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S-Adenosyl-L-Methionine: Macrocin *O*-Methyltransferase Activities in a Series of *Streptomyces fradiae* Mutants That Produce Different Levels of the Macrolide Antibiotic Tylosin

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A series of mutants of *Streptomyces fradiae* selected for increased production of the macrolide antibiotic tylosin was analyzed for levels of expression of macrocin *O*-methyltransferase, the enzyme which catalyzes the final step in the biosynthesis of tylosin. Increased tylosin production was accompanied by increased macrocin *O*-methyltransferase in some of the mutants. Increased expression of macrocin *O*-methyltransferase was due to more rapid early biosynthesis of the enzyme, to reduced decay of enzyme specific activity late in the fermentation, or to combinations of both. Mutant strains which showed rapid loss of enzyme specific activity late in the fermentation converted large amounts of tylosin to relomycin. The most productive mutants, which synthesized elevated levels of macrocin *O*-methyltransferase, also produced large amounts of macrocin, the substrate for the enzyme. Incomplete conversion of macrocin to tylosin by these mutants may be due to substrate and product inhibition (E. T. Seno and R. H. Baltz, Antimicrob. Agents Chemother. **20**:370–377, 1981). The results suggest that both the levels of precursors and the levels of expression of tylosin biosynthetic enzymes are important for efficient production of tylosin.

Mutation induction and selection have been useful approaches to yield improvement in antibiotic-producing microorganisms (8, 13). However, in many cases mutants have been selected for increased antibiotic production with little or no knowledge of the genes involved. Understanding which gene mutations have beneficial effects on antibiotic productivity should facilitate rational designs of enrichment and selection schemes and should also be useful to identify specific applications for genetic recombination and gene cloning in strain development. The antibiotic-producing *Streptomyces* spp. are of particular interest in this regard since highly efficient techniques for genetic recombination by protoplast fusion (1–3, 9–12) and gene cloning are now available (6, 7, 16, 17). To apply current genetic methodologies most efficiently to increase antibiotic productivity, it is advantageous to define what limits productivity. Limiting factors may include the availability of precursors or cofactors from primary metabolism and specific activities of antibiotic biosynthetic enzymes. To determine which are limiting, specific probes for expression of antibiotic genes may be required.

We have been investigating the genetics and biochemistry of tylosin biosynthesis in *Streptomyces fradiae* and have isolated a series of mutants blocked in specific tylosin biosynthetic steps. We have determined a probable biosynthetic pathway (4, 5, 14; E. T. Seno, J. Stonesifer, G. M. Wild, and R. H. Baltz, manuscript in preparation) and have begun to map the tylosin genes (2, 5). An efficient in vitro assay for macrocin *O*-methyltransferase, the enzyme which catalyzes the terminal conversion of macrocin to tylosin, has also been developed (14). This enzyme carries out the *O*-methylation of the 3″ ′-hydroxyl of macrocin, but will not *O*-methylate the 2″ ′-hydroxyl of demethylmacrocin (14). Macrocin *O*-methyltransferase is inhibited by its substrate, product, and other tylosin-like compounds which contain mycinose or demethyl analogs of mycinose (14). Also, a mutant selected for elevated tylosin production after successive steps of mutation produced elevated levels of this enzyme (14).

In this report, we describe the results of an extensive analysis of macrocin *O*-methyltransferase levels and antibiotic production in a series of mutants selected for increased tylosin production. We demonstrate a pronounced correlation between increased tylosin production and increased macrocin *O*-methyltransferase specific activity in many, but not all, of the mutants.

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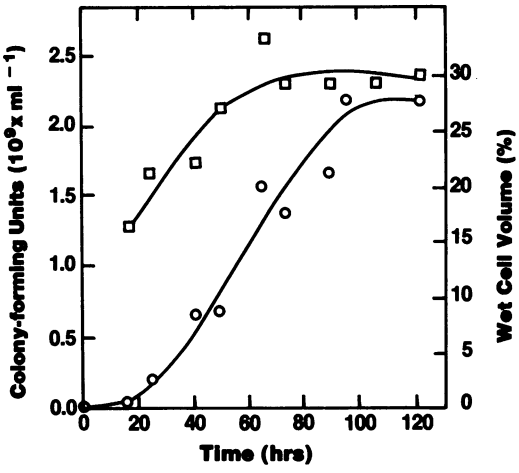

FIG. 1. Cell growth during a typical tylosin fermentation. *S. fradiae* cells were grown under fermentation conditions, and at various times cell mass was estimated by wet cell volume (□) and cell viable counts after ultrasonic treatment of mycelia (○).

TABLE 1. *S. fradiae* strains used in this study

Strain no.[a]	Designation	Source[b]
1	T59235	Recloned from original soil isolate (ATCC 19609)
2	NA171	HNO_2 mutagenesis of T59235
3	NC199	HNO_2 mutagenesis of NA171
4	OD198	UV mutagenesis of NC199
5	TPH72	HNO_2 mutagenesis of OD198
6	TPQ96	Spontaneous mutant of TPH72
7	TTM85	UV mutagenesis of TPQ96
8	C4	MNNG mutagenesis of TTM85
9	T482	MNNG mutagenesis of C4

[a] The strains were selected in sequence for increased tylosin production. The strains are listed as 1 through 9 to facilitate presentation of comparative data in the figures and text.

[b] MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

MATERIALS AND METHODS

Microorganisms. The tylosin-producing *S. fradiae* strains used in this study are listed in Table 1.

Media. AS-1 medium was as described previously (2). Complex fermentation medium and complex vegetative medium were as described previously (4).

Determination of tylosin and related macrolides. Macrolide concentrations in fermentation broths were determined by absorbance at 290 nm as described previously (4). Antibiotic activities were determined by an automated turbidimetric assay (4). The distribution of macrolide components in fermentation broths was determined by thin-layer chromatography (4).

Fermentation conditions. Growth conditions for the production of vegetative inoculum and fermentation conditions were as described previously (4, 14).

Determination of macrocin O-methyltransferase activity. The preparation of cell-free extracts and the assay for macrocin O-methyltransferase activity were as described previously (14).

Growth determinations. Samples (1 ml) of fermentation broth were diluted into 9 ml of Trypticase soy broth (BBL Microbiology Systems) and subjected to ultrasonic vibration as described previously (1). The sonicated cell suspensions were diluted, plated on AS-1 agar plates, and incubated at 32°C for about 7 days, and colonies were counted. Wet cell volumes were determined after centrifugation of fermentation broths as described previously (15).

RESULTS

Cell growth during fermentation. During a typical tylosin fermentation in complex medium, *S. fradiae* cell mass increased during the first 48 h and then remained relatively constant (Fig. 1). The number of colony-forming units, however, did not reach a maximum until about 96 h. Although the growth rates of all of the mutants described in this study have not been compared extensively in complex fermentation medium, several have been compared in soluble Trypticase soy broth. All had doubling times of about 2.0 to 2.5 h at 32°C (unpublished data).

Tylosin productivity in mutant strains of *S. fradiae*. A series of *S. fradiae* mutant strains with progressively elevated tylosin productivity was developed by mutagenesis and random selection. The strains are listed in Table 1 and are numbered from 1 to 9 for convenience. When these strains were fermented in complex medium, very little tylosin was produced in the first 24 h. Rapid tylosin biosynthesis began at about 48 h and continued for an additional 48 h before slowing somewhat (Fig. 2). Figure 2a and b show the relative antibiotic yields as measured by biological activity and UV absorbance assays, respectively.

The strains differed in initial tylosin biosynthetic rates and the most productive strains (7 through 9) also retained higher biosynthetic rates late in the fermentation. The latter observation was more pronounced when fermentation productivity was determined by biological activity rather than by UV absorbance. The apparent assay discrepancies can be explained by differences in the ratios of the tylosin-like compounds produced by the different strains (see below).

Three biologically active tylosin factors are normally produced during fermentations: tylosin, the major product of the fermentation; macrocin, a precursor of tylosin; and relomycin, a side product due to reduction of the C-20 formyl group of tylosin to a hydroxymethyl group (5, 15). Tylosin and macrocin have similar biological activities, whereas relomycin is only about 25% as active as tylosin against most test microorganisms. Figure 3a shows a typical fermenta-

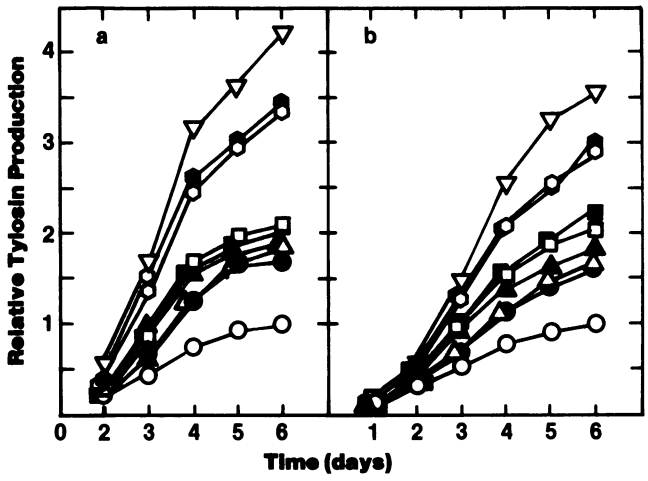

FIG. 2. Production of tylosin by *S. fradiae* strains. The *S. fradiae* strains 1 (○), 2 (●), 3 (△), 4 (▲), 5 (□), 6 (■), 7 (◇), 8 (◆), and 9 (▽) were grown under fermentation conditions, and tylosin production was determined by automated turbidimetric (a) and UV absorbance (b) assays. All antibiotic activities were normalized to the maximum amount produced by strain 1 (○).

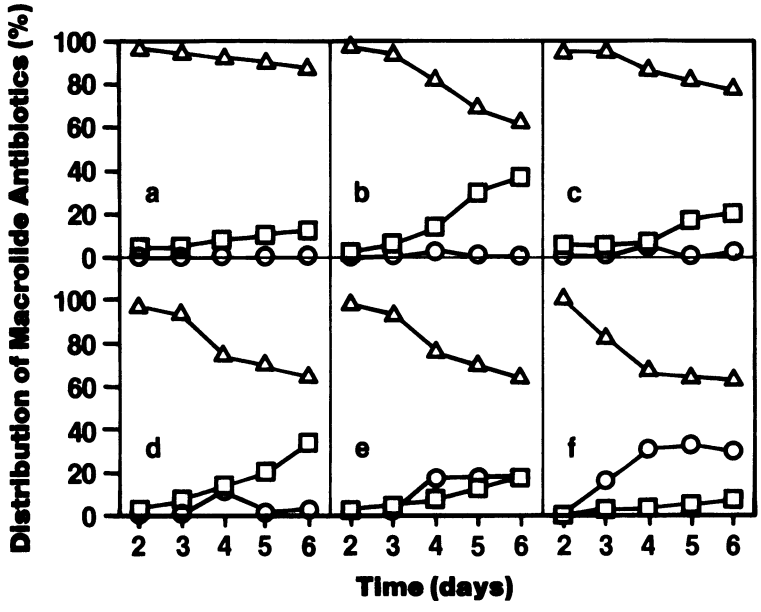

FIG. 3. Distribution of tylosin (△), macrocin (○), and relomycin (□) during fermentations with different *S. fradiae* strains. Fermentation broths from *S. fradiae* strains 1 (a), 4 (b), 5 (c), 6 (d), 7 (e), and 9 (f) from the experiment described in Fig. 2 were analyzed by thin-layer chromatography for amounts of tylosin, macrocin, and relomycin. The data from strains 2 and 3 (not shown) were nearly identical to those of strain 1, whereas the data from strain 8 (not shown) were essentially the same as those from strain 7.

tion pattern observed with strains 1 through 3. Early in the fermentation, 96% of the UV-absorbing macrolide component was tylosin. The percentage of tylosin slowly diminished during the fermentation to about 88%, with a corresponding increase of relomycin to 12%. Selection for increased antibiotic production was accompanied generally by increased conversion of tylosin to the less active relomycin in strains 4, 5, and 6 (Fig. 3b, c, and d). This conversion explains why the increased productivity of these strains is more apparent with the UV absorbance assay than with the biological assay (Fig. 2). This trend was reversed in strains 7 and 8 in which selection for higher antibiotic production was accompanied by decreased for-

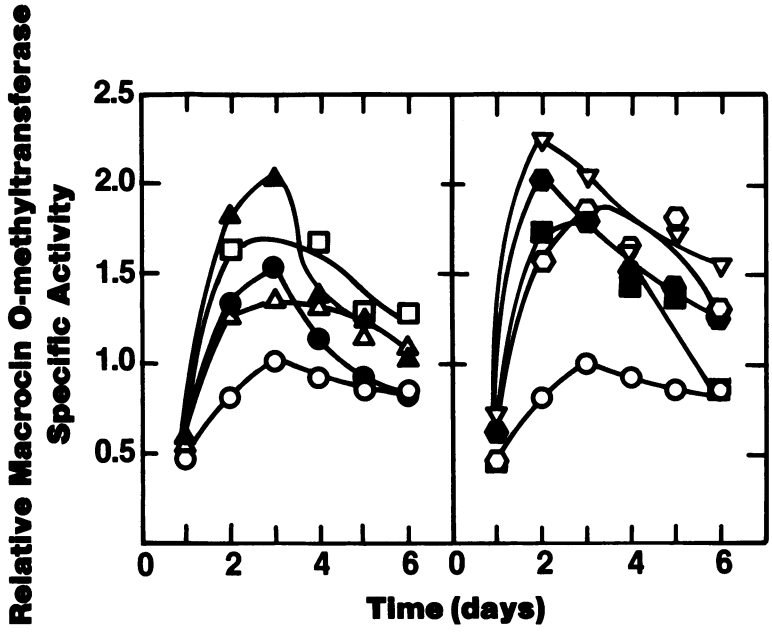

FIG. 4. Relative levels of macrocin O-methyltransferase during fermentation with various *S. fradiae* strains. Mycelia from *S. fradiae* strains 1 (○), 2 (●), 3 (△), 4 (▲), 5 (□), 6 (■), 7 (◇), 8 (◆), and 9 (▽) were grown under fermentation conditions (see Fig. 2 and 3 for tylosin production data) and fractured by ultrasonic treatment, and cell-free extracts were assayed for macrocin O-methyltransferase specific activities. All specific activities were normalized to the maximum specific activity of strain 1.

mation of relomycin but increased accumulation of macrocin (Fig. 3e). Strain 9 converted very little tylosin to relomycin, but accumulated relatively large amounts of macrocin (Fig. 3f). The shift toward increased macrocin accumulation and decreased relomycin formation in strains 7, 8, and 9 is reflected in the large increases in biological activities observed in the transitions from strains 6 to 7 and from strain 8 to 9 (Fig. 2).

Macrocin *O*-methyltransferase activities in *S. fradiae* strains. The levels of macrocin O-methyltransferase were measured during fermentations of the *S. fradiae* strains discussed above to determine whether any of the mutations which caused tylosin yield increases also caused enhanced expression of this tylosin-specific enzyme. Figure 4 shows the relative specific activities of this enzyme during fermentation. With strain 1, the specific activity increased between 24 and 72 h and then slowly declined. Strain 2 expressed a more rapid initial rate of enzyme synthesis, and produced about 50% more enzyme by 72 h. However, the macrocin O-methyltransferase specific activity declined to the level of strain 1 by 6 days. The enzyme activity in strain 3 increased at about the same rate as in strain 2, but higher levels were maintained late in the fermentation. The early rate of synthesis of the enzyme in strain 4 was significantly higher than that of strain 3, but the specific activity declined rapidly beyond 3 days and was about equivalent to that of strain 3 at 6 days. Strains 5, 6, and 7 had enzyme specific activity patterns similar to that of strain 4, except that generally higher enzyme levels were maintained later in the fermentation. Strain 8 produced macrocin O-methyltransferase very rapidly early in the fermentation cycle, but showed no advantage over strain 7 from days 3 to 6. Strain 9 exhibited the highest early rate of macrocin O-methyltransferase synthesis and maintained higher levels of enzyme specific activity than the other strains late in the fermentation. The enzyme specific activities of strain 9 at 2 days and 6 days were about 2.5 and 2.0 times the specific activities of strain 1 at these times. Similar experiments have been carried out several times, and comparable results were obtained.

Figure 5 shows the relative tylosin productivity and average macrocin O-methyltransferase specific activities for strains 1 through 9. Increases in tylosin productivity were accompanied by increases in macrocin O-methyltransferase specific activities with several mutants. However, some mutants did not appear to be altered in average O-methyltransferase specific activity, and the ratio of enzyme activity to tylosin productivity declined in these mutants. In two cases (strains 3 and 4), the ratios of macrocin O-methyltransferase to tylosin pro-

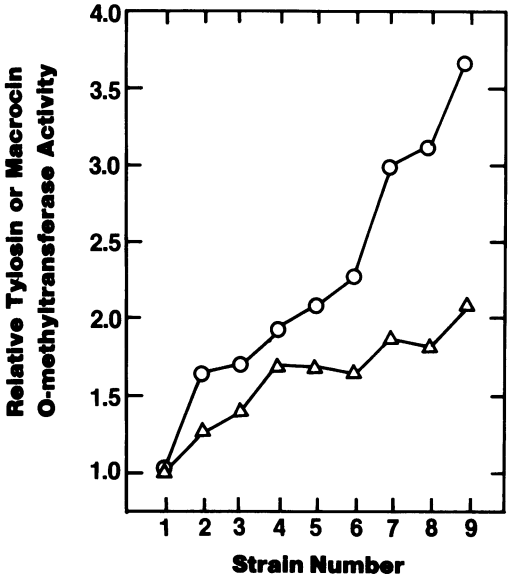

FIG. 5. Comparison of final tylosin productivities and average macrocin *O*-methyltransferase specific activities in *S. fradiae* strains. Macrocin *O*-methyltransferase specific activities were averaged between 2 and 6 days and normalized to the average specific activity of strain 1 (△). Final tylosin yields were determined by the UV absorbance assay (○) and normalized to that of strain 1.

duced increased over those of the preceding strains, and in one case (strain 9) the ratio was not altered (i.e., the rate of increase of tylosin productivity was proportional to the increase in macrocin *O*-methyltransferase activity). From the initial to the final strain in this series, the tylosin productivity increased about 3.7-fold, whereas the macrocin *O*-methyltransferase specific activity increased about 2.1-fold.

DISCUSSION

Macrocin *O*-methyltransferase is the enzyme which carries out the terminal step in biosynthesis of the macrolide antibiotic tylosin (4, 15). We have shown previously that macrocin *O*-methyltransferase activities can be measured accurately in crude extracts of *S. fradiae* (14), and that the macrocin *O*-methyltransferase specific activity profiles throughout the fermentation cycle are highly reproducible (4). Therefore, we felt that this enzyme would be a suitable probe for a comparative analysis of various mutants to determine whether there is a correlation between the levels of expression of antibiotic specific enzymes and the levels of antibiotic produced. Macrocin *O*-methyltransferase was produced most rapidly during a period in the fermentation cycle when cell mass approached a maximum, but when colony-forming units after ultrasonic treatment of mycelia were increasing. The increasing ratio of colony-forming units to cell mass during this period may be due to diminution of cell size (perhaps by completion of cross-wall formation), to an accumulation of cell wall material which renders the cells more resistant to ultrasonic breakage, or to other factors. These possibilities have not been further investigated. The period for rapid biosynthesis of macrocin *O*-methyltransferase preceded the initiation of rapid tylosin biosynthesis by about 24 h.

The results of this study show that increased production of tylosin is often, but not always, accompanied by increased specific activity of macrocin *O*-methyltransferase. Increased macrocin *O*-methyltransferase activity was due to the following: (i) increased rate of biosynthesis of *O*-methyltransferase between 24 and 72 h, (ii) decreased rate of loss of *O*-methyltransferase activity between 72 and 144 h, (iii) more rapid attainment of maximum specific activity (by 48 h), or (iv) combinations of i, ii, and iii. The cumulative effects of i, ii, and iii enabled certain strains which produced high levels of tylosin to maintain higher average *O*-methyltransferase specific activities between 24 and 144 h than preceding strains. Since some increases in tylosin productivity were not accompanied by increased macrocin *O*-methyltransferase specific activities, we infer that some mutations associated with tylosin yield increases are associated with other factors such as precursor availability or cofactor levels. Therefore, the level of tylosin specific enzymes may be limiting in certain strains, whereas the availability of precursors, cofactors, etc., may be limiting in other strains. This suggests that large increases in tylosin productivity may be achieved in single mutational steps only after at least one of these constraints is eliminated. This might be achieved if the tylosin-specific enzyme levels could be coordinately amplified by tandem gene duplication (13) or by gene cloning (6, 16, 17). Alternatively, the apparent constraint(s) in primary metabolism might be eliminated by identifying the rate-limiting step(s) in tylonolide (4, 5) biosynthesis and by genetically modifying the producing strain to alleviate the constraint(s).

Two other interesting observations were made with respect to tylosin biosynthesis in this series of strains. The first was that strains that produced elevated levels of macrocin *O*-methyltransferase early in the fermentation cycle, but that rapidly lost *O*-methyltransferase specific activity later in the cycle, also produced tylosin very rapidly early, but accumulated substantial levels of relomycin late in the fermentation. This is consistent with the conclusion that relomycin is not a precursor to tylosin, but is an enzymatic

degradation product of tylosin due to reduction of the C-20 formyl group to a hydroxymethyl group (4, 5, 15). Therefore, this reaction seems to occur predominately when tylosin is being synthesized at less than maximum rates. We have shown that this reduction reaction is carried out very rapidly by atypical *tylG* mutants which do not carry out any tylosin biosynthetic reactions and which are devoid of macrocin *O*-methyltransferase (4). Thus, the tylosin aldehyde reductase enzyme does not appear to be associated with normal tylosin biosynthesis, but the function of this enzyme remains obscure.

The second observation was that although the more developed strains of *S. fradiae* produced increased levels of macrocin *O*-methyltransferase over earlier strains, they also accumulated higher levels of macrocin during the fermentation. Since not all of the mutations resulted in enhanced macrocin *O*-methyltransferase specific activities, the most productive strains had a lower ratio of macrocin *O*-methyltransferase to tylosin produced. Therefore, this conversion appears to have become limiting in these strains. In addition, macrocin *O*-methyltransferase is inhibited by substrate, product, and substrate or product analogs (14), and thus the higher-producing strains may be inhibited in this conversion, particularly late in the fermentation cycle after high levels of these compounds have accumulated.

Identification of (at least) dual constraints on tylosin yields makes this system a difficult but appealing model for genetic study and manipulation. The apparent rate limitation in the terminal macrocin *O*-methyltransferase reaction (4, 5, 15) in highly productive *S. fradiae* strains is also an interesting model for manipulation by gene cloning, amplification, and possibly site-directed mutagenesis to alter the catalytic properties of the enzyme (3).

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