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Mutation and Cloning of *eryG*, the Structural Gene for Erythromycin *O*-Methyltransferase from *Saccharopolyspora erythraea*, and Expression of *eryG* in *Escherichia coli*

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A mutant strain derived by chemical mutagenesis of *Saccharopolyspora erythraea* (formerly known as *Streptomyces erythreus*) was isolated that accumulated erythromycin C and, to a lesser extent, its precursor, erythromycin D, with little or no production of erythromycin A or erythromycin B (the 3″-O-methylation products of erythromycin C and D, respectively). This mutant lacked detectable erythromycin *O*-methyltransferase activity with erythromycin C, erythromycin D, or the analogs 2-norerythromycin C and 2-norerythromycin D as substrates. A 4.5-kilobase DNA fragment from *S. erythraea* originating approximately 5 kilobases from the erythromycin resistance gene *ermE* was identified that regenerated the parental phenotype and restored erythromycin *O*-methyltransferase activity when transformed into the erythromycin *O*-methyltransferase-negative mutant. Erythromycin *O*-methyltransferase activity was detected when the 4.5-kilobase fragment was fused to the *lacZ* promoter and introduced into *Escherichia coli*. The activity was dependent on the orientation of the DNA relative to *lacZ*. We have designated this genotype *eryG* in agreement with Weber et al. (J. M. Weber, B. Schoner, and R. Losick, Gene 75:235–241, 1989). It thus appears that a single enzyme catalyzes all of the 3″-O-methylation reactions of the erythromycin biosynthetic pathway in *S. erythraea* and that *eryG* codes for the structural gene of this enzyme.

The antibiotic erythromycins (Fig. 1) are synthesized by *Saccharopolyspora erythraea* through the formation of a 14-membered macrolide ring and successive addition of two sugar residues: first L-mycarose at C-3, followed by D-desosamine at C-5 to form erythromycin D (7, 22). After erythromycin D, the pathway is branched, forming an apparent metabolic grid (Fig. 2). Erythromycin D can be hydroxylated at C-12 to form erythromycin C or methylated at C-3″ to form erythromycin B. Whereas the formation of erythromycin A from erythromycin C by methylation at C-3″ has been demonstrated clearly, the formation of erythromycin A from erythromycin B has been less certain, with conflicting reports in the literature (7). Erythromycins E and F are derived from erythromycin A and are present in only minor amounts (13, 14). The major product and most biologically active molecule is erythromycin A.

Mutant strains defective in erythromycin biosynthesis have been isolated frequently over the past 25 years (15, 16, 18, 25). In general, these strains can be classified into three types: (i) those that produce no detectable macrolide products, (ii) those that accumulate the aglycone erythronolide B, and (iii) those that accumulate the monoglycone 3-α-mycarosylerythronolide B. Gene-enzyme relationships in these mutants are largely unknown because of the number and complexity of steps involved. Strains with mutations in the late portion of the erythromycin pathway, past erythromycin D, are of interest for several reasons. First, these strains would assist in the clarification of the number and nature of the enzymes involved in the putative metabolic grid between erythromycin D and erythromycin A. Second, the mutant strains would provide valuable genetic background for the identification and cloning of erythromycin biosynthesis genes. Mutations in the late portion of the pathway are difficult to find. The antibiotic activity of the intermediate erythromycins (B, C, and D) eliminates a phenotypic selection based on the loss of antibiotic production. To isolate late-pathway mutants, we first developed and characterized an in vitro assay for erythromycin *O*-methyltransferase (EMT) activity (6), and then we conducted an empirical screen for mutants of *S. erythraea* based on the thin-layer chromatography (TLC) methods of Kibwage et al. (10). A mutant strain was isolated that failed to produce erythromycin A, accumulated erythromycin C, and lacked EMT activity. A 4.5-kilobase (kb) DNA fragment from *S. erythraea* was identified that regenerated the parental phenotype when transformed into the EMT-negative mutant. Finally, we detected EMT activity when the 4.5-kb fragment was fused to the *lacZ* promoter and introduced into *Escherichia coli*. The activity was dependent on the orientation of the DNA relative to *lacZ*.

A preliminary account of some of this work has been reported previously (8).

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* HB101 and JM103 (4) and plasmids pUC9, pUC18, and pUC19 (26) were obtained from commercial sources (Bethesda Research Laboratories, Inc., and Pharmacia Fine Chemicals). An erythromycin-sensitive *Bacillus subtilis* strain and a spontaneous thiostrepton-resistant mutant of *Staphylococcus aureus* were used in the bioassay of antibiotic production. *S. erythraea* strains were from the Northern Regional Research Laboratory (NRRL 2338), American Type Culture Collection (ATCC 11635), and our own collection (CA340, ER850, ER900). Strain ATCC 11635 is a nonpigmented derivative of NRRL 2338. Strain CA340, a former Abbott production strain, was derived from ATCC 11635 by a series of physical and chemical mutagenic treatments and screening for im-

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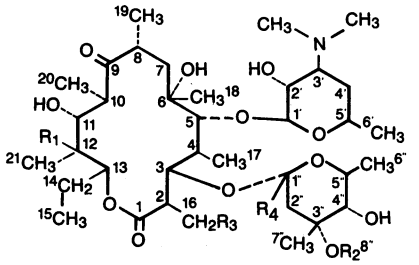

Erythromycin		R_1	R_2	R_3	R_4
1	A	OH	CH_3	H	H
2	B	H	CH_3	H	H
3	C	OH	H	H	H
4	D	H	H	H	H
5	E	OH	CH_3	—O—	
6	F	OH	CH_3	OH	H

FIG. 1. Erythromycins produced by *S. erythraea*.

proved erythromycin production. Strain ER850 was derived from CA340 by similar methods. The derivation of ER900 from ER850 is described below.

Plasmid p7A2 was composed of pNJ1 (17), a shuttle cosmid capable of replication in *S. erythraea* and *E. coli*, and a 41-kb segment of *S. erythraea* chromosomal DNA containing the erythromycin resistance gene *ermE*. Plasmid pGM406 contains the 4.5-kb *Eco*RI-*Bam*HI fragment subcloned from p7A2 and was constructed as follows. The 4.5-kb *Eco*RI-*Bam*HI fragment from p7A2 was first subcloned into pBR322 (obtained from commercial sources) to generate plasmid pGM403. Then pGM403 was digested with *Kpn*I, treated with calf intestinal alkaline phosphatase, and ligated into *Kpn*I-digested plasmid pIJ702 (D. Hopwood, John Innes Institute) to produce the *E. coli-S. erythraea* shuttle vector pGM406. A partial restriction map of p7A2 and pGM406, showing the DNA from *S. erythraea*, is shown in Fig. 5.

Culture conditions, transformation, and DNA isolation. Growth and transformation of *E. coli* and *S. erythraea* were as described previously (5, 12). Selection of *S. erythraea* transformants was carried out with 3.5 ml of overlay agar containing 100 μg of thiostrepton per ml and Trypticase soy broth (BBL Microbiology Systems) with 0.7% Bacto-Agar (Difco Laboratories). Plasmid DNA was isolated by the alkaline sodium dodecyl sulfate method of Birnboim and Doly (3) with slight modifications. *S. erythraea* protoplasts were prepared before the alkaline lysis step. High-molecular-weight cellular DNA from *S. erythraea* strains was isolated by spooling from ethanol precipitation of detergent-lysed protoplasts.

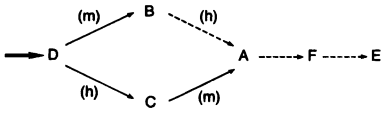

FIG. 2. Late portion of the erythromycin biosynthesis pathway. Capital letters refer to the erythromycins from Fig. 1. m, 3″-O-methylations; h, 12-hydroxylations. Dashed lines indicate relatively slow reaction rates.

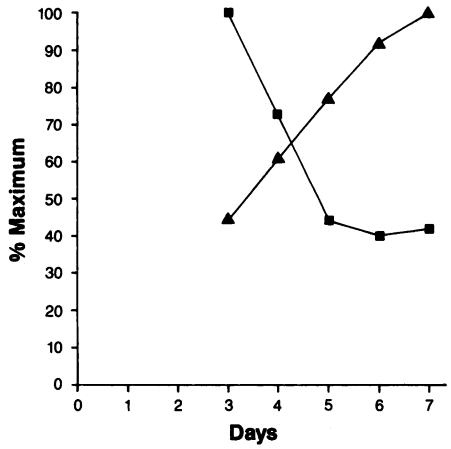

FIG. 3. Erythromycin and EMT production by *S. erythraea* grown in medium SCM. Permeabilized mycelia were used for EMT activity measurements. Erythromycin was measured by HPLC and represents primarily (>90%) erythromycin A. Symbols: ■, EMT activity; ▲, erythromycin production.

TLC screen. Survivors of standard mutagenic treatments of ER850 were fermented in 25- by 150-mm test tubes in the medium of Martin and Rosenbrook (16). After 5 days, the broth was filtered, spotted onto silica gel TLC plates, and developed with solvent system V of Kibwage et al. (10). Anisaldehyde-sulfuric acid-ethanol spray (1:1:9) reagent was used for detection (11); this reagent produces distinctive colors for the erythromycins A and C (grey-blue) and erythromycins B and D (violet-blue). Erythromycins A through D plus the precursors 3-α-mycarosylerythronolide B, erythronolide B, and 6-deoxyerythronolide B were analyzed qualitatively against standards. A similar type of screen was conducted by Baltz and Seno (1) to isolate mutants of *S. fradiae* blocked in the synthesis of the macrolide antibiotic tylosin.

EMT assay. *S. erythraea* strains were grown in SCM medium (17) for 72 h. EMT was assayed by using either permeabilized mycelia or cell extracts prepared by sonication as the source of enzyme. Permeabilized mycelial suspensions were prepared by the freeze-thaw and toluene-ethanol method of Basabe et al. (2), which was originally developed for the fungus *Neurospora crassa*. Buffer RB was 50 mM potassium 3-(*N*-morpholino)propanesulfonate–5 mM EDTA–1 mM phenylmethylsulfonyl fluoride (pH 7.2). Cell extracts were prepared by sonication of mycelia that had been washed and suspended in RB. Activity was measured by following the incorporation of radioactivity from [*methyl*-^{3}H]*S*-adenosylmethionine into a solvent-extractable final product. The assay mix contained 75 mM potassium phosphate (pH 7.5), 0.75 mM EDTA, 0.75 mM dithiothreitol, 0.5 to 0.6 mM erythromycin substrate, 0.1 mM [*methyl*-^{3}H]*S*-adenosylmethionine (3,000 to 5,000 dpm/nmol), and 15% glycerol in a final volume of 1.0 ml. The reaction was initiated by the addition of permeabilized mycelia (0.1 ml) and terminated after 60 min at 32°C by the addition of 0.05 ml of formaldehyde. The final product was extracted with 2 ml of ethyl acetate immediately after the addition of 0.025 ml of 5 N NaOH. A sample of the ethyl acetate extract was used for scintillation counting with external standard correction. The product of the reaction with erythromycin C as the substrate was identified as erythromycin A by TLC as described above and by high-pressure liquid chromatography (HPLC) (23).

TABLE 1. EMT activity produced by strains of *S. erythraea* with increased levels of erythromycin production

Strain	Erythromycin[a] (mg/ml)	EMT activity[b] (nmol/min per mg)
NRRL 2338	0.05	0.015
ATCC 11635	0.22	0.087
CA340	0.65	0.129

[a] Yield after 72 h, determined by HPLC.
[b] Determined by using sonic extracts of cells from 72-h cultures.

EMT activity in *E. coli* was detected by using cell extracts prepared as follows. Seed cultures (10 ml of L broth; 10-g/liter Difco tryptone, 5-g/liter Difco yeast extract, 0.5-g/liter sodium chloride, and 1-g/liter glucose in 25-mm test tubes) were inoculated from agar stab cultures and allowed to grow overnight at 37°C with agitation. The next morning, 1 ml of a seed culture was dispensed to each of five tubes of fresh L broth (10 ml) containing 1 mM isopropyl-β-D-thiogalactoside and incubated for 4 h. Identical cultures were combined, and the cells were pelleted by low-speed centrifugation at 4°C. Cells were washed once with cold RB, suspended in 1 ml of RB, and disrupted by sonication. Cell debris was removed by centrifugation (20 min, 10,000 × *g*, 4°C). The remainder of the EMT assay was as described above with 0.1 ml of extract; the reaction was allowed to proceed for 18 to 20 h. Erythromycin A was measured radiochemically after HPLC separation (23). Ethyl acetate extracts of the reaction mixes were concentrated 10-fold before injection. The protein concentration of the extracts was measured by using commercial bicinchoninic acid reagents (Pierce Chemical Co.).

RESULTS AND DISCUSSION

EMT activity and erythromycin production. We sought first to associate the activity in vitro of EMT with erythromycin production in vivo by comparing the time course of production of EMT with that of erythromycin and by measuring the specific activity of EMT from strains with increased antibiotic yields. Figure 3 shows the activity of EMT during the fermentation of *S. erythraea* CA340. Maximal activity was found at or near the onset of antibiotic production and then declined. The profile for EMT was similar to profiles of other putative erythromycin biosynthesis enzymes, methylmalonyl coenzyme A mutase (9) and 6-deoxyerythronolide B hydroxylase (7). Mutants isolated for increased antibiotic production by standard chemical mutagenesis procedures had increased levels of EMT activity (Table 1). Antibiotic production was increased approximately 13-fold in strain CA340, whereas EMT activity increased approximately 9-fold. Both erythromycin and EMT levels were measured after 72 h, near the peak of EMT activity. The data in Fig. 3 and Table 1 strongly suggest an involvement of EMT enzyme activity, as measured in vitro, in antibiotic biosynthesis. A comparison of EMT with other antibiotic *O*-methyltransferases showed similarities. Avermectin *O*-methyltransferase, which is involved in avermectin biosynthesis in *Streptomyces avermitilis* (19, 20), and macrocin *O*-methyltransferase, which is involved in tylosin biosynthesis in *Streptomyces fradiae* (21), have similar fermentation profiles. The activities of avermectin and macrocin *O*-methyltransferases also increase with the selection of mutant strains with improved antibiotic titers (19, 21). All of the *O*-methyltransferases appear to be involved in the terminal steps of antibiotic biosynthesis.

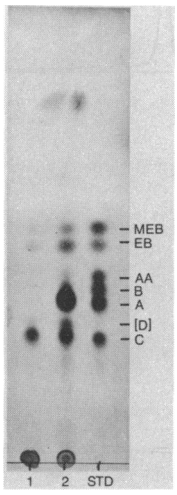

FIG. 4. TLC of the erythromycins produced by mutant strain ER900 (lane 1) and the parent culture, ER850 (lane 2). Abbreviations are as used in Fig. 1, plus the following: STD, standards; EB, erythronolide B; MEB, 3-α-mycarosyl erythronolide B; AA, anhydroerythromycin A (an acid degradation product). The relative position of erythromycin D ([D]) was known from previous work.

TABLE 2. EMT activity produced by the erythromycin C-accumulating mutant strain, ER900, and the parent culture, ER850

Strain	Substrate[a]	Relative enzyme activity[b]
ER850	Erythromycin C	1.00
	Erythromycin D	0.51
	2-Norerythromycin C	0.34
	2-Norerythromycin D	0.33
ER900	Erythromycin C	0.0
	Erythromycin D	0.0
	2-Norerythromycin C	0.0
	2-Norerythromycin D	0.0

[a] 2-Norerythromycin analogs of erythromycin C and D were supplied by J. McAlpine of Abbott Laboratories.
[b] Determined by using permeabilized mycelia from 72-h cultures.

Isolation and characterization of an EMT-negative mutant. Of 15,000 isolates screened by TLC, over 200 isolates with apparent mutations in the erythromycin pathway were isolated. Most of the isolates failed to produce any macrolide intermediate. Among these were strains that grew poorly and most likely did not have true biosynthetic blocks. Seven mutants were isolated that accumulated erythronolide B, and eight were isolated that accumulated 3-α-mycarosylerythronolide B. Most likely, these strains have defects in antibiotic sugar biosynthesis or transfer to the macrolide ring. Only one late-pathway mutant, ER900, was isolated. This strain, a survivor of nitrosoguanidine mutagenesis, was

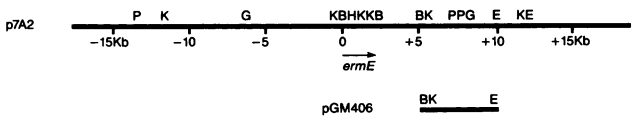

FIG. 5. Partial restriction map of p7A2 and pGM406. B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I.

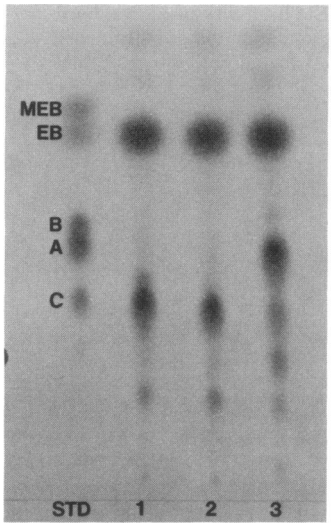

FIG. 6. TLC of the erythromycins produced by mutant strain ER900 and transformants. Lanes: STD, standards as described in the legends to Fig. 1 and 4; 1, ER900; 2, ER900 transformed with vector alone; 3, ER900 transformed with p7A2.

found to accumulate erythromycin C and, to a lesser extent, erythromycin D; no erythromycin A or B was detectable by TLC (Fig. 4). We designated this mutation in the erythromycin biosynthetic pathway *eryG900*, in agreement with the results of an independently isolated mutant of identical phenotype derived by using insertional mutagenesis by Weber et al. (24).

We measured the EMT activity in vitro of strain ER900 and its parent (Table 2). The parent strain displayed EMT activity with all substrates. No EMT activity was detected with ER900 with either erythromycin D or erythromycin C as the substrate, confirming the expectations derived from the phenotype. Moreover, no activity was detected with the 2-norerythromycin analogs of erythromycins D and C, indicating that the production of 2-norerythromycins by genetically manipulated *S. erythraea* (17) uses the same late-pathway enzymes as normal erythromycin biosynthesis. These results, and the results of Corcoran (6) with a partially purified enzyme preparation, indicate that *S. erythraea* produces a single EMT catalyzing all erythromycin 3″-O-methylation reactions.

Complementation of *eryG900*. A partial restriction map of p7A2 is shown in Fig. 5. p7A2 was introduced into the EMT-negative mutant strain ER900 by protoplast-assisted transformation (5). Thiostrepton-resistant transformants were fermented for erythromycin production and analyzed by TLC. In each of two separate experiments, one trans-

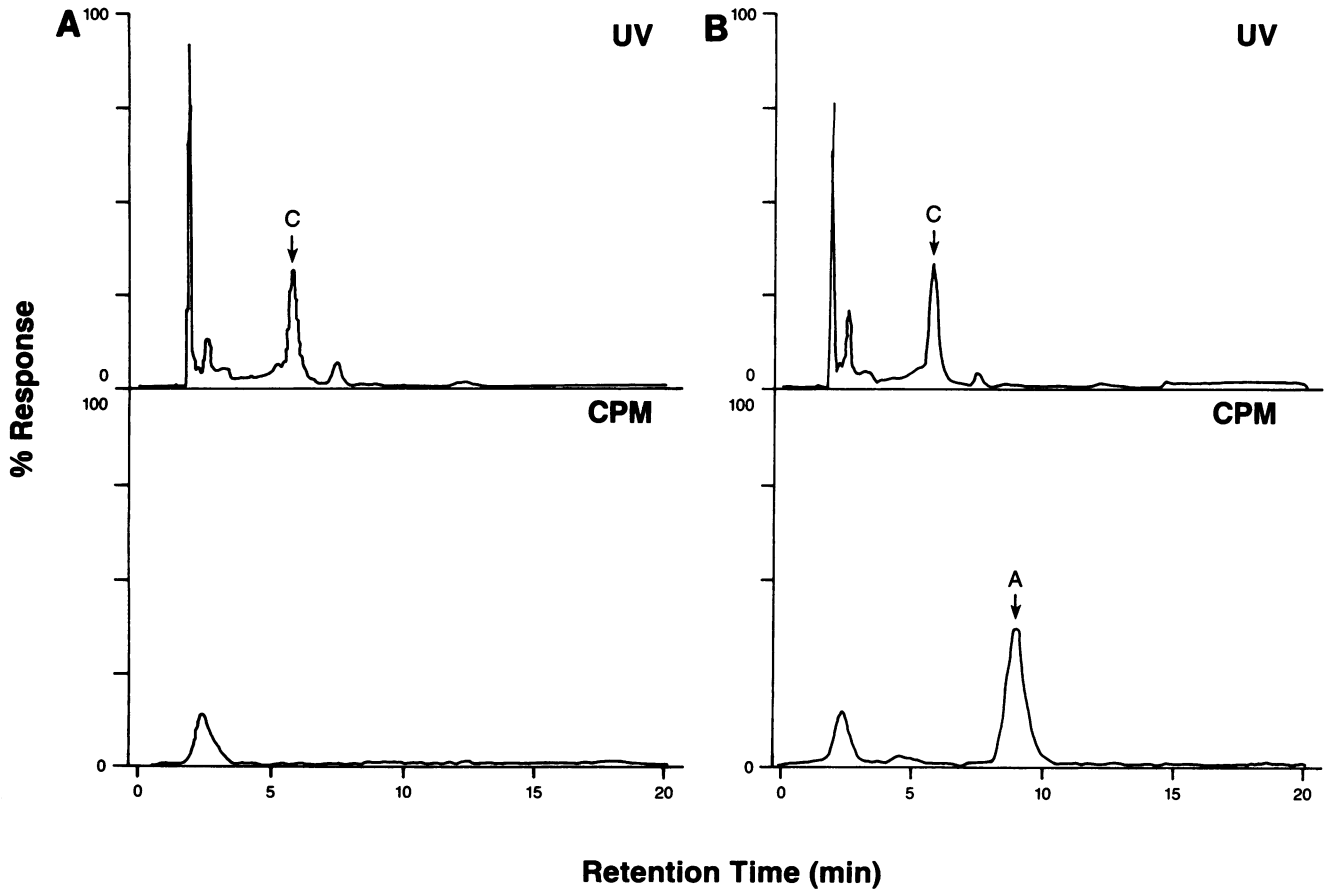

FIG. 7. HPLC of the EMT reaction products with mutant strain ER900 (A) and the p7A2 transformant (B). Letters with arrows refer to erythromycins C and A. Identification was based on the retention times of known standards.

TABLE 3. Expression of *S. erythraea eryG* in *E. coli*: EMT activity in cell extracts

Plasmid[a]	EMT activity[b] (nmol/min per mg)	Protein (mg/ml)
pTEG18	0.002	6.8
pTEG18	0.004	6.5
pTEG18	0.003	7.8
pTEG19	ND	5.9
pTEG19	ND	6.3
pUC18	ND	5.8
pUC19	ND	6.2

[a] Derivatives of *E. coli* JM103 containing the indicated plasmids were used.
[b] Determined by HPLC with radiochemical detection. ND, None detected.

formant was isolated that exhibited the restoration of erythromycin A production with a concomitant loss of erythromycin C accumulation. The TLC profile of one transformant is shown in Fig. 6. Transformation with the vector alone did not produce this effect. The isolate tested in Fig. 6 was tested for EMT activity. EMT activity was detectable only from the p7A2 transformant and the parent culture; the reaction product was verified as erythromycin A by HPLC with a radioactive flow detector (Fig. 7). The work of Weber et al. (24) suggested that *eryG* is located on a 4.5-kb *Eco*RI-*Bam*HI fragment located downstream from *ermE* (Fig. 5). This fragment was subcloned from p7A2 into pIJ702 and used to transform ER900. Two thiostrepton-resistant transformants were isolated that had regained the ability to produce erythromycin A as detected by TLC.

Expression of *eryG* in *E. coli*. The 4.5-kb *Eco*RI-*Bam*HI fragment containing the putative *eryG* gene was subcloned for expression in *E. coli*. It was inserted into pUC18 and pUC19 digested with both *Eco*RI and *Bam*HI. The resultant plasmids were designated pTEG18 and pTEG19, respectively. Expression of the insert by transcriptional readthrough from the *lac* promoter should be orientation dependent. The plasmids were introduced by transformation into *E. coli* JM103. Cultures were grown for approximately 16 h in L broth and then diluted 10-fold into fresh L broth containing 1 mM inducer (isopropyl-β-D-thiogalactoside). After 4 h, the cultures were harvested and assayed for EMT activity. Activity was detected from the pTEG18-containing culture but not from the pTEG19-containing culture (Table 3). Activity was low, and therefore the assays were allowed to continue for 18 to 20 h. The production of erythromycin A was verified and quantitated by HPLC with a radioactive flow monitor. Duplicate assays were spiked with nonradioactive erythromycin A before HPLC (UV and radioactivity detection) to confirm the identity of the peak. Controls omitting substrate or enzyme solution were negative. These results present strong evidence for the residence of the structural gene for EMT (*eryG*) on the 4.5-kb fragment. The orientation of the fragment in pTEG18 is consistent with the transcriptional mapping data of Weber et al. (24). Further characterization of *eryG* will require more extensive subcloning and sequencing.

ACKNOWLEDGMENTS

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