APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2758–2761
0099-2240/91/092758-04$02.00/0

Vol. 57, No. 9

NOTES

Cloning Vectors, Mutagenesis, and Gene Disruption (*ermR*) for the Erythromycin-Producing Bacterium *Aeromicrobium erythreum*

ERIC S. MILLER

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695

Received 19 March 1991/Accepted 29 June 1991

Genetic systems for study of *Aeromicrobium erythreum*, a gram-positive, G+C-rich (72%) bacterium with the capacity for erythromycin biosynthesis, are described. High-copy-number plasmids suitable as gene cloning vectors include derivatives of the *Streptomyces* plasmids pIJ101, pVE1, and pJV1. pIJ101 derivatives with missense substitutions at the *rep* gene *Bam*HI site do not replicate in *A. erythreum*. Ethyl methanesulfonate treatment generated several amino acid auxotrophs and non-erythromycin-producing (Ery^-) strains. Using the Ery^- strain AR1807 as a recipient for plasmid-directed integrative recombination, the chromosomal *ermR* gene (encoding 23S rRNA methyltransferase) was disrupted. Phenotypic characterizations demonstrated that *ermR* is the sole determinant of macrolide antibiotic resistance in *A. erythreum*.

Erythromycin is a clinically important macrolide antibiotic produced by *Saccharopolyspora erythraea* and, although less appreciated, by the unicellular, gram-positive bacterium *Aeromicrobium erythreum* (formerly *Arthrobacter* sp. strain NRRL B-3381; 6, 17). Biosynthesis of the erythromycin 14-membered lactone appears to occur by a series of repeated condensations that resemble the reactions characteristic of fatty acid synthesis (18), whereas resistance to the antibiotic usually occurs by methylation of a specific adenosine residue in 23S rRNA (2, 27). Expression of the corresponding macrolide-lincosamide-streptogramin (MLS) resistance genes in antibiotic-producing bacteria is either constitutive (e.g., *carB*, *ermE*) or under translational attenuation control (e.g., *ermSF*) (3, 5, 10, 23). The taxonomic position of *A. erythreum* among the nonsporulating G+C-rich actinomycetes (17) and its capacity for erythromycin production throughout growth have prompted studies of antibiotic resistance in this bacterium (20, 21). To enhance genetic analysis of erythromycin metabolism and other metabolic processes in *A. erythreum*, a survey of potential plasmid cloning vectors, a chemical mutagenesis procedure, and an approach to directed chromosomal-gene disruption were undertaken. The erythromycin resistance gene *ermR* was used as a model system to develop the gene disruption procedure. (Following the recommendation of Kamimiya and Weisblum [10], *ermR* denotes the 23S rRNA methyltransferase gene of *A. erythreum* [*ermA* in references 20 and 21].)

Plasmid cloning vehicles. Plasmid DNA was introduced into the *A. erythreum* wild-type strain AR18 by using a protoplast transformation procedure (20). Protoplasts were combined with 1 to 2 μg of the plasmid DNAs indicated in Table 1, and the transformation frequencies were determined by counting antibiotic-resistant colonies of AR18 that appeared in 4 to 7 days at 32°C on thiostrepton-containing R2YE medium (8). Most derivatives of the high-copy-number streptomycete plasmid pIJ101 replicate in *A. erythreum*, as expected from the previous description of *A. erythreum* transformation with pIJ702 (20). Other pIJ101-derived plasmids (i.e., pIJ364 and pIJ424) carry additional antibiotic resistance genes (*vph* and *aph*, respectively) and can be used as gene-cloning vectors in *A. erythreum* (however, see below). Derivatives of the high-copy-number plasmids pVE1 and pJV1, originating from *Streptomyces venezuelae* (15, 19) and *Streptomyces phaeochromogenes* (1), respectively, were readily introduced into *A. erythreum* (Table 1). At 10^4 transformants per μg of DNA, pVE28 and pVE138 transformed at a 10-fold-higher frequency than did the other vectors. There is no indication of a restriction barrier to transformation in *A. erythreum*, as similar frequencies were observed using DNA from *A. erythreum*, *Streptomyces lividans*, or *Escherichia coli*. Low-copy-number streptomycete plasmids were also tested (Table 1), but none of the SLP1 (22) or SCP2* (13, 14) constructs could be introduced into *A. erythreum*. Minimal replicons of SCP2* with increased copy numbers (pHJL210, pHJL302, and pHJL401; 13) also failed to replicate in *A. erythreum*.

pIJ101 *rep* substitutions prevent replication in *A. erythreum*. Plasmids disrupted at the native *Bam*HI restriction site in the pIJ101 *rep* gene (11) fail to replicate in *Streptomyces* spp. (12). However, versatile streptomycete cloning vectors which have a nucleotide substitution that destroys the *Bam*HI recognition sequence in *rep* (4) have been constructed (7, 8). Two such plasmids, pIJ680 and pIJ697, failed to transform *A. erythreum*. To test whether the substitution at the pIJ101 *Bam*HI site affected replication in *A. erythreum*, transformation was examined with two plasmid sets. One set, pIJ424 and pIJ425, has the wild-type *Bam*HI sequence, whereas the second pair, pIJ486 and pIJ487, has the *rep* nucleotide substitution (4, 7, 8). Both pIJ424 and pIJ425 transformed *A. erythreum* AR18 as efficiently as other functional pIJ101 derivatives, whereas neither pIJ486 nor pIJ487 transformed AR18. The results show that although the resulting amino acid substitution in the Rep protein does not affect pIJ101 replication in *Streptomyces* spp., this allele is nonfunctional in *A. erythreum*. Although these plasmids are not suitable cloning vectors for *A. erythreum*, the conditional replication observed with *Streptomyces* and *Aeromicrobium* spp. provides a genetic system for

TABLE 1. Potential cloning vectors for *A. erythreum*

Plasmid (origin)[a]	Transformation frequency	Selection[b]
pIJ101 (*S. lividans*)		
pIJ303	10^3	*tsr*
pIJ364	10^3	*tsr vph*
pIJ424	10^3	*tsr* (*aph*II, promoter probe)
pIJ425	10^3	*tsr* (*aph*II, promoter probe)
pIJ486	<10	*tsr* (*aph*II, promoter probe)
pIJ487	<10	*tsr* (*aph*II, promoter probe)
pIJ643	10^3	*tsr vph*
pIJ680	<10	*tsr aph*I
pIJ697	<10	*tsr* (*amp*, *E. coli*)
pIJ702	10^3	*tsr*
pVE1 (*S. venezuelae*)		
pVE28	10^4	*tsr*
pVE30	10^3	*tsr*
pVE209	10^3	*aph*I
pVE138	10^4	*tsr vph* (*amp*, *E. coli*)
pJV1 (*S. phaeochromogenes*)		
pWOR125	10^3	*tsr*
pWOR191	10^3	*tsr*
SLP1 (*S. coelicolor*)		
pIJ61	<10	*tsr aph*I
SCP2* (*S. coelicolor*)		
pIJ903	<10	*tsr* (*amp*, *E. coli*)
pIJ916	<10	*tsr*
pIJ922	<10	*tsr*
pHJL210[c]	<10	*tsr aph*I (*amp*, *E. coli*)
pHJL302[c]	<10	*tsr* (*amp*, *E. coli*)
pHJL401[c]	<10	*tsr* (*amp*, *E. coli*)

[a] Many of these plasmids and their properties have been reviewed elsewhere (8). See text for additional references.
[b] Where possible, all transformations used thiostrepton for selection. Additional markers indicated also function in *A. erythreum*.
[c] Derivatives of SCP2* with increased copy number (13).

domain analysis of Rep or for identification of protein interactions in pIJ101 DNA replication.

Isolation of amino acid auxotrophs and non-erythromycin-producing strains. *A. erythreum* AR18 cells grown for 24 to 36 h at 32°C in TYE broth (21) were incubated in 60 mM ethyl methanesulfonate (EMS) for 4 h, diluted 100-fold into TYE for overnight growth, and then spread onto TYE agar medium for colony isolation. Colonies were screened for amino acid auxotrophy by examining growth on minimal medium (21) supplemented with biotin, nicotinic acid, and thiamine, each at 2 μg/ml, with or without added amino acids at 50 μg/ml each. Erythromycin production was similarly determined by spot testing colonies for growth inhibition of *Bacillus subtilis* BD170. From a single round of EMS treatment, mutants were obtained that were phenotypically Arg^-, His^-, ILV^- (requiring all three branched-chain amino acids), Ile^- Thr^-, Leu^-, Met^-, Met^- Cys^-, Ser^- Gly^-, and Val^-, totaling nine separate classes. Four colonies that displayed no or limited antimicrobial activity and were thus deficient in erythromycin synthesis were also identified. Media from cocultured Ery^- isolates failed to produce inhibition zones on strain BD170, indicating an absence of cosynthesis. Strain AR1807, a distinctly Ery^- isolate, was used in subsequent experiments. The 10 mutant phenotypes isolated among the 640 colonies screened (1.6%) demonstrate that EMS is an effective mutagen of *A. erythreum*.

***ermR* replacement vectors and isolation of chromosomal recombinants.** Disruption of *ermR* in *A. erythreum* required the cloned gene (21) and used an Ery^- recipient strain,

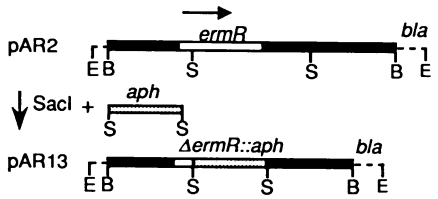

FIG. 1. Integrative recombination vectors. *aph* was isolated from pUC4-KISS (Pharmacia LKB, Piscataway, N.J.) following *Sac*I digestion and ligated to pAR2 (21) that had also been digested with *Sac*I. pAR13 contains *aph* in place of the deleted 1.5-kbp fragment containing the distal portion of *ermR*. Symbols are as follows: open boxes, *ermR* coding region, with arrow showing the direction of transcription; filled boxes, *A. erythreum* DNA; stippled boxes, *aph*; dashed lines, pUC8 DNA linearized at the *Eco*RI site (not to scale). Letters indicate restriction enzyme cleavage sites (B, *Bam*HI; E, *Eco*RI; and S, *Sac*I).

AR1807. The *E. coli* plasmid pUC8 (24), which does not replicate in *A. erythreum*, was used as the vector for delivery of disrupted *ermR* into recipient cells. Because *A. erythreum* had been shown to be kanamycin sensitive (17) and because plasmids such as pVE209 that carry *aph* (Table 1) confer kanamycin resistance on *A. erythreum*, the *aph* gene of Tn*903* was chosen as the selectable marker (kanamycin or neomycin resistance) for recombination. The *ermR* replacement vector pAR13 (Δ*ermR*::*aph*) was constructed by replacing a 1.5-kbp *Sac*I fragment of pAR2 (Fig. 1), containing 0.8 kbp of *ermR* and 0.7 kbp of DNA beyond the 3′ end of *ermR*, with an approximately 1.3-kbp *Sac*I fragment containing *aph*.

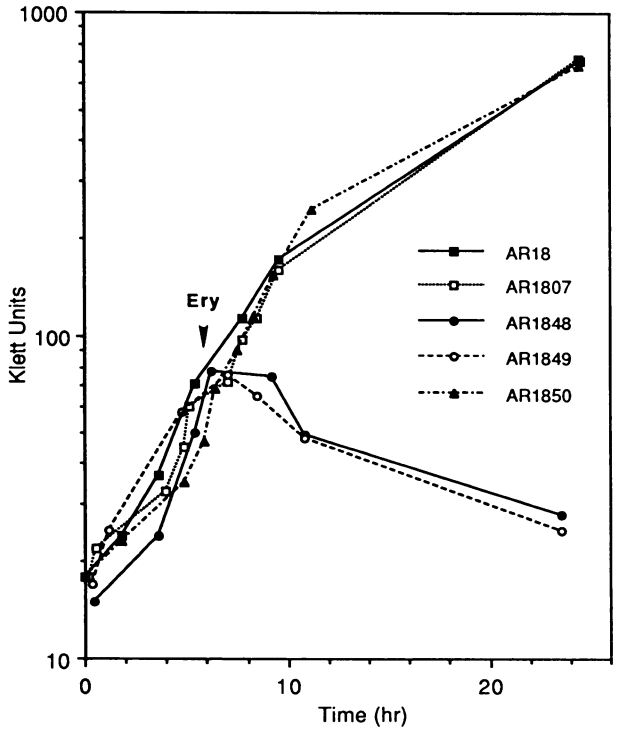

FIG. 2. Growth and erythromycin sensitivity of recombinant strains. Cultures were diluted into TYE broth and incubated at 32°C with aeration, and erythromycin (50 μg/ml) was added at the indicated time (arrowhead).

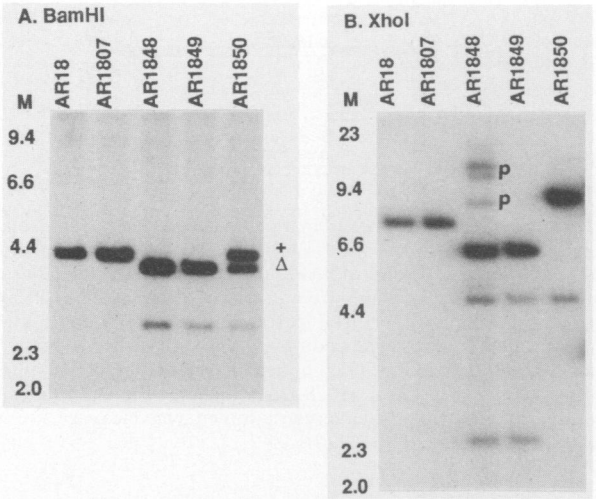

FIG. 3. Southern blot analysis of *A. erythreum* strains. Chromosomal DNA was treated with either *Bam*HI (A) or *Xho*I (B) and probed with the ^{32}P-labeled 4-kbp *Bam*HI fragment from pAR2. Weaker hybridization signals in panel A are vector (pUC8) sequences. Symbols: +, 4-kbp *ermR*$^+$ fragment; Δ, 3.8-kbp Δ*ermR*::*aph* fragment; p, fragments due to partial digestion with *Xho*I; M, positions of marker DNAs. Sizes (at left) are in kilobase pairs.

Protoplasts of strain AR1807 were prepared (20), transformed with pAR13, and spread onto R2YE regeneration medium (8). After 15 to 24 h at 32°C, the cells were overlaid with soft agar containing neomycin sulfate at 80 μg/ml. After approximately 4 days at 32°C, rare Neo^r colonies were obtained. Three colonies grew from transformations of AR1807 with 2 μg of pAR13 DNA. Two of these isolates were Kan^r Erm^s (strains AR1848 and AR1849), whereas one was Kan^r Erm^r (strain AR1850). Identical transformation conditions produced 1,500 thiostrepton-resistant transformants per μg of pIJ702 DNA. The fre-quency of integrative recombinants obtained per microgram of DNA was therefore at least 2 to 3 orders below that of replicative transformation.

Growth properties of Kan^r recombinants. Figure 2 shows that wild-type and gene replacement strains grew in TYE broth with a doubling time of approximately 2 h, demonstrating that integration of the exogenous DNA did not significantly alter cell growth. To examine whether *ermR* encodes the sole methyltransferase responsible for MLS resistance in *A. erythreum*, the sensitivity of strains AR1848 and AR1849 (Δ*ermR*::*aph*) to MLS was tested. AR18, AR1807, and AR1850 (Ery$^-$ Erm^r) were resistant to clindamycin, erythromycin, spiramycin, and tylosin (some sensitivity to tylosin was observed at high concentrations), whereas the *ermR*-disrupted strains AR1848 and AR1849 were highly sensitive to erythromycin (Fig. 2) and the other macrolide antibiotics (determined by disk diffusion assays). Therefore, unlike some of the other macrolide-producing bacteria (5, 10), *A. erythreum* does not have multiple resistance determinants. For the nonproducing parent strain AR1807, there was no delay in the onset of the erythromycin resistance phenotype, confirming that *ermR* is constitutively expressed.

Chromosome arrangement of recombinant strains. Southern blots (8) were used to examine the region of recombination at *ermR*. Chromosomal DNA prepared from each strain was treated with *Bam*HI and, following electrophoresis through 0.8% agarose, was transferred to a GeneScreen (Dupont) blotting membrane. An approximately 4-kbp *Bam*HI fragment purified from pAR2 (Fig. 1) was used as the ^{32}P-labeled probe (16). Results in Fig. 3 show that the probe hybridized to the 4-kbp *ermR* region of the wild-type strain and to an identical-size fragment from the Ery$^-$ strain AR1807. No apparent alteration in the AR1807 *ermR* region was detected as a consequence of EMS mutagenesis of AR18. Specific chromosomal alterations were detected in the Kan^r recombinant strains. The strong hybridization signal at 3.8 kbp for AR1848 and AR1849 was the expected size for replacement of wild-type *ermR* with Δ*ermR*::*aph* of pAR13. Strain AR1850, which was also obtained by recombination with pAR13, displayed two predominant hybridization signals, i.e., one corresponding to the wild-type allele and the other corresponding to the disrupted allele. The weak hybridization signal (Fig. 3) observed for all Kan^r strains but absent in the nontransformed strains is the expected size (2.7 kbp) of *Bam*HI-treated vector DNA from pAR13 (also see Fig. 1). An additional restriction endonu-

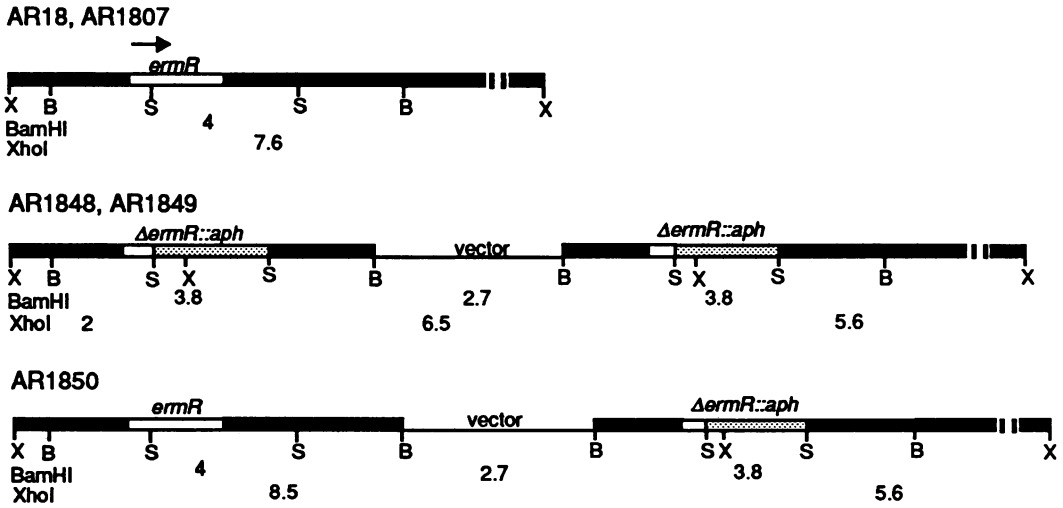

FIG. 4. Chromosome arrangement at *ermR*. Configurations were deduced from the restriction enzyme maps of the integration vectors (Fig. 1) and the results of Southern blot analyses (Fig. 3). Symbols are as in Fig. 1. Numbers below the maps are the sizes (in kilobase pairs) of the respective *Bam*HI (B) and *Xho*I (X) fragments observed in Fig. 3. S, *Sac*I.

clease (*Xho*I) was used in Southern blot analysis to further characterize the *ermR* region. These data confirmed that pAR13 vector sequences were present in the recombinant genomes as a likely consequence of a single cross-over event. Figure 4 summarizes the deduced organization of the *ermR* region of each strain. The AR1848 and AR1849 configurations appear to arise by integration, with subsequent sequence conversion of *ermR* to Δ*ermR*::*aph*. These data account for the growth properties shown in Fig. 2.

Using this approach, *ermR*-encoded methyltransferase was shown to be the only determinant of MLS resistance in *A. erythreum*. This strategy is similar to that used for other microorganisms, whereby recombinants are selected by using a tagged gene carried on a plasmid (9, 25, 26). Recent observations that the described *ermR* deletion strains can be complemented by the *Saccharopolyspora erythraea* erythromycin resistance gene *ermE* (contained on pIJ425; data not shown) suggest that expression of heterologous macrolide antibiotic genes can be studied in *A. erythreum*. The cloning vectors, EMS mutagenesis, and chromosomal gene disruption described herein should facilitate genetic analysis of this nondifferentiating, antibiotic-producing bacterium.

I thank Dana Agnell and Charles Trunks for their technical assistance at various stages of this work. Leslie Barnett assisted with the EMS mutagenesis, and both she and Anne Roberts provided thoughtful discussions. I am especially grateful to Sydney Brenner for sharing his laboratory during my introduction to *A. erythreum* and to David Hopwood, Tobias Kieser, Mervyn Bibb, Tanya McNeil, and Jeff Larson for providing plasmids. Thiostrepton and viomycin were the kind gifts of S. J. Lucania (Squibb, New Brunswick, N.J.) and E. Friend (Pfizer, Kent, United Kingdom), respectively.

This work was supported by grant 87-G-00710 from the North Carolina Biotechnology Center and by the North Carolina Agricultural Research Service (NC06024).

REFERENCES

1. **Bailey, C. R., C. J. Bruton, M. J. Butler, K. F. Chater, J. E. Harris, and D. A. Hopwood.** 1986. Properties of *in vitro* recombinant derivatives of pJV1, a multi-copy plasmid from *Streptomyces phaeochromogenes*. J. Gen. Microbiol. **132**:2071–2078.
2. **Corcoran, J. W.** 1984. Mode of action and resistance mechanisms of macrolides, p. 231–259. *In* S. Omura (ed.), Macrolide antibiotics. Chemistry, biology and practice. Academic Press, Inc., Orlando, Fla.
3. **Dubnau, D.** 1984. Translational attenuation: the regulation of bacterial resistance to the macrolide-lincosamide-streptogramin B antibiotics. Crit. Rev. Biochem. **16**:103–132.
4. **Eckhardt, T. (Lederle Laboratories).** Personal communication.
5. **Epp, J. K., S. G. Burgett, and B. E. Schoner.** 1987. Cloning and nucleotide sequence of a carbomycin-resistance gene from *Streptomyces thermotolerans*. Gene **53**:73–83.
6. **French, J. C., J. D. Howells, and L. E. Anderson.** December 1970. U.S. patent 3,551,294.
7. **Hopwood, D. A., M. J. Bibb, K. F. Chater, and T. Kieser.** 1987. Plasmid and phage cloning vectors for gene cloning and analysis in *Streptomyces*. Methods Enzymol. **153**:116–166.
8. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of streptomyces. A laboratory manual. John Innes Foundation, Norwich, England.
9. **Husson, R. N., B. E. James, and R. A. Young.** 1990. Gene replacement and expression of foreign DNA in mycobacteria. J. Bacteriol. **172**:519–524.
10. **Kamimiya, S., and B. Weisblum.** 1988. Translational control of *ermSF*, an inducible resistance determinant encoding rRNA *N*-methyltransferase from *Streptomyces fradiae*. J. Bacteriol. **170**:1800–1811.
11. **Kendall, K. J., and S. N. Cohen.** 1988. Complete nucleotide sequence of the *Streptomyces lividans* plasmid pIJ101 and correlation of the sequence with genetic properties. J. Bacteriol. **170**:4634–4651.
12. **Kieser, T., D. A. Hopwood, H. M. Wright, and C. J. Thompson.** 1982. pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. **185**:223–238.
13. **Larson, J. L., and C. L. Hershberger.** 1986. The minimal replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. Plasmid **15**:199–209.
14. **Lydiate, D. J., F. Malpartida, and D. A. Hopwood.** 1985. The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene **35**:223–235.
15. **MacNeil, T., and P. H. Gibbons.** 1986. Characterization of the *Streptomyces* plasmid pVE1. Plasmid **16**:182–194.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Miller, E. S., C. R. Woese, and S. Brenner.** 1991. Description of the erythromycin-producing bacterium *Arthrobacter* sp. strain NRRL B-3381 as *Aeromicrobium erythreum* gen. nov., sp. nov. Int. J. Syst. Bacteriol. **41**:363–368.
18. **Omura, S.** 1984. Macrolide antibiotics. Chemistry, biology and practice. Academic Press, Inc., Orlando, Fla.
19. **Paradiso, M. J., G. Roberts, S. L. Streicher, and R. B. Goldberg.** 1987. Characterization of suppressible mutations in the viomycin phosphotransferase gene of the *Streptomyces* enteric plasmid pVE138. J. Bacteriol. **169**:1325–1327.
20. **Roberts, A. N., L. Barnett, and S. Brenner.** 1987. Transformation of *Arthrobacter* and studies on the transcription of the *Arthrobacter ermA* gene in *Streptomyces lividans* and *Escherichia coli*. Biochem. J. **243**:431–436.
21. **Roberts, A. N., G. S. Hudson, and S. Brenner.** 1985. An erythromycin-resistance gene from an erythromycin-producing strain of *Arthrobacter* sp. Gene **35**:259–270.
22. **Thompson, C. J., T. Kieser, J. M. Ward, and D. A. Hopwood.** 1982. Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. Gene **20**:51–62.
23. **Uchiyama, H., and B. Weisblum.** 1985. N-methyl transferase of *Streptomyces erythraeus* that confers resistance to the macrolide-lincosamide-streptogramin B antibiotics: amino acid sequence and its homology to cognate R-factor enzymes from pathogenic bacilli and cocci. Gene **38**:103–110.
24. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
25. **Weber, J. M., and R. Losick.** 1988. The use of a chromosome integration vector to map erythromycin resistance and production genes in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). Gene **68**:173–180.
26. **Winans, S. C., S. J. Elledge, J. H. Krueger, and G. C. Walker.** 1985. Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. J. Bacteriol. **161**:1219–1221.
27. **Zalacain, M., and E. Cundliffe.** 1989. Methylation of 23S rRNA caused by *tlrA* (*ermSF*), a tylosin resistance determinant from *Streptomyces fradiae*. J. Bacteriol. **171**:4254–4260.