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Binding of Novel Macrolide Structures to Macrolides-Lincosamides-Streptogramin B-Resistant Ribosomes Inhibits Protein Synthesis and Bacterial Growth

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Dimethylation of adenine 2058 in 23S rRNA renders bacteria resistant to macrolides, lincosamides, and streptogramin B (MLS resistance), because the antibiotic binding site on the altered 50S ribosomal subunit is no longer accessible. We now report that certain 6-*O*-methyl-11,12-cyclic carbamate derivatives of erythromycin are able to bind to dimethylated MLS-resistant 50S ribosomal subunits, thus inhibiting protein synthesis and cell growth. One of these novel structures, an 11-deoxy-11-(carboxyamino)-6-*O*-methylerythromycin A 11,12-(cyclic ester) derivative, structure 1a, was studied in detail. It inhibited in vitro protein synthesis in extracts prepared from both susceptible and MLS-resistant *Bacillus subtilis* with 50% inhibitory concentrations of 0.4 and 20 μM, respectively. The derivative bound specifically to a single site on the 50S subunit of MLS-resistant ribosomes prepared from *B. subtilis* and *Staphylococcus aureus*, and no binding to 30S subunits was observed. The association rate constant of derivative 1a with sensitive and resistant ribosomes was 100- and 500-fold slower, respectively, than that of the parent compound, erythromycin, with sensitive ribosomes. The dissociation rate constant of 1a from sensitive and resistant ribosomes was 50- to 100-fold slower than the rate of erythromycin dissociation from sensitive ribosomes. Furthermore, 1a binding to sensitive 50S subunits led to induction of *ermC* and *ermD*, while binding to resistant 50S subunits did not, showing that perturbation of sensitive and resistant 50S subunit function by 1a differs. These data demonstrated that 1a is unique in its interaction with MLS-resistant ribosomes and that this interaction causes a novel allosteric perturbation of ribosome function.

There are several theoretical mechanisms by which bacteria could exhibit resistance to the inhibitory effects of macrolide antibiotics. These fall into four classes: (i) lack of macrolide entrance into the cell, (ii) chemical inactivation of the macrolide, (iii) lack of binding of the macrolide to the ribosome target, and (iv) lack of an inhibitory response upon binding to the ribosome target. Evidence for the existence of actual mechanisms falling into classes i to iii has been published (see below), and the most definitive studies concern mechanisms from classes ii and iii. The only report of a class iv mechanism of resistance concerns erythromycin-dependent growth of *Escherichia coli* containing the *mac* allele (31, 35).

Several reports suggest that resistance to macrolides can involve lack of entrance into the cell of either macrolide producers (11) or bacterial pathogens (16, 34). However, there are also studies which demonstrate that macrolides (specifically erythromycin) enter into cells by passive diffusion of the un-ionized form across the lipid bilayer of the cell membrane (7, 27). Thus, accumulation is temperature dependent due to phase changes in the lipid bilayer and energy independent (7, 27), and in the absence of access to the intracellular binding site (using macrolide-lincosamide-streptogramin B [MLS]-resistant bacteria), drug does not accumulate, but rather only equilibrates with the extracellular drug concentration (R. C. Goldman, unpublished observations). It would thus appear difficult to reconcile these data with a mechanism of resistance due to lack of passive diffusion across the lipid bilayer. Since none of the studies suggesting resistance due to lack of drug entrance into the cell have concurrently demonstrated the mechanism involved, further studies are required before these observations can be generally accepted. In contrast, resistance due to drug modification is well documented. Degradation of macrolides by plasmid-coded esterases renders the drug inactive and imparts resistance to bacteria carrying such genes, designated *ere* (1). In addition, various macrolides are phosphorylated on the 2'-hydroxyl group by extracts of *Streptomyces coelicolor* (36), and recently a similar enzymatic activity was found in a clinical isolate of *Escherichia coli* (25).

Although macrolide resistance can occur due to low-frequency mutational changes in ribosomal proteins (29) in laboratory strains, the most frequent form of resistance observed clinically is MLS resistance (9). The mechanism involves enzymatic dimethylation (15) of adenine 2058 in the 23S rRNA sequence (30) by a specific methylase, resulting in 50S ribosomal subunits which will not bind the antibiotics; however, the exact structural mechanism by which dimethylation precludes binding is unknown, as is the stage in ribosome synthesis and assembly when dimethylation occurs in vivo. Expression of the methylase gene can be constitutive or inducible at the level of translational control (8, 33). In either case, constitutive or inducible expression, dimethylation alters ribosome structure in a manner which precludes antibiotic binding. The methylase genes (*erm* genes) apparently arose in antibiotic producers and subsequently spread to both gram-negative and gram-positive bacterial pathogens (2).

The two logical approaches to overcoming MLS resistance are inhibiting dimethylation (somewhat analogous to inhibitors of β-lactamase) and modifying the antibiotic so as to regain access to the structurally modified ribosomal binding site. We considered the latter approach to be valid in

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the following context. The chemical modification resulting in MLS resistance is rather modest (incorporation of two methyl groups) and has no apparent effect on normal ribosome functions. However, a subtle conformational change in the ribosome probably does occur, as this is the most logical explanation for lack of binding of structurally unrelated macrolides, lincosamides, and streptogramins B. These considerations lead us to conclude that a minor change in ribosome structure occurs upon dimethylation which acts either (i) as a gate to preclude access of antibiotic to chemical groups on the ribosome, which would still be in a proper conformation to interact with the antibiotic and promote binding and perturbation of ribosome function; or (ii) simply to cause a modest alteration in the binding site, rendering it incompatible with antibiotic conformation. A series of macrolide derivatives were thus synthesized which contained additional structural features that could potentially interact with the MLS-resistant ribosome in a unique manner to initiate ribosome binding. In this report, we demonstrate that certain 6-*O*-methyl-11,12-cyclic carbamate analogs of erythromycin do regain access to a binding site on the 50S subunit, resulting in inhibition of protein synthesis and thus growth of MLS-resistant bacteria. The activities of these and other analogs of erythromycin against MLS-resistant *Streptococcus pyogenes* were reported recently (10).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Bacillus subtilis* BD170 (*trpC2 thr-5*) containing plasmid pE194 was obtained from D. Dubnau, Public Health Research Institute of the City of New York, New York, N.Y. Plasmid pE194 contains the inducible *ermC* gene which codes for methylase. *Staphylococcus epidermidis* 958-1 (MLS susceptible) and 958-3 (MLS resistant due to the presence of plasmid pNE131) were obtained from J. Parisi, University of Missouri-Columbia. Plasmid pNE131 is known to code for constitutive MLS resistance (16). *Staphylococcus aureus* RN1389 (MLS resistant) was obtained from B. Weisblum, University of Wisconsin, Madison. Bacteria were grown in VY broth (23) at 30 or 37°C as indicated. *B. subtilis* was transformed with plasmid DNA as described before (4).

Determination of MIC. Cells in the log phase of growth were inoculated into microdilution wells (10^6 cells per well) containing serially diluted antibiotic. Plates were incubated at 37°C for 18 to 24 h and then scored. The concentration of antibiotic which yielded no visible growth was defined as the MIC.

Selection of a pE194 variant with both increased copy number and constitutive expression of the *ermC* gene product. *B. subtilis* containing plasmid pE194 was grown in medium lacking antibiotic, and 5×10^8 cells were spread onto plates containing 10 μg of tylosin (a noninducing macrolide) per ml. Plates were incubated at 30°C, and tylosin-resistant colonies were streaked to single colonies on medium containing 10 μg of tylosin per ml. Plasmid DNA was prepared (34) and analyzed by electrophoresis in agarose gels (6). One of the resistant strains (tylosin MIC, 16 μg/ml) contained elevated amounts of plasmid DNA and was used to select a constitutive variant as follows. Cells (10^7) were mixed with soft agar (0.75% agar, wt/vol) and overlaid to agar plates. Several paper disks (6 mm) containing 15 μg of tylosin each were applied to the plate, which was incubated at 30°C for 24 h. Colonies which appeared within a clear zone of growth inhibition were picked, streaked to a single colony, and characterized for macrolide resistance. One strain with resistance to >1,000 μg of tylosin per ml was selected for further study.

Preparation of ribosomes. Cells were grown at 37°C to late log phase, harvested, and washed as described previously (24) to remove extracellular protease. *B. subtilis* cells were suspended in buffer A (10 mM Tris hydrochloride, pH 7.5, containing 10 mM $MgCl_2$, 60 mM NH_4Cl, 5 mM EDTA, 5 mM mercaptoethanol, 10% glycerol [vol/vol], and 2 mM phenylmethylsulfonyl fluoride) and lysed by three passages through a French pressure cell at 18,000 lb/in^2. *S. aureus* cells were lysed with lysostaphin (10 U of lysostaphin per ml, 0.15 g [wet weight] of cells per ml) at 30°C for 1 h. DNase was added to 2 μg/ml, and the lysate was incubated on ice for 15 min. Lysates were treated twice with hemoglobin-Sepharose (24), and ribosomes were isolated by differential centrifugation (twice at 30,000 × *g* for 30 min to remove cell walls and membranes, followed by centrifugation of the supernatant at 100,000 × *g* for 4 h to pellet ribosomes). Ribosomes were washed with either buffer A alone or buffer A containing 1 M NH_4Cl, yielding washed or high-salt-washed ribosomes, respectively. Ribosomes were stored in buffer A in small samples at −80°C. Although some experiments were conducted with high-salt-washed ribosomes, all of the experimental data shown in this report were obtained with ribosomes which were not washed with 1 M NH_4Cl (see Discussion).

Preparation of the in vitro protein synthesis system. Bacteria were grown at 30°C to late log phase, harvested, washed as described for isolation of ribosomes, and frozen at −80°C. Cell pellets were thawed, suspended in buffer A (about 0.5 g [wet weight]/ml), lysed, and processed as described for preparation of ribosomes up to the 30,000 × *g* centrifugation. The upper two-thirds of supernatant was removed as the S30 extract and stored in frozen samples at −80°C.

Poly(A) (Sigma Chemical Co.)-directed protein synthesis was conducted as described previously (19). S30 extract was added to a final concentration of 100 to 300 μg of protein per reaction. The final reaction volume was 0.125 ml containing 13 μM [^{14}C]lysine (Amersham CFB.69, >11 GBq/mmol), and reactions were started by the addition of poly(A) (100 μg per reaction, final concentration). Duplicate 25-μl samples were removed at 5 and 10 min and added to 0.5 ml of cold 5% trichloroacetic acid containing 100 μg of lysine per ml and 0.5 g of tungstic acid (adjusted to pH 2 with NaOH) per liter. Although tungstic acid addition was required to precipitate polylysine peptides efficiently (12), we found that addition of carrier polylysine was not required. Samples were collected on 0.45-μm nitrocellulose filters, washed twice with 5 ml of cold 5% trichloroacetic acid–tungstic acid (0.5 g/liter) solution and once with cold 95% ethanol, dried, and assayed by liquid scintillation counting.

Spermidine optimization was performed by setting up a series of reactions with varied concentrations of spermidine from 0.5 to 10 mM. The spermidine concentration that produced the maximum linear incorporation of radioactivity over 10 min was deemed optimum and was used for subsequent experiments. Inhibition of protein synthesis was assessed by setting up a series of reactions with varied amounts of antibiotic added, preincubating at 37°C for 10 min, and starting reactions by addition of poly(A). Samples were withdrawn at 5 and 10 min. Controls included no antibiotic addition (100% incorporation rate) and no poly(A) addition (background due to endogenous mRNA).

Separation of ribosomal subunits and determination of the stoichiometry of macrolide binding to ribosomes. Some 5 to 10

A_{260} units of 70S ribosomes was incubated at room temperature for 30 min with radiolabeled macrolide in 200 μl of 10 mM Tris hydrochloride buffer, pH 7.2, containing 50 mM NH_4Cl and 0.1 mM $MgCl_2$. An additional 200 μl of buffer was added, and the sample was applied to a linear 10 to 30% (wt/vol) sucrose gradient (12 ml) prepared with the same buffer. Samples were overlaid with light mineral oil and centrifuged for 5 h at 39,000 rpm in a Beckman SW41 rotor at 4°C. Gradients were fractionated from the bottom, and fractions (0.3 to 0.4 ml) were collected. Buffer (0.7 ml) was added, and the A_{260} per fraction was recorded. The entire fraction was then added to scintillation vials containing 9 ml of InstaGel (United Packard), and radioactivity was determined by liquid scintillation counting. Disintegrations per minute were determined by quench correction and used to calculate picomoles of radiolabeled antibiotic. A_{260} values (22) were used to quantitate the amounts of ribosomal subunits present (1 A_{260} = 45 pmol of 50S; 1 A_{260} = 101 pmol of 30S), and the stoichiometry of macrolide binding to ribosomal subunits was calculated by using these data. Stoichiometry of binding to 70S ribosome complexes (22) was determined by incubating a constant amount of 70S subunits (1 A_{260} = 31 pmol of 70S) with an increasing amount of macrolide, followed by analysis of the amount of macrolide bound by the filter-binding or spun column method as specified. One molecule of macrolide was found bound per 70S ribosome, or 50S subunit, using the above A_{260} values.

Analysis of macrolide binding to ribosomes. Parameters of macrolide binding to ribosomes were determined by several methods. The nitrocellulose filter-binding method (26) was used in analysis of macrolide interaction with ribosomes, and competition studies were performed as described previously (32), using 0.6 μM [^{3}H]erythromycin, 0.2 μM ribosomes, and incubation for 4 h. Quenching of ^3H disintegrations on nitrocellulose filters was determined by flame combustion analysis to ^3H_2O, using a Packard combustion analyzer (United Packard). Since binding of the radiolabeled 6-*O*-methyl-11,12-cyclic carbamate derivative to nitrocellulose and glass-fiber filters precluded accurate analysis, a spun column method was devised. Sephadex G-50 in 10 mM Tris hydrochloride, pH 7.2, containing 4 mM $MgCl_2$, 100 mM KCl, and 10 mM NH_4Cl was packed by centrifugation (1,600 × *g* for 2 min) into 1-ml disposable syringes fitted with a porous polystyrene disk. Samples (100 μl) containing ribosomes plus bound and free radiolabeled macrolide were applied to the top of freshly prepared spun columns. Columns were immediately centrifuged for 2 min at 1,600 × *g*, and the void volume was collected into 1.5-ml Microfuge tubes placed in the bottom of a 15-ml Corex centrifuge tube. Samples from the Microfuge tubes were transferred to scintillation vials, and radioactivity was determined.

The rate of the forward reaction (formation of the macrolide-ribosome complex) was calculated by using the formula $1/(B_0 - A_0)\ \ln[A_0(B_0 - x)/B_0(A_0 - x)] = k_1 t$, where B_0 is the concentration of free drug at time zero; A_0 is the concentration of free ribosomes at time zero; x is the concentration of drug-ribosome complex formed at time t; and k_1 is the forward rate constant in molar^{-1} minute^{-1} (14). This equation describes the forward rate for a second-order reaction, and initial linear rates were used to estimate the rate constant. Ribosome and erythromycin concentrations were reduced to 40 to 60 nM to slow the rate of complex formation. Triplicate 5-ml samples were collected at intervals over a 30-min time period by filtering through 0.45-μm nitrocellulose filters (Millipore Corp.). Excess erythromycin was then added to assure that all ribosomes contained bound erythromycin, thus giving an accurate measure of the total number of ribosomes present. Ribosome and compound 1a concentrations were in the range of 1 to 2 μM (0.1-ml volumes), and samples were processed by the spun column procedure (see above).

Rates of dissociation of drug-ribosome complexes were determined by either adding a 50- to 100-fold excess of unlabeled derivative or removing free drug, with subsequent monitoring of ribosome-bound drug with time. The dissociation rate constant was calculated by using the formula $\ln[(RD)/(RD_0)] = -k_{-1}t$, where RD_0 is the concentration of macrolide-ribosome complex at time zero; RD is the concentration of macrolide-ribosome complex at time t after addition of an excess of unlabeled macrolide; and k_{-1} is the reverse rate constant in minute^{-1} (14).

Data were plotted and rate constants were determined from slopes for both forward and reverse rate constants. Since the forward rate will decline with time due to any dissociation of the complex which occurs during the course of the binding reaction, initial rates were used in calculation of k_1. Dissociation constants (K_d) were calculated by using the formula $K_d = k_{-1}/k_1$ and are expressed in terms of molarity. K_d values were also estimated by the method of Scatchard (28), where $(RD)/(L) = [(R_t) - (RD)]K_d^{-1}$ and RD is the concentration of the ribosome-macrolide complex; R_t is the total concentration of binding sites; (L) is the concentration of free drug; and K_d^{-1} is the slope.

Selection of spontaneous resistance to macrolides. *B. subtilis* BD170 lacking pE194 was grown overnight at 37°C, and 10^8 cells were spread onto agar plates containing 5 μg of erythromycin per ml or its 6-*O*-methyl-11,12-cyclic carbamate derivative, 1a. Resistant colonies were picked and streaked to single colonies for further characterization.

Macrolide derivatives. Erythromycin A base and niddamycin were prepared at Abbott Laboratories as fermentation products. Tylosin was obtained from Sigma. The synthesis of 6-*O*-methyl-11,12-cyclic carbamate derivatives is described elsewhere (3; W. R. Baker and J. D. Clark, U.S. patent 4,742,049, 3 May 1988). Radiolabeled macrolides were synthesized at Abbott Laboratories. The specific activities of [^{3}H]erythromycin and 1a were 38.7 and 12.6 mCi/mmol, respectively. The structures of erythromycin A and key derivatives are shown in Fig. 1.

RESULTS

Selection of pE194 with increased copy number and constitutive expression of the *ermC* methylase. We constructed a *B. subtilis* strain which would contain fully dimethylated ribosomes in the absence of any added macrolide for use in our study of macrolide interaction with dimethylated ribosome. This involved selection for a pE194 derivative with elevated copy number as described previously (34). Such a variant was isolated following plating of *B. subtilis* BD170 containing pE194 onto plates containing the noninducing macrolide tylosin; this variant contained elevated amounts of pE194 DNA by agarose gel electrophoresis (data not shown) and had a tylosin MIC of 16 μg/ml. The *ermC* gene was still inducible, because the tylosin MIC increased to >1,000 μg/ml in the presence of an inducing concentration (0.05 μg/ml) of erythromycin. A second alteration giving constitutive expression of resistance was selected by picking colonies growing in the zone of inhibition created by 15 μg of tylosin applied to a 6-mm paper disk. Several levels of constitutive resistance to tylosin were found among such isolates, ranging from 100 to >1,000 μg/ml. One isolate

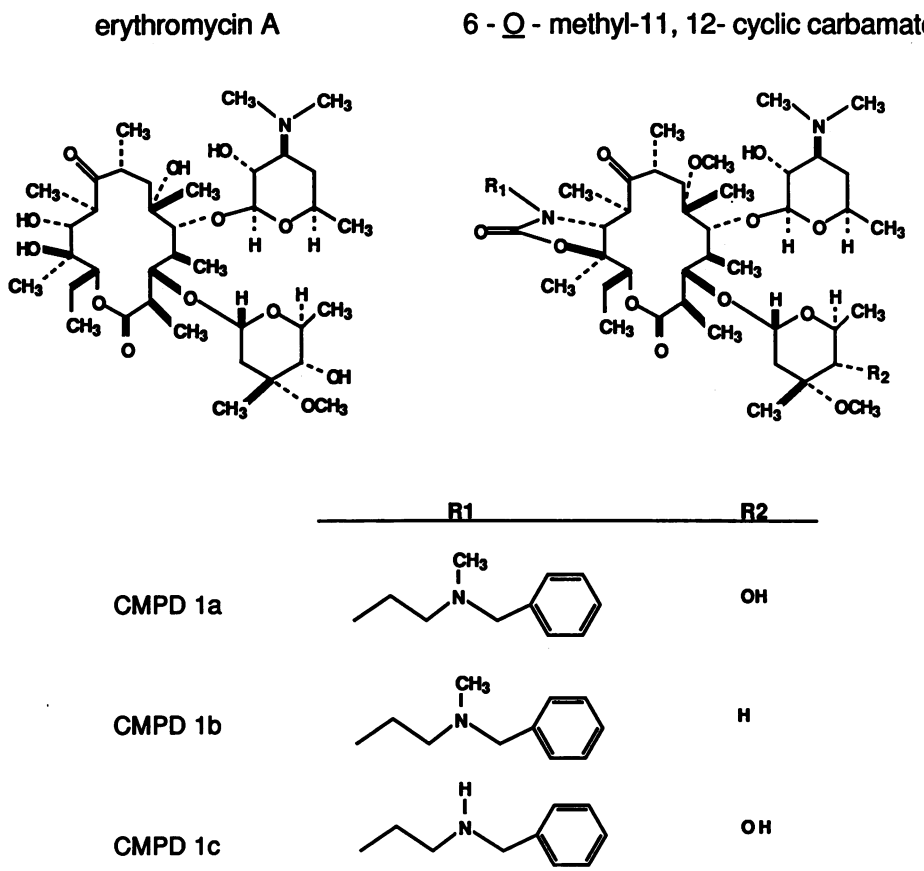

FIG. 1. Structures of erythromycin A and 6-*O*-methyl-11,12-cyclic carbamate derivatives 1a, 1b, and 1c.

(tylosin MIC of >1,000 μg/ml) was selected for further use. This strain now contained a plasmid (pE194-3A) with an elevated (5- to 10-fold) plasmid copy number and which constitutively produced methylase as well. Although elevated plasmid copy number was maintained during at least three subcultures, we always started experiments from a frozen stock culture. Plasmid pE194 is maintained at a copy number of approximately 10 per cell in *B. subtilis* (34), and previously selected *cop* mutants showed plasmid levels of 50 to 100 per cell, the same range of increase we observe.

Inhibition of growth of constitutively MLS-resistant *B. subtilis* by 6-*O*-methyl-11,12-cyclic carbamate derivatives of erhythromycin. *B. subtilis* containing an inducible or constitutive *ermC* determinant was resistant to >8,000 μg of erythromycin per ml; in striking contrast, certain 6-*O*-methyl-11,12-cyclic carbamate derivatives inhibited growth at 30 μg/ml (Table 1). Although 16-membered macrolides such as tylosin and niddamycin are active against inducible MLS bacteria due to lack of *erm* methylase induction, the 6-*O*-methyl-11,12-cyclic carbamate derivatives 1a and 1b were unique in their abilities to inhibit growth of constitutively MLS-resistant bacteria (Table 1). The abilities of 6-*O*-methyl-11,12-cyclic carbamates to inhibit growth of susceptible *B. subtilis* and *S. epidermidis* were similar to those of erythromycin, tylosin, and niddamycin (Table 1). Although these data are consistent with the hypothesis that 6-*O*-methyl-11,12-cyclic carbamates interact with MLS-resistant ribosomes, they do not rule out nonspecific inhibition as the reason for growth inhibition. Therefore, the interaction of these novel macrolides with in vitro protein synthesis systems derived from both susceptible and constitutively MLS-resistant *B. subtilis* was examined.

TABLE 1. Inhibitory activities of macrolides[a]

Strain	MIC (μg/ml)				
	Niddamycin	Tylosin	Erythromycin	Compound 1a	Compound 1b
B. subtilis BD170 (susceptible)	0.2	0.5	0.5	0.5	0.5
B. subtilis BD170 (constitutive MLS resistance; pE194-3A)	>1,000	>1,000	>8,000	30	30
S. epidermidis 958-1 (susceptible)	0.8	0.1	0.2	0.2	0.1
S. epidermidis 958-3 (constitutive MLS resistance; pNE131)	>1,000	>1,000	>8,000	30	30
S. aureus RN1389 (MLS resistant)	ND[b]	ND	5,000	32	32

[a] See Fig. 1.
[b] ND, Not determined.

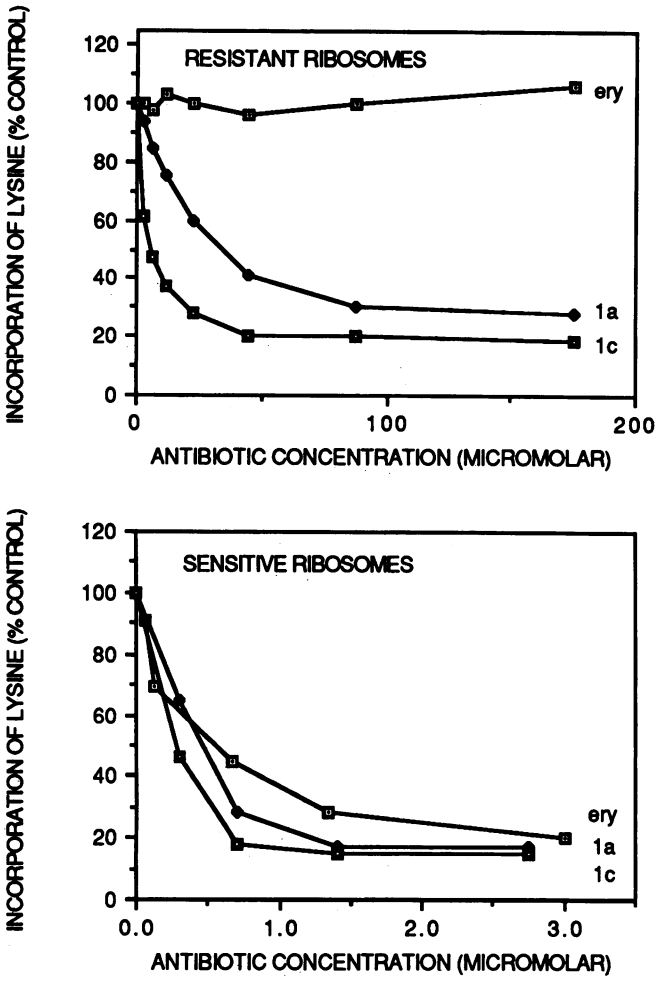

FIG. 2. Inhibition of in vitro protein synthesis by macrolide derivatives. S30 extracts were prepared from susceptible or MLS-resistant (containing plasmid pE194-3A) *B. subtilis*, and macrolide derivatives were tested for ability to inhibit incorporation of [^{14}C]lysine into protein. The control value (100%) represents the amount of lysine incorporated in the absence of antibiotic (20,000 cpm at 10-min incubation). ery, Erythromycin.

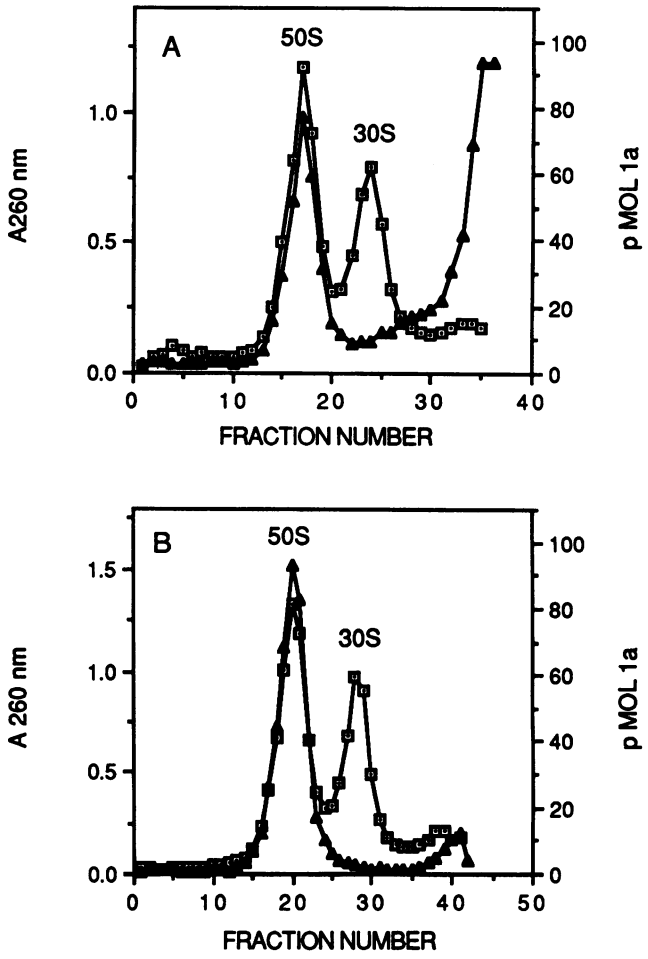

FIG. 3. Binding of 1a to MLS-resistant and -sensitive ribosomal subunits from *B. subtilis*. (A) MLS-resistant ribosomes (2 μM) were incubated with radiolabeled 1a (5.8 μM) in 200 μl of ribosome buffer for 1 h at room temperature. (B) Sensitive ribosomes (2.1 μM) were incubated with radiolabeled 1a (2.2 μM) in 200 μl of ribosome buffer for 1 h at room temperature. Both samples were applied to linear 15 to 30% sucrose gradients for separation of ribosomal subunits. Gradients were fractionated and monitored for A_{260} (□) and radioactivity. The amount of 1a per fraction (▲) was calculated after correcting for quenching.

Inhibition of in vitro protein synthesis by 6-*O*-methyl-11,12-cyclic carbamate derivatives of erythromycin. Certain 6-*O*-methyl-11,12-cyclic carbamate derivatives (1a and 1c) were unique in their abilities to inhibit protein synthesis by MLS-resistant ribosomes (Fig. 2). In contrast, erythromycin (Fig. 2), tylosin, and niddamycin (data not shown) were completely ineffective at inhibiting protein synthesis by MLS-resistant ribosomes, as expected from the in vivo MIC data (Table 1). All of the macrolides tested were able to inhibit in vitro protein synthesis by normal sensitive ribosomes (erythromycin, 1a, and 1c [Fig. 2] and niddamycin and tylosin [data not shown]). In all cases, the degree of inhibition was dose dependent, and 1a and 1c were more potent inhibitors of protein synthesis by sensitive ribosomes compared with MLS-resistant ribosomes. Although these data are consistent with the hypothesis that 6-*O*-methyl-11,12-cyclic carbamate bind to MLS-resistant ribosomes, inhibiting protein synthesis and thus cell growth, they do not rule out nonspecific effects on in vitro protein synthesis. Therefore, direct interaction of one 6-*O*-methyl-11,12-cyclic carbamate derivative, 1a, with both normal and MLS-resistant ribosomes was examined.

Binding of erythromycin and 1a to normal and MLS-resistant ribosomes and ribosomal subunits. The radiolabeled 6-*O*-methyl-11,12-cyclic carbamate derivative, 1a, interacted specifically with the 50S MLS-resistant ribosomal subunit. Ribosomes (70S) were incubated with an excess of drug, and subunits were dissociated and separated on a sucrose gradient. Radiolabeled drug was bound specifically to the 50S MLS-resistant subunit, not to the 30S subunit, and remaining unbound drug was at the top of the gradient (Fig. 3A). Derivative 1a also bound specifically to the sensitive 50S ribosomal subunit (Fig. 3B). The stoichiometry of binding to 50S subunits was approximately 1. In contrast, radiolabeled erythromycin did not bind to either the 50S or the 30S MLS-resistant ribosomal subunit (Fig. 4A), but as expected, did bind to sensitive 50S subunits (Fig. 4B) by similar analysis. Derivative 1a also bound to both the MLS-resistant

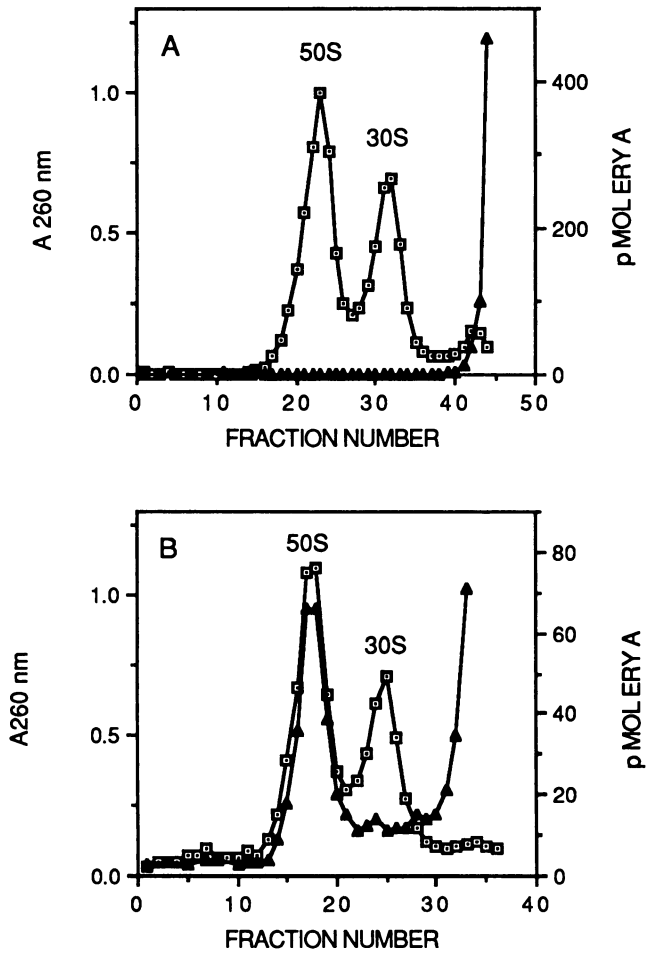

FIG. 4. Binding of erythromycin to MLS-resistant and sensitive ribosomal subunits from *B. subtilis*. (A) MLS-resistant ribosomes (2 μM) incubated with radiolabeled erythromycin (5 μM). (B) Sensitive ribosomes (2 μM) incubated with radiolabeled erythromycin (3 μM). Incubations and separation of ribosomal subunits were as given in the legend to Fig. 3. Symbols: □, A_{260}; ▲, picomoles of erythromycin.

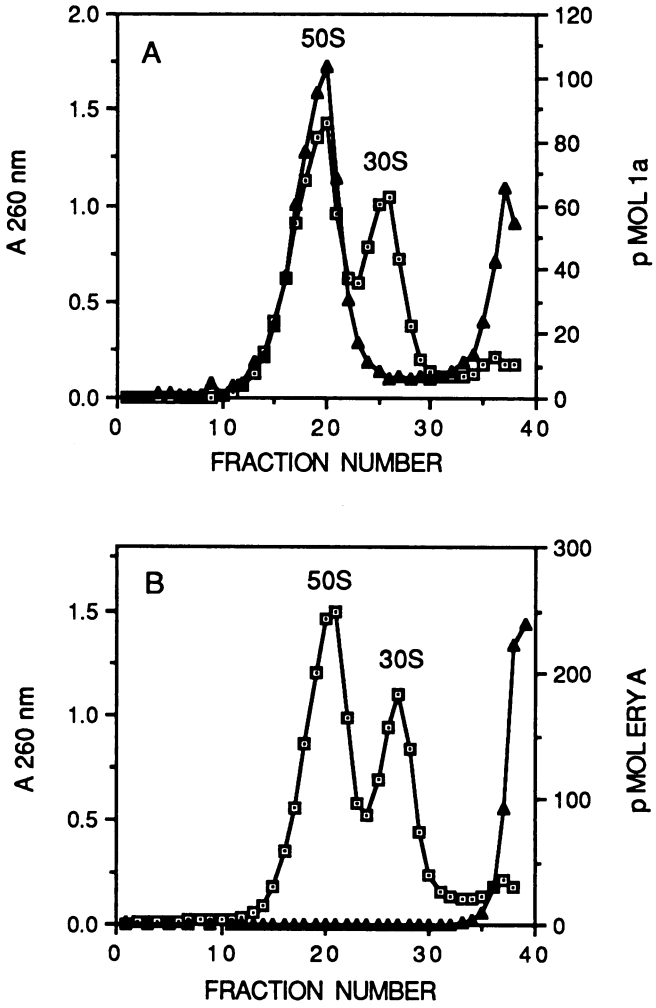

FIG. 5. Binding of 1a and erythromycin to MLS-resistant ribosomal subunits from *S. aureus*. (A) MLS-resistant ribosomes (2.5 μM) incubated with radiolabeled 1a (4.8 μM). (B) MLS-resistant ribosomes (2.5 μM) incubated with radiolabeled erythromycin (5 μM). Incubations and separation of ribosomal subunits were as given in the legend to Fig. 3. Symbols: □, A_{260}; ▲, picomoles of erythromycin.

(Fig. 5A) and -sensitive (not shown) 50S ribosomal subunits isolated from *S. aureus*, and as expected, erythromycin did not bind to the MLS-resistant 50S (Fig. 5B), but did bind to the sensitive 50S subunit (not shown).

These results were confirmed by using isolated ribosomal subunits from constitutively MLS-resistant *B. subtilis*. The traditional filter-binding assay for macrolide interaction with ribosomes was not applicable to studying the interaction of 6-*O*-methyl-11,12-cyclic carbamate because of nonspecific binding to filters. We therefore developed a spun column procedure for separation of ribosome-bound and free drug. In this case, ribosomes elute in the void volume fraction due to their large size and are quantitatively recovered. In contrast, free drug is included in the column matrix and only trace amounts elute in the void fraction in the absence of ribosomes. The radiolabeled 6-*O*-methyl-11,12-cyclic carbamate derivative, 1a, bound specifically to the isolated 50S MLS-resistant ribosomal subunit, and no significant binding was detected to isolated 30S subunits (Fig. 6); the slight binding observed with 30S subunits (Fig. 6) was due to slight contamination with 50S subunits (data not shown). The stoichiometry of binding to the 50S MLS-resistant ribosomal subunit was estimated at 1.

Additional studies of binding to 70S ribosomal complexes yielded stoichiometries of one macrolide per 70S complex for erythromycin A binding to sensitive ribosomes (filter-binding method) and for 1a binding to sensitive or MLS-resistant ribosomes (spun column method). In contrast, no binding of erythromycin A was observed to MLS-resistant ribosomes (<1 pmol of erythromycin A per 100 pmol of ribosome) by filter binding even when the erythromycin concentration was increased to 40 μM. At this concentration 10 pmol of erythromycin A was found bound to 100 pmol of 70S complex, using the spun column method. This difference between methods was likely due to the ability of the spun column procedure to detect weaker binding than will the filter-binding method. However, both methods show that erythromycin binds poorly, if at all, to dimethylated ribosomes from *B. subtilis*. Compound 1a was able to compete for the erythromycin-binding site on sensitive ribosomes with a 50% inhibitory concentration (IC_{50}) of 0.8 μM. The

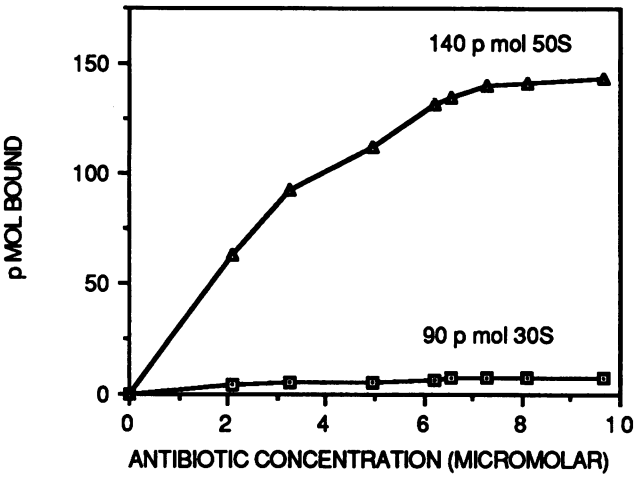

FIG. 6. Binding of 1a to isolated 30 and 50S MLS-resistant ribosomal subunits isolated from *B. subtilis*. Ribosomal subunits were separated on a 15 to 30% linear sucrose gradient, collected by centrifugation at 100,000 × *g* for 3 h, and suspended in ribosome-binding buffer. Subunits (140 pmol of 50S, 90 pmol of 30S) were incubated in 0.5 ml of buffer containing increasing concentrations of radiolabeled 1a for 1 h. Subunits and bound 1a were collected by centrifugation through 1-ml G50 columns. The amount of 1a bound was determined by liquid scintillation counting.

IC_{50} of erythromycin itself was 0.6 μM under the conditions used (0.6 μM [^{3}H]erythromycin, 0.2 μM concentrations of sensitive ribosomes, and 4-h incubation at 37°C).

Kinetics of interaction of macrolides with sensitive and MLS-resistant ribosomes. The kinetics of interaction of the 6-*O*-methyl-11,12-cyclic carbamate derivative, 1a, with sensitive or MLS-resistant ribosomes was significantly different than the interaction of its parent structure, erythromycin, with sensitive ribosomes prepared from *B. subtilis* (Table 2). The association rate constant for the 1a-ribosome complex, using either sensitive or MLS-resistant ribosomes, was several hundredfold slower than formation of the erythromycin-sensitive ribosome complex (Table 2). The dissociation rate constant of the 1a-ribosome complex, using sensitive or MLS-resistant ribosomes, was also dramatically slower (50- to 100-fold) than the dissociation of the erythromycin-sensitive ribosome complex. Addition of sodium dodecyl sulfate to ribosomes containing bound 1a, followed by gel electrophoresis, gave no evidence of any covalent (<5%) linkage of 1a to ribosomal components. We have not acertained whether 1a released from ribosomes is modified, but have no evidence which suggests that any alterations occur.

The K_d for equilibrium binding can be calculated by using the forward and reverse rate constants, k_1 and k_{-1} (Table 2). The K_d values for erythromycin and 1a for the sensitive ribosome are nearly identical, as are their MICs for growth inhibition of susceptible *B. subtilis* (Table 1) and their IC_{50}s for inhibition of in vitro protein synthesis (Fig. 2). In addition, 1a had the same IC_{50} for competition against erythromycin as did erythromycin itself (see above), indicating a similar equilibrium binding affinity. However, as mentioned above (Table 2), both the forward and the reverse rate constants of 1a for the sensitive ribosome are drastically different than the rate constants for the parent molecule erythromycin. Although erythromycin does not bind to MLS-resistant ribosomes, 1a does, and again the rate constants differ dramatically from those characteristic of the erythromycin-sensitive ribosome interaction. In this case, the forward rate constant is even slower for 1a interaction with the MLS-resistant ribosome than it is for that with the sensitive ribosome. Thus, the K_d is even weaker, as predicted by the poorer MIC of 1a on the constitutively MLS-resistant *B. subtilis* strain compared with the susceptible strain (Table 1) and the higher IC_{50} for inhibition of in vitro protein synthesis from the MLS-resistant strain compared with the susceptible strain (Fig. 2).

Selection of macrolide-resistant derivatives from susceptible *B. subtilis*. Macrolide-resistant derivatives of the susceptible strain BD170 were obtained by plating cells on medium containing erythromycin or derivative 1a. The frequency of resistance to both macrolides was identical when selection used cells grown from a single colony; however, as expected, this frequency varied when cells from independent single colonies were used (range, 1×10^{-7} to 5×10^{-9}). The MICs of erythromycin and 1a were 25 and 50 μg/ml, respectively, for all resistant mutants tested regardless of which agent was used for selection.

DISCUSSION

Compound 1a, a 6-*O*-methyl-11,12-cyclic carbamate derivative of erythromycin, binds to dimethylated MLS-resistant ribosomes, inhibiting protein synthesis and thus growth of constitutively MLS-resistant bacteria. We utilized two sets of bacteria (*S. epidermidis* and *B. subtilis*) constructed by transformation, and in the case of *B. subtilis* we constructed a strain which maximally expressed the *ermC* methylase due to elevated plasmid copy number and constitutive expression. Binding was specific for a single site on the MLS-resistant 50S ribosomal subunit isolated from *B. subtilis* and *S. aureus*. In contrast, the parent structure, erythromycin, from which compound 1a was synthetically derived did not bind to MLS-resistant ribosomes or inhibit growth of constitutively MLS-resistant bacteria. These data are consistent with the hypothesis that 1a has regained access to the altered macrolide-binding site present on the dimethylated MLS-resistant 50S ribosomal subunit. We used ribosomes which were not washed with 1 M NH_4Cl for the detailed experiments reported above, because we thought this would more

TABLE 2. Kinetics of erythromycin and compound 1a interaction with sensitive and MLS-resistant ribosomes[a]

Ribosomes	k_1 (liters mol^{-1} min^{-1})		k_{-1} (min^{-1})		K_d (mol liter^{-1})	
	Erythromycin	Compound 1a	Erythromycin	Compound 1a	Erythromycin	Compound 1a
Sensitive	4.9×10^7	4.5×10^5	0.067	0.0005	1.4×10^{-9}	1.1×10^{-9}
Resistant	NA[b]	0.9×10^5	NA	0.0012	NA	1.3×10^{-8}

[a] Washed ribosomes were prepared from susceptible and MLS-resistant (containing plasmid pE194-3A) *B. subtilis* cells, and the rate of complex formation, or dissociation, was determined in triplicate. Data were analyzed as given in the text to calculate the forward (k_1) and reverse (k_{-1}) rate constants. K_d was calculated as follows: $K_d = k_{-1}/k_1$.

[b] NA, Not applicable (erythromycin does not bind).

effectively mimic the intracellular state of the ribosome. When less detailed experiments were performed with ribosomes washed with 1 M NH_4Cl, only minor differences in ribosome-macrolide interactions were observed (data not shown).

Derivative 1a does bind to the macrolide-binding site on sensitive ribosomes isolated from *B. subtilis*, based on the following data. Derivative 1a will compete for erythromycin binding to sensitive ribosomes with an IC_{50} of 0.8 μM under conditions in which the IC_{50} for erythromycin itself is 0.6 μM. Furthermore, selection for resistance to erythromycin or 1a, using susceptible *B. subtilis*, yielded identical frequencies of resistance in any given experiment (as expected, absolute frequencies varied in each experiment), and cross-resistance of isolates was identical regardless of which macrolide derivative was used for selection. These results would be expected if both structures bind at the same site on sensitive ribosomes. In addition, a single specific change in ribosomal protein L17 appears to be involved in spontaneous resistance to erythromycin (29), and our data indicate that this same change is selected by derivative 1a.

We are only aware of one method in the literature in which the kinetics of macrolide interaction with ribosomes was studied directly (17, 18), and in this case the macrolide, 9(*S*)-erythromycylamine, was derivatized with the bulky fluorescent group 5-fluorescein isothiocyanate. In this case, the kinetic constants were altered significantly compared with the parent macrolide. Since the association rate constant had never been directly measured for the parent macrolide erythromycin, but only calculated (13, 26), we thought it advisable to attempt such direct measurements. We recently devised a procedure to measure the initial rate of formation of the macrolide-ribosome complex directly, allowing calculation of the second-order rate constant for the reaction, by using ribosomes isolated from various gram-negative and gram-positive bacteria (R. Goldman and C. Doran, manuscript in preparation). We have applied this procedure to the analysis of ribosome interaction with the macrolide derivatives described in this report.

The forward rate constant of erythromycin binding to sensitive *B. subtilis* ribosomes was 4.9×10^7 liters mol^{-1} min^{-1}; the reverse rate constant was 0.067 min^{-1}, yielding a K_d of 1.4×10^{-9} M at equilibrium (Table 2). The value measured directly for k_1 is significantly greater than values estimated for k_1 by previous indirect measurements with *E. coli* ribosomes (13, 26); the K_d value we calculated, using our measured k_{-1} (Table 2), also indicates tighter equilibrium binding than previously reported for erythromycin binding to *E. coli* ribosomes. Our data are not unique to the *B. subtilis* ribosome, however, as we have recently measured k_1 and k_{-1} of erythromycin for ribosomes prepared from *E. coli* and found k_1 to also be faster than estimated previously (13, 26), thus yielding a lower calculated K_d (i.e., tighter binding [Goldman and Doran, in preparation]). We have attempted to analyze some of our equilibrium binding data by Scatchard analysis; however, accurate measurement of free macrolide was impossible under the conditions used, due to the strong affinity of macrolide for the ribosome-binding site.

Significant differences were observed between k_1 and k_{-1} for the interaction of compound 1a with sensitive and MLS-resistant ribosomes from *B. subtilis* when compared with the same values measured for the interaction of the parent structure erythromycin with sensitive ribosomes. The association rate constants for compound 1a binding to both sensitive and MLS-resistant ribosomes is 100- and 500-fold slower, respectively, than the k_1 value for erythromycin binding to sensitive ribosomes. Since k_1 for erythromycin interaction with sensitive ribosomes is of the same order of magnitude as that reported for other receptor-ligand interactions (37), the slower value of k_1 for 1a interaction with both sensitive and MLS-resistant ribosomes shows that the interaction of 1a with ribosomes is different than that of the parent structure erythromycin and is unique in terms of interaction kinetics. However, interaction with the sensitive ribosome does occur at the macrolide-binding site because (i) 1a will compete for erythromycin binding to sensitive ribosomes, (ii) selection for resistance to erythromycin or 1a yields bacteria with identical cross-resistance patterns, and (iii) interaction of 1a with sensitive ribosomes causes induction of both *ermC* and *ermD* regulatory systems (S. Kadam and R. Goldman, manuscript in preparation). This latter observation shows that both 1a and erythromycin interaction with sensitive *B. subtilis* ribosomes perturb ribosome function similarly, leading to induction of inducible *ermC* and *ermD* regulatory systems. In addition, k_{-1} is also 50- to 100-fold slower for the dissociation rate constant of the 1a-ribosome complex compared with the k_{-1} value for the erythromycin-sensitive ribosome complex. The slow dissociation rate constant of 1a for sensitive ribosomes also occurs in vivo, leading to a more bactericidal response and to a prolonged postantibiotic effect when compared with the parent structure erythromycin (R. C. Goldman and S. K. Kadam, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 928, 1988).

Derivative 1a appears to interact with ribosomes in a unique manner. Since 1a interacts at the normal macrolide-binding site on sensitive ribosomes with similar affinity, but with very slow association and dissociation rate constants compared with the parent structure erythromycin, its unique chemical structure must be limiting the rate of complex formation and dissociation at the normal macrolide-binding site. Similarly, association and dissociation rate constants of 1a for dimethylated MLS-resistant ribosomes are slow, but in this case there is a 10-fold decrease in binding affinity compared with binding to sensitive ribosomes. Although we are pursuing identification of the precise binding site of 1a on the MLS-resistant ribosome, we as yet have no definitive data demonstrating that binding occurs at a site analogous to the macrolide site on sensitive ribosomes. Chloramphenicol will partially compete for erythromycin binding to sensitive ribosomes, as well as for 1a binding to MLS-resistant ribosomes, but the high concentrations required do not allow firm conclusions to be drawn (unpublished data).

Dimethylation of adenine 2058 is a rather modest change in ribosome structure, and all of the other structural components of the macrolide-binding site, other ribosomal proteins, and rRNA are still present and functioning normally in MLS-resistant ribosomes (we observe no change in the rate of protein synthesis in vitro [unpublished data]). Thus, it is possible that 1a regains access to a sequestered or conformationally altered macrolide-binding site which exists on the MLS-resistant ribosome. The aromatic ring introduced into 1a is required for activity against MLS-resistant organisms (10; unpublished data) and thus plays an important role in binding. This could involve the recently described role of aromatic rings as hydrogen bond acceptors (3-kcal [ca. 12.5 kJ]/mol stabilizing enthalpy) in protein-drug complexes (20). Our data are most consistent with the hypothesis that 1a interaction with the MLS-resistant ribosome initiates a conformational change which renders the macrolide-binding site available for ligand interaction, because (i) k_1 and k_{-1} are slow for 1a interaction with both sensitive and MLS-resis-

tant ribosomes, (ii) 1a interacts at the macrolide-binding site on sensitive ribosomes, (iii) a rate-limiting induced conformational change during complex formation would explain the slow dissociation rate of the complex in terms of the thermodynamics of microscopic reversibility, and (iv) preliminary molecular modeling studies show that the macrolide ring and sugars are in the same conformation in both erythromycin and compound 1a.

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