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Site-directed mutagenesis of the binding site for ribosomal protein S8 within 16S ribosomal RNA from *Escherichia coli*

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ABSTRACT

Twelve specific alterations have been introduced into the binding site for ribosomal protein S8 in *Escherichia coli* 16S rRNA. Appropriate rDNA segments were first cloned into bacteriophage M13 vectors and subjected to bisulfite and oligonucleotide-directed mutagenesis *in vitro*. Subsequently, the mutagenized sequences were placed within the *rrn*B operon of plasmid pNO1301 and the mutant plasmids were used to transform *E. coli* recipients. The growth rates of cells containing the mutant plasmids were determined and compared with that of cells containing the wild-type plasmid. Only those mutations which occurred at highly conserved positions, or were expected to disrupt the secondary structure of the binding site, increased the doubling time appreciably. The most striking changes in growth rate resulted from mutations that altered a small internal loop within the S8 binding site. This structure is phylogenetically conserved in prokaryotic 16S rRNAs and may play a direct role in S8-16S rRNA recognition and interaction.

INTRODUCTION

The advent of efficient methods for site-directed mutagenesis *in vitro* has opened new avenues for the study of structure-function relationships in bacterial ribosomal RNAs (rRNAs). The introduction of specific alterations into *Escherichia coli* rRNA operons cloned in multicopy plasmids has made it possible to produce cells in which approximately 60% of the rRNA is transcribed from mutant operons (1). The utility of this approach has already been demonstrated in studies on rRNA processing and protein-rRNA interaction (2-8). While most previous experiments were performed under conditions that should have resulted in random mutagenesis throughout a defined rRNA segment, only a small subset of the possible mutations were recovered in many cases. In particular, it is likely that mutants with very deleterious rRNA defects were often not detected, as the selective techniques employed made the isolation of colonies with severely impaired growth difficult. Thus, to study the consequences of specific nucleotide changes, it would be advantageous to first construct the mutant rRNA genes under non-selective conditions, and then

introduce them into host cells for an assessment of their physiological effects.

E. coli ribosomal protein S8 is involved in the early steps of assembly of the 30S ribosomal subunit (9). In this capacity, it interacts strongly with a specific binding site within the central domain of 16S rRNA that encompasses residues 583 to 653 (10-13). From a comparative analysis of the sequences with which S8 associates in a variety of eubacterial and archaebacterial 16S rRNAs, a number of conserved structural features were identified within the site of protein attachment (14). On the assumption that these features--which include helical segments of defined length, interior loops of constant size and location, and specific nucleotide sequences and base pairs in comparable positions--play an important role in S8-16S rRNA interaction, it should be possible to change the affinity of the protein for its binding site by introducing genetic alterations into the rRNA molecules. Accordingly, we have utilized bisulfite and oligonucleotide-directed mutagenesis *in vitro* to construct a set of mutations within the segment of the gene for *E. coli* 16S rRNA that encodes the binding site for protein S8 (15-18). Among the many potential targets, phylogenetically conserved features of the S8 binding site were given special priority. In all cases, mutagenesis was performed on fragments of the 16S rRNA gene that had been subcloned into M13 bacteriophage vectors. The mutagenized gene fragments were then cloned into the *rrn*B operon of plasmid pNO1301 (1), replacing the corresponding wild-type sequences. The resulting plasmids were used to transform *E. coli* in order to test the expression of the mutant genes. Although several of the mutations had little effect on the rate of cell growth, alterations in several of the evolutionarily conserved bases of the S8 binding site led to severe growth deficiencies that were manifested by large increases in the doubling time of the recipient cells.

MATERIALS AND METHODS

Bacterial Strains

E. coli JM101 has been previously described (19); *E. coli* CAG1574 (*araD139*, Δ(*ara leu*)7697, Δ*lacX74*, *galU*$^-$, *galK*$^-$, *hsd* r$^-$, *hsd* m$^+$, *strA*, *recA56*, *srl*$^-$, *tet*^{S}) was obtained from Dr. R. L. Course (University of Georgia). All strains were stored in 50% glycerol at -70°C.

Plasmids and Bacteriophages

Plasmid pNO1301, a derivative of pBR322 containing the *rrn*B ribosomal RNA operon of *E. coli* (1), was kindly provided by Dr. M. Nomura (University of

California, Irvine). Bacteriophage M13mp9 (19) was used to construct all vectors employed for mutagenesis and DNA sequencing.

Enzymes and Radioisotopes

T4 DNA ligase was purchased from New England Biolabs. *E. coli* DNA polymerase I (large fragment) and restriction endonucleases were obtained from Bethesda Research Laboratories, International Biotechnologies, Inc., or New England Biolabs. [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from New England Nuclear and ICN, respectively.

Gel Electrophoresis

DNA restriction fragments were separated on 0.6 to 1.0% agarose gels containing 100 mM Tris-100 mM H_3BO_4, pH 8.3, and 2 mM EDTA (TBE buffer). Purified DNA fragments were recovered from gels by electrophoresing them onto DEAE-nitrocellulose membranes (Schleicher and Schuell) placed in the path of the desired bands. DNA was eluted from the membranes with 1.0 M NaCl and precipitated with ethanol (20).

Mutagenesis

Mutagenesis with 3 M sodium bisulfite was performed as described by Everett and Chambon (16) with modifications according to Kalderon *et. al* (17). Single-stranded DNA from phage M13 , containing residues 615 to 1385 of the 16S rRNA gene, was mixed with linear, duplex phage DNA from which the target region had been deleted. The mixture was denatured with NaOH, neutralized with HCl and allowed to reanneal for 2.5 hours at 63°C which resulted in circular, partial duplexes with single-stranded gaps spanning the region to be mutagenized. These molecules were treated with 3 M sodium bisulfite and 2 mM hydroquinone under mineral oil at 37°C for 0.5 to 3 hours. Bisulfite was removed by two successive centrifugations through 2-ml columns of Sephadex G-50 (Pharmacia). The DNA was then incubated in 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 200 mM NaCl overnight at 37°C, precipitated with ethanol, and resuspended in polymerase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1mM DTT, 1 mM dNTPs). 0.5 units of DNA polymerase I (large fragment) were added to fill in the gap and prevent excision of uracil residues by the host. Aliquots of this mixture were used to transfect *E. coli* JM101. The resulting M13 plaques were screened for base changes by DNA sequencing as described below.

Oligonucleotide-directed mutagenesis was performed according to Zoller and Smith (18). An oligonucleotide capable of directing the desired changes in rRNA structure was kindly synthesized for us by Dr. R. J. Cedergren (University of Montreal). The oligonucleotide was first phosphorylated at the 5' end using ATP and T4 polynucleotide kinase. The mutagenic primer was

hybridized to a single-stranded M13 vector, extended *in vitro* with DNA polymerase I (large fragment) and treated with T4 DNA ligase. This preparation was enriched for covalently-closed, circular DNA by centrifugation through alkaline sucrose density gradients. Enriched fractions were neutralized with HCl and used to transfect *E. coli* JM101. Screening of the resulting plaques was performed by differential hybridization of the mutagenic primer, end-labeled with ^{32}P, to phage DNA fixed on nitrocellulose filters (18, 21). The filters were washed with 6X SSC buffer (0.9 M NaCl, 0.09 M Na^+ citrate, pH 7.0) at successively higher temperatures; autoradiography of the filters was performed between each wash. Only mutant phages which contained sequences exactly complementary to the mutagenic oligonucleotide retained the mutagenic primer at 40°C. Potential mutants identified in this way were confirmed by DNA sequence analysis.

DNA Sequence Analysis

DNA sequencing was performed by the dideoxynucleotide chain termination method (22). The universal pentadecamer primer (New England Biolabs) was used for sequencing DNA segments close to the priming site. Alternatively, an *Eco*RI/*Bgl*II restriction fragment of plasmid pNO1301, corresponding to nucleotides 679 to 708 of the 16S rRNA, was used for sequencing regions distant from the universal priming site. Sequences were determined by electrophoresis of the reaction mixtures on 8% polyacrylamide gels in TBE buffer followed by autoradiography at -70°C with Kodak XAR-5 film.

RESULTS

Isolation of mutations in the binding site for protein S8

As the first step in the construction of vectors for *in vitro* mutagenesis, a *Sma*I restriction fragment from the *rrn*B operon, corresponding to nucleotides 615 to 1385 in mature 16S rRNA, was cloned into bacteriophage M13mp9 in both orientations to yield M13-770 and M13-077 (Fig. 1). Bisulfite mutagenesis was used to produce C⇒T and G⇒A transitions throughout the 3′ half of the S8 binding site. To obtain the gapped duplex molecules needed for mutagenesis, two additional vectors, M13-770ΔH and M13-077ΔE, were constructed by removing small restriction fragments from M13-770 and M13-077, respectively, that span the protein binding site (Fig. 1). As shown in Figure 2, hybridization of M13-770ΔH with M13-770 permitted the production of C⇒T transitions in the RNA-like strand. Annealing of M13-077ΔE and M13-077 allowed the generation of C⇒T transitions in the complementary strand which resulted in G⇒A transitions in the RNA-like strand. After treatment with

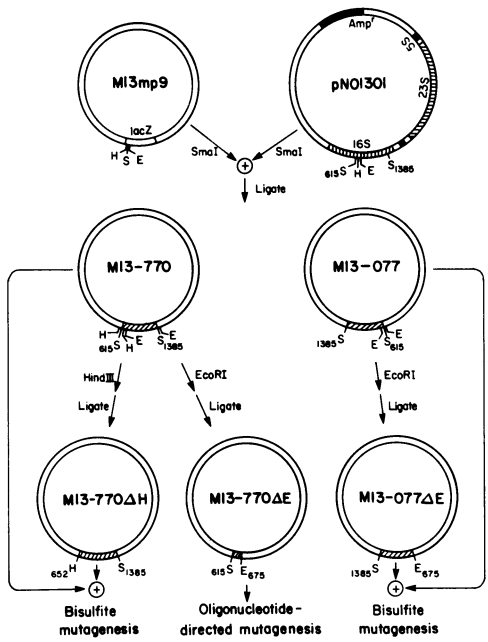

Figure 1. Construction of the bacteriophage M13 subclones used for mutagenesis. The coordinates correspond to nucleotides in mature *E. coli* 16S rRNA. E=*Eco*RI, S=*Sma*I, H=*Hin*dIII.

bisulfite, repair, and propagation, DNA was isolated from individual plaques and screened directly for mutations by DNA sequencing. Given the high efficiency of bisulfite mutagenesis, single point mutants were obtained without the imposition of biological selection (Fig. 3).

Oligonucleotide-directed mutagenesis was utilized to induce specific changes within the highly conserved internal loop that contains the putative recognition features for the binding of S8 (14). The mutagenic primer was designed to delete the conserved dimer, A_{642}-C_{643}, from the loop and replace it with a single A, C, G or U(T) residue (Fig. 4). Another subclone, M13-770ΔE, encompassing the DNA segment that corresponds to bases 615 to 674 in 16S rRNA, was constructed for this purpose (Fig. 1). Use of M13-770ΔE eliminated the possibility of adventitious mutations in neighboring regions of the cloned DNA and facilitated DNA sequencing by bringing the target region closer to the universal primer site. Following *in vitro* synthesis of covalently-closed, double-stranded M13 DNA and subsequent transformation of *E. coli*, phage plaques were screened for the desired alterations by differential

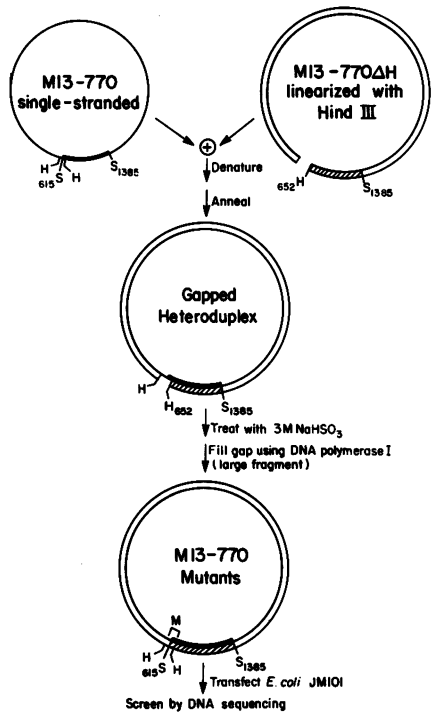

Figure 2. Strategy of bisulfite mutagenesis. The thickened line represents the segment of 16S rDNA in single-stranded M13-770. M13-770ΔH was linearized with *Hin*dIII and hybridized to single-stranded M13-770, yielding a partial duplex that was treated with sodium bisulfite, repaired, propagated and screened for mutations by DNA sequencing. S=*Sma*I, H=*Hin*dIII, M=location of the bisulfite mutations.

hybridization to the mutagenic primer (Fig. 5). As verified by direct sequencing of DNA from positive plaques, all four types of mutants were recovered (see Fig. 4). *Hin*dIII fragments containing the mutations were cloned into M13-770ΔH before insertion into the *rrn*B operon.

Introduction of mutations into the *rrn*B operon

Mutationally altered rDNA segments were cloned into the *rrn*B operon of plasmid pNO1301 by a procedure that minimized the recovery of wild-type plasmids and made possible the isolation of transformants with severely debilitating phenotypes. In most cases, restriction fragments containing mutations were used to replace the corresponding wild-type sequence in pNO1301 via the tripartite reconstruction illustrated in Figure 6. The 6.4-kb *Bgl*II/*Pst*I and 3.8-kb *Pst*I/*Sma*I restriction fragments from pNO1301 were

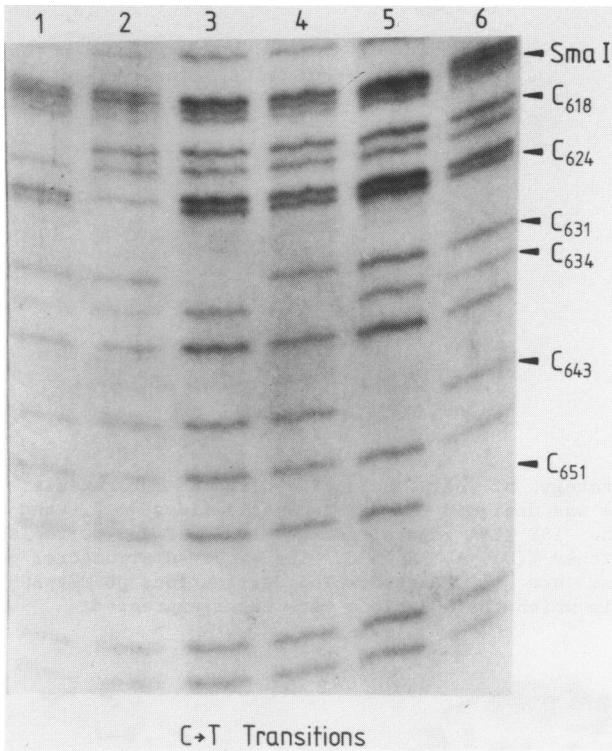

Figure 3. Screening of bacteriophage DNA by dideoxynucleotide DNA sequencing after bisulfite mutagenesis. Only the dideoxy G reaction was used to identify mutations. Six different C⇒T transitions in the portion of the 16S rRNA gene encoding the S8 binding site are shown. The coordinates correspond to nucleotides in mature 16S rRNA.

purified electrophoretically and mixed with a *Sma*I + *Bgl*II + *Bgl*I digest of mutant M13 replicative-form DNA in the presence of T4 DNA ligase. Inclusion of *Bgl*I in the digest disrupted the competing *Sma*I/*Bgl*II fragments derived from M13-770 and thereby enhanced recovery of the desired product. The reconstituted plasmid was then used to transform *E. coli* to ampicillin resistance. Nearly all of the transformants contained the 90-bp *Sma*I/*Bgl*II fragment from the mutant phage between the two restriction fragments derived from pNO1301. The presence of the mutations in each of the fully reconstructed plasmids was confirmed either by restriction analysis, when the mutation destroyed a specific restriction site, or by DNA sequence analysis of the appropriate rDNA fragment subcloned into M13 (Fig. 7).

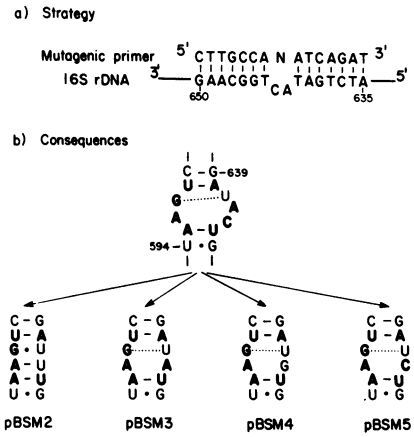

Figure 4. Strategy of oligonucleotide-directed mutagenesis. The mutagenic oligonucleotide was designed to delete nucleotides A_{642} and C_{643} from a segment of the 16S rRNA gene cloned in M13-770ΔE and to replace them with a single base, either T(U), A, G or C. The expected structural consequences of these mutations are illustrated. The designations pBSM2-pBSM5 refer to the *rrn*B plasmids in which the mutations were later expressed.

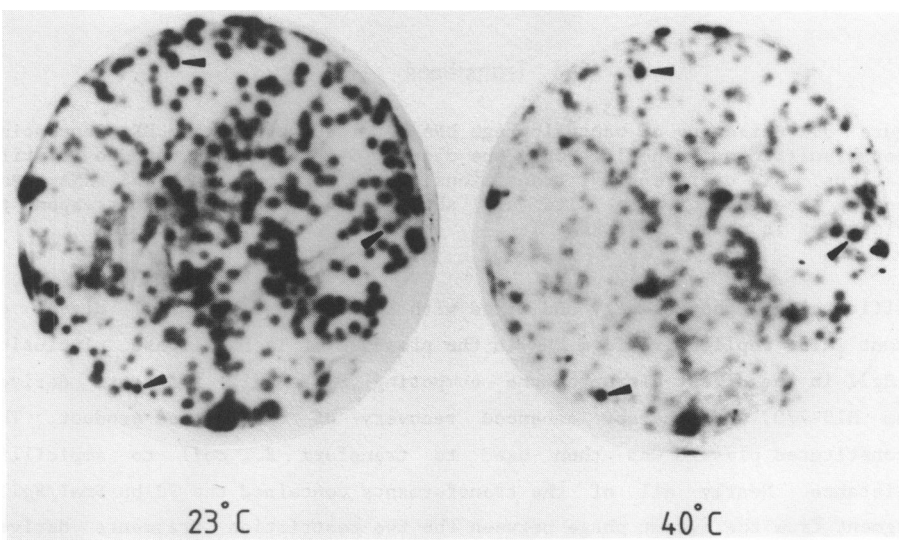

Figure 5. Screening for oligonucleotide-directed mutations by differential hybridization. The mutagenic primer was end-labeled with ^{32}P and hybridized to bacteriophage DNA immobilized on a nitrocellulose filter. The filter was washed at 23°C and 40°C to discriminate between mismatched and perfectly paired hybrids. Mutant phages preferentially retained the primer at the higher temperature. The arrows point to mutant phage plaques.

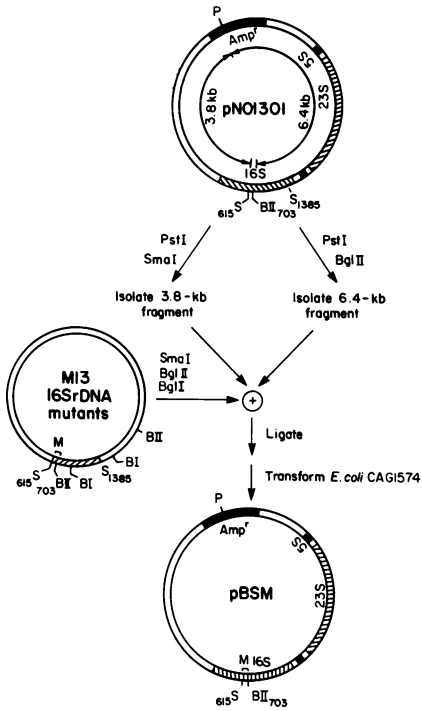

Figure 6. Tripartite cloning strategy. Fragments of DNA containing mutations produced in M13 phage vectors were placed in proper context within the 16S rRNA gene through the scheme outlined above. Two restriction fragments from plasmid pNO1301 were isolated and mixed with a digest of mutant M13 DNA. Only *SmaI/BglII* fragments from the phages could fill the gap between the fragments from pNO1301 and thus allow them to recircularize. Competing fragments from the M13 vector were eliminated by digestion with *BglI*. S=*SmaI*, BI=*BglI*, BII=*BglII*, P=*PstI*, M=location of the mutations in the various clones.

Characterization of the site-directed mutants

Using the techniques described above, 12 different point mutations were introduced into the portion of the 16S rRNA gene that encodes the binding site for protein S8. Their locations, as well as the structural alterations they produce, are indicated in the secondary-structure model of the S8 binding region depicted in Figure 8. Transformation of *E. coli* CAG1574 with the mutant plasmids (pBSM) led to consistent and reproducible changes in the growth rate of the host cells. On solid medium, for instance, cells transformed with certain plasmids produced large colonies within 24 hours after plating, while those transformed with other plasmids yielded colonies of similar size only after 48 hours. These differences were confirmed and

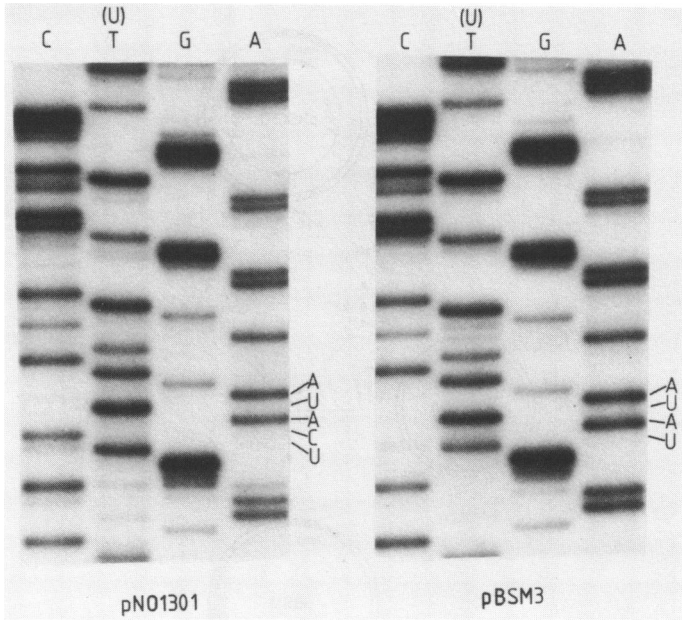

Figure 7. Analysis of the mutant plasmids by DNA sequencing. The persistence of the mutations in the mutant plasmids was verified by dideoxynucleotide DNA sequencing. In order to prepare template DNA for sequence analysis, restriction fragments containing the mutations were cloned from each plasmid into M13mp9 (17). This figure shows the sequence from wild-type (pNO1301) and mutant (pBSM3) plasmids; nucleotide C_{643} has been deleted from pBSM3.

quantitated by growth-rate measurements in liquid culture where the doubling times of cells containing mutant plasmids ranged from a minimum value of 37 minutes, characteristic of cells containing wild-type plasmids, to a maximum of 105 minutes (Table 1).

Growth of the recipient cells was not significantly perturbed by the presence of 16S rRNA molecules containing single base substitutions in helical segments of the S8 binding site as long as base pairing was maintained. Thus, the replacement of G-C by G•U base pairs at positions 588/651 (pBSM9), 604/634 (pBSM7) and 616/624 (pBSM11), and of a U•G by a U-A base pair at position 594/645 (pBSM13), had little or no influence on growth rate (Table 1, Fig. 8). Similarly, certain alterations in the single-stranded segments of the S8 binding region had little effect on the doubling time of the host cells. Transformants containing U instead of C at position 631, for example, grew at the wild-type rate (Table 1). Although this base occurs within a sequence protected from RNase digestion by S8, it is not conserved phylogenetically and

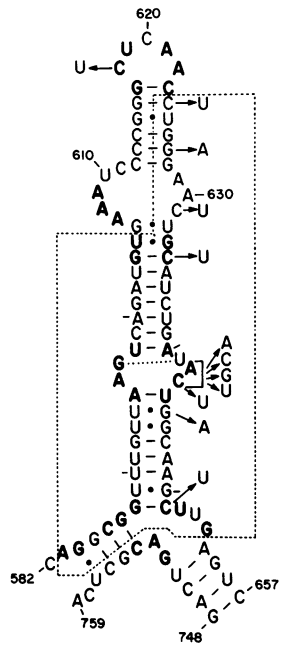

Figure 8. Location of site-directed mutations within the secondary structure of the S8 binding region. The dashed line encloses the rRNA segments protected by S8 from nuclease digestion (13). Bases shown in boldface are phylogenetically conserved in bacterial 16S rRNAs (14).

may not be directly involved in S8 recognition. Replacement of C by U at position 618 slightly increased the doubling time of the recipient cells despite the fact that it is not protected by S8 from RNase digestion (10-13).

Moderate to severe impairment of host-cell growth resulted from the introduction of plasmids with alterations at positions 642 and 643. These bases form part of the highly conserved internal loop that lies between the two main helical segments of the S8 binding site (Fig. 8). When the C residue at position 643 was replaced by U (pBSM1), the doubling time of cells containing the mutant 16S rRNA increased almost two-fold (Table 1). Still lower growth rates were observed if a single base was deleted from the internal loop (Fig. 8, Table 1). Removal of A_{642} (pBSM5) or C_{643} (pBSM3) led to a 2.5-fold increase in doubling time, whereas replacement of A_{642}-C_{643} with G (pBSM4) or U (pBSM2) appeared even more deleterious to host cells harboring the mutant alleles. Cells transformed with pBSM12, which contains a G⇒A transition at position 627, also exhibited a significant increase in growth rate (Table 1). This alteration disrupts a C-G base pair and may destabilize the apical helix shown in Fig. 8.

Table 1. Doubling times of *E. coli* cells transformed with wild-type and mutant plasmids.

Plasmid	Location of mutation in 16S rRNA	Base change	Doubling time (min)*
pNO1301	-	-	37
pBSM1	643	C⇒U	63 (3)
pBSM2	642-643	AC⇒U	105 (15)
pBSM3	642-643	AC⇒A	87 (3)
pBSM4	642-643	AC⇒G	96 (6)
pBSM5	642-643	AC⇒C	92 (4)
pBSM6	631	C⇒U	37 (2)
pBSM7	634	C⇒U	37 (2)
pBSM8	618	C⇒U	46 (2)
pBSM9	651	C⇒U	43 (1)
pBSM11	624	C⇒U	36 (2)
pBSM12	627	G⇒A	61 (5)
pBSM13	645	G⇒A	37 (3)

* Cells were scraped from agar plates, suspended in liquid medium containing 8 g/l yeast extract, 5 g/l tryptone, 5 g/l NaCl and 200 mg/l ampicillin at an initial OD_{600} of approximately 0.1 and incubated at 37°C for at least 3 doublings. All measurements were made on exponentially growing cells and were repeated a minimum of five times for each mutant. The observed range about the average doubling time is indicated in parentheses.

DISCUSSION

Ribosomal protein S8 plays a critical role in the early stages of 30S subunit assembly by binding to the central domain of the 16S rRNA cooperatively with several other small-subunit proteins (9). Mutations that perturb the interaction of S8 with 16S rRNA might therefore be expected to have deleterious effects upon ribosome assembly and, consequently, upon cell growth. The binding site for S8 within the 16S rRNA has been extensively characterized by nuclease protection experiments (10-13) and has been localized to an imperfect helix comprising nucleotides 583 to 605 and 624 to 653 (ref 13; see Fig. 8). The emphasis in the study of the S8 binding site has now shifted toward the identification of specific bases recognized by the protein. The importance of certain structural features in promoting this interaction is suggested by their conservation in more than ten prokaryotic 16S rRNAs capable of associating with *E. coli* S8 (14). Among the conserved features are the length of the helical segments, the location and size of the internal loops and the identity of particular base sequences and base pairs. In addition, chemical modification experiments have delineated the nucleotides in *E. coli* 16S rRNA required in unmodified form for S8-16S rRNA interaction (23). The location of these nucleotides correlates very well with the

phylogenetically conserved features of the 'S8 helix'. In an effort to alter the affinity of 16S rRNA for S8, we have used the information available on the S8 binding site as a basis for targeting mutations *in vitro*. Specifically, we have employed bisulfite and oligonucleotide-directed mutagenesis to construct a series of nucleotide substitutions and single base deletions within the segment of the 16S rRNA gene coding for the site with which S8 interacts. One of the most interesting features of the 'S8 helix' is the small internal loop encompassing bases 596-597 and 641-643 (Fig. 8). This loop--along with the nucleotides within it--is almost universally conserved in bacterial 16S rRNAs (24). Moreover, S8 has been cross-linked to bases on the 5' side of the loop (25), indicating a close physical association of the protein with this segment of the rRNA. As these data suggest a prominent role for the internal loop in S8-16S rRNA interaction, it was chosen as the primary site for mutagenesis.

To examine the physiological effects of the site-directed mutations, they were cloned into the *rrn*B rRNA operon of the multicopy plasmid pNO1301 and introduced into the appropriate *E. coli* hosts by transformation. As pNO1301 is maintained at up to 14 copies per cell, approximately 60% of the 16S rRNA produced should result from transcription of the mutant rRNA genes (1). The influence of the mutations on the growth rate of transformed cells varied from severe to none whatsoever (Table 1). The most debilitating effects were observed when alterations were made in the conserved internal loop defined by the sequences 596 to 597 and 641 to 643 (Fig. 8). For example, the doubling time of cells harboring a plasmid in which C_{643} was replaced by a uridine residue was over 50% longer than that of cells containing the wild-type plasmid. More substantial changes in the structure of the internal loop led to even greater decreases in host-cell growth rate. The deletion of A_{642} or C_{643}, or their substitution by a single G residue, provoked more than a two-fold increase in doubling time. The replacement of A_{642}-C_{643} by a U resulted in the slowest growth rate found in any of the mutants. It is noteworthy that the substituted U residue should be able to base pair with A_{596}, closing the loop and thereby eliminating altogether one of the most highly conserved features of the S8 binding site. It is quite likely that the replacement of phylogenetically conserved bases within the internal loop directly decreases the affinity of the mutant 16S rRNAs for protein S8 through the removal of key recognition signals. Further, the single-base deletions almost certainly alter the three-dimensional structure of the 'S8 helix'. Quanitative analysis of complex formation between S8 and the altered rRNA molecules indicates that all of the mutations within the conserved internal loop do in fact reduce the

association constant for the S8-16S rRNA interaction by at least two orders of magnitude (R.J. Gregory, P.B. Cahill and R.A. Zimmermann, in preparation). Our observations therefore suggest very strongly that the loop structure itself, as well as the individual bases within it, are required for proper 16S rRNA function.

A G⇒A transition at position 627, which should disrupt the helix comprised of nucleotides 612-617 and 623-628, also results in a significant reduction in the growth rate of cells that harbor the relevant plasmid. Although G_{627} is not phylogenetically conserved, it lies within the S8 binding site and is one of the bases required in unmodified form for interaction with the protein S8 (13, 23). The effect of the G⇒A shift on doubling time may therefore be a consequence of defective S8-16S rRNA association. On the other hand, it has been suggested that nucleotides 620 to 626 may be involved in a tertiary interaction with nucleotides 1420 to 1426 (26). The alternative interactions may be part of a structural switch and the mutation at position 627 could disturb the equilibrium between the two conformations, leading to an impairment of cell growth. A similar conclusion was reached in the case of a mutant 16S rRNA from which a single base at position 615 was deleted (7, 26). Although the presence of the altered rRNA caused an increase in host-cell doubling time *in vivo*, the deletion did not appear to interfere with the binding of S8 to 16S rRNA *in vitro*.

Mutation of three bases that are highly conserved in prokaryotic 16S rRNAs had little effect upon growth rate. Two of these resulted from C⇒U transitions at positions 634 and 651 within the S8 binding site. The relatively benign consequences of these mutations may stem from their structurally conservative nature, namely, the replacement a G-C base pair with a G•U base pair. The third mutation, a C⇒U transition at position 618, occurred outside the region that S8 protects from RNase digestion although it is conserved in prokaryotic 16S rRNAs and may play a role in rRNA function distinct from protein binding (24). Three of the mutant plasmids had no discernible effect upon the growth rate of transformed cells (Table 1). The common feature of the latter mutants is that the nucleotide substitutions do not materially alter base pairing nor do they occur at highly conserved positions.

On the basis of the foregoing, it appears likely that the greatly increased doubling times exhibited by cells containing certain of our mutant plasmids are due primarily to decreases in the affinity of the altered 16S rRNAs for protein S8. In an earlier report, it was shown that mutant 16S

rRNAs lacking defined segments of the central domain were unable to bind several ribosomal proteins that normally interact with the deleted sequences (7). *In vivo* these mutations led to slow growth, defective subunit assembly and incomplete rRNA processing (2, 4, 7). As the alterations reported here are also located at a site with which a major RNA-binding protein associates, it is probable that subunit assembly and rRNA processing are impaired in the present case as well. Furthermore, protein S8 appears to serve an important role in maintaining the balanced synthesis of ribosomal components by regulating the expression of eight ribosomal proteins encoded within the *spc* operon (27). Autogenous regulation is thought to occur at the level of translation of the polycistronic *spc* operon mRNA, presumably through the interaction of S8 with a site on the message that is structurally homologous to its binding site in 16S rRNA (28). Production of S8 in excess of 16S rRNA should therefore result in binding of the protein to the *spc* mRNA, slowing or halting its translation. In the same vein, we speculate that the presence of mutant 16S rRNAs with reduced affinity for S8 may lead to the inhibition of *spc* mRNA translation. Lower levels of the several 30S and 50S proteins encoded by the *spc* operon would in turn impede the assembly of both ribosomal subunits, even if they contained wild-type transcripts from the chromosomal rRNA genes. The involvement of S8 in autogenous regulation could thereby amplify the deleterious effects of the altered 16S rRNAs on ribosome synthesis in cells harboring the mutant plasmids. Although direct evidence on these matters is not yet available, the physiological characterization of cells harboring the mutant plasmids will be a main goal of our future experiments.

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