Essential Role of Endogenously Synthesized Tylosin for Induction of *ermSF* in *Streptomyces fradiae*

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We compared *ermSF* induction in wild-type *Streptomyces fradiae* NRRL B-2702 and that in GS-14, a *tylA* mutant which cannot synthesize tylosin. Our findings suggest that (i) endogenously synthesized tylosin plays an obligatory role in *ermSF* induction and (ii) tylosin, or a biosynthetic intermediate beyond tylactone, has an "autocrine" function that induces *ermSF* synthesis, thereby enabling *S. fradiae* to resist higher levels of tylosin.

*Streptomyces fradiae* NRRL B-2702 produces the 16-membered ring macrolide antibiotic tylosin and possesses four genes which confer tylosin resistance, namely, *tlrA* (synonymous with *ermSF*), *tlrB*, *tlrC*, and *tlrD* (3, 5, 6, 10, 12). These genes specify, respectively, the following products: (i) TlrA (ErmSF), a 23S rRNA adenine N°,N°-dimethyltransferase which is expressed inducibly; (ii) TlrB, a product with an unknown function that is presumed to be constitutively expressed; (iii) TlrC, a tylosin efflux ATPase that is presumed to be constitutively expressed; and (iv) TlrD, a 23S rRNA adenine N°-monomethyltransferase which is constitutively expressed.

Resistance mediated by the methylating enzymes TlrA and TlrD, which act on 23S rRNA, results from the successive modification of adenine to form, first, N°-monomethyladenine (by TlrD) and then N°,N°-dimethyladenine (by TlrA, ErmSF) at a site in the peptidyl transferase cleft which corresponds to Escherichia coli coordinate A2058 (6, 7). The constitutive monomethylating activity, catalyzed by TlrD, confers low-level macrolide resistance, while the second methylation, catalyzed by TlrA, leads to a higher level of resistance (6). As a consequence of methylation, the ribosome binds macrolides, lincosamides, and streptogramin type B antibiotics with reduced affinity. (For recent reviews of the *erm* gene family and its relationship to induced macrolide-lincosamide-streptogramin B resistance, see references 8, 13, and 14.)

In a previous study (4), we noted the presence of both N°-monomethyl and dimethyl adenine in 23S rRNA from *S. fradiae* NRRL B-2702 grown to the late logarithmic phase in the absence of added antibiotic. Since *ermSF* has been shown to be inducible (5, 6), we inferred that endogenously synthesized tylosin may act as an inducer. To test this hypothesis, we measured both the concentration of tylosin in the medium and expression of *ermSF* to determine whether a connection between the two could be established.

Tylosin production by *S. fradiae* NRRL B-2702 was measured as a function of the age of the culture. A 5% inoculum of *S. fradiae* NRRL B-2702 mycelium from a 3-day culture in LB medium (11) was transferred to 50 ml of fresh LB medium in 250-ml shaker flasks and incubated on a rotary shaker at 30°C. Samples of the culture were collected on days 1, 2, 4, and 6 and clarified by centrifugation, and 100 μl was loaded into wells for bioassay of antibiotic activity by the well diffusion method by using *Bacillus subtilis* 168 as the indicator lawn.

Solutions of tylosin tartrate containing the concentrations indicated in Fig. 1 (100 μl) were tested in parallel.

A maximum level of tylosin production was reached on day 4, at which time the growth-inhibitory activity of the medium was equivalent to 20 to 30 μg of tylosin/ml, based on an agar well bioassay, as shown in Fig. 1a and b. For each of the samples taken, the pH of the fermentation broth was adjusted to 9.0 by addition of a 1 M Tris OH solution, and the alkalized broth was extracted with an equal volume of ethyl acetate. A sample of the ethyl acetate phase was analyzed by thin-layer chromatography on silicic acid using a solvent system based on that of Betina (2) which contained benzene-chloroform (65:35 [vol/vol]). The *R* _f_ for tylosin in this system was approximately 0.5. Antibiotic activity in the ethyl acetate phase was demonstrated by bioautography with a *B. subtilis* 168 indicator overlaid on the thin-layer plate. The inhibitory activities of both the tylosin standard and the fermentation beer had the same mobility (data not shown).

The time course of the methylase concentration was determined as a function of the age of the culture. Cultures of wild-type *S. fradiae* NRRL B-2702 and a *tylA* mutant which cannot synthesize tylosin due to a block between tylactone and 20-dihydroxy-23-deoxy-O-mycaminosyl-tylolinide (1) were tested. A 1% inoculum of *S. fradiae* NRRL B-2702 mycelium from a 3-day culture in LB medium was transferred to 100 ml of fresh LB medium in a 250-ml shaker flask and incubated on a rotary shaker at 30°C. Samples were collected on days 1, 2, 4, and 6 and stored frozen at –70°C until analyzed by the immunoblot method (11). Frozen mycelium was thawed and treated with lysozyme (1 mg/g of cell paste), and from the resultant digest, 120 μg of protein per lane was fractionated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunoreactive protein was allowed to react successively with polyclonal anti-ErmSF antibodies raised in rabbits (primary antibody), with a polyclonal goat anti-rabbit alkaline phosphatase conjugate (secondary antibody), and finally with the phosphatase chromogenic substrate combination 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium. The experimental conditions used for gel lanes 1–10 of Fig. 2 are summarized in Table 1, which also includes a quantification of the relative concentrations of ErmSF as a function of fermentation time. The results are shown in Fig. 2a, lanes 1 to 10, and summarized in Table 1. Increased methylase protein in NRRL 2883 was detectable on day 4, and a higher level was seen on day 6. None was detectable in GS-14.

To determine the inducibility of *ermSF* in response to exogenously added tylosin, 1% inocula of *S. fradiae* NRRL B-2702 and GS-14 mycelium were prepared as described above. On 
day 2, tylosin was added to the cultures to a concentration of 0, 5, or 10 µg/ml, as indicated in the legend to Fig. 2, and incubation was continued for an additional day. Samples were collected on day 3 and stored frozen at −70°C until analyzed by the immunoblot method. The experimental conditions used for gel lanes 11 to 18 of Fig. 2 are summarized in Table 1, which also includes a quantification of the relative concentrations of ErmSF induced by addition of exogenous tylosin to the growth medium. The results shown in Fig. 2b, lanes 11 to 18, and summarized quantitatively in Table 1 indicate tylosin dose-dependent synthesis of ErmSF methylase in both strains. Thus, the failure of strain GS-14 to synthesize ErmSF spontaneously during its normal cycle of growth cannot be ascribed to its failure to respond to tylosin.

Our findings suggest an obligatory role for endogenously synthesized tylosin in *S. fradiae* in the controlled expression of *ermSF*, which, together with products of the genes *tlrB*, *tlrC*, and *tlrD*, contributes to the complete pattern of resistance that this organism develops during the progress of tylosin fermentation. We can integrate these findings with those reported previously (6, 9) as follows. During the earliest stage of antibiotic production, *tlrC* and *tlrD*, and possibly *tlrB*, are expressed constitutively and provide a low but sufficient level of resistance that allows the organism to survive the low concentrations which it initially produces. As higher concentrations of tylosin are produced by *S. fradiae*, the partial susceptibility of its ribosomes to tylosin allows them to stall while synthesizing the *ermSF* leader peptide and thus trigger induction of *ermSF*. The resultant activation of *ermSF* increases the cellular level of dimethylated adenine in 23S rRNA and makes the ribosomes maximally resistant to tylosin.

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**TABLE 1. Induction of *ermSF* as a function of time and the concentration of tylosin added to the culture**

| Lane | Sample | Experimental condition | Relative ErmSF concn (%) |
|------|--------|------------------------|--------------------------|
| 1    | *S. fradiae* NRRL 2883 | Day 1                 | <100                     |
| 2    | *S. fradiae* NRRL 2883 | Day 2                 | <100                     |
| 3    | *S. fradiae* NRRL 2883 | Day 4                 | 223                      |
| 4    | *S. fradiae* NRRL 2883 | Day 6                 | 929                      |
| 5    | *S. fradiae* GS-14     | Day 1                 | <100                     |
| 6    | *S. fradiae* GS-14     | Day 2                 | <100                     |
| 7    | *S. fradiae* GS-14     | Day 4                 | <100                     |
| 8    | *S. fradiae* GS-14     | Day 6                 | <100                     |
| 9    | *E. coli*(pET23b)     | Negative control      |                          |
| 10   | *E. coli*(pHJJ103)    | Positive control and marker |                      |
| 11   | *S. fradiae* NRRL 2883 | 0 µg of tylosin/ml | <100                     |
| 12   | *S. fradiae* NRRL 2883 | 5 µg of tylosin/ml | 693                      |
| 13   | *S. fradiae* NRRL 2883 | 10 µg of tylosin/ml | 2,521                    |
| 14   | *S. fradiae* GS-14     | 0 µg of tylosin/ml | 217                      |
| 15   | *S. fradiae* GS-14     | 5 µg of tylosin/ml | 708                      |
| 16   | *S. fradiae* GS-14     | 10 µg of tylosin/ml | 1,314                    |
| 17   | *E. coli*(pET23b)     | Same as lane 9       |                          |
| 18   | *E. coli*(pHJJ103)    | Same as lane 10      |                          |

*S. fradiae* extracts were fractionated by polyacrylamide gel electrophoresis as described in the legend to Fig. 2.
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