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Biochemistry

Cloning genes for the biosynthesis of a macrolide antibiotic

(amplifiable unit of DNA/bacteriophage λ Charon 4/recombinant DNA/synthetic oligonucleotides/tylosin)

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ABSTRACT **Macrocin-*O*-methyltransferase (MacOMeTase) catalyzes the final enzymatic step in the biosynthesis of tylosin in *Streptomyces fradiae*. A 44-base mixed oligonucleotide probe containing only guanosine and cytidine in the third position of degenerate codons was synthesized based on the amino acid sequence of the amino terminus of MacOMeTase. Plaque blot hybridization to a bacteriophage λ library and colony blot hybridization to a cosmid library of *S. fradiae* DNA identified recombinants that contained overlapping fragments of chromosomal DNA. The nucleotide sequence of the cloned DNA verified that the DNA contained the coding sequence for MacOMeTase. Recombinant plasmids transformed mutants blocked in tylosin biosynthesis and complemented *tylF* (the structural gene for MacOMeTase) and *tyl* mutations of eight other classes.**

Streptomycetes are the focus of efforts to develop recombinant DNA cloning techniques (1–4) in order to isolate antibiotic biosynthetic genes (5–9), generate hybrid antibiotics (10, 11), isolate regulatory genes (12), and investigate the molecular biology of differentiation (13). Tylosin is a commercially important veterinary antibiotic used to treat infections caused by Gram-positive bacteria and mycoplasma. Tylosin biosynthesis in *Streptomyces fradiae* is one of the best understood biosynthetic pathways for a commercially important antibiotic (14); therefore, it provides a good system to apply recombinant DNA methodology (15). Tylosin is a macrolide antibiotic composed of a 16-member lactone (tylactone) with three attached sugars (Fig. 1). Genetic and biochemical studies (26) have identified nine classes of mutants blocked in tylosin biosynthesis. Studies with these mutants identified the most probable pathway for the bioconversion of the intermediate, tylactone, to tylosin through a series of macrolide intermediates (Fig. 1) (14–16). Members of the mutant class *tylF* accumulate the precursor, macrocin, and lack MacOMeTase enzyme activity (17). This report describes the isolation of the structural gene† for MacOMeTase and the identification of eight other closely linked tylosin biosynthetic genes.

MATERIALS AND METHODS

Strains, Growth Conditions, and Biosynthesis Assays. *Escherichia coli* strains C600 (ref. 18; ATCC 33525), SF8 (19), NM539 (20), and JM109 (21) were used as hosts for recombinant plasmids and bacteriophage. *E. coli* cultures were grown in L broth (22). For infection with bacteriophage λ, *E. coli* NM539 was grown in L broth without glucose. Both L broth and L broth without glucose were solidified with 1.5% Bacto Agar (Difco) to grow isolated colonies. The media contained ampicillin at 50 μg/ml and tetracycline at 10 μg/ml to select for antibiotic resistance when appropriate. Frozen competent *E. coli* was transformed by the $CaCl_2$–heat shock method (23). *Streptomyces lividans* TK23 and TK24 (24) were used as streptomycete hosts for recombinant plasmids. *S. lividans* cultures were grown and transformed by standard procedures (25). The media contained thiostrepton at 25 μg/ml to select for thiostrepton resistance when appropriate. *S. fradiae* strains and blocked mutants are listed in Table 1. Most of the mutants have been described (26, 27); however, unreported mutants are described in Table 1. *S. fradiae* cultures were grown, protoplasted, and regenerated as described (28). *S. fradiae* transformation used a previously described method (29). Recombinant plasmids containing cloned *S. fradiae* DNA were originally isolated in *E. coli*; then they were introduced by transformation into a highly transformable strain, *S. fradiae* M1 (28). Plasmid DNA from *S. fradiae* M1 transformed the *tyl*-blocked mutants and other *S. fradiae* strains at high frequencies. Growth conditions for the production of tylosin or tylosin intermediates have been described (26). The media contained thiostrepton at 25 μg/ml to select for thiostrepton resistance when appropriate. The macrolide compounds were identified by high-performance liquid chromatography (30) and thin-layer chromatography (26). MacOMeTase activity was assayed as described (30).

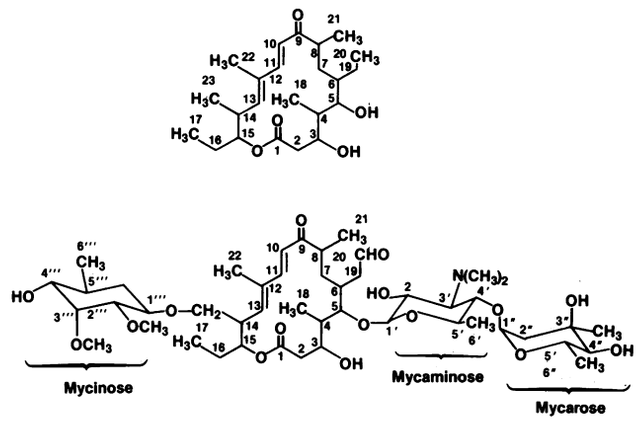

FIG. 1. Structures of tylactone (*Upper*) and tylosin (*Lower*). The names and brackets identify the sugars attached by glycosidic linkages.

Genomic Libraries and Screens. The genomic library of *S. fradiae* DNA, partially digested with *Alu* I and *Hae* III and coupled with an *Eco*RI linker before ligation with vector DNA of bacteriophage λ Charon 4, has been described (31). The library was screened by plaque blot hybridization (32). A cosmid library of *Mbo* I-digested *S. fradiae* DNA was

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Abbreviations: AUD, amplifiable unit of DNA; MacOMeTase, macrocin-*O*-methyltransferase.

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†The sequence of the 5′ end of this gene is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03008).

Table 1. *S. fradiae* strains

Strain	Relevant genotype	Phenotype
GS14	*tylA14*	Fails to add or synthesize *tyl* sugars
GS33	*tylL33*	Fails to add or synthesize *tyl* sugars
GS50	*tylB50*	Fails to add or synthesize mycaminose
GS51	*tylM51*	Fails to add or synthesize mycaminose
GS62	*tylM62*	Fails to add or synthesize mycaminose
GS52	*tylC52*	Fails to add or synthesize mycarose
GS85	*tylK85*	Fails to add or synthesize mycarose
GS48	*tylD48*	Fails to add or synthesize 6-deoxyallose
GS76	*tylD48*	Fails to add or synthesize 6-deoxyallose
	tylH76	Fails to oxidize C-23
GS77	*tylD48*	Fails to add or synthesize 6-deoxyallose
	tylI77	Fails to oxidize C-20
GS87	*tylJ87*	Fails to add or synthesize 6-deoxyallose
GS88	*tylJ88*	Fails to add or synthesize 6-deoxyallose
GS16	*tylE16*	Accumulates demethylmacrocin
GS75	*tylE75*	Accumulates demethylmacrocin
GS15	*tylF15*	Lacks macrocin-*O*-methyltransferase
GS28	*tylF28*	Lacks macrocin-*O*-methyltransferase
GS3	*tylG3*	Fails to synthesize tylactone
GS13	*tylG13*	Fails to synthesize tylactone
GS22	*tylG22*	Fails to synthesize tylactone
GS53	*tylG53*	Fails to synthesize tylactone
GS73	*tylG73*	Fails to synthesize tylactone
GS18	*tylG18*	Fails to synthesize tylactone
JS85		Pleiotrophic Tyl^- and Tyl^s
JS87		Pleiotrophic Tyl^- and Tyl^s
M1	*spo1*	Sporulation defective, readily transformed

These mutants have been described (26–28) except for GS5, GS33, GS50, GS51, GS62, GS75, GS85, GS87, and GS88, which were isolated and characterized by similar procedures.

prepared in the vector pKC462a as described (9) using *S. fradiae* DNA instead of *Streptomyces erythreus* DNA (N. Rao, R. Stanzak, and B. Schoner, personal communication). The library was screened by colony blot hybridization (33).

Plasmids, DNA Manipulations, and Restriction Mapping. Plasmids pUC19 (34), pHJL210, pHJL302, and pHJL401 (35) were used as vectors to subclone DNA fragments from isolated genomic libraries. DNA isolation, plasmid purification, restriction analysis, and construction of recombinant plasmids were performed using established procedures (35–38). Bacteriophage DNA was isolated by standard methods (38). Restriction sites in the recombinant bacteriophage DNA were mapped as described (39). Southern blot hybridization (40) of restricted DNA was used in conjunction with restriction mapping to test colinearity between cloned DNA and the *S. fradiae* genome. Fig. 2 contains descriptions of the recombinant plasmids used in this study.

Oligonucleotides were synthesized by the phosphotriester method on a SAM I automated DNA synthesizer from Biosearch (San Rafael, CA) and were purified by gel electrophoresis in 15% polyacrylamide and 7.0 M urea. Synthetic oligonucleotides were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (38). Deoxyribonucleotide sequences were determined by the chemical cleavage method (41). Amino acid sequences were determined using an Applied Biosystems (Foster City, CA) gas phase protein sequencer model 470A updated to run program 03RPTH. Amino acids were detected using on-line HPLC model 120 (42).

RESULTS

Cloning and Characterization of Recombinant Plasmids. The amino acid sequence near the amino terminus of MacOMeTase was used to design synthetic hybridization probes to identify the gene for the enzyme. MacOMeTase is a 65-kDa enzyme that contains two identical 32-kDa subunits. Full details of MacOMeTase purification and characterization will be published elsewhere. Amino acid sequence determination identified 35 amino acids near the amino terminus of the protein. The amino acid sequence was used to generate all of the possible codons for this portion of the MacOMeTase structural gene. Streptomycete DNA contains about 70% guanosine + cytidine, and a guanosine or cytidine occurs in the third position of more than 90% of the amino acid codons used in the small number of genes sequenced from the genus (43). A mixed oligonucleotide probe was synthesized using only guanosine or cytidine at the third position of codons. The 44-base sequence was derived from positions 20–33⅓ of the amino acid sequence within the box in Fig. 3 and represented the peptide sequence with the least codon degeneracy. The probe contained a mixture of 64 oligonucleotides.

The synthetic probe hybridized to Southern blots of *S. fradiae* DNA and identified a single 3.7-kilobase (kb) *Sal* I fragment and a single 5.9-kb *Bam*HI fragment. Furthermore, the probe hybridized to DNA from the *S. fradiae* genomic library, but it did not hybridize to the bacteriophage λ vector Charon 4 (data not shown).

Hybridization of the synthetic probe to plaque blots of 17,000 recombinant plaques identified 16 recombinants. Restriction maps of the cloned fragments were constructed to determine the extent of overlap among the recombinants. Southern blot hybridization of restricted recombinant bacteriophage DNA and *S. fradiae* DNA confirmed colinearity between 10 cloned fragments and *S. fradiae* DNA (Fig. 2). The 10 overlapping recombinants contained 27 kb of DNA.

The 44-base mixed oligonucleotide probe was hybridized to blots of 1610 colonies of a cosmid library containing *S. fradiae* genomic DNA in the cosmid pKC462a. Restriction mapping and Southern blot hybridization confirmed colinearity between four recombinant cosmids and the *S. fradiae* DNA (Fig. 2). The four overlapping cosmid recombinants contained 58 kb of *S. fradiae* DNA and included the 27 kb of DNA obtained in the bacteriophage λ recombinants. Fig. 2 summarizes the restriction map of *S. fradiae* DNA surrounding the sequence that hybridized to the synthetic probe.

Identification of the MacOMeTase Coding Sequence. A 1.2-kb *Eco*RI–*Sal* I fragment hybridized to the synthetic probe and was subcloned into pUC19 for DNA sequence determination. The *Eco*RI site originated from the synthetic linker used to construct the genomic library. The nucleotide sequence of an internal 326-base-pair *Bst*EII–*Mlu* I fragment that hybridized to the synthetic probe is shown in Fig. 3. The observed DNA sequence at bases 217–319 matches a DNA sequence predicted by the amino acid sequence (Fig. 3). Therefore, the cloned DNA contains a nucleotide sequence coding for at least part of MacOMeTase. The natural MacOMeTase sequence uses a CAT codon at bases 299–301 for a histidine instead of the assumed CAC codon and generates a single mismatch between the probe and the observed nucleotide sequence.

Confirmation that the cloned DNA contained the entire coding sequence for MacOMeTase came from expression of MacOMeTase in *S. lividans*. pHJL288 contains a 2.3-kb DNA fragment with homology to the oligonucleotide probe cloned into pHJL302. *S. lividans* expresses a low level of MacOMeTase activity when transformed with pHJL288. The parent strain without the plasmid or with the vector does not contain MacOMeTase activity. pHJL302 is an ultrahigh-copy-number plasmid with 1000–2000 copies per cell in *S. lividans*. Therefore, it was surprising that the level of MacOMeTase in the *S. lividans* transformant was only about 10% of the level in control strains of *S. fradiae* with a single copy of the gene (data not shown).

Linkage of the MacOMeTase Coding Sequence to Previously Cloned DNA. The DNA including and surrounding the MacOMeTase coding sequence was subcloned into plasmid

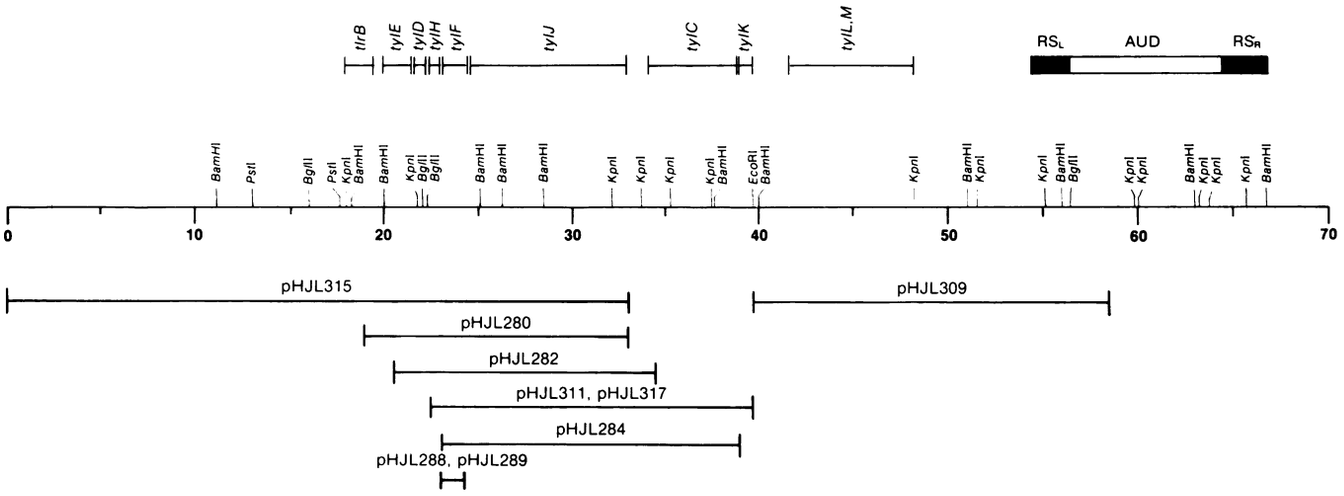

FIG. 2. Genomic map of the tylosin gene cluster. Use of the synthetic probe resulted in the isolation of recombinant bacteriophages and cosmids that contained DNA complementary to the probe. Restriction mapping and Southern blot hybridization confirmed colinearity between the recombinants and the *S. fradiae* genome. The cloned *S. fradiae* inserts were subcloned into pHJL210 to generate pHJL280, pHJL282, pHJL284, pHJL289, pHJL309, and pHJL311, into pHJL302 to generate pHJL288, or into pHJL401 to generate pHJL315 and pHJL317. The line at the middle of the figure shows the restriction map of 70 kb of DNA including the sequence that hybridizes to the synthetic probe. The locations of genes as determined in this report or previously identified structural features are aligned above the restriction map. Genotypes are explained in Table 1 and complementation results are summarized in Table 2. RS_L and RS_R indicate the directly repeated sequence at each end of AUD.

shuttle vectors pHJL210 and pHJL401 (Fig. 2). *Eco*RI sites bracket the cloned inserts in both the bacteriophage and cosmid clones, whereas the cloned *S. fradiae* DNA contains a single natural *Eco*RI site within this region. Therefore, all but one of the cloned inserts was subcloned intact. The cosmid insert containing the *Eco*RI site was subcloned as two fragments.

Southern blot hybridization was used to test for linkage between the newly isolated recombinant plasmids and previously cloned *S. fradiae* DNA sequences. The cloned DNA fragment in pHJL280 hybridized to a DNA sequence expressing tylosin resistance, *tlrB*.‡ The DNA insert in pHJL309 hybridized with a DNA fragment containing the left end of the amplifiable unit of DNA (AUD) (31). The AUD sequence is 12.7 kb long with a directly repeated 2.2-kb sequence at each end. Amplification of AUD generates 500 copies of the repeating sequence in a tandem array. The distance between *tlrB* and AUD was approximately 35 kb. The mapping results are summarized in Fig. 2.

Identification of Linked Tylosin Biosynthetic Genes by Restoration of Tylosin Biosynthesis in *tyl* Mutants. GS15 and GS28 (*tylF*) are blocked in tylosin biosynthesis and are deficient in MacOMeTase activity. Transformants of GS15 and GS28 with any of the plasmids containing the MacOMeTase coding sequence restored MacOMeTase activity and tylosin production (Table 2). Approximately 800 to 900 nucleotides are needed to code for the 32-kDa MacOMeTase subunit. Therefore, the results with pHJL289 in *tylF* mutants GS15 and GS28 suggest that *tylF* is the MacOMeTase structural gene or it is very close to the MacOMeTase structural gene, because pHJL289 contains the MacOMeTase coding sequence within a 2.3-kb insert.

Using similar methods, the eight other published classes of tylosin blocked mutants (*tylA*, *tylB*, *tylC*, *tylD*, *tylE*, *tylG*, *tylH*, and *tylI*; Table 1) and previously unpublished *tyl* mutants (Table 1) were tested for restoration of tylosin biosynthesis (Table 2). Cloned DNA fragments allowed tylosin biosynthesis in strains with the previously identified mutations (*tylC52*, *tylD48*, *tylE16*, and *tylH76*) and the unreported mutations (*tylJ88*, *tylK85*, *tylL33*, and *tylM62*). The pattern in which the inserts in pHJL280, pHJL282, pHJL284, and pHJL311 restored tylosin biosynthesis in the strains bearing the *tylD48*, *tylE16*, *tylF*, and *tylH76* mutations indicates that these four presumptive genes are clustered in an approximately 6-kb DNA sequence in the order shown in Fig. 2. *tylE75*, which confers a phenotype similar to that of *tylE16*, was restored to tylosin biosynthesis by pHJL280. GS88 confers a phenotype similar to that of GS48, but the GS88 mutant was restored to tylosin biosynthesis by both pHJL280 and pHJL284, suggesting that the mutation in GS88 defines a locus, *tylJ*, that is independent of *tylD*. Both GS52 and GS85 produce desmycosin (TylC phenotype), a shunt metabolite that accumulates because of a deficiency in mycarose synthesis or addition (26). The apparent complementation of GS52 by pHJL284 but not by pHJL280 or pHJL282 positioned the *tylC* gene near the right end of pHJL284. The failure of pHJL284 to complement GS85 and the restoration of tylosin synthesis in GS85 by pHJL311 suggested that a second locus, *tylK*, causes accumulation of desmycosin when the gene is mutated. The presumptive *tylK* gene is near the right end of the pHJL311 insert. Of course the data do not exclude the possibility that *tylC* and *tylK* are different alleles of the same gene with the mutations at widely separated sites in the gene. Five independent mutants, GS14, GS33, GS50, GS51, and GS62, accumulate the tylosin precursor, tylactone. They can be divided into two phenotypic classes based on their ability to cosynthesize tylosin in mixed cultures with GS48 and GS52 (ref. 26 and E.T.S., unpublished results). GS14 and GS33 define one class whereas GS50, GS51, and GS62 define the other class. pHJL309 restored tylosin biosynthesis in strains containing the mutations in GS33, GS51, and GS62, but it did not restore tylosin biosynthesis in strains containing the mutations in GS14 and GS50. The results suggest that at least four loci are represented by the five mutants. They are designated *tylA* and *tylL* for the first phenotypic class and *tylB* and *tylM* for the second phenotypic class. *tylA* and *tylB* were not complemented by any of the cloned DNA fragments (Table 2); however, pHJL309 (Table 2) and the subcloned *Kpn* I–*Bam*HI fragment of pHJL309 (B. Schoner and R. Stanzak, personal communication) restored

‡Birmingham, V. B., Cox, K. & Seno, E., Fifth International Symposium on Genetics of Industrial Microorganisms, Sept. 14–20, 1986, Split, Yugoslavia, p. 133 (abstr.).

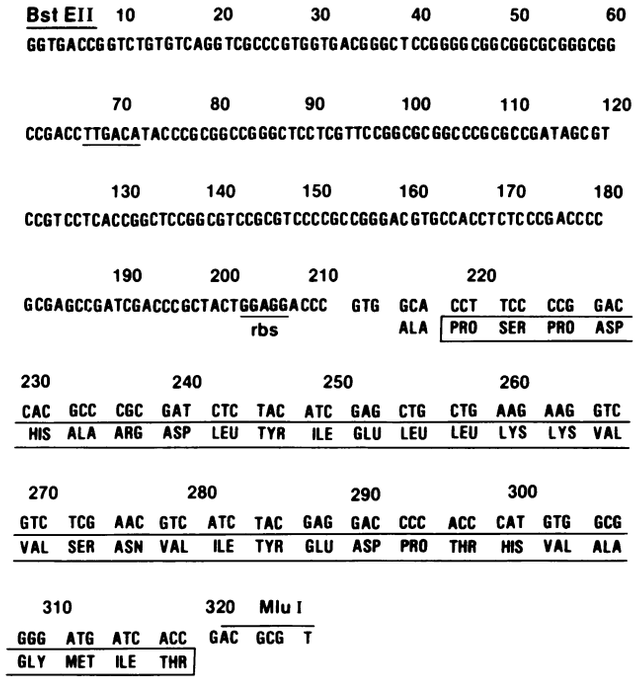

FIG. 3. Nucleotide sequence at the 5′ end of the *tylF* gene. The nucleotide sequence is shown for a 323-bp *Bst*EII–*Mlu* I fragment that hybridizes to the oligonucleotide probe. The nucleotide sequence of both strands was determined by the chemical cleavage method, but only the coding strand is shown for convenience. The theoretical translation product is shown below the nucleotide sequence. The amino acid sequence within the box agrees with the determined amino acid sequence near the amino terminus of MacOMeTase. The amino-terminal alanine is consistent with the data but is excluded from the box because the amino acid sequence data was inconclusive for this position. The underlined sequence labeled rbs contains a potential ribosome binding site that is complementary to the sequence near the 3′ end of *S. lividans* 16S ribosomal RNA (44). The sequence underlined at base pairs 67–72 matches the consensus −35 promoter sequence (45). The DNA does not contain a properly spaced −10 consensus promoter sequence. Furthermore, the data do not identify a promoter but merely show a similarity to promoter sequences in other bacteria.

tylosin biosynthesis in strains containing the *tylL* and *tylM* mutations (data not shown). The map locations of *tylC, tylD, tylE, tylF, tylH, tylJ, tylK, tylL*, and *tylM* are shown in Fig. 2.

None of the tested recombinant plasmids complemented the *tylA, tylB*, or *tylI* mutations, several *tylG* mutations, or the mutations in the pleiotropic Tyl^- and Tyl^s mutants, JS85 and JS87. These experiments tested the ability of recombinant plasmids to restore tylosin biosynthesis in blocked mutants; however, the results do not distinguish between trans complementation and recombinational repair of the mutations. Nine of the gene assignments (*tylA, tylB, tylC, tylD, tylE, tylF, tylG, tylH*, and *tylI*) were made previously (14). Our results actually identify the relative positions of mutations, but the map in Fig. 2 uses the previously assigned gene designations. *tylJ, tylK, tylL*, and *tylM* mutations are phenotypically identical to the *tylD, tylC, tylA*, and *tylB* mutations, respectively. *tylJ* and *tylD* appear to represent mutations in different genes because *tylH* and *tylF* map between them, but we cannot exclude the possibility that *tylK, tylL*, and *tylM* are alleles of *tylC, tylA*, and *tylB*, respectively.

DISCUSSION

Oligonucleotide probes derived from the amino acid sequence near the amino terminus of MacOMeTase hybridized specifically to restriction fragments and recombinant clones containing *S. fradiae* DNA. Inclusion of only guanosine + cytidine in the degenerate position of codons reduced the number of oligonucleotides in the mixed probe and improved the specificity of the probe. Shorter 17-base mixed probes were originally synthesized based on the amino-terminal amino acid sequence of MacOMeTase, but they did not specifically hybridize to restriction fragments of *S. fradiae* DNA or recombinant clones in the *S. fradiae* genomic libraries (S.E.F. and C.L.H., unpublished results). The 17-base probes showed either nonspecific binding to many bands or no hybridization. We could not identify conditions that allowed hybridization to only a few bands with the 17-base oligonucleotides. Therefore, long oligonucleotide probes were essential for the success of this approach.

Table 2. Complementation of *tyl* mutants

Strain	Complementing plasmids/noncomplementing plasmids
GS14	−/pHJL280, pHJL309
GS33	pHJL309/pHJL284
GS50	−/pHJL280, pHJL284, pHJL309, pHJL311, pHJL315, pHJL317
GS51	pHJL309/pHJL284, pHJL315
GS62	pHJL309/pHJL280, pHJL311
GS52	pHJL284, pHJL311/pHJL280, pHJL282, pHJL309, pHJL315
GS85	pHJL311/pHJL280, pHJL284, pHJL309, pHJL315
GS48	pHJL280, pHJL282/pHJL284, pHJL317
GS76	pHJL280, pHJL282/pHJL284, pHJL311, pHJL317
GS77	pHJL280/pHJL284, pHJL309, pHJL311
GS87	pHJL280, pHJL284, pHJL315/−
GS88	pHJL280, pHJL282, pHJL284, pHJL311, pHJL315, pHJL317/−
GS16	pHJL280/pHJL282, pHJL284, pHJL311, pHJL317
GS75	pHJL280/−
GS15	pHJL280, pHJL282, pHJL284, pHJL289, pHJL311, pHJL315, pHJL317/pHJL309
GS28	pHJL280, pHJL284, pHJL289/−
GS76	pHJL280, pHJL282, pHJL311, pHJL317/pHJL284
GS77	−/pHJL280, pHJL282, pHJL309, pHJL311
GS3	−/pHJL284, pHJL309
GS13	−/pHJL280, pHJL284, pHJL311
GS22	−/pHJL280, pHJL284, pHJL309, pHJL311, pHJL315
GS53	−/pHJL280, pHJL284, pHJL309
GS73	−/pHJL280, pHJL284, pHJL315
GS18	−/pHJL280, pHJL284, pHJL311, pHJL315, pHJL317
JS85	−/pHJL280, pHJL284, pHJL311, pHJL315, pHJL317
JS87	−/pHJL280, pHJL284, pHJL309, pHJL311, pHJL315, pHJL317

Plasmid entries indicate clear unambiguous results with the named plasmid. −, None of the tested plasmids gave the indicated result either complementing or noncomplementing.

Analysis of the nucleotide sequence indicates that the cloned DNA contains at least part of the *tylF* gene that codes for MacOMeTase because the amino acid sequence predicted from 102 nucleotides of coding sequence corresponds exactly to the observed amino acid sequence near the amino terminus of MacOMeTase. Furthermore, the 2.3-kb insert in the ultrahigh-copy-number plasmid pHJL288 expressed a low level of MacOMeTase in *S. lividans*, indicating that the cloned DNA contains the entire coding sequence for MacOMeTase.

The nucleotide sequence contains a GTG potential translation initiation site at bases 212–214 (Fig. 3). The partial amino acid sequence of MacOMeTase begins with an alanine that is situated one amino acid from the predicted start of the translation product. The missing methionine at the amino terminus of the isolated protein could reflect *in vivo* processing or *in vitro* proteolysis at the amino terminus of MacOMeTase. Identification of the translation initiation site

is supported by the presence of the GGAGG sequence that is complementary to the 3′ end of *S. lividans* 16S rRNA and provides a potential ribosome binding site (44) 4 bases in the 5′ direction from the GTG at bases 212–214. Translation in *E. coli* occasionally initiates at GTG, but translation initiates at GTG more frequently in streptomycetes (43).

The cloned sequence around *tylF* contains approximately 60 kb of DNA that overlaps previously cloned sequences from *S. fradiae* (31, ‡). The linkage map spans approximately 70 kb of contiguous DNA when the previously cloned sequences are aligned with the recombinant fragments obtained in this study. A tylosin resistance gene, *tlrB*, was found approximately 6 kb to the left of *tylF* in a region containing three other tylosin biosynthetic genes: *tylD*, *tylE*, and *tylH*. Linkage of antibiotic resistance genes to antibiotic biosynthetic genes has been documented in other antibiotic biosynthetic pathways (5, 9, 46). Also linked to the tylosin biosynthetic genes is a region of known genetic instability (31). This region was designated as AUD and lies about 35 kb to the right of *tylF*. AUD can amplify to 500 copies per genome when cells are protoplasted and allowed to regenerate (37). Mutants were identified that contain extensive deletions adjacent to and in some cases including AUD (27, 47). These mutants also had their tylosin biosynthetic genes deleted (S.E.F. and C.L.H., unpublished observations). The close physical linkage between AUD and the tylosin biosynthetic genes agrees with the observed genetic linkage between AUD and tylosin biosynthesis (27).

The locations of the cloned genes (Fig. 2) suggest that clustered genes exhibit related functions in tylosin biosynthesis. *tylD*, *tylE*, *tylF*, *tylH*, and *tylJ* catalyze the following reactions at C-23 of the tylactone ring (Fig. 1): C-23 oxidation, biosynthesis or attachment of 6-deoxy-*D*-allose (the precursor of mycinose), and methylations of the attached sugar residue. Four of the genes (*tylD*, *tylE*, *tylF*, and *tylH*) are clustered on a 6-kb DNA sequence. The *tylC* and *tylK* gene cluster concerns mycarose biosynthesis or addition, whereas the *tylL* and *tylM* gene cluster controls mycaminose biosynthesis or addition. DNA sequences complementing the *tylA*, *tylB*, *tylG*, and *tylI* mutations were not found. These mutations block tylactone biosynthesis (*tylG*), prevent the attachment or biosynthesis of all tylosin sugars (*tylA*) or just mycaminose (*tylB*), or block oxidation at the C-20 position of tylactone (*tylI*). The failure to identify these genes suggests that they are not present on the cloned DNA. All of the complementing sequences were located in the 35-kb of DNA between *tlrB* and AUD. Flanking DNA did not complement any of the *tyl* mutations. Therefore, genes for tylosin biosynthesis may be located in more than one region of the *S. fradiae* genome. Further cloning and complementation studies will be needed to identify the remaining genes for tylosin biosynthesis.

Appreciation is expressed to R. Belagaje for providing 17-base synthetic oligonucleotides that were used in the initial attempts to identify specific hybridization probes to screen the genomic libraries and for helpful suggestions about synthesis and purification of the 44-base probes used to identify the *tylF* gene. We also thank N. Rao, R. Stanzak, and B. Schoner for making the *S. fradiae* cosmid library available before publication of the vector and library and also Paul Rosteck for helpful suggestions. The art work was provided by W. Kruse.

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