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The Macrolide-Lincosamide-Streptogramin B Resistance Phenotypes Characterized by Using a Specifically Deleted, Antibiotic-Sensitive Strain of *Streptomyces lividans*

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Genes conferring resistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics via ribosomal modification are widespread in bacteria, including clinical isolates and MLS-producing actinomycetes. Such *erm*-type genes encode enzymes that mono- or dimethylate residue A-2058 of 23S rRNA. The different phenotypes resulting from monomethylation (MLS-I phenotype, conferred by *erm* type I genes) or dimethylation (MLS-II phenotype due to *erm* type II genes) have been characterized by introducing *tlrD* or *ermE*, respectively, into an MLS-sensitive derivative of *Streptomyces lividans* TK21. This strain (designated OS456) was generated by specific replacement of the endogenous resistance genes *lrm* and *mgt*. The MLS-I phenotype is characterized by high-level resistance to lincomycin with only marginal resistance to macrolides such as chalcomycin or tylosin, whereas the MLS-II phenotype involves high-level resistance to all MLS drugs. Mono- and dimethylated ribosomes were introduced into a cell-free protein-synthesizing system prepared from *S. lividans* and compared with unmodified particles in their response to antibiotics. There was no simple correlation between the relative potencies of MLS drugs at the level of the target site (i.e., the ribosome) and their antibacterial activities expressed as MICs.

Streptomyces lividans TK21, a plasmid-free derivative of strain 66, is widely used as a host organism during interspecific or intergeneric cloning of genes isolated from actinomycetes. However, the presence of a tandem pair of resistance determinants in the *S. lividans* genome confers an inducible macrolide-lincosamide-streptogramin B (MLS) resistance character that has hindered previous attempts to analyze the different phenotypes conferred by cloned MLS resistance genes.

MLS resistance was first observed in clinical isolates of *Staphylococcus* (in which it was inducible by erythromycin) and is due to specific methylation of 23S rRNA (for a review, see reference 24). Since then, MLS resistance determinants, commonly referred to as *erm*-type genes (denoting erythromycin resistance), have been described for a broad range of bacteria, both gram positive and gram negative, including actinomycetes that produce MLS antibiotics (see reference 24). However, *erm* gene products do not all act identically, even though they all target nucleoside A-2058 of 23S rRNA (21) (for ease of reference, the *Escherichia coli* numbering scheme is used to identify the methylated nucleoside). The archetypal staphylococcal ErmC protein catalyzes N^6,N^6-dimethylation of residue A-2058, as does the product of *ermE* from the erythromycin producer *Saccharopolyspora erythraea* (21), whereas other *erm*-type genes (such as *tlrD* from the tylosin producer *Streptomyces fradiae*) encode enzymes that modify A-2058 via N^6-monomethylation (27). The resistance character of *S. lividans* is derived, in part, from the latter effect, due to the product of an *erm*-type gene, *lrm*. However, immediately downstream of *lrm* in the *S. lividans* genome lies another resistance gene (*mgt*, denoting macrolide glycosyl transferase), whose product inactivates a broad range of macrolides via 2′-O-glycosylation using UDP-glucose as a cosubstrate (4, 10, 11).

Expression of *lrm-mgt* in *S. lividans* is induced by various macrolides, but this is superimposed on a variable background level of resistance. Typically, around 5% of uninduced mycelial units that would plate successfully on drug-free medium will also grow on high concentrations of lincomycin and will replate with a similar low efficiency on lincomycin-containing medium (10). Such leaky expression of *lrm-mgt* allowed direct selection for the gene pair, without deliberate induction, in a shotgun cloning protocol utilizing a multicopy vector (10). On the other hand, this phenomenon has repeatedly frustrated our attempts to study the phenotypes conferred by MLS resistance genes cloned in *S. lividans* and has necessitated the use of alternative hosts, such as *Streptomyces griseofuscus* (25) or *Streptomyces albus* (14). Technical difficulties associated with the use of such nonstandard hosts led us to the present work, the initial aim of which was to generate a strain of *S. lividans* lacking both Lrm and Mgt activities via gene replacement. Then, having introduced previously characterized *erm*-type genes into the modified strain, we proposed to examine the stepwise development of the MLS resistance character following mono- or dimethylation of rRNA. This has not been possible in staphylococcal or other hosts, since very few *erm*-type gene products have been characterized in sufficient detail and almost all of these originated in actinomycetes. For example, no *erm*-type gene encoding an rRNA monomethylase has been definitively identified outside the order *Actinomycetales* (for a review, see reference 24).

In presenting our results, we have introduced the following nomenclature: *erm*-type genes whose products monomethylate 23S rRNA at residue 2058 are designated *erm* type I, whereas those that encode dimethylation of residue 2058 are *erm* type

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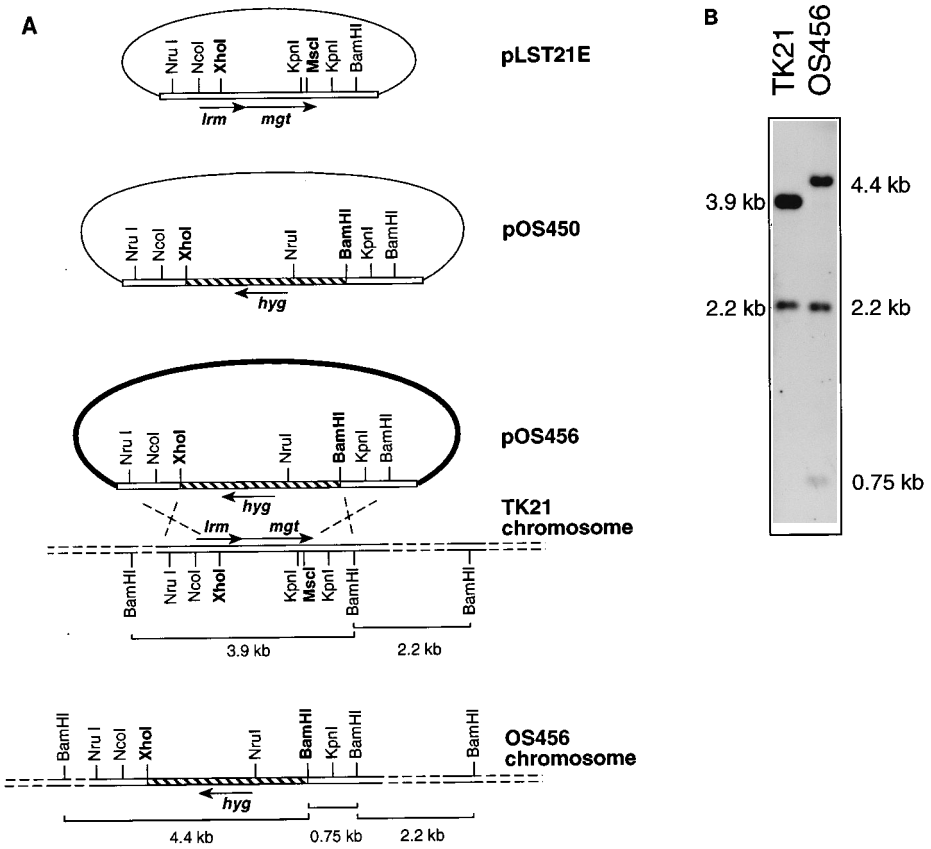

FIG. 1. Gene replacement. (A) Schematic representation of the replacement of part of the *lrm-mgt* gene pair of *S. lividans* TK21 with the ΩHYG cassette. The 1.4-kb *Xho*I-*Msc*I fragment was excised from the pUC18-based plasmid pLST21E, and the residual plasmid DNA was blunt ended with Klenow enzyme and ligated with the ΩHYG cassette (a 2.6-kb *Bam*HI fragment, similarly blunt ended, containing the hygromycin resistance gene, *hyg*, from *Streptomyces hygroscopicus*). This recreated the *Xho*I site at the left-hand end of the inserted cassette (in the orientation shown) and generated a *Bam*HI site at the other end. The entire insert of *S. lividans* DNA, interrupted by the ΩHYG cassette, was then excised from the resultant plasmid (pOS450) and introduced into pGM160, generating pOS456. Double homologous recombination between pOS456 and the genome of *S. lividans* TK21, generating strain OS456, is illustrated. —, pUC DNA; ■, pGM160 DNA; ▭, *S. lividans* DNA; ▨, ΩHYG cassette DNA. (B) Southern blot hybridization analysis revealing interruption of *lrm-mgt*. *Bam*HI digests of total genomic DNA from *S. lividans* TK21 and OS456 were probed with the 6.15-kb insert from pOS450. The sizes of hybridizing *Bam*HI fragments are shown alongside the autoradiogram, and their genomic locations are indicated in panel A.

II. The resistance phenotypes conferred by these genes are quite different and are designated MLS-I and MLS-II, respectively.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *S. lividans* TK21 (9) and its derivative OS456 (generated in this study) were maintained and grown on NE agar (20) or R2YE agar (8). Liquid cultures were grown in YEME medium (8) supplemented with 5 mM $MgCl_2$ and 0.5% (wt/vol) polyethylene glycol 6000. Transformation of protoplasts was carried out according to standard protocols (8) using the following plasmids: pLST912 carrying *tlrD* (27), pLST391 carrying *ermE* (25), pIJ702 (13), and pOS456 (this study). For maintenance of pLST391 or pIJ702, thiostrepton (5 μg/ml) or nosiheptide (5 μg/ml) was included in the medium. Lincomyin (50 μg/ml) was used for pLST912 and hygromycin B (200 μg/ml) was used to maintain pOS456. Manipulation of DNA in *E. coli* was performed according to standard protocols (19). Other media used were tryptic soy broth (TSB) (Difco) and tryptic soy agar (Difco).

Antibiotics and MIC determinations. Antibiotics were obtained as follows: carbomycin from Pfizer Central Research, Groton, Conn.; chalcomycin, erythromycin, and lincomycin from Sigma; hygromycin B from Boehringer Mannheim; nosiheptide, spiramycin, and pristinamycin IA (the last is identical to streptogramin B) from Rhône-Poulenc Rorer, Vitry, France: rosaramicin from Schering-Plough, Union, N.J.; thiostrepton from the Squibb Institute, Princeton, N.J.; and tylosin from Lilly Research Laboratories, Indianapolis, Ind. MICs were determined by spreading fresh spores of *S. lividans* strains on NE agar and incubating the cultures at 30°C for 3 days.

Cell-free protein synthesis. Cell-free protein synthesis was carried out with a coupled transcription-translation system. Salt-washed ribosomes were prepared from various strains of *S. lividans* and used together with protein synthesis initiation factors plus postribosomal 100,000 × *g* supernatant (S100) from strain OS456. These materials were prepared as described elsewhere (2, 22). Plasmid DNA (pUC18) was added as the template for transcription (the S100 contains RNA polymerase), and translation of the resultant mRNA was measured as the incorporation of [^{35}S]methionine into newly synthesized protein.

RESULTS AND DISCUSSION

Gene replacement inactivating the *lrm* and *mgt* genes of *S. lividans*. The *lrm-mgt* gene pair of *S. lividans* TK21 was originally isolated as a 3.6-kb *Sau*3AI DNA fragment and inserted into the *Bgl*II site of pIJ702, thereby generating pLST21 (10). Digestion of pLST21 with *Bcl*I released the *S. lividans* DNA together with flanking *mel* DNA as a 4.9-kb fragment, which was inserted into the *Bam*HI site of pUC18 to give pLST21E (Fig. 1A). This plasmid possesses unique sites for *Xho*I and *Msc*I that lie approximately centrally within *lrm* and close to the downstream end of *mgt*, respectively. Accordingly, a 1.4-kb *Xho*I-*Msc*I fragment could be released and replaced by the ΩHYG cassette (1) to generate pOS450 (Fig. 1A). The ΩHYG cassette was derived from the Ω interposon (17, 18) by replacement of the streptomycin-spectinomycin resistance gene by the hygromycin resistance determinant, *hyg* (28). In ΩHYG, *hyg* is flanked on either side by transcriptional and translational stop

TABLE 1. MICs of MLS antibiotics for various strains of *S. lividans*

Antibiotic	MICs (μg/ml) for:			
	TK21 (uninduced)	OS456	OS456 *tlrD*	OS456 *ermE*
Tylosin	30–50	0.1–0.5	1–5	1,000–2,500
Spiramycin	50–100	10–25	50–500	2,500–5,000
Carbomycin	50–100	5–10	100–250	>20,000
Rosaramicin	30–50	1–5	50–75	1,000–2,500
Erythromycin	30–50	0.1–0.5	10–25	2,500–5,000
Chalcomycin	10–25	1–5	1–5	15,000–20,000
Lincomycin	30–50	10–25	2,500–5,000	>20,000
Streptogramin B (Pristinamycin IA)	25–50	25–50	500–750	>20,000

signals and confers resistance to hygromycin B both in *E. coli* and in *Streptomyces* spp. The ΩHYG cassette, flanked on either side by about 1 kb of *S. lividans* DNA, was then excised from pOS450 and introduced into pGM160 (15), a shuttle vector whose replication is temperature sensitive in *Streptomyces* spp., to generate pOS456 (Fig. 1A). Plasmid pGM160 confers resistance to thiostrepton and gentamicin in *Streptomyces* spp., whereas, when pOS456 was used to transform protoplasts of *S. lividans* TK21, it additionally conferred resistance to hygromycin B (200 μg/ml). One such transformant was grown in TSB with thiostrepton (5 μg/ml) for 2 days at 30°C (a permissive temperature for pOS456 replication) and was then used to inoculate drug-free TSB. This culture was grown for 3 days at 39°C (nonpermissive for pOS456 replication) before samples were diluted and plated on TSB with and without hygromycin (200 μg/ml). About 20% of the CFU were resistant to hygromycin, and, of these, about 4% were sensitive to thiostrepton following replica plating. Since the thiostrepton-sensitive organisms were also sensitive to gentamicin, they had presumably lost the pGM160 moiety of pOS456 via a double crossover recombination event. These clones were also considerably more sensitive than *S. lividans* TK21 to spiramycin, suggesting that the replacement event had affected the *lrm-mgt* genes. This was confirmed in hybridization analysis in which Southern blots of total genomic DNA from one of the clones and from strain TK21 (each digested separately with nine different enzymes) were probed and compared. The probe was the 6.15-kb insert from pOS450, and the results (some of which are shown in Fig. 1B) suggested that a double homologous recombination event had resulted in replacement of the *lrm* and *mgt* genes of the *S. lividans* chromosome by their disrupted counterparts from pOS456. The disrupted strain was designated *S. lividans* OS456.

Antibiotic sensitivities of *S. lividans* OS456 and TK21. Strain OS456 was much more sensitive than TK21 to MLS antibiotics (Table 1). MLS resistance encoded by *lrm-mgt* in strain TK21 is normally inducible (e.g., by erythromycin), but the difference in sensitivity between OS456 and uninduced TK21 suggested that some expression of *lrm* and/or *mgt* was occurring in the latter strain. This was confirmed when ribosomes from the two strains were assayed in a coupled transcription-translation system (used to monitor cell-free protein synthesis) with and without drugs. Ribosomes from uninduced TK21 were more resistant than those from OS456 to MLS antibiotics (in particular, lincomycin), presumably because of production of the Lrm protein, and extracts from uninduced TK21 contained substantial levels of macrolide glycosyl transferase (Mgt) activity that could not be detected in OS456 extracts (data not shown). Evidently, leaky expression of *lrm-mgt* occurs in a small (and, on a day-to-day basis, perhaps variable) proportion of the mycelial units of strain TK21, resulting in the routine preparation of mixed populations of ribosomal particles that differ in their response to antibiotics. No explanation for leaky expression of *lrm-mgt* in *S. lividans* is proposed here, since the mechanism of induction has not been determined, although analogy with the regulation of other *erm*-type genes suggests that translational or transcriptional attenuation might well be involved (14; for a review, see reference 24).

The MLS resistance phenotypes. Introduction of *erm* type I and *erm* type II genes into *S. lividans* OS456 allowed comparison of the MLS-I and MLS-II resistance phenotypes in the absence of non-*erm* genes, such as *mgt*. The genes used were *tlrD* (*erm* type I) from the tylosin producer *S. fradiae* (27), and *ermE* (type II) from *Saccharopolyspora erythraea* (21, 23), producer of erythromycin. Both are constitutively expressed, and both were introduced into strain OS456 on pIJ702-derived vectors. The *erm* type I resistance mechanism generated high-level resistance to lincomycin which, for most practical purposes, bordered on insensitivity (Table 1), and the level of resistance to streptogramin B (pristinamycin IA) was also quite high. However, the response to macrolides was drug specific and less dramatic. Each MIC is expressed as a range, from the highest drug concentration tested that allowed growth to the lowest concentration that did not allow growth. For several macrolides, regardless of their relative potencies, the midpoint of that range was elevated about 10- to 20-fold because of the *erm* type I mechanism, but, even so, the resultant MICs of some drugs were still below those seen for uninduced TK21, which emphasizes the role of *mgt* in conferring resistance to macrolides in *S. lividans*. This was particularly evident with chalcomycin, for which there was little or no elevation of the MIC caused by *tlrD*. Chalcomycin normally induces expression of *lrm-mgt* in *S. lividans* and is also one of the preferred substrates for the Mgt enzyme (4, 10). In summary, the MLS-I resistance phenotype relates principally to lincomycin, with much lower levels of macrolide resistance and an intermediate effect on the response to streptogramin B.

The *erm* type II mechanism rendered *S. lividans* OS456 virtually insensitive to MLS antibiotics, except at concentrations far in excess of those normally employed to test resistance levels. Therefore, the principal difference between the MLS-I and MLS-II phenotypes concerns the relative levels of macrolide resistance.

Analysis of ribosomes in vitro. Ribosomes from *S. lividans* OS456 *tlrD*$^+$ and OS456 *ermE*$^+$ were compared with control OS456 particles in a coupled transcription-translation system (set up to monitor protein synthesis) in which all other components were derived from the control strain. Acting on control ribosomes, MLS antibiotics fell into two distinct groups (Table 2): those that inhibited protein synthesis by around 50% at 50 μg/ml and those that exerted similar, or even higher, levels of inhibition at concentrations as low as 0.5 μg/ml. The former group included lincomycin, streptogramin B, and macrolides with monosaccharidic substituents on C-5 of the lactone ring (including erythromycin, rosaramicin, and chalcomycin), whereas macrolides with disaccharides at C-5 were at least 2 orders of magnitude more potent in vitro. Yet again, macrolide antibiotics are seen not to have a common mode of action (for a review, see reference 6). Moreover, there was no simple correlation between the relative potencies of MLS drugs at the level of the target site (i.e., the ribosome) and their antibacterial activities expressed as MICs (Tables 1 and 2). Thus, although tylosin and erythromycin had similar MICs for strain OS456, tylosin was at least 100 times more active than erythromycin against ribosomes in vitro. In marked contrast,

TABLE 2. Inhibition of cell-free protein synthesis, in a coupled transcription-translation system, by MLS antibiotics

Antibiotic	% Inhibition of synthesis (drug concn) in ribosomes from[a]:		
	OS456	OS456 *tlrD*$^+$	OS456 *ermE*$^+$
Tylosin	90 (0.5)	70 (0.5)	75 (50)
Spiramycin	90 (0.5)	50 (0.5)	0 (50)
Carbomycin	80 (0.5)	50 (0.5)	0 (50)
Rosaramicin	45 (50)	10 (50)	0–5 (50)
Erythromycin	55 (50)	35 (50)	0–5 (50)
Chalcomycin	40 (50)	25 (50)	0–5 (50)
Lincomycin	65 (50)	0 (50)	0 (50)
Streptogramin B (Pristinamycin IA)	60 (50)	10 (50)	0 (50)

[a] All other components of the coupled transcription-translation system were derived from control strain OS456. Drug concentrations are given in micrograms per milliliter.

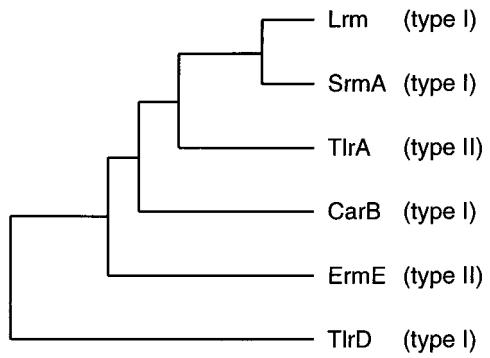

FIG. 2. Dendrogram of deduced Erm-type protein sequences from actinomycetes. Only *erm* gene products which have been biochemically characterized as type I or type II are shown. The protein sequences were analyzed with the program PILEUP from the sequence analysis software package from Genetics Computer Group, Madison, Wis. The sequences are from the following references: Lrm, 10; SrmA, 16; TlrA (synonym ErmSF), 12; CarB, 5; ErmE, 23; and TlrD, 7.

erythromycin was more potent than spiramycin in vivo but much less so in vitro. Since coupled transcription-translation represents the nearest cell-free approximation to the conditions of intracellular translation, these results presumably imply that different macrolides are taken up and/or accumulated by *S. lividans* with different efficiencies.

Ribosomes from the *erm* type I strain were highly resistant to lincomycin and streptogramin B, in keeping with the corresponding MICs. However, the results with macrolides emphasize that MICs (or relative MICs) cannot be used to predict (changes in) the ribosomal response to drugs. This is most clearly seen with carbomycin and erythromycin, for which the *erm* type I mechanism resulted in substantial increases in the respective MICs with only marginal changes in ribosomal sensitivity. Evidently, small effects at the level of the ribosome can lead to large effects at the level of the whole cell in this context, and changes in the one parameter cannot be used to predict changes in the other quantitatively.

No such complications were attached to the results with *erm* type II ribosomes. Little or no inhibition of their activity was observed in vitro with MLS antibiotics except tylosin, which mirrored the high levels of resistance of the intact organisms.

Concluding remarks. Arrangement of the deduced Erm protein sequences into a dendrogram (24) strongly suggests that the *erm*-type genes are homologous and broadly separates them into those from clinical isolates and those from actinomycetes (mainly antibiotic producers). However, since the vast majority of the known *erm* genes cannot be assigned to type I or II (because their products have not been characterized biochemically), such a comparison could not reveal possible systematic differences between products of the two types. Most of the *erm* gene products that have been enzymatically characterized are from MLS-producing actinomycetes. Thus, dimethylases are encoded by *ermE* from *Saccharopolyspora erythraea* (21) and *tlrA* (synonym, *ermSF*) from the tylosin producer *S. fradiae* (25). These are type II genes that confer the MLS-II phenotype. Monomethylases are encoded by *tlrD* from *S. fradiae* (27), *clr* from celesticetin-producing *Streptomyces caelestis* (3), *carB* from the carbomycin producer *Streptomyces thermotolerans* (26), and *srmA* from *Streptomyces ambofaciens*, producer of spiramycin (16), in addition to *lrm* from *S. lividans* (11). These *erm* type I genes confer the MLS-I phenotype. However, even when sequence comparisons are restricted to these actinomycete proteins, the dendrogram does not separate them into type I and type II enzymes (Fig. 2), nor is it possible to discern within the gene sequences any motifs that would allow the construction of probes specific for type I or type II genes. Now, however, *erm*-type genes may be classified according to the phenotypes they confer. The MLS-I phenotype is characterized by high-level resistance to lincomycin with only marginal resistance to chalcomycin or tylosin, whereas the MLS-II phenotype involves high-level resistance to all MLS drugs.

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