ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1981, p. 214-225
0066-4804/81/080214-12$02.00/0

Vol. 20, No. 2

Properties of *Streptomyces fradiae* Mutants Blocked in Biosynthesis of the Macrolide Antibiotic Tylosin

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Received 30 January 1981/Accepted 29 April 1981

We isolated numerous mutants of *Streptomyces fradiae* blocked in tylosin biosynthesis after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. These mutants were classified into nine groups, based upon the tylosin-like compounds produced and upon cofermentation analyses. More than 80% of the mutants isolated produced no tylosin-like compounds, and the majority of these were blocked only in the formation of tylactone. Four classes of mutants blocked in the biosynthesis or addition of tylosin sugars were isolated; *tylA* mutants were blocked in the formation of all three tylosin sugars, whereas *tylB*, *tylC*, and *tylD* mutants were blocked specifically in the biosynthesis or the addition of mycaminose, mycarose, and 6-deoxy-D-allose, respectively. Two classes of mutants (*tylH* and *tylI*) blocked in specific oxidations of tylactone and two classes (*tylE* and *tylF*) blocked in specific O-methylations of demethylmacrocin and macrocin were also characterized. Cofermentation and bioconversion studies with these mutants suggested the following relationships: (i) the tylosin sugars are derived from a common intermediate; (ii) tylactone is the first intermediate which can be excreted in appreciable quantities; (iii) the addition of mycaminose to the C-5 hydroxyl group of tylactone must precede oxidations at C-20 and C-23; (iv) oxidation at C-20 normally precedes the attachment of mycarose to the 4' hydroxyl position of mycaminose; and (v) 6-deoxy-D-allose is added to the C-23 hydroxyl position of the lactone and subsequently O-methylated at 2''' and 3''' positions. The O-methylations appear to be the final two steps in tylosin biosynthesis, and the 2''' O-methylation must occur before the 3''' O-methylation can take place. All of the *tyl* mutants except the *tylG* mutants produced relatively high levels of tylosin-like intermediates or shunt products. Mutants blocked in specific steps other than 3''' O-methylation, including a mutant blocked in 2''' O-methylation of demethylmacrocin, produced normal levels of macrocin *O*-methyltransferase. Mutants apparently containing specific tylosin structural gene mutations produced normal levels of aerial mycelia and spores, produced low levels of tylosin aldehyde reductase, and were resistant to high levels of tylosin. However, three atypical *tylG* mutants produced no tylosin-like compounds, could not cosynthesize tylosin with any other *tyl* mutant, could not bioconvert tylactone or macrocin to tylosin, and produced no macrocin *O*-methyltransferase. These three mutants produced elevated levels of tylosin aldehyde reductase. In addition, one was very succeptible to tylosin and did not produce aerial mycelia or spores.

Tylosin is a complex macrolide antibiotic which is produced commercially by a strain of *Streptomyces fradiae* (13, 26); it is also produced by strains of *Streptomyces rimosus* (22) and *Streptomyces hygroscopicus* (11). Tylosin is composed of a 16-member branched lactone (tylonolide) and three sugars (mycarose, mycaminose, and mycinose) (14, 15, 18). ^{13}C nuclear magnetic resonance studies (19, 20) have shown that the lactone is derived from two acetates, five propionates, and one butyrate, and incorporation patterns have suggested that the formyl group (C-20) and the hydroxymethyl group (C-23) (Fig. 1) are derived from methyl groups, presumably by oxidation of the lactone (tylactone) after ring closure. Also, formation of the lactone appears to be carried out by a mechanism similar to the mechanism of long-chain fatty acid biosynthesis since it is inhibited by the antibiotic cerulenin (17). Previous studies with other macrolide-producing *Streptomyces* strains indicated that mycarose, mycaminose, and mycinose are derived from glucose (9) and

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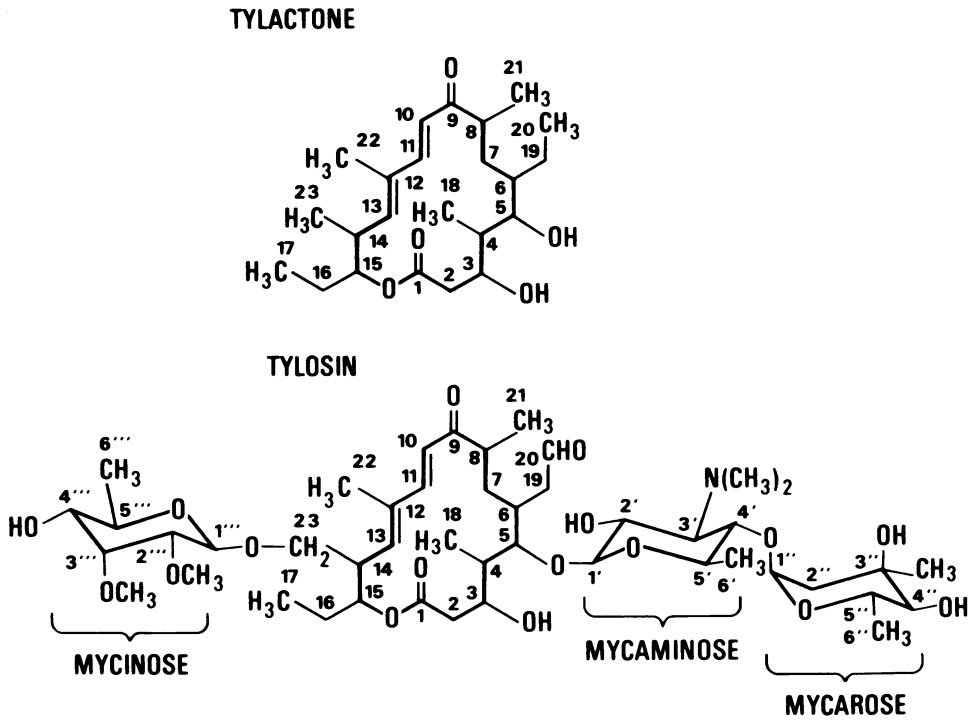

FIG. 1. *Structures of tylactone and tylosin.*

that the N-methyl groups of mycaminose and the C-methyl group of mycarose are derived from L-methionine (8, 18, 21). Pape and Brillinger (22) have shown that thymidine diphospho (TDP)-glucose can be converted to [^{14}C]TDP-mycarose by cell-free extracts of a tylosin-producing strain of *S. rimosus* in the presence of *S*-adenosyl-L-[*methyl*-^{14}C]methionine. These authors also showed that TDP-glucose is converted to a second methylated TDP sugar. Matern et al. (12) purified TDP-D-glucose oxidoreductase from this strain and showed that this enzyme converts TDP-L-glucose to TDP-4-keto-6-deoxy-D-glucose. Since the specific activity of this enzyme increased during stationary phase (idiophase), it appeared to be involved in the conversion of TDP-D-glucose to TDP-mycarose and perhaps to at least one other TDP sugar required in tylosin biosynthesis.

The tylosin-producing strain of *S. fradiae* also produces macrocin, which lacks the 3‴ O-methyl group, and relomycin, which contains a hydroxymethyl group rather than a formyl group at C-20 (10, 26). In vivo bioconversion studies have suggested that macrocin is a direct precursor of tylosin and that subsequently tylosin is converted to relomycin by reduction of the C-20 formyl group to a hydroxymethyl group (26). In vitro enzymatic studies have shown that macrocin can be converted to tylosin by O-methylation with *S*-adenosyl-L-methionine or L-methionine plus adenosine triphosphate as a methyl donor (26). Seno et al. (26) have also shown that lactenocin, which lacks mycarose and the 3‴ O-methyl group of tylosin, and desmycosin, which lacks only mycarose, can also be converted to tylosin in vivo, and have proposed that lactenocin is an intermediate which can be converted to tylosin by either of two routes. Other bioconversion studies have suggested that *O*-mycaminosyl tylonolide, which lacks both mycarose and mycinose, may be a direct precursor of tylosin (17).

We have been interested in applying various genetic techniques, including gene recombination, site-directed mutagenesis, and gene amplification (23), to the manipulation of tylosin productivity in *S. fradiae*. To facilitate these approaches, we first developed an efficient procedure for genetic recombination by protoplast fusion (1, 2). We also wanted to define more precisely the biosynthetic pathway of tylosin, the rate-limiting step(s), and the locations and possible clustering patterns of tylosin genes. To aid in these studies, we isolated mutants of *S. fradiae* blocked in specific steps in tylosin biosynthesis.

In this paper we describe the isolation and

properties of many *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced mutants blocked in tylosin biosynthesis and discuss the properties of these mutants in relation to the biosynthetic pathway to tylosin. We also determined the levels of macrocin *O*-methyltransferase, tylosin aldehyde reductase, and tylosin resistance in these mutants and discuss the possible physiological significance of these findings.

MATERIALS AND METHODS

Media and growth conditions. *S. fradiae* C4 and mutants of this strain induced by MNNG were grown in Trypticase soy broth and fragmented by ultrasound as described previously (1). AS-1 agar medium and CDA agar medium were prepared as described previously (2). Complex fermentation medium contained 2% beet molasses, 1.5% corn meal, 0.9% fish meal, 0.9% corn gluten, 0.1% sodium chloride, 0.04% ammonium phosphate (dibasic), 0.2% calcium carbonate, and 3% crude soybean oil. The pH of this medium was adjusted to 7.1 with 1 N NaOH. Complex vegetative medium contained 1% (wt/vol) corn steep liquor, 0.5% yeast extract, 0.5% soybean grits, 0.3% calcium carbonate, and 0.45% crude soybean oil; the pH was adjusted to 7.8 with 1 N NaOH.

MNNG mutagenesis. A 50-ml volume of Trypticase soy broth in a 250-ml Erlenmeyer flask was inoculated with 0.5 ml of vegetative mycelium preserved in liquid nitrogen. This culture was incubated at 32°C on a New Brunswick water bath shaker for about 24 h, as described previously (1). A sample of the culture was homogenized in a tissue grinder, and the mycelia were fragmented by ultrasonic vibration into approximately single-cell units, as described previously (1); 0.5 ml of the resulting cell suspension was inoculated into 50 ml of Trypticase soy broth and grown for about 17 h under the same conditions to an absorbance at 600 nm of about 2 to 4. The culture was again homogenized and sonicated, and then it was diluted into 50 ml of Trypticase soy broth to an absorbance at 600 nm of 0.1 to 0.2. This culture was incubated for an additional 3 h at 32°C. Then the pH was adjusted to 8.5 with 2% sodium hydroxide, MNNG was added to a final concentration of 400 to 800 μg/ml, and the culture was incubated at 37°C in a New Brunswick water bath shaker at about 250 to 300 rpm. After 20 min, 1 ml of culture was diluted into 19 ml of Trypticase soy broth chilled to 0°C to determine the cell count. The remaining cells were washed free of MNNG by centrifugation in a clinical centrifuge for 5 min. The supernatant was decanted, and the cell pellet was resuspended in 50 ml of Trypticase soy broth. The cultures were grown at 32°C overnight to allow segregation and then homogenized and sonicated. Mycelial fragments (cells) were diluted, plated onto AS-1 agar, and incubated at 32°C for about 10 days to obtain mutant clones.

Determination of tylosin and other macrolide antibiotic concentrations. Fermentation broths were mixed with equal volumes of methanol and filtered through Whatman no. 1 paper. Filtrates were further diluted, and macrolide antibiotic concentrations were estimated by absorbance at 290 nm, using purified tylosin as a reference standard. Interference from pigments and other substances absorbing at 290 nm was minimized by subtracting the absorbance at 330 nm.

The distributions of tylosin-like macrolide antibiotics in fermentation broths were estimated by separating the macrolide components by thin-layer chromatography and determining the peak areas obtained by scanning thin-layer chromatography plates for substances absorbing at 290 nm. Compounds not previously identified were purified, and their structures were determined by nuclear magnetic resonance spectroscopy (D. Dorman and G. M. Wild, unpublished data). The R_f values of the tylosin-like compounds in ethyl acetate-diethylamine (95:5) are listed in Table 1.

The antimicrobial activities contained in fermentation broths were determined by an automated turbidometric assay in which *Staphylococcus aureus* was used as the test organism.

Detection of mutants blocked in tylosin biosynthesis. Fermentations (see below) were carried out with about 6,000 individual colonies from MNNG-mutagenized cells, and fermentation broths were spotted onto silica gel thin-layer chromatography plates and developed as described previously (26). The developed plates were dipped into a solution containing 3 g of vanillin, 97 ml of methanol, and 3 ml of concentrated sulfuric acid and then heated at 60°C for 90 min. Under these conditions, macrolide components appeared as blue or black spots. Standard solutions containing tylosin and other tylosin-like macrolides, such as macrocin, relomycin, and desmycosin (26), were run on each plate to help identify mutants blocked in tylosin production. Fermentation broth samples containing no tylosin-like components or containing macrolide antibiotics other than tylosin were chromatographed again, and the plates were analyzed on a Schoeffel thin-layer plate scanner for material absorbing at 283 nm. Putative mutants were grown in complex vegetative medium broth, and fermentation analyses were repeated to confirm the mutant phenotypes. Cultures of confirmed mutants were preserved in liquid nitrogen for further work.

Identification of tylosin-like intermediates and branch products. Some of the mutants accumulated tylosin-like macrolide antibiotics which have been described previously (e.g., macrocin or desmycosin). The identities of these compounds were confirmed by cochromatography with purified compounds. Compounds not previously described were identified by their chromatographic properties on thin-layer chromatographic plates and were purified and characterized by nuclear magnetic resonance and mass spectral analysis (G. M. Wild, R. L. Hamill, and D. Dorman, unpublished data).

Fermentation and cofermentation conditions. Strains were obtained as vegetative cultures preserved in 1-ml volumes in liquid nitrogen. The frozen cells were thawed rapidly in warm water, and 0.5 ml of each culture was inoculated into 150 ml of complex vegetative medium in a 500-ml cotton-plugged Erlenmeyer flask to prepare the inocula. These cultures were incubated at 29°C for 48 h on a closed box shaker at 300

TABLE 1. *Structures of tylosin and other tylosin-like macrolide antibiotics*

Compound	Oxidation level		Mycaminose	Mycarose	6-Deoxy-D-allose	O-methyl groups present		R_f[b]
	C-20	C-23[a]				2‴	3‴	
Tylactone	CH_3	CH_3	–	–	–	–	–	0.93
Tylonolide	CHO	CH_2OH	–	–	–	–	–	ND[c]
20-Deoxy-20-dihydro-*O*-mycaminosyl tylonolide	CH_3	CH_2OH	+	–	–	–	–	0.39
20-Dihydro-23-deoxy-*O*-mycaminosyl tylonolide	CH_2OH	CH_3	+	–	–	–	–	0.37
20-Dihydro-*O*-mycaminosyl tylonolide	CH_2OH	CH_2OH	+	–	–	–	–	0.18
23-Deoxy-*O*-mycaminosyl tylonolide	CHO	CH_3	+	–	–	–	–	0.62
O-Mycaminosyl tylonolide	CHO	CH_2OH	+	–	–	–	–	0.48
23-Deoxydemycinosyl tylosin	CHO	CH_3	+	+	–	–	–	0.79
Demycinosyl tylosin	CHO	CH_2OH	+	+	–	–	–	0.56
Demethylmacrocin	CHO	CH_2OR	+	+	+	–	–	0.10
Lactenocin	CHO	CH_2OR	+	–	+	+	–	0.27
Macrocin	CHO	CH_2OR	+	+	+	+	–	0.29
Desmycosin	CHO	CH_2OR	+	–	+	+	+	0.54
Relomycin	CH_2OH	CH_2OR	+	+	+	+	+	0.38
Tylosin	CHO	CH_2OR	+	+	+	+	+	0.67

[a] R = 6-Deoxy-D-allose, 3-demethylmycinose, or mycinose.
[b] R_f values were determined by thin-layer chromatography in ethyl acetate-diethylamine (95:5).
[c] ND, Not determined.

rpm; then 0.5 ml of each culture was inoculated into 7 ml of complex fermentation medium in a 50-ml plastic bottle, and fermentations were carried out for 7 days at 29°C, as described above.

For random screening of mutagenized clones, colonies from mutant clones were picked from AS-1 agar plates, added directly to complex fermentation medium, and incubated for 7 days at 29°C.

Cofermentation of tylosin by pairs of mutants blocked in tylosin production was assessed by inoculating fermentation bottles containing 7 ml of complex fermentation medium with 0.25 ml of a vegetative culture of each mutant. Fermentations were carried out for 7 days, and fermentation broths were analyzed for the presence of tylosin by thin-layer chromatography.

Bioconversion of tylosin-like compounds. Mutants blocked before macrolide ring closure were tested for the ability to convert tylactone (Fig. 1) and macrocin to tylosin and for the ability to reduce the tylosin C-20 aldehyde group to the alcohol of relomycin. Each compound (concentration, 2 mM) was added to duplicate fermentation broth cultures of each mutant 48 h after inoculation. The fermentations were continued for an additional 3 days and then analyzed for the presence of tylosin or other tylosin-like products by thin-layer chromatography. Tylosin aldehyde reductase activity was determined as the percentage of tylosin converted to relomycin during the incubation period.

Tylosin susceptibility. Mutants were tested for tylosin susceptibility by patching ultrasonic fragments of mycelia diluted 10-fold onto AS-1 medium containing 400 μg of tylosin per ml. The efficiency of plating of each *S. fradiae* mutant on tylosin was determined by growing the mutant in Trypticase soy broth for 16 h and then fragmenting the mycelia by ultrasound; mycelial fragments (cells) were diluted and plated onto AS-1 agar plates containing varying concentrations of tylosin. The plates were incubated for 7 days at 32°C, and the numbers of colony-forming units were determined.

Aerial mycelium formation, sporulation, and auxotrophy. Aerial mycelium formation was scored visually by the appearance of colonies growing on AS-1 agar plates, and spore formation was determined by phase-contrast microscopy. Auxotrophy was defined by the inability of the mutants to grow on CDA agar plates (2).

Macrocin *O*-methyltransferase activity. *S*-Adenosyl-L-methionine:macrocin *O*-methyltransferase activities were determined by a procedure similar to a previously described procedure (26). This enzyme assay measured the transfer of [^{14}C]methyl from *S*-adenosyl-L-[*methyl*-^{14}C]methionine to macrocin to produce tylosin. Enzyme specific activities are expressed as picomoles of [^{14}C]methyl incorporated per minute per milligram of protein. A detailed description of the modified macrocin *O*-methyltransferase assay procedure is presented elsewhere (Seno and Baltz, Antimicrob. Agents Chemother., in press).

RESULTS

MNNG mutagenesis. After a survey of several mutagenic agents, including ultraviolet light, methyl methane sulfonate, ethyl methane sulfonate, and MNNG, for induction of forward mutations to auxotrophy, to rifamycin resistance, or to streptomycin resistance in *S. fradiae*, it was apparent that MNNG was superior to the other mutagens tested (R. H. Baltz, J. Stonesifer, and E. T. Seno, unpublished data). For instance, we could readily obtain several percent stable auxotrophs and rifamycin-resistant clones

at frequencies of 100- to 1,000-fold over the spontaneous background after MNNG mutagenesis. We screened about 6,000 clones of *S. fradiae* which survived MNNG treatment and isolated 72 mutants blocked in tylosin biosynthesis.

Locations of genetic blocks. Figure 1 shows the structures of tylosin and tylactone (produced by *tylA* and *tylB* mutants [see below]), and Table 1 summarizes the salient structural features of these and the other tylosin-like macrolide antibiotics discussed here. Table 2 summarizes the biochemical blocks and compounds produced by the various classes of *S. fradiae* mutants isolated. We obtained mutants blocked in the formation or addition of individual tylosin sugars (*tylB*, *tylC*, and *tylD*), as well as mutants (*tylA*) blocked in the addition of all three sugars (see below). Two types of O-methylation mutants (*tylE* and *tylF*) and two oxidation mutants (*tylH* and *tylI*) were also isolated. In addition, numerous mutants (59 of 72) were apparently blocked in early biosynthetic steps preceding macrolide ring closure (*tylG*). Unlike the other mutants, none of the *tylG* mutants produced detectable tylosin intermediates or shunt metabolites.

Tylosin intermediates or shunt metabolites produced by blocked mutants. Table 3 lists the tylosin-like compounds produced by certain specific mutants containing single or multiple blocks in tylosin biosynthesis. Most of the mutants produced less tylosin-like macrolide antibiotics than the control strain, and none of the mutants produced more than 2% tylosin. All of the mutants studied in detail (Table 3) also appeared to be at least fairly stable, since no tylosin-producing spontaneous revertants were detected. Also, after *S. fradiae* GS48 (*tylD48*) was mutagenized with MNNG, no tylosin-producing revertants were detected among 1,600 clones tested by fermentation analysis. However, a more quantitative assessment of spontaneous and mutagen-induced reversion frequencies was impractical since tylosin-producing revertants could not be identified selectively.

All of the *tylA* and *tylB* mutants produced tylactone (Fig. 1 and Tables 2 and 3) as a predominant factor and thus could not carry out any oxidations of C-20 and C-23 positions or additions of any of the tylosin sugars to tylactone. However, the *tylB* mutant produced some 5-mycarosyl tylactone as an apparent shunt product. *tylC* and *tylD* mutants were blocked in the biosynthesis or addition of mycarose and 6-deoxy-D-allose (a precursor of mycinose [see below]), respectively, but were capable of carrying out all other biosynthetic steps. Thus, *tylC* mutants accumulated desmycosin (lacking only mycarose), whereas *tylD* mutants accumulated demycinosyl tylosin (lacking mycinose).

The *tylE* mutant accumulated demethylmacrocin and therefore was unable to carry out O-methylations of the 2‴ and 3‴ positions (Fig. 1 and Table 1). Accumulation of demethylmacrocin in the *tylE* mutant indicates that both O-methylations took place after 6-deoxy-D-allose was added to the lactone ring. However, the *tylF* mutants were deficient only in 3‴ O-methylation and accumulated macrocin, an apparent precursor of tylosin (26).

The mutants containing *tylH* and *tylI* mutants were derived from GS48 (*tylD48*) and therefore contained at least two mutations. The *tylD tylH* double mutant accumulated 23-deoxydemycinosyl tylosin and therefore could not oxidize the C-23 methyl position of lactone (Fig. 1 and Table 1), but this mutant carried out all other possible biosynthetic steps. The *tylD tylI* double mutant produced nearly equal amounts of 20-deoxy-20-dihydro-*O*-mycaminosyl tylonolide and 20-deoxy-20-dihydrodemycinosyl tylosin, indicating that it was blocked in oxidation of the C-20

TABLE 2. *Summary of classes of S. fradiae mutants blocked in tylosin biosynthesis*

Mutant class	Biochemical block	Compound produced	No. of mutants isolated
tylA	Mycaminose, mycarose, and 6-deoxy-D-allose	Tylactone	2[a]
tylB	Mycaminose[b]	Tylactone	1
tylC	Mycarose[b]	Desmycosin	4
tylD	6-Deoxy-D-allose[b]	Demycinosyl tylosin	1
tylE	2‴ O-methylation	Demethylmacrocin	1
tylF	3‴ O-methylation	Macrocin	2
tylG	Tylactone formation	None	59
tylH	C-23 oxidation	23-Deoxydemycinosyl tylosin	1
tylI	C-20 oxidation(s)	Not known[c]	1

[a] One of the mutants (GS25) was isolated as a *tylG* mutant and subsequently was shown to also contain a *tylA* mutation (see Tables 3 and 4).

[b] May be blocked in sugar biosynthesis or in glycosyltransferase reactions.

[c] No *tylI* single mutant is yet available. The *tylI tylD* double mutant produces 20-deoxy-20-dihydro-*O*-mycaminosyl tylonolide and 20-deoxy-20-dihydrodemycinosyl tylosin.

TABLE 3. *Tylosin-like compounds and macrocin O-methyltransferase activities produced by mutants blocked in tylosin biosynthesis*

Strain	Major tylosin-like macrolide antibiotic produced	Relative macrolide antibiotic production	Distribution of products (%)			Relative macrocin O-methyltransferase activity[b]	
			Major compound	Tylosin	Others[a]	Maximum	Avg
C4	Tylosin	100	74	74	26	100	100
GS14 (*tylA14*)	Tylactone	45	70	<1	30	111	101
GS50 (*tylB50*)	Tylactone	35	76	<1	24	128	121
GS52 (*tylD52*)	Desmycosin	75	81	<1	19	146	134
GS48 (*tylD48*)	Demycinosyl tylosin	59	92	<1	18	130	123
GS16 (*tylE16*)	Demethylmacrocin	100	88	2	10	208	185
GS15 (*tylF15*)	Macrocin	91	71	<1	29	7	4
GS28 (*tylF28*)	Macrocin	~100	57	2	41	61	40
GS76 (*tylH76 tylD48*)	23-Deoxydemycinosyl tylosin	42	98	2	0	130	133
GS77 (*tylI77 tylD48*)	20-Deoxy-20-dihydro-*O*-mycaminosyl tylonolide and 20-Deoxy-20-dihydrodemycinosyl tylosin	62	79[c]	<1	21	131	113
GS5 (*tylG5*)		<1				112	115
GS13 (*tylG13*)		<1				82	80
GS20 (*tylG20*)		<1				89	85
GS22 (*tylG22*)		<1				118	104
GS53 (*tylG53*)		<1				114	95
GS73 (*tylG73*)		<1				109	111
GS25 (*tylG25 tylA78*)		<1				90	85
GS18 (*tylG18*)		<1				0.7	1
GS40 (*tylG40*)		<1				0.4	0.8
GS41 (*tylG41*)		<1				0.3	1.3

[a] In most cases, a substantial fraction of the minor factors produced were C20 dihydro analogs of the major compound. The minor factors produced by the *tylA* mutants have not been identified. The major additional factor produced by the *tylB* mutant was 5-mycarosyl tylactone (G. M. Wild, R. L. Hamill, E. T. Seno, and R. H. Baltz, unpublished data). GS28 accumulated a large amount of demethylmacrocin in addition to 20-dihydromacrocin.

[b] Maximum macrocin *O*-methyltransferase activities were present after 3 or 4 days of fermentation (see Fig. 2). Average macrocin *O*-methyltransferase activities were determined from days 1 through 6 of fermentation (Fig. 2).

[c] Approximately equal amounts of the two major products comprised 79% of the total.

methyl group of lactone as well as in formation or addition of 6-deoxy-D-allose (because of the *tylD* mutation) and was capable of adding mycarose to the 4′ of mycaminose with moderate efficiency. All *tylG* mutants did not produce detectable levels of tylosin-like compounds.

Cofermentation of tylosin by blocked mutants. To determine the extent of the genetic defects present in the blocked mutants, pairs of blocked mutants were cofermented in complex fermentation medium, and fermentation broths were screened for tylosin production (Table 4). With the exception of the *tylA* mutants, all classes of mutants apparently blocked in single steps beyond tylactone formation produced tylosin in pairwise combinations. The *tylA* mutants cofermented tylosin with mutants blocked in either O-methylations or oxidations, but did not coferment tylosin with *tylB*, *tylC*, and *tylD* mutants. The *tylC* and *tylD* mutants were clearly blocked in either biosynthesis or addition of mycarose and 6-deoxy-D-allose, respectively, since they produced desmycosin and demycinosyl tylosin (Fig. 1 and Table 2). The *tylB* mutant produced tylactone (like the *tylA* mutant), but was capable of cosynthesizing tylosin with all mutants other than *tylA*. Thus, it appeared that the *tylB* mutant was blocked in the formation or addition of mycaminose and that the *tylA* mutants were blocked in the formation of all three sugars. These observations suggest that addition of mycaminose is the essential first step in the conversion of tylactone to tylosin.

A total of 10 mutants classified as *tylG* were cofermented with each other and with typical mutants from all other classes (Table 4); none of these *tylG* mutants produced detectable tylosin when they were cofermented with other *tylG* mutants. Six of the mutants (GS5, GS13, GS20, GS22, GS53, and GS73) cosynthesized tylosin with all eight other classes of mutants. However, GS18, GS40, and GS41 did not cosynthesize tylosin with any of the other eight classes of mutants. GS25 cofermented tylosin with *tylE* and *tylF* mutants but not with *tylA*, *tylB*, *tylC*, and *tylD* mutants. Thus, GS25 appeared to con-

TABLE 4. *Cosynthesis of tylosin by pairs of S. fradiae mutants blocked in tylosin biosynthesis*

Strain	Cofermentation with:										
	GS14	GS50	GS52	GS48	GS16	GS15	GS5[a]	GS18[b]	GS25	GS76	GS77
GS14 (*tylA14*)	−	−	−	−	+	+	+	−	−	−	−
GS50 (*tylB50*)	−	−	+	+	+	+	+	−	−	+	+
GS52 (*tylC52*)	−	+	−	+	+	+	+	−	−	+	+
GS48 (*tylD48*)	−	+	+	−	+	+	+	−	−	−	−
GS16 (*tylE16*)	+	+	+	+	−	+	+	−	+	+	+
GS15 (*tylF15*)	+	+	+	+	+	−	+	−	+	+	+
GS5 (*tylG5*)	+	+	+	+	+	+	−	−	−	+	+
GS18 (*tylG18*)	−	−	−	−	−	−	−	−	−	−	−
GS25 (*tylG25 tylA78*)	−	−	−	−	+	+	−	−	−	I[c]	I
GS76 (*tylH76 tylD48*)	−	+	+	−	+	+	+	−	I	−	−
GS77 (*tylI77 tylD48*)	−	+	+	−	+	+	+	−	I	−	−

[a] Identical responses were obtained with the *tylG* mutants GS13, GS20, GS22, GS53, and GS73.
[b] Identical responses were obtained with the *tylG* mutants GS40 and GS41.
[c] I, Results inconclusive.

tain at least two classes of mutations (*tylG* and *tylA*).

Macrocin *O*-methyltransferase activities in blocked mutants. Figure 2 shows the specific activities of macrocin *O*-methyltransferase during fermentations by a series of blocked mutants. Table 3 shows the relative macrocin *O*-methyltransferase activities for all of the mutants studied. All of the mutants blocked in the biosynthesis or addition of tylosin sugars (Fig. 2a) produced enzyme-specific activity profiles similar to the activity profile of the parent strain (Table 3 and Fig. 2c). In all cases, macrocin *O*-methyltransferase specific activities increased between 1 and 3 days and then declined during the next 3 days. Similar profiles were obtained with mutants blocked in specific oxidations and with all of the *tylG* mutants (blocked in lactone formation) which cosynthesized tylosin with other blocked mutants. However, GS18, GS40, and GS41, which did not cosynthesize tylosin with any other blocked mutant, did not produce detectable levels of macrocin *O*-methyltransferase (Fig. 2b and Table 3). However, all of the mutants blocked in tylosin sugars or in specific oxidations produced significantly less total tylosin-like compounds than the parent strain (Table 3).

The mutants blocked in specific O-methylations displayed several interesting patterns. Strain GS16 (*tylE16*), which accumulated demethylmacrocin lacking both 2‴ and 3‴ O-methyl groups, produced normal levels of macrocin *O*-methyltransferase during the first 2 days of fermentation. However, the specific activity continued to increase for an additional 2 days (Fig. 2c), thus allowing GS16 to accumulate about twice the normal specific activity for macrocin *O*-methyltransferase and to maintain nearly twice the normal enzyme level during the final 2 days of fermentation.

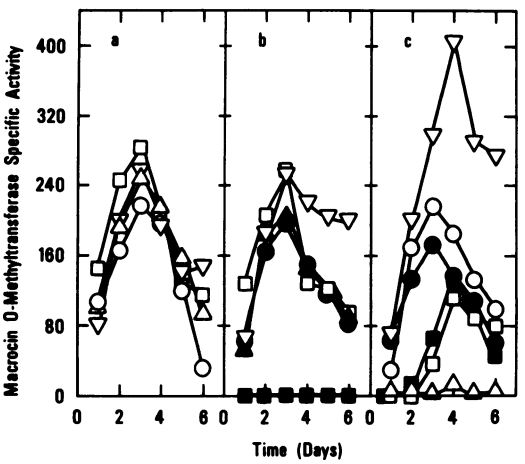

FIG. 2. *Macrocin O-methyltransferase activities in S. fradiae blocked mutants during fermentation in complex fermentation medium. Specific activities were determined as picomoles of [^{14}C]methyl incorporated from S-adenosyl-L-[methyl-^{14}C]methionine into tylosin per minute per milligram of protein. (a) Symbols: ○, GS14 (tylA14); △, GS50 (tylB50); □, GS52 (tylC52); ▽, GS48 (tylD48). (b) Symbols: ●, GS5 (tylG5); ▲, GS22 (tylG22); □, GS76 (tylH76 tylD48); ▽, GS77 (tylI77 tylD48); ■, GS18 (tylG18). (c) Symbols: ○ and ●, C4; △, GS15 (tylF15); □ and ■, GS28 (tylF28); ▽, GS16 (tylE16). Experiments represented by open and solid symbols were carried out at different times.*

Strain GS15, which carried a *tylF* mutation and accumulated macrocin (Table 3), the substrate for macrocin *O*-methyltransferase, produced nearly undetectable levels of macrocin *O*-methyltransferase during fermentation.

GS28, which also carried a *tylF* mutation, did not produce detectable macrocin *O*-methyltransferase activity during the first 2 days of fermentation (Fig. 2c), but produced approximately 50% of the control level of macrocin *O*-

methyltransferase by 4 days. This mutant produced very low levels of tylosin (Table 2).

Aerial mycelium, spore formation, and auxotrophy. All of the strains listed in Table 3 except GS40 and GS41 produced normal amounts of aerial mycelia and aerial spores on AS-1 medium; GS40 and GS41 produced no detectable aerial mycelia or spores. Only 1 of 72 mutants tested contained an auxotrophic mutation in addition to a specific *tyl* mutation.

Tylosin aldehyde reduction and other bioconversions. All of the mutants listed in Table 3 which contained blocks in biosynthetic steps beyond formation of tylactone and most of those blocked before tylactone formation converted tylosin to relomycin very slowly by reducing the C-20 aldehyde to an alcohol function. This slow reduction is characteristic of tylosin-producing strains (26). However, the three *tylG* mutants (GS18, GS40, and GS41) which appeared to be completely devoid of tylosin biosynthetic enzymes (see above) converted tylosin to relomycin and macrocin to 20-dihydromacrocin rapidly (Table 5). However, none of these mutants was able to convert tylactone to any other compound. GS22, GS25, and GS73 converted macrocin to tylosin efficiently and thus were similar to normal tylosin-producing strains (26), and GS22 and GS73 converted tylactone to tylosin efficiently. GS25 converted tylactone to tylosin poorly, indicating that it contained both *tylG* and *tylA* mutations (Table 4). Since GS25 converted 11% of the tylactone to tylosin, it could not contain a deletion mutation spanning both loci.

Tylosin resistance. All of the mutants listed in Table 3 except GS40 grew on AS-1 medium containing 400 μg of tylosin per ml in patch tests. In subsequent tests of plating efficiency, GS40 was susceptible to low levels of tylosin (Fig. 3); however, a fraction of the cells (about 4×10^{-5}) initiated colony formation. Subsequent tests of these clones indicated that they were phenotypically resistant but genotypically susceptible.

TABLE 5. *Bioconversion of tylosin, macrocin, and tylactone by tylG mutants of S. fradiae*

Strain	Compound added	Distribution of bioconversion products (%)				
		Tylosin	Relomycin	Macrocin	20-Dihydromacrocin	Tylactone
GS22	Tylosin	87	13	0	0	0
	Macrocin	86	10	4	0	0
	Tylactone	83	17	0	0	0
GS25	Tylosin	82	18	0	0	0
	Macrocin	94	6	0	0	0
	Tylactone	11	0	0	0	89
GS73	Tylosin	91	9	0	0	0
	Macrocin	93	4	3	0	0
	Tylactone	83	17	0	0	0
GS18	Tylosin	36	64	0	0	0
	Macrocin	0	0	44	56	0
	Tylactone	0	0	0	0	>99
GS40	Tylosin	27	73	0	0	0
	Macrocin	0	0	44	56	0
	Tylactone	0	0	0	0	>99
GS41	Tylosin	16	84	0	0	0
	Macrocin	0	0	19	81	0
	Tylactone	0	0	0	0	>99

DISCUSSION

MNNG is a potent mutagenic agent for a variety of microorganisms (3, 5), including the actinomycete *Streptomyces coelicolor* (4, 24). MNNG produces multiple closely linked mutations in many bacteria (3, 5, 24).

We found that MNNG is also a potent mutagenic agent for *S. fradiae* and used it to induce mutations in genes associated with tylosin biosynthesis. With treatments resulting in about 90 to 99% inactivation, more than 1% of the surviving cells contained mutations which blocked one or more steps in tylosin biosynthesis. Among these were mutants blocked in formation of tylactone, in oxidation of the C-20 or C-23 position of tylactone, in formation or addition of the three tylosin sugars, and in O-methylations of the 6-deoxy-D-allose and 3‴-demethylmycinose covalently linked to the lactone ring. Of the 19 mutants studied in detail, only 1 (GS25) clearly contained two distinct mutations in tylosin genes. However, three mutants appeared to be blocked in many biosynthetic steps, but the phenotypes of these mutants were probably not caused by multiple closely linked mutations (see below). Of the 72 blocked mutants studied, only 1 contained an additional auxotrophic mutation, and 2 lacked the ability to produce aerial mycelia and spores. Thus, we did not obtain a large number of mutants carrying easily identifiable multiple mutations. However, many multiple mutations in tylosin genes could go undetected. For instance, multiple mutations in genes coding for enzymes involved in tylactone formation cannot be distinguished from single mutations by cofermentation or bioconversion experiments, since intermediates of tylactone were not detected and are probably bound covalently to an acyl carrier protein (see below). Also, multiple mutations in specific sugar pathway genes cannot be distinguished from single mutations. Therefore, a more critical analysis of the frequency of comutation in tylosin genes awaits the development of a complementation assay which does not depend on excretion of intermediates.

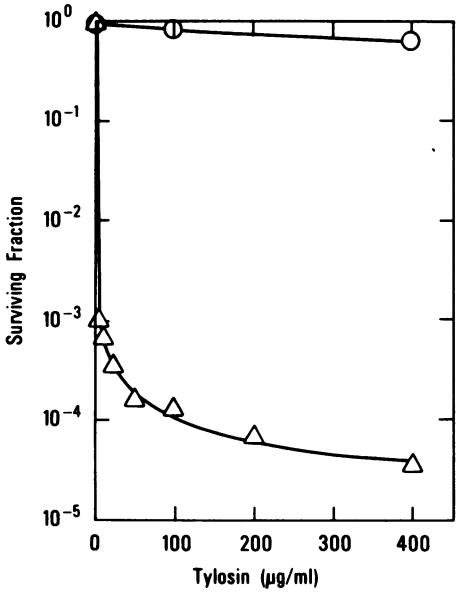

FIG. 3. *Efficiency of plating strains C4* (○) *and GS40 (tylG40)* (△) *on AS-1 media containing different concentrations of tylosin.*

An analysis of the properties of the mutants isolated and characterized in this study provided new information on the biosynthetic pathway to tylosin. Tylosin is composed of a 16-member lactone (tylonolide) to which three sugars (mycaminose, mycinose, and mycarose) are attached (Fig. 1). The lactone is derived from two acetates, five propionates and one butyrate (19, 20), presumably by condensation of one propionyl-*S*-coenzyme A molecule (as primer) with two malonyl-*S*-coenzyme A molecules, four methylmalonyl-*S*-coenzyme A molecules, and one ethylmalonyl-*S*-coenzyme A molecule by a scheme analogous to that involved in fatty acid biosynthesis (18). A prediction of this scheme is that the C-20 and C-23 positions should originate as methyl groups derived from ethylmalonyl-*S*-coenzyme A and methylmalonyl-*S*-coenzyme A, respectively. We confirmed this by isolating *tylA* and *tylB* mutants which accumulated tylactone (Fig. 1), a compound which was readily converted to tylosin by certain *tylG* mutants blocked in tylactone formation. Also, tylactone was the least complex molecule to accumulate in any of the blocked mutants and thus appears to be the first stable intermediate excreted by *S. fradiae*. If tylactone is in fact synthesized by a multienzyme complex analogous to fatty acid synthetase, one would predict that intermediates would be bound covalently to an acyl carrier protein and that only the final product might be released. The lack of positive cosynthesis with *tylG* mutants, which must comprise a heterologous collection of mutants blocked in many different steps in tylactone formation, is consistent with this hypothesis.

The mutants designated *tylG* accounted for more than 80% of the blocked mutants isolated. Of the 10 *tylG* mutants analyzed for cosynthesis with other blocked mutants, 7 appeared to be blocked only in tylactone formation and were capable of carrying out all other tylosin biosynthetic reactions. If each step in tylactone formation is carried out by a specific enzyme, about 21 steps can be postulated (i.e., 4 acyl transferase reactions, 8 condensations, 5 β-keto reductions, 2 dehydrations, and 2 enoyl reductions). Since fatty acid synthetase complexes are generally comprised of many fewer enzymes, some of which carry out more than one specific conversion, tylactone synthetase could be composed of fewer enzymes. It is likely that a roughly equal number of reactions are required to carry out the remaining steps in the biosynthesis of tylosin, including about 12 postulated steps to convert glucose to TDP-4-keto-6-deoxy-D-glucose and subsequently to TDP-mycarose, TDP-6-deoxy-D-allose, and TDP-mycaminose (see below), 2 oxidations of C-20, 1 oxidation of C-23, 2 O-methylations, and 3 glycosyltransferase reactions. Thus, mutations in genes coding for specific enzymes involved in tylactone formation are clearly induced at higher frequencies than mutations in other specific tylosin genes. This may be accounted for if more sites in these genes are mutable (or fewer sites can accumulate silent mutations) because of subtle protein-protein interactions involved in a multienzyme tylactone synthetase. Alternatively, these genes may be intrinsically more mutable.

Tylosin contains three sugars, mycarose, mycinose, and mycaminose (Fig. 1). We identified four classes of mutants blocked in tylosin sugar biosynthesis or in glycosyltransferase reactions. *tylB*, *tylC*, and *tylD* mutants are blocked specifically in the formation or addition of mycaminose, mycarose, and 6-deoxy-D-allose (a precursor of mycinose), respectively. Although a *tylB* mutant has been shown by cofermentation analysis to be capable of carrying out oxidations of C-20 and C-23, additions of 6-deoxy-D-allose and mycarose, and O-methylations of the 2‴ and 3‴ positions of 6-deoxy-D-allose, it produced primarily unmodified tylactone. Therefore, the addition of mycaminose to tylactone must precede all other biosynthetic steps involved in conversion of tylactone to tylosin. A similar scheme has been demonstrated for the biosynthesis of platenomycin (7).

On the other hand, the *tylC* and *tylD* mutants

carried out all of the steps except addition of mycarose and addition of 6-deoxy-D-allose, respectively, and produced desmycosin or demycinosyl tylosin. Therefore, these glycosyl transfers do not necessarily proceed in a particular order, and neither is required to proceed before either C-20 or C-23 oxidation.

The *tylA* mutants were blocked in the formation or addition of all three tylosin sugars and, like the *tylB* mutant, produced tylactone. The TylA phenotype is probably not caused by deletions spanning several genes involved in biosynthesis or glycosyl transfer of the sugars since GS25, which was blocked in tylactone formation and in the formation or addition of all three tylosin sugars, was capable of converting a small amount of exogenously added tylactone to tylosin. Thus, GS25 appears to contain a *tylA* point mutation. A single point mutation could block the formation of all three sugars if all three are derived from a common intermediate. Two likely intermediates are TDP-D-glucose and TDP-4-keto-6-deoxy-D-glucose (12, 22).

We isolated two classes of mutants blocked in oxidation of tylactone. The *tylH* mutant, which was blocked in the oxidation of C-23 methyl to hydroxymethyl, was capable of carrying out all other biosynthetic steps (except addition of 6-deoxy-D-allose and subsequent O-methylations) and produced the biologically active compound 23-deoxydemycinosyl tylosin. Thus, oxidation of C-23 does not have to precede addition of mycaminose to tylactone (as expected from above), oxidation of C-20 methyl to formyl, or addition of mycarose to the 4' hydroxyl of mycaminose. The *tylI tylD* double mutant was blocked in the oxidation of C-20 methyl to formyl and in the formation or addition of 6-deoxy-D-allose and produced 20-deoxy-20-dihydro-*O*-mycaminosyl tylonolide and 20-deoxy-20-dihydrodemycinosyl tylosin. Thus, oxidation of C-20 does not necessarily precede oxidation of C-23. However, a cofermentation analysis indicated that the *tylI* mutant was proficient in the biosynthesis and addition of mycarose but incapable of efficient addition of mycarose to endogenously produced 20-deoxy-20-dihydro-*O*-mycaminosyl tylonolide. Thus, it appears that the oxidation of methyl to formyl at C-20 normally precedes the addition of mycarose in tylosin biosynthesis. However, we cannot rule out the possibility that the mutant containing the *tylI* mutation might also contain a leaky *tylC* mutation, which could account for the poor addition of mycarose. This will be discussed elsewhere (E. T. Seno, R. H. Baltz, J. Stonesifer, and G. M. Wild, manuscript in preparation). Also, since the *tylI* mutant contained an additional *tylD* mutation, we could not determine whether C-20 oxidation normally precedes the addition of 6-deoxy-D-allose to the C-23 hydroxymethyl position.

The tylosin sugars contain five methyl groups (Fig. 1). It has been shown previously that the C-methyl group of mycarose and the N-methyl groups of mycaminose in other macrolide-producing *Streptomyces* strains are derived from L-methionine (8, 18, 21). Also, Seno et al. (26) have shown that the 3''' hydroxyl group of macrocin can be O-methylated in vitro by cell-free extracts of *S. fradiae* containing *S*-adenosyl-L-[*methyl*-^{14}C]methionine or L-[*methyl*-^{14}C]methionine plus adenosine triphosphate as a methyl donor to produce tylosin. These authors proposed that 3''' O-methylation of macrocin and addition of mycarose to desmycosin are alternative terminal steps in tylosin biosynthesis. We isolated two classes of mutants blocked in sugar methylations; both were blocked in specific O-methylations of precursors of mycinose. The *tylE* mutant accumulated demethylmacrocin, which contained 6-deoxy-D-allose instead of mycinose. Production of this compound indicated that 6-deoxy-D-allose can be added efficiently to the C-23 hydroxymethyl position of the lactone ring before either O-methylation. Cofermentation analysis indicated that the *tylE* mutant was proficient in all tylosin biosynthetic steps except 2''' O-methylation of the 6-deoxy-D-allose moiety of demethylmacrocin. In vitro measurements of *O*-methyltransferase activity with macrocin as the substrate also indicated that the *tylE* mutant produced high levels of macrocin *O*-methyltransferase. Thus, demethylmacrocin does not appear to be a substrate for macrocin *O*-methyltransferase, and 2''' O-methylation must occur before 3''' O-methylation. This conclusion was further supported by in vitro *O*-methyltransferase studies with the mutants blocked in either step (Seno and Baltz, in press). It is interesting that demethylmacrocin acts as a potent inhibitor of macrocin *O*-methyltransferase (Seno and Baltz, in press).

The *tylE* mutants were blocked specifically in macrocin *O*-methyltransferase activity and accumulated macrocin (a tylosin analog lacking only the 3''' O-methyl group). The *tylF15* mutant produced nearly undetectable levels of macrocin *O*-methyltransferase activity (Fig. 2 and Table 3) but high levels of demethylmacrocin *O*-methyltransferase activity (Seno and Baltz, in press). Thus, the 2''' and 3''' *O*-methyltransferases show high degrees of specificity. The fact that both demethylmacrocin and macrocin can be converted rapidly to tylosin by *tylG* mutants (Seno et al., manuscript in preparation) suggests that both of these compounds are precursors of

tylosin and that both O-methylations normally occur after 6-deoxy-D-allose is attached to the lactone ring. Also, dimethylation of 6-deoxy-D-allose after it is linked covalently to the macrolide ring might explain why only two TDP sugars containing [^{14}C]methyl derived from L-[*methyl*-^{14}C]methionine were detected in a tylosin-producing strain of *S. rimosus* (22). One TDP sugar was clearly TDP-mycarose, whereas the other may have been TDP-mycaminose.

The *tylF28* mutant produced little macrocin *O*-methyltransferase early in fermentation, but normal levels late in the fermentation. However, it did not produce appreciable amounts of tylosin. This mutant also accumulated quantities of demethylmacrocin, suggesting that it may be deficient in demethylmacrocin *O*-methyltransferase also. Further work to determine whether this mutant contains a mutation in a regulatory sequence controlling both enzymes awaits development of a quantitative assay for demethylmacrocin *O*-methyltransferase.

Most of the *tyl* mutants shared certain similarities, although three *tylG* mutants showed marked differences. All of the mutants other than the *tylG* mutants produced relatively high levels of intermediates or shunt metabolites, ranging from 35 to 100% of normal tylosin yields. The lower relative production of tylosin-like compounds by many *tyl* mutants may be due to second-site mutations resulting from high levels of MNNG mutagenesis (Baltz et al., unpublished data).

All of the mutants blocked only in biosynthesis or addition of tylosin sugars, in specific oxidations, or in tylactone formation produced normal or elevated levels of macrocin *O*-methyltransferase activity. Thus, it appears that no specific tylosin intermediate or shunt product is required in high concentrations to induce this enzyme. However, we cannot rule out the possibility that all of these mutants might produce trace amounts of an inducing tylosin-like compound. The *tylE* mutant (blocked in demethylmacrocin *O*-methyltransferase) produced twice the normal maximum specific activity of macrocin *O*-methyltransferase, even though this strain was incapable of using this enzyme unless macrocin was provided exogenously. The product of the *tylE* mutant, demethylmacrocin, could act as a specific stimulator of macrocin *O*-methyltransferase biosynthesis or could inhibit macrocin O-methyltransferase turnover, perhaps by binding to the enzyme (Seno and Baltz, in press).

The three *tylG* mutants (GS18, GS40, and GS41) which could not cosynthesize tylosin with any other blocked mutant and could not convert exogenously added tylactone or macrocin to tylosin did not produce detectable levels of macrocin *O*-methyltransferase.

Tylosin is reduced slowly to a less active antibiotic, relomycin (which contains C-20 hydroxymethyl instead of formyl), by *S. fradiae* during normal fermentations (26). This reaction is also carried out by a tylosin-producing strain of *S. hygroscopicus* (11) and by several other species of *Streptomyces* and *Nocardia* (6, 16). All of the blocked mutants capable of carrying out any steps in tylosin biosynthesis converted tylosin to relomycin very slowly. However, the three *tylG* mutants which were incapable of carrying out any tylosin biosynthetic steps in cofermentation, bioconversion, or in vitro O-methylation experiments converted tylosin to relomycin and macrocin to 20-dihydromacrocin very rapidly. Since these mutants could not carry out the reverse reaction (oxidation of relomycin to tylosin) or coferment tylosin with a mutant blocked in C-20 oxidation, it appears that tylosin aldehyde reductase is a separate enzyme and is not involved in tylosin biosynthesis.

All of the *tyl* mutants except GS40 were resistant to 400 μg of tylosin per ml in patch tests on AS-1 agar. GS40 was susceptible to very low levels of tylosin in a more quantitative efficiency-of-plating test. GS40 was also devoid of aerial mycelia and spore formation. These combined phenotypes were similar to certain variants of *Streptomyces riticulli* which have undergone plasmid rearrangements (25). The two other *tylG* mutants which did not cosynthesize tylosin with any other *tyl* mutants and produced no macrocin *O*-methyltransferase activities were resistant to tylosin in patch tests, and one produced aerial mycelia and spores. These atypical *tylG* mutants may contain substantial deletions of tylosin genes or mutations in regulatory sequences and are being characterized further.

The isolation and characterization of mutants blocked in tylosin biosynthesis have increased our understanding of the biosynthetic pathway of tylosin. However, the precise sequence of reactions after addition of mycaminose to tylactone have not been elucidated completely. We have used these mutants to probe the specific rates of particular bioconversions in more detail and will report these findings elsewhere (Seno et al., manuscript in preparation).

These mutants have been used to produce tylosin intermediates and shunt products which contain antibiotic activities. In turn, these compounds can be used as intermediates in bioconversion and chemical conversion reactions to produce novel antibiotics (23). The tylosin-blocked mutants are also being used in genetic mapping experiments by protoplast fusion and

conjugation (1, 2; R. H. Baltz, E. T. Seno, J. Stonesifer, P. Matsushima, and G. Wild, *in Microbiology—1981*, in press) and should aid in identifying potential tylosin operons which might be manipulated genetically by promoter mutations or by gene amplification by tandem duplication or gene cloning (23).

ACKNOWLEDGMENTS

We thank E. Gary for technical assistance, G. M. Wild for fruitful discussions and for providing purified tylosin intermediates and shunt metabolites, D. Dorman for structural determinations, D. A. Hopwood and J. W. Corcoran for comments, and J. Treadway for carefully typing the manuscript.

LITERATURE CITED

1. **Baltz, R. H.** 1978. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. J. Gen. Microbiol. **107**:93–102.
2. **Baltz, R. H.** 1980. Genetic recombination by protoplast fusion in *Streptomyces*. Dev. Ind. Microbiol. **21**:43–54.
3. **Cerdá-Olmedo, E., and R. Ruiz-Vázques.** 1979. Nitrosoguanidine mutagenesis, p. 15–20. *In* O. K. Sebek and A. I. Laskin (ed.), Genetics of industrial microorganisms. American Society for Microbiology, Washington, D.C.
4. **Delić, V., D. A. Hopwood, and E. J. Friend.** 1970. Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in *Streptomyces coelicolor*. Mutat. Res. **9**:167–182.
5. **Drake, J. W., and R. H. Baltz.** 1976. The biochemistry of mutagenesis. Annu. Rev. Biochem. **45**:11–37.
6. **Feldman, L. I., I. K. Dill, C. E. Holmlund, H. A. Whaley, E. L. Patterson, and N. Bohonas.** 1964. Microbial transformation of macrolide antibiotics, p. 54–57. Antimicrob. Agents Chemother. 1963.
7. **Furamai, T., K. Takeda, and M. Suzuki.** 1975. Studies on the biosynthesis of basic 16-membered macrolide antibiotics, platenomycins. IV. Biosynthesis of platenomycins. J. Antibiot. **28**:789–797.
8. **Gersch, D., H. Bocker, and H. Thrum.** 1977. Biosynthetic studies on the macrolide antibiotic turimycin using ^{14}C-labeled precursors. J. Antibiot. **30**:488–493.
9. **Grisebach, H.** 1978. Biosynthesis of macrolide antibiotics, p. 113–127. *In* R. Hütter, T. Leisinger, J. Nüesch, and W. Wehrli (ed.), Antibiotics and other secondary metabolites. Biosynthesis and production. Academic Press, Inc., London.
10. **Hamill, R. L., and W. M. Stark.** 1964. Macrocin, a new antibiotic, and lactenocin, an active degradation product. J. Antibiot. **17**:133–139.
11. **Jensen, A. L., M. A. Darken, J. S. Schultz, and A. J. Shay.** 1964. Relomycin: flask and tank fermentation studies, p. 49–53. Antimicrob. Agents Chemother. 1963.
12. **Matern, H., G. U. Brillinger, and H. Pape.** 1973. Metabolic products of microorganisms. 114. Thymidine diphospho-D-glucose oxidoreductase from *Streptomyces rimosus*. Arch. Microbiol. **88**:37–48.
13. **McGuire, J. M., W. S. Boniece, C. E. Higgens, M. M. Hoehn, W. M. Stark, J. Westhead, and R. N. Wolfe.** 1961. Tylosin, a new antibiotic. I. Microbiological studies. Antibiot. Chemother. **11**:320–327.
14. **Morin, R. B., and M. Gorman.** 1964. The partial structure of tylosin, a macrolide antibiotic. Tetrahedron Lett. **34**:2339–2345.
15. **Morin, R. B., M. Gorman, R. L. Hamill, and P. V. Demarco.** 1970. The structure of tylosin. Tetrahedron Lett. **54**:4737–4740.
16. **Nakahama, K., and S. Igarasi.** 1974. Microbial conversion of antibiotics. IV. Reduction of maridomycin. J. Antibiot. **27**:605–609.
17. **Ōmura, S., C. Kitao, J. Miyazawa, H. Imai, and H. Takeshima.** 1978. Bioconversion and biosynthesis of 16-membered macrolide antibiotic, tylosin, using enzyme inhibitor: cerulenin. J. Antibiot. **31**:254–256.
18. **Ōmura, S., and A. Nakagawa.** 1975. Chemical and biological studies on 16-membered macrolide antibiotics. J. Antibiot. **28**:401–433.
19. **Ōmura, S., A. Nakagawa, H. Takeshima, J. Miyazawa, C. Kitao, F. Piriou, and G. Lukacs.** 1975. A ^{13}C nuclear magnetic resonance study of the biosynthesis of the 16-membered macrolide antibiotic tylosin. Tetrahedron Lett. **50**:4503–4506.
20. **Ōmura, S., H. Takeshima, A. Nakagawa, J. Miyazawa, F. Piriou, and G. Lakacs.** 1977. Studies on the biosynthesis of 16-membered macrolide antibiotics using carbon-13 nuclear magnetic resonance spectroscopy. Biochemistry **16**:2860–2866.
21. **Ono, H., S. Harada, and T. Kishi.** 1974. Maridomycin, a new macrolide antibiotic. VII. Incorporation of labeled precursors into maridomycin and preparation of ^{14}C-labeled 9-propionylmaridomycin. J. Antibiot. **27**:442–448.
22. **Pape, H., and G. U. Brillinger.** 1973. Metabolic products of microorganisms. 113. Biosynthesis of thymidine diphospho mycarose in a cell-free system from *Streptomyces rimosus*. Arch. Microbiol. **88**:25–35.
23. **Queener, S. W., and R. H. Baltz.** 1979. Genetics of industrial microorganisms. Annu. Rep. Ferment. Processes **3**:5–45.
24. **Randazzo, R., G. Sciandrello, A. Carere, M. Bignami, A. Velcici, and G. Sermonti.** 1976. Localized mutagenesis in *Streptomyces coelicolor* A3(2). Mutant. Res. **36**:291–302.
25. **Schrempf, H., and W. Goebel.** 1979. Functions of plasmid genes in *Streptomyces riticuli*, p. 259–268. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier, New York.
26. **Seno, E. T., R. L. Pieper, and F. M. Huber.** 1977. Terminal stages in the biosynthesis of tylosin. Antimicrob. Agents Chemother. **11**:455–461.