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Ribosomal Protein Gene Sequence Changes in Erythromycin-Resistant Mutants of *Escherichia coli*

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The genes for ribosomal proteins L4 and L22 from two erythromycin-resistant mutants of *Escherichia coli* have been isolated and sequenced. In the L4 mutant, an A-to-G transition in codon 63 predicted a Lys-to-Glu change in the protein. In the L22 strain, a 9-bp deletion removed codons 82 to 84, eliminating the sequence Met-Lys-Arg from the protein. Consistent with these DNA changes, in comparison with wild-type proteins, both mutant proteins had reduced first-dimension mobilities in two-dimensional polyacrylamide gels. Complementation of each mutation by a wild-type gene on a plasmid vector resulted in increased erythromycin sensitivity in the partial-diploid strains. The fraction of ribosomes containing the mutant form of the protein was increased by growth in the presence of erythromycin. Erythromycin binding was increased by the fraction of wild-type protein present in the ribosome population. The strain with the L4 mutation was found to be cold sensitive for growth at 20°C, and 50S-subunit assembly was impaired at this temperature. The mutated sequences are highly conserved in the corresponding proteins from a number of species. The results indicate the participation of these proteins in the interaction of erythromycin with the ribosome.

The interaction of antimicrobial agents with the bacterial ribosome has been a significant area of research for many years because of its relevance to infectious diseases (20, 39). Erythromycin and other macrolide antibiotics in particular have been widely studied for their effects on the functions of the ribosome during translation (12). These compounds bind strongly to the 50S ribosomal subunit of both gram-positive (12, 35) and gram-negative (46) cells and interfere with the elongation of the nascent peptide chain (9, 13, 52).

Several studies have focused on the 50S-subunit proteins involved in erythromycin binding. Reconstitution experiments identified proteins L15 and L16 as critical for drug binding to the subunit and demonstrated weak binding of erythromycin to isolated protein L15 (55). Affinity labeling studies with erythromycin derivatives have identified a strong interaction of the drug with protein L22 and weaker associations with proteins L2, L4, and L15 (2). Each of these proteins has been shown to be essential for reconstitution of the peptidyltransferase activity of the 50S subunit (24). Recent studies have identified the location of several of these proteins at a common region in the 50S-subunit structure (11).

The involvement of rRNA in erythromycin activity has been indicated by a number of recent reports identifying 23S rRNA mutations leading to erythromycin resistance in *Escherichia coli* (15, 18, 50) and other organisms (14). These sequence changes are located exclusively in domain V of the RNA secondary structure except for a deletion affecting domain II of the molecule. Mutagenesis experiments have suggested a tertiary interaction between domains II and V in the 23S RNA (15, 16). Proteins L4 and L22 interact with each other in binding to 23S RNA at sequences associated with domains I and II near the 5′ end of the 23S RNA (26, 59).

Resistance to erythromycin can also result from changes in ribosomal proteins in *E. coli* (1, 42–45, 58) and in *Bacillus* species (48, 49, 56). Whereas the RNA sequence changes have been specifically identified, no sequence information about the alterations in ribosomal proteins leading to erythromycin resistance is presently available. Phenotypic effects of alterations in ribosomal proteins L4 and L22 in two resistant mutants of *E. coli* were described many years ago (1, 58). We have recently acquired these mutants and have determined the specific DNA sequence changes leading to antibiotic resistance. Our results suggest that specific regions of these ribosomal proteins are involved in the interaction of the drug with the large ribosomal subunit. This information will be helpful in determining the mechanism of erythromycin inhibition of bacterial protein synthesis.

MATERIALS AND METHODS

Strains and genetic manipulations. The erythromycin-resistant mutant strains N281 and N282 were obtained from David Apirion (1). The wild-type strain used (SK901) has been described previously (7). Plasmid complementation in these strains was conducted by the method of Silhavy et al. (51). Plasmids pLF1.0 and pLF4.6, carrying wild-type copies of the *rplV*$^+$ and *rplD*$^+$ genes, respectively, were used for the complementation tests (57). Plasmid DNA was isolated from cells with the Elu-Quik DNA purification kit (Schleicher and Schuell) by the procedure recommended by the manufacturer. Growth rates were measured for cells growing at 37°C in L broth or tryptone broth (38) containing ampicillin at 50 μg/ml and erythromycin at several different concentrations.

Ribosomal protein gel electrophoresis. Ribosomes were isolated from cells grown in L broth as described previously (8). Ribosomes from the plasmid-complemented strains were isolated from cells grown in the presence of ampicillin (50 μg/ml) and in the presence and absence of erythromycin (300 μg/ml). Ribosomal proteins were extracted with acetic acid (25) and collected by acetone precipitation (5). The proteins were separated by two-dimensional gel electrophoresis, with the first dimension at pH 5.5, by the method of Kenny et al. (29).

PCR amplification and DNA sequencing. Genomic DNA

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was isolated from cells as described elsewhere (4). Oligonucleotide primers for sequences from the S10 operon (61) were identified by the Oligo program (W. Rychlik, National Biosciences) and were synthesized by K. Lohman. The sequences of the 25-mer primers used for PCR amplification of the *rplV* gene are 5′-CGGTGGAAAGCGGAGACAAGAAGCC-3′ (forward) and 5′-ACCAGTTTTGCGTCCAGTTCAGG CT-3′ (reverse). The 15-mer primers used for *rplV* sequencing are 5′-GCGACGCTGCTGATA-3′ (forward) and 5′-CCCAG GCGAATACCA-3′ (reverse). For *rplD* amplification and sequencing, the 25-mer primers used are 5′-GGCAAGAAAAT GGCAGGTCAGATGG-3′ (forward) 5′-TTCCATCGCAGT AGACGCTTTTTCA-3′ (reverse). PCR amplification with a thermostable *Taq* polymerase (Promega) was conducted for 30 cycles with 10 pmol of primers and 1 μg of genomic DNA (17). Amplification consisted of melting at 94°C for 2 min, annealing at 55°C for 2 min, and primer extension at 72°C for 5 min. The product was isolated from a 1.15% agarose gel by electroelution using an IBI apparatus and the method provided by the manufacturer.

Double-stranded DNA sequencing of the gel-purified PCR product was performed with Sequenase V. 2.0 (U.S. Biochemicals), using the primers described above and the protocol supplied by the manufacturer. Both strands of two independent PCR products from the wild type and both mutants were sequenced in order to establish a consensus sequence. Protein sequence homologies were examined with the Macintosh program Gene Jockey (Biosoft).

Quantitation of plasmid DNA and ribosomal proteins. Two-dimensional protein gels and photographic negatives of plasmid gels were scanned into the Bioimage analysis system (Millipore), and areas of the gel spots were integrated by using the software included. Two or three protein gels were scanned for each determination, and the resulting areas were averaged.

Ribosome binding assays. Erythromycin binding assays were conducted by the method of Teraoka (54), using [*N*-methyl-^{14}C]erythromycin (54 mCi/mM; New England Nuclear) and 4 A_{260} units (96 pmol) of 70S ribosomes. Binding was done at 37°C for 15 min in a buffer containing 50 mM Tris-HCl (pH 7.8), 16 mM Mg acetate, and 80 mM KCl.

Ribosomal subunit assembly analysis. Ribosomal subunit assembly at 20°C was examined by a method described previously (7). Briefly, cells growing in tryptone broth at 37°C in a shaking water bath were shifted down to 20°C, and after 1 h they were labeled with [^{3}H]uridine (1 μCi/ml with 2 μg of uridine per ml) for 7 to 8 h. The cells were collected by centrifugation and lysed, and the subunits were separated by sucrose gradient centrifugation as previously described (7). The A_{260} and the ^{3}H radioactivity were measured for each gradient fraction.

RESULTS

Ribosomal proteins were isolated from both erythromycin-resistant mutants and were examined by two-dimensional gel electrophoresis. The alteration of protein L22 in strain N281 had been demonstrated previously by Wittmann et al. (58), using a different gel system, but no altered mobility of protein L4 was reported by those authors. Electrophoresis at pH 5.5 in the first-dimension gel system of Kenny et al. (29) revealed changes in the mobilities of the mutant proteins L4 and L22, as Fig. 1 indicates. For both altered proteins, the reduced first-dimension mobility suggested that they were less basic molecules. Protein L22 was not changed in strain N282 with the L4 alteration, and L4 was not different in strain N281 with the L22 change.

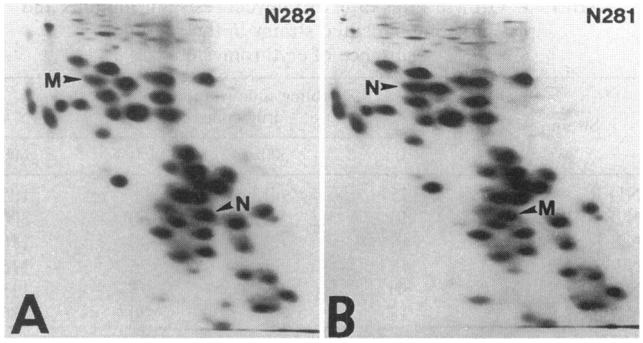

FIG. 1. Two-dimensional polyacrylamide gels of ribosomal proteins from erythromycin-resistant mutants. The first-dimension electrophoresis was done at pH 5.5 (29). (A) Proteins from N282. Arrowheads indicate the positions of the normal L22 (N) and mutant L4 (M) proteins. (B) Proteins from N281. Arrowheads indicate the positions of the normal L4 (N) and mutant L22 (M) proteins.

The *rplD* and *rplV* genes coding for proteins L4 and L22, respectively, were amplified by the PCR method from genomic DNA isolated from both wild-type and mutant cells. The PCR products were sequenced to determine the mutational changes in the genes conferring erythromycin resistance. The wild-type sequences for these two genes were found to be the same as those determined previously by Zurawski and Zurawski (61). In the *rplD* sequence for protein L4 in strain N282, an A-to-G transition was found in the first position of codon 63 (Fig. 2A). This change would predict a Lys-to-Glu change in the L4 sequence and would account for the reduced gel mobility of the mutant protein at pH 5.5. Sequencing of the *rplV* gene for protein L22 in mutant N281 revealed a 9-bp deletion eliminating codons 82 to 84 in the gene, which would result in the loss of the tripeptide sequence Met-Lys-Arg from the L22 protein (Fig. 2B). This loss would reduce the gel mobility of this protein at pH 5.5, similar to the L4 alteration. A 4-bp direct repeat (GCAT) was found in the wild-type DNA sequence flanking the deletion site.

A.

	60 65
rp L4	-Trp-Arg-Gln--Lys-Gly-Thr-Gly-
rplD	...TGG CGC CAG AAA GGC ACC GGC...
rplD 1	...TGG CGC CAG GAA GGC ACC GGC...
rp L4 (ery^R)	-Trp-Arg-Gln-Glu-Gly-Thr-Gly-

B.

	80 85
rp L22	-Pro-Ser-Met-Lys-Arg-Ile-Met-
rplV	...CCG AGC ATG AAG CGC ATT ATG...
rplV 1	...CCG AGC ATT ATG...
rp L22 (ery^R)	-Pro-Ser--Ile-Met-

FIG. 2. DNA and ribosomal protein (rp) sequence changes in erythromycin-resistant mutants N282 (*rplD1*) (A) and N281 (*rplV1*) (B) of *E. coli*. In panel A, the Glu found in the mutant protein (boldface) is indicated. In panel B, the deleted Met-Lys-Arg sequence (boldface) and the direct repeat GCAT (double-underlined) are shown.

TABLE 1. Growth rates of erythromycin-resistant mutants and plasmid-complemented strains in the presence and absence of erythromycin

Strain	Doubling time (min) at the indicated drug concn (μg/ml)			
	0	300	450	600
N281	45	60	90	105
N281(pLF1.0)	40	85	160	300
N281(pLF4.6)	45	65	90	110
N282	45	55	75	115
N282(pLF1.0)	50	65	75	115
N282(pLF4.6)	35	65	70	130

Complementation of the mutants with plasmids bearing wild-type alleles of the appropriate genes was performed. *Eco*RI DNA fragments from the S10 ribosomal protein operon were cloned into pBR325 to give plasmids pLF1.0, containing only the *rplV*$^+$ gene, and pLF4.6, containing the *rplB*$^+$, *rplD*$^+$, and *rplW*$^+$ genes for proteins L2, L4, and L23, respectively (57). The growth rates of the mutant cells with and without complementing plasmids were measured in the presence and absence of erythromycin. As Table 1 indicates, both strains showed reduced growth rates with increasing concentrations of erythromycin. The mutants grew about 2.5 times more slowly at 600 μg/ml. Each strain became more sensitive to erythromycin in the presence of the wild-type gene. N281(pLF1.0) grew 7.5 times more slowly at this final concentration, while N282(pLF4.6) grew 3.7 times more slowly. In comparison with N281, N281(pLF1.0) was more affected by higher concentrations of the drug than N282(pLF4.6) was in comparison with N282. In each mutant, the noncomplementing plasmid had no effect on the growth rate in the presence of erythromycin (Table 1). These results reflect the known dominance of erythromycin sensitivity over resistance in merodiploid cells (40).

TABLE 2. Ribosomal protein distribution and erythromycin binding for wild-type, mutant, and complemented strains

Strain	Erythromycin[a]	Protein distribution[b]		Erythromycin binding[c]
		Wild type	Mutant	
SK901	−	100	0	75 (13)
N282	−	0	100	3.5 (4)
N282(pLF4.6)	+	16 (4.9)	84 (4.9)	7.4 (5)
N282(pLF4.6)	−	72 (2.4)	28 (2.4)	55 (8)
N281	−	0	100	61 (7)
N281(pLF1.0)	+	37 (1.1)	63 (1.1)	33 (13)
N281(pLF1.0)	−	100	0	69 (8)

[a] Cells were grown without (−) or with (+) erythromycin (300 μg/ml).
[b] Fractions of wild-type and mutant proteins in two-dimensional gels, with standard deviations in parentheses.
[c] Percent [^{14}C]erythromycin binding (pmol/100 pmol of 70S subunit). Values are averages for the numbers of assays indicated in parentheses.

Ribosomes were isolated from the complemented mutant cells grown in the absence and presence of erythromycin, and the protein contents were examined by gel electrophoresis and image analysis. Ribosomes from strain N282(pLF4.6) contained predominately wild-type L4 protein (72%) when cells were grown without erythromycin (Fig. 3A) and predominately mutant protein (84%) when grown with the drug (Fig. 3B; Table 2). Plasmid pLF4.6 also contains the genes for proteins L2 and L23. No differences were found in the relative amounts of these two proteins in cells grown with or without the drug. Ribosomes from strain N281(pLF1.0) cells grown without erythromycin contained only wild-type L22 protein (Fig. 3C). In the presence of the antibiotic, a mixed ribosome population

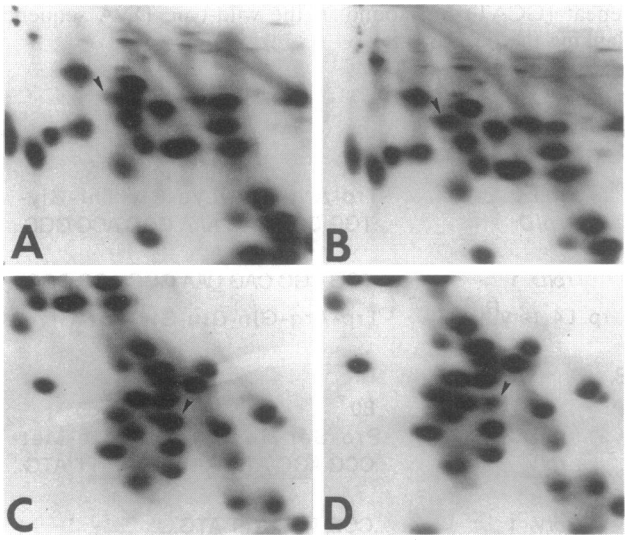

FIG. 3. Two-dimensional polyacrylamide gels of ribosomal proteins from plasmid-complemented erythromycin-resistant mutants. Regions of the gels containing the mutant proteins have been enlarged. (A and B) Proteins from N282(pLF4.6) grown without and with erythromycin (300 μg/ml), respectively. (C and D) Proteins from N281(pLF1.0) grown without and with erythromycin (300 μg/ml), respectively. Arrowheads indicate the positions of the respective proteins in the complemented mutant strains.

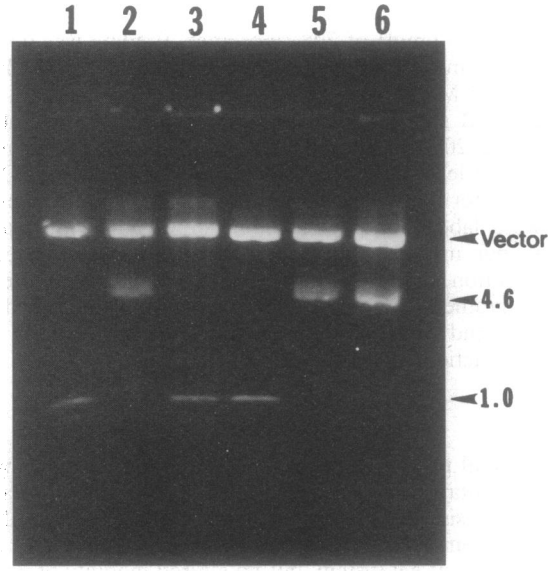

FIG. 4. Relative plasmid yields from wild-type and complemented mutant cells grown with and without erythromycin. Plasmid DNA was digested with *Eco*RI to release the insert, and the fragments were separated on a 1% agarose gel. Lane 1, SK901(pLF1.0); lane 2, SK901(pLF4.6); lane 3, N281(pLF1.0) without erythromycin; lane 4, N281(pLF1.0) with erythromycin (300 μg/ml); lane 5, N282(pLF4.6) without erythromycin; lane 6, N282(pLF4.6) with erythromycin (300 μg/ml). Vector, pBR325; 4.6, 2.2-kb *Eco*RI fragment from pLF4.6; 1.0, 0.5-kb *Eco*RI fragment from pLF1.0.

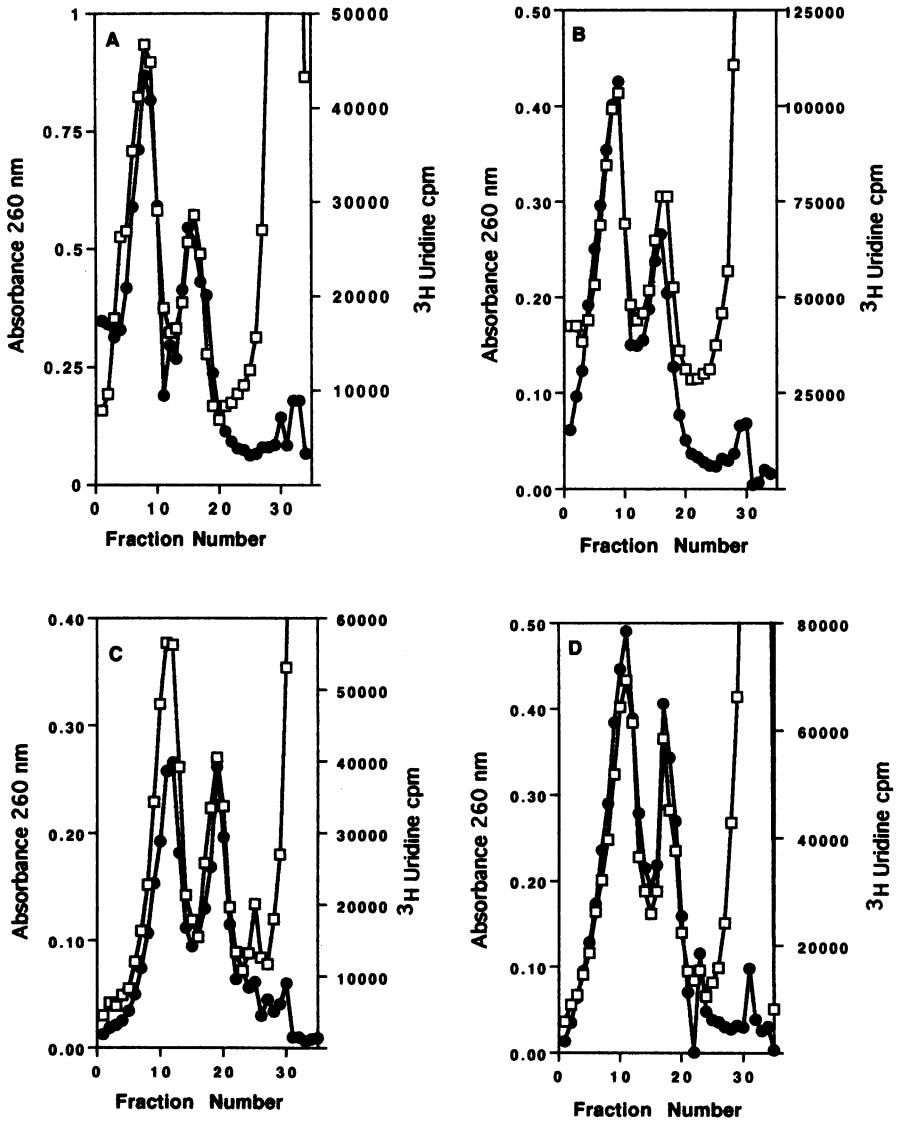

FIG. 5. Sucrose gradient sedimentation profiles of ribosomal subunits from wild-type and mutant strains grown at 20°C. The A_{260} (□) and [^{3}H]uridine radioactivity (●) for each fraction are indicated. (A) SK901; (B) N281; (C) N282; (D) N282(pLF4.6).

was found, with 63% of the ribosomes containing the mutant L22 and 37% showing the wild-type protein (Fig. 3D; Table 2).

The amount of plasmid DNA was estimated for cells growing with or without erythromycin. Figure 4 shows the relative amounts of plasmid DNA isolated from equal numbers of wild-type and mutant cells. A scan of this gel showed that there was less than a 20% difference in plasmid yield from either wild-type or complemented mutant cells grown with or without erythromycin.

Erythromycin binding to ribosomes from wild-type and mutant cells was measured under the conditions used previously to examine these ribosomes (54, 58). As Table 2 indicates, control ribosomes showed a binding stoichiometry of 0.75, and as observed previously, the N281 ribosomes were still able to bind erythromycin very well (0.61) while the N282 ribosomes showed no appreciable binding (58). Ribosomes from the merodiploid strain N282(pLF4.6) bound erythromycin in proportion to the amount of wild-type protein present in the ribosomes (Table 2). Ribosomes from complemented N281 cells grown without the drug bound erythromycin slightly better than the noncomplemented mutant (0.69 versus 0.61), but ribosomes from the diploid cells grown with erythromycin did not bind the drug well (0.33 mol/mol of 70S ribosomes).

As determined by replica plating, strain N282 would not form colonies at 20°C, whereas slow growth was observed for wild-type cells and strains N281 and N282(pLF4.6) under the same conditions. This observation is consistent with a subunit assembly defect in the mutant at the reduced temperature (23). Subunit assembly at 20°C was examined in each strain. No effect on subunit formation was found for wild-type cells or for strain N281 (Fig. 5A and B). The specific activity ratio (^{3}H counts per minute per A_{260} unit) for 50 to 30S subunits was 1.0 in each case. The L4 mutant cells showed a specific defect in the assembly of the large ribosomal subunit at this temperature (Fig. 5C), with the specific activity ratio reduced to 0.8. Complementation with the wild-type L4 gene on plasmid pLF4.6 abolished the assembly defect and permitted the equivalent formation of both subunits (Fig. 5D).

TABLE 3. Ribosomal protein sequence similarities

Protein and organism	Sequence[a]	% Similarity[b]	Reference
L4			
E. coli	PWRQKGTGR	100 (201)	61
Y. pseudotuberculosis	PWRQKGTGR	94.5 (210)	22
M. capricolum	PWKQKGTGL	35.8 (193)	41
L22			
E. coli	GPSMKRIMP	100 (110)	61
M. capricolum	GPTLKRFRP	54.1 (109)	41
B. stearothermophilus	GPTLKRFRP	52.8 (108)	30
Pea (chloro.)[c]	GKTLKRVRA	43.6 (110)	21
Liverwort (chloro.)	GTFFKRFQP	41.6 (101)	19
C. paradoxa	GPTLKRFRP	41.4 (99)	37
Red alga (chloro.)	GPKLKRFQP	40.6 (101)	28
Spinach (chloro.)	GITLKKVKP	38.1 (97)	60
Tobacco (chloro.)	GTTVKKLKP	37.6 (101)	53
Rice (chloro.)	STIMNKFRP	33.3 (102)	27
Rat (liver)	APKMRRRSG	32 (109)	32
Halobacterium m.	VGESQGRKP	17.8 (90)	3

[a] Residues altered in erythromycin-resistant *E. coli* ribosomal proteins are underlined. Identical sequences in other organisms are in boldface.

[b] Percent overall sequence homology, with the total number of amino acids in each protein given in parentheses.

[c] chloro., chloroplast.

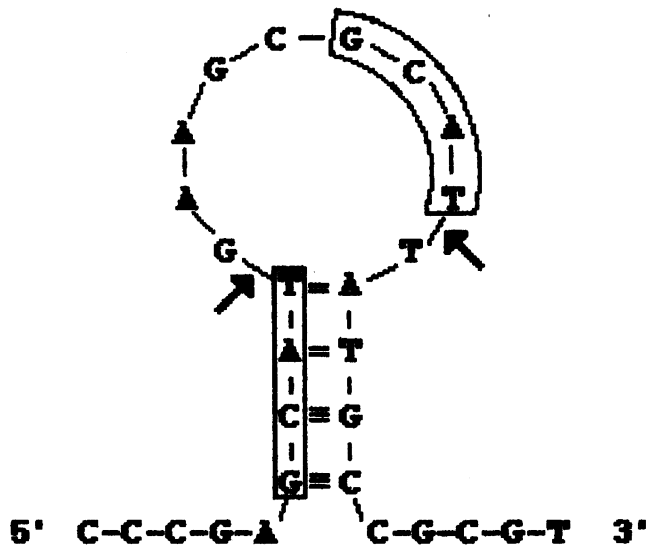

FIG. 6. Proposed transient secondary structure of the DNA sequence in the *rplV* gene surrounding the site of the deletion. A loop of 10 nucleotides, including the nine bases in the deletion, is shown, with arrows indicating potential cleavage sites. The GCAT direct repeats are boxed.

The predicted amino acid sequences in L4 and L22 associated with erythromycin resistance were compared with sequences reported for homologous ribosomal proteins from other organisms. These results are presented in Table 3. The sequence around the altered site in the L4 sequence is highly conserved, showing complete identity in *Yersinia pseudotuberculosis* and nearly complete identity in *Mycoplasma capricolum* (Table 3). For protein L22, a strong conservation of the sequence around the Met-Lys-Arg deletion site was found for L22 homologs in two other prokaryotes (*Bacillus stearothermophilus* and *Mycoplasma capricolum*). In *Cyanophora paradoxa* and in six chloroplast genes, additional significant similarity was observed (Table 3). Ribosomes from these cells and plastids are known to be sensitive to erythromycin (36, 42). Little similarity to L22-like sequences from archaebacteria and rats was found (3, 32).

DISCUSSION

This work represents the first description of the molecular basis of erythromycin resistance associated with ribosomal protein gene sequence changes. In the wild-type *rplD* sequence, we found one difference from that reported by Zurawski and Zurawski (61), a CAG codon for Gln at position 30 instead of the CAA sequence reported previously. Our sequence of the *rplV* gene agrees exactly with the sequence reported by those authors. The deletion in the *rplV* gene in N281 may have arisen by nuclease cleavage and ligation after the formation of a single-stranded stem-loop structure surrounding the affected sequence. Figure 6 shows this predicted structure with the loop containing all of the deleted nucleotides. This is predicted to be a quasi-stable structure with a ΔG of -1.2 kcal/mol (ca. -5.0 kJ/mol). Formation of this structure by slipped mispairing of direct repeats in one or both of the DNA strands during replication or transcription could account for the creation of this mutant (31, 47).

The high degree of sequence conservation seen around the mutated regions of both proteins suggests that these sites are important for ribosomal activities. These observations suggest that ribosomes from other prokaryotes or from mitochondria that have significant sequence homology to *E. coli* ribosomal proteins L4 and L22 should be sensitive to inhibition by erythromycin, as has been previously demonstrated (33).

These strains were isolated as spontaneous mutants resistant to high concentrations of erythromycin (1). Some functions of the ribosomes from these cells have been previously examined (58). Ribosomes containing the altered L4 protein bound erythromycin poorly and had a reduced peptidyltransferase activity. Significantly, neither of these features was found for ribosomes containing the L22 mutation. Our results with strain N282 show that the amount of erythromycin bound to the 50S subunit was proportional to the amount of wild-type L4 protein present. This shows that L4 is specifically involved in the association of erythromycin with the ribosome, presumably through the sequence where the mutation was identified. The binding we observed was comparable to that demonstrated previously for wild-type and N282 mutant ribosomes (54, 58).

The interaction of erythromycin with ribosomes from strain N281 is less clear. It was previously shown that ribosomes from this resistant mutant still bind erythromycin (58), and we have confirmed this observation. Plasmid-complemented cells grown without the drug contained almost exclusively wild-type L22 protein and also bound the drug almost as well as wild-type 70S particles. Curiously, ribosomes from this diploid strain grown with erythromycin did not bind the macrolide well, showing a binding stoichiometry of only 0.33 in numerous assays. We have no clear explanation for this finding. This level of binding is slightly above that expected if only ribosomes with wild-type L22 protein are binding the drug ($37\% \times 0.75 = 0.28$). Polyribosomes in these cells must consist of mixtures of individual 70S ribosomes containing either wild-type or mutant L22 proteins. Since the diploid cells grew more slowly in the presence of erythromycin than noncomplemented cells did (Table 1), the inhibitory effect of erythromycin in vivo seems apparent.

The complementation results show that the presence of wild-type protein in the 50S subunits of the mutant strains

leads to erythromycin sensitivity, with the growth rate diminished in proportion to the wild-type-protein content of the particle. In particular, the reduction in growth rate for the complemented mutants at 300 μg of erythromycin per ml [40% for N281(pLF1.0) and 18% for N282(pLF4.6); Table 1] is reflected closely in the amount of wild-type L22 or L4 protein present in the ribosomes (37 and 16%, respectively; Table 2) from cells grown at this same drug concentration.

Presumably, this reflects a change in the amounts of these proteins assembled into the 50S particle, since the relative amounts of either plasmid were in wild-type cells and in the mutants growing with or without erythromycin were not different. This is consistent with transcription of mRNA for these proteins from the chloramphenicol transacetylase promoter in this plasmid, which is not under ribosomal protein control (6) but is controlled by catabolite repression (34). A major effect of L4 overproduction on autogenous regulation of expression of the chromosomal S10 operon is unlikely, since the presence of the pLF4.6 plasmid in either wild-type cells or strain N281 did not influence the growth rate or erythromycin sensitivity. This suggests that the change in ribosomal protein content is related to the number of 50S subunits able to assimilate either form of the protein in the presence of erythromycin.

We have shown elsewhere that erythromycin specifically inhibits the assembly of the 50S subunit in growing wild-type *E. coli* cells (10). Mutant strain N281 with the L22 alteration is partially resistant to this effect and requires about a fivefold-greater concentration of erythromycin for assembly to be affected. Strain N282 is virtually immune to inhibition of assembly by erythromycin. In diploid cells growing in the absence of erythromycin, wild-type L4 and L22 proteins are presumably preferentially assembled into new 50S subunits, as Table 2 indicates. Mutant proteins may be at some type of assembly disadvantage, as shown by the cold-sensitive assembly defect in the L4 mutant strain. In the presence of erythromycin, incorporation of wild-type proteins into the particle would lead to erythromycin sensitivity in translation and to a reduction in cellular growth rate, as shown in Table 1. This suggests that mutant 50S subunits would be preferentially formed in the presence of the antibiotic (Table 2). We intend to examine erythromycin effects on ribosomal protein-RNA interactions and to measure the relative rates of ribosomal protein synthesis in these complemented mutants to more closely examine this possibility.

In conclusion, we have identified critical parts of the sequences of two different ribosomal proteins necessary for the interaction of the macrolide antibiotic erythromycin with the bacterial ribosome. It seems clear that the binding of erythromycin to the 50S subunit involves sequences in both proteins L4 and L22 and nucleotides in domain V of the 23S RNA secondary structure (15, 18, 50). These observations should help in identifying the critical amino acid and nucleotide residues involved in the binding of this compound and its inhibitory effects on translation and ribosome formation.

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REFERENCES

1. **Apirion, D.** 1967. Three genes that affect *Escherichia coli* ribosomes. J. Mol. Biol. **30**:255–275.
2. **Arevalo, M. A., F. Tejedor, F. Polo, and J. P. G. Ballesta.** 1988. Protein components of the erythromycin binding site in bacterial protein synthesis. J. Biol. Chem. **263**:58–63.
3. **Arndt, E., W. Kroemer, and T. Hatakeyama.** 1990. Organization and nucleotide sequence of a gene cluster coding for eight ribosomal proteins in the archaebacterium *Halobacterium marismortui*. J. Biol. Chem. **265**:3034–3039.
4. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl (ed.).** 1989. Short protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
5. **Barritault, D. S., A. Expert-Bezancon, M.-F. Guerin, and D. Hayes.** 1976. The use of acetone precipitation in the isolation of ribosomal proteins. Eur. J. Biochem. **63**:131–135.
6. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of pBR322 carrying unique EcoR1 sites for selection of EcoR1 generated recombinant DNA molecules. Gene **4**:121–136.
7. **Champney, W. S.** 1979. Localized mutagenesis for the isolation of temperature-sensitive mutants of *Escherichia coli* affected in protein synthesis. Genetics **91**:215–227.
8. **Champney, W. S.** 1980. Protein synthesis defects in temperature-sensitive mutants of *Escherichia coli* with altered ribosomal proteins. Biochim. Biophys. Acta **609**:464–474.
9. **Chinali, G., E. Nyssen, M. DiGiambattista, and C. Cocito.** 1988. Action of erythromycin and virginiamycin S on polypeptide synthesis in cell-free systems. Biochim. Biophys. Acta **951**:42–52.
10. **Chittum, H. S., and W. S. Champney.** Erythromycin inhibits the assembly of the large ribosomal subunit in *Escherichia coli*. Curr. Microbiol., in press.
11. **Cooperman, B. S., C. J. Weitzmann, and C. L. Fernandez.** 1990. Antibiotic probes of *Escherichia coli* peptidyltransferase, p. 491–501. *In* W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner (ed.), The ribosome: structure, function, and evolution. American Society for Microbiology, Washington, D.C.
12. **Corcoran, J. W.** 1984. Mode of action and resistance mechanisms of macrolides, p. 231–259. *In* S. Omura (ed.), Macrolide antibiotics. Academic Press, Orlando, Fla.
13. **Cundliffe, E.** 1987. On the nature of antibiotic binding sites in ribosomes. Biochimie **69**:863–869.
14. **Cundliffe, E.** 1990. Recognition sites for antibiotics within rRNA, p. 479–490. *In* W. E. Hill, A. Dahlberg, R. A. Garrett, P. B. Moore, D. Schlessinger, and J. R. Warner (ed.), The ribosome: structure, function, and evolution. American Society for Microbiology, Washington, D.C.
15. **Douthwaite, S.** 1992. Functional interactions within 23S rRNA involving the peptidyltransferase center. J. Bacteriol. **174**:1333–1338.
16. **Douthwaite, S., J. B. Prince, and H. F. Noller.** 1985. Evidence for functional interaction between domains II and V of 23S ribosomal RNA from an erythromycin-resistant mutant. Proc. Natl. Acad. Sci. USA **82**:8330–8334.
17. **Ehrlich, H. A., D. H. Gelfand, and R. S. Saiki.** 1988. Specific DNA amplification. Nature (London) **331**:461–462.
18. **Ettayebi, M., S. M. Prasad, and E. A. Morgan.** 1985. Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*. J. Bacteriol. **162**:551–557.
19. **Fukuzawa, H., T. Kohchi, T. Sano, H. Shirai, K. Umesono, H. Inokuchi, H. Ozeki, and K. Ohyama.** 1988. Structure and organization of *Marchantia polymorpha* chloroplast genome. III. Gene organization of the large single copy region from *rbcL* to *trnI* (CAU). J. Mol. Biol. **203**:333–351.
20. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring.** 1981. The molecular basis of antibiotic action. John Wiley & Sons, New York.
21. **Gantt, J. S., S. L. Baldauf, P. J. Calie, N. F. Weeden, and J. D. Palmer.** 1989. Transfer of rpl22 to the nucleus greatly preceded its loss from the chloroplast and involved the gain of an intron. EMBO J. **10**:3073–3078.
22. **Gross, U., J. H. Chen, D. H. Kono, J. G. Lobo, and D. T. Y. Yu.** 1989. High degree of conservation between *Yersinia pseudotuberculosis* and *Escherichia coli*. Nucleic Acids Res. **17**:3601–3602.
23. **Guthrie, C., H. Nashimoto, and M. Nomura.** 1969. Structure and function of *E. coli* ribosomes. VIII. Cold-sensitive mutants defec-

tive in ribosomal assembly. Proc. Natl. Acad. Sci. USA **63**:384–391.
24. **Hampl, H., H. Schultze, and K. H. Nierhaus.** 1981. Ribosomal components from *Escherichia coli* 50S subunits involved in the reconstitution of peptidyltransferase activity. J. Biol. Chem. **256**: 2284–2288.
25. **Hardy, S. J. S., C. G. Kurland, P. Voynow, and G. Mora.** 1969. The ribosomal proteins of *Escherichia coli*. I. Purification of the 30S ribosomal proteins. Biochemistry **8**:2897–2905.
26. **Herold, M., and K. H. Nierhaus.** 1987. Incorporation of six additional proteins to complete the assembly map of the 50S subunit from *Escherichia coli* ribosomes. J. Biol. Chem. **262**:8826–8833.
27. **Hiratsuka, J., H. Shimada, R. Whittier, T. Ishibashi, M. Sakamoto, M. Mori, C. Kondo, Y. Honji, C. R. Sun, B. Y. Meng, Y. Q. Li, A. Kanno, Y. Nishizawa, A. Hirai, K. Shinozaki, and M. Sugiura.** 1989. The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of cereals. Mol. Gen. Genet. **217**:185–194.
28. **Kao, J. S., and M. Wu.** 1990. The sequence of the plastid encoded rpl22 protein in marine macroalgae *Gracilaria tenuistipitata*. Nucleic Acids Res. **18**:3067.
29. **Kenny, J. W., J. M. Lambert, and R. R. Traut.** 1979. Cross-linking of ribosomes using 2-iminothiolane (methyl-4 mercaptobutyrimidate) and identification of cross-linked proteins by diagonal polyacrylamide sodium dodecyl gel electrophoresis. Methods Enzymol. **59**:534–550.
30. **Kromer, W. J., T. Hatakeyama, and M. Kimura.** 1990. Nucleotide sequences of *Bacillus stearothermophilus* ribosomal protein genes: part of the S10 operon. Biol. Chem. Hoppe-Seyler **371**:631–636.
31. **Kunkel, T. A.** 1990. Misalignment-mediated DNA synthesis errors. Biochemistry **29**:8003–8011.
32. **Laine, R. O., P. J. Laipis, N. F. Shay, and M. S. Kilberg.** 1991. Identification of an amino acid-regulated mRNA from rat liver as the mammalian equivalent of bacterial ribosomal protein L22. J. Biol. Chem. **266**:16969–16972.
33. **Lamb, A. J., G. D. Clark-Walker, and A. W. Linnane.** 1968. The differentiation of mitochondrial and cytoplasmic protein synthesizing systems *in vitro* by antibiotics. Biochim. Biophys. Acta **161**:415–427.
34. **LeGrice, S. F. J., and H. Matzura.** 1981. Binding of RNA polymerase and the catabolite gene activator protein within the *cat* promoter in *Escherichia coli*. J. Mol. Biol. **150**:185–196.
35. **Mao, J. C.-H., and M. Putterman.** 1969. The intermolecular complex of erythromycin and the ribosome. J. Mol. Biol. **44**:347–361.
36. **Mets, L. J., and L. Bogorad.** 1971. Mendelian and uniparental alterations in erythromycin binding by plastid ribosomes. Science **174**:707–709.
37. **Michalowski, C. B., B. Pfanzagal, W. Loeffelhardt, and H. J. Bohnert.** 1990. The cyanelle S10 spc ribosomal protein gene operon from *Cyanophora paradoxa*. Mol. Gen. Genet. **224**:222–231.
38. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Nakayama, I.** 1984. Macrolides in clinical practice, p. 261–300. *In* S. Omura (ed.), Macrolide antibiotics. Academic Press, Orlando, Fla.
40. **Nomura, M., and F. Engbaek.** 1972. Expression of ribosomal protein genes as analyzed by bacteriophage Mu-induced mutations. Proc. Natl. Acad. Sci. USA **69**:1526–1530.
41. **Ohkubo, S., A. Muto, Y. Kawauchi, F. Yamao, and S. Osawa.** 1987. The ribosomal protein gene cluster of *Mycoplasma capricolum*. Mol. Gen. Genet. **210**:314–322.
42. **Omura, S. (ed.).** 1984. Macrolide antibiotics. Academic Press, Orlando, Fla.
43. **Pardo, D., and R. Rosset.** 1974. Genetic studies of erythromycin-resistant mutants of *Escherichia coli*. Mol. Gen. Genet. **135**:257–268.
44. **Pardo, D., and R. Rosset.** 1977. New ribosomal mutation which affects the two ribosomal subunits in *Escherichia coli*. Mol. Gen. Genet. **153**:199–204.
45. **Pardo, D., and R. Rosset.** 1977. Properties of ribosomes from erythromycin resistant mutants of *Escherichia coli*. Mol. Gen. Genet. **156**:267–271.
46. **Pestka, S.** 1974. Binding of [^{14}C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Agents Chemother. **6**:474–478.
47. **Ripley, L. S.** 1982. Model for the participation of quasi-palindromic DNA sequences in frameshift mutation. Proc. Natl. Acad. Sci. USA **79**:4128–4132.
48. **Schnier, J., H.-S. Gewitz, S.-E. Behrens, A. Lee, C. Ginther, and T. Leighton.** 1990. Isolation and characterization of *Bacillus stearothermophilus* 30S and 50S ribosomal protein mutations. J. Bacteriol. **172**:7306–7309.
49. **Sharrock, R. A., T. Leighton, and H. G. Wittmann.** 1981. Macrolide and aminoglycoside antibiotic resistance mutations in the *Bacillus subtilis* ribosome resulting in temperature-sensitive sporulation. Mol. Gen. Genet. **183**:538–543.
50. **Sigmund, C. D., and E. A. Morgan.** 1982. Erythromycin resistance due to a mutation in a ribosomal RNA operon of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79**:5602–5606.
51. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Tai, P.-C., and B. D. Davis.** 1979. Action of antibiotics on chain-initiating and on chain-elongating ribosomes. Methods Enzymol. **59**:851–862.
53. **Tanaka, M., T. Wakasugi, M. Sugita, K. Shinozaki, and M. Sugiura.** 1986. Genes for the eight ribosomal proteins are clustered on the chloroplast genome of tobacco (*Nicotiana tabacum*): similarity to the S10 and spc operons of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:6030–6034.
54. **Teraoka, H.** 1970. A reversible change in the ability of *Escherichia coli* ribosomes to bind to erythromycin. J. Mol. Biol. **48**:511–515.
55. **Teraoka, H., and K. H. Nierhaus.** 1978. Proteins from *Escherichia coli* ribosomes involved in the binding of erythromycin. J. Mol. Biol. **126**:185–193.
56. **Tipper, D. J., C. W. Johnson, C. L. Ginther, T. Leighton, and H. G. Wittmann.** 1977. Erythromycin resistant mutations in *Bacillus subtilis* cause temperature sensitive sporulation. Mol. Gen. Genet. **150**:147–159.
57. **Watson, J. C., and S. J. Surzycki.** 1983. Both the chloroplast and nuclear genomes of *Chlamydomonas reinhardi* share homology with *Escherichia coli* genes for transcriptional and translational components. Curr. Genet. **7**:201–210.
58. **Wittmann, H. G., G. Stoffler, D. Apirion, L. Rosen, K. Tanaka, M. Tamaki, R. Takata, S. Dekio, E. Otaka, and S. Osawa.** 1973. Biochemical and genetic studies on two different types of erythromycin resistant mutants of *Escherichia coli* with altered ribosomal proteins. Mol. Gen. Genet. **127**:175–189.
59. **Zengel, J. M., and L. Lindahl.** 1993. Domain I of 23S rRNA competes with a paused transcription complex for ribosomal protein L4 of *Escherichia coli*. Nucleic Acids Res. **21**:2429–2435.
60. **Zhou, D. X., F. M. Quigley, O. Massenet, and R. Mache.** 1989. Cotranscription of the S10- and spc-like operons in spinach chloroplasts and identification of three of their gene products. Mol. Gen. Genet. **216**:439–445.
61. **Zurawski, G., and S. M. Zurawski.** 1985. Structure of the *Escherichia coli* S10 ribosomal protein operon. Nucleic Acids Res. **13**:4521–4526.