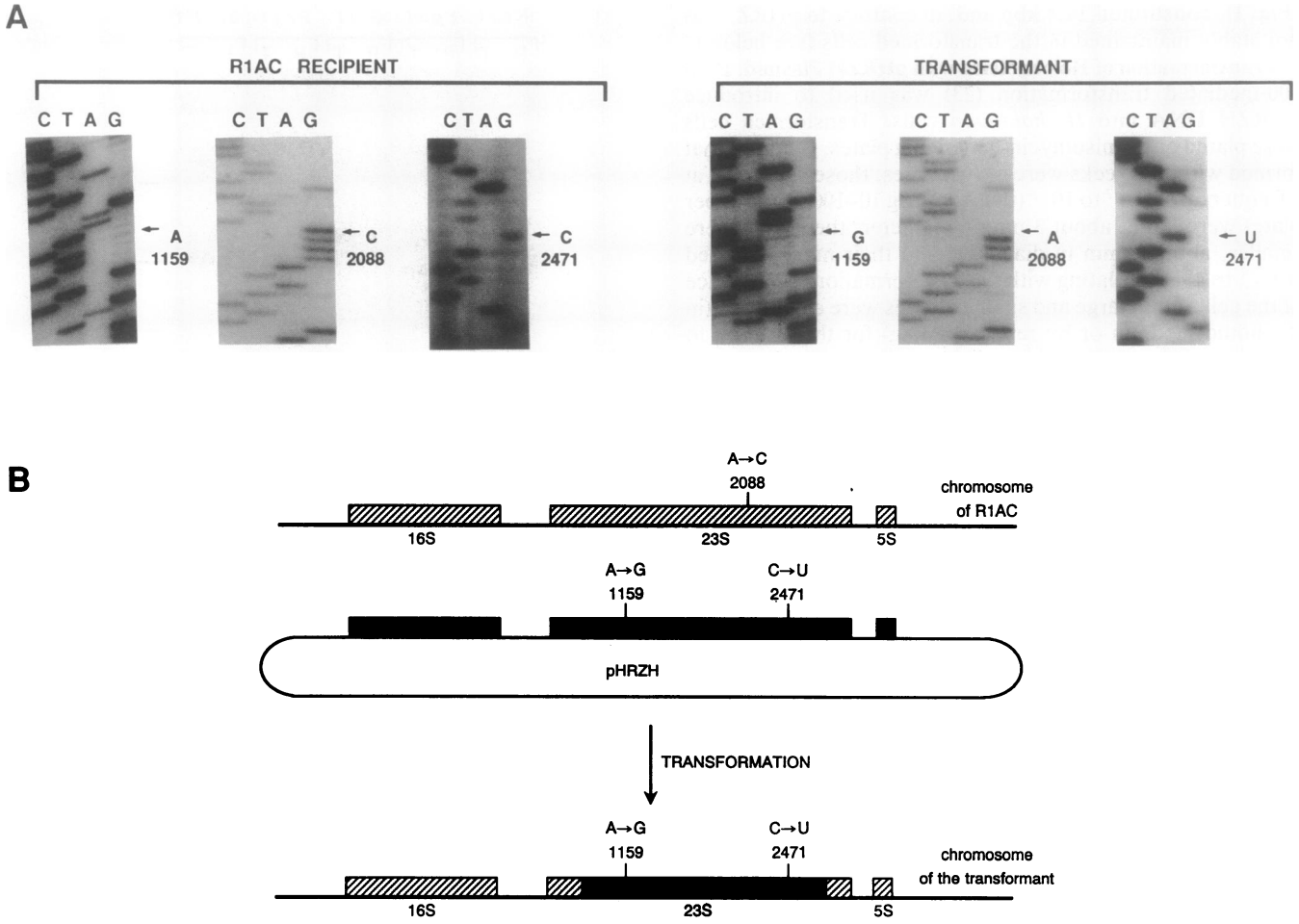

FIG. 3. (*A*) Reverse transcriptase sequencing of the 23S rRNA region around positions 1159, 2088, and 2471 of both the R1AC recipient cells and the R1AC cells transformed with pHRZH plasmid. Ribonucleotides at the mutation sites are shown and numbered. (*B*) Schematic representation of the results of transformation of R1AC cells with pHRZH plasmid. Chromosomal rRNA genes are shown by hatched bars and pHRZH-borne rRNA genes are shown by solid bars. Nucleotide mutations present in the corresponding DNAs are indicated. Exact borders of the fragment transferred from the plasmid to the chromosome are unknown and shown arbitrarily.

rRNA genes concomitantly with the selective markers, the approach can be used to design the nucleotide sequence of the single-copy chromosomal rRNA operon.

The vector construct, pHRZH, consists of three functional parts (Fig. 1): (*i*) a fragment of *H. halobium* DNA containing the entire rRNA operon, including the selective mutations, which acts as a donor of the mutated rRNA gene segment; (*ii*) the pBR322 moiety, which permits vector replication and ampicillin selection in *E. coli*; this renders possible the application of standard genetic engineering and *in vitro* mutagenesis techniques; and (*iii*) a 1736-bp sequence of pHSB1 plasmid from *Halobacterium* sp. SB3, which abolishes pHRZH maintenance in the transformed halobacterial cells and thereby promotes selection of recombinants.

Two nucleotide substitutions, 1312 bp apart, are present in the *H. halobium* 23S rRNA gene on the pHRZH plasmid; they render transformed halobacteria resistant to the two ribosomal drugs anisomycin and thiostrepton. The use of two antibiotic-resistance markers in the vector is essential for selecting authentic transformants and for discriminating them from single spontaneous point mutants. The latter appear with a very high frequency (10^{-7} to 10^{-8}) when selected on either anisomycin or thiostrepton (ref. 19; A.S.M., unpublished data). However, since two independent mutations must occur spontaneously within the same cell to render it sensitive to both antibiotics, the frequency of such an event should be extremely low (10^{-14} to 10^{-16}) such that only true transformants would grow in the presence of both selective drugs.

A two-step procedure was developed to select for *H. halobium* cells transformed with pHRZH. After transformation, cells are initially plated onto anisomycin-containing plates, on which transformants as well as spontaneous Ani^r mutants can grow. Next, all Ani^r colonies are tested for their resistance to thiostrepton either by replica plating or by growing in liquid culture containing the drug, which permits identification of true Ani^r Ths^r transformants. It is noteworthy that the sizes of the colonies of Ani^r Ths^r transformants on the first, anisomycin-containing, plate were reproducibly smaller than those of spontaneous Ani^r thiostrepton-sensitive mutants. (We now use the colony-size criterion as a preliminary screen for transformants.) However, the reduced colony size did not reflect a slower growth of transformants since in liquid culture, or after replating, the growth rate was indistinguishable from that of the Ani^r mutants. Rather it reflected the delayed growth of the transformants on the anisomycin selective plate. This delay may indicate that the transformed cells resume their growth and division only after the cellular pool of wild-type ribosomes has been partly replaced by mutant ones. Clearly, no lag in the growth of spontaneous Ani^r mutants would be expected because they preexist in the culture and have 100% mutant ribosomes at the time of plating.

A similar line of argument might explain the observation that double-resistant colonies rarely appeared, either when

transformed cells were plated directly on agar medium containing both antibiotics or when the cells were plated first onto thiostrepton- and then onto anisomycin-containing plates. Possibly, the ratio of mutant to wild-type ribosomes required for protein synthesis to resume is higher for thiostrepton than for anisomycin, and the time needed to reach this ratio is longer for the former than the period during which the cell can survive without protein synthesis.

An important feature of the pHRZH vector is that it ensures selection of the cells in which recombination between the plasmid-borne and chromosomal rRNA operons has occurred. Such recombination accompanied by rapid plasmid loss will eventually lead to a homogeneous population of the mutated ribosomes in the cell. The presence of the pHSB1 sequence is crucial for this. The plasmid construct lacking pHSB1 but otherwise identical to pHRZH was quite stable in halobacteria (A.S.M., N. I. Derckacheva, and V.K.K., unpublished results); however, introduction of the pHSB1 sequence abolished the vector maintenance in *H. halobium* as indicated by Southern analyses (see Fig. 2) and colony hybridization screening of several hundreds of transformants (not shown). It is likely, therefore, that the simultaneous presence of pHSB1 and rRNA-operon-associated replicons in the same plasmid is unfavorable for its replication or partitioning, resulting in its instability. Thus, since the vector plasmid is rapidly lost the selective markers can be inherited only if they recombine into the chromosomal rRNA operon. Whatever the mechanism of marker transfer, its efficiency seems to depend on the size of the homologous DNA segments in the plasmid and chromosome. When the plasmid-borne rRNA-encoding segment was reduced to 4.5 kb, including the 16S/23S spacer, 23S rRNA, and 5S rRNA genes, the efficiency of transformation/recombination dropped by about 1 order of magnitude, compared with pHRZH (P. Østergaard, personal communication). The exact size of the plasmid DNA fragment that recombines into the chromosome is unknown, but it probably lies in the range 1.3 kb (the distance between *ths* and *ani* loci) to 10 kb (the size of the plasmid-borne rDNA fragment).

Transformation of halobacteria with linear fragments of the chromosomal DNA has recently been reported (30). In our hands, however, transformation of *H. halobium* with pHRZH plasmid opened at different restriction sites (located close to or far from the selective mutations) produced much lower numbers of transformants (if any) than the covalently closed pHRZH (not shown); thus the topological state of pHRZH or its transient replication is important for plasmid-to-chromosome marker transfer.

The major advantage of the pHRZH-based transformation/recombination system lies in its potential for rRNA designing: introducing desired mutations into the single-copy chromosomal rRNA operon. This was tested by curing the *H. halobium* mutant R1AC (20) of an A → C transversion at position 2088 in its 23S rRNA gene. After transformation of the R1AC cells with the pHRZH vector the appearance of the Ani^r and Ths^r mutations in the chromosomal rRNA operon was paralleled by a restoration of the wild-type sequence at position 2088 of the 23S rRNA gene and thus in all the cellular 23S rRNA molecules (see Fig. 3). Similarly, mutation(s) can be engineered in pHRZH-borne rRNA genes, using standard *in vitro* mutagenesis techniques, and transferred to the chromosomal rRNA operon of *H. halobium* concomitantly with the selectable Ani^r and Ths^r markers. The system can also be extended to more complex mutations; thus recently the pHRZH vector was successfully used to generate a 4-bp insertion into the 23S rRNA gene of *H. halobium* (A.S.M. and H. F. Noller, unpublished results). The approach can, in principle, be applied to other organisms with single sets of rRNA genes, including the hyperthermophilic archaea, which have the added advantage of a high thermal stability of their ribosomes.

We are grateful to A. Bogdanov and P. Forterre for valuable discussions and to L. Baratova for moral support throughout the course of this work. We thank T. Powers for critical reading of the manuscript. This work was supported by Russian Biogen grants to A.S.M. and grants from the Danish Science Research Council to R.A.G.; A.S.M. thanks the Federation of European Biochemical Societies and the Plasmid Foundation, Denmark, for support during his stay at the laboratory of R.A.G.

1. Crick, F. H. C. (1968) *J. Mol. Biol.* **38**, 367–379.
2. Noller, H. F. (1991) *Annu. Rev. Biochem.* **60**, 191–227.
3. Sollner-Webb, B. & Mougey, E. B. (1991) *Trends Biochem. Sci.* **16**, 58–62.
4. Dujon, B. (1981) in *Molecular Biology of Yeast Saccharomyces: Life Cycle and Inheritance*, eds. Strathern, J. N., Jones, E. & Broach, J. R. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 505–635.
5. Sweeney, R. & Yao, M. C. (1989) *EMBO J.* **8**, 933–938.
6. Woese, C. R., Kandler, O. & Wheelis, M. L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4576–4579.
7. Brown, J. W., Daniels, C. J. & Reeve, J. N. (1989) *CRC Crit. Rev. Microbiol.* **16**, 287–338.
8. Garrett, R. A., Dalgaard, J., Larsen, N., Kjems, J. & Mankin, A. S. (1991) *Trends Biochem. Sci.* **16**, 22–26.
9. Lam, W. L. & Doolittle, W. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5478–5482.
10. Blaseio, U. & Pfeifer, F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6772–6776.
11. Holmes, M. L. & Dyall-Smith, M. L. (1990) *J. Bacteriol.* **172**, 756–761.
12. Hofman, J. D., Lau, R. H. & Doolittle, W. F. (1979) *Nucleic Acids Res.* **7**, 1321–1333.
13. Mankin, A. S., Teterina, N. L., Rubtsov, P. M., Baratova, L. A. & Kagramanova, V. K. (1984) *Nucleic Acids Res.* **12**, 6537–6546.
14. Mankin, A. S. & Kagramanova, V. K. (1986) *Mol. Gen. Genet.* **202**, 152–161.
15. Hui, I. & Dennis, P. P. (1985) *J. Biol. Chem.* **260**, 899–906.
16. Chant, J. & Dennis, P. P. (1986) *EMBO J.* **5**, 1091–1097.
17. Mankin, A. S. & Kagramanova, V. K. (1988) *Nucleic Acids Res.* **16**, 4679–4692.
18. Hummel, H. & Böck, A. (1987) *Nucleic Acids Res.* **15**, 2431–2443.
19. Hummel, H. & Böck, A. (1987) *Biochimie* **69**, 857–861.
20. Mankin, A. S. & Garrett, R. A. (1991) *J. Bacteriol.* **173**, 3559–3563.
21. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
22. Stoeckenius, W. & Kunau, W. H. (1968) *J. Cell. Biol.* **38**, 337–357.
23. Cline, S. W., Lam, W. L., Charlebois, R. L., Schalkwyk, L. C. & Doolittle, W. F. (1989) *Can. J. Microbiol.* **35**, 148–152.
24. Ebert, K., Goebel, W. & Pfeifer, F. (1984) *Mol. Gen. Genet.* **194**, 91–97.
25. Kagramanova, V. K., Derckacheva, N. I. & Mankin, A. S. (1988) *Nucleic Acids Res.* **16**, 4158.
26. Hackett, N. R. & DasSarma, S. (1989) *Can. J. Microbiol.* **35**, 86–91.
27. Kagramanova, V. K., Derckacheva, N. I. & Mankin, A. S. (1989) *Can. J. Microbiol.* **35**, 160–163.
28. Conover, B. K. & Doolittle, W. F. (1990) *J. Bacteriol.* **172**, 3244–3248.
29. Egebjerg, J., Leffers, H., Christiansen, A., Andersen, H. & Garrett, R. A. (1987) *J. Mol. Biol.* **196**, 125–136.
30. Cline, S. W., Schalkwyk, L. C. & Doolittle, W. F. (1989) *J. Bacteriol.* **17**, 4987–4991.
31. Dahlberg, A. H. (1989) *Cell* **57**, 525–529.