A novel site of antibiotic action in the ribosome: Interaction of evernimicin with the large ribosomal subunit

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Evernimicin (Evn), an oligosaccharide antibiotic, interacts with the large ribosomal subunit and inhibits bacterial protein synthesis. RNA probing demonstrated that the drug protects a specific set of nucleotides in the loops of hairpins 89 and 91 of 23S rRNA in bacterial and archaeal ribosomes. Spontaneous Evn-resistant mutants of *Halobacterium halobium* contained mutations in hairpins 89 and 91 of 23S rRNA. In the ribosome tertiary structure, rRNA residues involved in interaction with the drug form a tight cluster that delineates the drug-binding site. Resistance mutations in the bacterial ribosomal protein L16, which is shown to be homologous to archaeal protein L10e, cluster to the same region as the rRNA mutations. The Evn-binding site overlaps with the binding site of initiation factor 2. Evn inhibits activity of initiation factor 2 *in vitro*, suggesting that the drug interferes with formation of the 70S initiation complex. The site of Evn binding and its mode of action are distinct from other ribosome-targeted antibiotics. This antibiotic target site can potentially be used for the development of new antibacterial drugs.

rRNA | ribosomal protein | antibiotics | L16 | drug resistance

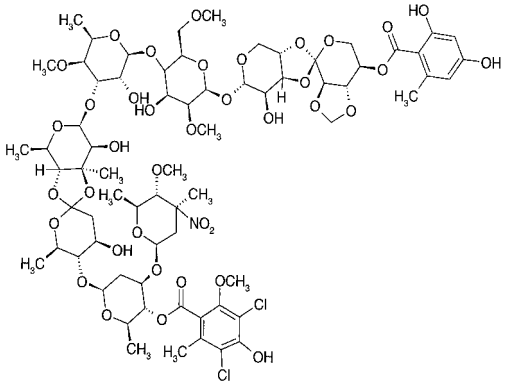

Fig. 1. Structure of Evn.

A large number of natural and synthetic antibiotics inhibit protein synthesis. The majority of these drugs act on ribosome (1). RNA accounts for two-thirds of the ribosomal weight and is responsible for the most critical functions of the ribosome, including decoding of genetic information and catalysis of peptide bond formation (2–4). Therefore, it is not surprising that ribosome-targeted antibiotics interact primarily with rRNA. The enormous size of the ribosome provides numerous possibilities for small molecules to bind to different segments of rRNA and/or ribosomal proteins. Remarkably, however, only a few sites are used by known antibiotics. Thus, in the large ribosomal subunit, most of the clinically important antibiotics interact with various segments of the central loop of domain V; these drugs inhibit either activity of the catalytic peptidyl transferase center or prevent growth of the nascent peptide chain (1, 5).

Extensive use of antibiotics has led to the appearance and rapid spread of drug-resistant pathogens. Unfortunately, because of the overlap of binding sites of different drugs on the ribosome, a single resistance mechanism can confer resistance to several different antibiotics (6). Because of this phenomenon a significant effort is invested in identifying compounds that will be effective against organisms that have developed resistance to previously used drugs. It is expected that drugs that bind to "new" ribosomal sites may constitute the next generation of ribosome-targeted antibiotics. An interesting group of such compounds are the oligosaccharide orthosomycins, the best-characterized member of which is evernimicin (Evn) (Fig. 1). Evn inhibits bacterial protein synthesis by binding to a single high affinity site on the large ribosomal subunit (7). Importantly, bacteria that have developed resistance to other drugs in current clinical usage do not exhibit cross-resistance to Evn. Furthermore, other ribosome-targeted drugs, except for the structurally similar drug avilamycin, do not compete with Evn for binding to the ribosome, suggesting that it interacts with a novel ribosomal site (7). The precise location of a Evn-binding site on the ribosome has not been determined. Evn-resistant mutants of *Streptococcus pneumoniae* were isolated that contained either single amino acid substitutions in the ribosomal protein L16 or nucleotide substitutions in the 23S rRNA (8, 9). However, it was not determined whether the mutations interfered with drug binding and, in the case of rRNA mutations, the possibility that second site mutations contributed to the resistance was not ruled out. The precise mechanism of Evn action also remains obscure, although it was suggested that the drug inhibits elongation of translation (7). Finally, Evn inhibited assembly of the large ribosomal subunit (10); however, the mechanism underlying such inhibition is poorly understood.

Here we present results of biochemical and genetic experiments designed to define the Evn-binding site on the ribosome and to determine the mode of the drug action. We demonstrate that Evn interacts with a novel evolutionary conserved site in 23S rRNA that is not used by antibiotics of other classes. Biochemical data suggest that the drug inhibits protein synthesis by interfering with formation of the 70S initiation complex.

Materials and Methods

Preparation of Ribosomes. *Escherichia coli* ribosomes were prepared from the RNase I^- *E. coli* strain MRE 600 according to ref. 11. The only deviation from the published procedure was that after pelleting ribosomes through sucrose cushion containing 0.5 M NH_4Cl no additional salt washes were done. Ribosomal subunits were isolated according to ref. 12. *Halobacterium*

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Abbreviations: Evn, evernimicin; Ani, anisomycin; DMS, dimethyl sulfate; IF, initiation factor.

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halobium ribosomes were prepared from the exponential cultures of strain R1 (13) as described (14).

Footprinting of Evn on *E. coli* and *H. halobium* Ribosomes. Nonformulated Evn was dissolved in DMSO. Footprinting of Evn on *E. coli* ribosomes was performed in 50 μl buffer E (80 mM potassium cacodylate, pH 7.2/20 mM $MgCl_2$/100 mM NH_4Cl/1.5 mM DTT) containing ribosomes at 200 nM and Evn at final concentrations of 10 μM and 50 μM. After a 10-min incubation at 37°C dimethyl sulfate (DMS) or kethoxal probing was performed according to refs. 15 and 16.

Footprinting of Evn on *H. halobium* ribosomes was performed in 50 μl buffer H (80 mM potassium cacodylate, pH 7.2/100 mM $MgCl_2$/3 M KCl/1.5 mM DTT). Ribosomes and Evn were added as above and rRNA probing with DMS or kethoxal was performed as described (17).

Isolation of Evn-Resistant Mutants of *H. halobium*. *H. halobium* cells were grown at 37°C in liquid media or on agar plates as described (18, 19). For selection of *H. halobium* mutants Evn was solubilized as the clinical formulation at 620 μg/ml (as a control the same clinical formulation minus drug was added to all control plates and liquid cultures). It should be noted that because of the high salt content of the halobacterial growth medium a substantial portion of antibiotic precipitated under these conditions. Therefore the precise concentration of the drug in the selection plates was not known. A total of 10^8 cells of *H. halobium* were plated onto Evn-containing agar plates and colonies were analyzed after 7–10 days growth at 37°C. Twenty-three individual colonies were picked, and mutations were mapped by sequencing as follows. A segment of 23S rDNA was PCR-amplified directly from Evn^r *H. halobium* colonies by using a forward primer, GGCCCGGTGAACTGTACG (positions 2004–2021) and reverse primer, GTTCCTCTCGTACTATACG (positions 2649–2667). PCR products were purified by using a PCR purification kit (Promega) and sequenced by using *fmol* DNA sequencing system (Promega) without additional cloning.

Evn sensitivity of the wild-type and mutant cells was determined by E test (20). Specifically, 10^8 cells from an exponentially growing *H. halobium* culture were mixed with 5 ml of soft (0.6%) agar formed in *H. halobium* medium and poured onto a 1.5% agar plate. E strips with Evn concentrations ranging from 0.016 to 256 μg/ml (AB Biodisk, Solna, Sweden) were applied, and the minimal inhibitory concentrations were read after 5 days incubation at 37°C.

Introduction of A2471C and G2527A Mutations into Wild-Type *H. halobium*. A total of 10^8 cells of Evn^r *H. halobium* A2471C or G2527A mutants were plated onto agar plate containing 10 μg/ml anisomycin (Ani). Ani^r mutants arose after 7 days incubation and were shown to carry the C-to-T transition at position 2452 of the 23S rDNA gene (21) in addition to the original A2471C or G2527A mutations. A 2-kb fragment was amplified from the Ani^r/Evn^r double mutants by using primers CTGTTAATATTCCAGTGCCACC and GGCCACAACGAGCAACCCCAC and was used to transform wild-type *H. halobium* (18, 19). Transformants were selected on Ani (10 μg/ml) and clones that acquired both the Ani^r mutation C2452T, and either A2471C or G2527A were identified by direct sequencing. The Evn sensitivity of the transformants was tested by using E strips.

Peptidyl Transferase Assays. The effect of Evn on the peptidyl transferase activity was analyzed by using 70S ribosomes in a "standard" peptidyl transferase assay (22) or "fragment reaction" catalyzed by isolated large ribosomal subunits (23).

Initiation Factor IF2 Activity Assay. Construction of an *E. coli* strain overexpressing IF2 translation IF will be described elsewhere. IF2 was isolated by using the method described in ref. 24. A mRNA transcript with the sequence GGGAAUUCGGGCCCUUGUUAACAAUUAAGGAGGUAUACUAUGAAUGCAAUAAAUAACUGCAG$(A)_{21}$ (25) was prepared by *in vitro* transcription and purified according to ref. 26. IF2-dependent puromycin assay was performed as described (27). Specifically, 40 μl buffer A (50 mM Tris·HCl, pH 7.7/100 mM NH_4Cl/7 mM $MgCl_2$/1 mM DTT) containing 30 pmol of 30S subunits, 50 pmol of IF2, 15 pmol of f[^{3}H]Met-tRNA (170 dpm/pmol), 50 pmol of mRNA, and varying concentrations of Evn was incubated 15 min at 37°C. Ten microliters of buffer A supplemented with 1 mM GTP/5 mM puromycin and containing 15 pmol 50S ribosomal subunits was added, and the incubation continued for 15 min at 37°C. The reaction was stopped by the addition of 10 μl of 10 M NaOH. After 20-min incubation at 37°C, 200 μl of 1 M KH_2PO_4 (pH 7) were added, fMet-puromycin was extracted with 1 ml of ethyl acetate, and the radioactivity present in ethyl acetate phase was determined by liquid scintillation counting.

Similarity Search Between Bacterial Ribosomal Protein L16 and *Haloarcula marismortui* Ribosomal Proteins. The amino acid sequences of *H. marismortui* large ribosomal subunit proteins were obtained from the Protein Data Bank file containing atomic coordinates of the *H. marismortui* 50S subunit (accession number 1FFK) (28). Protein sequences were "fused" into a continuous amino acid sequence, and the best match between amino acid sequence of *S. pneumoniae* ribosomal protein L16 and the "fused" *H. marismortui* sequence was found by using pairwise sequence alignment algorithm (29) available at the Baylor College of Medicine Search Launcher web site, http://dot.imgen.bcm.tmc.edu.

Mapping of rRNA and Protein Mutations Within the Tertiary Structure of 50S Ribosomal Subunit. Atomic coordinates for rRNA and proteins of *H. marismortui* large ribosomal subunit (Protein Data Bank accession number 1FFK) (28) were used to map Evn resistance mutations in the tertiary structure of the large ribosomal subunits. The computer program RASMOL (30) was used for data analysis and figure preparation.

Results

Footprinting of Evn on Bacterial and Archaeal Ribosomes. The site of Evn binding in 23S rRNA was mapped by using RNA probing (15). Evn was complexed with *E. coli* 70S ribosomes, and accessibility of rRNA bases to DMS or kethoxal was analyzed. Primer extension analysis revealed that Evn protected a specific set of adenine residues in the hairpin 89, including A2468, A2469, A2476, A2478, and A2482. In addition, A2534 was protected in hairpin 91 (Fig. 2). Unexpectedly, we also observed an Evn-sensitive reverse transcriptase stop at position U2533 in the DMS-modified samples. DMS does not modify uridine residues (16) and mass spectroscopy and biochemical studies indicated an absence of posttranscriptional modifications at this position in 23S rRNA (ref. 31; J. A. McCloskey, P. F. Crain, and A.S.M., unpublished results). Therefore, we believe that the band corresponding to U2533 resulted from reverse transcriptase stuttering, which in turn depended on modification of A2534. No other protections were detected in either the 23S rRNA or the 5S rRNA. Although we cannot exclude the possibility that some of the observed protections resulted from changes in rRNA conformation induced by Evn binding, the most straightforward explanation of the footprinting results is that the drug forms specific tight contacts with at least some of the protected residues. Thus, rRNA must be an important component of a Evn binding site. A similar set of nucleotides to those seen protected in the *E. coli* ribosome also was protected by the drug in ribosomes from an Archaeon *H. halobium* (data not shown). In the latter case, an additional weak protection of G2529 in the hairpin 91 also was observed. The fact that the drug interacts in a similar fashion with ribosomes from evolutionary distant species indicates that the

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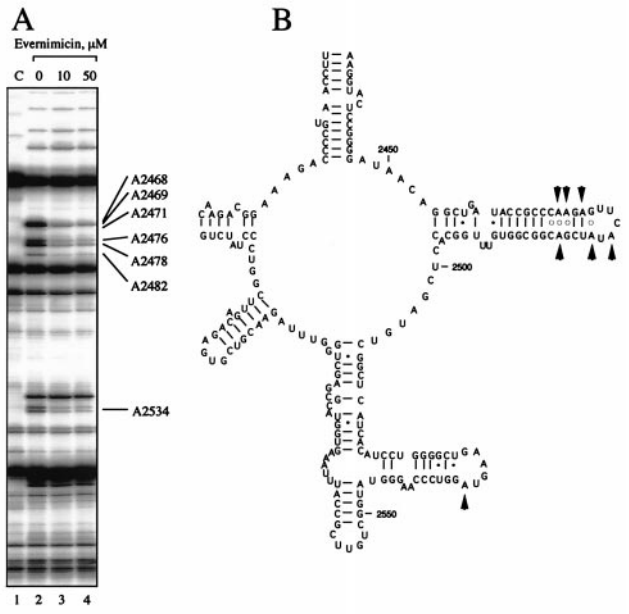

Fig. 2. Footprinting of Evn on the ribosome. (*A*) The primer extension gel that illustrates the effect of Evn on DMS modification of nucleotides in hairpins 89 and 91 of *E. coli* 23S rRNA. Lane 1, unmodified control; lane 2, ribosomes modified with DMS in the absence of Evn; lanes 3 and 4, ribosomes modified with DMS in the presence of 10 μM or 50 μM Evn, respectively. Nucleotides protected by Evn are indicated by lines. (*B*) Secondary structure of a segment of domain V of *E. coli* 23S rRNA containing hairpins 89 and 91 (28, 50). Nucleotides protected by Evn are indicated by arrowheads.

Evn binding site is evolutionary conserved, highlighting its possible functional importance.

Isolation of Evn-Resistant Mutants of *H. halobium*. Footprinting revealed the interaction of Evn with archaeal ribosomes. Furthermore, antibiotic sensitivity testing demonstrated that Evn efficiently inhibited growth of *H. halobium*. This organism, which possesses a single-copy rRNA operon in its genome (32, 33), was successfully used for the isolation of antibiotic resistance mutations in rRNA (reviewed in ref. 19). Therefore, to further define the Evn-binding site we isolated and characterized a number of Evn-resistant mutants of *H. halobium*. Spontaneous mutants were obtained by plating 10^8 *H. halobium* cells on Evn-containing agar plates. Approximately 40 colonies of different sizes appeared on the plate after 2 weeks incubation at 37°C. Mutations were mapped in 23 randomly picked resistant clones (34); all 23 clones had mutations in the 23S rRNA. This result clearly showed that rRNA constitutes the major component of the drug-binding site. Consistent with the footprinting data, all of the mutations (A2471G, A2471C, A2478C, U2479C, C2480A, C2480U, G2527A, U2528C, and G2535A, *E. coli* numeration) were clustered within hairpins 89 and 91 (Table 1 and Fig. 3). The mutants showed growth characteristics comparable to those of the wild-type cells, demonstrating that none of the mutations interfered severely with the ribosome function. The level of Evn resistance of isolated mutants was determined by E test (Table 1) (20). The mutants all exhibited an increase in minimal inhibitory concentration of at least 2 orders of magnitude whereas several mutations in hairpin 89 increased resistance beyond the limit of detection via E test. In RNA probing experiments, the drug failed to protect any RNA residues in ribosomes isolated from the Evn-resistant mutants (data not shown), presumably because the 23S rRNA mutations prevent drug binding.

Engineering the A2471C and G2527A Mutations in 23S rRNA Gene of Wild-Type *H. halobium*. Mutations conferring resistance to Evn arose in *H. halobium* at a frequency of *ca.* 10^{-7}; this frequency is

Table 1. Phenotype of Evn^r *H. halobium* mutants

Mutation	Number of isolates	MIC (μg/ml)*	Doubling time (min)†
Wild type	—	0.047	500 ± 10
A2471G	3	96	540 ± 40
A2471C	5	>256	560 ± 55
A2478C	2	>256	500 ± 25
U2479C	2	>256	520 ± 10
C2480A	6	>256	460 ± 70
C2480U	1	>256	570 ± 20
G2527A	1	8–12	490 ± 10
U2528C	1	8	510 ± 20
G2535A	2	8	500 ± 20

*Minimal inhibitory concentration (MIC).
†An average of three independent experiments.

consistent with single point mutations (19, 21). To rule out the possibility that a second site mutation contributed to the resistance we separately introduced two representative Evn^r mutations, A2471C, located in hairpin 89, and G2527A, located in hairpin 91, into wild-type *H. halobium* cells. To achieve this, double mutants were selected in which the chosen Evn mutations were combined with a mutation conferring resistance to the antibiotic Ani (21). A 2-kb segment of the rRNA operon containing Ani^r and Evn^r mutations was amplified from these strains and used to transform wild-type *H. halobium* to Ani resistance. DNA sequencing confirmed that the single rRNA operon of the transformed cells had acquired the Ani^r C2452U mutation together with A2471C or G2527A mutations. As can be seen in Fig. 4, the Evn resistance of the Ani^r/Evn^r transformants was identical to the resistance of the original mutants whereas the C2452U Ani^r mutation alone did not confer resistance to Evn. Because these double mutants were never exposed to Evn during transformation, they could not have accumulated any additional mutation(s) that could have contributed to Evn resistance. Therefore, the results of this experiment demonstrate that single nucleotide substitutions in hairpin 89 or 91 of the 23S rRNA are sufficient to confer Evn resistance.

Location of Evn Resistance Mutations in the Spatial Structure of the Large Ribosomal Subunit. The nucleotide atomic coordinates in the 2.4-Å x-ray structure of 50S subunit from *H. marismortui* were used to determine location of the rRNA mutations in the ribosome tertiary structure (28). *H. marismortui* and *H. halobium* are closely related archaea and therefore, *H. halobium* mutations could be directly "plotted" in the *H. marismortui* 50S subunit structure. As shown in Fig. 3, in the spatial structure of the 50S subunit, helices 89 and 91 are juxtaposed and sites of the mutations in two helices form a tight cluster, which apparently delineates the drug-binding site in 23S rRNA. The site is "sandwiched" between the helices and is readily accessible for the drug from the interface site of the 50S subunit. Access to the site from the "back" of helices 89 and 91 is obstructed by their tight packing against other elements of the 50S subunit structure; in particular, it is shielded by helix 97 and protein L6. Therefore, interaction of the Evn molecule with 23S rRNA apparently includes contacts from the interface side of the subunit.

Evn Resistance Mutations in Protein L16 Map Close to the rRNA Site Involved in the Drug Binding. Substitutions of isoleucine at position 52 in ribosomal protein L16 were reported to reduce susceptibility of *S. pneumoniae* to Evn (8). Similarly, mutations at several neighboring positions in L16 recently were shown to confer Evn resistance in *Staphylococcus aureus*, *S. pneumoniae*, and *Enterococcus faecium* (P.M.M., unpublished results) (Fig. 5). The relative position of the L16 Evn^r mutations and rRNA mutations was unclear because no close homologue of the bacterial L16 was

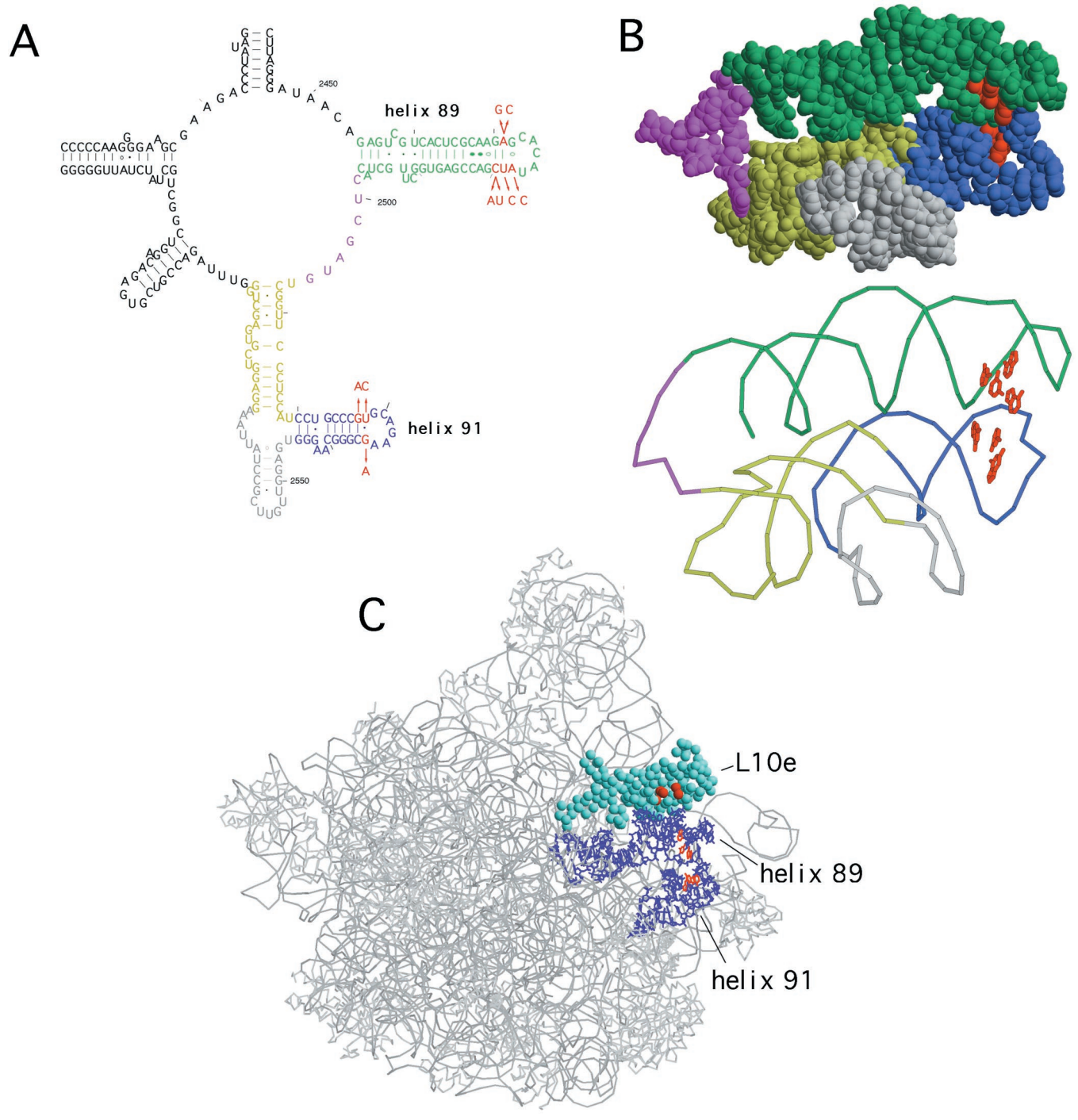

Fig. 3. *H. halobium* mutations conferring resistance to Evn. (*A*) Secondary structure of the segment of *H. halobium* 23S rRNA encompassing the central loop of domain V and the neighboring regions (28, 50). Nucleotide substitutions conferring Evn resistance are shown in red. (*B*) Three-dimensional arrangement of Evn^r mutations in the ribosome (28). The rRNA region 2454–2585 is shown in a space-fill (*Upper*) or backbone (*Lower*) representation. Coloring of the hairpins is the same as in *A*. The nitrogen bases of the nucleotides, whose mutation confer Evn resistance, are shown in red. (*C*) Spatial arrangement of Evn resistance mutations in both rRNA and ribosomal protein L10e. The large ribosomal subunit is shown in a crown projection with the interface side of the 50S subunit toward the reader (28). rRNA and ribosomal proteins are outlined in gray. Hairpins 89 and 91 are shown blue in a ''stick'' representation, and the α-carbon atoms of the protein L10e chain are shown in cyan in space-fill representation. The sites corresponding to the location of mutations that confer Evn resistance are shown in red.

identified in the 50S subunit of an archaeon *H. marismortui* whose crystallographic structure is available (28). However, considering the important role protein L16 plays in the ribosome (35, 36), it was likely that one of those *H. marismortui* ribosomal proteins, for which no bacterial homologues was found, fulfils L16 functions and occupies its space in the archaeal ribosome. Bacterial L16 belongs to the same cluster of orthologous groups of proteins as archaeal L10e (37, 38), a protein for which no obvious bacterial counterpart was identified (28). Comparative analysis revealed significant similarity between the highly con-

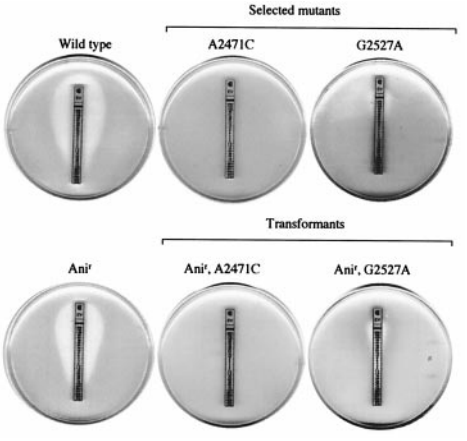

Fig. 4. Confirmation that single nucleotide substitutions in the 23S rRNA are sufficient to confer Evn resistance. Single base pair changes in the 23S rRNA were introduced into a wild-type *H. halobium* strain via linkage to a single base pair change conferring resistance to Ani. After transformation and selection of Ani resistance colonies, the susceptibility of the transformants to Evn was assayed by E test (see *Materials and Methods*). Ani^r indicates cells that contained an Ani resistance mutation C2452T.

served central region of L16 and *H. marismortui* protein L10e (Fig. 5). In the *H. marismortui* 50S subunit x-ray structure, L10e is located immediately adjacent to the hairpin 89 (Fig. 3*C*), and it is likely, therefore, that a similar space is occupied in bacterial ribosome by L16. Indeed, protein L16 can be photolabeled in the *E. coli* ribosome by a probe attached to oligonucleotides complementary to positions 2448–2458 in 23S rRNA or to the hairpin 89 loop (39, 40). Because the site of Evn-resistance mutations in bacterial L16 falls within the region of homology with L10e, it was possible to identify analogous positions in the L10e structure (Fig. 5). In the ribosome tertiary structure, these amino acid positions (shown in red in Fig. 3*C*) localize to the same region as the site of Evn resistance mutations in rRNA.

Mode of Action of Evn. Antibiotics that bind to the large ribosomal subunit can potentially inhibit functions of the ribosomal peptidyl transferase center, interfere with binding or functioning of translation factors, or obstruct growth of the nascent peptide. No inhibition of peptidyl transferase activity was observed by using two independent assays (22, 23) (data not shown). In contrast, Evn dramatically inhibited activity of IF2 in a translation initiation assay (27). In this assay, formyl-methionyl initiator tRNA is bound to the 30S ribosomal subunit in the absence or in the presence of IF2 and varying concentrations of Evn after addition of the large ribosomal subunit and puromycin (Fig. 6). Formation of fMet-puromycin reflects successful assembly of the 70S initiation complex. Evn did not inhibit the IF2-independent reaction. However, the stimulatory action of IF2 was completely abolished by levels of Evn (>1 μg/ml) that were previously shown to dramatically reduced cell translation

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          60          70          80          90          100
          |           |           |           |           |
L10e  IRHNALEAARNAANRFVQNSGAAANYKFRIRKFPFHVIREQDGDGMRAPFG
      :    .:::: :  :.    : .    :  . . ...:    : :  :: :
L16   ITNRQIEAARIAMTRYMKRGGKVWIKIFPHKSYTAKAIGVRMGSGKGAPEG
           ***  **
          |           |           |           |           |
          50          60          70          80          90
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Fig. 5. The regions of similarity between *S. pneumoniae* L16 and *H. marismortui* L10e sequences. Identical amino acids in L16 and L10e sequences are indicated by colons, and chemically similar residues are marked by single dots. Amino acid positions in protein L16 where Evn^r mutations were found in *S. pneumoniae*, *S. aureus*, and *E. faecium* are shown in bold and marked by *, and the corresponding positions in *H. marismortui* L10e are shown in bold.

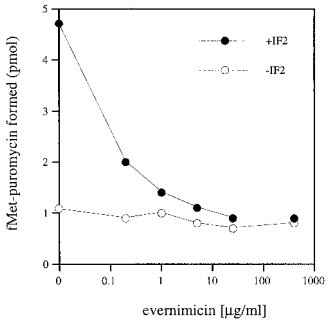

Fig. 6. (*A*) Effect of Evn on IF2-dependent formation of 70S initiation complex. [^{3}H]fMet-tRNA was bound to the mRNA-programmed 30S subunits in the presence (●) or absence (○) of IF2 and varying concentration of Evn. After addition of 50S subunits and puromycin, the amount of fMet-puromycin formed was determined by ethyl acetate extraction and liquid scintillation counting.

(7). In addition, Evn did not interfere with IF2-dependent formation of 30S initiation complex (data not shown) suggesting that the drug may prevent interaction of IF2 with the large ribosomal subunit and/or interfere with formation of 70S initiation complex. In agreement with this conclusion, a brief (20 min) treatment of sensitive bacterial cells with Evn led to a reduction in the amount of 70S ribosomes in the cell (data not shown). This reduction may result from interference with initiation complex formation.

Discussion

The experiments described in this paper were designed to characterize the site of binding and the mechanism of action of the ribosome-targeted antibiotic Evn. Both biochemical (RNA footprinting) and genetic (mutant selection) analyses demonstrated that 23S rRNA is an essential component of Evn-binding site. A number of RNA bases in the hairpins 89 and 91 were protected by the drug from chemical modification in bacterial as well as in archaeal ribosomes. These data suggest that Evn forms intimate contacts with at least some of these residues. This conclusion is further corroborated by mutational data; nucleotide substitutions in the same rRNA segments conferred high levels of Evn resistance in *H. halobium*. All of the Evn-resistant mutants of *H. halobium* that were analyzed had mutations in the rRNA, again strongly suggesting that hairpins 89 and 91 of 23S rRNA comprise the main component of the drug-binding site. Independently, four mutations that conferred Evn resistance in *S. pneumoniae* were identified in hairpins 89 and 91 of 23S rRNA (9), thus confirming our conclusion that the Evn-binding site is conserved between Bacteria and Archaea. It should be noted, however, that in the case of *S. pneumoniae*, due to the prolonged exposure of cells to the drug, the possibility remained that second site mutations could contribute to the resistance. Usage of an alternative selective marker (Ani), and the efficient transformation system available for *H. halobium* (18, 41), allowed us to demonstrate that single nucleotide substitutions in the hairpins 89 and 91 are sufficient to confer Evn resistance.

Both Evn footprints and resistance mutations indicate that two specific elements of 23S rRNA, hairpins 89 and 91, participate in the drug binding. Previously only one Evn-binding site was found in the ribosome, and we would therefore predict that hairpins 89 and 91 should form a single drug-binding pocket. When the sites of *H. halobium* mutations were contoured in the x-ray structure of the 50S subunit of a closely related *H. marismortui* (Fig. 3), they all were organized into a tight cluster. Interestingly, mutations in hairpin 89 confer the highest level of the drug resistance (Table 1), and the footprinting studies revealed that the majority of Evn footprints also are found in this hairpin. Therefore, the strongest interaction between Evn and the ribosome apparently occurs within this hairpin whereas it appears that hairpin 91 makes a smaller contri-

bution to drug binding. Although the information provided by mutational data and chemical probing was not sufficient to accurately model the structure of the ribosome-drug complex, it is possible that planar benzene rings of the drug molecule may intercalate between bases of hairpins 89 and 91.

Comparative analysis revealed sequence similarities between a conserved segment of bacterial protein L16 and archaeal L10e. This finding allowed us to localize the positions of Evn resistance mutations identified in L16, to the same region of the ribosome as the mutations in hairpins 89 and 91 of 23S rRNA. The α-carbon atoms of corresponding amino acids are separated by *ca.* 19 Å from the nearest mutation in the hairpin 89. In a fully extended conformation the Evn molecule is 37 Å long; it is therefore conceivable that the drug molecule interacting with hairpins 89 and 91 can reach the site of protein mutations. Alternatively, the protein L16 mutations may cause localized alterations in the rRNA structure, particularly in hairpin 89, and thereby interfere with Evn binding in an allosteric manner.

The Evn-binding site is located at a distance from the peptidyl transferase catalytic center (4). Therefore, it was not surprising to find that Evn did not inhibit ribosomal peptidyl transferase activity. In contrast, Evn efficiently inhibited IF2-dependent placement of fMet-tRNA in the 70S initiation complex. IF2 is involved in several steps of translation initiation. It stimulates binding of fMet-tRNA to the 30S subunit; subsequently, it promotes binding of 50S subunit to the 30S initiation complex and adjustment of fMet-tRNA in the P-site so that it can participate in the formation of the first peptide bond (27, 42). IF2 interacts with both the small and large ribosomal subunits (43–46). Furthermore, on the large subunit, IF2 footprints overlap with those of Evn in the hairpin 89 (A. La Teana and A. Dahlberg, personal communication). Therefore, Evn may inhibit ribosomal function by blocking the interaction of IF2 with the large ribosomal subunit. Experimental data strongly support this hypothesis. Evn strongly inhibited IF2-dependent formation of the 70S initiation complex, which resulted in reduced reactivity of fMet-tRNA toward puromycin (Fig. 6). In contrast, Evn did not interfere with formation of 30S initiation complex. *In vivo*, brief treatment with Evn reduced the amount of 70S ribosomes in the cells, compatible with the idea that the drug inhibits formation of 70S initiation complex. It was proposed previously that the main target of Evn action is elongation of protein synthesis (7). Our data do not necessarily contradict this proposal because some antibiotics inhibit initiation of translation at low concentrations and at higher concentrations can interfere with elongation (1). The finding that Evn interferes with the activity of IF2 makes this drug a useful tool for studies of IF2 functions.

The majority of clinically useful antibiotics interact with only a few sites in the large ribosomal subunit confined primarily to the peptidyl transferase center and entrance to the nascent peptide exit tunnel (5, 47). In contrast, Evn binds to a novel site in the ribosome, which is not used by any other therapeutically important drugs. This observation explains why bacterial strains that developed resistance to other ribosome-targeted antibiotics remain sensitive to Evn. The only exception is the structurally similar drug avilamycin, which competes with Evn for binding and whose use as a growth promotant in animal feed led to appearance of resistant strains that also exhibit cross-resistance to Evn (48, 49). Noteworthy, all of the spontaneous avilamycin-resistant mutants contained mutations in L16 protein gene, not in rRNA. This is not surprising given the multiplicity of rRNA genes in most pathogens. Indeed, all of the Evn-resistance mutants with rRNA gene mutations have been generated only in artificial systems or using model organisms like *H. halobium*.

The important practical implication of our findings is that they reveal a site in the ribosome where binding of a small drug molecule (not necessarily of oligosaccharide nature) may interfere with translation. Screening of chemical libraries or rational design can be now used to identify new structurally dissimilar compounds, with superior pharmacological properties, that can inhibit protein synthesis by interacting with this site in the ribosome. Targeting such compounds specifically to rRNA component of the Evn-binding site may prevent cross-resistance with mutants containing mutations in ribosomal protein genes and may significantly delay appearance of resistant strains.

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