JOURNAL OF BACTERIOLOGY, Sept. 1983, p. 989–994
0021-9193/83/090989-06$02.00/0

Vol. 155, No. 3

# Spectinomycin Resistance Due to a Mutation in an rRNA Operon of *Escherichia coli*

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Received 28 February 1983/Accepted 6 June 1983

A spectinomycin resistance mutation was isolated in an *Escherichia coli* rRNA operon (*rrnH*) located on a multicopy plasmid. Cell-free protein-synthesizing extracts made from cells containing the plasmid were partially resistant to spectinomycin. Although spectinomycin is an aminoglycoside antibiotic, the mutation did not confer resistance to any other aminoglycoside antibiotic tested.

A variety of aminoglycoside antibiotics interact with the small ribosomal subunit and interfere with protein synthesis in vivo and in vitro. The aminoglycoside antibiotics can be placed into groups on the basis of structural similarities. The major groups are the streptomycin group (also includes dihydrostreptomycin and bluensomycin), the neomycin group (also includes neamine, nebramine, and paromomycin), the kanamycin group (also includes amikacin and tobramycin), the gentamicin group, and the groups represented by hygromycin, apramycin, spectinomycin, and kasugamycin (reviewed in reference 14).

Spectinomycin binds to and inhibits functions of the small ribosomal subunit (3, 8). Spectinomycin resistance mutations have been isolated that are in the gene for ribosomal protein S5 and result in ribosomes that fail to bind spectinomycin (8, 36). Alterations of amino acids in a different region of S5 are associated with neamine resistance (36). The alteration of S5 in at least one spectinomycin-resistant ribosome alleviates the restriction of translational misreading caused by streptomycin (19). A mutation in the gene for S5 can also result in ribosomal misreading with the retention of spectinomycin sensitivity (6). Antibiotics of the streptomycin, neomycin, kasugamycin, gentamicin, hygromycin, and apramycin groups of aminoglycoside antibiotics also cause misreading of mRNA (5, 9–12, 18, 27, 32, 37). Mutations in the gene for ribosomal protein S12 can give rise to ribosomes that are both streptomycin resistant and altered in their fidelity of translation (31). These observations and others on antibiotic resistance mutations, antibiotic binding, and ribosome structure (reviewed in reference 14) suggest that most aminoglycoside antibiotics interfere with a restricted region of the ribosome that is probably involved with the entry of tRNA into the ribosome A site. Kasugamycin inhibition of ribosomes also involves rRNA structure near tRNA-binding sites (15, 28). Although spectinomycin does not cause misreading, the association of spectinomycin resistance and misreading mutations in the gene for S5 suggests that spectinomycin probably acts in the same general region of the small ribosomal subunit as other aminoglycoside antibiotics.

Some evidence suggests that the binding of some aminoglycoside antibiotics to ribosomes or ribosomal perturbations caused by certain aminoglycoside antibiotics involves the RNA component of the small ribosomal subunit. Kasugamycin resistance of *Escherichia coli* is often due to mutations affecting a 16S rRNA methylase (15). Paromomycin resistance of yeasts can be due to a sequence alteration in the mitochondrial small subunit rRNA (21). Streptomycin has been shown to bind preferentially to purified 16S rRNA (but not to 23S rRNA) (2). Since aminoglycoside antibiotics share common elements in their modes of action, it remains possible that these antibiotics require shared rRNA determinants for their binding or inhibition of ribosome function.

In these experiments, we used a modification of a previously described method (29) to isolate a spectinomycin resistance mutation in an *rrnH* operon located on a multicopy plasmid. This mutation should be useful in locating the site of spectinomycin action on rRNA and should help correlate the location of ribosomal protein S5 and a region of rRNA. In addition, the mutation is likely to be in the gene for 16S rRNA, as the site of action of spectinomycin is on the small ribosomal subunit. The isolation of this mutation should therefore greatly extend the usefulness of *rrn* genetics previously aided only by the macrolide-lincomycin resistance mutation in a 23S

rRNA gene (29; unpublished data) and nonsense suppressor mutations in a distal *rrn* tRNA gene (25).

## MATERIALS AND METHODS

**Antibiotics.** Paromomycin sulfate and bluensomycin sulfate were gifts from Warner-Lambert and The Upjohn Co., respectively. Apramycin, hygromycin, nebramine, and tobramycin were gifts from Eli Lilly & Co. Streptomycin sulfate, kasugamycin hemisulfate, spectinomycin dihydrochloride, kanamycin sulfate (95% kanamycin A and 5% kanamycin B), gentamicin sulfate, neomycin sulfate (90 to 95% neomycin B and the remainder neomycin C), and amikacin free base were obtained from Sigma Chemical Co.

**Bacterial strains and plasmids.** The bacterial strains EM2 F$^+$ *ilv-1 his-29 pro* Tsx$^r$ *trpA9605 trpR*, EM2(pLC7-21), and W3110 F$^-$ Nal$^r$ have been described previously (29). Strains EM348 F$^-$ *srl*::Tn*10* RecA$^+$ and EM349 F$^-$ *srl*::Tn*10 recA56* were made by P1 transduction of Tn*10* from MC1024 *araD139* Δ(*ara-leu*)*7697* Δ(*lacZ*)M15 *galU galK rpsL recA56 srl*::Tn*10* (obtained from M. Casadaban) into the nalidixic acid-sensitive parent of strain W3110. pLC7-21 contains the *rrnH* operon of *E. coli* on a ColE1 vector. pSPC-1 is a derivative of pLC7-21 that confers spectinomycin resistance, and pSPC-D1 was made by the deletion of a *SalI* restriction nuclease fragment from *rrn* DNA of pSPC-1 (see below). All plasmids used in these experiments confer colicin E1 immunity and are mobilizable by F.

**Isolation of mutants.** The procedure previously described for the isolation of mutations in *rrn* operons (29) was used with appropriate modifications. Briefly, a lawn of strain EM2 (pLC7-21) on Luria broth (LB) agar containing 15 μg of spectinomycin per ml was mutagenized with a methanesulfonic acid ethyl ester. A concentration of 15 μg of spectinomycin per ml was used because it strongly but incompletely inhibited the growth of strain EM2(pLC7-21), thereby allowing phenotypic expression but still conferring a selective advantage to cells having the desired mutation in pLC7-21. Mutant cells, therefore, formed prominent colonies on a weak lawn of cells. The cells were washed off the plate, diluted to an optical density at 550 nm of 0.2 in LB plus 15 μg of spectinomycin per ml, and grown overnight. The cells were then washed, diluted in LB, and mated with strain W3110 overnight. The mating mixture was then diluted 1:20 with LB, grown overnight on LB plus 20 μg of nalidixic acid per ml and colicin E1, and plated on LB agar containing 25 μg of spectinomycin per ml, 20 μg of nalidixic acid per ml, and colicin E1. The colonies that arose were confirmed to be strain W3110 harboring a plasmid by testing for nutritional markers, colicin E1 immunity, and phage BF23 sensitivity. About 20 colonies were initially screened for a plasmid location of the spectinomycin resistance mutation by testing for concerted loss of colicin immunity and spectinomycin resistance after growth in LB plus acridine orange (24). About half of the isolates showed concerted loss of colicin immunity and spectinomycin resistance. Most of these isolates also grew when restreaked onto LB agar plus 1,600 μg of spectinomycin per ml, whereas strain W3110 (pLC7-21) did not grow at all on medium containing spectinomycin at concentrations of 50 μg or more per ml. The plasmid in one of these strains was designated pSPC-1 and was further characterized as described below. The rationale used for the design of this procedure for the isolation of mutants has been previously described in detail (29).

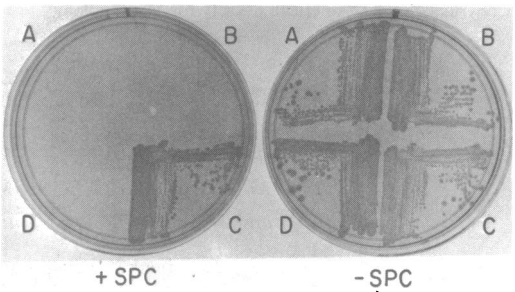


FIG. 1. Growth of bacterial strains in the presence or absence of 50 μg of spectinomycin per ml. The plate without spectinomycin was incubated for 24 h at 37°C; the plate with spectinomycin was incubated for 48 h. Strains: A, W3110; B, W3110(pLC7-21); C, W3110(pSPC-1); D, W3110(pSPC-D1).

**Other procedures.** Common bacterial manipulations and recombinant DNA procedures were done as described previously (23, 24, 29). Protein synthesis directed by polyinosine was assayed by using preincubated S30 protein-synthesizing extracts as described previously (1), except that [$^3$H]valine was used to monitor incorporation, and each reaction was 50 μl in volume. Values obtained from reactions without polyinosine were subtracted from values of reactions with added RNA. Each point in the graph is the average of values obtained from at least two separate reactions. The points without spectinomycin are the averages of eight separate reactions. Polyinosine was obtained from Miles Laboratories, Inc.

## RESULTS

**Characterization of pSPC-1.** As described above, a spectinomycin resistance mutation was isolated in strain W3110 harboring a multicopy ColE1 plasmid that carries *rrnH*. Concomitant loss of colicin immunity and spectinomycin resistance after growth of strain W3110(pSPC-1) in the absence of selection for the plasmid indicated that the plasmid carried a mutation necessary for spectinomycin resistance. Strain W3110(pSPC-1) grew at concentrations of at least 1,600 μg of spectinomycin per ml with nearly 100% plating efficiency, whereas strain W3110 carrying the parental plasmid (pLC7-21) failed to grow at 50 μg of spectinomycin per ml. A concentration of 50 μg of spectinomycin per ml provided good distinction between cells containing pLC7-21 and pSPC-1 (Fig. 1) and was used in all subsequent experiments described below. At 50 μg of spectinomycin per ml, the frequency of mutation of strain W3110(pLC7-21) to spectinomycin resistance was ca. $10^{-8}$ to $10^{-9}$.

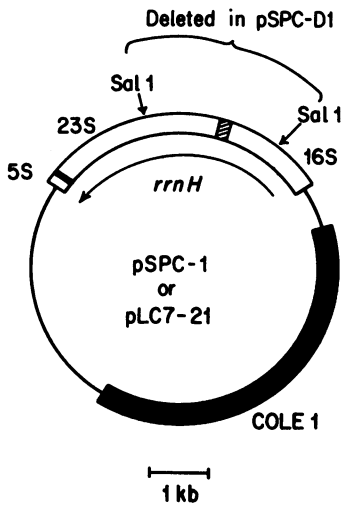


FIG. 2. Structures of pLC7-21, pSPC-1, and pSPC-D1. ColE1 DNA is indicated by the closed block; nonribosomal bacterial chromosomal DNA is indicated by single lines; *rrnH* DNA is indicated by the open block. The stippled area and the cross-hatched area represent precursor-specific sequences.

pSPC-1 and pLC7-21 were purified from strains W3110(pSPC-1) and W3110(pLC7-21) and used to transform strain W3110 to colicin immunity. All colicin-immune colonies resulting from transformation with pSPC-1 tested as resistant to spectinomycin, but none of the colonies transformed with pLC7-21 was spectinomycin resistant. After the transformation of strain W3110 with pSPC-1, the transformed culture was allowed to increase 10-fold in optical density and was then plated onto LB agar plus spectinomycin. All resulting colonies tested as colicin immune. Similar results were obtained by using strain EM2 instead of strain W3110. These results indicate that pSPC-1 carries a mutation or mutations sufficient for spectinomycin resistance.

An isogenic $RecA^+$-$RecA^-$ pair of bacterial strains, EM348 and EM349, was also transformed with pLC7-21 and pSPC-1. In both of these strains, pSPC-1, but not pLC7-21, conferred spectinomycin resistance when tested by selection for colicin immunity and screening for spectinomycin resistance or when tested by direct selection for spectinomycin resistance after phenotypic expression, followed by screening for colicin immunity. Therefore, the appearance of the spectinomycin resistance phenotype does not require that chromosomal *rrn* operons acquire the spectinomycin resistance mutation by recombination. These experiments demonstrate that the spectinomycin resistance mutation on pSPC-1 is dominant or codominant over the seven chromosomal *rrn* operons.

**Deletion of *rrnH* DNA.** An internal deletion in *rrnH* DNA on pSPC-1 was created by use of the restriction endonuclease *Sal*I (Fig. 2). In the resulting deletion plasmid (pSPC-D1), some of the 16S and 23S rRNA genes, as well as genes for $tRNA_1^{Ile}$ and $tRNA_{1B}^{Ala}$ located between the 16S and 23S rRNA genes, were removed. The genes for 5S rRNA and $tRNA^{Asp}$ remained intact at the distal end of *rrnH*. The deletion in pSPC-D1 completely abolished spectinomycin resistance when pSPC-D1 was tested by transformation into strain W3110 as described above (Fig. 1). Therefore, the spectinomycin resistance mutation is in *rrnH* on pSPC-1. It cannot be concluded from these results that the mutation is or is not in any particular gene or genes of *rrnH*, as the deletion completely or partially deletes four genes of *rrnH* and may prevent synthesis of functional RNA from the two intact genes downstream from the deletion.

**In vitro protein synthesis.** To determine whether the mutation on pSPC-1 results in spectinomycin resistance by causing synthesis of spectinomycin-resistant protein-synthesizing machinery or by causing spectinomycin to be excluded from the cells, in vitro protein-synthesizing extracts were prepared from strains EM2(pLC7-21) and EM2(pSPC-1) grown in LB medium in the absence of spectinomycin. The effect of spectinomycin on the synthesis of protein from polyinosine was then examined (Fig. 3). The results indicate that pSPC-1 results in partial spectinomycin resistance of the protein synthetic capacity of the extracts. The results are consistent with the possibility that 15% of the ribosomes are completely resistant to spectinomycin at concentrations of between 10 and 100 μg of spectinomycin per ml or that a larger percentage of ribosomes is partially spectinomycin resistant. It is possible that the sensitive

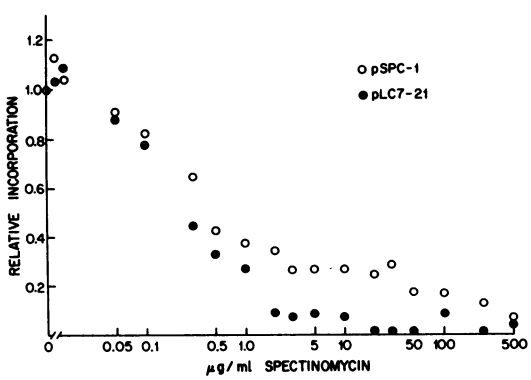


FIG. 3. Synthesis of polyvaline from polyinosine in the presence of various concentrations of spectinomycin.

ribosomes interfere with translation by resistant ribosomes (see below), thereby resulting in an underestimation of the level of spectinomycin-resistant ribosomes. This level of resistance is similar to the erythromycin resistance of protein-synthesizing extracts prepared from cells containing a plasmid with an erythromycin resistance mutation in the 23S rRNA gene of *rrnH* (29; unpublished data). Since inhibition of protein synthesis by spectinomycin in intact cells containing ColE1 replicons can result in an increase in plasmid copy number (unpublished data), it is possible that the percentage of spectinomycin-resistant protein biosynthesis is greater when cells containing pSPC-1 are grown in the presence of spectinomycin.

**Other antibiotics.** The resistance of strains W3110(pLC7-21) and W3110(pSPC-1) to other aminoglycoside antibiotics was tested at antibiotic levels that were partially as well as completely inhibitory to growth. The mutation in pSPC-1 did not confer resistance to any other aminoglycoside antibiotic tested. The antibiotics tested were kasugamycin, apramycin, streptomycin, tobramycin, paromomycin, neomycin, nebramine, kanamycin, hygromycin, amikacin, bluensomycin, and gentamicin.

## DISCUSSION

A spectinomycin resistance mutation was isolated in *rrnH* located on a multicopy plasmid. Deletion analysis confirmed that the mutation is in *rrnH*. A cell-free protein-synthesizing extract made from cells containing the mutant *rrnH* operon is partially resistant to spectinomycin. Because the known site of action of spectinomycin is the small ribosomal subunit (3, 8, 36), the mutation is probably in the 16S rRNA gene. DNA sequence analysis is needed to establish the exact location of the mutation.

Spectinomycin inhibits mRNA movement on the ribosome at high drug concentrations (1, 4). At lower drug concentrations, spectinomycin inhibits only translocation events that occur during or immediately after initiation (35). Previously isolated spectinomycin resistance mutations affecting the ribosome are in the gene for protein S5 of the small ribosomal subunit (8, 36). Ribosomal ambiguity or spectinomycin resistance mutations in the gene for S5 alter the fidelity of protein synthesis (29, 33). S5 is, therefore, probably involved with the entry of tRNA into the ribosome or the recognition of mRNA by tRNA. It does not seem likely that the spectinomycin resistance mutation in *rrnH* alters the function of protein S5 by altering the binding of S5 to rRNA, as direct binding of protein S5 to rRNA has not been detected (26, 38). The precise nucleotide change(s) of the spectinomycin resistance mutation in *rrnH* will identify a region of rRNA that probably is located near S5 in the ribosome and may be involved in the functional activities of the ribosome inhibited by spectinomycin or associated with S5. Determination of whether spectinomycin binds to the mutant ribosomes will require ribosome preparations with a higher percentage of mutant ribosomes than presently available. However, owing to the complexity of ribosome structure, a mutation that confers spectinomycin resistance need not be near the site at which the antibiotic binds, affect antibiotic binding, or be located at a position in the ribosome directly involved in the functions inhibited by spectinomycin or associated with protein S5.

The spectinomycin resistance mutation in *rrnH* does not confer resistance to any of a wide variety of other aminoglycoside antibiotics. Other mutations in *rrn* operons that confer resistance to aminoglycoside antibiotics must be isolated and characterized before it can be ascertained whether spectinomycin and other aminoglycoside antibiotics require common rRNA determinants for their action on the ribosome.

The spectinomycin resistance mutation described in this paper and the erythromycin resistance mutation previously isolated (29) are dominant or codominant over the seven chromosomal *rrn* operons presumably coding for sensitive ribosomes. This contrasts with spectinomycin and erythromycin resistance mutations in ribosomal protein genes, which are recessive or at best weakly codominant to wild-type genes (33, 35). It has been proposed that the resistant-type ribosomal proteins are defective in ribosomal assembly, and therefore, resistant/sensitive partial diploids are sensitive because only a small fraction of the ribosomes are of the resistant type (7). However, this explanation for the dominance of sensitivity has been challenged because a substantial fraction of the ribosomes has the resistant-type protein in some resistant or sensitive partial diploids (30). Other models constructed to explain the dominance of sensitivity propose that the sensitive ribosomes in resistant or sensitive partial diploids stably or cyclically block initiating or translocating ribosomes, thereby sequestering nearly all mRNA molecules or resistant ribosomes that accumulate behind sensitive ribosomes (20, 22, 33–35). It is noteworthy that sensitivity is usually dominant over resistance for all or nearly all antibiotics that affect the translational machinery (13, 16, 33–35) and for rifampin resistance mutations in RNA polymerase genes (17), implying that some common principle might exist in the way antibiotics interfere with the transcriptional and translational machinery. If antibiotic-induced blockage by sensitive ribosomes or RNA poly-

merase molecules is responsible for the dominance of sensitivity, then the existence of dominant or codominant antibiotic resistance mutations in *rrn* operons (29; see above) and a dominant rifampin resistance mutation in an RNA polymerase gene (17) suggests that the dominance of sensitivity must involve more than the existence of sensitive ribosomes or RNA polymerase molecules in sensitive or resistant partial diploids. Perhaps a certain threshold level or percentage of correct and appropriate antibiotic-resistant transcription or translation is necessary for a resistance mutation to be dominant. The level of correct and appropriate transcription and translation cannot be determined easily by measurement of the relative proportion of resistant and sensitive ribosomes or RNA polymerase molecules. The threshold could be exceeded by a large percentage of partially defective resistant ribosomes or RNA polymerase molecules or by highly correct and appropriate translation or transcription by a smaller percentage of resistant ribosomes or RNA polymerase molecules.

## ACKNOWLEDGMENTS

We thank Karen Travis for typing the manuscript.

This work was supported by a Junior Faculty Research Career Development Award from the American Cancer Society and a Public Health Service grant 1RO1GM27664-03 from the National Institutes of Health to E.M.

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