The enzyme which confers resistance to erythromycin in the producing organism Streptomyces erythraeus dimethylates a single adenine residue in Bacillus stearothermophilus 23 S rRNA. This corresponds to residue Ade 2058 in Escherichia coli 23 S RNA. The methylase responsible for resistance to macrolides, lincosycin, and streptogramin B-related antibiotics in Staphylococcus aureus also acts at this site.

Erythromycin, a macrolide antibiotic, inhibits protein synthesis by binding to a single site on the larger subunit of the prokaryotic ribosome. The drug is produced by Streptomycetes erythraeus, which is resistant not only to its product but also to other macrolides, to lincosycin, and to antibiotics related to streptogramin B. This so-called "MLS-resistance" phenotype was first observed in clinical isolates of Staphylococcus aureus, where it was inducible by erythromycin, and was characterized by Weisblum (for review, see Ref. 1). He demonstrated a causal connection between N^6-dimethylation of adenine within 235 rRNA and reduced affinity of ribosomes for MLS antibiotics. Since then, the MLS-resistance phenotype has been encountered in various Gram-positive bacteria and has been closely studied in a strain of Bacillus subtilis harboring the staphylococcal plasmid pE194 (2, 3).

Elsewhere, we have shown that self-defence in S. erythraeus and the MLS-resistance phenotype in S. aureus result from closely similar, if not identical, processes. A methylase was prepared from S. erythraeus, and studies of its action in vitro established that N^6-dimethylation of a single adenine residue in 23 S rRNA was sufficient to render ribosomes resistant to specific MLS antibiotics (4). Also, by cloning fragments of the S. erythraeus genome in Streptomyces lividans, it was shown that the methylase gene can act as an MLS-resistance determinant in vivo (5). Here, we have determined the site of action of the "erythromycin-resistance methylase" of S. erythraeus within 23 S rRNA and have examined its relationship to that of the staphylococcal MLS-resistance methylase.

Experimental Procedures

Bacterial Strains—The organisms used in this work were Streptomycetes coelicor A3 (2), S. erythraeus NRRL 2338, Bacillus stearothermophilus NCIB 8924, Escherichia coli MR1 600, and B. subtilis 430 containing staphylococcal plasmid pE194. The latter organism was kindly supplied by Dr. David Dubnau (Department of Microbiology, The Public Health Research Institute of the City of New York, Inc.). Plasmid pE194 specifies resistance to MLS antibiotics; this phenotype is inducible by erythromycin.

Preparation of Ribosomal Subunits and rRNA—Ribosomal subunits and total RNA from 70 S ribosomes were prepared as described elsewhere (4); 23 S rRNA was prepared as described below for [35S] RNA.

Preparation of Uniformly Labeled [35S]rRNA—This was carried out in 20 ml of buffered salt solution supplemented with 0.8 ml each of 10% (w/v) Difco casamino acids and 10% (w/v) Difco Bactopeptone plus 0.4 ml of 20% (w/v) glucose, 0.2 ml of 0.1 M MgSO4, and 0.2 ml of 0.13% (w/v) K2HPO4. Buffed salt solution contained, (per liter) 1.5 g of KCl, 5 g of NaCl, 1 g of NH4Cl, 13.2 g of Tris-HCl, and 1.9 g of Tris base. The final pH was 7.4. Components of the medium were sterilized separately, and solutions of casamino acids and peptone were freed of phosphate prior to autoclaving (8). The medium was inoculated with approximately 2 × 106 cells of B. stearothermophilus in the exponential phase of growth, 10 ml of [35S]phosphate was added, and the culture was shaken at 60°C. Cells were harvested after about six generations of growth (A550nm = 0.3) and were washed by recentrifugation in containing 10 mM Tris-HCl (pH 7.6 at 20°C), 10 mM MgCl2, 50 mM NaCl, 0.5 mM EDTA, and 3 mM mercaptoethanol. They were then resuspended in 2 ml of buffer to which were added lysozyme, pancreatic DNase, and Triton X-100 at final concentrations of 2.5 mg/ml, 5 µg/ml, and 0.05% (w/v), respectively. After incubation at 37°C for 2 min, the lysate was centrifuged at 200,000 × g for 90 min. The pellet was resuspended in 1 ml of buffer containing 10 mM Tris-HCl (pH 7.6 at 20°C), 10 mM Na3EDTA, 50 mM LiCl, 0.2% (w/v) sodium dodecyl sulfate and layered onto a 35-ml 10-30% sucrose gradient made in similar buffer lacking sodium dodecyl sulfate. Centrifugation was at 25,000 rpm for 20 h at 15°C in a Beckman SW 27 rotor. Fractions containing 23 S RNA were pooled, and the RNA was precipitated with 2.5 volumes of ethanol at −20°C, precipitated twice from 0.5 M sodium acetate, and stored frozen at −20°C. Typically, 1 mCi of 23 S [35S] RNA was obtained at a specific activity of about 8 µCi/ pmol. Unlabeled 23 S RNA was prepared similarly from cells grown in Tryptic Soy Broth (Difco).

Methylation of 23 S RNA in Vitro—Incubation mixtures (300 µl, pH 7.5) contained 50 mM HEPES-KOH, 10 mM Tris-HCl, 5 mM MgCl2, 200 mM NH4Cl, 3% (w/v) glycerol, 290 pmol of 23 S RNA, 60 µCi of S-adenosyl-L-[methyl-35S] methionine (15 Ci/mmol), and 60 units of erythromycin-resistance methylase from S. erythraeus. This partially purified enzyme was prepared as previously described (4). Incubation was at 37°C for 30 min, after which the methylated RNA was extracted twice with phenol and three times with ether and precipitated with ethanol. Stoichiometries of methylation were determined as described elsewhere (4).

Partial Digestion of Methylated 23 S RNA—Methylated RNA (250 pmol) was dissolved in 60 µl of buffer containing 30 mM Tris-HCl (pH 7.6 at 20°C), 300 mM KCl, MgCl2 (10 mM final concentration) was then added to give TMK buffer. After 15 min at 37°C, the RNA was chilled to 0°C, and 125 units of RNase T1 (Sankyo) was added.
After 30 min at 0 °C, 10 volumes of TMK buffer was added, followed by 0.1 volume of 2% (w/v) bentonite (Serva). The digest was then extracted three times with TMK-saturated phenol (with back extraction of the phenol layers) and precipitated with ethanol at -20 °C prior to fractionation by gel electrophoresis.

Preparative Gel Electrophoresis—RNA fragments were resolved by two-dimensional polyacrylamide gel electrophoresis as described by Pedersen and Haseltine (9) except that the thickness of our gels was 1.5 mm in the first dimension and 1.0 mm in the second. One-dimensional electrophoresis in 8 M urea, 20% (w/v) acrylamide gels was used both preparatively and analytically (see Fig. 1). Such gels were 0.5 mm thick and 35 cm long. They were prepared by adding (50 ml) 10 g of acrylamide, 0.4 g of bisacrylamide, containing 0.75 M Tris, 0.68 M boric acid, and 10 mM Na2EDTA, and 0.3 ml of 10% volumes of TMK buffer was added, followed by 0.1 volume of 2% (w/v) bentonite (Serva). The digest was then extracted three times with TMK-saturated phenol (with back extraction of the phenol layers) and precipitated with ethanol at -20 °C prior to fractionation by gel electrophoresis.

After 30 min at 0 °C, the digest was resolved by gel electrophoresis using reagents supplied by Bethesda Research Laboratories, except for RNase T1, which was obtained from Sankyo. Sequencing gels were prepared as above except that the acrylamide concentration was 10%.

Nucleotide Sequence Analysis—Fingerprinting of uniformly labeled [32P]RNA and determination of base compositions of oligonucleotides were performed as previously described (10). Gel sequencing of 5′-end-labeled RNA was carried out according to Donis-Keller et al. (11) using reagents supplied by Betheha Research Laboratories, except for RNase T1, which was obtained from Sankyo. Sequencing gels were prepared as above except that the acrylamide concentration was 25%.

RESULTS

When the erythromycin-resistance methylase from S. erythraeus acts in vitro upon RNA from various species of Bacillus or Streptomyces, the stoichiometry of methylation is usually within the range of 1.5-2.0 methyl groups incorporated per 23 S RNA molecule. Typical data are given in Table I. Since 23 S rRNA from Gram-positive bacteria in general (and from B. stearothermophilus in particular) does not contain monomethyladenine (6), we have argued previously (4) that such stoichiometries represent the conversion of a single adenine residue to N6,N′-dimethyladenine. Ideally, we wished to study the action of the enzyme upon E. coli 23 S rRNA, but, as shown in Table I, the latter was not such a good substrate for methylation. We have no ready explanation for this observation. However, since most of the nucleotide sequence of B. stearothermophilus 23 S RNA is known (7), we decided to determine the site of action of the S. erythraeus methylase using that RNA as substrate. Before doing so, we wished to ascertain whether the S. erythraeus methylase and the staphylococcal MLS-resistance methylase act at a common site. To do this, we prepared total ribosomal RNA from S. coelicolor carrying the staphylococcal plasmid pE194 before and after induction of the MLS-resistance phenotype with erythromycin (20 μg/ml). As shown in Table I, RNA from the uninduced culture was an excellent substrate for the S. erythraeus methylase, whereas that from induced cells was not. Since the Streptomyces enzyme acts at a single site within 23 S RNA, the simplest explanation for these data is that it was the staphylococcal methylase act at the same site. However, they do not exclude the possibility that the latter enzyme might also act at additional sites, although we have no reason to suspect that it does.

Preparation of a Methylated Fragment of 23 S RNA—In order to localize the site of action of the S. erythraeus methylase, it was first necessary to obtain a fragment of 23 S RNA containing the methylated residue and long enough to possess a unique sequence. Accordingly, 23 S RNA from B. stearothermophilus was radioactively methylated in vitro by incubation with the S. erythraeus enzyme together with S-adenosyl-L-[methyl-3H]methionine as cofactor. This material was then subjected to partial digestion by T1 ribonuclease followed by electrophoresis in polyacrylamide-urea gels. Only one methylated oligonucleotide was evident under our chosen conditions (Fig. 1, track 2). This was estimated to be about 20-25 residues long by comparison with the mobilities of tracker dyes. When this procedure was repeated using radioactively methylated RNA uniformly labeled with 32P, no single band in the T1 digest obviously corresponded to the methylated fragment (Fig. 1, track 1). Therefore, this digest was subjected to two-dimensional gel electrophoresis, and the approximate position of the methylated fragment was predicted from its mobility in the individual dimensions of the two-dimensional system. Oligonucleotides were eluted from this portion of the two-dimensional gel and subjected to double label counting. A fraction greatly enriched in 3H radioactivity was thus identified and was further resolved in a polyacrylamide-urea gel. Autoradiography revealed a major [32P]-labeled band containing all the 3H radioactivity, plus several minor contaminants of closely similar mobility (results not shown). The major component was eluted and rerun on a similar gel. It appeared homogeneous and co-migrated with the original 3H-labeled RNA fragment (Fig. 1, tracks 2 and 3). This purified oligonucleotide was then subjected to “fingerprint” analysis.

Fingerprint Analysis of the Methylated Oligonucleotide—The purified, methylated oligonucleotide was digested to completion with T1 ribonuclease and subjected to two-dimensional electrophoresis, as previously described (10). The resultant fingerprint revealed six 32P-labeled oligonucleotides (Fig. 2). These were eluted and their base compositions determined following digestion with RNase T2. Double label counting of the six oligonucleotides showed that only one of them (AAAG) was methylated and that there was only 1 residue of dimethyladenine/tetranucleotide. This finding is consistent with the earlier observation that the staphylococcal MLS-resistance methylase also acts within the oligonucleotide sequence AAAG (12).

To establish which Ade residue had been methylated by the S. erythraeus enzyme, radioactively methylated 23 S RNA was first digested to completion with RNase T1, to produce the methylated tetranucleotide ApApApGp. This was further digested with endonuclease P1, which liberates nucleoside 5′-phosphates. During paper chromatography, all the [methyl-3H]radioactivity co-migrated with N6,N′-dimethyladenosine (RI of approximately 0.65) under conditions where nucleotides did not move (results not shown). In control experiments, when either nuclease was omitted, all the radioactivity remained at the origin. We therefore concluded that the 5′-terminal residue of oligonucleotide AAAG had been methylated at position 23 S RNA.

Gel Sequencing of the Methylated RNA Fragment—The methyl-3H-fragment of 23 S RNA was prepared as described above using a small amount of the purified 32P-labeled mate-
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**FIG. 1.** Analytical gel electrophoresis of methylated oligonucleotides. Track 1, methylated 23 S \(^{32}P\)RNA partially digested with RNase T\(_1\); track 2, \(^3\)H-methylated 23 S RNA partially digested with RNase T\(_1\); track 3, purified methylated oligonucleotide uniformly labeled with \(^{32}P\); track 4, purified methylated oligonucleotide after \(\Upsilon\) end labeling with \(^{32}P\)phosphate. The gel contained 20\% (w/v) acrylamide and 8 M urea. The positions of the origin (O) and the dye xylene cyanol (XC) are indicated. The gel was impregnated with EN\(^3\)HANCE (New England Nuclear), and dried, and bands were visualized by fluorography.

**FIG. 2.** Two-dimensional fingerprint of the methylated oligonucleotide uniformly labeled with \(^{32}P\). \(m^6A, N^6,N^6\)-dimethyladenosine.

Material eluted from such a gel was subjected to 5' end labeling with \(^{32}P\)phosphate and was fractionated on a polyacrylamide-urea gel. The major component was eluted and its purity checked by gel electrophoresis (Fig. 1, track 4). Since this pure oligonucleotide possessed an extra terminal phosphate group, it migrated slightly ahead of those visualized in Fig. 1, tracks 2 and 3. The nucleotide sequence was established on sequencing gels following partial enzymic digestion (data not shown)\(^2\) and is given in Fig. 3a. The data obtained by fingerprint analysis can be fitted perfectly to this sequence provided that residue 19 (from the 5' end), which was not identified on sequencing gels, is assumed to be Ap. This is a necessary assumption since the oligonucleotide AAAG cannot otherwise be accommodated. Presumably, this position in the sequence was not cleaved by RNase U\(_2\) or Phy M because the Ade residue in question was dimethylated.

The sequence determined here for the methylated fragment of *B. stearothermophilus* 23 S RNA (Fig. 3a) is obviously homologous with that of *E. coli* 23 S between residues 2040 and 2061 (Fig. 3b). Part of the sequence of the corresponding region of *B. stearothermophilus* 23 S RNA has been published (7), and it is identical with our sequence. We therefore conclude that the adenine residue converted to \(N^6,N^6\)-dimethyladenine by the erythromycin-resistance methylase of *S. erythraeus* corresponds to residue Ade-2058 in *E. coli* 23 S rRNA.

\(^2\) Sequence data were provided to the referee.
Presumably, when the *S. erythraeus* methylase acts upon *E. coli* 23 S RNA (Table I), residue Ade-2058 is modified, although we have not established this directly.

**DISCUSSION**

Obvious similarities exist between the action and effects of the erythromycin-resistance methylase of *S. erythraeus* and those of the staphylococcal MLS-resistance methylase. However, there are also some apparent differences. The *Streptomyces* enzyme acts *in vitro* at a single site in 23 S rRNA, whereas results obtained *in vivo* suggested that the staphylococcal enzyme might act at more than one site (12). Some confusion also exists regarding substrate specificity. The *Streptomyces* enzyme, acting *in vitro*, methylates free 23 S rRNA quantitatively but is totally inactive on 50 S ribosomal subunits (Table I). Similarly, Weisblum (1) concluded that the staphylococcal enzyme probably does not methylate mature ribosomal subunits in *vivo*. In contrast, Shivakumar and Dubnau (13) claimed that the staphylococcal methylase acts *in vitro* both on 23 S RNA and on 50 S subunits, although not on 70 S ribosomes. However, we consider that the rather low levels of methylation reported by the latter authors do not unequivocally establish the substrate specificity of the staphylococcal methylase.

Considerable interest has been aroused by the translational attenuation model for inducibility of the MLS-resistance phenotype in *Staphylococcus* (14, 15). It is therefore relevant to ask whether the *Streptomyces* methylase gene is similarly controlled. This question cannot, at present, be answered definitively, although resistance in *S. erythraeus* is not obviously inducible. Now that the methylase gene from *S. erythraeus* has been cloned in *S. lividans* (5), it may be possible to resolve this matter.

Co-resistance to the MLS antibiotics can be rationalized on the basis that such drugs all bind to closely related ribosomal sites. For example, erythromycin and lincomycin compete with each other and with chloramphenicol for binding to the 50 S ribosomal subunit (16). It is therefore not surprising that methylation of a single site in 23 S rRNA can lead to reduced affinity for the various MLS antibiotics although, interestingly, the binding of chloramphenicol is not obviously affected.

Given the extensive sequence homology between 16S rRNA molecules from prokaryotes and mitochondria, it has been possible to identify in *E. coli* 23 S rRNA sites corresponding to those at which single base substitutions in mitochondrial rRNA result in antibiotic resistance. These sites are positions 2447, 2451, 2503, and 2504 in the case of chloramphenicol resistance (17, 18) and, significantly in the present context, position 2058 for erythromycin resistance (19). As shown in Fig. 3, current models for the secondary structure of 23 S RNA place these 5 residues close together (7, 20, 21). Again, these data are compatible with a model whereby chloramphenicol and the MLS antibiotics bind to a common ribosomal domain which presumably includes the region of 23 S RNA represented in Fig. 3. Additionally, that domain should also include protein L16, which has been implicated in the ribosomal binding of chloramphenicol, erythromycin, and virginiamycin S (an MLS antibiotic), and protein L15 which binds erythromycin, albeit weakly, in free solution (22-24).
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Although construction of detailed models based upon the presumed modes of action of these drugs would at present be premature (for a review, see Ref. 25), it does seem probable that this domain is somehow involved in the peptidytransferase center of the ribosome.

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