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Induction of *ermSV* by 16-Membered-Ring Macrolide Antibiotics

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The *erm* family of 23S rRNA adenine-N^6-methyltransferases confers resistance to all macrolide-lincosamide-streptograminB (MLS) antibiotics, but not all MLS antibiotics induce synthesis of Erm methyltransferase with equal efficiency in a given organism. The induction efficiency of a test panel of MLS antibiotics was studied by using two translational attenuator-*lac* reporter gene fusion constructs, one based on *ermSV* from *Streptomyces viridochromogenes* NRRL 2860 and the other based on *ermC* from *Staphylococcus aureus* RN2442. Four types of responses which were correlated with the macrolide ring size were seen, as follows: group 1, both *ermSV* and *ermC* were induced by the 14-membered-ring macrolides erythromycin, lankamycin, and matromycin, as well as by the lincosamide celesticetin; group 2, neither *ermSV* nor *ermC* was induced by the 12-membered-ring macrolide methymycin or by the lincosamide lincomycin or the streptogramin type B antibiotic ostreogrycin B; group 3, *ermSV* was selectively induced over *ermC* by the 16-membered-ring macrolides carbomycin, chalcomycin, cirramycin, kitasamycin, maridomycin, and tylosin; and group 4, *ermC* was selectively induced over *ermSV* by the 14-membered-ring macrolide megalomicin. These data suggest that the leader peptide determines the specificity of induction by different classes of MLS antibiotics and that for a given attenuator, a major factor which determines whether a given macrolide induces resistance is its size.

The *erm* family of rRNA methylases is one of the major groups of genes responsible for inducible erythromycin resistance, and within this group, *ermC* from *Staphylococcus aureus* is one of the most intensively studied. The biochemical basis for resistance has been shown to be a methylation of 23S rRNA by N^6-methyltransferase, and its regulation by translational or transcriptional attenuation has been reviewed (7, 19, 32, 33). The most effective inducers of the system in *S. aureus* include the 14-membered-ring macrolide erythromycin and the lincosamide celesticetin. Sixteen-membered-ring macrolides have been notable for their lack of inducing activity in *S. aureus.*

Little is known about the molecular basis for the distinction between inducers and noninducers, except that 16-membered-ring macrolides are generally considered noninducers and that we have located a consecutive series of four crucial amino acid residues in the *ermC* leader peptide at which amino acid changes markedly affected induction by erythromycin (20, 21). In addition, we showed that site-specific mutational alteration of single amino acids in this critical region of the *ermC* leader peptide could alter the relative effectiveness with which three test antibiotics (erythromycin, megalomicin, and celesticetin) induced *ermC* (21). On the basis of these findings we have proposed that the amino acid sequence of the *ermC* leader peptide acting jointly with the bound antibiotic determines whether induction of *ermC* will occur (20, 21, 33).

In the initial characterization of *ermC* and *ermSV* by assays based on inhibition zone morphology and on increased plating efficiency, we reported that *ermSV* in *Streptomyces viridochromogenes*, unlike *ermC* in *S. aureus*, can be induced by the 16-membered-ring macrolide tylosin (9). Induction of *ermSF* by tylosin was also reported by Kelemen et al. (17). If *ermSV* expression is controlled by an attenuator in a way similar to that for *ermC* expression, one would expect that if one 16-membered-ring macrolide induced *ermSV*, others might behave similarly.

To address the question of *erm* induction by 16-membered-ring macrolides, we constructed a *lac* fusion of *ermSV* and reinserted it into its original *S. viridochromogenes* background and tested induction by different groups of macrolide-lincosamide-streptogramin (MLS) antibiotics. For comparison, we included an *ermC* reporter fusion to *E. coli lacZ* and tested its response to the same set of test antibiotics.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the study and their derivations are presented in Table 1.

Construction of *ermSV*::*lac* reporter plasmid pSK611. (i) Plasmid pSK201 from pUC19 and *ermSV*. Plasmid pUC19 was digested in its multiple cloning site (mcs) with *Sal*I and was ligated with total DNA extracted from *S. viridochromogenes* NRRL 2860 digested with *Xho*I (Fig. 1a). The resultant library was probed for its ability to hybridize with *ermE* (30, 31), and plasmid DNA from one of several positive candidates, pSK201 (Fig. 1b), was selected for further analysis and sequencing. The pSK201 *Xho*I insert, which was 2 kb in length, was sequenced, revealing a promoter, a leader sequence encoding a potential attenuator and leader peptide open reading frame, and an *erm* open reading frame. Upon excision with *Hin*dIII and *Bam*HI and insertion into pHJL401 (18), the cloned fragment conferred inducible MLS resistance on *Streptomyces griseofuscus* (data not shown). The DNA sequence of *ermSV* (16) was deposited in GeneBank, and its detailed analysis will be described separately.

(ii) Plasmid pSK611 from plasmid pSK201 and plasmid pS13. The *ermSV* methylase open reading frame was translationally fused at the *Streptomyces lividans lac* determinant of plasmid pS13 (8). To accomplish this, plasmid pSK201 DNA, which was digested to completion with *Sph*I and partially with *Sma*I (Fig. 1b), was ligated with plasmid pS13 DNA and digested with *Xmn*I and *Sph*I (Fig. 1c). The *Xmn*I-*Sph*I promoterless *lac*-containing cassette is described elsewhere (Fig. 6 in reference 8). Ligation between the *Sma*I and *Xmn*I sites results in the in-phase fusion of *ermSV* with *lac*. The resultant ligation product was introduced into *Escherichia coli* NM522 by transformation, followed by selection for ampicillin-resistant transformants. Plasmid DNA preparations from individual transformants were checked for size and distribution of restriction sites, yielding plasmid pSK611 (Fig. 1d). Finally, plasmid pSK611 was introduced into *S. viridochromogenes* AR1 (*lac* mutant) by transformation, followed by selection for thiostrepton resistance.

Induction of reporter constructs. (i) *ermC*::*lacZ.* *Bacillus subtilis* MM222 (carrying plasmid pMM222) was tested for induction of β-galactosidase by antibiotics, as described previously (20).

(ii) *ermSV*::*lac.* A 100-ml stationary-phase culture of *S. viridochromogenes* SK611 (carrying plasmid pSK611) was frozen as 10 aliquots of 10 ml each for use as an inoculum by 100-fold dilution into YEME growth medium (13). The growth medium was supplemented with thiostrepton (20 μg/ml) to maintain

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TABLE 1. Strains and plasmids used in the study

Strain or plasmid	Description	Reference
Strains		
S. viridochromogenes NRRL 2860	Source of 2-kb *Xho*I DNA fragment which contains inducible *ermSV*	12
S. viridochromogenes AR1	*lac*-negative derivative from NRRL 2860 by nitrous acid mutagenesis followed by screening	This work
S. viridochromogenes SK611	From *S. viridochromogenes* AR1 transformed with pSK611 DNA	This work
E. coli NM522	Transformable *E. coli* host lacking restriction used as host to establish NRRL 2860 library in pUC19	11
B. subtilis MM222	From *B. subtilis* 168 transformed with plasmid pMM222 DNA	20
Plasmids		
pUC19	Vector for establishing cloned *S. viridochromogenes* NRRL 2860 library in *E. coli*	22
pS13	*S. lividans-E. coli* shuttle and expression vector with *lac* reporter gene; it contains pUC9- and pSL7-derived replicons	8
pIJ43	Source of probe used to locate *ermSV* by DNA sequence homology with *ermE*	30, 31
pSK201	pUC19(*Sal*I)::*ermSV*(*Xho*I) from pUC19(*Sal*I) and *S. viridochromogenes* NRRL 2860 total DNA digested with *Xho*I (see Fig. 1)	This work
pSK611	*E. coli-S. viridochromogenes* shuttle vector with *ermSV*::*lac* reporter, pSK201, and pS13 (see Fig. 1)	This work
pMM222	*E. coli-B. subtilis* shuttle vector carrying *ermC*::*lacZ* reporter	20

pSK611 and with test-inducing antibiotics at concentrations of between 1 ng/ml and 100 μg/ml, as indicated in Fig. 2a. Cultures were incubated with shaking for 7.5 h at 30°C and were frozen at −20°C until they were tested the next day. To measure β-galactosidase activity, the method of Miller (23) was used either directly or as adapted by Nicholson and Setlow (24) for fluorogenic assay with methylumbelliferyl β-D-galactoside (MUG) as the substrate. One-milliliter samples were centrifuged in a 1.5-ml Eppendorf tube for 5 min, and the supernatant was decanted. In the MUG-based assay, the pellet was resuspended in 1 ml of Z-buffer (23), followed by the addition of 100 μl of MUG stock solution (0.4 mg/ml of dimethyl sulfoxide), and the resultant mixture was incubated for 30 min at 30°C. Samples (200 μl) were dispensed into individual wells of a 96-well microtiter plate; each well contained 20 μl of 2 M Na_2CO_3. MUG fluorescence was read at 450 nm. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) was used as the substrate for the *ermC* determinations, whereas MUG was used for the *ermSV* determinations, in which greater sensitivity was needed.

Analysis of *erm* inducibility. The inducibility coefficient (maximum β-galactosidase activity in cells obtained by incubation with inducer at a concentration of between 1 ng and 100 μg of inducer per ml divided by the β-galactosidase activity obtained by incubation with the inducer at a concentration of 1 ng/ml) was calculated for both the *ermSV* and *ermC* fusion constructs. The inducibility coefficients for *ermSV* on the ordinate versus inducibility coefficients for *ermC* on the abscissa were plotted.

Antibiotics. The following antibiotics from the indicated suppliers were used: carbomycin (Pfizer), celesticetin (Upjohn), chalcomycin (Parke-Davis), cirramycin (Bristol), erythromycin (Sigma), kitasamycin (Ayerst), lankamycin (W. Keller-Schierlein), lincomycin (Upjohn), maridomycin (Takeda), matromycin (Pfizer), megalomicin (Schering), methymycin (Olin-Mathieson), ostreogrycinB (Glaxo), thiostrepton (Squibb), and tylosin (Lilly). All of the samples except erythromycin were gifts from the named suppliers; erythromycin was purchased.

RESULTS AND DISCUSSION

The *ermC* and *ermSV* reporter strains were induced and tested with their respective β-galactosidase substrates: ONPG for *B. subtilis* and MUG for *S. viridochromogenes*. The results are presented in Fig. 2a for *ermC* expressed in *B. subtilis* and for *ermSV* expressed in *S. viridochromogenes* SK611. Erythromycin and celesticetin, which have been characterized previ-

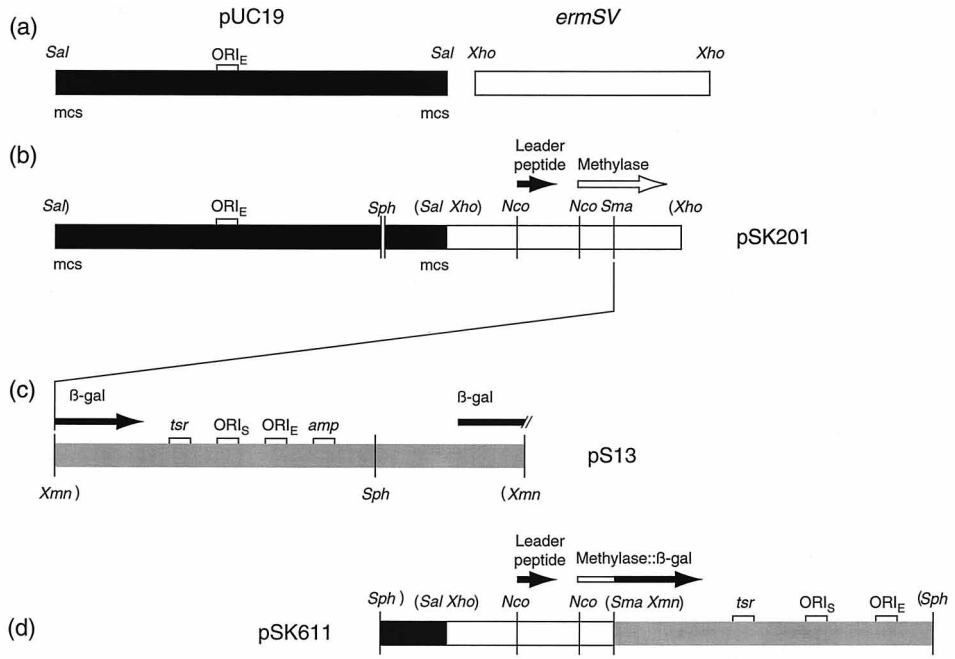

FIG. 1. Construction of plasmid pSK611, an *ermSV*::*lac* reporter. The reporter strain *S. viridochromogenes* SK611 was obtained by transformation of *S. viridochromogenes* AR1 with plasmid pSK611. β-gal, β-galactosidase. See text for details.

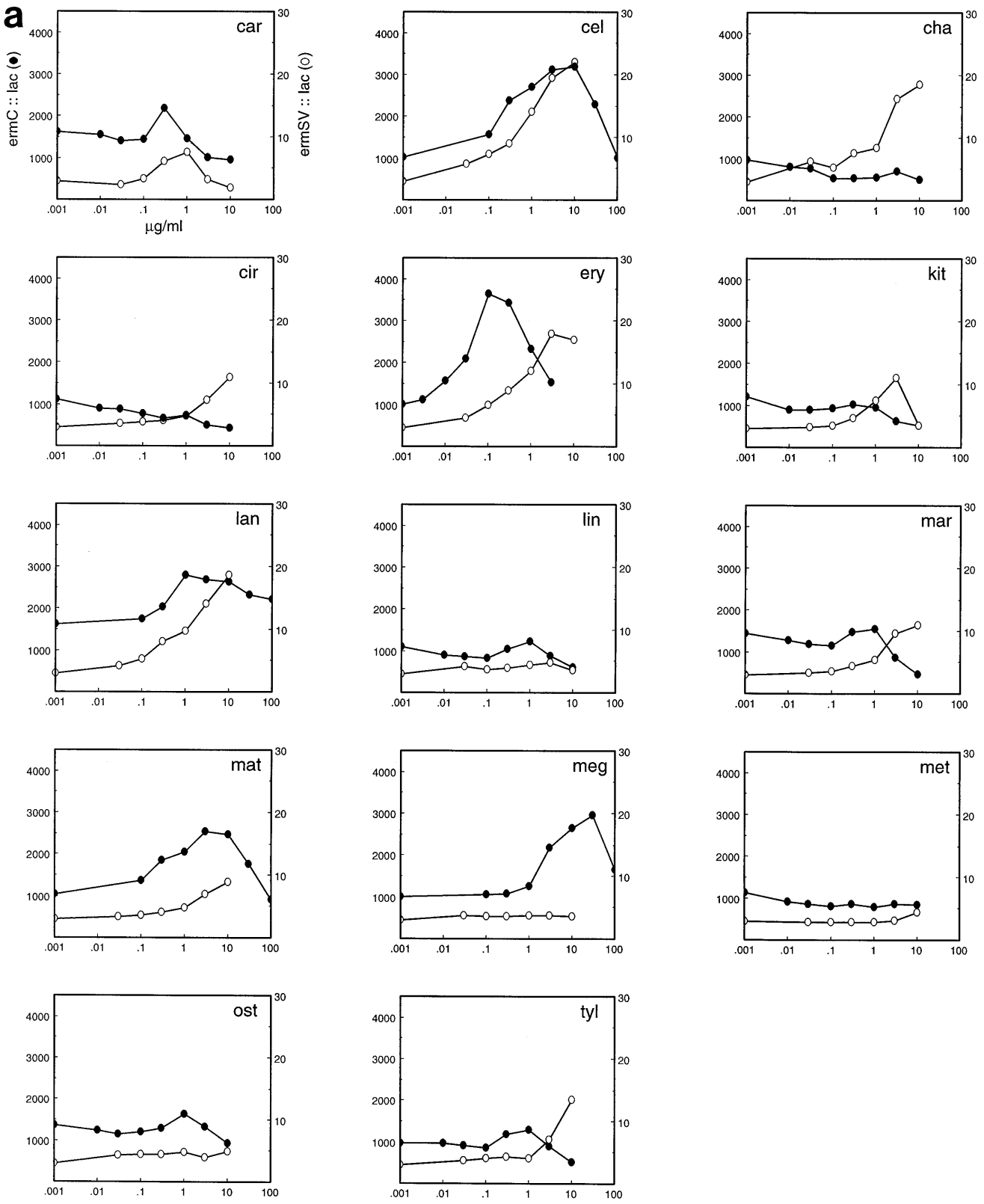

FIG. 2. Induction of reporter constructs. (a) *ermSV*::*lac*-carrying. *S. viridochromogenes* SK611 (○) and *ermC*::*lacZ*-carrying *B. subtilis* MM222 (●) were tested for induction as a function of antibiotic concentration. See text for details. (b) Inducibility of *ermC* and *ermSV* was calculated for each of the antibiotics tested and was graphed as a two-dimensional scatter plot, with the inducibility coefficient of *ermSV* plotted on the ordinate and the inducibility coefficient of *ermC* plotted on the abscissa. Abbreviations: car, carbomycin; cel, celesticetin; cha, chalcomycin; cir, cirramycin; ery, erythromycin; kit, kitasamycin (synonym, leucomycin); lan, lankamycin; lin, lincomycin; mar, maridomycin; mat, matromycin (synonym, oleandomycin); meg, megalomicin; met, methymycin; ost, ostreogrycin B; and tyl, tylosin.

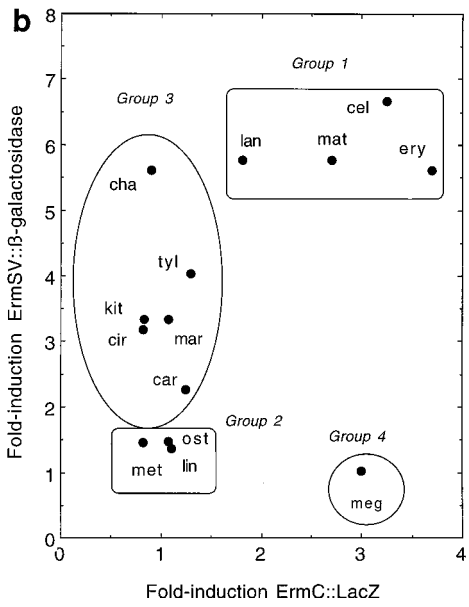

FIG. 2—*Continued.*

ously as effective inducers of resistance in *S. aureus* carrying *ermA* (1), gave the highest peak responses in both series of β-galactosidase assays.

To compare the induction of *ermC* and *ermSV*, we calculated an inducibility index for each antibiotic in each of the two *erm* systems. The calculated values were used as coordinates and were plotted two-dimensionally (Fig. 2b), with the inducibility index in *ermSV* plotted on the ordinate and the inducibility index in *ermC* plotted on the abscissa. The macrolides were then grouped according to size, with group 1 comprising the 14-membered-ring macrolides erythromycin, matromycin, and lankamycin, together with celesticetin because of its proximity. A second group, group 2, consisted of the 12-membered-ring macrolide methymycin, together with lincomycin and ostreogrycin B, because of their proximity. On the plot shown in Fig. 2b, group 1 and group 2 are diagonal elements and contain antibiotics which are either active or inactive in both systems, with group 1 antibiotics active in both the *ermC* and *ermSV* systems and group 2 antibiotics active in neither system. Group 3 consists of the 16-membered-ring macrolides chalcomycin, tylosin, kitasamycin, cirramycin, maridomycin, and carbomycin. These antibiotics form a cluster which favors induction of *ermSV* over *ermC*, and finally, group 4, megalomicin, a 14-membered-ring macrolide, appeared to favor *ermC* strongly over *ermSV*. The features of megalomicin which distinguish it from group 1 as defined here are unclear. With respect to the other groups, the clustering of macrolide antibiotics according to size is apparently correlated with their activities as inducers.

Previous studies indicated that 14-membered-ring macrolides induce MLS resistance, whereas 16-membered-ring macrolides do not (26, 27). An exception to this generalization appeared with the observation that tylosin could induce both *ermSV* (9) and *ermSF* (17). Our present data indicate that induction by tylosin is not an exception but that 16-membered-ring macrolides as a group are able to induce *erm* expression. The data presented in Fig. 2b demonstrated that 16-membered-ring macrolides can induce the *ermSV* but not the *ermC* reporter system. Induction of *erm* methylases involves interaction between antibiotic molecules, the nascent leader peptide, and the ribosome. We do not know the relative contributions of ribosomal structure and leader peptide to the observed induction of *ermSV*; however, our previous results that mutations in the leader peptide can affect the specificity of induction by different antibiotics (21) is consistent with the possibility that the *ermSV* leader peptide determines induction by 16-membered-ring macrolides.

Disk assay methods that follow bacterial cell growth in the presence of antibiotic are commonly used to study the induction of resistance. By this approach, we could not detect induction of resistance by the 16-membered-ring macrolides carbomycin, kitasamycin, and maridomycin (9), contrary to the results obtained with the reporter system (Fig. 2a). We cannot account for the discrepancies between growth-based and reporter-based assays. It is possible that multiple mechanisms of resistance known to occur commonly in *Streptomyces* (2, 3, 5, 6, 14, 15, 25, 28, 29, 30, 34, 35) are responsible.

Naturally occurring macrolide antibiotics which act on the ribosome belong mainly to three size classes, namely, 12-, 14-, or 16-membered-rings (for a review, see references 4 and 10). When tested for activity, the 12-membered-ring macrolide methymycin did not induce either of the two *erm* gene fusions described above, nor has inducing activity by members of this antibiotic group been reported. In contrast, the 14-membered-ring macrolides, excluding megalomicin, induced both *ermC* and *ermSV*, while 16-membered-ring macrolides selectively induce *ermSV*. Inhibition of protein synthesis, per se, cannot serve as a basis for induction because the equally potent 16-membered-ring macrolides do not induce *ermC*. These observations indicate that for a given attenuator system, induction is mostly determined by the ring size of the antibiotic.

Of the 14-membered-ring macrolides, lankamycin falls closest to the 16-membered-ring cluster (see Fig. 2b). In contrast to the other 14-membered-ring macrolides which have ethyl substituents at C-13, lankamycin has a four-carbon unit substituent at this position (4). It is precisely on this face of the molecule that the additional two carbon atoms, C-14 and C-15, that distinguish between the 14- and 16-membered-ring macrolides are located. Lankamycin also lacks the positive charge characteristic of other 14-membered-ring macrolides, which suggests that electrical charge is not intrinsically significant for the action of macrolides and leaves macrolide ring size as a significant determinant.

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