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A Family of r-Determinants in *Streptomyces* spp. That Specifies Inducible Resistance to Macrolide, Lincosamide, and Streptogramin Type B Antibiotics

YUKIO FUJISAWA† AND BERNARD WEISBLUM*

Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

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Inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics in *Streptomyces* spp. comprises a family of diverse phenotypes in which characteristic subsets of the macrolide-lincosamide-streptogramin antibiotics induce resistance mediated by mono- or dimethylation of adenine, or both, in 23S ribosomal ribonucleic acid. In these studies, diverse patterns of induction specificity in *Streptomyces* and associated ribosomal ribonucleic acid changes are described. In *Streptomyces fradiae* NRRL 2702 erythromycin induced resistance to vernamycin B, whereas in *Streptomyces hygroscopicus* IFO 12995, the reverse was found: vernamycin B induced resistance to erythromycin. In a *Streptomyces viridochromogenes* (NRRL 2860) model system studied in detail, tylosin induced resistance to erythromycin associated with N^6-monomethylation of 23S ribosomal ribonucleic acid, whereas in *Staphylococcus aureus*, erythromycin induced resistance to tylosin mediated by N^6-dimethylation of adenine. Inducible macrolide-lincosamide-streptogramin resistance was found in *S. fradiae* NRRL 2702 and *S. hygroscopicus* IFO 12995, which synthesize the macrolides tylosin and maridomycin, respectively, as well as in the lincosamide producer *Streptomyces lincolnensis* NRRL 2936 and the streptogramin type B producer *Streptomyces diastaticus* NRRL 2560. A wide range of different macrolides including chalcomycin, tylosin, and cirramycin induced resistance when tested in an appropriate system. Lincomycin was active as inducer in *S. lincolnensis*, the organism by which it is produced, and streptogramin type B antibiotics induced resistance in *S. fradiae*, *S. hygroscopicus*, and the streptogramin type B producer *S. diastaticus*. Patterns of adenine methylation found included (i) lincomycin-induced monomethylation in *S. lincolnensis* (and constitutive monomethylation in a mutant selected with maridomycin), (ii) concurrent equimolar levels of adenine mono- plus dimethylation in *S. hygroscopicus*, (iii) monomethylation in *S. fradiae* (and dimethylation in a mutant selected with erythromycin), and (iv) adenine dimethylation in *S. diastaticus* induced by ostreogrycin B.

Studies of inducible MLS (macrolide, lincosamide, and streptogramin) resistance in *Staphylococcus aureus* have focused on the requirements for induction by erythromycin, which initially appeared to be the only MLS antibiotic (excluding oleandomycin) with demonstrable inducing activity (21, 23). Underlying these studies has been the question, What property distinguishes erythromycin from other MLS antibiotics and endows it with optimal inducing activity? A step in the right direction was taken by Pestka et al. (17), who examined 53 erythromycin analogs and noted a correlation between inducing activity and ribosome binding activity. As the number and diversity of MLS-resistant organisms studied have increased, however, it has become clear that other MLS antibiotics could also induce. Dixon and Lipinski (7), Hyder and Streitfeld (11), and Malke (15) have reported that both lincomycin and erythromycin can induce *Streptococcus pyogenes*, and Allen (1) showed that the lincosamide celesticetin (but not lincomycin) could induce MLS resistance in *S. aureus*. Moreover, Tanaka and Weisblum (19) reported that a mutant of *S. aureus* selected with carbomycin was inducible by both carbomycin and lincomycin, but not by erythromycin. Additional divergence from the *S. aureus* pattern was suggested by studies of MLS resistance in *Streptomyces* spp. reported by Graham and Weisblum (9), who noted the presence of N^6-

† Present address: Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan.

monomethyladenine in *Streptomyces* strains that synthesize macrolide antibiotics, presumably associated with resistance to the antibiotics produced by these organisms.

The present studies were therefore undertaken to determine the extent of divergence from the "classical" case of *S. aureus*. As we shall show, inducible MLS resistance actually comprises a family of distinct phenotypes characterized by specific MLS subsets active as inducers of mono- or dimethylation or both, depending on the organism, and that *S. aureus* constitutes one of many special cases. In the present study we (i) determine the requirements for induction of adenine monomethylation and establish its association with MLS resistance in a *Streptomyces viridochromogenes* model system; (ii) characterize the diversity of induction patterns by testing several additional strains of *Streptomyces* for induction by a set of MLS antibiotics; and (iii) propose a model to account for the diversity of induction specificities in *Streptomyces* spp. based on the nucleotide sequence of the control region for MLS resistance in an *S. aureus* determinant.

MATERIALS AND METHODS

Bacterial strains. *S. viridochromogenes* NRRL 2860, provided by J. C. Ensign, who has described the properties of this organism in a review (8), was grown in enriched medium containing (per liter) 5 g of yeast extract (Difco) and 5 g of tryptone (Difco). *Streptomyces hygroscopicus* IFO 12995, a producer of the macrolide antibiotic maridomycin, was obtained from the collection of the Takeda Co. The remaining *Streptomyces* strains were NRRL stock cultures used in our previous studies.

Determination of resistance on solid medium. Mycelial suspensions of cells grown in enriched medium were disrupted by sonic oscillation for 10 s to obtain a homogeneous suspension for preparation of pour plates by the agar overlay method. The disks used to determine resistance contained 20 μg of test antibiotic.

Induction studies. To test induction in liquid medium, a sonicated cell suspension containing 10^6 colony-forming units per ml suspended in growth medium was incubated under the conditions of time and inducer concentration described. For *S. viridochromogenes*, the induction medium was sampled, and suitable dilutions were plated onto medium containing 50 μg of tylosin per ml to determine the concentration of induced colony-forming units in the medium, and onto antibiotic-free medium to determine the total viable colony-forming units.

Selection of mutants. For selecting mutants, MLS antibiotics producing large clear inhibition zones were used. The rationale behind this choice is that MLS antibiotics showing small inhibition zones probably induce in situ, and selection using such antibiotics would have a high probability of inducing the culture rather than selecting desired constitutively resistant mutants.

Altered methylation of 23S rRNA. 23S rRNA was labeled with either [2-^{3}H]adenine or [*methyl*-^{14}C]methionine in the presence or absence of inducer, as required. Inocula were grown in enriched medium supplemented with 1 g of K_2HPO_4 and 2 g of glucose per liter. For labeling experiments, actively growing mycelia from 100 ml of enriched medium were collected by centrifugation and suspended in 100 ml of M-9 medium containing (per liter): Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; 0.1 M $MgSO_4$, 10 ml; 0.01 M $CaCl_2$, 10 ml; and 50% glucose, 10 ml. The M-9 medium was additionally supplemented with 2% Casamino Acids and [^{3}H]adenine (specific activity, 15,000 Ci/mol; final concentration, 4 μCi/ml). Cells were labeled by incubation overnight (generally 14 to 18 h), collected, washed with 0.15 M NaCl solution, and stored at −20°C until used. Cells were disrupted by sonic oscillation, and labeled 23S rRNA was purified from the 50S ribosome subunits (obtained by sucrose density gradient centrifugation) by extraction with phenol, followed by depurination and separation of the resultant purines by column chromatography on Dowex-50X8 and paper chromatography in an isopropanol-ammonia-water (85:1.3:15) system as previously described (13). To label cells with [*methyl*-^{14}C]methionine, enriched medium diluted fivefold with water and supplemented with methionine (specific activity, 50 Ci/mol, 0.1 μCi/ml) was used. *methyl*-^{14}C-labeled 23S rRNA was digested with RNase A, and the resultant digest was fractionated with DEAE paper by the two-dimensional method of Brownlee et al. (3).

RESULTS

We first characterized the requirements for induction by using *S. viridochromogenes*, based on the fact that this organism is not known to produce any MLS antibiotics, a factor which if present might tend to mask induction by exogenously added inducers. The disk sensitivity method was used initially for determination of induced-resistance specificity profiles.

Patterns of induction in *S. viridochromogenes*. The ability of tylosin to induce resistance to erythromycin and to carbomycin (Fig. 1A) is manifested by the distorted erythromycin and carbomycin inhibition zones on the side facing the tylosin disk; the distorted inhibition zone results from the fact that cells induced on solid medium by tylosin, which diffuses from the disk, are capable of growing closer to the erythromycin or carbomycin disk, whereas the minimal inhibition zone surrounding the tylosin disk reflects the effectiveness of tylosin as inducer of resistance to itself.

We have shown previously that MLS antibiotics lacking inducing activity can be used to select constitutively resistant *S. aureus* mutants (23). As shown in Fig. 1B, a mutant selected for resistance to carbomycin shows coresistance to all remaining members of the test panel of MLS antibiotics. When wild-type inducible *S. aureus*

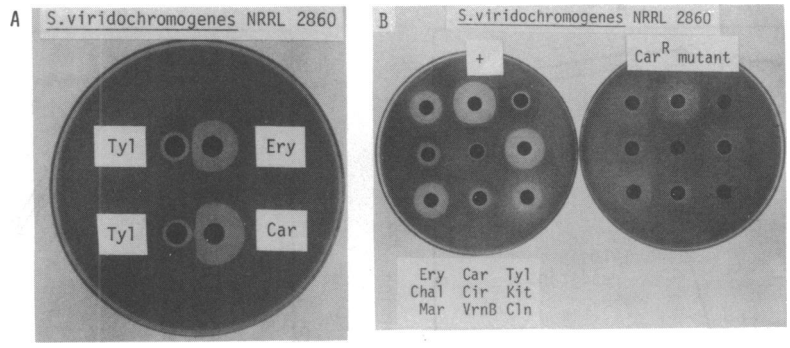

FIG. 1. *Specificity of induction and constitutive MLS resistance. Sonicated suspensions of S. viridochromogenes NRRL 2860 and a constitutive mutant derived from this strain were plated by the agar overlay method as indicated and tested for their response to antibiotic disks. (A) Tylosin (Tyl) induces resistance to erythromycin (Ery) and carbomycin (Car). (B) A spontaneous constitutive mutant selected for resistance to carbomycin (10 μg/ml) shows increased resistance to a set of MLS test antibiotics, as indicated in the figure. Abbreviations: Chal, chalcomycin; Cir, cirramycin; Kit, kitasamycin; Mar, maridomycin; VrnB, vernamycin B; Cln, clindamycin. Disks contained 20 μg of the named antibiotic.*

was tested similarly for induction, it was noted that erythromycin induced resistance to tylosin, whereas neither carbomycin nor tylosin had demonstrable inducing activity (19). We conclude that both *S. viridochromogenes* and *S. aureus* behave in a formally similar manner when tested for induction, but with differences in inducer specificity that remain to be explained.

Requirements for induction in *S. viridochromogenes*. The process of induction in *S. viridochromogenes* has both a time and inducer concentration dependence. To obtain reproducible quantitative data, it was necessary to prepare a homogeneous cell suspension. Baltz (2) has shown that a 100-fold-increased level in the number of colony-forming units is obtained if a mycelial suspension of *Streptomyces fradiae* is disrupted by sonication for 5 to 10 s before plating, and we employed this method without modification. Under the conditions used, optimal induction, measured in terms of the appearance of colony-forming units, requires exposure for 1 h to a concentration of tylosin in the range of 0.3 to 0.6 μg/ml (Fig. 2).

Altered methylation of 23S rRNA in resistant cells. [*methyl*-^{14}C]methionine was used as the source of label, followed by digestion of the labeled 23S rRNA sample with RNase A, two-dimensional fractionation of the resultant digest, and autoradiography (Fig. 3). Two additional spots in the rRNA from induced cells, indicated with arrows, were seen: one distinctly labeled fragment in the lower part of Fig. 3A, and a second, partially resolved spot in the upper part of this figure. We have not attempted to maximize the resolution of the larger, partially resolved fragment from its neighbors; however, the results suggest the possibility of at least two inducibly methylatable sites in 23S rRNA. The base compositions of these oligomers were not investigated further, but from our previous studies in *S. aureus* (12) we were able to locate the methylated adenine residue in a similar type of fragment.

Quantitative data are obtainable by labeling cells uniformly with [2-^{3}H]adenine, which allows us to determine the average number of adenine residues that have been methylated. We assume that all adenine residues in 23S rRNA are labeled to the same average specific activity, and that the relative amount of each derivatized form of adenine present in 23S rRNA is reflected in the relative amount of radioactivity present in that component. Since 23S rRNA contains approximately 800 adenine residues, one methylated adenine per 23S rRNA would comprise 0.12% of the total adenine fraction. Results of such an assay (Fig. 4, summarized in Table 1) indicate the presence of 0.13 and 0.17% monomethyl adenine relative to total adenine for the inducible and constitutively resistant strains, respectively. The apparent discrepancy between the results shown in Fig. 3 and 4 remains to be reconciled.

Reduced erythromycin binding by resistant cells. 50S subunits from uninduced, induced, and constitutively resistant *S. viridochromogenes* were tested for their ability to bind erythromycin. The extent of association between erythromycin and 50S ribosome subunits was measured by adsorption of the complex to nitrocellulose membrane filters. Results of the assay (data not shown) were similar to those obtained in our previous studies of erythromycin binding

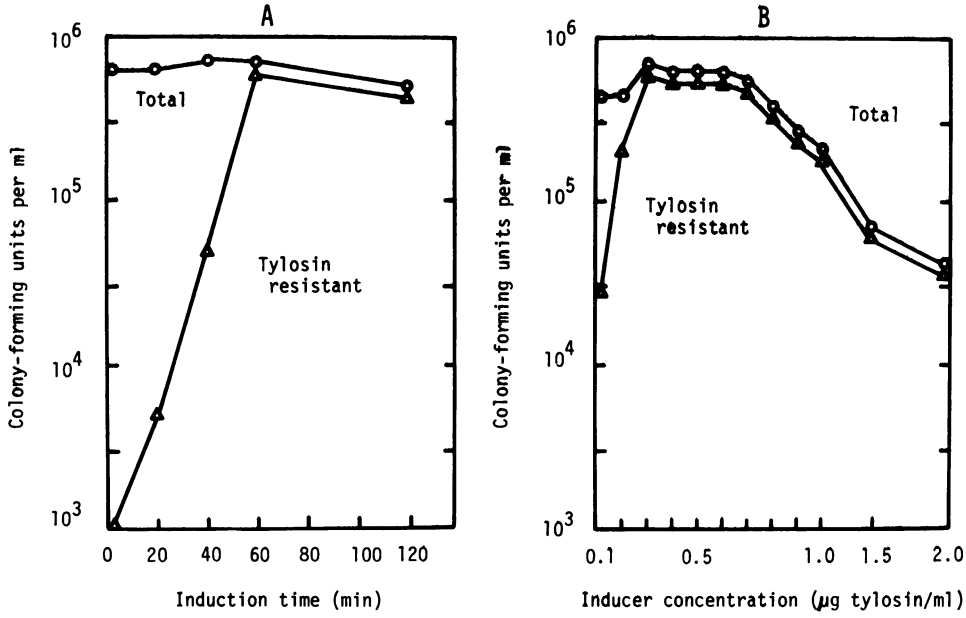

FIG. 2. *Time course and inducer concentration requirements for induction. (A) Time course of induction. Tylosin (1 μg/ml) was added to a sonicated suspension of actively growing cells. Samples were withdrawn and plated on antibiotic-free medium and on medium containing 50 μg of tylosin per ml. (B) Concentration dependence of induction. Cells were induced with tylosin at concentrations between 0.1 and 2.0 μg/ml. After incubation for 60 min, portions were withdrawn and plated as described for Fig. 2.*

by 50S subunits from uninduced, induced, and constitutively resistant *S. aureus* (23). We conclude that tylosin-inducible MLS resistance in *S. viridochromogenes* formally resembles erythromycin-inducible MLS resistance in *S. aureus* at the ribosome level.

Variety of MLS resistance phenotypes in *Streptomyces* spp. We next surveyed a group of selected *Streptomyces* strains to determine whether additional specificities of induction might be present. Of several possible tests for induction, we concentrated our effort on two: (i) the disk assay to determine specificity of induction, and (ii) the [^{3}H]adenine methylation assay, to determine the altered adenine methylation phenotype and to obtain an estimate of the number of altered sites. Results of the methylation assay are presented graphically and in tabular form. The strains chosen produce MLS antibiotics; however, in the case of *S. viridochromogenes* (as well as other examples cited), MLS resistance is not restricted in distribution to MLS producers and seems to occur in many *Streptomyces* strains that were tested. A wide range of phenotypes was found in our survey. We therefore present the data below in a form that illustrates the diversity encountered, rather than exhaustively documenting requirements for induction in all the samples.

***Streptomyces lincolnensis* NRRL 2936.** Examination of the response of *S. lincolnensis* to antibiotic disks revealed the distorted inhibition zones characteristic of induction (Fig. 5A). Cirramycin induced resistance to carbomycin and (more distinctly) to maridomycin. A more pronounced flattening of the carbomycin inhibition zone in response to cirramycin can be seen in Fig. 5B. To determine whether a common mechanism is responsible for resistance to the several MLS antibiotics to which *S. lincolnensis* appears sensitive, a mutant selected with maridomycin was tested (Fig. 5B). The mutant selected showed increased resistance to the set of test antibiotics.

We checked for the presence of methyl adenine in *S. lincolnensis* grown in the absence of antibiotic and in the presence of an inducing concentration of lincomycin, and in the constitutively resistant strain selected with maridomycin. Results parallel to those obtained in *S. virodochromogenes* were observed (Fig. 6 and Table 1), suggesting that adenine methylation similarly mediates lincomycin resistance in *S. lincolnensis*.

***S. fradiae* NRRL 2702.** Erythromycin induced resistance to lankamycin and to vernamycin B (Fig. 7A) in *S. fradiae*, although for reasons stated below we felt that it should have

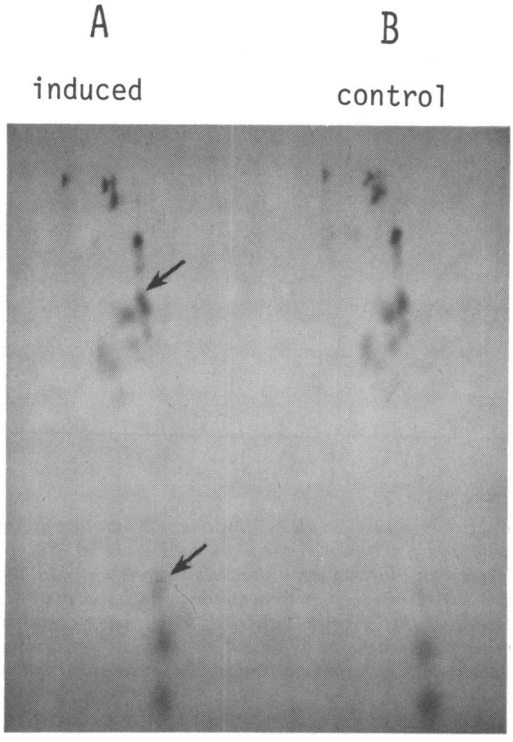

FIG. 3. *Fingerprint analysis of 23S rRNA labeled with [methyl-^{14}C]methionine. methyl-^{14}C-labeled 23S rRNA was prepared as described and digested with RNase A, and the resulting oligonucleotides, fractionated by the method described by Brownlee et al. (3), were located by autoradiography. The arrows indicate the position of new oligonucleotides present in the RNA from induced cells: a larger partially resolved oligomer and a clearly resolved smaller one. (A) 23S rRNA from induced cells; (B) 23S rRNA from uninduced controls.*

relatively weaker inducing activity compared to most of the antibiotics in our test panel. Examination of the response of *S. fradiae* to our test group of antibiotics (Fig. 7B) showed resistance to most of the members of this group with the exception of erythromycin and vernamycin B, to which it appeared to be sensitive. We interpret the absence of a significant inhibition zone for seven of the nine antibiotics tested to indicate a high level of inducing ability and a relatively lower level for the remaining two antibiotics tested, erythromycin and vernamycin B. We therefore also used erythromycin to select for constitutively resistant mutants and obtained a strain with higher levels of resistance to both erythromycin and vernamycin B. The use of vernamycin B for this selection might have been a more logical choice.

Examination of the methylation pattern in 23S rRNA revealed the presence of monomethyl adenine in the uninduced control, as in our previous study (9), in which we also noted the presence of monomethyl adenine in 23S rRNA from *Streptomyces cirratus* ATCC 21731, an organism that produces the macrolide antibiotic cirramycin. In studying *S. fradiae* further, however, we made the unexpected observation that dimethyl adenine was the predominant methylated form present in the mutant selected with erythromycin (Fig. 8 and Table 1). The apparent shift from mono- to dimethylation raises the question of whether one or two enzymes are responsible for the observed rRNA methylation patterns. The smaller zone diameter seen in the erythromycin-resistant mutant is reminiscent of the partially constitutive mutants of *S. aureus* found in earlier studies (23), and we interpret this pattern of mutation to reflect a nucleotide change that only partially destabilizes the r-determinant control region, as discussed in further detail below.

***S. hygroscopicus* IFO 12995.** In testing the specificity of induction we found that either of the two streptogramin B antibiotics, vernamycin B and ostreogrycin B, induced resistance to erythromycin (Fig. 9A). This observation is noteworthy since it represents the first time we have seen induction by a member of the streptogramin B family. Erythromycin was used in an attempt to select constitutively resistant mutants, and the strain obtained in this way became coresistant to the remaining members of the test group as shown in Fig. 9B.

Equimolar concentrations of mono- and dimethyl adenine were found in 23S rRNA from both the uninduced control and the constitutively resistant mutant (Fig. 10 and Table 1). Whereas rRNA from uninduced *S. hygroscopicus* conforms to the pattern of methylation found for other macrolide producers, namely, the presence of monomethyl adenine, the simultaneous presence of dimethyl adenine provides an additional variant methylation phenotype, possibly due to the presence of two methylating enzymes. As discussed below, we feel that macrolide-producing strains may synthesize their own inducers and that this may account for the observed presence of methylated adenine in macrolide-producing strains apparently unrelated to induction by exogenously added inducer.

***Streptomyces diastaticus* NRRL 2560.** Cirramycin clearly induced resistance in *S. diastaticus* to kitasamycin and maridomycin (Fig. 11A). The same disk assay did not show induction by a streptogramin B-type antibiotic; this finding might not be expected from the fact that

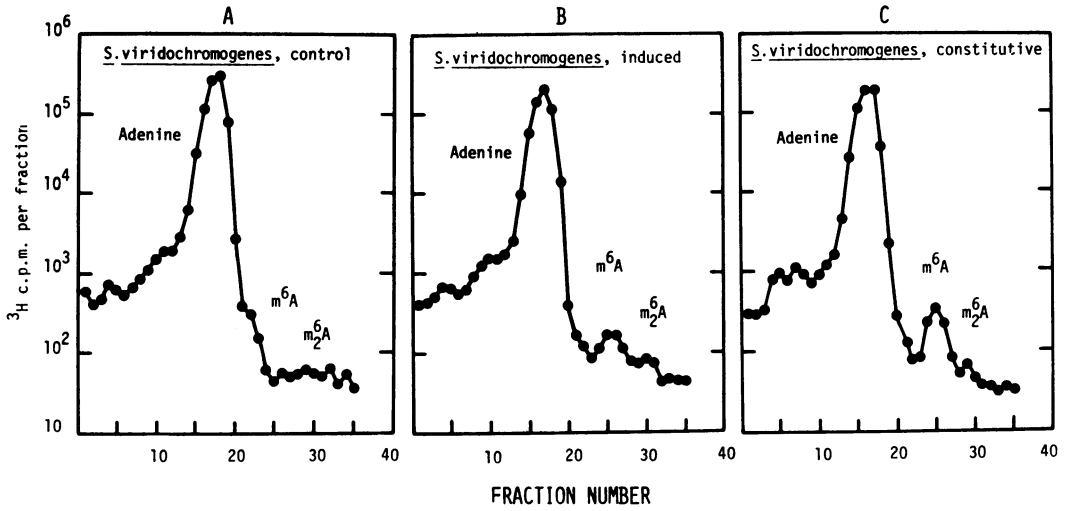

FIG. 4. *Separation of methylated adenines from S. viridochromogenes 23S rRNA by paper chromatography. Pooled fractions from 23S rRNA, labeled with [^{3}H]adenine and fractionated through the Dowex-50X8 step, containing adenine plus methylated adenines, were lyophilized and further fractionated by paper chromatography (with added carrier adenine plus methylated adenines) using a solvent system consisting of isopropanol-ammonia-water (85:1.3:15, vol/vol). The chromatogram was cut into 1-cm-wide strips, which were counted directly in a liquid scintillation counter. (A) Control, uninduced; (B) induced by growth in medium containing 1 μg of tylosin per ml; (C) constitutively resistant cells selected with carbomycin and grown in the absence of added antibiotic.*

TABLE 1. *Methylated adenine residues in 23S rRNA*[a]

Species	cpm in adenine	cpm in monomethyl adenine (% relative to adenine)	cpm in dimethyl adenine (% relative to adenine)
S. viridochromogenes	826,000	276 (0.03)	243 (0.03)
Tylosin induced	554,000	740 (**0.13**)	283 (0.05)
Carbomycin resistant	581,000	984 (**0.17**)	208 (0.04)
S. lincolnensis	380,000	211 (0.05)	198 (0.05)
Lincomycin induced	867,000	642 (0.07)	247 (0.03)
Maridomycin resistant	426,000	844 (**0.20**)	168 (0.04)
S. fradiae	232,000	522 (**0.23**)	195 (0.08)
Erythromycin resistant	579,000	386 (0.06)	949 (**0.16**)
S. hygroscopicus	575,000	994 (**0.17**)	1367 (**0.24**)
Erythromycin resistant	153,000	513 (**0.34**)	494 (**0.32**)

[a] Adenine and its methylated derivatives were isolated from 23S rRNA as described in Fig. 4. Radioactivity (counts per minute, cpm) in each of the methylated adenine fractions was determined, and the results were tabulated; 0.12% relative to adenine corresponds to 1 residue per 23S. Boldface data indicate experimental values corresponding to one or more methylated adenine residues per 23S' rRNA.

S. diastaticus synthesizes the virginiamycin complex, a group of streptogramin antibiotics. However, in view of a relatively small inhibition zone surrounding the vernamycin B disk (Fig. 11B), we inferred that vernamycin B might nevertheless have inducing activity, and we used it in the assay of induced methylation. The results (Fig. 12) suggest the apparent induction of a low level of dimethyl adenine formation which remains to be optimized. This observation is of interest since it provides an additional example of induction in a producing organism by an antibiotic produced by that organism. Moreover, these results further suggest the existence of inducing activity in a streptogramin B-type antibiotic.

Other *Streptomyces* spp. By means of the disk method we have examined additional *Streptomyces* strains specifically chosen because of their role in the synthesis of antibiotics that do

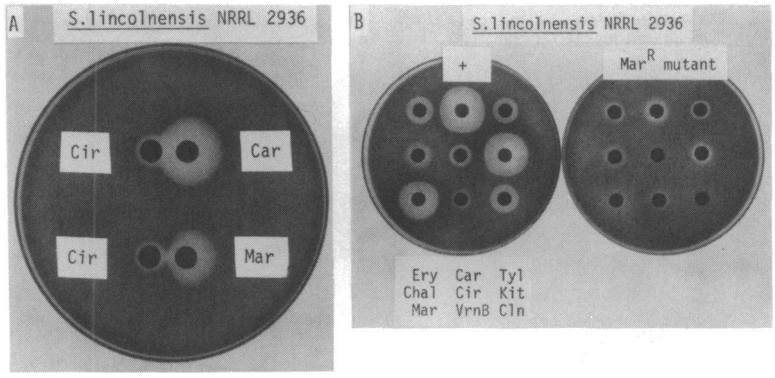

FIG. 5. *Inducible resistance in S. lincolnensis: disk sensitivity test. (A) Induction of resistance to carbomycin and maridomycin by cirramycin; (B) resistance to MLS antibiotics in the wild-type strain and in a constitutive mutant selected with maridomycin at 10 μg/ml. Drug abbreviations as described for Fig. 1.*

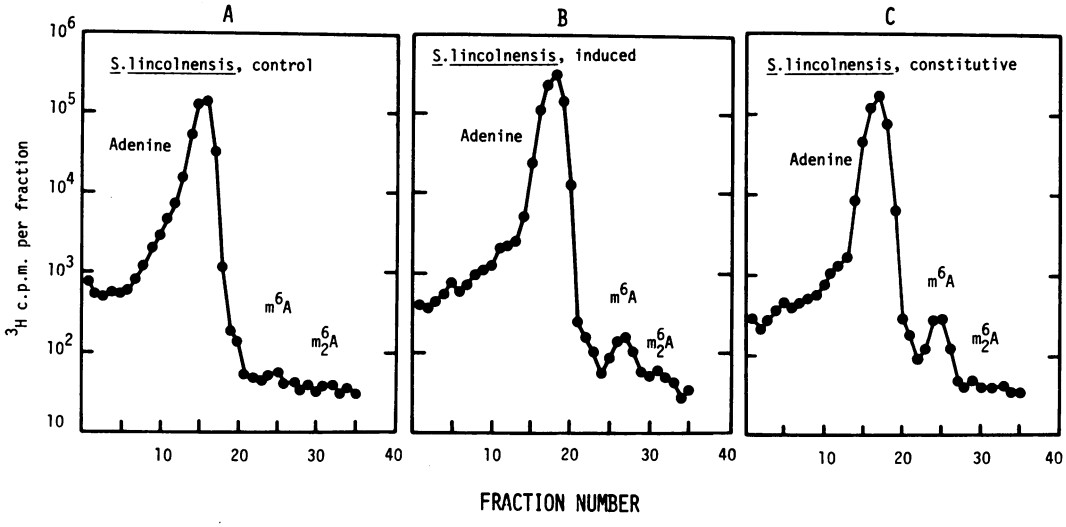

FIG. 6. *Inducible resistance in S. lincolnensis: methylation of 23S rRNA. (A) Wild type, uninduced controls; (B) wild type, induced by growth in medium containing 10 μg of lincomycin per ml; (C) constitutively resistant mutant selected with maridomycin at 10 μg/ml.*

not belong to the MLS families. Examination of *Streptomyces rimosus* NRRL 2234 and *Streptomyces griseus* NRRL B-1965, the organisms used to synthesize oxytetracycline and streptomycin, respectively, reveals that chalcomycin induces resistance to maridomycin in both these strains (data not shown).

DISCUSSION

In the *Streptomyces* spp. we see inducible MLS resistance in its most diverse and general form, and our main task is to explain the molecular basis for this diversity. Our findings prompt a redefinition of the MLS phenotype in terms of the fact that macrolides, lincosamides, and streptogramin B-type antibiotics can all induce resistance, and that the induced ribosomal alteration involves either mono- or dimethylation or both. This broader definition of the inducible MLS resistance phenotype subsumes all the variant phenotypes found to date.

The studies reported above were first undertaken to determine whether a causal relation could be shown between adenine monomethylation and MLS resistance. The use of an inducible strain, *S. viridochromogenes*, one not known to produce MLS antibiotics, provided the model system that enabled us to do this with equal facility as in *S. aureus*. To prove, however, that an organism does not synthesize compounds

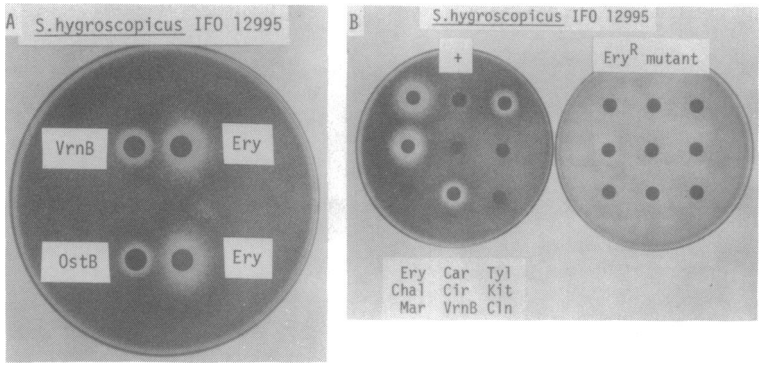

FIG. 7. *Inducible resistance in S. fradiae: disk sensitivity test. (A) Induction of resistance to lankamycin (Lank) and vernamycin B by erythromycin; (B) resistance to MLS antibiotics in the wild-type strain and in a constitutive mutant selected with erythromycin at 10 μg/ml. Drug abbreviations as described for Fig. 1.*

with inducing activity may be difficult, so that the use of a strain not known to produce any MLS antibiotics is the optimal choice at this time.

S. viridochromogenes provided us with an additional example of induction specificity in which erythromycin was not the most potent inducer. It was previously shown that erythromycin was not the sole inducer in staphylococci and streptococci (1, 7, 11, 15, 19). Although erythromycin has maximal inducing activity in certain cases, this did not appear to us to be the case universally after we examined the *Streptomyces* spp.

In the course of our studies of MLS resistance we have been faced with the problem of explaining the basis for induction specificity. In studies of a series of 53 erythromycin analogs Pestka et al. (17) first noted a correlation between ribosome binding and inducing activity, and they inferred that formation of a ribosome-erythromycin complex constituted a significant step in induction. More recent studies of MLS resistance by Shivakumar et al. (18) and from our laboratory (10) have suggested that the process of induction requires sensitive ribosomes and that formation of a complex between erythromycin and a sensitive ribosome plays a key role in the induction process.

On the basis of the DNA sequence of the control region for inducible resistance specified by plasmid pE194, including sequences of 11 constitutive mutants, we proposed an explicit translational attenuation model for induction of MLS resistance (10) which we believe is also utilized in regulating MLS resistance in *Streptomyces* spp. The significant feature of the model of induction pertinent to our present work is the experimental fact that members belonging to the three classes of antibiotics to which methylated

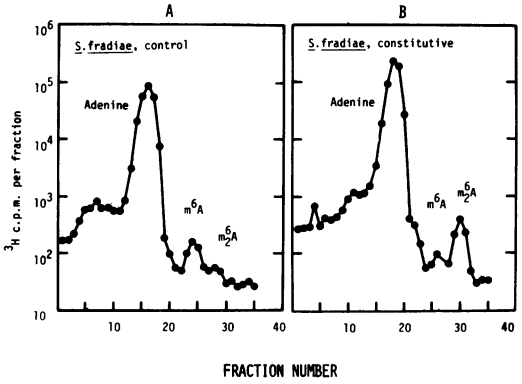

FIG. 8. *Inducible resistance in S. fradiae: methylation of 23S rRNA. (A) Wild type, uninduced control; (B) constitutive mutant selected with erythromycin at 10 μg/ml.*

ribosomes are resistant can, in principle, show inducing activity. Whereas our model helps to understand why any MLS antibiotic can have inducing activity, we can only speculate why it is that only a limited number can be shown to induce in any given organism.

Sorting out the variables that determine induction specificity will depend on distinguishing among: (i) primary effects on the induction mechanism, (ii) secondary effects such as antibiotic transport or conversion to either active or inactive substances, and (iii) incidental variations or differences in ribosome structure involving primary sequence differences or secondary sequence modifications of rRNA or ribosomal proteins. A system which may provide useful information involves transformation of *Bacillus subtilis* with plasmids from different sources and comparison of the induction specificities of the two plasmids in the new host cell with the re-

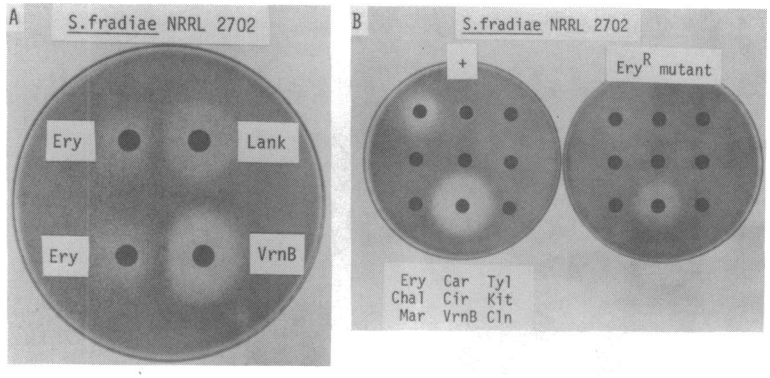

FIG. 9. *Inducible resistance in S. hygroscopicus: disk sensitivity test. (A) Induction of resistance to erythromycin by vernamycin B and ostreogrycin; (B) resistance to MLS antibiotics in the wild type and in a constitutive mutant selected with erythromycin at 10 μg/ml. Drug abbreviations as for Fig. 1.*

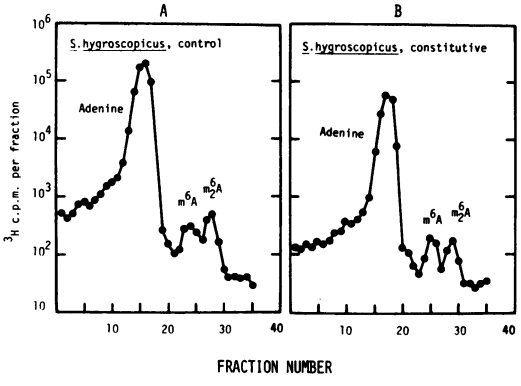

FIG. 10. *Inducible resistance in S. hygroscopicus: methylation of 23S rRNA. (A) Wild type, control; (B) constitutive mutant selected with erythromycin at 10 μg/ml.*

spective specificities found in the original host. In one such comparison we noted that pAM77 from *Streptococcus sanguis* and pE194 from *S. aureus*, when introduced into *B. subtilis* by transformation, gave phenotypes (disk assay) that resemble those seen in the original hosts (data not shown); both erythromycin and lincomycin induced in the former, whereas erythromycin alone induced in the latter. This suggests that the induction specificity is inherent in the DNA and not in the host cell background. In terms of the explicit model for control of MLS resistance we have postulated (10), the amino acid composition of the control region peptide and its variability make a major contribution to the specificity of induction, mediated by the different relative potencies of MLS antibiotics in inhibiting incorporation of specific amino acids. For example, in experiments performed using polyuridylate:polycytidylate-directed in vitro polypeptide synthesis as a model test system, we reported that leucine incorporation was strongly inhibited by lincomycin and minimally inhibited by erythromycin (4).

In our experience with *Streptomyces* spp. we observed that many false negatives, but no false positives, were obtained in testing for induction by the disk method. In terms of the model for induction that we have proposed, formation of a complex between the ribosome and the inducing antibiotic comprises the critical step in induction; i.e., the sensitive ribosome is the macromolecular sensor that determines the presence of an inducing stimulus in the environment. It has been shown previously that 50S subunit inhibitors generally can interact competitively in their binding to the ribosome (reviewed by Vazquez [20]). This would apply to the MLS antibiotics, whose binding to the ribosome appears to share overlapping specificities.

In our previous studies of *S. aureus* we have noted that inducible cells exposed to a noninducing MLS antibiotic can respond in at least four ways. The types of mutants obtained include: (i) "true constitutives," coresistant to all MLS antibiotics tested, by the disk assay (23); (ii) "partial constitutives," comprising a varied group of phenotypes involving reduced zone size for some test antibiotics, turbid inhibition zones for others, and no change for still others (23); (iii) "altered specificity of induction," as in the case of the carbomycin- and lincomycin-inducible mutant (19); and (iv) "high plasmid copy number," as in the case of pE194 mutants selected for resistance to tylosin in a *B. subtilis* background (22). The first three of these categories seem to have counterparts in *Streptomyces* spp.

In several instances, induction tested by the disk method produced only minimal distortion of the respective inhibition zones, which has raised some question about the validity of our

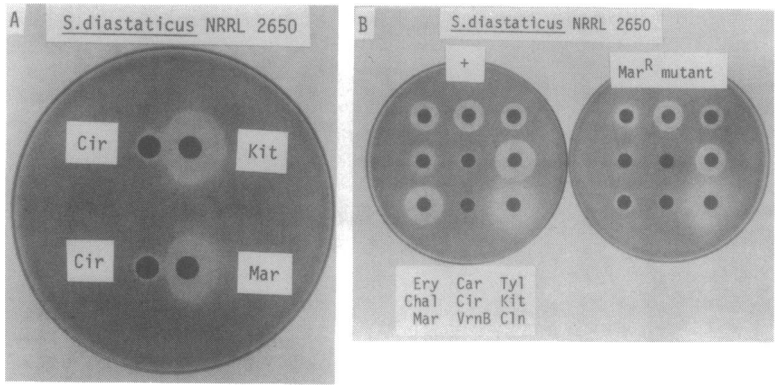

FIG. 11. *Inducible resistance in S. diastaticus: disk sensitivity test. (A) Induction of resistance to kitasamycin and maridomycin by cirramycin; (B) resistance to MLS antibiotics in the wild type and in a constitutive mutant selected with maridomycin at 10 μg/ml. Drug abbreviations as for Fig. 1.*

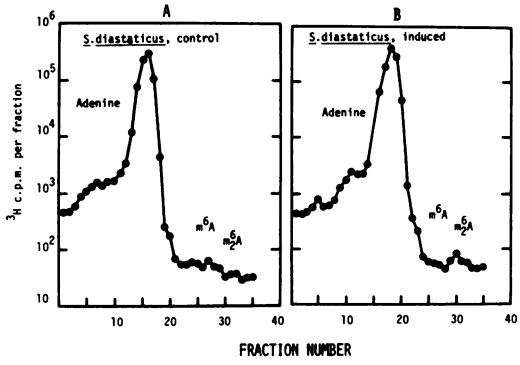

FIG. 12. *Inducible resistance in S. diastaticus: methylation of 23S rRNA. (A) Wild type, uninduced control; (B) wild type, induced by growth in medium containing 10 μg of ostreogrycin B per ml.*

interpretation. The ability of one MLS antibiotic to induce resistance to another requires not only that the inducer have optimal specificity, but also that the inducing member of the test pair bind more strongly to the ribosome than the antibiotic to which resistance is induced, or else the net effect would be dominated by noninduction characteristic of the more strongly binding antibiotic.

Naturally occurring mechanisms of resistance to MLS antibiotics unrelated to rRNA methylation might produce spurious effects in studies of induction. Such mechanisms include phosphorylation of clindamycin by *Streptomyces coelicolor* as described by Coats (5) and hydrolytic cleavage of pristinamycin I (a streptogramin type B antibiotic) by *S. aureus* as described by LeGoffic et al. (14). We have not yet checked for the extent to which these alternative mechanisms may operate in the producing strains, and whether they act instead of or in addition to methylation, if at all, is not yet known.

In the case of the macrolide producers, we have found the presence of methylated adenine in cells grown under noninducing conditions (i.e., in the absence of exogenously added inducer) and apparent inducibility of these strains when tested by the disk method. We attempt to reconcile these apparently inconsistent findings by suggesting the possibility that such strains may synthesize endogenous inducers. This must be the case for *S. lincolnensis*, since we have shown that the ribosomes lack methylated adenine until exposed to exogenously added lincomycin. We propose that under conditions used for antibiotic production, lincomycin or some congener such as celesticetin, produced initially at subinhibitory levels, may serve to mediate the metabolic switch that occurs as part of the commitment to antibiotic production. The difference between *S. lincolnensis* and the macrolide producers may reflect differences in regulation of the synthesis of endogenous inducers.

Induction of adenine monomethylation in *S. lincolnensis* by exogenously added lincomycin emerges as one of the most significant findings in this study. A similar situation may obtain in the streptogramin B producer *S. diastaticus*. These observations suggest, more generally, a possible link between production of other, if not all, MLS antibiotics and rRNA methylation. This can be tested; if the development of resistance to MLS antibiotics by rRNA methylation constitutes a limiting step in the synthesis of these antibiotics, we would expect to find more efficient production of MLS antibiotics by constitutively resistant derivatives of inducible parent producing strains.

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