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Evidence for functional interaction between domains II and V of 23S ribosomal RNA from an erythromycin-resistant mutant

(*in vitro* mutagenesis/ribosomal RNA genetics/peptidyl transferase)

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ABSTRACT **A mutation affording low levels of erythromycin resistance has been obtained by *in vitro* hydroxylamine mutagenesis of a cloned ribosomal RNA operon from *Escherichia coli*. The site of the mutational event responsible for antibiotic resistance was localized to the gene region encoding domain II of 23S rRNA by replacement of restriction fragments in the wild-type plasmid by corresponding fragments from the mutant plasmid. DNA sequencing showed that positions 1219–1230 of the 23S rRNA gene are deleted in the mutant. Since all previously characterized rRNA mutations conferring resistance to erythromycin show changes exclusively in domain V, our present findings provide direct evidence for functional interaction between domains II and V of 23S rRNA.**

Efforts to understand the role of rRNA in translation have led to the identification of several putative functional sites in 16S- and 23S-like rRNAs (reviewed in ref. 1). Our present knowledge of the structural organization of these sites is limited to a description of their secondary structures and, in some cases, their locations relative to the electron microscopic models for the ribosomal subunits (reviewed in ref. 2). Deeper insight into the mechanisms of rRNA function would undoubtedly result from information relevant to the three-dimensional arrangement of these sites.

The secondary structure models for the rRNAs suggest that they are organized into discrete structural domains (1). The strong phylogenetic conservation of these domains raises the question as to whether this has some important functional basis, as has been established for many proteins. Specifically, is it possible that there is functional interaction between rRNA domains during the course of protein synthesis? One of the most direct approaches, chemical crosslinking, has provided evidence for close proximity between specific regions of different rRNA domains (reviewed in ref. 3).

Genetic approaches, which are potentially among the most powerful in exploring the functional and structural organization of rRNA, have yet to be extensively exploited in this context. To a great extent, this is because of the difficulty of performing genetic analysis on an essential gene that is present in seven copies in the genome of *Escherichia coli*, the reference organism for study of ribosomes. This problem has been alleviated by using rRNA operons that have been cloned in high-copy-number plasmids. This approach has permitted isolation of rRNA mutants from *E. coli* that show observable phenotypic changes (4), including antibiotic resistance (5).

In this paper we describe an erythromycin-resistant mutant, obtained by random *in vitro* mutagenesis of a cloned rRNA operon. The site of mutation lies in domain II of 23S rRNA, remote from all previously identified erythromycin-resistant rRNA mutations, which have been localized to a small region in domain V (6, 7). We take this to be direct evidence for functional interaction between two different domains in a rRNA.

MATERIALS AND METHODS

Cell Strains and Medium. *E. coli* strain DH1 [F^-, *recA1*, *endA1*, *gyrA96*, *thi1*, *hsdR17* (r_k^-, m_k^+), *supE44*, λ^-] (8) was used as a plasmid host. *E. coli* strain JM103 [Δ(*lac-pro*), *thi*, *strA*, *supE*, *endA*, *sbcB*, *hsdR*$^-$, F′*traD36*, *proAB*, *lacI*^{q}, *lacZ* Δ*M15*] (9) was employed for growth of M13 recombinants. LB medium contained 10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride, and 10 mg of thiamine per liter; all incubations were at 37°C. Antibiotics were obtained from Sigma.

***In Vitro* DNA Manipulations.** All enzymes used in the digestion, ligation, and sequencing of DNA were purchased from New England Biolabs or Bethesda Research Laboratories and were used according to the suppliers' recommendations. Deoxy- and dideoxynucleoside triphosphates were purchased from P-L Biochemicals. All gel systems were buffered with 50 mM Tris borate, pH 8.3/1 mM EDTA. Elution of DNA fragments from agarose gels, cell transformations using calcium chloride, and plasmid preparations by alkaline lysis and by cleared lysate formation in conjunction with cesium chloride gradients were all performed by standard procedures described and cited in ref. 8.

***In Vitro* Mutagenesis.** The 11.9-kilobase-pair (kbp) plasmid pKK3535, containing the entire *rrnB* operon of *E. coli* (ref. 10; Fig. 1*a*), was employed as a target for hydroxylamine mutagenesis. A modified procedure described in ref. 11 was used to create on average one mutation per plasmid. A 100-μl mixture of ethylene glycol:water, 50:50 (vol/vol), containing 1 M hydroxylamine, 35 mM potassium phosphate (pH 5.2), 0.3 M sodium acetate, and 10 μg of plasmid DNA was incubated at 65°C; 50-μl aliquots were removed after 10 and 30 min and diluted to 200 μl with 0.3 M sodium acetate. The DNA was recovered by ethanol precipitation and redissolved at 0.05 mg/ml in 20 mM Tris·HCl, pH 8.0/1 mM EDTA.

Selection for Resistant Mutants. Competent cells were transformed with up to 100 ng of mutagenized plasmid, plated onto LB agar containing either (*i*) 20 μg of ampicillin per ml or (*ii*) 20 μg of ampicillin per ml and 100 μg of erythromycin per ml, and grown for 20 and 48 hr, respectively. Ampicillin was maintained in all media during the initial selection stages as, in the absence of this antibiotic, nontransformed cells were faster growing and capable of withstanding higher erythromycin concentrations than cells transformed with pKK3535 [DH1(pKK3535)]. The erythromycin concentration in growth media was raised in a stepwise manner to provide an increasingly selective advantage to cells that had acquired erythromycin resistance (5). Colonies from the

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Abbreviation: kbp, kilobase pair(s).

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ampicillin/erythromycin plates were transferred to LB broth with ampicillin at 20 μg/ml and erythromycin at 100 μg/ml, incubated for 24 hr, and plated onto LB agar containing erythromycin at 200 μg/ml. Colonies that formed on the latter plates after 48 hr were transferred to LB broth with 200 μg of erythromycin per ml. After 24 hr, cells were spread onto LB agar with erythromycin at 300 μg/ml as a final selection for resistant mutants. Transformation of fresh cells affirmed that the genotype for resistance was indeed plasmid encoded and not due to any change in the host cells. Gel analyses of plasmids and their restriction products confirmed that they corresponded in size and structure to pKK3535. Cells containing the plasmid pKK3535-118 from the 10-min hydroxylamine reaction remained viable longest and this plasmid was therefore particularly suitable for analysis.

Localization of a Mutational Change in pKK3535-118. All plasmid DNA manipulations, represented in schematic form in Fig. 2, were performed with pairs of restriction fragments, each cleaved within the ampicillin gene and at one other site. Subsequent selection for transformed cells on ampicillin plates minimized the occurrence of new recombinant plasmids with undesired deletions. After localizing the mutation to a 4.6-kbp region by *Pvu* I/*Sac* I fragment *in vitro* recombination in wild-type and mutant pKK3535 (Fig. 2*a*), a series of smaller plasmids was constructed to facilitate further recombinations. pSK41 and pSK51 were formed by combined *Xba* I and *Nae* I digestion of pKK3535 and pKK3535-118, respectively; the protruding ends were filled in with Klenow fragment and the four dNTPs; the 7.7-kbp digestion products were isolated and recircularized to include a *Bgl* II linker (Fig. 1*b*). The 11.9-kbp plasmids pKK3535A, -B, -C, -D, -E, and -F, the latter four formed via recombination in pSK plasmids, were analyzed for size and composition by restriction endonuclease digestion and gel electrophoresis. The growth rates of transformed cells were subsequently measured in the presence of erythromycin to track the portion of pKK3535-118 associated with erythromycin resistance. DNA sequencing was carried out in the M13 bacteriophage vectors mp8 and mp9 (12) by the dideoxynucleotide method (13).

RESULTS

Growth Characteristics of DH1/pKK3535-118. Random *in vitro* hydroxylamine mutagenesis of a cloned rRNA operon has produced a mutation conferring resistance to erythromycin. Cells bearing the mutant plasmid pKK3535-118 grow at approximately twice the erythromycin level tolerated by cells carrying the wild-type plasmid pKK3535. Compared with cells transformed with wild-type plasmid, mutant plasmid-transformed cells formed smaller colonies on ampicillin plates without erythromycin and exhibited longer doubling times in liquid medium (Table 1). We did not detect cross-resistance to several other antibiotics that inhibit ribosome function: on LB agar containing ampicillin at 25 μg/ml and either chloramphenicol at 1 μg/ml or hygromycin at 20 μg/ml or streptomycin at 2 μg/ml, DH1(pKK3535-118) formed slightly smaller colonies than DH1(pKK3535). No significant growth rate difference between DH1(pKK3535) and DH1-(pKK3535-118) could be detected in liquid medium with chloramphenicol (Table 1).

Localization of the Site of Mutation. The site of the mutation in pKK3535-118 conferring drug resistance was localized to a fragment convenient for sequencing by a series of *in vitro* DNA manipulations. Three main steps were necessary.

(*i*) Exchanging the 4.6-kbp *Sac* I/*Pvu* I fragments between pKK3535 and pKK3535-118 formed recombinants pKK-3535A and pKK3535B (Fig. 2*a*). pKK3535B, but not pKK-3535A, shows a drug-resistant phenotype identical to that of pKK3535-118. Erythromycin resistance was thus concluded to be encoded by the 4.6-kbp *Sac* I/*Pvu* I fragment of pKK-3535-118.

(*ii*) The second stage required the construction of pSK41 and pSK51 (Fig. 1*b*). These small plasmids offer two advantages: first, they lack the strong plasmid-encoded *rrnB* promoters, enabling cells to grow quicker and increasing the plasmid yield, and second, several multiple restriction sites have been eliminated, generating unique sites and thus simplifying recombination schemes. Exchanging the *Pst* I/*Sal* I fragments from pSK41 and pSK51 formed plasmids pSK62 and pSK71 (Fig. 2*b*). Ligation of the 4.6-kbp *Pvu* I/*Sac* I fragments from these latter plasmids to the 7.2-kbp

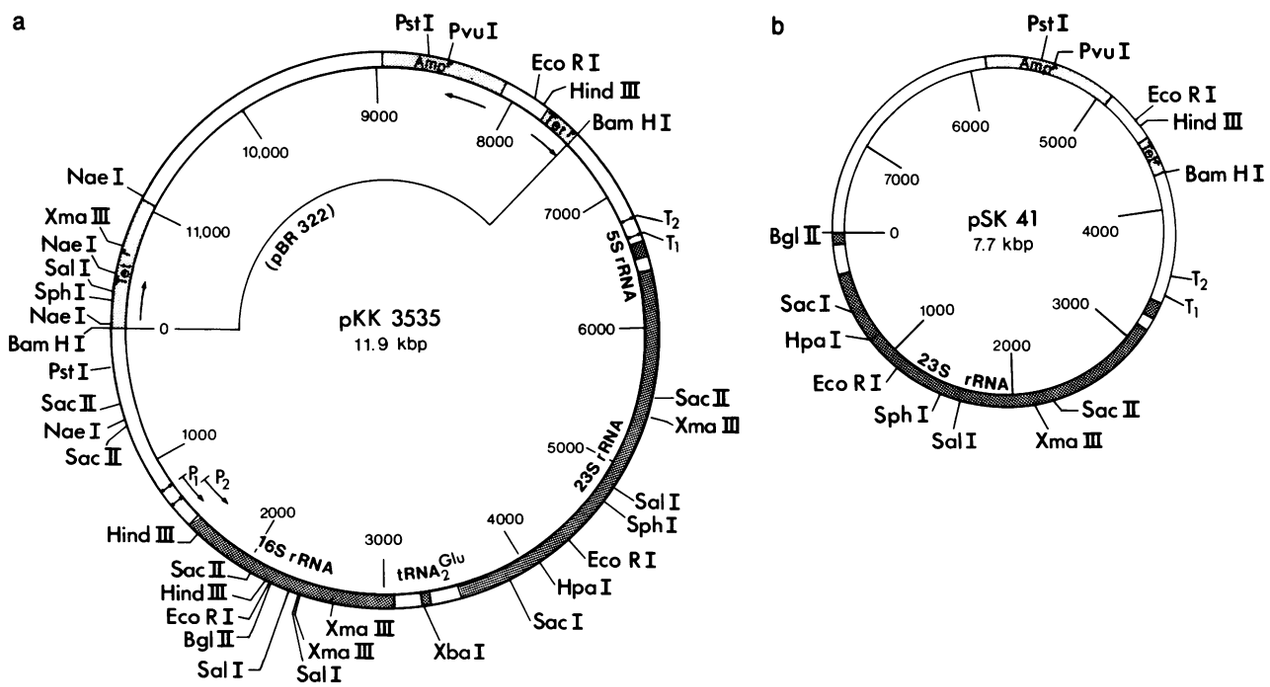

FIG. 1. Major restriction endonuclease sites in pKK3535 (10) (*a*) and pSK41 (*b*). The restriction maps of these plasmids are identical to pKK3535-118 and pSK51, respectively, except for the *Sph* I site that is missing from the 23S rRNA gene in the latter plasmids.

Table 1. Effects of antibiotics on the doubling times of cells containing mutant or wild-type plasmids

Antibiotic, μg/ml			Doubling time, min	
Ampicillin	Chloramphenicol	Erythromycin	DH1(pKK3535, -A, -D, and -E) (wild-type)	DH1(pKK3535-118, -B, -C, and -F) (mutant)
25	—	—	40 ± 1.2	46 ± 1.8
25	—	80	∞	72 ± 4.7
—	—	150	∞	101 ± 6.2
25	1	—	94 ± 9.1	88 ± 9.2

Doubling times were calculated from growth curves at optical densities between 0.1 and 0.4 at 650 nm. Each growth curve was determined an average of eight times. Standard errors are given. ∞ indicates that cells did not attain an optical density of 0.4.

Pvu I/*Sac* I fragment of pKK3535 created plasmids pKK3535C and pKK3535D (Fig. 2*c*). Of these two plasmids, only pKK3535C affords erythromycin resistance. The functional mutation was thus confined to the 1-kbp *Sac* I/*Sal* I fragment of pKK3535-118.

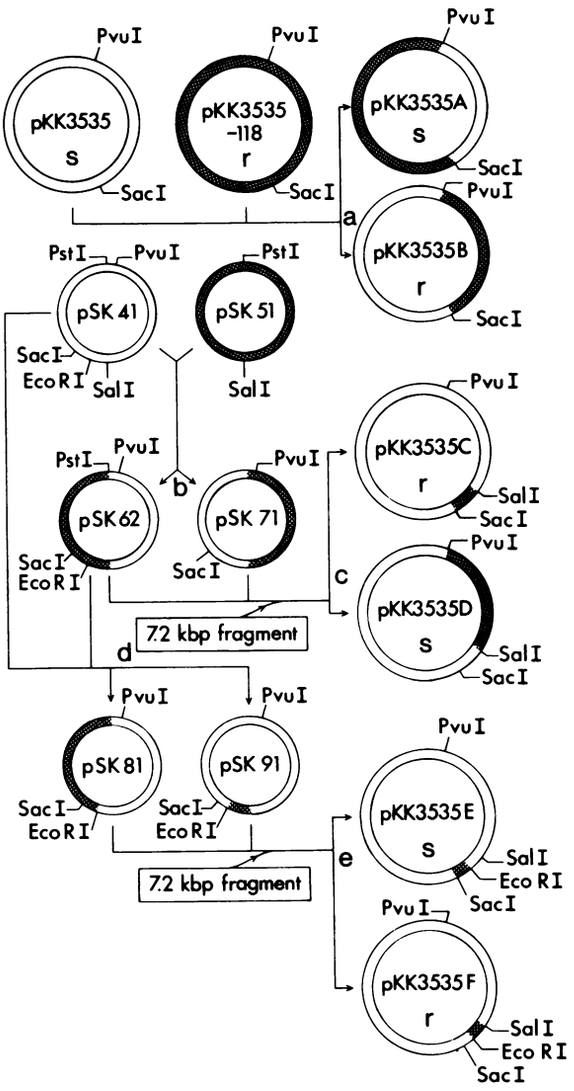

FIG. 2. Recombinations by which the 0.5-kbp fragment coding for erythromycin resistance was determined. pKK3535-type plasmids exhibiting erythromycin-resistant or -sensitive phenotypes are indicated by "r" and "s," respectively. The shaded regions of DNA originate from the mutant plasmid pKK3535-118.

(*iii*) In the final series of recombinations, pSK41 and pSK62 were cleaved by *Pst* I followed by either partial or complete *Eco*RI digestion to isolate the 4.3-kbp and 3.4-kbp *Pst* I/*Eco*RI fragments, respectively. The fragments were ligated to form pSK81 and pSK91 (Fig. 2*d*). Ligation of the 4.6-kbp *Pvu* I/*Sac* I fragments from these plasmids with the 7.2-kbp *Pvu* I/*Sac* I fragment of pKK3535 formed plasmids pKK3535E and pKK3535F. pKK3535F (but not pKK3535E) shows the mutant phenotype, establishing that erythromycin resistance is encoded by the 0.5-kbp *Eco*RI/*Sal* I portion of the 23S rRNA gene.

Dideoxynucleotide sequencing of both strands of the 0.5-kbp *Eco*RI/*Sal* I fragment revealed that a 12-base deletion had occurred in a region overlapping the *Sph* I site corresponding to positions 1219–1230 of 23S rRNA (Fig. 3*a*). No other changes were detected. Restriction digestion analysis with *Sph* I together with *Eco*RI or *Hin*dIII confirmed that the 23S rRNA gene *Sph* I site is missing from pKK3535-118, -B, -C, and -F (Fig. 3*b*).

DISCUSSION

The hydroxylamine-induced mutation responsible for erythromycin resistance has been identified as a 12-nucleotide deletion comprising positions 1219–1230 of the *rrnB* 23S rRNA gene. Although hydroxylamine mutagenesis normally causes cytidine to uridine transitions, this is not incompatible with the result observed here. The deleted sequence (Fig. 3*a*)

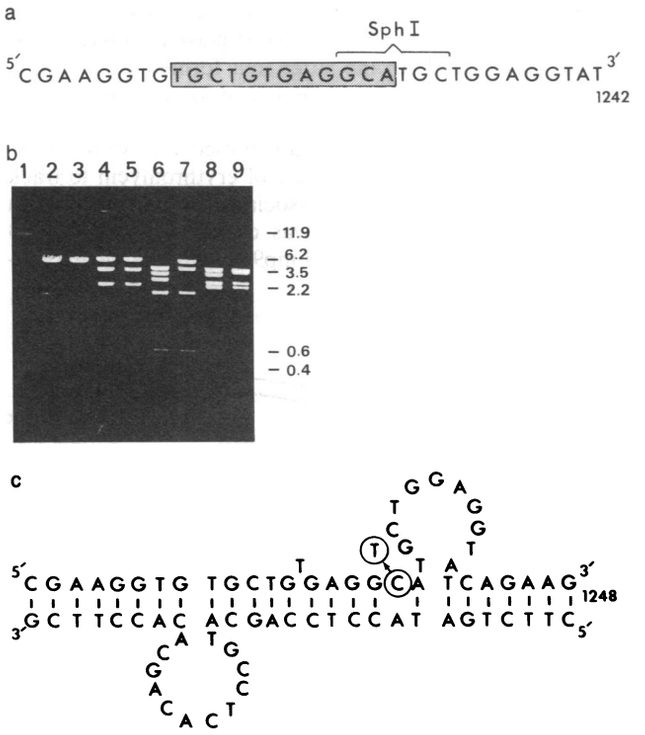

FIG. 3. (*a*) Sequence of the portion of the wild-type 23S rRNA gene within which the 12-base pKK3535-118 deletion occurred (shaded area). (*b*) Analysis of pKK3535-type plasmids on a 0.8% agarose gel. Lane 1, pKK3535, undigested. Lanes 2–9, pKK3535 and pKK3535-118 digested with *Hin*dIII (lanes 2 and 3, respectively), *Eco*RI (lanes 4 and 5, respectively), *Hin*dIII/*Sph* I (lanes 6 and 7, respectively), and *Eco*RI/*Sph* I (lanes 8 and 9, respectively). The restriction patterns of pKK3535A, -D, and -E were identical to pKK3535, and those of pKK3535B, -C, and -F were identical to pKK3535-118 (data not shown). Sizes are given in kbp. (*c*) One possible mechanism by which the deletion could have occurred involving transition of cytidine-1229 and rearrangement of the base pairing of the two strands.

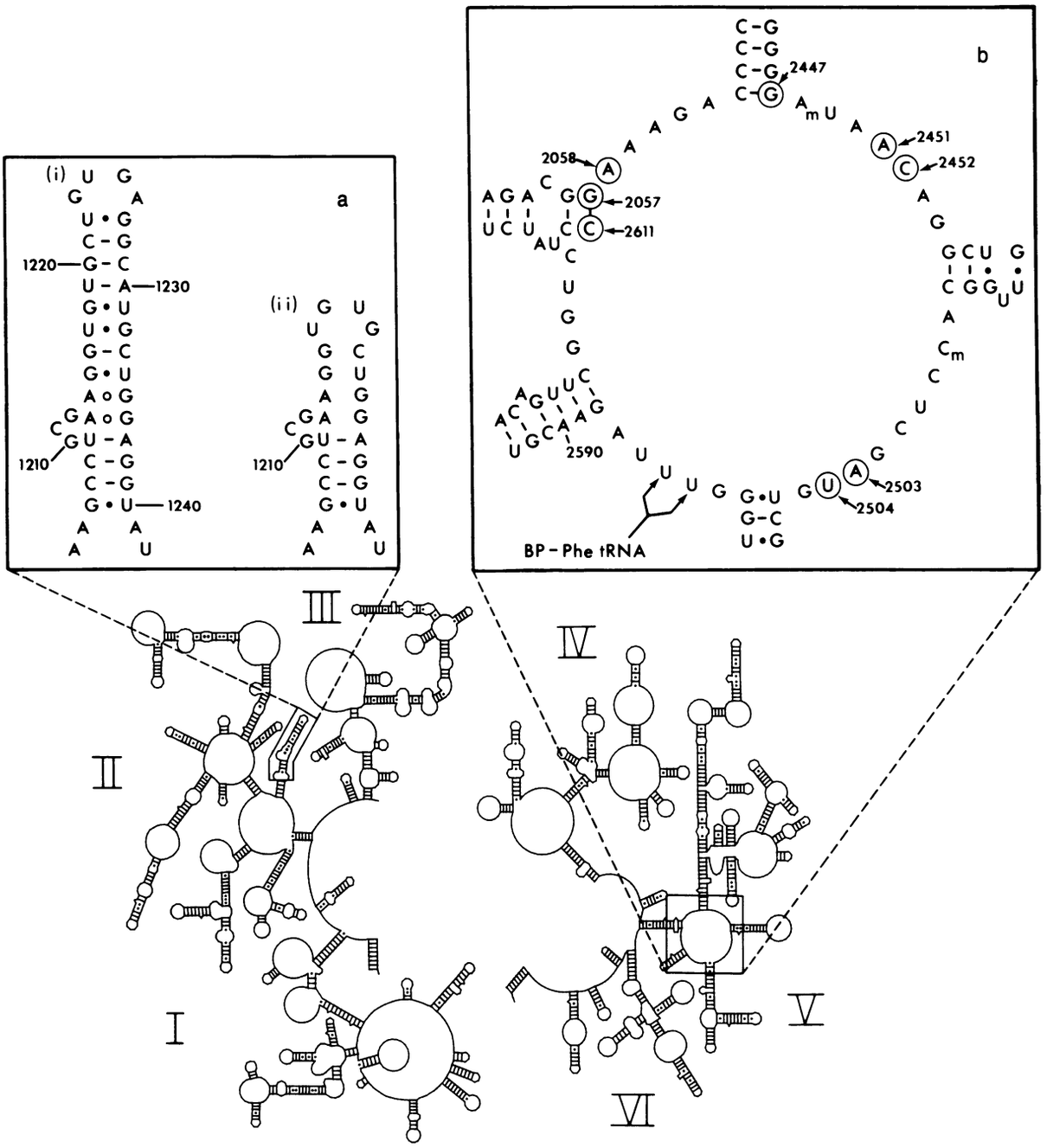

FIG. 4. Schematic representation of *E. coli* 23S RNA secondary structure (18). (*a*) Expanded view of the (*i*) wild-type and (*ii*) mutagenized stem 1213/1236 in domain II. (*b*) Expanded view of the central loop of domain V showing sites of mutation conferring resistance to erythromycin (guanosine-2057, adenosine-2058, and cytidine-2611) and chloramphenicol (guanosine-2057, guanosine-2447, adenosine-2451, cytidine-2452, adenosine-2503, and uridine-2504) in domain V, reviewed in refs. 6 and 7. BP-Phe tRNA, sites of affinity labeling by benzophenone-derivatized Phe-tRNA (19).

is part of a tandem, directly repeated sequence in the 23S rRNA gene. Hydroxylamine modification of one or both strands of the DNA in this region could result in looping out of two portions of the helix favoring the alternative base pairing shown (Fig. 3*c*). Subsequent repair events could generate the observed deletion.

Although the deletion mutation confers a selective advantage on cells in the presence of erythromycin, in the absence of this antibiotic the mutation is detrimental. This is apparent from the slower growth of DH1(pKK3535-118) compared with DH1(pKK3535) in LB/ampicillin. Dahlberg and coworkers have demonstrated that even single base deletions in the large rRNA genes can be inhibitory to cell growth (4). These observations indicate that plasmid-encoded *rrnB* is expressed in the absence of erythromycin. In addition, it has been suggested that amplification of the plasmid copy number, and thus plasmid-encoded products, is induced by low levels of erythromycin (5). In cells containing plasmid-encoded rRNA mutants conferring erythromycin resistance, this would lead to a fuller expression of resistance to the drug.

Complete inhibition of protein synthesis is effected by a single erythromycin molecule per ribosome, but the mechanism of inhibition remains unclear (14). Acquisition of erythromycin resistance through changes in rRNA has previously been associated solely with a confined region in the central loop of domain V of the large subunit rRNA (Fig. 4). The high degree of phylogenetic conservation of sequence in this region indicates that the integrity of the primary structure is

of extreme functional importance. Transition, transversion, or methylation of adenosine-2058, or homologous nucleotide, has been shown to confer resistance to erythromycin in yeast mitochondria (15), *E. coli* (16), and *Bacillus stearothermophilus* (17). In yeast mitochondria, erythromycin resistance has also been obtained by a cytidine to guanosine transversion of the base equivalent to *E. coli* cytidine-2611 (6) that has been proposed to base pair to guanosine-2057 (18). Transition of the latter base produces dual resistance to erythromycin and chloramphenicol in *E. coli* (7). Within this region, benzophenone-derivatized Phe-tRNA has been photocrosslinked to uridine-2584 and uridine-2585 (19). As the crosslinked phenylalanine derivative could still participate in peptide bond formation, and because several point mutations causing chloramphenicol resistance have been mapped in close proximity (Fig. 4), it seems likely that this sequence is an integral component of the peptidyl transferase site (19). UV crosslinking studies by Brimacombe and co-workers have demonstrated that the sequences 571–577 and 739–748 in domain II are proximal to positions 2030–2032 and 2609–2618, respectively, in domain V (20). These structural data are supported by the result presented here, which, additionally, establishes a functional link between domains II and V of 23S rRNA.

Understanding how the observed deletion results in erythromycin resistance would be a first step in explaining the functional connection between domains II and V. The simplest explanation, that the deleted stem constitutes part of the antibiotic binding site, seems unlikely. Erythromycin inhibits protein synthesis in a phylogenetically diverse range of bacteria and organelles and we would therefore expect the ribosomal binding site of erythromycin to be conserved. However, the primary structure of the stem between positions 1213 and 1236 shows high phylogenetic variability. In addition, although cells possessing the uridine-2058 transversion in 23S RNA can withstand at least 10 times the erythromycin level that prevents detectable growth of wild-type cells (5), deletion of positions 1219–1230 enables cells to tolerate only twice the levels of antibiotic as wild-type cells. Although differences in *E. coli* host strains could influence these measurements, the two forms of erythromycin resistance appear to be qualitatively distinct. These latter observations are more consistent with the idea that the resistance observed here is the indirect consequence of perturbation of higher-order structure. One plausible explanation for such perturbation is that the deleted helix is involved in a direct RNA–RNA tertiary interaction. An alternative explanation is suggested by the secondary structure of helix 1213–1236 (Fig. 4). This helix shows guanosine-adenosine juxtapositions intermingled with guanosine-uridine and Watson–Crick base pairs, a pattern which is shared by some helices that have been shown to bind r-proteins (21). The helix containing residues 1219–1230 could be part of the binding site for a protein that is involved in quaternary interactions bridging domains II and V in 23S RNA.

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