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The tylosin resistance gene *tlrB* of *Streptomyces fradiae* encodes a methyltransferase that targets G748 in 23S rRNA

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Summary

***tlrB* is one of four resistance genes encoded in the operon for biosynthesis of the macrolide tylosin in antibiotic-producing strains of *Streptomyces fradiae*. Introduction of *tlrB* into *Streptomyces lividans* similarly confers tylosin resistance. Biochemical analysis of the rRNA from the two *Streptomyces* species indicates that *in vivo* TlrB modifies nucleotide G748 within helix 35 of 23S rRNA. Purified recombinant TlrB retains its activity and specificity *in vitro* and modifies G748 in 23S rRNA as well as in a 74 nucleotide RNA containing helix 35 and surrounding structures. Modification is dependent on the presence of the methyl group donor, S-adenosyl methionine. Analysis of the 74-mer RNA substrate by biochemical and mass spectrometric methods shows that TlrB adds a single methyl group to the base of G748. Homologues of TlrB in other bacteria have been revealed through database searches, indicating that TlrB is the first member to be described in a new subclass of rRNA methyltransferases that are implicated in macrolide drug resistance.**

Introduction

Tylosin is a 16-membered ring macrolide antibiotic that is widely used in veterinary medicine and as a growth promoter in livestock. Similar to other macrolide drugs, tylosin inhibits bacterial protein synthesis by binding to the large (50S) ribosomal subunit at, or close to, the site of peptide bond formation (reviewed by Vázquez, 1979; Gale *et al*., 1981; Bryskier *et al*., 1993). The main site of macrolide contact is with 23S rRNA adenosine 2058 (A2058) and adjacent nucleotides, which are situated within the peptidyl transferase loop of domain V of the rRNA (Moazed and Noller, 1987). Recent evidence on the tertiary folding of 23S rRNA indicates that the spatial position of the peptidyl transferase loop is close to that of hairpin 35 within domain II of the rRNA (Gutell, 1996; Baranov *et al*., 1998). The proximity of these two regions is further emphasized by chemical protection studies on a group of 14-membered ring macrolides and ketolide derivatives, which show that these drugs simultaneously interact with A2058 in domain V and with A752 in hairpin 35 of domain II (Hansen *et al*., 1999a; Xiong *et al*., 1999).

Tylosin is a natural product of secondary metabolism in some strains of the actinomycete *Streptomyces fradiae*. Drug-producing strains of *S. fradiae* encode four distinct resistance genes that enable the bacterium to avoid inhibition of its own ribosomes. *tlrA* (*ermSF*) and *tlrD* belong to the *erm* family of methyltransferases and have recently been reclassified as *ermS* and *ermN* respectively (Roberts *et al*., 1999). TlrD (ErmN) is constitutively expressed and adds a single methyl group at the N6 position of A2058 (Zalacain and Cundliffe, 1991; Gandecha and Cundliffe, 1996). Monomethylation of A2058 confers high-level resistance to lincosamide antibiotics, but lower level resistance to macrolides and streptogramin B antibiotics (Weisblum, 1995). The expression of *tlrA* is induced by endogenously synthesized tylosin (Memili and Weisblum, 1997). TlrA (ErmS) dimethylates the N6 of A2058, either by adding an extra methyl group to the monomethylated residue or by directly dimethylating the unmodified residue (Kamimiya and Weisblum, 1988; Zalacain and Cundliffe, 1989; Kelemen *et al*., 1994). Dimethylation at A2058 confers the so-called MLS resistance phenotype, characterized by high resistance to all macrolide, lincosamide and streptogramin B antibiotics (Weisblum, 1995).

The other two resistance genes, *tlrB* and *tlrC*, flank the tylosin synthesis gene cluster (*tyl*), which spans 85 kb and contains 43 genes (Baltz and Seno, 1988; Bate *et al*., 1999). The N- and C-terminal halves of TlrC are extensively homologous to one another, suggesting that tandem duplication was involved in the evolution of the gene. TlrC shows similarity to many prokaryotic and eukaryotic membrane proteins, with highest levels of homology in the ATP-binding domains, suggesting that it

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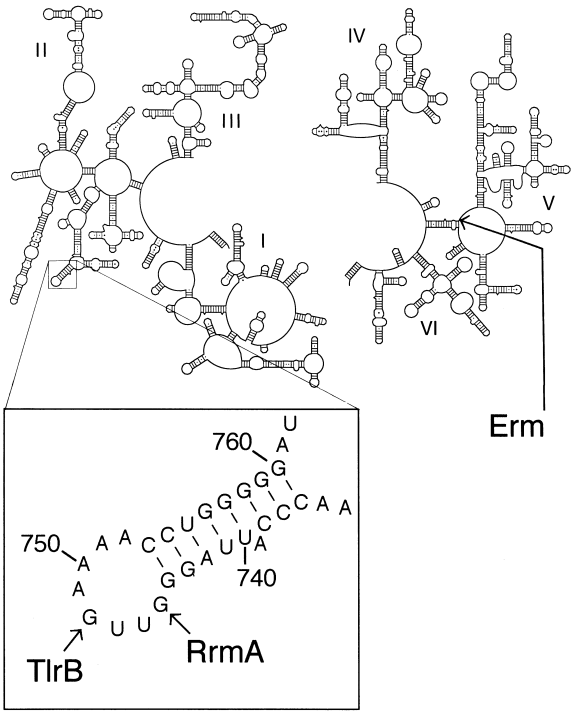


Fig. 1. Schematic representation of the secondary structure of bacterial 23S rRNA (Noller, 1984; Gutell *et al*., 1994). The locations of the six rRNA domains are labelled. The target nucleotides for methylation by RrmA (at G745) and TlrB (at G748) are shown in the enlarged segment of helix 35 in domain II. The sequence of helix 35 is that of *S. lividans*, as deduced from the gels in Fig. 2. Also indicated is the methylation site of Erm (the methyltransferase family to which TlrA and TlrD belong) at A2058 in domain V of the rRNA.

may be part of an ATP-dependent system for tylosin efflux (Rosteck *et al*., 1991; Schoner *et al*., 1992).

The remaining resistance gene, *tlrB*, was recently sequenced independently by two groups (Fouces *et al*., 1999; Wilson and Cundliffe, 1999) and shows significant similarity to *myrA*, a resistance determinant found in *Micromonospora griseorubida*, the actinomycete that produces the macrolide mycinamicin. Slightly lower, but nevertheless significant, similarity has also been noted to an *Escherichia coli* open reading frame (ORF30) (Wilson and Cundliffe, 1999). Conserved S-adenosyl-methionine (SAM) binding motifs are clearly distinguishable in TlrB, MyrA and the ORF30 protein, suggesting that they, like Erm, are methyltransferases. However, the mode of action of TlrB is distinctly different from that of Erm because, although *tlrB* confers tylosin resistance, it offers no resistance to erythromycin or lincomycin (Fouces *et al*., 1999; Wilson and Cundliffe, 1999). Recombinant TlrB was recently reported to inactivate tylosin *in vitro*, supposedly by methylation of the drug (Wilson and Cundliffe, 1999). However, the putative methylated product could be neither isolated nor characterized.

In independent studies, the TlrB homologue ORF30 has now been conclusively identified as the SAM-dependent methyltransferase RrmA, which methylates the N1 position of G745 in *E. coli* 23S rRNA (Gustafsson and Persson, 1998). Nucleotide G745 is located in hairpin 35, where it potentially interacts with A752 at the end of the hairpin stem structure (Fig. 1; Noller, 1984; Gutell *et al*., 1994). As position A752 is strongly implicated in playing a role in macrolide binding (Hansen *et al*., 1999a; Xiong *et al*., 1999), it became intriguingly apparent that TlrB might confer tylosin resistance by methylating a nucleotide that serves as part of the drug binding site within the loop of hairpin 35.

In this study, we present biochemical, genetic and mass spectrometric evidence that *tlrB* encodes an RNA methyltransferase that specifically monomethylates the base of nucleotide G748 in 23S rRNA. This is the first report of nucleotide G748 being a target for enzymatic methylation and links this nucleotide with a significant role in tylosin binding and resistance.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference
Strains		
E. coli TG1	*supE hsd*5 *thi* (*lac-proAB*) F′[*traD*36*proAB*$^+$ *lacI*^{q} *lacZDM15*]	Sambrook *et al.* (1989)
E. coli ET12567	*dam, dcm* strain for vector transfer	MacNeil *et al.* (1992)
S. fradiae 19609	Wild type, tylosin producer	Lee and Lee (1993)
S. lividans 1326	Wild type	Hopwood *et al.* (1985)
Plasmids		
pUHE24-2	Cloning/expression vector in *E. coli*, Amp^r.	Bujard *et al.* (1987)
pHM10a	Contains P_E and synthetic RBS; conjugative vector in *Streptomyces* and extrachromosomal in *E. coli*; Hyg^r and Amp^r	Motamedi *et al.* (1995)
pHJL401	Shuttle vector between *Streptomyces and E. coli*; Ts^r and Amp^r; lacks P_E	Larson and Hershberger (1986)
pSD14c	*tlrB* gene cloned in pUHE24-2; Amp^r.	This study
pSD24	*tlrB* cloned in pHM10a under P_E, Hyg^r and Amp^r	This study
pSD44	*tlrB* (including P_E) cloned in pHJL401; Ts^r and Amp^r	This study

Amp^r, ampicillin resistant; Hyg^r, hygromycin B resistant; Kan^r, kanamycin resistant; Ts^r, thiostrepton resistant; RBS, Shine–Dalgarno ribosome binding site.

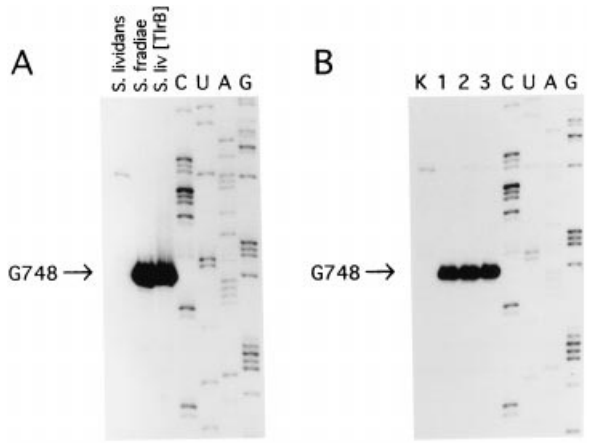


Fig. 2. TlrB methylation of *S. lividans* 23S rRNA. Autoradiographs of sequencing gels showing reverse transcriptase extension on rRNA from the mf11 primer.
A. Analysis of *in vivo* rRNA methylation in *S. fradiae* and in *S. lividans* harbouring plasmid pSD44 (S. liv [TlrB]); the control rRNA sample (left lane) was from *S. lividans* cells without *tlrB* (harbouring pHJL401). Most of the radiolabelling in the control sample was in longer run-off transcripts at the top of the gel (not shown).
B. Analysis of *S. lividans* rRNA after incubation *in vitro* with TlrB methyltransferase. Lane K, no SAM added to the reaction; lanes 1–3, incubated with 2 mM SAM. TlrB was isolated from *E. coli*/pSD14c (lane 1) or from *S. lividans*/pSD44 and lysed in imidazole–lysozyme buffer or in protoplast buffer (lanes 2 and 3 respectively). Dideoxy sequencing reactions (lanes C, U, A and G) were performed on an unmodified *S. lividans* rRNA template.

Results

TlrB confers tylosin resistance in Streptomyces lividans

The *tlrB* gene was amplified by polymerase chain reaction (PCR) from the chromosomal DNA of a tylosin-producing strain of *S. fradiae*. The primers used in the PCR reaction result in the addition of an extra sequence to the end of the *tlrB* gene, which encodes a tail of six histidines at the C-terminus of the recombinant protein, enabling rapid purification of TlrB from cell lysates by Ni-affinity chromatography. The gene was inserted into an *E. coli* expression vector to form plasmid pSD14c. Most *E. coli* strains are refractory to tylosin, so the phenotype conferred by *tlrB* was determined by placing the gene under the control of the constitutive *ermE* promoter in the shuttle vector pHJL401 (generating plasmid pSD44, Table 1) and expressing TlrB in *S. lividans.* The minimal inhibitory concentration (MIC) of tylosin for spores of *S. lividans* pSD44 transformants was 250 μg tylosin ml^{-1}. The control spores of *S. lividans* harbouring pHJL401 had a MIC of 32–64 μg tylosin ml^{-1}.

Purification of TlrB

TlrB with the histidine tag was produced in *E. coli* and *S. lividans* and purified by Ni-affinity chromatography. SDS–PAGE showed that the recombinant TlrB protein was approximately 85% pure when isolated from *E. coli* cells harbouring pSD14c, and over 95% pure when isolated from *S. lividans* harbouring pSD44. The protein has a molecular weight of approximately 32 kDa, which, including approximately 1 kDa for the histidine tail, is consistent with the expected value (data not shown). Recombinant TlrB was also constructed with an N-terminal histidine tag and was expressed in *S. lividans*. The substrate specificity of TlrB (described below) was tested *in vivo* and was unaffected by the position of the tag (data not shown). *In vitro* studies were continued with the C-terminal-tagged protein.

In vivo *modification of* Streptomyces *23S rRNA by TlrB*

RNA was isolated from *S. lividans* with or without plasmid pSD44, and the entire 23S rRNA sequence was screened by reverse transcriptase using 14 primers evenly spaced throughout the rRNA sequence. The only detectable difference in the 23S rRNAs from cells with or without TlrB was found using primer mf11. There was a complete stop of transcription at position 749 (*E. coli* numbering) on rRNA from *S. lividans* containing pSD44, whereas there was no such stop in the control sample from *S. lividans* without this plasmid (Fig. 2A). The reverse transcriptase stop was interpreted as being caused by modification of residue G748 by TlrB. 23S rRNA from *S. fradiae*, which contains its own endogenous TlrB, was screened from the same primers. The *S. fradiae* 23S rRNA also showed a complete stop of transcription at position 749 (Fig. 2A). These data indicate that TlrB was responsible for altering the 23S rRNA structure at G748 in a manner that blocks reverse transcriptase extension. Additional data were collected to identify the type of alteration introduced at G748.

In vitro *methylation of* S. lividans *23S rRNA and the 74-mer RNA*

Unmodified *S. lividans* 23S rRNA was incubated *in vitro* with recombinant TlrB in the presence of the methyl donor SAM. Analysis of the rRNA, from the same set of primers used for the *in vivo*-modified rRNA, similarly showed a complete stop in reverse transcriptase extension at position 749 (Fig. 2B), consistent with a modification at G748. The modification at G748 is dependent on the presence of SAM in the incubation mixture, which is consistent with this modification being a methylation. The origin of purified TlrB (from *E. coli* or *S. lividans*), or its method of purification, did not affect the specificity of the enzyme *in vitro* (Fig. 2B). No other new stops were evident at other 23S rRNA positions

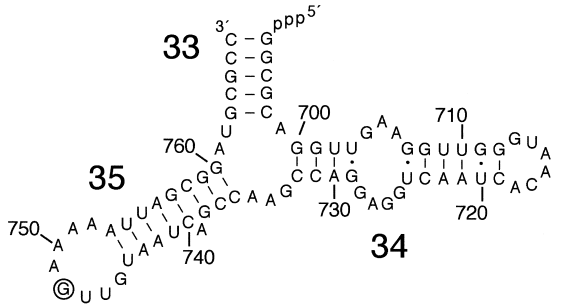
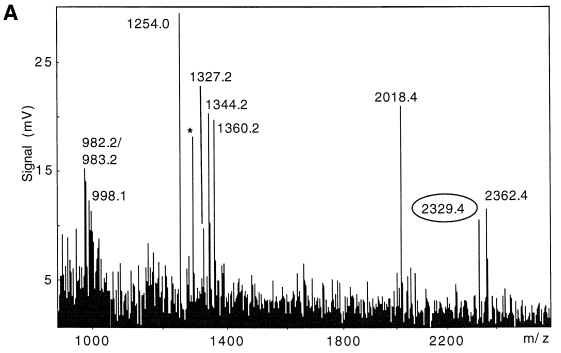


Fig. 3. Structure of the 74-mer RNA synthesized by *in vitro* transcription. The structure of the RNA corresponds to hairpins 34 and 35 and a portion of helix 33 in *E. coli* 23S rRNA. The basepairing in helix 33 at the 3′- and 5′-termini of the transcript has been altered from the *E. coli* sequence to increase the stability of the secondary structure. The site of TlrB methylation at G748 is encircled.

A



B

Nucleotide position	Mass/charge (m/z)	Sequence
715-717	982.2	AAC
721-723	982.2	AAC
742-744	983.2	AAU
738-740	998.2	GAC
756-758	998.2	AGC
694- 696	1254.1	GGC
733-736	1327.2	GAAC
699-702	1344.2	AGGU
759-762	1344.2	GGAU
711-714	1360.2	GGGU
704-709	2018.3	GAAGGU
748-754	2315.4	GAAAAAU
725-731	2362.4	GGAGGAC

Fig. 4. A. MALDI time-of-flight mass spectrum of the 74-mer RNA after methylation by TlrB and digestion with RNase A. The empirically determined mass/charge (m/z) ratios of the fragments are indicated. The fragment at m/z 2329.4 corresponds to GAAAAAU with a methyl group. The residual unmethylated fragment at m/z 2315.4 disappears into the background noise after TlrB treatment. The fragment at m/z 1254.0 contains the 5′-triphosphate and is therefore prone to form a potassium salt (marked *).
B. Table of the fragments predicted after RNase A digestion of the unmodified 74-mer RNA (only trinucleotides and larger are shown). The m/z ratios are calculated for singly protonated fragments that have masses increased by 1.0 Da in comparison with neutral fragments.

compared with control rRNA that had not been treated with TlrB.

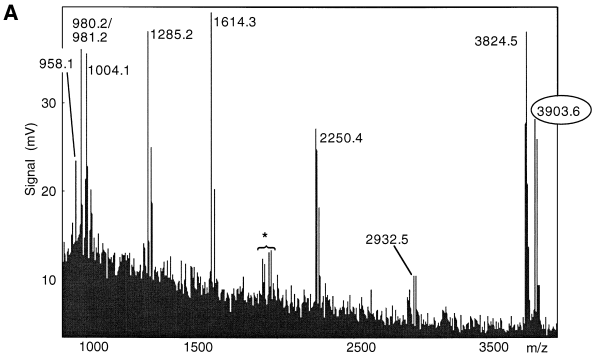
Some questions concerning the type of modification remained to be clarified. First, the formal possibility remained that the TlrB-specific reverse transcriptase stop could be caused by some other mechanism, such as hydrolysis of the rRNA chain between nucleotides 748 and 749. Secondly, if the modification proved to be a methylation (as the *in vitro* SAM dependence data suggested), then we needed to ascertain how many methyl groups were added to G748. These questions were answered unequivocally using a smaller RNA substrate for the enzyme and analysing the reaction product by both reverse transcriptase extension and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

A truncated 74 nucleotide RNA, corresponding to the *E. coli* 23S rRNA sequence spanning helix 35 and adjacent structures, was transcribed *in vitro* (Fig. 3). Reverse transcriptase extension analysis of the 74-mer after treatment with recombinant TlrB showed a $>80\%$ stop of transcription at the position equivalent to 749 (data not shown). Thus, TlrB exhibits identical specificity *in vitro* for the truncated RNA substrate of 74 nucleotides as it does *in vivo* and *in vitro* for the complete *Streptomyces* 23S rRNA.

MALDI-MS analysis of RNA

The exact position and the number of methyl groups added to the RNA by TlrB were determined by MALDI-MS. Sets of well-defined RNA fragments were generated by complete RNase A or RNase T1 digestion of the 74-mer RNA transcript. The addition of methyl or other chemical groups to the RNA alters the masses of one or more RNase digestion fragments within a set. Figure 4B displays the expected fragment sizes derived from RNase A digestion of unmodified 74-mer RNA. The observed masses of RNase fragments generated from RNA untreated with TlrB perfectly matched these expected sizes (data not shown). However, after TlrB treatment, the fragment 5′-GAAAAAU-3′ at the expected m/z 2315.4 was only present as a minor signal. At the same time, a new and stronger signal appeared at m/z 2329.4 (Fig. 4A). This increase in mass by 14 Da strongly suggests that the fragment covering positions 748–754 is modified with a single methyl group (theoretical value plus 14.0 m/z). The degree of *in vitro* methylation judged from Fig. 4A is over 75%, which is in good agreement with the results obtained by primer extension analysis. It should be noted, however, that the strength of the MS method lies in its ability accurately to determine molecular masses, which are defined by the position of the spectrum peaks. Using the peak height to quantify relative amounts

A



B

Nucleotide position	Mass/charge (m/z)	Sequence
702-704	958.1	UUG
709-711	958.1	UUG
746-748	958.1	UUG
698-700	980.2	CAG
761-763	981.2	AUG
705-707	1004.2	AAG
730-733	1285.2	ACCG
734-738	1614.2	AACCG
739-745	2250.3	ACUAAUG
749-757	2932.4	AAAAAUUAG
714-725	3824.5	UAACACUAACUG

Fig. 5. A. MALDI time-of-flight mass spectrum of the 74-mer RNA after methylation by TlrB and digestion with RNase T1. The empirically determined mass/charge (m/z) ratios of the fragments are indicated. As discussed in the text, the predominant fragments have 2′−3′ cyclic phosphates and appear in the spectra 18 Da before the corresponding fragment with a linear phosphate. The fragment AAAAAUUAG (positions 749–757) at m/z 2932.5 becomes much reduced after TlrB modification, and a new stronger signal appears at m/z 3903.6. This new peak corresponds to a composite fragment with the sequence UUG_{Me}AAAAAUUAG (positions 746–757). The minor peaks (marked *) are doubly charged versions of the 3824.5 and 3903.6 fragments.
B. Table of the fragments predicted after RNase T1 digestion of the unmodified 74-mer RNA (only trinucleotides and larger, with 2′−3′ cyclic phosphates, are shown). The m/z ratios are calculated as for Fig. 4B.

of fragments is a somewhat more capricious process (Kirpekar *et al.*, 2000) and is generally not as reliable as the reverse transcriptase technique.

The position of the methylated nucleotide was pinpointed by RNase T1 digestion analysis of the methylated 74-mer RNA. RNase T1 digestion resulted predominantly in fragments with 2′−3′ cyclic phosphates, as noted previously (Kirpekar *et al.*, 2000). The fragment AAAAAUUAG (positions 749–757) at m/z 2932.5 becomes much reduced after TlrB modification and was replaced by a signal at m/z 3903.6 (Fig. 5A). This is consistent with the presence of a single methylation on G748, which renders this position insensitive to RNase T1 cleavage and results in a composite fragment with the sequence UUG_{Me}AAAAAUUAG (positions 746–757) and a theoretical m/z of 3903.5.

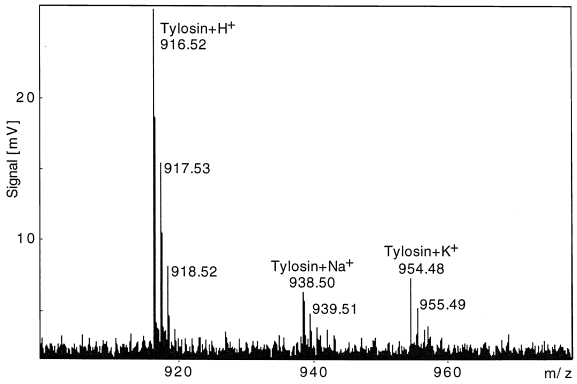


Fig. 6. MALDI time-of-flight mass spectrum of tylosin after incubation with TlrB and SAM. The major peak corresponds to the expected mass of unmodified tylosin (915.52 Da) plus a single proton. The distribution of natural carbon isotopes is evident in peaks of tylosin with one (917.53) and two (918.52) ^{13}C atoms. Sodium and potassium adducts of the drug are also clearly resolved. In comparison with the untreated tylosin control (not shown), no new peaks indicating drug modification or degradation are evident.

Analyses for tylosin modification

Tylosin was incubated *in vitro* with TlrB under conditions described previously to inactivate the drug (Wilson and Cundliffe, 1999). Parallel incubations without TlrB were performed as controls. The reactions were analysed directly by MALDI-MS with 3-hydroxypicolinic acid, a 2,3,4-/2,4,6-trihydroxyacetophenone mixture or α-cyano 4-hydroxycinnamic acid as matrix. Figure 6 shows the outcome of an analysis using the 2,3,4-/2,4,6-trihydroxyacetophenone mixture, after incubation of tylosin with TlrB. Tylosin gives a clear signal at the m/z of 916.52, which is the signal expected for unmodified drug, and sodium and potassium salts of unmodified tylosin are also distinct. There was no evidence of tylosin methylation (plus 14 Da or multiples thereof) or of new signals at lower m/z that could suggest drug breakdown. Analysis with the other two matrices yielded qualitatively identical results.

Tylosin was additionally analysed by a microbiological method after incubation of the drug with TlrB under conditions described previously (Wilson and Cundliffe, 1999), and also under the conditions described here as being effective for RNA methylation. Disk analyses of tylosin treated with TlrB showed no evidence of drug inactivation (data not shown).

Discussion

The *tlrB* gene from *S. fradiae* was cloned, and its function was studied in *S. lividans*. When expressed from the *ermE* promoter, *tlrB* confers moderated levels of resistance to the macrolide antibiotic tylosin. The resistance phenotype conferred in this study is similar to that

reported in other studies of *tlrB* in *S. lividans* (Fouces *et al*., 1999; Wilson and Cundliffe, 1999).

The 280-amino-acid sequence of TlrB was used in an NCBI database BLAST search (Altschul *et al*., 1997) to identify homologous proteins. Four sequences were initially identified as having significant homology with the TlrB protein. The product of *myrA*, a macrolide resistance gene from *Micromonospora griseorubida* (Inouye *et al*., 1994), shows the highest sequence identity (53%). The three other sequences display lower sequence identities: YxjB from *Bacillus subtilis* (28% identity); RrmA (ORF30) from *E. coli* (29%); and an open reading frame (ORF) from the *Corynebacterium glutamicum* plasmid, pAG1 (37%). YxjB and the pAG1 ORF have not previously been attributed a specific function. RrmA, on the other hand, has been defined as the methyltransferase responsible for N1 methylation of G745 in *E. coli* 23S rRNA (Isaksson, 1970; Gustafsson and Persson, 1998).

Analyses of *S. lividans* 23S rRNA by reverse transcriptase primer extension showed that TlrB specifically modifies position G748 within the rRNA of exponentially growing cells. Studies on the rRNA from *S. fradiae*, in which *tlrB* is endogenously expressed, revealed a modification at the same position. To identify the nature of the rRNA modification, recombinant TlrB was overexpressed in *E. coli* and purified to near homogeneity. Purification of the enzyme and substrate enabled us to study the reaction under controlled conditions *in vitro*. The recombinant protein is active *in vitro* and maintains its specificity for position G748 in *S. lividans* 23S rRNA. Modification of G748 by TlrB is dependent on the presence of the methyl group donor, SAM, strongly suggesting that nucleoside methylation is occurring.

However, the formal possibility still existed that the reverse transcriptase stop could result from a mechanism other than methylation (such as hydrolysis of the phosphodiester bond between 748 and 749 or induction of a stable RNA secondary structure). The function of TlrB was therefore assessed by independent techniques. Fragments of the 23S rRNA sequence spanning helix 35 and adjacent structures were transcribed *in vitro*, and the ability of TlrB to modify these was assessed by reverse transcriptase extension, as well as by RNase digestion in conjunction with MALDI-MS. One transcript of 74 nucleotides (Fig. 3) was a practicable compromise between small size and good methylatable properties. Smaller RNAs tend to be poorer substrates for the methyltransferase reaction, whereas larger RNAs complicate the mass spectrometric analyses. Reverse transcriptase extension analysis of the 74-mer showed that TlrB exhibits identical specificity for the truncated RNA as for the complete 23S rRNA. MALDI-MS analysis of the 74-mer RNA digestion products confirmed the site of methylation at G748 and also established that a single methyl group is added there by TlrB. Furthermore, the position of the methyl group can be localized to the N1 or exocyclic N2 of the guanine base, as neither guanine position N7 (Stern *et al*., 1988) nor 2′-O-ribose methylation (Maden *et al*., 1995) blocks the progress of reverse transcriptase under the assay conditions used here.

There are two main conclusions about the function of TlrB that can be drawn from the above findings. First, expression of *tlrB* in *Streptomyces* confers resistance to the macrolide tylosin; and secondly, TlrB specifically methylates 23S rRNA nucleotide G748. Thus, a causal link between G748 methylation and tylosin resistance is strongly suggested (but is not unequivocally established). An alternative function of TlrB, involving modification and inactivation of tylosin, has been suggested recently (Wilson and Cundliffe, 1999). If correct, TlrB would be unique among the methyltransferases in having two chemically distinct targets, an RNA and a macrolide. In our hands, using mass spectrometric and microbiological analytical techniques, TlrB does not modify tylosin. We cannot rule out that we have lost some form of TlrB activity in our studies, even though TlrB retains its rRNA methyltransferase activity under the conditions used.

To our knowledge, there has been no previous report of 23S rRNA position G748 being a target for enzymatic modification. However, the G748 methylating activity of TlrB is not an isolated case. Preliminary cloning and expression data on *myrA*, *yxjB* and the pAG1 *orf* gene indicate that each of these gene products specifically methylates position G748 (our unpublished data). The *myrA* gene has been linked to macrolide resistance (Inouye *et al*., 1994), and the pAG1 *orf* gene is encoded on a plasmid that also carries a tetracycline resistance determinant (Tauch *et al*., 1999). The helix 35 region of 23S rRNA, where G748 is situated, has already been implicated in macrolide interaction (Moazed and Noller, 1987; Hansen *et al*., 1999a; Xiong *et al*., 1999), as well as in macrolide resistance (Gregory and Dahlberg, 1999; Xiong *et al*., 1999). The two adjacent bases at positions 746 and 747 are additionally modified (to Ψ746 and T747) in *E. coli* (McCloskey and Crain, 1998), and modification presumably occurs at these positions in other species. The influence that these modifications have on the RNA structure and on the methylation of G745 and G748 is not presently known.

The mechanisms by which methylation of G745 or G748 influence the rRNA structure and function also remain to be determined. An equally intriguing question is how the methyltransferases recognize helix 35 and differentiate between positions G745 and G748. Closer analyses of larger sets of G745 and G748 methyltransferases will undoubtedly further our understanding of protein–RNA interactions. Most recent database searches reveal other homologues of these proteins,

Table 2. Oligodeoxynucleotides used in this study.

Primer	Sequence [5′→3′]	Corresponding positions in *E. coli* 23S rRNA
mf1a	AGACATATGCGGAAGAACGTCGTGCGA	NA
mf2	ACTTAAGCTTAgtgatggtgatggtgatgGGGCAGAGGGCGGCAGACGGTGAA	NA
mf11	TTTCGGGGGAGAACCAGCTA	804–822
mf12	TTCAAAG**N**CTCCCA**S**CTATCCT	2115–2136
mf30	ATCAACGGTTTCTGACTC	271–288
mf31	AGGTACTATTTCACTCCG	495–512
mf32	CCTGGTGGGTTCGGTCCT	728–745
mf33	CTGCGACTCCACATCCTT	1029–1046
mf34	CAGCTTGACCCAGGAACC	1311–1328
mf35	CGGCATCAGGTCTCAGAC	1511–1528
mf36	CTGGTCTCTGCGGCCACC	1747–1764
mf37	CATTCGTGCAGGTCGGA	1957–1973
mf38	CACTTACACTCAACACCT	2315–2332
mf39	ACCGACTCCAGCCCCAG	2522–2538
mf40	GCCAACCAGCCATGCCCT	2707–2724
mf41	AGGACAAGCCCTCGGCCT	2887–2904
SD45	GGCGCATCCGCTAATTTTTCAACATTAGTCGGTTCGGTCCTCCA-GTTAGTGTTACCCAACCTTCAACCTGCGcctatagtgagtcgtatta	694–768
SD48	GGCGCATCCGCTAATTT	767–751
ANPCR-1	TAATACGACTCACTATAGG	NA

The underlined sequences in primers mf1a and mf2 introduced *Nde*I and *Hin*dIII restriction sites, respectively, into the PCR fragment; the lower case nucleotides created a 6× histidine tag at the C-terminus of TlrB. In primer mf12, N represents any nucleotide, and S represents 50% C and 50% G. The lower case nucleotides in SD45 indicate the T7 promoter sequence and are complementary to ANPCR-1; the remaining sequence of SD45 corresponds to nucleotides 694–768 in *E. coli* 23S rRNA with minor changes described in Fig. 3. NA, not applicable.

which, on the basis of sequence comparison, appear to fall into (at least) two subclass. Here, we have described TlrB, which is the first member to be reported in the G748 subclass of rRNA methyltransferases.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain TG1 was used as a cloning host and for protein overexpression; *E. coli* strain ET12567 (MacNeil *et al*., 1992) was used for making unmethylated DNA for introduction into *Streptomyces*. Both TG1 and ET12567 were grown at 37°C in LB medium (Sambrook *et al*., 1989). *S. fradiae* ATCC-19609 and *S. lividans* 1326 were cultured at 30°C in YEME containing 10% sucrose (Hopwood *et al*., 1985). R5 supplemented with 0.2% $CuSO_4$ was used for regenerating protoplasts and, after the addition of thiostrepton (50 μg ml^{-1}), for selecting recombinants. For spore preparation, *S. lividans* was cultured on soy flour mannitol (SFM) agar, consisting of soy flour meal, mannitol and agar (2% each). Where appropriate, ampicillin and kanamycin were added to the *E. coli* media at concentrations of 100 μg ml^{-1} and 25 μg ml^{-1} respectively. Thiostrepton was used at 50 μg ml^{-1} for screening *S. lividans* transformants and at 10 μg ml^{-1} for maintaining plasmids.

The following plasmids were used: pUHE24-2, a descendent of the pDS plasmid family, contains the phage T7 promoter A1 (P_{A1}), a T5 ribosome binding site (RBS) (recognized by *E. coli* ribosomes) and two tandem *lac* operators (Bujard *et al*., 1987); pHM10a is a conjugative vector in *Streptomyces* (that can be maintained as an extrachromosomal plasmid in *E. coli*) and contains a strong constitutive *ermE* promoter (P_E), a synthetic RBS and the origin of transfer from plasmid RK2 (Motamedi *et al*., 1995); pHJL401 (Larson and Hershberger, 1986) is an *E. coli–Streptomyces* shuttle vector containing the ColE1 *ori* and SCP2* *ori*, but lacking P_E.

Cloning of tlrB

The primers used for PCR amplification, mf1a and mf2, were designed from the published *S. fradiae tlrB* gene sequence (Fouces *et al*., 1999; Wilson and Cundliffe, 1999). *S. fradiae* total DNA was used as the PCR template and was denatured with 0.4 M NaOH before use (Agarwal and Perl, 1993). The PCR reaction was carried out using *Taq* DNA polymerase (Promega) according to the supplier's recommendations, with the exception that DMSO was added to a final concentration of 10%. Reaction conditions involved 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 1 min and extending at 72°C for 1 min.

The amplified *tlrB* gene was cloned first into the *Nde*I–*Hin*dIII sites of the expression vector pUHE24-2 (the resultant plasmid was designated pSD14c). The *tlrB* fragment was then subcloned using the same enzymes into plasmid pHM10a under the control of the *ermE* promoter (forming plasmid pSD24). The *tlrB* gene cassette containing the *ermE* promoter was ligated into the *Eco*RI–*Hin*dIII sites of plasmid pHJL401 (creating plasmid pSD44; Table 1). The correctness of the *tlrB* sequence was confirmed by dideoxynucleotide sequencing. All the enzymes in this study were obtained from Roche Molecular Biochemicals, unless otherwise indicated, and were used according to the supplier's recommendations. DNA manipulations were carried using standard procedures for *E. coli* (Sambrook *et al*., 1989) and for *Streptomyces* (Hopwood *et al*., 1985). Plasmid DNA was isolated using a plasmid miniprep kit (Bio-Rad).

Tylosin resistance in S. lividans *expressing* tlrB

Plasmid pSD44 was purified from a *dam*, *dcm* strain of *E. coli* (ET12567), before being used to transform *S. lividans* protoplasts. The growth of colonies was screened on R5 agar plates containing thiostrepton. Single colonies were streaked onto SFM plates containing 50 μg ml^{-1} thiostrepton and cultured at 30°C. After 6 days, spores were collected and filtered (Hopwood *et al*., 1985), and then spread onto R5 plates containing serial dilutions of tylosin (Sigma). Spores of *S. lividans* harbouring vector pHJL401 were prepared in the same way and used as a control against which tylosin resistance was compared.

Screening of Streptomyces *rRNA for modifications*

S. lividans harbouring pHJL401 or pSD44 and plasmid-free *S. lividans* and *S. fradiae* were all grown in YEME to mid-log phase before harvesting the cells. Total RNA was isolated from cell pellets using a Qiagen RNeasy mini kit. 23S rRNA from these strains was screened by extension with reverse transcriptase (New England Biolabs) (Stern *et al*., 1988; Hansen *et al*., 1999b) from 14 oligodeoxynucleotide primers complementary to sequences approximately 200 nucleotides apart in the rRNA sequence (primers mf11 to mf41; Table 2). The primers were designed to be complementary to sequences that are conserved in both *S. lividans and S. fradiae* rRNAs. Primer extension products were analysed on denaturing 6% polyacrylamide gels alongside sequence lanes (Hansen *et al*., 1999b).

Purification of recombinant TlrB protein

E. coli TG1 cells containing plasmid pSD14c were grown at 22°C, and TlrB production was induced during early log phase by the addition of 1 mM IPTG (the formation of inclusion bodies was observed at 30°C and 37°C, so these higher temperatures were avoided). Six hours after induction, cells were harvested and lysed by sonication after 30 min pretreatment in 10 mM imidazole with 1 mg ml^{-1} lysozyme. TlrB was purified by affinity chromatography using Ni-NTA resin (Qiagen). The TG1 strain also harboured a derivative of the compatible, low-copy plasmid pREP-4 (Qiagen), which constitutively expresses the *lac* repressor protein (LacI), giving a tighter regulation of the *lac* promoter, and allows the cells to be grown at 37°C when no expression of TlrB is required. In addition, TlrB was expressed from pSD44 in *S. lividans* grown at 30°C. *S. lividans* cells were lysed by sonication after 1 h pretreatment in imidazole–lysozyme solution or in protoplast buffer (Hopwood *et al*., 1985). Recombinant TlrB in *E. coli* or *S. lividans* cell extracts was bound to Ni-NTA in 10 mM imidazole and was sequentially washed with 20 mM and 50 mM imidazole to remove other proteins before being eluted in purified form with 250 mM imidazole.

In vitro *methylation of 23S rRNA with TlrB*

Total RNA was extracted from plasmid-free *S. lividans* as described above. RNA methylation reactions were typically carried out for 30 min at 30°C in 50 μl of reaction mixture containing 100 mM Tris-HCl (pH 7.5), 2 mM $Mg(OAc)_2$, 40 mM NH_4Cl, 6 mM dithiothreitol (DTT), 2 mM SAM, 1 μg of rRNA and 0.1 μg of TlrB. Reactions were stopped by phenol–chloroform extraction, and rRNA was recovered by ethanol precipitation (Hansen *et al*., 1999b). The entire 23S rRNA sequence was screened by reverse transcriptase extension with the 14 primers as described above.

In vitro *methylation of 74 nucleotide RNA*

A deoxynucleotide template composed of 91 nucleotides (SD45) was constructed to encode a T7 phage promoter proximal to the 23S rRNA domain II sequence from 694 to 768 (Table 2). A second oligomer, ANPCR-1, was hybridized to the template oligomer to create a double-stranded region in the T7 promoter sequence. From this template, a 74 nucleotide RNA was transcribed using T7 RNA polymerase (Promega) in the presence of RNAguard (Pharmacia), according to the suppliers' recommendations. The 74-mer RNA transcript corresponds to helix 35 and adjacent structures of *E. coli* 23S rRNA. Several nucleotides outside helix 35 have been changed to give better secondary structure stability in the terminal helix of the transcript (Fig. 3).

The 74-mer RNA could be methylated by TlrB under the same conditions as described for *S. lividans* 23S rRNA. The corresponding *Streptomyces* 74-mer RNA structure would undoubtedly also have made an excellent substrate for TlrB. However, the high G+C composition of this sequence makes it less favourable for RNase A and RNase T1 analyses. Reaction conditions for methylation of the *E. coli* 74-mer were optimized and subsequently performed at 37°C for 30 min in 50 μl of buffer containing 20 mM HEPES (pH 7.6), 100 mM NH_4Cl, 6 mM β-mercaptoethanol, 10% glycerol, 50 mM Triton X-100, 175 ng of RNA and 0.1 μg of TlrB. RNA was extracted and recovered as described above. The position, type and degree of RNA modification was analysed by both reverse transcriptase primer extension and MALDI-MS.

Reverse transcriptase quantification of TlrB modification was carried out from primer SD48, which hybridizes to the sequence corresponding to 23S rRNA positions 751–767, and thus lies within three nucleotides 3′ of the predicted site of modification. This type of analysis was adapted from techniques described previously (Sigmund *et al*., 1988; Vester and Douthwaite, 1994). Briefly, the primer was extended by reverse transcriptase using the substrates dATP, dCTP, dTTP and ddGTP (1 mM each). The extension reaction is blocked by either methylation of the target guanine or (in unmethylated RNAs) incorporation of ddGTP eight nucleotides further downstream at the position equivalent to C740 (see Fig. 3). The cDNA extension products were separated on a denaturing 13% polyacrylamide gel. Band intensities were estimated by scanning on a Storm 840 phosphorimager (Molecular Dynamics), and the degree of methylation was calculated by comparison of these band intensities.

TlrB treatment of tylosin

Tylosin (as the tartrate salt; Sigma) and purified TlrB methyltransferase were incubated together *in vitro* under a

wide range of conditions. TlrB was purified from both *E. coli* and *S. lividans* (lysed in protoplast buffer). Tylosin was incubated with TlrB in the buffer described above for methylation of the 74-mer RNA and in the buffer described by Wilson and Cundliffe (1999). Aliquots of 10 μg of tylosin were incubated in 50 μl volumes with 5 mM SAM and 10 μg of TlrB for 20 h at 30°C. For all samples, 20 μl aliquots were spotted onto 6 mm Whatman disks placed on LB agar plates (Sambrook *et al*., 1989) that had been seeded with the indicator strain *Micrococcus luteus*. Untreated dilutions of tylosin were used as standards. The sizes of inhibition zones were measured after incubation at 30°C for 2 days. Treated and untreated drug samples were additionally analysed by MALDI-MS.

MALDI-MS analysis of RNA and tylosin

The unmethylated and TlrB-methylated 74 nucleotide RNA transcripts were purified on an in-house-fabricated reverse-phase column (Kirpekar *et al*., 1998) to remove abortive transcription products. The eluted full-length transcripts were dried and redissolved in water. RNA was completely digested with RNase A or RNase T1 and analysed by MALDI-MS as described previously (Kirpekar *et al*., 2000). Untreated tylosin and tylosin that had been incubated with TlrB were analysed by MALDI-MS without prior purification using the matrices 0.5 M 3-hydroxypicolinic acid in 50% acetonitrile, 0.3 M 2,4,6-trihydroxyacetophenone−0.15 M 2,3,4-trihydroxyacetophenone−30 mM $(NH_4)_2$-citrate in 80% acetone or 20 g l^{-1} α-cyano 4-hydroxycinnamic acid in 99% acetone. After air drying, the last two preparations were washed twice with 5 μl of water. Approximately 10 pmol of tylosin was used for each MS analysis. All spectra were recorded in positive ion mode on a Bruker reflex II MALDI mass spectrometer (Bruker-Daltonik).

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