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Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of *Escherichia coli*

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ABSTRACT

Recombinant DNA and classic genetic procedures were used to map a spectinomycin resistance mutation to a 121 base pair region of a 16S RNA gene and a macrolide-lincosamide-streptogramin type B resistance mutation to a 32 base pair region of a 23S RNA gene. DNA sequence analysis of these regions revealed that spectinomycin resistance results from a C/G to T/A transition at position 1192 of a 16S RNA gene. Resistance to macrolide, lincosamide and streptogramin type B antibiotics results from an A/T to T/A transversion at position 2058 of a 23S RNA gene. The alteration in 16S RNA is in a sequence that can participate in alternate base pairing arrangements that have been proposed to be involved in the translocation process. The alteration in 23S RNA identifies sequences important to peptidyl transfer.

INTRODUCTION

Ribosomal RNA operons of E. coli have not been extensively analyzed by genetics because of a lack of dominant selectable mutations in these operons. Mutations in rRNA operons have probably been difficult to isolate because there are seven rRNA operons in E. coli (1). Therefore, mutations in rRNA genes might escape detection because the mutations are recessive or alter only a small fraction of the ribosomes. An rRNA operon (*rrnH*) cloned on a multicopy plasmid increases the contribution of a single rRNA operon to ribosomes and allows isolation of dominant or co-dominant spectinomycin (Spc) and erythromycin (Ery) resistance mutations (2,3). In this paper we report the mapping, sequence analysis, and structural implications of these mutations.

MATERIALS AND METHODS

Bacterial Strains and Plasmids.

The E. coli K12 strains EM22 (*recA*) and EM2 (RecA$^+$) have been previously described (3, 4). DB11 is an antibiotic hypersensitive E. coli strain (5). pLC7-21 is a recombinant DNA plasmid that contains *rrnH* cloned

in ColE1. pERY-1 and pSPC-1 confer resistance to erythromycin and spectinomycin, respectively (2, 3), and were isolated by *in vivo* methanesulfonic acid ethyl ester mutagenesis of pLC7-21. pACYC184 and pACYC177 are multicopy plasmids that are compatible with ColE1 (6). pLC7-21, pERY-1, pSPC-1, pACYC177, and pACYC184 are described in Fig. 1.

Antibiotic Sensitivity Testing.

The sensitivity of EM2/pERY-1 and EM2/pLC7-21 to macrolide and lincosamide antibiotics was tested by streaking each strain on LB agar containing antibiotic concentrations which were partially or completely inhibitory to growth. Sensitivity of DB11/pLC7-21 and DB11/pERY-1 to streptogramin type B antibiotics was tested by making LB soft agar overlays containing each cell type and placing a 6mm diameter filter paper disk containing 1mg of osteogrycin B on the overlays. The osteogrycin B caused a 2mm zone of inhibition on the lawn of DB11/pLC7-21, but no visible zone of inhibition on DB11/pERY-1.

Recombinant DNA and DNA Sequencing.

Recombinant DNA procedures were performed according to Davis et al. (7). DNA was sequenced by the dideoxynucleotide chain termination method as described by Viera and Messing (8), using M13mp8 and M13mp9. DNA synthesis was primed by a 15 base primer (PL Biochemicals) or by a denatured DNA fragment produced by restriction nuclease digestion of pLC7-21.

RESULTS

The *rrnH* operon used in this study is transcribed from tandem promoters and has the gene order 16S - $tRNA^{ile}$ - $tRNA^{ala}$ - 23S - 5S - $tRNA^{asp}$ (1).

Resistance Profiles of pSPC-1 and pERY-1.

pSPC-1 was previously shown to confer resistance to Spc but not other aminoglycoside antibiotics (2), and does not confer resistance to tetracycline or inhibitors of the large ribosomal subunit (unpublished data). pERY-1 was previously shown to confer resistance to Ery (3). When tested as described in Materials and Methods, pERY-1 also conferred resistance to all other macrolide antibiotics tested (oleandomycin, niddamycin, tylosin, and spiramycin), to the lincosamide antibiotics lincomycin and clindamycin, and to the streptogramin type B antibiotic osteogrycin B. The antibiotic concentrations needed to inhibit growth of strains containing pLC7-21 vary at least ten-fold using different standard

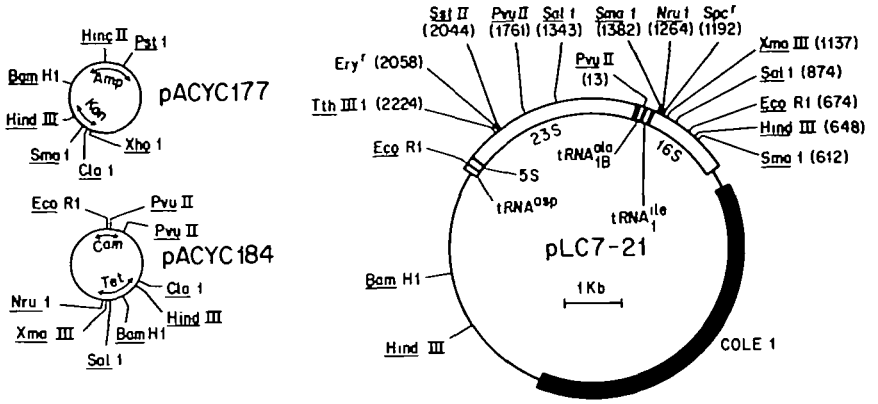

FIGURE 1. Plasmid structures. pSPC-1 and pERY-1 are identical to pLC7-21 except for point mutations at the indicated positions. Only six base-pair specificity restriction nuclease sites important for mapping are indicated.

laboratory strains. Therefore, absolute resistance levels are of little meaning. However, all strains containing pERY-1 grew at macrolide or lincosamide concentrations that were at least twice the concentrations that totally prevented growth when the strain contained pLC7-21. The strains W3110/pERY-1 and EM2/pERY-1 will grow at up to at least 500 μg/ml of all macrolide and lincosamide antibiotics tested. The mutation in pERY-1 did not confer resistance to chloramphenicol (Cam), capreomycin, P114A, fusidic acid, or a variety of antibiotic inhibitors of the small ribosomal subunit.

Mapping Procedures.

Mapping procedures were devised to map the Spc^r and Ery^r mutations. The mapping procedures included sequence reconstruction using restriction nucleases, plasmid-plasmid marker rescue, and shotgun recombination mapping. These methods are described below. When the mapping procedure did not require recombination the bacterial strain EM22 was used to prevent loss of mutations by recombination with the chromosome. When recombination was required, EM2 was used. Media contained Ery (300 μg/ml) or Spc (50 μg/ml) as indicated. The planning of mapping experiments and the identification of restriction nuclease fragments were facilitated by the published partial sequence of *rrnH* (9), complete sequence of *rrnB* (10), and knowledge of the restriction nuclease maps of pLC7-21, pSPC1, pERY-1, pACYC177, and ACYC184 (3, 6, 9, Fig. 1). The coordinates for 16S and 23S RNA genes used in Figs. 1-3 and Table 1 are numbered according to the sequence of *rrnB* (10) because the complete sequences of the 16S and 23S RNA

TABLE 1

Coordinates of Fragments	Method(s) Used
Spc^r MAPPING	
648 (16S, HindIII) - 7.2 Kb downstream (HindIII)	SRM, MR
874 (16S, Sal1) - 1343 (23S, Sal1)	SRM, MR
1137 (16S, XmaIII) - 1343 (23S, Sal1)	SRM, MR
874 (16S, HincII) - 605 (23S, HincII)	SRM, MR
674 (16S, EcoR1) - 13 (tRNA^{ala}, PvuII)	SRM, MR
612 (16S, Sma1) - 1382 (16S, Sma1)	MR
874 (16S, Sal1) - 1264 (16S, Nru1)	MR
961 (16S, Taq1) - 1321 (16S, Taq1)	SRM, MR
Ery^r MAPPING	
1343 (23S, Sal1) - 3.7 Kb downstream (BamH1)	MR
2044 (23S, SstII) - 2.9 Kb downstream (BamH1)	SR, MR
1761 (23S, PvuII) - 1.4 Kb downstream (EcoR1)	MR
1343 (23S, HincII) - 2201 (23S, HincII)	SRM, MR
1343 (23S, Sal1) - 2224 (23S, TthIII1)	SR, MR
1751 (23S, Taq1) - 2479 (23S, Taq1)	SRM, MR
1884 (23S, Sau3A) - 2391 (23S, Sau3A)	SRM, MR
1877 (23S, Alu1) - 2082 (23S, Alu1)	SRM, MR

Mapping of the Spc^r and Ery^r mutations. The indicated restriction nuclease fragments were mapped by one or more of the three mapping methods described in the text (SRM, shotgun recombination mapping; MR, plasmid-plasmid marker rescue; SR, sequence reconstruction).

genes of rrnH are not known. No differences in restriction nuclease recognition sites between the rRNA genes of rrnH and rrnB were observed in regions important to these mapping studies.

In this paper the numerical coordinates of restriction nuclease sites in 16S and 23S RNA genes refer to the first nucleotide of the recognition sequence of the restriction nucleases. The nucleotides are numbered from the start of the mature RNA products of the 16S and 23S RNA genes. When a restriction nuclease site used to produce a DNA fragment is not in an rRNA gene, the distance and direction from a site in the rRNA gene is indicated. The numbering system should be apparent from Fig. 1 and Table 1.

Plasmid-Plasmid Marker Rescue. Plasmid-plasmid marker rescue was used to test whether a specific restriction nuclease fragment contained the site of the Spc^r or Ery^r mutation. The restriction nuclease fragments to be tested were cloned into pACYC184 or pACYC177. The resulting pACYC derivatives were then transformed into EM2/pLC7-21. Transformants were selected on LB agar containing colicin E1 and the antibiotic to which the pACYC derivative conferred resistance. Transformants were then purified by streaking, grown to saturation in LB medium, and plated on LB agar containing Spc or Ery. If the cloned fragment contained the mutation, recombination between

pLC7-21 and the cloned restriction nuclease fragment resulted in transfer of the mutation from a promoterless or partial rRNA gene to the complete rrnH operon on pLC7-21. The restriction nuclease fragment present in the pACYC derivative was therefore judged to have the mutation if significantly more colonies grew on Ery or Spc as compared to control cultures containing pLC7-21 and either pACYC177 or pACYC184, or control cultures with pLC7-21 and a pACYC derivative containing the corresponding restriction nuclease fragment of pLC7-21. At least three different transformants and control transformants were tested to insure that differences in colony number were not merely due to statistical fluctuations.

Shotgun Recombination Mapping. Shotgun recombination mapping allowed simultaneous identification and isolation of restriction nuclease fragments containing the Spc^r or Ery^r mutation by virtue of the ability of a restriction nuclease fragment to participate in plasmid-plasmid marker rescue. This easy and rapid technique was particularly useful for fine scale mapping of the Spc^r and Ery^r mutations.

To map mutations by this method pSPC-1 or pERY-1 were digested with a restriction nuclease and the resulting mixture of fragments were ligated into restriction nuclease digested pACYC177 or pACYC184. The ligation mixture was then transformed into EM2/pLC7-21. Transformants were selected on LB agar containing colicin E1 and the antibiotic to which the pACYC derivative conferred resistance. After incubation for 48 hours to allow recombination and phenotypic expression, the resulting 1,000 - 10,000 colonies on each plate were replica plated onto medium containing colicin E1, the antibiotic to which the pACYC derivative conferred resistance, and either Spc or Ery. A fraction of the original colonies were capable of continued growth after replica plating due to plasmid-plasmid marker rescue between pLC7-21 and a fragment cloned into the pACYC plasmid. DNA fragments containing the Spc^r or Ery^r mutations were therefore selectively recovered in the pACYC derivatives in these colonies.

It was then necessary to purify the pACYC derivative with the cloned fragment. To accomplish this the colonies obtained above were purified by restreaking. The pACYC derivative and pLC7-21 in these colonies were separated from each other by transformation of EM22 with one microliter of a cleared lysate prepared from each isolate. Recipients selected for transformation by the pACYC derivative were then screened for colicin E1 sensitivity and used as a source of purified pACYC-derivative plasmid DNA. The presence of the mutation on the cloned fragment was then confirmed by

subjecting the intact plasmid to the plasmid-plasmid marker rescue test described above. In nearly every case the mutation was recovered on the purified pACYC derivative, probably because the recombination event that transferred the mutation to pLC7-21 involved only one of the many pACYC derivatives in the cell, or because the mutation was transferred by gene conversion. The fragment cloned in the pACYC derivative was then identified by restriction nuclease digestion of the purified DNA.

Shotgun recombination mapping was most useful when four base-pair specificity nucleases generated fragments from pSPC-1 or pERY-1 and six base-pair specificity nucleases generated termini in the pACYC plasmids. Enzyme combinations were chosen so that the cloned fragments could be precisely excised by the four base-pair specificity nuclease. Restriction nuclease combinations of this type that are useful with the pACYC plasmids are BamH1-Sau3A, PvuII-Alu1, Cla1-Taq1, and Nru1-Tha1 (Fig. 1).

Sequence Reconstruction. It was occasionally desirable to map a mutation to one side of a restriction nuclease site when the restriction nuclease did not yield DNA fragments that could be directly cloned in pACYC177 or pACYC184. In these cases hybrid fragments were constructed from DNA derived from two different plasmids. For example, the Ery^r mutation was mapped upstream of the TthIII1 site by constructing hybrid fragments with one Sal1 and one BamH1 terminus. The hybrid fragments consisted of pLC7-21 DNA on one side of the TthIII1 site and pERY-1 DNA on the other side (see Fig. 1). This fragment was then ligated into Sal1-BamH1 cleaved pACYC184 and tested for the Ery^r mutation by plasmid-plasmid marker rescue. Ery^r recombinants were obtained only when DNA upstream of the TthIII1 site came from pERY-1.

Results of Mapping.

The restriction nuclease fragments that were shown by these three mapping methods to carry the Spc^r or Ery^r mutations are listed in Table 1. The mapping methods identified a series of partially or completely overlapping restriction nuclease fragments that have the Spc^r and Ery^r mutations. The regions of overlap map the Spc^r mutation between an XmaIII recognition site at position 1137 and an Nru1 recognition site at position 1264 of the 16S RNA gene. The Ery^r mutation maps between an SstII site at position 2044 and an Alu1 site at position 2082 in the 23S RNA gene.

Sequencing the Mutations.

Restriction nuclease fragments from pERY-1, pSPC-1, and pLC7-21 were cloned into M13mp8 or M13mp9 and sequenced by the dideoxynucleotide chain

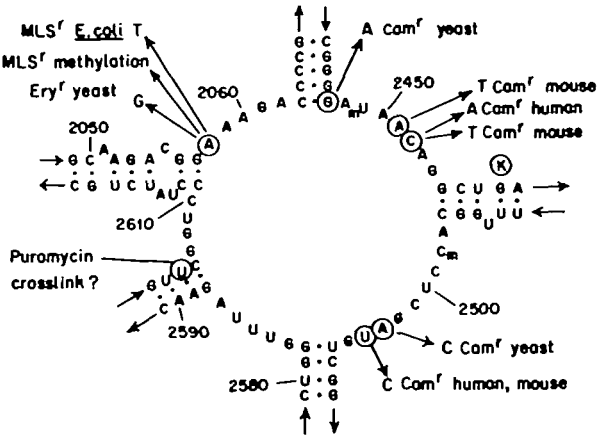

FIGURE 2. Proposed secondary structure for a portion of E. coli 23S RNA (27). Sites of mutations or interactions in RNA of bacteria or mitochondria that can be identified in homologous sequences of rrnB of E. coli are noted. The circled K indicates a guanosine protected from kethoxal modification by subunit association (28). Puromycin can be caused to crosslink to 23S rRNA (29). One of the three possible locations (29) for this crosslink is indicated.

termination method. Both strands were sequenced. Each DNA strand of a mapped region was sequenced in its entirety using a single clone constructed from M13mp8 or M13mp9. Sequences were read from DNA molecules less than 263 nucleotides in length (including attached primer sequences). Sequencing shows that Spc^r is due to a C to U base substitution at position 1192 of 16S RNA and MLS antibiotic resistance is due to an A to U base substitution at position 2058 of 23S RNA (Figs. 1-3). There were no other differences observed in the sequenced regions of pLC7-21 and pSPC-1 or pERY-1. rrnH on pLC7-21 was identical to rrnB (10) in the regions sequenced. The sequencing and mapping studies therefore rule out the possibility that other mutations are required for the resistance phenotypes.

DISCUSSION

We have shown that dominant or co-dominant Spc^r results from a mutation that alters position 1192 of 16S RNA, and dominant or co-dominant resistance to MLS antibiotics results from a mutation that alters position 2058 of 23S RNA. The location of the mutations were determined by genetic

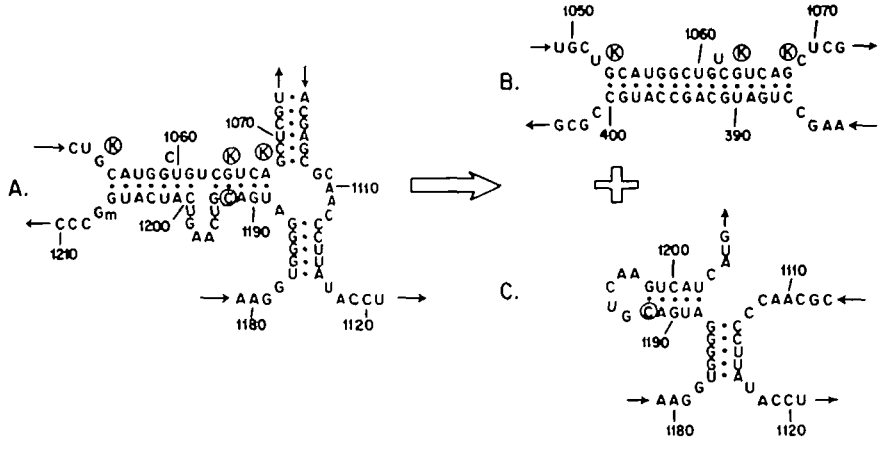

FIGURE 3. Proposed secondary structures in 16S RNA that may be affected by the Spc^r mutation at position 1192 (circled). A secondary structure present in 30S subunits (structure A) may undergo rearrangement during subunit association or translocation, resulting in alternate long-range base pairing of the sequences centered around position 1060 (structure B) and position 1190 (structure C). Structure A is redrawn from Stiegler et al. (25) with a minor modification suggested by recent psoralen crosslinking studies (30). Structure B (22) and C (25) are as previously proposed. Guanosines in 30S subunits that are protected from kethoxal modification by association of 30S subunits with 50S subunits (31) are indicated by circled Ks. The existence of this switch mechanism as originally proposed (22) has been brought into question by comparative sequence analysis (32). Structure B seems to be ruled out by this analysis. However, the switch may involve only structures A and C. Structure A is supported by comparative sequence analysis and structure C was not specifically analyzed. Structure C unambiguously exists in 16S rRNA in solution (22, 30).

mapping and DNA sequencing. These mutations result in ribosomes that are less sensitive to antibiotics *in vitro* (2,3).

Mutations in rRNA genes of mitochondria provide insight into the structure and function of the region around position 2058 of *E. coli* 23S RNA (Fig. 2). Ery^r yeast mitochondria have mutations at a position that corresponds to nucleotide 2058 of *E. coli* 23S RNA (11). Cam^r mutations in mitochondrial rRNA genes of yeast, mouse, or human cells are located in sequences analogous to sequences near position 2058 in the *E. coli* 23S RNA secondary structure (11-15). In addition, a plasmid-encoded inducible methylase that results in resistance to MLS antibiotics methylates *Bacillus stearothermophilus* 23S RNA at a position corresponding to nucleotide 2058 of *E. coli* 23S RNA (16). That the mutations and modifications leading to

Ery^r and Cam^r are in the same region of the ribosome is also supported by a mutation in *rrnH* of *E. coli* which confers both Ery^r and Cam^r (our unpublished data), extensive evidence that the binding sites for Cam and MLS antibiotics partially or totally overlap (17-19), and ribosome reconstitution studies that show ribosomal protein L16 is required for binding Cam and MLS antibiotics (19-21). Cam, MLS antibiotics, and L16 are all strongly associated with the peptidyl transferase function of the ribosome (19-21, 23). Therefore, position 2058 of 23S RNA is probably located in the peptidyl transferase center.

The Spc^r mutation that alters position 1192 of 16S RNA is in a region that may be involved in an intramolecular switch mechanism proposed to be part of the translocation machinery (Fig. 3). In this proposed mechanism translocation occurs when several regions of 16S RNA form alternate base pair interactions with other regions of 16S RNA (22). The Spc^r mutation changes a G/C base pair to a G/U base pair in two of these proposed switch structures (Fig. 3), with little affect on the relative stability of the various switch structures. Spc^r due to an alteration of one of these structures suggests that Spc may prevent certain switch structures from forming, thereby preventing translocation. This would be consistent with the suggestion that the primary action of Spc on ribosomes is the inhibition of ribosome movement on mRNA (23).

Spc is a member of one of several structurally dissimilar groups of aminoglycoside antibiotics, most or all of which bind to the small ribosomal subunit and alter properties associated with tRNA binding (23). 16S RNA sequences have been associated with resistance to three aminoglycoside antibiotics. This paper proves that a mutation at position 1192 of 16S RNA confers resistance to Spc but not other aminoglycosides (2). Yeast mitochondrial resistance to the aminoglycoside paromomycin results in an alteration at a position (24) that is not homologous in primary sequence to *E. coli* 16S RNA but which is analogous to a region around position 1409 of *E. coli* 16S RNA in comparative secondary structure models (25). Kasugamycin resistance results from alterations in post-transcriptional methylation of positions 1517-18 of 16S rRNA (26). These studies show that resistance to three structurally dissimilar aminoglycoside antibiotics results from alterations at three widely dispersed regions in the 16S RNA primary and proposed secondary structures. However, an overlap of aminoglycoside binding sites in the ribosome is still possible due to tertiary structure considerations.

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