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Molecular Analysis of *tlrB*, an Antibiotic-resistance Gene from Tylosin-producing *Streptomyces fradiae*, and Discovery of a Novel Resistance Mechanism

VANESSA T. W. WILSON and ERIC CUNDLIFFE*

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

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The *tlrB* gene, which confers inducible resistance to a range of macrolide antibiotics including biosynthetic precursors of tylosin, was isolated and sequenced. In the genome of *Streptomyces fradiae*, it lies between *pbp*, which encodes a putative penicillin-binding protein, and *tylN*, encoding a glycosyltransferase involved in tylosin biosynthesis. The TlrB protein was produced in *E. coli* as a fusion to MalE. The fusion protein, but not MalE alone, inactivates macrolides in the presence of *S*-adenosyl-methionine (SAM) but the modified product(s) has not been characterised.

Resistance to tylosin in the producing-organism, *Streptomyces fradiae*, is a complex phenomenon. Four distinct fragments of the genome confer resistance when expressed from multi-copy vectors in other hosts. Two of these resistance determinants (*tlrA* and *tlrD*) are *erm*-type genes (for review, see[1]) encoding methyltransferases that modify 23S rRNA and thereby render ribosomes resistant to MLS antibiotics, including tylosin. The TlrD protein is produced constitutively and generates N^6-monomethyladenosine at position 2058 (*E. coli* numbering scheme) within 23S rRNA[2]. This confers high level resistance to lincomycin plus lower, non-uniform, levels of resistance to macrolides. The TlrA protein is produced inducibly in *S. fradiae* and introduces a second methyl group into A-2058 (generating N^6,N^6-dimethylA at that site) thereby increasing the level of resistance to macrolides in general and tylosin in particular[3]. Expression of *tlrA* is controlled *via* transcriptional attenuation and involves ribosome-mediated changes in the conformation of the mRNA leader sequence, brought about by the action of inducers such as tylosin and its biosynthetic precursors[4]. The induction specificity of *tlrA* is dependent on the state of the ribosomes (*i.e.* on the manner in which they respond to inhibition by potential inducers) and is significantly influenced by constitutive expression of *tlrD*. For example, tylosin induces expression of *tlrA* in *S. fradiae* and in an engineered (*tlrA*$^+$, *tlrD*$^+$) strain of *Streptomyces albus* but not in *S. albus* (*tlrA*$^+$) lacking *tlrD*.

In addition to *tlrA* and *tlrD*, two other DNA fragments have been isolated from *S. fradiae* and shown to confer resistance to tylosin[5]. One fragment conferred resistance when introduced into *Streptomyces lividans* on the multi-copy plasmid pSVB25, a derivative of pIJ702, and was presumed to contain a resistance gene, designated *tlrB*. The other fragment restored tylosin-resistance to *S. fradiae* JS87, a sensitive mutant from which most (or all) of the biosynthetic gene cluster, has been deleted[6]. Sequence analysis revealed a gene, *tlrC* , the deduced product of which is an ATP-binding protein with a presumed role in tylosin-efflux[7]. However, prior to the present work, no putative function had been ascribed to the TlrB protein and the *tlrB* gene had not been analysed in detail.

Materials and Methods

Bacterial Strains and Growth Conditions

S. fradiae T59235 (known also as C373.1) was provided by Dr. EUGENE T. SENO (Lilly Research Laboratories, Indianapolis, IN) together with pHJL315[8] and pSVB25[5], both of which contain *tlrB* plus flanking *S. fradiae* DNA. *S. fradiae* was routinely grown at 30°C in

Tryptic Soy Broth (Difco) or on AS-1 agar plates[9]. *S. lividans* OS456, a specifically-deleted, macrolide-sensitive strain[10], was grown on NE agar[11], or in YEME liquid medium[12] supplemented with 10% (w/v) sucrose. Thiostrepton (5 μg/ml) or gentamicin (25 μg/ml) was added to media to maintain plasmids where applicable, and expression of *tlrB* was induced by addition of tylosin (2 μg/ml) to growth media. *E. coli* NM522[13] was used to propagate pUC18 and its derivatives. Plasmid DNA was isolated from *Streptomyces* spp. or *E. coli* and manipulated using standard protocols[12,14].

MIC Determinations

S. lividans OS456 containing pSVB25 was grown for 5 days at 30°C on NE agar plates containing thiostrepton (5 μg/ml) to maintain the plasmid, plus tylosin (2 μg/ml) to induce expression of *tlrB*. Spores were then collected and immediately plated onto NE agar containing macrolide antibiotics or lincomycin and growth was assessed after incubation at 30°C for 4 days.

DNA Sequencing

A 3.1 kb DNA fragment containing *tlrB*, generated by digestion of pSVB25 with *Sst*I plus *Bgl*II, was isolated from low melting point agarose following electrophoresis and ligated with pUC18 (similarly digested, and dephosphorylated with calf intestinal alkaline phosphatase), to generate pLST77. Restriction endonucleases and T4 DNA ligase were used in accordance with the manufacturer's guidelines (Gibco BRL). The sequence of the 3.1 kb DNA fragment was generated directly from double stranded templates by the dideoxy-chain termination method[15] using [α-^{35}S]-∂ATP and modified T7 DNA polymerase (Pharmacia). This was done from both strands in overlapping fashion following the generation of nested deletions (200~250 bp) using *Exo*III and S1. To overcome possible gel artefacts (compressions) due to the high G+C content of *Streptomyces* DNA, one strand was completely resequenced using 7-deaza ∂GTP mixes (Pharmacia). Products of DNA sequencing reactions were resolved by electrophoresis in 6% polyacrylamide-7 M urea gels.

Purification of MalE-TlrB Fusion Protein

PCR-amplification of *tlrB* was carried out as described elsewhere[16]. The primers, 5′ **GAATTC**GAGGATGAGGCCTTCGTCA 3′ and 5′ **TCTAGA**TCAGGGCAGAGGGCGGCA 3′, contained 5′ terminal sequences to generate *Eco*RI and *Xba*I restriction sites, respectively, at the ends of the amplified DNA to facilitate in-frame fusion of the amplified product with the MalE coding sequence in the expression vector pMal-p2 (New England Biolabs). *E. coli* TB1 was used as host, expression of the fusion protein was induced by addition of IPTG, and MalE-TlrB was purified from cell lysates by affinity chromatography, all according to the manufacturer's protocol. Samples of column eluates were analysed by SDS-PAGE on 10% (w/v) polyacrylamide gels (data not shown) and fractions containing the fusion protein were pooled.

Methyltransferase Assays

Tylosin (10 μg) and MalE-TlrB fusion protein (20 μg) were incubated at 30°C for 24 hours, with and without SAM (8 mM) in 25 μl of buffer containing (final concentrations) HEPES-KOH pH 7.5 (30 mM), $MgCl_2$ (6 mM) and KCl (60 mM). SAM-dependent inactivation of tylosin was revealed when paper discs impregnated with samples of the reaction mixtures were applied to LB-agar plates seeded with the indicator strain *Micrococcus luteus* T194. Radiotransfer assays were carried out under similar conditions but with 1 μCi of [methyl-^{3}H]-SAM (500 mCi/mmol; 18.5 GBq/mmol) and various macrolides as alternative acceptor substrates. After 24 hours at 30°C, samples (4 μl) were applied to plastic coated silica gel 60 F_{254} sheets (10 cm × 0.5 cm; Merck) for ascending thin-layer chromatography in solvent containing ethyl acetate, diethylamine, methanol (95 : 5 : 10). Following separation, the sheets were cut into strips (1 × 0.5 cm) and their radioactivity estimated by liquid-scintillation spectrometry. The Rf values of standard compounds (antibiotics and SAM, 10 μg each) were determined under short-wave UV illumination.

Targeted Disruption of *tlrB* in the *S. fradiae* Genome

A 2.3 kb *Pst*I-*Sst*I fragment, including the whole of *tlrB* plus flanking DNA (Fig. 1), was excised from pLST77 and ligated into pUC18 to generate pLST7715. This plasmid was linearized with *Bam*HI, treated with alkaline phosphatase, and ligated with a 2.3 kb *Bam*HI fragment from pHP45Ωhyg which contains the hygromycin B-resistance cassette, Ωhyg[17], derived from *Streptomyces hygroscopicus*. The ligation products were then introduced into *E. coli* NM522 and transformants, initially selected on LB agar plates containing ampicillin (100 μg ml^{-1}), were replica plated onto medium containing hygromycin (75 μg ml^{-1}). Plasmid (pLST7716) was isolated from several of the hygromycin-resistant colonies and restriction analysis confirmed the structure. The disrupted *tlrB* gene, together with flanking *S. fradiae*

DNA, was then excised from pLST7716 as a 4.6 kb *Eco*RI-*Pst*I fragment. This was done in 2 stages since there is an internal *Eco*RI site in Ωhyg. First, pLST7716 was digested to completion with *Pst*I, followed by partial digestion with *Eco*RI. The digestion products were then separated by agarose gel electrophoresis. The desired band (4.6 kb; located with respect to DNA size markers) was then excised from the agarose gel and ligated with pOJ260[18], previously restricted with *Pst*I and *Eco*RI, to generate pLST7717. This suicide vector (unable to replicate in *Streptomyces* spp.) was introduced into *S. fradiae via* conjugal transfer from *E. coli* S17-1, as described elsewhere[16]. Since pOJ260 (and therefore pLST7717) carries an apramycin-resistance determinant, replacement of *tlrB* with the disrupted gene *via* double recombination generated apramycin-sensitive strains that were distinguished from single recombinants (apramycin-resistant) by replica plating on AS-1 agar containing hygromycin B, with and without apramycin (25 $\mu g\,ml^{-1}$). An authentic double recombinant strain was then identified among several candidates by Southern blot hybridization analysis, carried out at high stringency 3 different probes. These were: a *Bam*HI-*Nco*I fragment (approximately 180 bp) derived from *tlrB*; Ωhyg DNA (2.3 kb); and a 0.9 kb *Sac*I fragment derived from the apramycin-resistance gene.

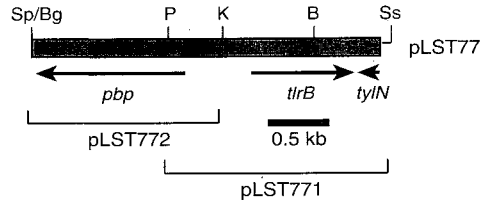
Tylosin-production Fermentation

This was carried out at 28°C in shake flasks as previously described[16]. The cultures were sampled at various times, mycelium was removed from each sample by filtration and portions of the filtrates were applied directly to antibiotic assay discs. Tylosin levels were estimated microbiologically on LB agar plates at 37°C using *Micrococcus luteus* as indicator strain. Zone sizes were compared with those generated in controls using calibrated amounts of tylosin.

Antibiotics

These were obtained from the following sources: lincomycin, The Upjohn Co. (Kalamazoo, Mich.); methymycin and thiostrepton, E.R. Squibb and Sons (Princeton, N.J.); oleandomycin, Pfizer Ltd. (Sandwich, U.K.); pikromycin, Pfizer (Groton, C.T.); rosaramicin, Schering-Plough Corp. (Union, N.J.); spiramycin, Rhône-Poulenc (Paris, France); erythromycin, gentamicin and ampicillin were purchased from Sigma. We are particularly grateful for the following kind gifts: angolamycin, Prof. W. KELLER-SCHIERLEIN (Eidgenössische Technische Hochschule, Zurich, Switzerland); mycinamicin II, Dr. M. INOUYE (Asahi Chemical Industry Co., Ltd., Japan); tylosin and its derivatives, Dr. H. A. KIRST, Eli Lilly and Co. (Indianapolis, IN).

Fig. 1. Restriction map of the *tlrB* region of the *S. fradiae* genome.



Sequencing revealed two complete orfs (now designated *pbp* and *tlrB*) plus one that was incomplete (part of *tylN*). The two complete orfs were subcloned separately in pIJ702, generating pLST771 and pLST772, in order to identify the resistance determinant. Restriction sites: Sp, *Sph*I; Bg, *Bgl*II; P, *Pst*I; K, *Kpn*I; B, *Bam*HI; Ss, *Sst*I.

Results and Discussion

DNA Manipulation and Sequence Analysis

S. fradiae DNA (2824 bp) derived from pSVB25 was sequenced (accession number AJ009971), revealing an incomplete open reading frame (subsequently characterised[19] as a tylosin biosynthetic gene and referred to as *tylN*) together with two others that were complete and divergently orientated (Fig. 1). These were subcloned separately, using the multicopy vector pIJ702, as a 2 kb *Pst*I-*Sst*I fragment (generating pLST771) and a 1.6 kb *Kpn*I-*Bgl*II fragment (generating pLST772) and introduced into *S. lividans* OS456. Only pLST771, containing the rightward-reading orf in the orientation given in Fig. 1, conferred inducible resistance to tylosin (data not shown) and evidently contained the *tlrB* resistance gene. This same *Pst*I-*Sst*I fragment was also introduced into the conjugal vector pSET152[18] thereby generating pLST7711, which was used to integrate *tlrB* into the genome of *S. lividans* (see below). The deduced product of the leftward-reading orf subcloned in pLST772 displays striking similarity to the much studied[20] D,D carboxypeptidase of *Streptomyces* R61, and to various other β-lactamases and penicillin-binding proteins, in-

Table 1. MIC values of various macrolides for *S. lividans* OS456 strains harbouring *tlrB*.

Antibiotic	MICs (μg/ml)			
	Control strain	A single copy *tlrB* own promoter	B multi-copy *tlrB* own promoter	C single copy *tlrB* with *ermEp**
Tylosin	1-2.5	30-40	50-60	200-500
Spiramycin	10-20	70-80	90-100	500-1000
Mycinamicin	50-60	150-200	150-200	200-500
Oleandomycin	1.2.5	20-40	20-40	200-500
Erythromycin	5-10	10-15	5-10	20-30
Rosaramicin	<1	<1	<1	<1
23-deoxy-OMT	1.2.5		10-15	
demycinosyl-tylosin	2.5-5		30-40	
macrocin	2.5-5		50-60	
demycarosyl-tylosin	1.2.5		50-60	

Spores were taken from NE agar plates containing an inducing concentration of tylosin, and re-plated on various drugs as shown. In controls, neither pIJ702 or an integrated copy of pSET152 affected the resistance levels of *S. lividans* OS456. Abbreviation: OMT, *O*-mycaminosyl-tylonolide.

cluding a "penicillin-recognising" alkaline endopeptidase[21] from *Bacillus cereus*. We discern no obvious role in tylosin biosynthesis for the product of this orf, which we temporarily refer to as "*pbp*". Searches of the protein identification data bases using the BLAST algorithm revealed only two significant matches (Fig. 2) to the deduced sequence of the TlrB protein (maximal size 280 amino acids, Mr 30,440). Strong, end-to-end similarity (51% sequence identity) was seen to the product of *myrA*, a macrolide-resistance determinant[22] from the mycinamicin producer, *Micromonospora griseorubida* and the other match was to the product of *orf30* from a *mukB* mutant of *E. coli*[23]. No functions have been proposed for these proteins.

The TlrB Resistance Phenotype

The levels of resistance to various antibiotics conferred by *tlrB* in *S. lividans* OS456 are shown in Table 1. Initially, *tlrB* was expressed from its own promoter, either as a single gene copy integrated at the chromosomal φc31 *attB* site (using pLST7711; Table 1, column A) or as multiple copies on the replicating vector pLST771 (Table 1, column B), and resistance was assessed following pre-growth on agar plates containing an inducing concentration of tylosin. The immediate conclusion was that *tlrB* did not confer resistance to lincomycin (*i.e.* did not confer MLS resistance) but was principally effective against tylosin, spiramycin and mycinamicin. Moreover, at least in *S. lividans*, the presence of additional copies of *tlrB* did not greatly enhance the levels of resistance conferred. However, *tlrB* conferred much higher resistance levels in *S. fradiae*. This was apparent when pSET152 was used to integrate a single copy of *tlrB* (with its own promoter) into the φc31 *attB* site of *S. fradiae* GS93, a sensitive strain from which the entire *tyl* gene cluster (including *tlrB*, *tlrC* and *tlrD*) has been deleted[24]. The MIC for tylosin was raised from below 1 μg/ml to above 500 μg/ml. [Strain GS93 still contains a functional copy of *tlrA* but this resistance gene is not inducible by tylosin in the absence of *tlrD*[4]. This was confirmed in the present experiments when GS93 (*tlrB*$^+$) failed to grow on lincomycin, even after exposure to tylosin.] These results suggested that *tlrB* might be more strongly

Fig. 2. Comparison of the amino acid sequences of TlrB and similar proteins.

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 TLRB  MRKNVVRYLR CPHCAAPLRS SD....RTLR CENGHTFDVA RQGYVNLLR.
 MYRA  MHPDLLPHLR CPVCGQPLHQ ADAAPPRALR CPAGHSFDIA RQGYVNLLT.
ORF30  ......MSFS CPLCHQPLSR EKN....SYI CPQRHQFDMA KEGYVNLLPV

 TLRB  ..RPTKLAAD TTDMVAARAA LLDSGHYAPL TE........ ....RLRGTS
 MYRA  ..GRAPHVGD TAEMIAAREE FLAAGHYDPF SAALATAAAR AVPRRVRPGD
ORF30  QHKRSRDPGD SAEMMQARRA FLDAGHYQPL RDAIVA.... ....QLRERL

                   MOTIF I                      *
 TLRB  AGAAGAGAPD CVVDIGGGTG HHLARVLEEF EDAEGLLLDM SKPAVRRAAR
 MYRA  GVGEPVAYPD LVVDAGAGTG RHLAAVLDAV PTAVGLALDV SKPALRRAAR
ORF30  DDKATA.... .VLDIGCGEG YYTHAFADAL PEITTFGLDV SKVAIKAAAK

                                                   MOTIF III
 TLRB  AHPRASSAVA DVWDTLPLRD GAAAMALNVF APRNPPEIRR ILRPGGTLLV
 MYRA  AHPRAGAAVC DTWGRLPLAD ATVAVLVNVF APRNGPEFRR VLRPDGALLV
ORF30  RYPQVTFCVA S.SHRLPFSD TSMDAIIRIY APCKAEELAR VVKPGGWVIT

 TLRB  VTPQQDHLAE LVDALGLLRV RDHKEGRLAE QLAPHFEAVG QERLRTTLRL
 MYRA  VTPTAEHLVE LVDRLGLLRV DPAKDARVAD SLTRHFEPAG QSTHRHRLQL
ORF30  ATPGPRHLME LK...GLIYN EVHLHAPHAE QLEG.FTLQQ SAELCYPMRL

 TLRB  DHDALGRVVA MGPSSWHQDP DELARRIAEL PGIHEVTLSV TFTVCRPLP*
 MYRA  TRKEVLTLVG M.PSAWHTDP ARLTARVAAL SEPVTVTAAV RLARYRPI..
ORF30  RGDEAVALLQ MTPFAWRAKP EVWQTLAAKE VFDCQTDFNI HL.WQRSY..
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Sequences within these proteins resemble the methyltransferase consensus motifs I and III and are shown in bold. The consensus sequences are hh(D/E)h**G**(G/C)**G**(T/P)**G** for motif I and LhxPGGhhh*h for motif III (where h = L, I, V and h* = L, I, V or A). Accession numbers: TlrA, AJ009971, this work; MyrA, D16099[22]; the product of *E. coli* "*orf30*" D90286[23].

expressed from its own promoter in *S. fradiae* than in *S. lividans* and, in support of this idea, greatly elevated resistance levels were observed in *S. lividans* (Table 1, column C) when a single copy of *tlrB* driven by the strong, constitutive promoter *ermEp**[25] was integrated into the ϕc31 *attB* site.

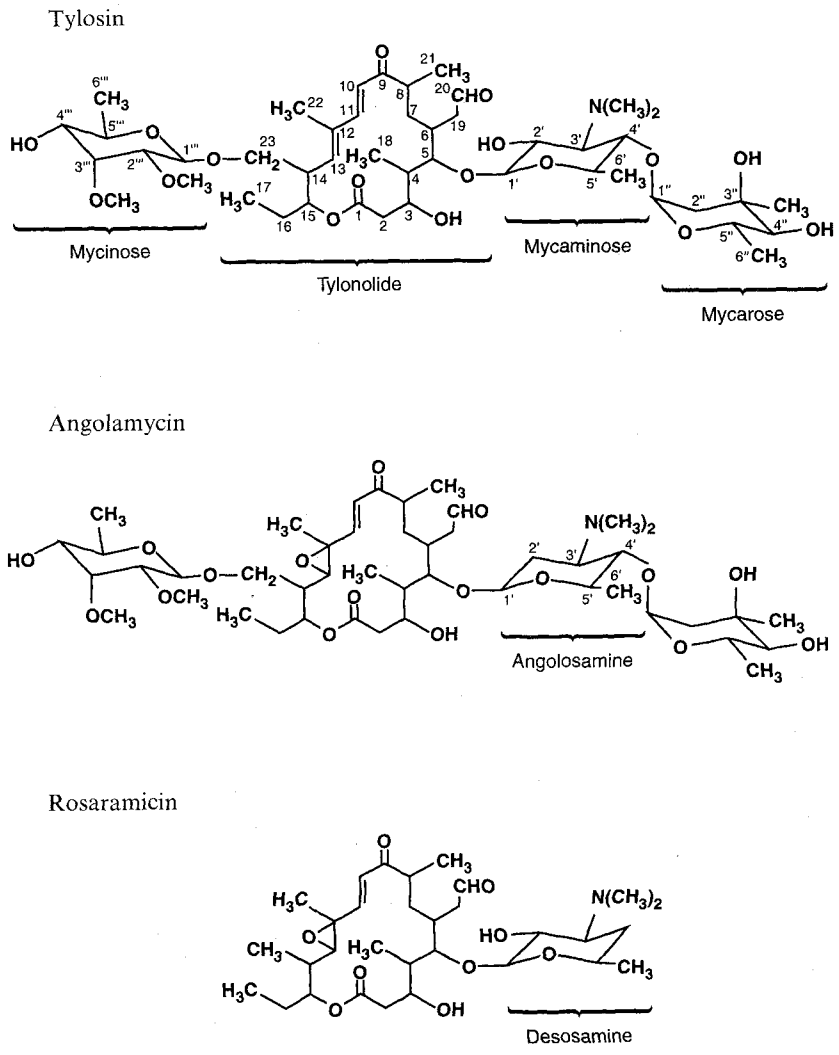
In further studies, *tlrB* conferred significant levels of resistance to glycosylated precursors of tylosin (for structures, see Fig. 3) and was particularly effective against the later intermediates such as 3‴-*O*-demethyltylosin (macrocin) and demycarosyl-tylosin (desmycosin).

Mode of Action of the TlrB Protein

Although data base searches did not indicate the likely action of the TlrB protein, closer inspection of the sequence revealed motifs characteristic of methyltransferases that utilize SAM as cofactor (Fig. 2). Three consensus sequence motifs have been described in such enzymes[26] and crystallographic data[27] indicate that motif I forms part of the binding pocket for SAM, within which the TlrB protein, like most other putative or authentic methyltransferases, displays the sequence G × G × G. Also present is the characteristic acidic amino acid about 19 residues downstream of motif I. Methyltransferase motif II is not obviously present in TlrB but there is a good match to motif III. Such considerations suggested that TlrB was probably capable of binding SAM and was, therefore, a candidate methyltransferase.

The possibility that *tlrB* might encode a ribosomal RNA methylase, similar to those responsible for MLS resistance, was quickly discounted since *tlrB* does not confer resistance to lincomycin. Moreover, when assayed in cell-free protein synthesis, ribosomes from an *S. lividans* (*tlrB*$^+$) strain were fully sensitive to tylosin, even after exposure of the strain to inducing levels of the drug (M. ZALACAIN and E.C., unpublished data). We therefore considered the alternative possibility that the methyl acceptor utilised by the TlrB protein might be tylosin itself, although SAM-dependent inactivation of antibiotics had not previously been reported. This hypothesis was strengthened when a *tlrB*$^+$ strain of *S. lividans* was found to inactivate tylosin in a microbiological assay

Fig. 3. Structures of macrolide antibiotics.



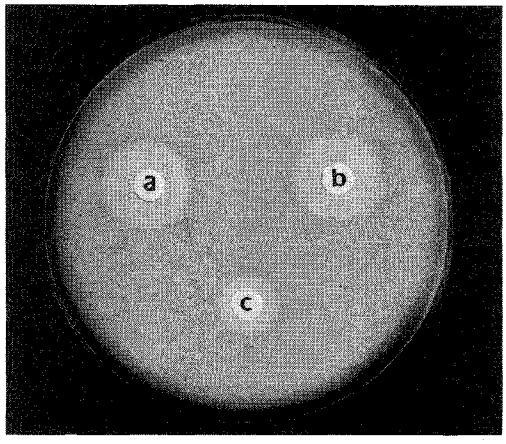
Tylactone, the non-glycosylated, polyketide precursor of tylosin is 20, 23-dideoxy-tylonolide.

using *M. luteus* as the test organism (data not shown). Subsequently, extracts of *S. lividans* (*tlrB*$^+$) were shown to inactivate tylosin during overnight incubation at 30°C. Such activity was lost following dialysis of extracts but was specifically restored by the (re)addition of SAM. The TlrB protein was therefore produced in *E. coli* as a fusion to the maltose-binding protein (MalE) and the fusion product, together with native MalE, was purified by affinity chromatography on an amylose column. Although the fusion product could not be cleaved at the MalE-TlrB junction using Factor Xa, the fusion protein (but not native MalE) inactivated tylosin *in vitro* in a SAM-dependent manner (Fig. 4)

Substrate Specificity of the TlrB Protein

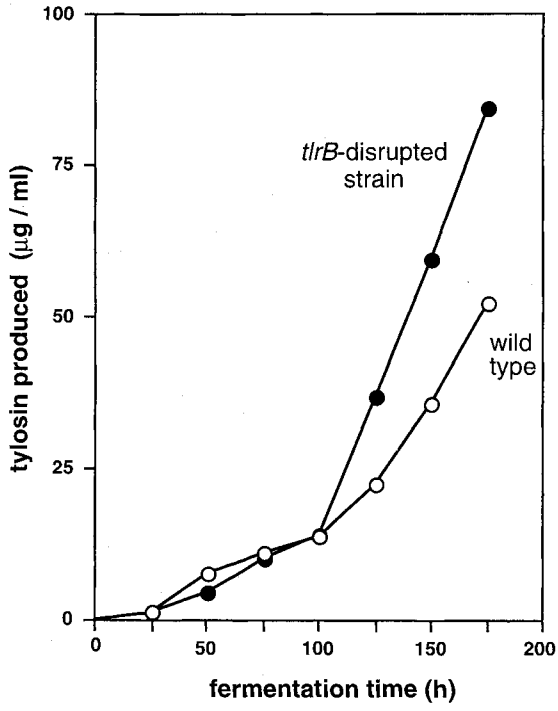
When [^{3}H-methyl]-SAM was incubated together with tylosin and the MalE-TlrB fusion protein, [^{3}H-methyl] radioactivity was incorporated into tylosin, as judged by thin-layer chromatography of the product(s). The availability of this rapid and convenient radiotransfer assay allowed the substrate specificity of the TlrB protein to be investigated in more detail (Table 2). Consistent with the TlrB resistance phenotype (Table 1), macrolides other than tylosin were also methylated, including glycosylated precursors of tylosin, but not tylactone. However, these data did not indicate the likely site(s) of

Fig. 4. SAM-dependent inactivation of tylosin by MalE-TLRB fusion proptein.



Tylosin was incubated with the fusion protein, with and without SAM, for 24 hours at 30°C, before samples of the incubation mixture were placed onto agar plates seeded with the indicator strain *M. luteus*. Key: a, control, MalE protein only; b, fusion protein minus SAM; c, fusion protein plus SAM.

Fig. 5. Effect of *tlrB*-disruption on tylosin yields.



Wild type and *tlrB*-disrupted strains of *S. fradiae* were fermented in shake flask cultures and levels of tylosin production were assayed microbiologically, using *M. luteus* as indicator strain, in samples taken at various times.

Table 2. Incorporation of [methyl-^{3}H] radioactivity from SAM into macrolides, catalysed by the MalE-TlrB fusion protein.

Substrate	Acceptor activity
Tylosin	+++
Angolamycin	+++
Carbomycin	+++
Oleandomycin	+++
Spiramycin	+++
Mycinamicin	+++
Demycarosyl-tylosin	+++
Macrocin	+++
Methymycin	+
Pikromycin	+
Erythromycin	+
Demycinosyl-tylosin	+
23-deoxy-OMT	+
Rosaramicin	-
Tylactone	-

Substrate activity: high (+++); low (+); insignificant (−).

modification, although the high substrate activity of angolamycin eliminated the possibility that modification might have occurred on the 2′-OH group, the site targeted by macrolide glycosyltransferases and phosphotransferases[1]. Curiously, rosaramicin was not a substrate for TlrB (see also Table 1) despite close structural similarity to 23-deoxy-OMT (Fig. 3).

Attempted Characterisation of TlrB-modified Tylosin

Tylosin was incubated overnight at 30°C with SAM plus the MalE-TlrB fusion protein and the reaction mixture was subjected to HPLC analysis. However, the methylated product(s) were not readily detectable, and may have labile under the conditions employed.

Possible Implications for Tylosin-yield Enhancement

Since the TlrB protein is capable of modifying tylosin and various of its biosynthetic precursors, it might reduce the final yield of tylosin in fermentations. This possibility was addressed *via* targeted disruption of *tlrB*, using the hygromycin-resistance gene cassette, Ωhyg[17]. Following gene replacement *via* double recombination, Southern blot hybridisation analysis (involving three different probes) was used to confirm the chromosomal insertion (data not shown). The *tlrB*-disrupted strain was then introduced into tylosin-production medium and found to produce significantly enhanced levels of antibiotic, as judged microbiologically using *M. luteus* as indicator strain (Fig. 5). Whether this effect would be repeatable in developed industrial strains remains to be seen.

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