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Genetics

Erythromycin resistance due to a mutation in a ribosomal RNA operon of *Escherichia coli*

(multicopy plasmid/deletion analysis/altered ribosomes/*rrnH*)

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ABSTRACT There are seven ribosomal RNA operons (*rrn* operons) in *Escherichia coli*. A single *rrn* operon was amplified by use of a multicopy recombinant plasmid containing a complete *rrnH* operon. *rrnH* thereby has the potential to contribute a greater fraction of the rRNA found in ribosomes. Erythromycin-resistant mutants were isolated from cells containing the plasmid, and at least one mutation to resistance was shown to reside in *rrnH* on the plasmid. Erythromycin resistance was retained when a major deletion was introduced into the 16S rRNA gene and was abolished by deletions that affect the 16S and 23S rRNA genes but do not alter the 5S rRNA gene or non-*rrnH* DNA. Cell-free S30 protein-synthesizing extracts from cells containing the mutant plasmid have an increased resistance to erythromycin. The selection procedure used to isolate erythromycin-resistance mutations in *rrnH* may allow, with minor modifications, the isolation of mutations in *rrn* operons that change resistance of the ribosome to other antibiotics or that alter other properties of ribosomes.

The presence of seven *rrn* operons in *Escherichia coli* may be responsible for the inability to isolate mutations in rRNA genes because a single mutant rRNA gene can contribute rRNA to only a fraction of the total ribosomes and might not result in an observable phenotypic change in the bacterium. Resistance to many antibiotics, including erythromycin, is recessive or weakly codominant to sensitivity when the resistance mutation alters ribosomal proteins (1–3). Dominance of sensitivity is best documented for streptomycin-resistance mutations, in which streptomycin-resistant/streptomycin-sensitive merodiploids contain ribosomes of both normal and altered types and are still nearly as sensitive to the bacteriocidal and bacteriostatic activities of streptomycin as are streptomycin-sensitive cells (1, 3). Therefore, a mutation in a rRNA gene also might go undetected due to dominance of sensitive ribosomes that contain rRNA synthesized from nonmutant *rrn* operons in the same cell.

In yeast mitochondria, which have only a single set of rRNA genes, mutations in the large rRNA gene can give rise to chloramphenicol resistance (*rib-1*), erythromycin resistance (*rib-3*), and erythromycin and spiromycin resistance (*rib-2*) (4, 5). It remains possible that paromomycin resistance in yeast mitochondria results from a mutation in the gene for the small rRNA (5). Chloramphenicol resistance has been shown, by sequence analysis, to result from a DNA sequence alteration in the rRNA genes of the mitochondria of yeast (6) and mice (7). Kasugamycin resistance in *E. coli* can result from a mutation in a methylase that modifies 16S rRNA (8). Thiostrepton resistance in *Streptomyces* (9) and erythromycin resistance in *Staphylococcus*, *Streptococcus* (10), and *Streptomyces* (11) are due to methylation of the 23S rRNA of these organisms. Mutations to viomycin resistance in *Mycobacterium smegmatis*, a bacterium that contains a single set of rRNA genes, can alter both large rRNAs in some way (12).

In all these cases the alterations of rRNA result either from a mutation in an rRNA gene in a system with a single set of rRNA genes or from the effect of an enzyme capable of modifying all rRNA molecules. These examples prove that rRNA is involved in the interaction of antibiotics with ribosomes and suggest that the previous failure to isolate antibiotic resistance mutations in *rrn* operons of *E. coli* was due to the repetition of rRNA genes.

We therefore sought to isolate mutations in an *rrn* operon which is present on a multicopy plasmid and can therefore contribute a greater percentage of the rRNA in cells. In this paper we report the isolation of an erythromycin-resistance mutation in *rrnH*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains used were EM2 (*ilvl his29 pro2 tsx trpA9605 trpR ara* F^+), W3110 ($Nal^R F^-$), and EM4 (a *recA* derivative of EM2). The plasmid used to obtain the mutation described in this paper is pLC7-21, which contains *rrnH* on a ColE1 vector. pLC7-21 confers immunity to colicin E1 and is mobilizable by F (13). The restriction nuclease maps of pLC7-21 and its derivatives were determined by standard methods (14) and are in agreement with the restriction nuclease cleavage sites predicted by the partial DNA sequence of *rrnH* (15–17) and by examination of the complete nucleotide sequence of *rrnB* (18). pERY-1 is a derivative of pLC7-21 that confers erythromycin resistance. pERY-D1 is derived from pERY-1 by *in vitro* deletion of DNA between the *Sal* I recognition sites in the 16S and 23S rRNA genes, and pERY-D2 is derived from pERY-1 by *in vitro* deletion of DNA between the two *Hin*dIII sites in the 16S rRNA gene (see Fig. 1). Colicin E1 was purified from W3110/ColE1 through the ammonium sulfate precipitation steps of Schwartz and Helinski (19) and stored over chloroform.

***In Vitro* DNA Manipulations.** Restriction nuclease digestion of DNA, ligation of DNA fragments with T4 ligase, purification of plasmid DNA by CsCl/ethidium bromide gradients, agarose gel electrophoresis of restriction nuclease fragments, and transformation of cells by using $CaCl_2$ were performed by standard procedures (20).

Isolation of Mutations. EM2/pLC7-21 and W3110 were first tested for sensitivity to erythromycin on Petri dishes containing LB (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) agar. Erythromycin at 70 μg/ml was found to greatly retard, but not prevent, growth of EM2/pLC7-21 and W3110; at 100 μg/ml it suppressed growth strongly but still incompletely; and at 200 μg/ml and greater it prevented detectable

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colony formation. To isolate mutants, 0.1 ml of an overnight culture of EM2/pLC7-21 was plated on LB plates containing erythromycin at 70 μg/ml and colicin E1. This erythromycin concentration was used at this stage to minimize possible loss of mutations that had not yet been phenotypically expressed while still providing a possible selective advantage to cells that had acquired the desired erythromycin-resistance mutation in *rrnH*.

A filter paper disk soaked in methanesulfonic acid ethyl ester was placed in the middle of the dish. After 1 day many colonies had appeared, overgrowing a weak lawn. The cells were washed off the plate, diluted to an OD_{550} of 0.2 in LB plus erythromycin (100 μg/ml), and grown overnight. Erythromycin at 100 μg/ml was used at this stage to allow additional opportunity for phenotypic expression under a stronger selection for erythromycin resistance. The cells were then centrifuged, washed with LB, resuspended in LB to an OD_{550} of 0.05, grown to an OD_{550} of 0.2, and then mixed with an equal volume of growing W3110 at the same cell density. The mating mixture was incubated overnight at 37°C in a thin layer at the bottom of a stationary flask, diluted 1:20 in LB plus nalidixic acid (20 μg/ml), grown overnight, and plated on LB plus colicin E1, nalidixic acid (20 μg/ml), and erythromycin (100 μg/ml). Many colonies grew on these plates, but none grew on controls with only EM2/pLC7-21 or W3110. All colonies were identified as W3110/pLC7-21 derivatives when checked for nonselected nutritional markers, immunity to colicin E1, sensitivity to phage BF23 (which shares the colicin E1 receptor), and resistance to 300 μg of erythromycin per ml. Only colonies resistant to this concentration of erythromycin were further analyzed.

Transformation and Segregation Tests. Several isolates resistant to erythromycin at 300 μg/ml were screened for linkage of colicin immunity and erythromycin resistance to identify erythromycin-resistance mutations on pLC7-21. Overnight LB broth cultures of individual mutants were diluted 1:100 and grown to stationary phase (in the absence of erythromycin) three consecutive times to allow plasmid loss. After plating on LB agar, a substantial fraction of the colonies were not immune to colicin E1. In all cases, erythromycin resistance concomitantly segregated or was retained with immunity to colicin E1. Plasmids were then purified from five of the erythromycin-resistant mutants and were used to transform EM2 to colicin immunity. In all cases, colicin-immune colonies were erythromycin resistant as an unselected trait.

It was possible to select directly for resistance to 300 μg of erythromycin per ml, but only after newly transformed cells had been allowed to increase 10- to 100-fold in number. All erythromycin-resistant colonies selected in this manner were found to be immune to colicin E1 as an unselected trait. One of the mutant plasmids was chosen for further study and was designated pERY-1. Erythromycin at 300 μg/ml adequately distinguished between erythromycin-sensitive cells and these mutants and this concentration was used for all subsequent experiments.

Protein Synthesis in Extracts. Cultures for making S30 protein-synthesizing extracts were begun by isolation of single colonies on LB plates containing colicin E1 or colicin E1 plus erythromycin. A colony was then used to inoculate 12 liters of LB containing 0.4% glucose. S30 extracts were prepared from the cells and pre-incubated according to Nathans (21). Each 50-μl *in vitro* protein-synthesizing reaction mixture contained 92.5 μg of S30 protein assayed according to Bradford (22), 1.0 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]leucine (500 Ci/mol), erythromycin when desired, and all other components specified by Nathans (21) except mRNA. The extract was incubated for 5 min at 37°C before addition of 25 μg of MS2 RNA (purchased from Boehringer). After incubation for an additional 40 min, the amount of [^{3}H]leucine incorporated into protein was determined (21). The protein synthesized in identical reaction mixtures without MS2 RNA was subtracted from each value.

RESULTS

Isolation of pERY-1. pLC7-21 is a recombinant plasmid (Fig. 1) that contains *rrnH* on a ColE1 vehicle (13). *rrnH* has the gene order promotor-promotor-16S rRNA-tRNA$^{Ile}_{1}$-tRNA$^{Ala}_{1B}$-23S rRNA-5S rRNA-tRNA$^{Asp}_{1}$-terminator (14–17). A mutation was isolated on pLC7-21 to give rise to pERY-1. Erythromycin resistance was found to cotransfer and to cosegregate with the colicin immunity encoded by pERY-1.

Characterization of pERY-1. In the presence of erythromycin at 300 μg/ml, EM2/pERY-1 grew well at 30°C, 37°C, or 42°C; EM2/pLC7-21 did not grow at all (Fig. 2). On medium without erythromycin, cells containing either pERY-1 or pLC7-21 grew at the same rate, but on medium with erythromycin cells containing pERY-1 grew noticeably slower than they did on the same medium without erythromycin. pERY-1 also conferred resistance to erythromycin when present in EM4, a *recA* derivative of EM2. Therefore, erythromycin resistance does not require alteration of chromosomal *rrn* sequences by recombination events or gene conversion events involving the plasmid. Because all colonies tested that arose from cells recently transformed with pERY-1 (selected by colicin immunity) were erythromycin resistant, it is unlikely that inactivation of chromosomal *rrn* sequences is a prerequisite for erythromycin resistance. Therefore, erythromycin resistance due to the mutation on pERY-1 is dominant or codominant over erythromycin sensitivity due to the seven chromosomal *rrn* sequences.

Although we have not analyzed in great detail the response of EM2/pERY-1 to increased levels of erythromycin, its plating efficiency was decreased at levels of erythromycin above 400 μg/ml, although surviving colonies grew reasonably well. Plating efficiency of EM2/pERY-1 was well above that of EM2/pLC7-21 up to at least 2,000 μg of erythromycin per ml. It is possible that a complex interplay among plasmid copy number, antibiotic-caused plasmid amplification, phenotypic lag in the percentage of antibiotic-resistant ribosomes, and inhibition of protein synthesis by erythromycin underlies the observed downward trend in plating efficiency with increasing concentrations of erythromycin.

Deletion Analysis of pERY-1. The erythromycin-resistance mutation in pERY-1 was localized by analysis of deletions pro-

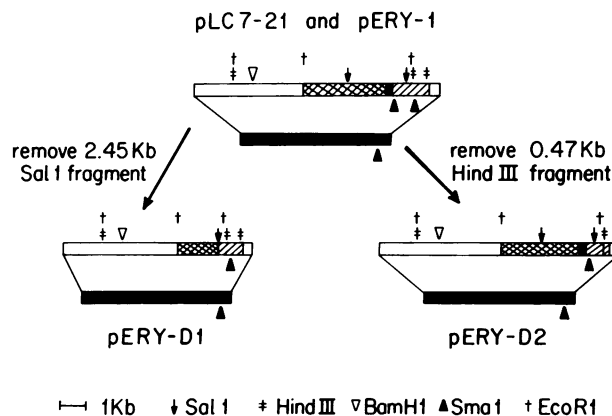

FIG. 1. Structure of plasmids used in this study. Black bars, ColE1 DNA; white bars, nonribosomal bacterial DNA; single hatching, 16S rRNA gene; and the cross-hatching, 23S rRNA gene. Transcription of *rrnH* is from right to left. Kb, kilobases.

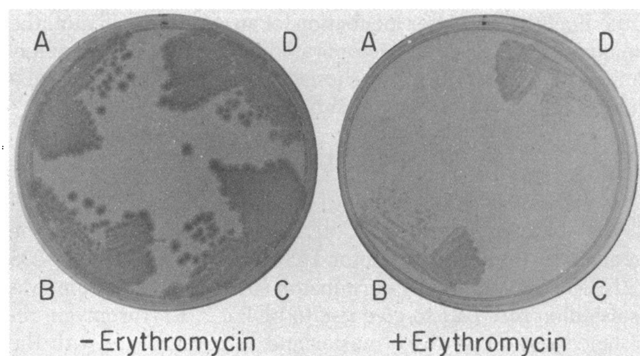

FIG. 2. Growth of bacterial strain EM2 containing plasmid pLC7-21 (A), pERY-1 (B), pERY-D1 (C), or pERY-D2 (D) on LB agar plus colicin E1 with and without erythromycin at 300 μg/ml. Plates were incubated for 2 days at 37°C.

duced *in vitro* by restriction nucleases (Fig. 1). Deletions of *rrn* operons on plasmids that retain the *rrn* promotors can occur readily only if the *rrn* termination sequences remain intact (unpublished data). pERY-1 was first digested to completion with *Sal* I and the resulting mixture of DNA fragments was then ligated at low DNA concentration to favor circularization of individual DNA fragments. The mixture of DNA was used to transform EM2. Many colicin-immune colonies arose, and all were erythromycin sensitive. The DNA from one of these (pERY-D1) was analyzed in detail by use of restriction nucleases and found to be the expected simple deletion of the *Sal I* fragment derived from the center of *rrnH* (Fig. 1). The deletion in pERY-D1 removed major portions of the 16S and 23S rRNA genes and all of the $tRNA_1^{Ile}$ and $tRNA_{1B}^{Ala}$ genes. The *rrnH* promotor, 5S rRNA gene, and $tRNA_1^{Asp}$ gene remained intact, but we have no evidence that functional 5S rRNA or $tRNA_1^{Asp}$ molecules are synthesized from the altered *rrnH* operon on pERY-D1 and therefore cannot conclude that the mutation is not in the 5S rRNA or $tRNA^{Asp}$ genes. The erythromycin sensitivity of cells containing pERY-D1 proves the erythromycin-resistance mutation is in *rrnH*.

pERY-1 was then digested to completion with *Hin*dIII, which cuts twice in the 16S rRNA gene and once outside of *rrnH*. The resulting mixture of DNA fragments was ligated under conditions that favor formation of new plasmids containing multiple *Hin*dIII fragments. The ligated DNA was transformed into EM2 and the products were selected for colicin immunity. The colonies were then replica plated onto medium containing 300 μg of erythromycin per ml. Ten percent of the colonies were erythromycin resistant. Plasmids from five erythromycin-resistant colonies were analyzed by restriction nucleases and found to have deletions of the 450-base-pair *Hin*dIII fragment containing a portion of the 16S rRNA gene, and had the *Hin*dIII fragment containing the distal portion of *rrnH* ligated in an orientation that allowed all remaining portions of *rrnH* to be expressed from the *rrnH* promotor.

One of these plasmids, pERY-D2 (Fig. 1), was analyzed in further detail. This selection for fragment retention in a single orientation was expected if the erythromycin-resistance mutation isolated in *rrnH* on pERY-1 was in the 23S or 5S rRNA genes or tRNA genes, but it also may have occurred if the *rrnH* transcription termination signal must properly terminate transcripts from the *rrnH* promotor so that transcription does not interfere with plasmid replication. The deletion in pERY-D2 proves that the mutation is not in the 16S rRNA gene. The properties of pERY-1, pERY-D1, and pERY-D2, in conjunction with previous observations on the mechanisms of erythromycin resistance and erythromycin action on ribosomes, strongly suggest, but do not absolutely prove, that the erythromycin-resistance mutation is in the 23S rRNA gene of *rrnH*.

The presence of pERY-D1 or pERY-D2 in cells may result in abortive assembly of ribosomal subunits due to the partial rRNA molecules produced from these plasmids. The deletion in pERY-D2 may cause an unbalanced synthesis of rRNA because this plasmid contains a major deletion in the 16S rRNA gene and probably produces 23S rRNA (because the plasmid confers erythromycin resistance). Adding erythromycin to cells containing pERY-1 or pERY-D2 probably will rapidly alter the ratio of small ribosomal subunits to functional large ribosomal subunits. In the absence of erythromycin, cells containing pERY-1, pERY-D1, or pERY-D2 grew at similar rates (Fig. 2). This indicates that either an imbalance of synthesis or accumulation of rRNA or ribosomes does not occur or that the imbalances that do occur under these conditions are not strongly inhibitory to cell growth. However, in the presence of erythromycin, cells containing pERY-D2 grew noticeably better than cells containing pERY-1. This may be because, for cells growing in erythromycin, the ratio of functional large ribosomal subunits to small ribosomal subunits is closer to unity in cells containing pERY-D2 than in cells containing pERY-1.

Protein Synthesis in S30 Extracts. When extracts were prepared from EM2/pERY-1 and EM2/pLC7-21 grown in the absence of erythromycin, protein synthesis by extracts from EM2/pERY-1 had increased resistance to erythromycin compared to extracts from EM2/pLC7-21 (Fig. 3). The response to erythromycin observed in extracts from EM2/pERY-1 is compatible with the existence of a fraction of ribosomes with increased resistance to erythromycin or the presence in these cells of a fraction (or all) of ribosomes partially resistant to erythromycin. The observed level of resistance in extracts may not completely reflect the level of erythromycin resistance in EM2/pERY-1 because it is possible that the fraction of erythromycin-resistant ribosomes in EM2/pERY-1 grown on erythromycin may be much higher than in these extracts due to erythromycin-induced plasmid amplification (see below) and selection against plasmid segregation. Because these experiments used S30 extracts, they show that the erythromycin resistance is not due to alteration of the permeability of cells to erythromycin. These *in vitro* experiments by themselves do not identify the ribosome

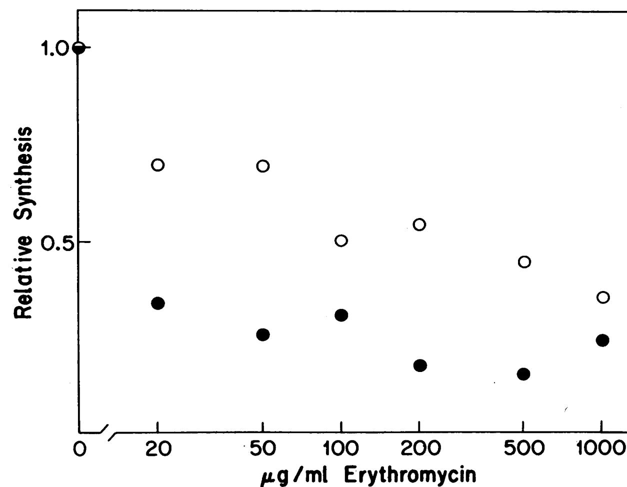

FIG. 3. Erythromycin sensitivity of protein synthesis directed by MS2 RNA in cell-free S30 extracts prepared from EM2/pLC7-21 (solid circles) and EM2/pERY-1 (open circles). The data are presented as [^{3}H]leucine incorporated into protein in extracts containing erythromycin relative to identical reactions without erythromycin. The data are summarized from three independent experiments.

or rRNA as the site of the alteration that confers erythromycin resistance.

DISCUSSION

An erythromycin-resistance mutation has been isolated on a multicopy plasmid that carries *rrnH* of *E. coli*. Cell-free S30 extracts from cells containing the mutant plasmid have an increased resistance to erythromycin. *In vitro* deletion analysis proves that the mutation is in *rrnH*. Erythromycin resistance is still expressed from plasmids that contain a major deletion of the 16S rRNA gene and is not expressed when the 5S rRNA gene and $tRNA_1^{Asp}$ gene of *rrnH* remain intact and the 16S and 23S rRNA genes and $tRNA_1^{Ile}$ and $tRNA_{1B}^{Ala}$ genes of *rrnH* are partially or completely deleted. The evidence is most consistent with the location of the erythromycin-resistance mutation in the 23S rRNA gene, but location of it in the 5S rRNA gene or tRNA genes cannot be completely ruled out until further genetic studies and sequence analysis are done.

Several aspects of the design of the selection procedure may have contributed to our success in isolating erythromycin-resistance mutations on this plasmid. Probably the most important aspect is the presence of a *rrn* operon on a multicopy plasmid. This allows a mutation in a single rRNA gene to eventually give rise to cells in which a substantial fraction of the total ribosomes can contain altered rRNA molecules. The second aspect which may be important is the fact that ColE1 plasmids amplify in the presence of at least some treatments that inhibit protein synthesis, including chloramphenicol (23), tetracycline, spectinomycin, and starvation for isoleucine (unpublished data). It therefore is possible that, in the presence of erythromycin, pERY-1 amplifies until it contributes a large share of the rRNA in ribosomes, a balance eventually being achieved when the fraction of erythromycin-resistant ribosomes is sufficient to prevent further erythromycin-induced plasmid amplification. The third aspect is elimination of erythromycin-resistance mutations not located on the plasmid, which was accomplished by selecting for simultaneous F-mediated plasmid mobilization of colicin immunity and erythromycin resistance. A fourth aspect which may have been important in isolation of the mutation was the recognition that there probably would be a long phenotypic lag between acquisition of a mutant plasmid and onset of significant resistance to erythromycin. For this reason, mutagenesis was initially followed by growth on medium containing a concentration of erythromycin that was strongly but incompletely inhibitory to growth of cells containing pLC7-21. This procedure allows cell growth, and therefore full phenotypic expression, under conditions such that the presence of even a single mutant copy of the plasmid may have conferred a growth advantage to the cell that harbored it. A fifth aspect was that the final selection for erythromycin resistance was done by using concentrations of erythromycin just above the threshold concentration that inhibits growth of cells containing pLC7-21. It was realized that a mutation in *rrnH* on pLC7-21 could have resulted in only a marginal increase in resistance to erythromycin (due to the seven chromosomal copies of *rrn* operons) if the mutation was codominant. Most antibiotic-resistance mutations in *E. coli* are recessive or weakly codominant (1–3). Indeed, although cells containing pERY-1 can grow at erythromycin concentrations of at least 2,000 μg/ml, the plating efficiency decreases markedly with increasing erythromycin concentration.

In *E. coli*, erythromycin is a bacteriostatic antibiotic (24) that binds with high affinity to a single site on the large ribosomal subunit (25). Erythromycin is probably an inhibitor of the peptidyltransferase activity of the large ribosomal subunit. However, the action of erythromycin appears to be complex when analyzed in the framework of existing models of ribosome action, and inhibition of other ribosome functions cannot be ruled out (9). Previous ribosomal mutations to erythromycin resistance were either in genes for ribosomal proteins L4 or L22 (26) or in another gene (*eryC*) that may affect both ribosomal subunits (27). Mutations in ribosomal proteins S5 and S12 of the small subunit can also affect erythromycin binding to the large subunit (28).

In ribosome reconstitution studies, ribosomal proteins L15 and L16 were necessary to bind erythromycin to the ribosome. Purified L15 alone can bind erythromycin, but with lower affinity than L15 and L16 in ribosomal particles (29). In the 50S ribosomal assembly map of Röhl and Nierhaus (30), L4 binds 23S rRNA strongly, L22 has a weak binding dependence on 23S rRNA, L22 and L15 have a strong binding dependence on the previous binding of L4, and L16 interacts weakly with 23S rRNA and depends strongly on previous binding of L15. L4, L15, and L16 are three of the five proteins identified by Röhl and Nierhaus (30) as proteins essential for reconstitution of peptidyltransferase activity.

Because these proteins are involved in erythromycin action on the ribosome and in some cases interact with rRNA, it seems likely that potentially complex interactions between rRNA and ribosomal proteins are involved in the action of erythromycin and in the function or structure of the peptidyltransferase region of the ribosome. Sequence analysis of the mutation isolated in *rrnH* in the present work and alterations in 23S rRNA molecules observed in erythromycin-resistant mitochondria and bacteria (4, 5, 10, 11) may partially clarify functional and structural interactions in the region of the ribosome affected by erythromycin.

When mutations to erythromycin resistance are in ribosomal protein genes, sensitivity is dominant over resistance (2). Because cells with pERY-1 can grow in the presence of erythromycin, the mutation on pERY-1 must be dominant or codominant, at least when on a multicopy plasmid. The reason for the difference in dominance between mutations in ribosomal protein genes and this mutation in *rrnH* remains to be determined. If the mutation proves to be in the 23S rRNA gene (as is likely but not completely proven; see above) then *E. coli* containing pERY-1 must be capable of growing in the presence of erythromycin when most or all protein synthesis is directed by ribosomes containing 23S rRNA from *rrnH*. This suggests that all 23S rRNA genes are functionally similar. The erythromycin resistance of cells containing pERY-D2, a plasmid with a 470-base-pair deletion in the 16S rRNA gene, suggests that erythromycin resistance (and therefore probably large ribosomal subunit assembly) using rRNA from *rrnH* is not dependent on any aspects of small ribosomal subunit assembly that are blocked by the deletion in the 16S rRNA gene of *rrnH*. However, there may be an assembly dependence of large subunits containing rRNA from *rrnH* on assembly of small subunits containing 16S rRNA from other *rrn* operons.

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