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Genetic Selection and DNA Sequences of 4.5S RNA Homologs

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A general strategy for cloning the functional homologs of an *Escherichia coli* gene was used to clone homologs of 4.5S RNA from other bacteria. The genes encoding these homologs were selected by their ability to complement a deletion of the gene for 4.5S RNA. DNA sequences of the regions encoding the homologs were determined. Since this approach does not require that the homologous genes hybridize with probes generated from the *E. coli* sequence, the sequences of the homologs were not all similar to the sequence of the *E. coli* gene. Despite the dissimilarity of the primary sequences of some of the homologs, all could be folded to obtain a similar structure.

4.5S RNA is a metabolically stable, 114-nucleotide RNA of the bacterium *Escherichia coli* (10). It is essential for *E. coli* growth (5) and is synthesized by a pathway common to many stable RNA species that participate in protein synthesis. That is, it is subject to stringent control (11) and is processed from a larger precursor by RNase P (1). Experiments conducted to identify the role of 4.5S RNA in the growth of *E. coli* indicate that its role is in protein synthesis. Extracts prepared from cells depleted of 4.5S RNA are defective in translation (3), and suppressors that reduce the requirement of 4.5S RNA for growth reside in the gene for protein synthesis, elongation factor G (4). In extracts prepared from such suppressor strains, the residual 4.5S RNA sediments with ribosomes in sucrose gradients, and this sedimentation is sensitive to the protein synthesis inhibitor puromycin (4).

To determine the structure of 4.5S RNA, we have cloned the functional 4.5S RNA homologs from other bacteria. Sequence analysis of the complementing clones indicates that 4.5S RNA may attain a structure similar to that of the SRP RNAs of eucaryotic protein secretion, as proposed by Poritz et al. (18) and by Struck et al. (21). The strategy we have used to select the functional homologs of 4.5S RNA should be applicable to other essential genes of *E. coli*.

MATERIALS AND METHODS

Media and genetic manipulations. YT medium (15) was used throughout. Ampicillin was used at a final concentration of 200 μg/ml, and isopropyl-β-D-thiogalactoside (IPTG) was used at 0.1 mM. Transformations were conducted as described previously (6). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Inc., and used as specified by the manufacturer. Plasmids were prepared as described previously (13).

Bacterial strains. *Bacillus subtilis* W168 was obtained from M. Perego. Strains S1192, S1608, and S1610 are derivatives of *E. coli* K-12. All alleles of *ffs*, the gene for 4.5S RNA, have been described previously (5). The genotypes of the *E. coli* strains are as follows: S1192, HfrH *lacI*^{q} *relA1 spoT1* ffs::*kan-591* [λ *imm*^{434} *c*$^+$ *nin5 Xho*I::Φ(P*tac-ffs*)] (4); S1608, F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsdR rpsL* ffs::*kan-591* [λ *c*I857 *nin5 Xho*I::Φ(P*tac-ffs*)]; and S1610, F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsdR rpsL ffs*::*kan*-591 λ*vir*^{r} [λ *c*I857 *nin5 Xho*I::Φ(P*tac-ffs*)].

Construction or source of genomic libraries. Genomic DNA from *B. subtilis* was prepared by isopycnic centrifugation in CsCl-ethidium bromide gradients. *Micrococcus lysodeikticus* DNA was purchased from Sigma Chemical Co. Genomic libraries from these two species were prepared by digesting the DNA with *Mbo*I. Complete digests were ligated with *Bam*HI-digested pGFIB-1 (14). The genomic library of *Pseudomonas aeruginosa* PAK (19) cloned in the multicopy plasmid pUC-18 (17) was obtained from S. Lory. The genomic library of *Legionella pneumophila* cloned in the cosmid vector pLAFR1 (8) was obtained from H. Shuman.

Selection by pulse curing. Genomic libraries from *B. subtilis*, *M. lysodeikticus*, and *P. aeruginosa* were introduced into S1610 by transformation. No heat pulse was used in the transformations, and transformants were selected at 30°C on YT-ampicillin agar. The transformants were pooled, a sample was grown to early log phase in YT-ampicillin broth at 30°C, and the prophage was cured as described previously (9). The cultures were incubated at 45°C for 5 min to denature the temperature-labile λ repressor and induce expression of the genes for prophage excision (24). After this period of induction, the cultures were cooled in an ice bath for 5 min to permit renaturation of the λ repressor. The cultures were then grown to saturation at 30°C to permit segregation of the excised, repressed λ prophages. Dilutions of the saturated cultures were spread on YT agar and incubated at 42°C. The 42°C-resistant survivors were purified, and plasmid DNA was prepared (13) and examined by restriction analysis. The plasmid DNA was also retransformed into S1610, and its ability to permit curing of the prophage was verified. The clones containing the smallest inserts were examined further. The initial clone from the *P. aeruginosa* library contained a 3.1-kilobase (kb) insert. Subcloning by *Sau*3A into pGFIB-1 yielded pSB1272 containing an 850-base-pair (bp) *Sau*3A fragment.

The 4.5S RNA homolog from *L. pneumophila* was isolated in a similar manner. In this case, the 4.5S RNA homolog was originally selected from a cosmid library by using strain S1608. S1608 was infected with the cosmid library of *L. pneumophila*, and transductants selected at 30°C on YT-ampicillin agar. The transductants were pooled and cured of the λ *c*I857 prophage in the presence of 10 mM EDTA to prevent reinfection by any released λ bacteriophage (9). The resulting cosmid clone from *L. pneumophila* was digested with *Sau*3A, and a 1-kb fragment containing the 4.5S RNA

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homolog was subcloned into the *Bam*HI site of pGFIB-1 and selected in S1610 to yield pSB1338.

DNA sequence analysis. Sequences of both strands were determined for all sequences reported. The sequences for *P. aeruginosa*-, *B. subtilis*-, and *M. lysodeikticus*-derived clones were determined by automated sequencing on an ABI 370A sequencer. Clones in vector pGFIB-1 were sequenced by using fluorescent-labeled M13 sequencing primer. Clones in pBluescript KS(−) and pBluescript SK(−) (Stratagene) were sequenced by using fluorescent-labeled reverse sequencing primer. The sequence from *L. pneumophila* was determined by using a Sequenase kit (U.S. Biochemicals Corp.) and the T3 primer with pSB1364 and primer 5′-TAGGAAGTGGGCACACTC-3′ with pSB1338. DNA sequences were compared by using Genepro sequence analysis software (Riverside Scientific).

RESULTS AND DISCUSSION

The 4.5S RNA homologs were selected by their ability to complement the deletion of the gene for 4.5S RNA in strain S1610 (see Materials and Methods). In this strain, all but the last 38 nucleotides of the chromosomal gene for 4.5S RNA is replaced with a gene for neomycin phosphotransferase, and the sole intact copy of the gene for 4.5S RNA is present on a λ *c*I857 prophage. Weisberg and Gallant (24) had found that λ *c*I857 prophages can be cured with approximately 80% efficiency after transient derepression of phage functions. The cured progeny are temperature resistant as a result of loss of the thermoinducible prophage. Since the prophage of S1610 contains the sole copy of an essential gene, fewer than 1 in 10^6 cells can form colonies at 42°C after transient induction. However, when harboring a multicopy plasmid bearing the gene for 4.5S RNA, more than 50% of the cells are able to form colonies at 42°C. The ability of multicopy plasmids encoding 4.5S RNA to permit cured cells to grow provided a selection adequate for the recovery of 4.5S RNA homologs from genomic libraries.

We used this strategy to select clones encoding 4.5S RNA homologs from the bacteria *P. aeruginosa*, *B. subtilis*, *M. lysodeikticus*, and *L. pneumophila*. These clones contain a single *Sau*3A or *Mbo*I fragment cloned in the multicopy plasmid vector pGFIB-1. The complementing clone derived from *P. aeruginosa*, pSB1272, contains an 850-bp *Sau*3A fragment, the complementing clone derived from *B. subtilis*, pSB1290, contains a 1.5-kb *Mbo*I fragment, the clone derived from *M. lysodeikticus*, pSB1311, contains a 282-bp *Mbo*I fragment, and the clone derived from *L. pneumophila*, pSB1338, contains a 1-kb *Sau*3A fragment.

All of the clones described above permit the appearance of temperature-resistant survivors from S1610. The vector, pGFIB-1, fails to permit this. To determine whether this stimulation of 42°C-resistant survivors is due to provision of 4.5S RNA function or to an effect on λ development, we examined a second 4.5S RNA phenotype. Strain S1192 (see Materials and Methods) has its sole copy of the gene for 4.5S RNA regulated by the *lac* repressor. Thus, S1192 requires IPTG to transcribe its gene for 4.5S RNA. If a plasmid allows S1610 to be cured of its prophage because the plasmid provides 4.5S RNA function, it will allow S1192 to grow without IPTG. Clones of all of the homologs described above, pSB1272, pSB1290, pSB1311, and pSB1338, allow S1192 to grow without IPTG. Furthermore, this growth without IPTG is not due to derepression of the *E. coli* 4.5S RNA gene, since the coordinately regulated *lac* operon is not induced.

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A GATCCAGCAG CAATGAACCG CTTTCGCGAA ATGGATTGCA AACGTAAGTG CTTGAAGTGG   60
  TTGTAACTTA CGCGCAAGCC GGTAGAATGG CGCGGCCTAC CCGAGGGTAG GCCGCAATGG  120
  TGGCCCTGTC GGTCCCCCCG CAACGATTAC CCGTCAACCT GGTCAGGTCC GGAAGGAAGC  180
  AGCCACAGCG GGAACATCGT GTGCCGGGGT GTGGCTGGCG GGGCCGCCTC CATTTCCTAA  240
  GTCCTTAATT CTAAAGGGCT TTCTCTGCTG AAAATCTCGA                         280

B TTACTCTTCG CTATTCAAAT ATGGTGACTC TATTGGTCCC CTCGCGACGA TAGATTGTGA   60
  ACCCCGTCAG GCCCCGAAGG GAGCAGCGGT AGCAGTTGAT GCGGGCGCCG GGGTGTGGCT  120
  CTTAGAGTCG CCGCCCAACT TGTGAAACAC GTATGAGCTA TCTGGCACTT GCCCGTAAAT  180
  GGCGACCACG CACATTTTCC CAACTGGTTG GCCAAGAGCA TATTAATAAA GCACTGATC   239
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FIG. 1. (A) Sequence of the complementing *Sau*3A-*Taq*I fragment from *P. aeruginosa*; (B) sequence derived from the complementing clones from *L. pneumophila*. The sequence shown in panel B represents the portion of the complementing clones for which both strands were sequenced.

The complementing region from *P. aeruginosa* was further localized to a 280-bp *Sau*3A-*Taq*I fragment by subcloning a *Taq*I fragment of pSB1272 into the *Cla*I site of pBluescript KS(−) and selecting 4.5S RNA function in S1610. Plasmids bearing the insert in both orientations, pSB1305 and pSB1307, complement S1610. Their ability to allow S1192 to grow without IPTG was not tested because the pBluescript vector contains the *lac* operator and partially induces transcription of *lac* and *ffs* in S1192.

The sequences of the inserts in pSB1305 and pSB1307 were determined (Fig. 1). This sequence starting at nucleotide 119 is 75% identical (86 of 114) with that encoding mature 4.5S RNA (10) if no gaps are permitted in the alignment. The gene isolated here is the same as the one isolated by Toschka et al. (23) by hybridization with an oligonucleotide probe derived from the *E. coli* 4.5S RNA sequence. These authors (23) found that *P. aeruginosa* contains a stable RNA that hybridizes to the cloned *Pseudomonas* gene. Sequence differences between that reported here and that described by Toschka et al. (23) are outside of the structural gene for the 4.5S RNA homolog and are probably due to differences in the *P. aeruginosa* strains used.

The complementing region from *B. subtilis* was identified by deletion analysis of pSB1290. The 1.5-kb *Mbo*I fragment in pSB1290 contains an *Xho*I and an *Eco*RI site such that the order of sites relative to the polylinker in pGFIB-1 is *Eco*RI, *Bam*HI (*Mbo*I), *Xho*I, *Eco*RI, *Bam*HI (*Mbo*I), *Sal*I. Deletion of either the *Eco*RI or the *Xho*I-*Sal*I fragments eliminated complementation behavior in both S1610 and S1192. This finding indicates that the 4.5S RNA homolog from *B. subtilis* enters the *Xho*I-*Eco*RI portion of the insert. The DNA sequence of part of the insert in pSB1290 showed it to contain the small cytoplasmic RNA (scRNA) gene (20). Since the scRNA gene contains an 88-bp *Xho*I-*Eco*RI fragment (22) and has been so well characterized (20, 22), we have not determined the sequence of both strands of our clone but used the sequence presented by Struck et al. (22) in developing a structure for 4.5S RNA. The sequence and structural similarity between scRNA and 4.5S RNA has been noticed earlier (22).

The complementing region from *L. pneumophila* was characterized primarily by sequence analysis. An 800-bp *Eco*RI-*Taq*I fragment from pSB1338 was subcloned into the *Eco*RI-*Cla*I sites of pBluescript SK(−). The resulting clone, pSB1364, complemented S1610. The sequences of both strands of part of this region were determined (Fig. 1). Since

Micrococcus lysodeikticus

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CCCGGGCCTCCCGCGGCCCGAATCGCCGCGCCCGCCGCCACCCTGCTAGACTCTTCGACG     60

                                                GGGGGCTCTGT      11

GCCCCTACGTGGTGTCATCTCGCTGAACTTCCCCCAGGACCGGAAGGTAGCAAGGGTAGG    120
       | |     |                    |||| |||||||| ||||     |
TGGTTCTCCCGCAACGCTACTCTGTTTACCAGGTCAGGTCCGGAAGGAAGCAGCCAAGGC     71

TGGGCTCTGGCAGGTGCGTGAGGGGTCTTCACGTTTCCCCAGGATC    166
 |   | | |   |  | |   || |  |||
AGATGACGCGTGTGCCGGGATGTAGCTGGCAGGGCCCCCACCC      114
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FIG. 2. Sequence of the complementing *Sma*I-*Mbo*I fragment from *M. lysodeikticus* shown above the sequence for mature *E. coli* 4.5S RNA. The sequence of one strand was determined from pSB1334; the sequence of the other was determined by recloning the *Bam*HI-*Sal*I fragment of pSB1311 into the *Bam*HI-*Sal*I sites of pBluescript KS(−). The resulting plasmid, pSB1345, complemented S1610, and the sequence of its insert was determined.

these sequences are 70% identical to that encoding mature 4.5S RNA (starting at nucleotide 23, 80 of 144 nucleotides are identical if no gaps are permitted), we did not further localize the complementing region within pSB1364. Instead, we conclude that the sequence presented in Fig. 1 represents the complementing portion of pSB1338 and pSB1364. Furthermore, the sequence of this region from *L. pneumophila* and the sequence of *E. coli* 4.5S RNA allow a similar structure to be formed (Fig. 3).

The complementing region from *M. lysodeikticus* was further localized within pSB1311 by deleting the *Sma*I fragments. The resulting clone, pSB1334, contains a 166-bp *Sma*I-*Mbo*I fragment and complements both S1610 and S1192. Although the *M. lysodeikticus* gene is able to complement the mutations in *E. coli*, there is little similarity between the sequence of the *E. coli* gene and that of the homolog from *M. lysodeikticus*. In fact, if no gaps are permitted in aligning the two sequences, only 33 of 114 nucleotides are identical (Fig. 2). This absence of extensive sequence similarity would render this homolog difficult to detect by a screen based on nucleic acid hybridization. However, both sequences allow a similar structure to be proposed (Fig. 3).

Since all of the homologs must mediate the function of 4.5S RNA in *E. coli*, it is likely that at least some portion of the molecules all attain a similar structure. In Fig. 3, we propose a structure that is consistent with the sequences of all of the complementing clones. Although considerably more evidence is necessary to identify the structure of 4.5S RNA, it is remarkable that all of the homologs can be folded to form a hairpin with a highly conserved apex. This structure for 4.5S RNA is similar to that proposed by Poritz et al. (18) and by Struck et al. (21). The structure proposed by Hsu et al. (10) cannot be attained by the homolog from *M. lysodeikticus*. The apical region has the most conserved primary sequence. The primary sequence is less conserved in the rest of the molecule, but the possibility of extensive base pairing is consistent with the high melting temperature observed for 4.5S RNA (2). In deriving the structure for the *L. pneumophila* sequence, we have allowed A · G base pairs within helices. Such base pairs have been observed in 16S rRNA (7, 16). A · G base pairs observed at the end of a helix are those at the top of the anticodon stem in tRNA. Since these involve modified G's (12), we have refrained from drawing A · G base pairs at the ends of helices. Allowing A · G base pairs at the ends of helices does not change the overall structure.

The similarity between the structure for 4.5S RNA that we derive here and that of SRP RNA (18, 21) is striking. We are

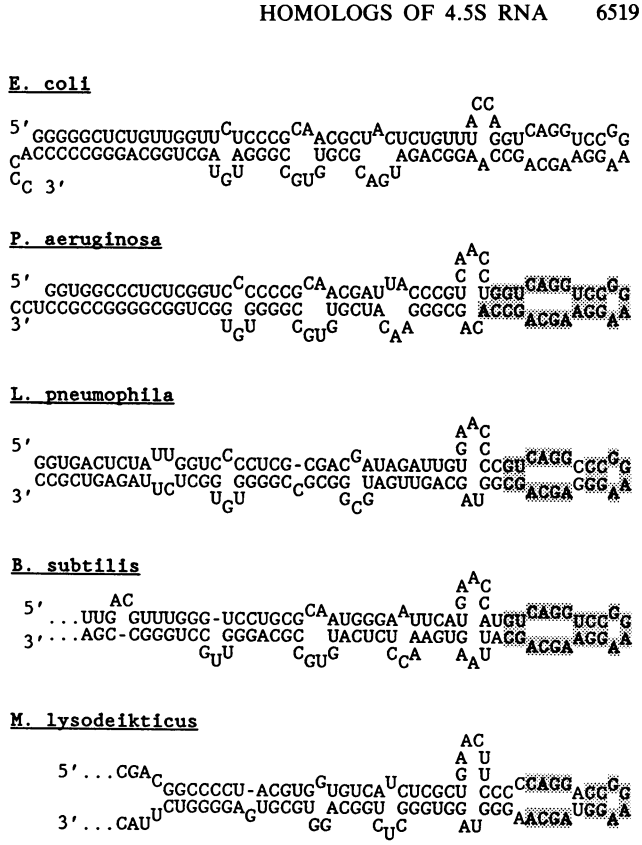

FIG. 3. Potential structures derived from the sequences of clones complementing 4.5S RNA of *E. coli*. The sequence of *E. coli* 4.5S RNA is that reported in reference 10, and the portion of *B. subtilis* scRNA displayed is that reported in reference 22. Shaded nucleotides are those of the apical region that are identical with those of *E. coli* 4.5S RNA.

unable to explain the significance of this finding, since all of the evidence regarding the function of 4.5S RNA indicates that its role is in translation (3, 4, 4a). We speculate that a similar structure is used in procaryotes for translation (4.5S RNA) and in eucaryotes for protein secretion (SRP RNA).

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