ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1995, p. 2141–2144 Vol. 39, No. 9
0066-4804/95/$04.00+0


# Macrolide Antibiotics Inhibit 50S Ribosomal Subunit Assembly in *Bacillus subtilis* and *Staphylococcus aureus*

W. SCOTT CHAMPNEY* AND ROBIN BURDINE

*Department of Biochemistry, J. H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614*

Received 20 March 1995/Returned for modification 15 May 1995/Accepted 13 July 1995

**Macrolide antibiotics are clinically important antibiotics which are effective inhibitors of protein biosynthesis in bacterial cells. We have recently shown that some of these compounds also inhibit 50S ribosomal subunit formation in *Escherichia coli*. Now we show that certain macrolides have the same effect in two gram-positive organisms, *Bacillus subtilis* and *Staphylococcus aureus*. Assembly in *B. subtilis* was prevented by erythromycin, clarithromycin, and azithromycin but not by oleandomycin. 50S subunit formation in *S. aureus* was prevented by each of seven structurally related 14-membered macrolides but not by lincomycin or two streptogramin antibiotics. Erythromycin treatment did not stimulate the breakdown of preformed 50S subunits in either organism. The formation of the 30S ribosomal subunit was also unaffected by these compounds. Assembly was also inhibited in a *B. subtilis* strain carrying a plasmid with the *ermC* gene that confers macrolide resistance by rRNA methylation. These results suggest that ribosomes contain an additional site for the inhibitory functions of macrolide antibiotics.**

Erythromycin and related macrolide antibiotics are widely used clinically against infections by gram-positive microorganisms (15). Their structures and functional activities have been extensively investigated (17). Erythromycin binds with a 1:1 stoichiometry to the 50S ribosomal subunit of gram-positive (4, 12, 16) and gram-negative cells (19). The inhibition of translation is associated with the peptidyltransferase activity of the 50S subunit (24). However, peptide bond formation is unaffected by this drug. Polyribosomes appear to function normally in the presence of this drug (1, 21), but their distribution on mRNA is influenced by erythromycin (7). These observations have led to the suggestion that erythromycin inhibits a step in translation between initiation and elongation (1, 21, 23) or affects the P site-dependent binding (2) or dissociation (13, 14) of peptidyl-tRNA. The exact mechanism of protein synthesis inhibition by macrolide antibiotics is still not understood (5, 6).

In addition to this well investigated effect on the process of translation, we have recently shown that erythromycin and azithromycin can also prevent the 50S ribosomal subunit from being assembled in growing *Escherichia coli* cells (3). Because macrolide antibiotics are used primarily against infections by gram-positive organisms, it was important to see if this feature of these drugs was also manifest in gram-positive cells. Now we show that three related macrolide antibiotics prevent 50S ribosome assembly in *Bacillus subtilis* cells and that seven macrolides affect assembly in *Staphylococcus aureus* cells. The results suggest that these compounds have two mechanisms of action as antimicrobial agents.

**Cell growth and uridine labeling.** *B. subtilis* W168 ($Trp^-$) (from John Laffan, East Tennessee State University) was grown in 10 ml of tryptone broth at 37°C in a rotary shaker. At a density of 20 in a Klett-Summerson colorimeter, antibiotics were added at the indicated concentrations. After 1 h, [$^3$H]uridine (1 μCi/ml; 2 μg/ml) was added to the culture. Cells were grown for 6 to 10 h in the presence of each drug, generally for at least two doublings. Cells were collected by centrifugation at 8,000 × *g* for 10 min, washed once with S buffer (10 mM Tris-HCl [pH 7.8], 0.5 mM magnesium acetate, 50 mM $NH_4Cl$), and stored frozen at −70°C. *B. subtilis* BD464(pBD9) (10) containing the *ermC* methylase gene from plasmid pE194 (8, 26) was obtained from the Bacillus Genetics Stock Center at Ohio State University. Cells were grown in tryptone broth containing 5 μg of kanamycin per ml with and without erythromycin at 1.5 mg/ml. Cells were labeled with [$^3$H]uridine as described above. *S. aureus* RN1786 (from Joyce Sutcliffe at Pfizer) was grown in 10 ml of tryptic soy broth and was labeled as described above.

To examine ribosomal subunit stability in both organisms, 1-ml cultures of cells were labeled with [$^3$H]uridine (1 μCi/ml) in the absence of carrier uridine for 2 to 3 h at 37°C. Cells were collected by centrifugation, resuspended in 10 ml of fresh broth containing uridine (2 μg/ml), and grown to 20 Klett units. Erythromycin was added to a final concentration of 0.4 μg/ml, and cells were grown through two doublings before being collected.

**Sucrose gradient analysis of ribosomal subunits.** Cell pellets were resuspended in 0.2 ml of S buffer. *B. subtilis* cells were lysed by the addition of lysozyme (250 μg/ml) and incubation at 37°C for 15 min with two subsequent cycles of freezing and thawing. DNase I (10 μg/ml) was added, and cells were incubated at room temperature for an additional 10 min. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. *S. aureus* cells were lysed by incubation with lysostaphin (50 μg/ml) in the presence of 2.5 mM phenylmethylsulfonyl fluoride and 40 U of RNasin (Promega). After a 20-min incubation at room temperature, cells were frozen and thawed three times. DNase I treatment and centrifugation were performed as described above.

Cell lysates from both organisms were layered on 5 to 20% sucrose gradients in S buffer. The gradients were centrifuged in an SW50.1 rotor at 175,000 × *g* for 2 h at 4°C. About 30 fractions were collected and brought to 1 ml with water. The $A_{260}$ was measured, 100 μg of bovine serum albumin was added, and then the fractions were made 5% in trichloroacetic acid. Precipitated material was collected on glass fiber filters, and [$^3$H]uridine radioactivity was measured by liquid scintilla-

* Corresponding author. Phone: (615) 929-6211. Fax: (615) 975-8235.

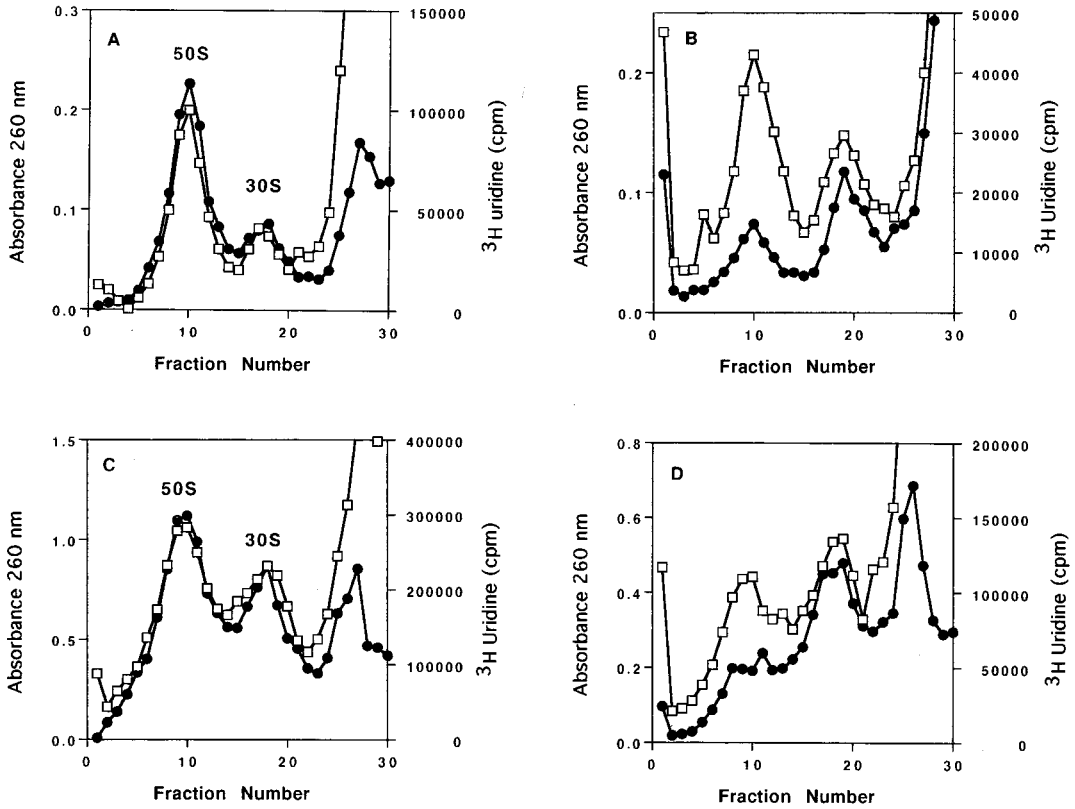


FIG. 1. Sucrose gradient profiles of lysates from cells labeled with [$^3$H]uridine in the presence (B and D) and absence (A and C) of erythromycin. The bottom of each gradient is on the left. Shown are the $A_{260}$ (□) and [$^3$H]uridine counts per minute (●) for *B. subtilis* cells labeled with [$^3$H]uridine for 3 h without erythromycin (A) and labeled for 6 h with erythromycin present (0.4 μg/ml) (B) and for *S. aureus* cells labeled with [$^3$H]uridine for 1.5 h without erythromycin (C) and for 4.25 h with [$^3$H]uridine in the presence of erythromycin (0.4 μg/ml).

tion counting. Specific activities ([$^3$H]uridine counts per minute/$A_{260}$) were calculated for five fractions around each subunit peak.

**Macrolide inhibition of 50S subunit assembly.** Ribosomal subunit formation in growing *B. subtilis* and *S. aureus* cells was examined by labeling control and antibiotic-inhibited cells with [$^3$H]uridine for several generations. Erythromycin at 0.4 μg/ml reduced the growth rate of each organism by more than 80%. Ribosomal subunits in cell lysates were separated by sucrose gradient centrifugation. Typical gradient profiles are shown in Fig. 1 for both control and erythromycin-treated *B. subtilis* and *S. aureus* cells. Specific activities ([$^3$H]uridine counts per minute/$A_{260}$) were calculated for each subunit to examine the effects of each drug on ribosome formation. In each control gradient, the specific activity ratio of 50S to 30S subunits was 1.0 (Table 1). For cells grown in erythromycin, the ratio was reduced to 0.69 for *B. subtilis* (Fig. 1B) and to 0.65 for *S. aureus* cells (Fig. 1D). The formation of the 30S ribosomal subunit was not significantly affected by erythromycin treatment. A relatively constant specific activity for the 30S subunit was found when the values were normalized to equivalent cell densities at harvest. Because of differences in growth rates, cells were not collected at the same density.

This effect on 50S subunit formation was not limited to erythromycin. Azithromycin and clarithromycin, derivatives of erythromycin, reduced growth rates substantially (Table 1) and were as effective as erythromycin in preventing 50S subunit formation in both organisms (Fig. 2). Three other 14-membered macrolides (roxithromycin, flurithromycin, and 14-hydroxyclarithromycin) inhibited 50S subunit formation in *S. aureus* cells to similar extents (Table 1). Oleandomycin did not influence subunit formation in *B. subtilis* cells but was an inhibitor of assembly in *S. aureus* cells (Table 1).

Lincosamide and streptogramin antibiotics have effects on protein synthesis similar to those of macrolides (14, 22), and

TABLE 1. Antibiotic effects on growth rates and 50S subunit assembly

| Organism | Antibiotic[a] | Concn (μg/ml) | Doubling time (h) | Sp act[b] |
|---|---|---|---|---|
| *B. subtilis* | None | | 1.2 | 1.09 (5) |
| | Erythromycin | 0.4 | 4.3 | 0.69 (2) |
| | Clarithromycin | 0.4 | 4.2 | 0.64 (2) |
| | Azithromycin | 1.2 | 4.8 | 0.31 (2) |
| | Oleandomycin | 30 | 4.6 | 1.03 (2) |
| *S. aureus* | None | | 0.7 | 1.04 (6) |
| | Erythromycin | 0.4 | 4.2 | 0.65 (2) |
| | Clarithromycin | 0.4 | 4.7 | 0.57 (2) |
| | Azithromycin | 1.5 | 3.5 | 0.67 (2) |
| | 14-Hydroxyclarithromycin | 2.0 | 6.0 | 0.70 (2) |
| | Flurithromycin | 2.0 | 4.8 | 0.74 (2) |
| | Roxithromycin | 2.0 | 4.5 | 0.89 (2) |
| | Oleandomycin | 30 | 3.9 | 0.80 (2) |
| | Lincomycin | 0.4 | 2.3 | 1.08 (2) |
| | Virginiamycin $M_1$ | 0.5 | 1.8 | 0.97 (2) |
| | Virginiamycin S | 75 | 2.7 | 1.03 (2) |

[a] Clarithromycin, roxithromycin, flurithromycin, and 14-hydroxyclarithromycin were gifts from Abbott Laboratories (Abbott Park, Ill.); azithromycin was a gift from Pfizer (Groton, Conn.); and virginiamycin S was a gift from SmithKline-RIT (Brussels, Belgium).

[b] Ratio of the 50S subunit to the 30S subunit. Numbers in parentheses are the numbers of experiments performed.

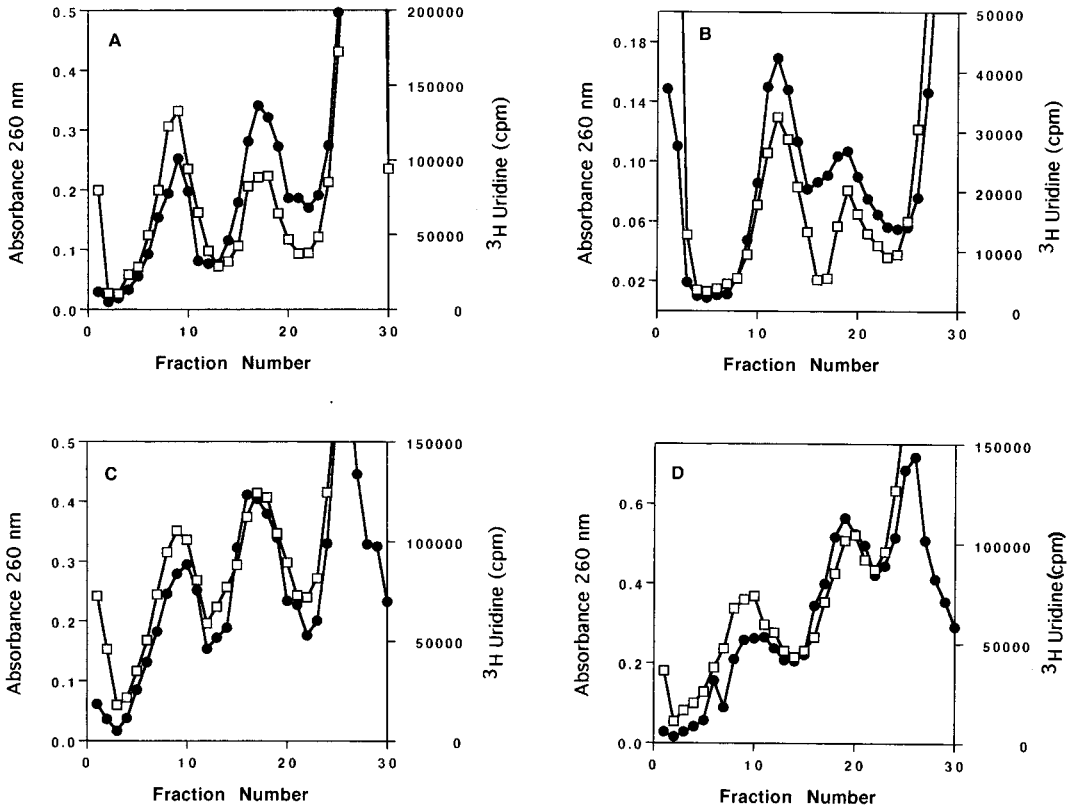


FIG. 2. Sucrose gradient profiles of lysates from cells labeled with [$^3$H]uridine in the presence of azithromycin (A and C) and clarithromycin (B and D). Shown are the $A_{260}$ (□) and [$^3$H]uridine counts per minute (●) for *B. subtilis* cells grown with azithromycin at 1.2 μg/ml for 5 h (A) and with clarithromycin at 0.4 μg/ml for 6 h (B) and for *S. aureus* cells grown in the presence of azithromycin at 1.5 μg/ml for 4.25 h (C) and in the presence of clarithromycin at 0.4 μg/ml for 5.25 h (D).

simultaneous resistance to all three types of compounds can be achieved by methylation of a 23S rRNA nucleotide (4, 20, 25). Lincomycin and the streptogramin antibiotics virginiamycin $M_1$ and virginiamycin S were significant inhibitors of cell growth, but they did not influence subunit formation in *S. aureus* cells (Table 1).

Erythromycin treatment of growing cells did not stimulate the breakdown of subunits formed in the absence of this antibiotic. Figure 3 indicates that a 2:1 ratio of [$^3$H]uridine radioactivity was found by comparing 50S and 30S subunits in both untreated cells and cells treated with erythromycin after [$^3$H] uridine labeling.

In two erythromycin-resistant strains of *E. coli*, the assembly effects of erythromycin were demonstrated at increased concentrations of this macrolide (3). A *B. subtilis* strain carrying the *ermC* gene for inducible erythromycin resistance on a plasmid vector (8, 10, 11) was examined for assembly defects. Erythromycin at 1.5 mg/ml reduced the growth rate by 90%, and assembly of the 50S subunit was impaired by this drug concentration (specific activity ratio = 0.55).

**Specificity of macrolide effects on subunit formation.** The results presented above indicate that two targets exist on the 50S ribosomal subunit for the inhibitory effects of macrolide antibiotics. Both the elongation step of polypeptide chain

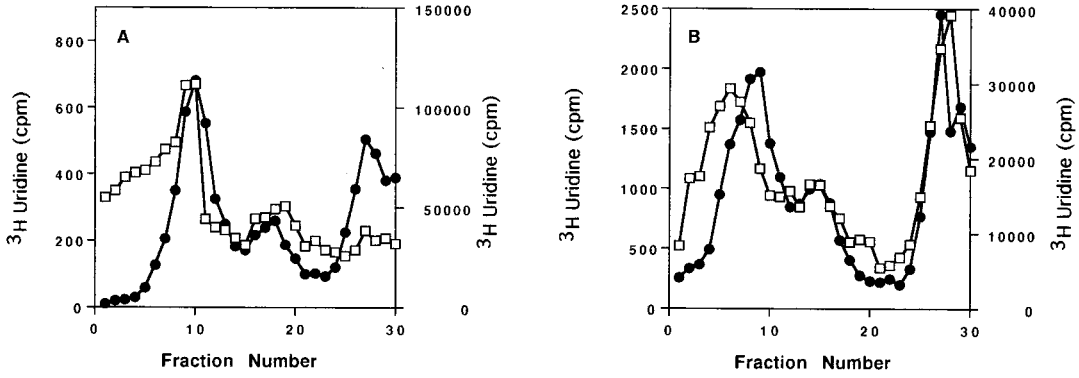


FIG. 3. Sucrose gradient profiles of cell lysates labeled with [$^3$H]uridine before the addition of erythromycin. (A) *B. subtilis* cells grown and labeled with [$^3$H]uridine without (●, left axis) or with erythromycin (0.4 μg/ml) for 4 h after labeling (□, right axis); (B) *S. aureus* cells grown and labeled with [$^3$H]uridine without (●, left axis) or with erythromycin (0.4 μg/ml) for 6.5 h after labeling (□, right axis).

growth and the initial steps of 50S subunit assembly are affected by these compounds. The macrolide effect on assembly is very specific. Seven related compounds affected assembly in *S. aureus* cells, compared with only three in *B. subtilis* cells and two in *E. coli* cells (3). In each of these organisms, 50S subunit degradation was not stimulated and 30S subunit formation was not influenced. Lincomycin and streptogramin antibiotics were ineffective in blocking assembly.

The suggestion of a second target for macrolide effects is strengthened by the observation that assembly can be prevented in erythromycin-resistant strains. Erythromycin prevented assembly in two ribosomal-protein mutants of *E. coli* (3) and in an *ermC* strain of *B. subtilis*.

Chloramphenicol is the only other well studied antibiotic which affects ribosomal subunit assembly (18). In contrast with our findings, however, growth inhibition by chloramphenicol results in the incomplete assembly of both ribosomal subunits, consistent with a generalized effect on the translation of mRNAs for all ribosomal proteins (9). There is no demonstrated specificity for an effect on 50S subunit formation, unlike the situation with erythromycin, in which 30S subunit assembly is unaffected by this macrolide.

The effectiveness of macrolide antibiotics against gram-positive bacteria is likely due to the presence of two ribosomal targets for these compounds. Our results suggest that other macrolides should be examined for effects on ribosome formation to establish the most effective features of the structure which promote this inhibitory activity.

## REFERENCES


1. **Andersson, S., and C. G. Kurland.** 1987. Elongating ribosomes are refractory to erythromycin. Biochimie (Paris) **69:**901–904.
2. **Chinali, G., E. Nyssen, M. DiGiambattista, and C. Cocito.** 1988. Action of erythromycin and virginiamycin S on polypeptide synthesis in cell-free systems. Biochim. Biophys. Acta **951:**42–52.
3. **Chittum, H. S., and W. S. Champney.** 1995. Erythromycin inhibits the assembly of the large ribosomal subunit in growing *Escherichia coli* cells. Curr. Microbiol. **30:**273–279.
4. **Corcoran, J. W.** 1984. Mode of action and resistance mechanisms of macrolides, p. 231–259. *In* S. Omura (ed.), Macrolide antibiotics. Academic Press, Orlando, Fla.
5. **Cundliffe, E.** 1980. Antibiotics and prokaryotic ribosomes: action, interaction and resistance, p. 555–581. *In* G. R. Craven, G. Chambliss, J. Davies, K. Davis, L. Kahan, and M. Nomura (ed.), Ribosomes, structure function and genetics. University Park Press, Baltimore.
6. **Cundliffe, E.** 1987. On the nature of antibiotic binding sites in ribosomes. Biochimie (Paris) **69:**863–869.
7. **Dahlberg, A. E., E. Lund, and N. O. Kjeldgaard.** 1973. Some effects of antibiotics on bacterial polyribosomes as studied by gel electrophoresis. J. Mol. Biol. **78:**627–636.
8. **Denoya, C., and D. Dubnau.** 1989. Mono- and dimethylating activities and kinetic studies of the ermC 23S rRNA methyltransferase. J. Biol. Chem. **264:**2615–2624.
9. **Dodd, J., J. M. Kolb, and M. Nomura.** 1991. Lack of complete cooperativity of ribosomal assembly in vitro and its possible relevance to in vivo assembly and the regulation of ribosomal gene expression. Biochimie (Paris) **73:**757–767.
10. **Gryczan, T., and D. Dubnau.** 1978. Construction and properties of chimeric plasmids in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **75:**1428–1432.
11. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150:**804–814.
12. **Mao, J. C.-H., and M. Putterman.** 1969. The intermolecular complex of erythromycin and the ribosome. J. Mol. Biol. **44:**347–361.
13. **Menninger, J.** 1985. Functional consequences of binding of macrolides to ribosomes. J. Antimicrob. Chemother. **16**(Suppl. A)**:**23–34.
14. **Menninger, J. R., R. A. Coleman, and L.-N. Tsai.** 1994. Erythromycin, lincosamides, peptidyl-tRNA dissociation, and ribosome editing. Mol. Gen. Genet. **243:**225–233.
15. **Nakayama, I.** 1984. Macrolides in clinical practice, p. 261–300. *In* S. Omura (ed.), Macrolide antibiotics. Academic Press, Orlando, Fla.
16. **Oleinick, L., and J. W. Corcoran.** 1969. Two types of binding of erythromycin to ribosomes from antibiotic-sensitive and -resistant *Bacillus subtilis* 168. J. Biol. Chem. **244:**727–735.
17. **Omura, S. (ed.).** 1984. Macrolide antibiotics. Academic Press, Inc., Orlando, Fla.
18. **Osawa, S., E. Otaka, T. Itoh, and T. Fukui.** 1969. Biosynthesis of 50s ribosomal subunit in *Escherichia coli*. J. Mol. Biol. **40:**321–351.
19. **Pestka, S.** 1974. Binding of [$^{14}$C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Agents Chemother. **6:**474–478.
20. **Skinner, R., E. Cundliffe, and F. J. Schmidt.** 1983. Site of action of ribosomal RNA methylase responsible for resistance to erythromycin and other antibiotics. J. Biol. Chem. **258:**12702–12706.
21. **Tai, P.-C., and B. D. Davis.** 1979. Action of antibiotics on chain-initiating and on chain-elongating ribosomes. Methods Enzymol. **59:**851–862.
22. **Vazquez, D.** 1979. Inhibitors of protein biosynthesis. Springer-Verlag, Berlin.
23. **Vester, B., and R. A. Garrett.** 1987. A plasmid-coded and site-directed mutation in *Escherichia coli* 23S RNA that confers resistance to erythromycin: implications for the mechanism of action of erythromycin. Biochimie (Paris) **69:**891–900.
24. **Vogel, Z., T. Vogel, A. Zamir, and D. Elson.** 1971. Correlation between the peptidyl transferase activity of the 50S ribosomal subunit and the ability of the subunit to interact with antibiotics. J. Mol. Biol. **60:**339–346.
25. **Weisblum, B.** 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. **39:**577–585.
26. **Weisblum, B., M. Y. Graham, T. Gryczan, and D. Dubnau.** 1979. Plasmid copy number control: isolation and characterization of high-copy-number mutants of plasmid pE194. J. Bacteriol. **137:**635–643.