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THE USE OF RECOMBINANT DNA TECHNIQUES TO STUDY TYLOSIN BIOSYNTHESIS AND RESISTANCE IN *STREPTOMYCES FRADIAE*[1]

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ABSTRACT.—A substantial amount of information on the biosynthesis of tylosin has been obtained over the past ten years. Physiological studies and experiments with tylosin-blocked (*tyl*) mutants have suggested the probable pathway by which tylactone is converted to tylosin. The development of recombinant DNA methodology for streptomycetes in general, and for *Streptomyces fradiae* in particular, has allowed us to apply gene cloning techniques in further studies of tylosin biosynthesis in *S. fradiae*. The macrocin *O*-methyltransferase (MOMT), which catalyzes the last step in tylosin biosynthesis, was purified, and the sequence of the 35 amino acids at its amino-terminus was determined. A synthetic 44 base oligonucleotide probe was constructed on the basis of the amino acid sequence. The probe was used to identify sequences containing the MOMT structural gene in bacteriophage and cosmid libraries of *S. fradiae* DNA. Complementation of *tyl* mutants with the cloned DNA sequences identified nine *tyl* biosynthetic genes (*tylC, D, E, F, H, J, K, L,* and *M*) in a 42 kb stretch of DNA. Genes complementing four mutant classes, *tylA, B, G,* and *I* were not found. A tylosin-resistance gene, *tlrB*, was located just left of the *tyl* gene cluster. Tylosin-sensitive mutants of *S. fradiae*, which were isolated from regenerated protoplasts and which have pleiotropic deficiencies in tylosin biosynthesis, contained deletions which included at least some of the identified *tyl* loci and one or both of two tylosin-resistance genes, *tlrB* and *tlrC*. Possible schemes for the functional organization of the *tyl* region of the *S. fradiae* genome are discussed.

Tylosin is a macrolide antibiotic produced by *Streptomyces fradiae*, which is used widely in the swine industry to promote growth. It is also used in veterinary medicine to treat infections caused by Gram-positive bacteria.

The tylosin molecule consists of a 16-membered branched lactone and three sugar residues: the amino sugar mycaminose, which is attached to the C5-hydroxyl position of the aglycone; the neutral sugar mycarose, which is attached to the 4-hydroxyl position of the mycaminose residue; and the neutral sugar mycinose, which is attached to the C23-hydroxyl position of the aglycone (Figure 1). Notable structural features of the aglycone are the aldehyde group at C20 and the ketone at C9 conjugated to double bonds at C10-C11 and C12-C13. The conjugated system is responsible for the tylosin molecule's absorbance maximum at 283 nm, a characteristic that is important in the detection and quantitation of tylosin and its intermediates.

The biosynthesis and regulation of tylosin have been of considerable interest to Eli Lilly and Company for more than a decade (1). Early studies by Omura and co-workers suggested that the tylosin aglycone was derived by condensation of two-, three-, and four-carbon subunits by a mechanism similar to that of fatty acid biosynthesis (Figure 2) (2). Thus, a propionyl-CoA primer (C15, C16, C17) would be extended by successive condensations of two methylmalonyl-CoA units, a malonyl-CoA unit, a methylmalonyl-CoA unit, an ethylmalonyl-CoA unit, a methylmalonyl-CoA unit, and a final malonyl-CoA unit as shown in Figure 2. Formation of the lactone would be completed by esterification between the C1 carboxyl group and the C15 hydroxyl group. This

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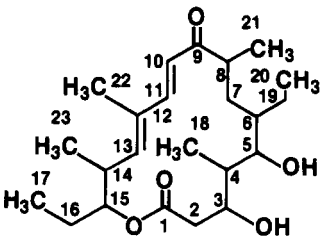

tylactone

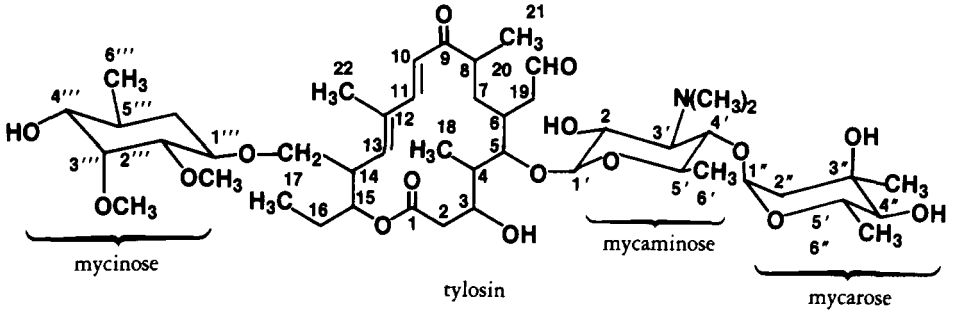

FIGURE 1. The structures of tylactone and tylosin

mechanism predicted that the original product of lactone formation, tylactone, would be fully reduced at positions C20 and C23. This prediction was confirmed by the isolation of blocked mutants of *S. fradiae*, which produced only tylactone (3, 4) (see below).

The enzymatic bioconversion of tylactone to tylosin involves seven modifications of the tylactone molecule (Figure 1): oxidations at C20 and C23; the attachment of mycaminose at the 5-hydroxyl position of tylactone, the attachment of mycarose to the 4′-hydroxyl position of the mycaminose residue, the attachment of 6-deoxy-D-allose to the C23-hydroxyl position; and methylations at the 2‴- and 3‴-hydroxyl positions of the 6-deoxy-D-allose residue (5).

Experiments in which radiolabeled macrocin (3‴-*O*-demethyltylosin, Figure 3) was introduced into tylosin fermentations demonstrated that macrocin was efficiently converted to tylosin (6). This suggested that macrocin was a precursor to tylosin and that

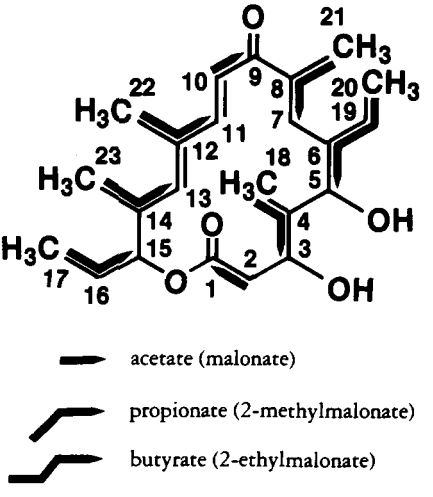

FIGURE 2. The biosynthetic origin of tylactone (2)

methylation of macrocin was the last step in the pathway. Cell-free extracts of *S. fradiae* were found to contain macrocin *O*-methyltransferase (MOMT) activity that could convert macrocin to tylosin in the presence of [^{14}C-methyl] S-adenosylmethionine (6-9).

These findings were confirmed and extended by genetic studies. Nine phenotypic classes (*tylA*↦*tylI*) of mutants blocked at different stages in tylosin biosynthesis were isolated and characterized (3). Complementation analysis with recently cloned *tyl* sequences (see below) has further subdivided each of four of these phenotypic classes into two genotypic classes yielding a total of at least 13 loci at which mutations block tylosin biosynthesis (Figure 3). The *tylF* mutations resulted in the loss of MOMT activity and the accumulation of macrocin. This confirmed the results of the earlier experiments which measured the conversion of radioactive macrocin to tylosin. The *tylE* mutations caused the loss of a second methylating enzyme activity, demethylmacrocin *O*-methyl-

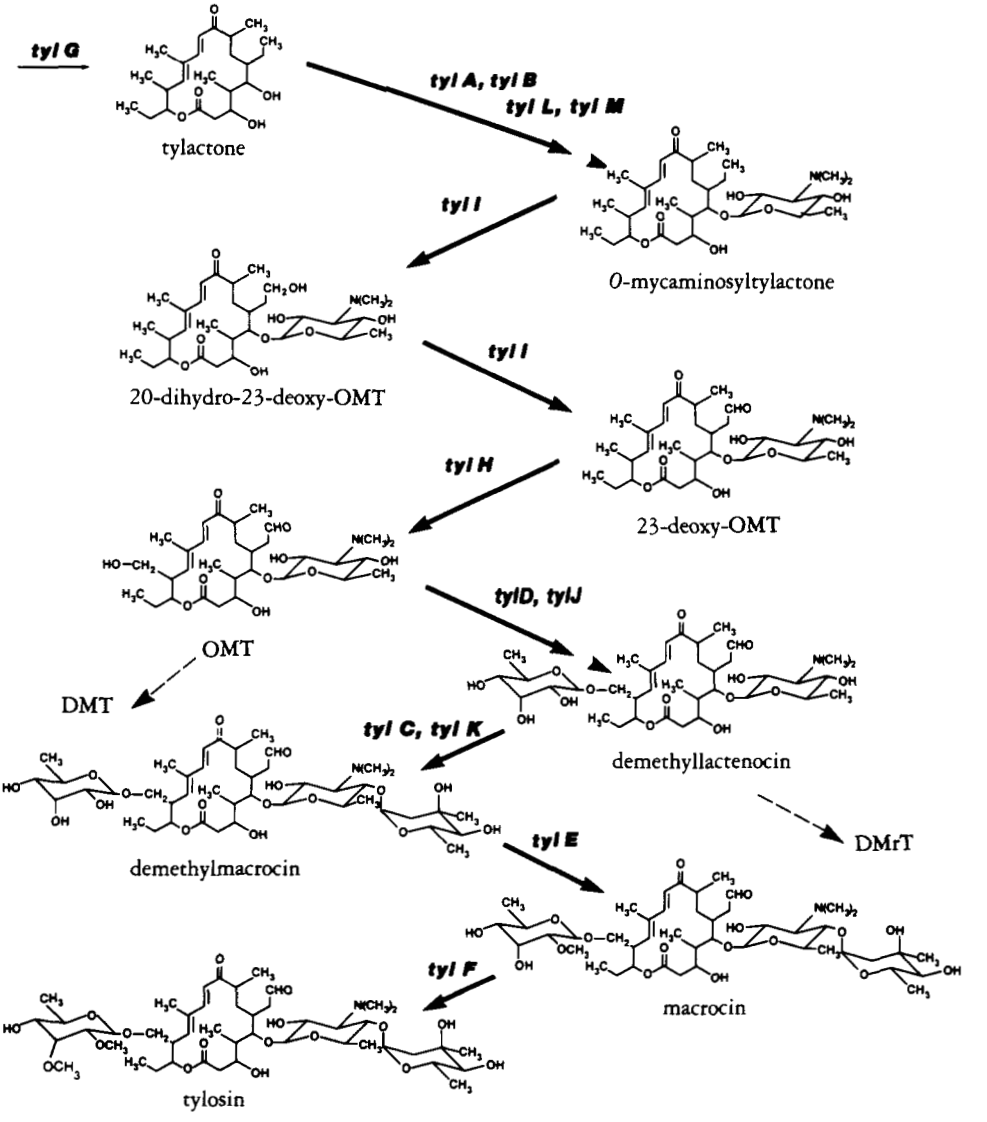

FIGURE 3. The probable biosynthetic pathway from tylactone to tylosin. OMT (*O*-mycaminosyltylonolide); DMT (demycinosyltylosin); DMrT (demycarosyltylosin). Mutations (*tyl*) blocking tylosin biosynthesis at different steps in the pathway are indicated.

transferase (DMOMT). The *tylE* mutants accumulated the substrate for this enzyme, 2‴-*O*-demethylmacrocin (DOMM). The isolation and characterization of these two mutant classes indicated that the last two steps in tylosin biosynthesis were catalyzed by two distinct *O*-methyltransferases.

Mutants of classes *tylA*, *tylL*, *tylB*, and *tylM* produce only tylactone (Figure 3). Cofermentation experiments, which determine the ability of pairs of mutants blocked at different steps to cosynthesize tylosin in mixed culture, suggested that the *tylA* and *tylL* mutants were deficient in the biosynthesis or attachment of all three tylosin sugars, while the *tylB* and *tylM* mutants were defective in only mycaminose biosynthesis or addition (3; E. Seno, unpublished data). This implied that the addition of mycaminose was the first step in the conversion of tylactone to tylosin. The *tylI* mutation prevents oxidation at C20, while the *tylH* mutation blocks oxidation at C23. The *tylD* and *tylJ* mutations block either the biosynthesis of 6-deoxy-D-allose or its attachment to the C23-hydroxyl position, while *tylC* and *tylK* mutations prevent the biosynthesis of mycarose, or its attachment to the 4′-hydroxyl position of the mycaminose residue. The *tylI*, *tylH*, *tylD*, *tylJ*, *tylC*, and *tylK* mutations result in the formation of substrates that can be acted upon by later enzymes in the pathway causing accumulation of shunt metabolites. For example, the *tylD* mutation results in accumulation of demycinosyltylosin (DMT) by addition of mycarose to *O*-mycaminosytylonolide (OMT) (Figure 3).

The *tylG* mutants represent the largest mutant class. They produce no detectable intermediates containing the chromophoric lactone of tylosin. These mutants are presumed to be defective in the tylactone synthase. Most of the *tylG* mutants tested can bioconvert tylactone to tylosin suggesting that they express all other tylosin biosynthetic enzymes. These mutants also contain parental levels of MOMT activity. Some *tylG* mutants, however, do not bioconvert tylactone and do not contain MOMT activity. Such mutants are likely to contain large deletions or mutations in regulatory genes.

The ability of most *tylG* mutants to bioconvert tylosin intermediates to tylosin was exploited to define the most likely pathway for the conversion of tylactone to tylosin (Figure 3) (5). The sequence of the biosynthetic reactions is: (a) the attachment of mycaminose to the C5-hydroxyl position of tylactone, requiring genes *tylA*, *tylB*, *tylL*, and *tylM*; (b) and (c) the oxidation of C20 from a methyl group to an aldehyde function, requiring *tylI*; (d) the oxidation of C23 to an hydroxymethyl group, requiring *tylH*; (e) the attachment of 6-deoxy-D-allose to the C23 position, requiring *tylD* and *tylJ*; (f) the addition of mycarose to the 4′-hydroxyl position of the mycaminose residue, requiring *tylC* and *tylK*; and (g) and (h) the methylation of the 2‴ (*tylE*) and 3‴ (*tylF*)-hydroxyl positions of the 6-deoxy-D-allose residue. The steps that involve the addition of a sugar residue require an active glycosyl transferase enzyme and all of the enzymes required for the biosynthesis of the respective sugar.

The development of plasmid transformation methodology (10, 11) and plasmid cloning vectors (12-16) for *Streptomyces coelicolor*, *Streptomyces lividans*, and some other streptomycetes suggested that recombinant DNA technology might be used to gain additional information on tylosin biosynthesis in *S. fradiae*. The well-characterized *tyl* mutants would provide a good system for the identification of cloned *tyl* biosynthetic genes. Unfortunately, the transformation protocols established for other streptomycetes did not allow detectable transformation of *S. fradiae* protoplasts. The development of alternative procedures yielding high frequency plasmid transformation of *S. fradiae* protoplasts (17; K. Cox and E.T. Seno, unpublished data) and the construction of improved cloning vectors (18) were critical in the application of gene cloning technology of tylosin biosynthesis in *S. fradiae*.

Several approaches to cloning *tyl* genes were considered: (a) shotgun cloning for *S.*

fradiae DNA sequences complementing *tyl* mutations; (b) construction of a DNA probe based upon the N-terminal amino acid sequence of the MOMT enzyme; and (c) cloning the tylosin-resistance gene from *S. fradiae* for use as a probe to locate nearby *tyl* biosynthetic genes. The last approach was based on the results of earlier genetic experiments which suggested that structural genes for tylosin biosynthesis were closely linked to genes controlling tylosin resistance (19).

The second and third approaches, being potentially more direct, were pursued in parrallel. Since the use of a probe required a genomic library of the target organism, a library of *S. fradiae* DNA was constructed in the bacteriophage lambda vector Charon 4 (20). A second *S. fradiae* genomic library was later constructed in a bifunctional (*Escherichia coli—Streptomyces*) cosmid vector pKC462a (21).

To construct a synthetic DNA probe for the MOMT gene, the enzyme was purified to homogenity as assessed by SDS-PAGE, and the sequence of the 35 amino acids at the amino terminus of the enzyme was determined. A 44-base mixed oligonucleotide probe containing only guanine (G) and cytosine (C) in the third positions of redundant codons was synthesized using the region of the amino acid sequence with the least codon degeneracy (Figure 4). The use of only G or C in the third codon positions was based on the observation that the frequency of G or C in the third position of cloned streptomycete structural genes has been greater than 90% (22-24). This is expected based upon the unusually high G+C content (73%) of streptomycete DNA (25). Hybridization of the MOMT probe to the Charon 4 library resulted in the isolation of ten recombinant bacteriophages containing overlapping *S. fradiae* DNA inserts which were shown to be colinear with the *S. fradiae* genomic DNA. Three of the inserts were used in further exper-

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
?	PRO	SER	PRO	ASP	HIS	ALA	ARG	ASP	LEU	TYR	ILE	GLU	LEU	LEU	LYS	LYS	VAL	VAL
	CAC	AGC	CCA	GAC	CAC	GCA	AGA	GAC	CTA	TAC	ATA	GAA	CTA	CTA	AAA	AAA	GTA	GTA
	CCC	AGT	CCC	GAT	CAT	GCC	AGG	GAT	CTC	TAT	ATC	GAG	CTC	CTC	AAG	AAG	GTC	GTC
	CCG	TCA	CCG			GCG	CGA		CTG		ATT		CTG	CTG			GTG	GTG
	CCT	TCC	CTT			GCT	CGC		CTT				CTT	CTT			GTT	GTT
		TCG					CGG		TTA				TTA	TTA				
		TCT					CGT		TTG				TTG	TTG				

20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
SER	ASN	VAL	ILE	TYR	GLU	ASP	PRO	THR	HIS	VAL	ALA	GLY	MET	ILE	THR

44 base probe

AGC	AAC	GTG	ATC	TAC	GAG	GAC	CCG	ACG	CAC	GTG	GCG	GGG	ATG	ATC	ACA
AGT	AAT	GTC	ATA	TAT	GAA	GAT	CCC	ACC	CAT	GTC	GCC	GGC		ATA	ACG
TCA		GTA	ATT				CCA	ACA		GTA	GCA	GGA		ATT	ACG
TCC		GTT					CTT	ACT		GTT	GCT	GGT			ACT
TCG															
TCT															

FIGURE 4. The aminoterminal amino acid sequence of the macrocin *O*-methyltransferase (MOMT) and the possible triplet codons specifying each amino acid. The extent and composition of the mixed 44-base DNA probe is indicated.

iments, and these are shown in Figure 5. The probe was also used to identify DNA sequences containing the MOMT gene in the cosmid library of *S. fradiae* DNA. Two cosmids, pMOMT3 and pMOMT4, were isolated which contained inserts that included the DNA sequences obtained from the bacteriophage library and which extended farther leftward and rightward (Figure 5).

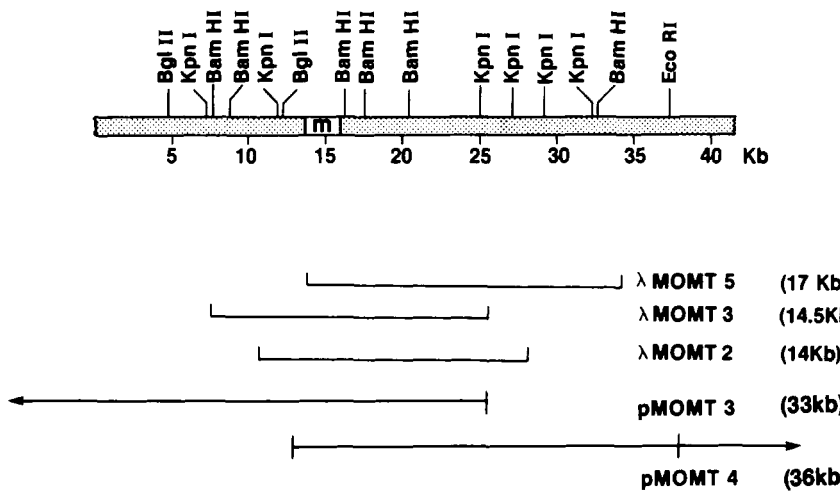

FIGURE 5. Representative DNA inserts homologous to the MOMT probe (see Figure 4) identified in libraries of *Streptomyces fradiae* DNA in the Lambda Charon 4 vector (λMOMT2, 3, and 5) and in the cosmid vector, pKC462a (pMOMT3 and 4). Synthetic *Eco*RI sites introduced in the construction of the libraries form the termini of the inserts. The location of the single natural *Eco*RI site in pMOMT4 is indicated. A restriction endonuclease cleavage map of the genomic DNA represented by the Charon 4 inserts is also shown.

An *Eco*R1-*Sal*I fragment (1.1 kb) from the left end of the λMOMT5 insert was subcloned and shown to hybridize to the 44-base DNA probe. The sequence with homology to the probe was further localized to a 320 base pair *Bst*EII-*Mlu*I fragment. This fragment was sequenced and shown to contain an open reading frame with a deduced N-terminal amino acid sequence identical to that determined for the MOMT protein. This suggested that the DNA fragments identified by homology with the DNA probe actually contained the MOMT structural gene.

To test the DNA sequences containing the MOMT structural gene for complementation of the *tylF* mutations and other *tyl* mutations, the bacteriophage and cosmid library inserts were subcloned into plasmid vectors known to transform *S. fradiae* efficiently. Both the bacteriophage and cosmid libraries were constructed to allow excision of the inserts by cleavage with *Eco*RI. Streptomycete DNA contains relatively few sites for *Eco*RI, and thus, the inserts can frequently be excised intact. The *S. fradiae* DNA inserts from the three recombinant bacteriophages, λMOMT2, λMOMT3, and λMOMT5, were subcloned into a bifunctional *Streptomyces—E. coli* plasmid vector, pHJL210 (18) forming plasmids pHJL282, pHJL280, and pHJL284, respectively (Figure 6). The insert from pMOMT4 contained an internal *Eco*RI site, and it was subcloned in two pieces in the same vector to create pHJL311 (leftward fragment) and pHJL309 (rightward fragment). The insert from pMOMT3 was subcloned intact into a similar bifunctional plasmid vector, pHJL401 (18), to form pHJL315. In addition, a 2.3 kb *Eco*RI-*Bam*HI fragment from the left end of the λMOMT5 insert was subcloned into pHJL401 to form pHJL289. This fragment contained the MOMT structural gene.

S. fradiae exhibits strong restriction against plasmids prepared from heterologous hosts (26,27). Since the subcloned inserts were identified in *E. coli*, the recombinant

plasmids were first introduced into a more efficiently transformable *S. fradiae* strain, M1 (28). After confirmation of plasmid structure, the plasmids were introduced into representatives of the nine original phenotypic classes of *tyl* mutants (3). The transformants were grown under conditions favorable for tylosin biosynthesis and plasmid maintenance, and the culture broths were assayed by tlc for the presence of tylosin or the mutant precursors (3). The results are summarized in Figure 6.

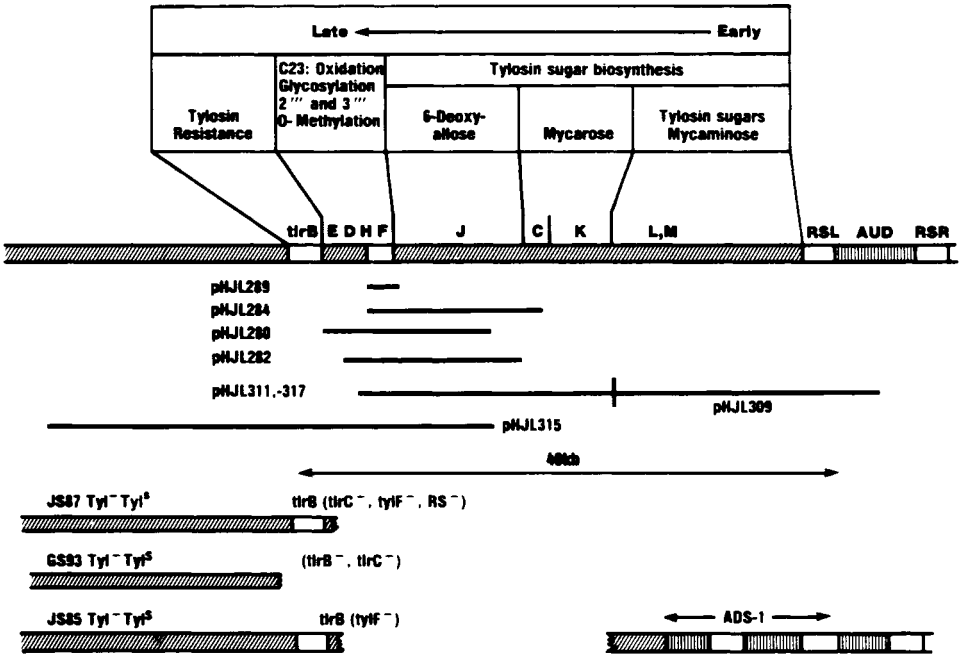

FIGURE 6. The region of the *Streptomyces fradiae* genome containing the cloned tylosin biosynthetic genes (*E, D, H, F, J, C, K, L,* and *M*). The locations of the tylosin-resistance gene, *tlrB*, and the amplifiable DNA sequence, AUD, are shown. RSL and RSR are direct repeat sequences flanking AUD on the left and the right, respectively. Possible schemes for the functional organization of the tylosin biosynthetic and resistance genes are indicated at the top of the figure. Recombinant plasmids containing *S. fradiae* DNA inserts complementing *tyl* mutations are shown; their origins from the bacteriophage and cosmid DNA libraries are explained in the text. JS87, GS93, and JS85 are pleiotropic Tyl⁻Tyl^s mutants of *S. fradiae* generated by protoplast formation and regeneration. The locations of deletions and a DNA amplification (ADS-1) in these mutants are shown. Genetic loci in parentheses have been shown to be deleted in the respective mutants.

All of the *S. fradiae* DNA fragments containing the MOMT gene (i.e., all but pHJL309) restored tylosin biosynthesis in the two *tylF* mutants GS15 and GS28. The restoration of tylosin biosynthesis in GS15 and GS28 by pHJL289 indicated that the *tylF* mutations are located in, or very near to, the MOMT structural gene. The order of the tightly clustered *tylE*, *tylD*, *tylH*, and *tylF* genes was unambiguously determined by the complementation pattern of the respective mutants with pHJL280, pHJL282, pHJL311, and pKJL284. Recombinant plasmid, pHJL309, restored tylosin biosynthesis in the *tylL* and *tylM* mutants but not in the phenotypically similar *tylA* and *tylB* mutants.

All of the *tyl* mutations that were complemented are involved in the bioconversion of tylactone to tylosin. Genes complementing mutations blocking tylactone formation (*tylG*), mutations blocking further conversion of tylactone in the *tylA* and *tylB* mutants, and the mutation (*tylI*) blocking oxidation at C20 have not been located. These four classes as a group affect early steps in tylosin biosynthesis (noting of course the

overlap between the phenotypically identical *tylA* and *tylL*, and *tylB* and *tylM* mutations). Therefore, it is possible that a cluster of early *tyl* genes is located elsewhere.

The locations of the genes complementing the nine classes of *tyl* mutations suggest a grouping of genes with related functions (Figure 6). The *tylE, tylD, tylH,* and *tylF* genes, which are clustered on a 6 kb *Bam*HI fragment, involve the C23 position of tylosin and the 6-deoxy-D-allose residue attached at that position (see Figure 1). Thus, *tylH* is necessary for oxidation of C23 to the hydroxymethyl group; *tylD* involves 6-deoxy-D-allose biosythesis or its attachment to the C23 hydroxymethyl group; *tylE* and *tylF* are required for the methylations at the 2‴- and 3‴ -hydroxyl positions, respectively, of the attached 6-deoxy-D-allose residue. In addition, *tylJ*, which may be close to *tylF*, is also likely to be involved in 6-deoxy-D-allose biosynthesis or attachment. (There is probably only one gene required for attachment of each sugar residue to the core of the tylosin molecule. If *tylD* codes for the 6-deoxy-D-allose glycosylating enzyme, then *tylJ* would very likely be involved in biosynthesis of the sugar and vice-versa.) Similarly, *tylC* and *tylK* involve mycarose biosynthesis or attachment.

Another interpretation of the *tyl* gene organization on the cloned DNA is the location of genes coding for late steps in the bioconversion of tylactone to tylosin at the left end of the cluster and genes determining earlier steps nearer to the right end (Figure 6; refer to Figure 3). The *tylE* and *tylF* genes, whose products catalyze the last two steps in tylosin biosynthesis, are located near the left end of the gene cluster. The *tylL* and *tylM* genes, which are required for the first reaction in the conversion of tylactone to tylosin, are located near the right end of the *tyl* cluster.

The introduction of cloned DNA fragments containing the MOMT gene restored MOMT activity to the *tylF* mutants GS15 and GS28 (Table 1). Similarly, introduction of the cloned *tylE* gene on pHJL280 into the *tylE* mutant GS16 restored DMOMT activity. In both cases, the specific activities of the enzymes were twofold to fivefold those of the parental control strain, presumably reflecting the multicopy (10-20 copies/cell) nature of the pHJL210 and pHJL401 replicons. Interestingly, pHJL280 did not cause expression of MOMT activity in *S. lividans, Streptomyces griseofuscus,* or the Tyl$^-$Tyl^s *S. fradiae* mutant JS87. A small amount of MOMT activity (about 10% of control) was expressed in *S. lividans* containing the MOMT gene cloned in an ultrahigh copy *Streptomyces* plasmid, pHJL288. These results suggest that the MOMT gene is poorly expressed in *S. lividans, S. griseofuscus,* and *S. fradiae* JS87, possibly due to a requirement for a positive regulator of expression.

The insert in pMOMT4 (pHJL311/pHJL309) was found to overlap a DNA fragment containing a previously identified *S. fradiae* DNA sequence, AUD (Amplified Unit of DNA, 29) (Figure 6). The AUD, which is bounded by two directed repeat sequence RS_L and RS_R, is altered in two *S. fradiae* mutants, JS85 and JS87, which have pleiotropic deficiencies in tylosin biosynthesis (29, 30; Fishman and Hershberger, Abstr. Annu. Meet. Am. Soc. Microbiol., 1983). Both mutants were isolated after protoplast formation and regeneration (30). JS85, which is phenotypically Tyl^s, contains a 500-fold amplification of AUD and is deleted for the MOMT gene. JS87 is sensitive to tylosin and is deleted for AUD and two other copies of RS known to be present in the parental genome. The MOMT gene is also deleted in JS87. The location of *tyl* biosynthetic genes near AUD was consistent with the results of previous genetic experiments that suggested close linkage between these loci (19).

The insert in λMOMT3 (pHJL280) overlapped a cloned fragment (pSVB11) containing a tylosin-resistance gene, *tlrB*, which was isolated by shotgun cloning *S. fradiae* DNA fragments into *S. lividans* and screening the transformants for tylosin resistance (Figure 6) (V. Birmingham, K. Cox, and E. Seno. Abstracts, Fifth International Symposium on the Genetics of Industrial Microorganisms, Split, Yugoslavia, 1986). The

TABLE 1. Macrocin *O*-methyltransferase (MOMT) and Demethylmacrocin *O*-methyltransferase (DMOMT) Activities from Cloned *tylF* and *tylE* Genes

Strain[a]		Phenotype	Plasmid	Relative Specific Activity[b]	
				MOMT	DMOMT
Streptomyces fradiae	373.10	Tyl^+Tyl^R	—	1	1
	GS15	TylF	pHJL210	0	n.t.[c]
	GS15	TylF	pHJL280	2-5	n.t.
	GS15	TylF	pHJL289	2-3	n.t.
	GS28	TylF	pHJL210	0	n.t.
	GS28	TylF	pHJL280	2-5	n.t.
	GS16	TylE	pHJL210	n.t.	0
	GS16	TylE	pHJL280	n.t.	2-5
	JS87	Tyl^-Tyl^s	pHJL210	0	n.t.
	JS87	Tyl^-Tyl^s	pHJL280	0	n.t.
	JS87	Tyl^-Tyl^s	pHJL284	0	n.t.
	JS87	Tyl^-Tyl^s	pHJL311	0	n.t
	JS87	Tyl^-Tyl^s	pHJL317	0	n.t.
	JS87	Tyl^-Tyl^s	pHJL315	0	n.t.
Streptomyces lividans	TK23	Tyl^-Tyl^s	pHJL280	0	n.t.
			pHJL288	0.1	n.t.
Streptomyces griseofuscus	C581		pHJL280	0	n.t.

[a]All strains were grown in tylosin fermentation medium (3) containing 20 μg/ml of thiostrepton for plasmid maintenance. *Streptomyces lividans* and *Streptomyces griseofuscus* were also grown in TS broth (33).

[b]MOMT and DMOMT activities were determined at daily intervals between two and six days after inoculation. MOMT was measured by a published assay (9). DMOMT was measured by a similar assay except that demethylmacrocin rather than macrocin was used as the substrate, and the rate of formation of both macrocin and tylosin was used in the DMOMT activity calculation. Relative specific activity values represent the range of activities relative to control which were found over the course of the five day experiment.

[c]n.t.: not tested.

tlrB gene is present in JS85 and JS87 but is deleted in GS93, a Tyl^-Tyl^s *S. fradiae* mutant phenotypically similar to JS87, which was also isolated from regenerated protoplasts. Therefore, the location of *tyl* genes in this region of the *S. fradiae* genome is consistent with the sites of deletions in mutants pleiotropically defective in tylosin biosynthesis and resistance.

The close linkage of *tlrB* to the tylosin biosynthetic genes indicates that the approach of cloning the tylosin-resistance gene as a probe to locate the biosynthetic genes should have been successful. Experiments done to clone the tylosin-resistance gene from *S. fradiae*, however, resulted in the isolation of three unique DNA sequences, *tlrA*, *tlrB*, and *tlrC* which were associated with tylosin-resistance phenotypes in *S. lividans* (*tlrA* and *tlrB*) or in JS87 (*tlrB* and *tlrC*) (V. Birmingham, K. Cox, and E. Seno. Abstracts, Fifth International Symposium on the Genetics of Industrial Microorganisms, Split, Yugoslavia, 1986). The *tlrA* and *tlrB* genes were isolated by cloning *S. fradiae* DNA fragments into *S. lividans* and screening the transformants for resistance to tylosin (500 μg/ml). The *tlrC* gene was discovered by cloning *S. fradiae* DNA fragments into JS87 and screening for tylosin resistance. The *tlrA* gene was the first *S. fradiae* Tyl^R gene isolated, and it conferred a significantly higher level of tylosin resistance in *S. lividans* than did the *tlrB* gene (31). DNA sequences near *tlrA* were isolated from the bacteriophage lambda library and found not to complement mutations of the classes *tylA*, *tylB*, *tylC*, *tylD*, *tylF*, and *tylG* (31). Southern hybridizations (32) revealed no apparent alteration of *tlrA* in Tyl^s mutants of *S. fradiae*, but *tlrB* was deleted in GS93, and *tlrC* was deleted in both JS87 and GS93 (Figure 6). Moreover, *tlrA* conferred

no tylosin resistance in JS87, while *tlrB* and *tlrC* both restored substantial tylosin resistance in JS87. Therefore, *tlrB* and *tlrC* are likely to be important for tylosin resistance in *S. fradiae*, but the role of *tlrA* is uncertain.

The information that has been presented here is the result of a largely collaborative effort by scientists in the Biochemical Development Division of Eli Lilly and Company and the Molecular and Cellular Biology Division of Lilly Research Laboratories. The work is the culmination of more than a decade of commitment by the management of Eli Lilly and Company to research on the biochemistry, genetics, and molecular biology of tylosin biosynthesis in *S. fradiae*.

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