Analysis of the Tunicamycin Biosynthetic Gene Cluster of *Streptomyces chartreusis* Reveals New Insights into Tunicamycin Production and Immunity

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**ABSTRACT**

The tunicamycin biosynthetic gene cluster of *Streptomyces chartreusis* consists of 14 genes (*tunA* to *tunN*) with a high degree of apparent translational coupling. Transcriptional analysis revealed that all of these genes are likely to be transcribed as a single operon from two promoters, *tunp1* and *tunp2*. In-frame deletion analysis revealed that just six of these genes (*tunABCDEH*) are essential for tunicamycin production in the heterologous host *Streptomyces coelicolor*, while five (*tunFGKLN*) with likely counterparts in primary metabolism are not necessary, but presumably ensure efficient production of the antibiotic at the onset of tunicamycin biosynthesis. Three genes are implicated in immunity, namely, *tunI* and *tunJ*, which encode a two-component ABC transporter presumably required for export of the antibiotic, and *tunM*, which encodes a putative S-adenosylmethionine (SAM)-dependent methyltransferase. Expression of *tunIJ* or *tunM* in *S. coelicolor* conferred resistance to exogenous tunicamycin. The results presented here provide new insights into tunicamycin biosynthesis and immunity.

**KEYWORDS**

tunicamycin, biosynthesis, immunity, antibiotic, *Streptomyces*

The tunicamycins are fatty acyl nucleoside antibiotics produced by several actinomycetes, mostly *Streptomyces* species, including *Streptomyces chartreusis* (1, 2). They consist of a unique 11-carbon core (tunicamine) decorated with uracil, N-acetylglucosamine (GlcNAc), and variable fatty acyl moieties (Fig. 1). They are potent inhibitors of cell wall biosynthesis in Gram-positive bacteria, where they target MraY, which is required for the production of the peptidoglycan precursor Lipid I (3, 4), and TarO, MnaA, and Cap5P, which are involved in teichoic acid biosynthesis (5). They also inhibit protein N-glycosylation in eukaryotes (6), targeting dolichyl-phosphate alpha-N-acetyl-glucosaminyl-phosphotransferase (DPAGT1, also known as GlcNAC-1-P transferase) and resulting in severe toxicity. While the bacterial (e.g., MraY) and human (DPAGT1) targets are similar, in principle, it may be possible to design tunicamycin variants that specifically inhibit the bacterial proteins. A better understanding of tunicamycin biosynthesis and of the genes responsible for the individual steps in its production could play an important role in the delivery of such analogues. In an earlier work, we cloned and sequenced the tunicamycin biosynthetic gene cluster (*tun*) from *S. chartreusis*, expressed it heterologously in *Streptomyces coelicolor*, and proposed a biosynthetic pathway based largely on homology of the encoded gene products with proteins of known function (7). The cluster contains 14 genes, *tunA* to *tunN*, with many apparently translationally coupled to the preceding gene (Fig. 1). Subsequent *in vitro* studies of TunA and TunF combined with the deletion of *tunB* provided experimental evidence for their specific roles in tunicamycin biosynthesis (8). Here, we report...
mutational analyses of the other 13 genes in the tun cluster, which, together with transcriptional characterizations, provide new insights into tunicamycin biosynthesis and immunity.

RESULTS

Transcriptional analysis of the tun gene cluster. Inspection of the tun gene cluster (Fig. 1) revealed that of the 14 genes, 10 appeared to be translationally coupled to the gene upstream. This, together with a maximal intergenic spacing of 39 bp, suggested that the entire tun gene cluster might be expressed in a single operon. To address this possibility, RNA was prepared from a lawn of S. coelicolor M1152 containing the cloned tun gene cluster present on a 12.9-kb SacI fragment of pIJ12003a (7) and used in reverse transcription (RT)-PCR experiments with primer pairs corresponding to tun sequences located approximately 100 nt from the ends and beginnings of adjacent genes (see Table S1 in the supplemental material). Amplification of cDNA spanning each of the gene junctions (Fig. 1) was indeed consistent with transcription of the tun genes in a single operon.

To locate the potential transcriptional start site(s) of this likely tun operon, 5′ rapid amplification of cDNA ends (RACE) experiments were carried out using the S. coelicolor RNA preparation, and also RNA from S. chartreusis using five different primers, RACE1 to RACE5 (Table S1). Two putative transcriptional starts sites (tunp1 and tunp2) were identified in RNA isolated from S. chartreusis and located within the Sacl fragment; while the tunp2 transcript was not observed in RNA from S. coelicolor/pIJ12003a, transcription initiation at the promoter, appr, of the apramycin resistance gene present in the cloning vector was detected (Fig. 2). To assess whether these 5′ transcript ends represented promoter activity in vivo (as opposed to mRNA processing or degradation), PCR fragments were generated that contained each individual putative promoter element, inserted into the β-glucuronidase reporter plasmid pGUS (9) and introduced into S. coelicolor M1152. Growth on R5 agar containing the β-glucosiduronic acid-derived substrate X-gluc confirmed promoter activity for tunp1 and appr, but not for tunp2.
antiporter, transcriptional start site of the apramycin resistance (apr) gene; p1 and p2, transcriptional start sites of the putative tun operon. (Fig. 3), consistent with the lack of a detectable tunp2 transcript in S. coelicolor M1152/puJ12003a.

Antibiotic production in S. coelicolor under conditions of nitrogen limitation is dependent on the intracellular signaling molecular ppGpp (10). To assess whether tun

![Sequence of the tun promoter region showing the transcriptional start sites identified by 5' RACE. Putative −10 and −35 regions, where discernible, are overscored in blue. The locations of the complementary primers used for 5' RACE are indicated beneath the sequence by red arrows. Nucleotides 1 to 100 are derived from the cloning vector pRT802, used to construct puJ12003a, and the SacI site at the end of the cloned tun gene fragment is shown in magenta. The N-terminal amino acid sequence of TunA is shown in green. aprp, transcriptional start site of the apramycin resistance (apr) gene; p1 and p2, transcriptional start sites of the putative tun operon.](image)

![Gus reporter assays on R5 agar of promoter fragments initiating transcription of the tun gene cluster in S. coelicolor M1152 (negative, pGUS without an insert). Putative promoters contained within the inserted PCR fragments are indicated by aprp (putative promoter of the apramycin resistance gene apr) and p1 and p2, putative promoters of the likely tun operon. Gus activity results in the production of an insoluble indigo-blue precipitate, which appears green on yellow R5 agar plates. (Bottom) Schematic showing the relative positions of the three identified transcriptional start sites and the extent of the sequences present in each inserted PCR fragment. aprp, transcriptional start site of the apramycin resistance gene (apr); p1 and p2, transcriptional start sites of the putative tun operon.](image)
gene transcription was dependent on ppGpp, the same β-glucuronidase promoter fusions were introduced into S. coelicolor M145 and its ΔrelA mutant M571, and the resulting exconjugants were assayed for promoter activity on supplemented minimal medium solid (SMMS) agar containing X-gluc; no differences in promoter activity were observed between the two strains (data not shown).

**Deletion analysis of the tun gene cluster.** Previous work showed that deletion of tunB abolished tunicamycin production and provided new insights into its likely role in tunicamycin biosynthesis (8).

To assess the validity of the rest of the proposed tunicamycin biosynthetic pathway, in-frame deletion mutations were made by PCR-targeting for all 13 of the remaining putative biosynthetic genes, and the mutated plasmid derivatives were introduced into S. coelicolor M1152 by conjugation from *Escherichia coli*. The resulting strains were then assayed for antimicrobial activity, using *Bacillus subtilis* EC1524 as an indicator strain (Fig. 4). Individual deletion of tunACDEH abolished activity, while individual obliteration of tunFGK significantly or markedly reduced it; mutations in tunLMN had no significant effects on the sizes of the zones of inhibition. Liquid chromatography-mass spectrometry (LC-MS) analyses of culture supernatants obtained from the individual tunFGKLMN mutants confirmed retention of tunicamycin biosynthesis (very low for tunF), while supernatants from the mutants lacking antimicrobial activity failed to show the typical characteristic tunicamycin mass spectrum of the wild-type gene cluster (see reference 7; data not shown).

To confirm that the loss of antibiotic production in the tunACDEH mutants and the reduction in the tunFGK deletion strains reflected the in-frame deletion of individual genes, and not polar effects on the expression of downstream genes, PCR fragments containing each of the deleted genes were cloned individually in pIJ12551 (tunAC-DEGHK) or in pSET152 (tunF) under the control of the constitutive ermE promoter and introduced into the S. coelicolor derivative containing the corresponding mutated tun gene cluster. With the exception of tunA (see Discussion), antimicrobial activity against *B. subtilis* was restored in the nonproducing strains and was markedly increased in the tunFGK mutants (Fig. 4), confirming that the loss or reduction of antibiotic activity did indeed reflect the inactivation of individual genes.

**FIG 4** Bioassays of *S. coelicolor* M1152 derivatives containing a mutated tun gene cluster together with the empty vector pRT802 (left) or complementation construct (right). M1152 derivatives containing pJ12003a or pRT802 as positive and negative controls, respectively, are also shown (center bottom). N/A, not applicable.
To identify possible biosynthetic intermediates, culture supernatants and acid extracts of the mycelia of the tunCDEH mutants were also assessed by mass spectrometry for the production of the predicted biosynthetic intermediates that would be expected to accumulate based on the proposed biosynthetic pathway. However, none of the predicted molecular ions could be detected (data not shown). A comparative untargeted metabolomics analysis (11) of these strains also failed to show differential accumulation in any of the mutants of any metabolites with masses compatible with tunicamycin-related molecules.

An attempt was then made to identify the production of tunicamycin biosynthetic intermediates by cross-feeding and coculture experiments, using all possible pairwise combinations of mutants (including the tunB mutant) in which antibiotic activity had been lost. Bioactivity was assessed by overlaying the cross-feeding and coculture plates with a lawn of B. subtilis cells. In no case was antibiotic production restored (data not shown).

Immunity to tunicamycin. In addition to making in-frame deletion mutations in the tun genes thought to be involved in tunicamycin biosynthesis, we also attempted to make similar mutations in tunIJ, encoding a putative ABC transporter and believed to be required for export of the intracellular antibiotic. Attempts were made to make in-frame deletions of tunIJ together and of each gene individually, and the effect of the mutations on tunicamycin production was assessed as before, using B. subtilis EC1524 as the indicator strain. In contrast to the other tun gene mutations, exconjugants with deletions of tunIJ or tunI took up to 2 weeks to emerge and were few in number.

When both genes were deleted simultaneously, two mutant phenotypes were obtained, a marked reduction in antibiotic activity (the ΔtunIJ-A phenotype) and a complete loss of activity (the ΔtunIJ-B phenotype). Introduction of pIJ12551::tunIJ into each of the mutants had no significant effect on their phenotypes (Fig. 5a).

Sequencing of the tun gene cluster in the ΔtunIJ-A mutant revealed insertion of a T toward the end of tunG (immediately after the codon for aspartate 171 of the 203-amino acid [aa] TunG) that would result in the production of a TunGH fusion protein. While earlier deletion of tunG reduced tunicamycin production, mutation of tunH, which encodes a putative UDP-tunicamyl-uracil pyrophosphatase, abolished it. Thus, the frameshift mutation may have resulted in a fusion protein with little or no TunH activity and may have also markedly reduced or abolished translation initiation at the natural tunH start codon, thus resulting in markedly reduced levels of tunicamycin production.

Sequencing of the tun gene cluster in the ΔtunIJ-B mutant revealed a frameshift mutation in tunD, which is predicted to encode the glycosyltransferase required for addition of an N-acetylglucosamine moiety to tunicamyl-uracil. The insertion of a G after the codon for threonine 279, while leaving glycine 280 and proline 281 unchanged, would replace the C-terminal 191-aa residues of the 472-aa TunD protein with 107 residues of presumably nonfunctional protein (see Fig. S1 in the supplemental material), likely accounting for the lack of bioactivity and failure to complement the mutation.

In-frame deletion of tunI alone yielded the same two mutant phenotypes (A and B, marked reduction in and complete loss of antibiotic activity, respectively) observed as when tunI and tunJ were deleted simultaneously; introduction of pIJ12551::tunI into each of the mutants had no significant effect on their phenotypes (Fig. 5b). Sequencing of the tun gene cluster in the ΔtunIJ-A mutant revealed a single nucleotide change, a T to C transition found four nucleotides upstream of the GTG start codon of tunA. To confirm that this mutation was indeed responsible for the low level of antibiotic activity, this same mutation was introduced into pIJ12003a by replacing the 2.4-kb SacI-Stul region of the wild-type tun gene cluster with a PCR fragment generated from the mutated plasmid. The resulting construct (pBDW177) was confirmed by sequencing and introduced into S. coelicolor M1152, whereupon it gave the same phenotype as the tunI-A mutant, i.e., a much-reduced level of antimicrobial activity (see Fig. S2 in the supplemental material). Sequencing of the tun gene cluster in the ΔtunIJ-B mutant
revealed a G to A missense mutation in tunC that would result in a Gly to Asp substitution at position 70 of the putative 318-aa N-acyltransferase, presumably resulting in loss of enzyme function and lack of tunicamycin production.

In-frame deletion of tunJ alone resulted in loss of antibiotic production that could not be complemented by introduction of pIJ12551::tunJ (Fig. 5c); subsequent sequencing of the tun gene cluster in two of these possibly clonal mutants revealed the insertion of a copy of IS10 toward the end of tunD (one nucleotide after the codon for glycine 436 of TunD) that must have occurred when pIJ12003a was passaged through E. coli for mutant construction. Insertion of IS10 occurred after nucleotide 12871 of GenBank accession number HQ172897 and resulted in the duplication of residues

FIG 5 Bioassays of agar plugs of S. coelicolor M1152 containing the wild-type tun gene cluster (pIJ12003a), and failed attempts to complement the tunI, tunJ, and tunI mutants with wild-type versions of the deleted gene(s). In each case, B. subtilis EC1524 was used as the indicator strain. pIJ12551, the empty vector used in the complementation experiments. In panel B, note that while the pIJ12003a ΔtunI-A mutant clone generally gave a small zone of inhibition (see Fig. S2 in the supplemental material), it failed to do so in this particular assay.
of the S. coelicolor M1152 exconjugants containing the tunJ deletion was normal for an E. coli-Streptomyces conjugation, in contrast to the prolonged period required for emergence of the tunI suppressor mutants, and was consistent with insertion of IS10 into tunD in E. coli.

In all five cases, the unexpected mutations were presumably not only responsible for the lack of bioactivity, but also for the failure to complement the tunIJ mutants with wild-type copies of the genes.

Both tunIJ and tunM confer immunity to tunicamycin in S. coelicolor. The results obtained above suggested that in addition to playing a role in the export of tunicamycin, tunIJ also played a role in conferring immunity to the antibiotic in the producing organism, and that their deletion resulted in lethality or the selection of mutations that abolished or markedly reduced the level of tunicamycin production. To assess the potential role of these genes in immunity, S. coelicolor derivatives containing the wild-type tun gene cluster and derivatives from which tunIJ, tunK, tunL, tunM, or tunN had been deleted were plated as lawns on R5 agar, and their susceptibility to tunicamycin was assessed. While the wild-type gene cluster conferred complete immunity to exogenous tunicamycin, deletion of tunIJ and, surprisingly, of tunL and tunM, resulted in increased sensitivity, although all three deletion mutants were noticeably more resistant than the strain containing the empty vector (pRT802) (Fig. 6a). Deletion of tunK or tunN had no effect on susceptibility to tunicamycin.

To assess whether expression of tunIJ, tunL, or tunM alone could confer immunity to tunicamycin in S. coelicolor, the pIJ12551 expression constructs containing each of the genes that had been used in the earlier complementation assays were introduced into S. coelicolor M1152 by conjugation, and the resulting strains were used in tunicamycin susceptibility assays. Expression of either tunIJ or tunM from the ermE* promoter conferred complete immunity, while expression of tunL resulted in the same level of susceptibility as that of the vector control (Fig. 6b). We therefore assume that the enhanced susceptibility to tunicamycin shown by the tunL deletion mutant observed above reflected a polar effect on tunM expression.

DISCUSSION

Although the presence of promoter elements within the cluster cannot be excluded, the results presented in this paper suggest that all of the genes in the tunicamycin biosynthetic gene cluster are cotranscribed in S. chartreusis from two promoters, one of which, tunp2, is not utilized in S. coelicolor. The latter presumably reflects elements of regulation that are missing in the heterologous host or the absence of cis-acting regulatory sequences upstream of tunp2 in the cloned SacI fragment that are required for its activation. With the exception of the 5’ tunA, all of the genes required for wild-type levels of tunicamycin biosynthesis appear to be translationally coupled to the preceding gene. It is conceivable that such an arrangement ensures near stoichiometric production of each of the corresponding proteins, which, interestingly, would be consistent with the production of a large multifunctional enzyme complex. Given that tunIJ also exhibit this coupling, it is also conceivable that such a complex could be located at the cell membrane. Its existence would also be consistent with the inability to detect biosynthetic intermediates predicted to accumulate in the tunCDEH mutants either by mass spectrometry analysis or by cross-feeding, i.e., the lack of any one component could result in inactivation of the entire complex (or indeed of a subcomplex).

Repeated attempts to complement the tunA mutant using both the ermE* and native tunA promoter (data not shown) failed. Similar observations were made for mibA, the 5’ gene in a polycistronic mRNA that is essential for microbisporicin biosynthesis in Microbispora corallina (Lucy Foulston, personal communication). It is conceivable that this reflects an essential element of posttranscriptional control that operates on the 5’ end of the tun operon transcript that is absent in the tunA deletion mutant.

The deletion analysis reported here and previously (8) has demonstrated that of the
14 genes contained within the tun gene cluster just six (tunABCDEH) are essential for tunicamycin production in S. coelicolor, each of which can be assigned a likely biosynthetic role (Fig. 7) (7, 8), while tunI and tunJ are required for immunity. Of the others, tunFGKLN all have putative roles also carried out by homologues involved in primary metabolism, potentially explaining their nonessentiality. This would require that the primary metabolic enzymes were capable of substituting for their Tun counterparts if the latter were indeed present in the hypothetical tunicamycin biosynthetic complex (see above); alternatively, the complex might only contain the essential enzymes, TunABCDEH. The presence of tunFGKN within the tun gene cluster may be to ensure adequate precursor supply and/or flux at the onset of tunicamycin production, feeding the likely critical C-C bond-forming step (tunFGN) and efficient acylation from the endogenous fatty acid pool during biosynthesis (tunKL) (see Fig. 7 and references 7, 8 for a detailed discussion of tunicamycin biosynthesis). Indeed, deletion of tunF, tunG, or tunK resulted in a reproducible reduction in antibiotic activity (Fig. 4). The ability of the tunL mutant clone to produce tunicamycin in S. coelicolor M1152 contrasts with the results reported by Chen et al. (12), where tunicamycin production was abolished when a tunL-deleted gene cluster was introduced into Streptomyces lividans TK24. However, since TunL is a putative phospholipid phosphatase with homologues...
encoded by the S. coelicolor genome (e.g., SCO1102 and SCO0402) that are likely involved in primary metabolism, it is conceivable that this disparity reflects differences in the heterologous hosts and growth conditions used in the two studies. Given the very low level of tunicamycin production in the tunF mutant, it is also possible that a homologous host epimerase (e.g., SCO3137 and/or SCO2988) is responsible for partial suppression of tunF ablation. The deletion analysis reported here rules out the previously assigned role for TunM in tunicamycin biosynthesis (8). However, the ability of tunM to confer resistance to tunicamycin in S. coelicolor, and the increased levels of tunicamycin susceptibility observed after deletion of tunM from the tun gene cluster in S. coelicolor, suggest that it may instead play a role in immunity in the producing organism. Intriguingly, TunM encodes a putative SAM-dependent methyltransferase and, although unprecedented to our knowledge, it is conceivable that it mediates its effect by modification of intracellular tunicamycin to provide an immunity mechanism, although such a nonreversible modification would require a corresponding demethylation for activation, perhaps after export. Conceivably, such demethylation enzymes might exist in the natural host’s key target organisms. Alternatively, self-resistance might be mediated by methylation of endogenous enzyme (or other molecular) targets of the antibiotic (or indeed by modification of one of their substrates in a manner that would afford sufficient substrate protection to prevent tunicamycin from binding to any corresponding complex).
MATERIALS AND METHODS

Strains and general methods. The strains used in this study are listed in Table 1. Escherichia coli strains were grown and manipulated following standard methods (14, 23), with E. coli DH5α (15) used as the general cloning host. Bacillus subtilis EC1524 (13) was grown in Luria Bertani broth (23). Streptomyces strains were grown and manipulated as described previously (7, 24). Plasmids and oligonucleotides are described in Tables 1 and S1, respectively. Tunicamycin was obtained from Abcam Biochemicals and dissolved in 100% dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml prior to use.

Transcriptional analysis. RNA was prepared from lawns of mycelium approximately 30 mm in diameter of S. coelicolor M1522/pIJ12003a and S. chartreusis cells grown for 2 days on R5 and DNA agar (18, 24), respectively, using a bead-beater and a Qiagen RNeasy kit (Qiagen, Crawley, United Kingdom). RT-PCR analysis was carried out on the S. coelicolor RNA sample; cDNA was prepared using a Qiagen RT-PCR kit (version 2.0; Invitrogen, Paisley, United Kingdom) following the manufacturer’s instructions. Briefly, first-strand cDNA synthesis was carried out using 5 μg of RNA, reverse transcriptase and the oligonucleotide primer RACE1 (Table S1). cDNA was purified using the SNAP columns provided in the kit, and poly(dC) tails were added.

| Strain or plasmid | Description | Reference and/or source |
|-------------------|-------------|-------------------------|
| **Strains**       |             |                         |
| B. subtilis EC1524| Bioassay strain | 13                      |
| E. coli BW25113/pIJ790| E. coli containing λ Red plasmid | 14          |
| E. coli DH5α      | General cloning host | 15                      |
| E. coli BT340     | FLP recombinase strain | 14                      |
| E. coli ET12567/pIJ28002 | Conjugation of plasmids into S. coelicolor M1522 | 16          |
| E. coli ET12567/pR9406 | Conjugation of plasmids into S. coelicolor M1522 | 17; David Figurski, personal communication |
| S. coelicolor M1522| Heterologous expression strain | 18                      |
| **Plasmids**      |             |                         |
| pBlueScript II KS | General cloning vector | Agilent Technologies |
| pGUS              | β-glucuronidase reporter plasmid | 9                       |
| pIJ773            | PCR template for apramycin resistance cassette | 14                      |
| pBluescript KS     | λBT1 integrative vector; used as source of ermE’p | 19                      |
| pIJ12003a         | pRT802 containing the tun cluster on a 12.9 kb SacI fragment | 7                       |
| pIJ12541          | pIJ12003a with tunB deleted | 8                       |
| pIJ12551          | ΔC31 integrative expression vector with ermE’ promoter | 20                      |
| prRT802           | ΔBT1 integrative vector | 21                      |
| pSET152           | ΔC31 integrative vector | 22                      |
| pBDW91            | Effectively pIJ12003a with tunA deleted | This study |
| pBDW92            | Effectively pIJ12003a with tunC deleted | This study |
| pBDW36            | Effectively pIJ12003a with tunD deleted | This study |
| pBDW37            | Effectively pIJ12003a with tunE deleted | This study |
| pIJ12542          | pIJ12003a with tunF deleted | This study |
| pBDW38            | Effectively pIJ12003a with tunG deleted | This study |
| pBDW39            | Effectively pIJ12003a with tunH deleted | This study |
| pBDW40            | Effectively pIJ12003a with tunI deleted | This study |
| pBDW41            | Effectively pIJ12003a with tunJ deleted | This study |
| pBDW42            | Effectively pIJ12003a with tunL deleted | This study |
| pBDW43            | Effectively pIJ12003a with tunK deleted | This study |
| pBDW44            | Effectively pIJ12003a with tunM deleted | This study |
| pBDW45            | Effectively pIJ12003a with tunN deleted | This study |
| pBDW46            | Effectively pIJ12003a with tunO deleted | This study |
| pBDW58            | pIJ12551 tunA complementation construct | This study |
| pBDW59            | pIJ12551 tunC complementation construct | This study |
| pBDW60            | pIJ12551 tunD complementation construct | This study |
| pBDW61            | pIJ12551 tunE complementation construct | This study |
| pIJ12544          | pSET152 ermE’p::tunF complementation construct | This study |
| pBDW62            | pIJ12551 tunG complementation construct | This study |
| pBDW65            | pIJ12551 tunH complementation construct | This study |
| pBDW66            | pIJ12551 tunI complementation construct | This study |
| pBDW155           | pIJ12551 tunK complementation construct | This study |
| pBDW153           | pIJ12551 tunL complementation construct | This study |
| pBDW177           | pIJ12003a carrying the tunL-A mutation in tunA | This study |
to the 3’ ends using terminal deoxynucleotidyl transferase. PCR amplification of the tailored cDNA was initially carried out using the 5’ RACE abridged anchor primer with the first-strand primer RACE2 or RACE4 (Table S1). A dilution of the PCR mixture was then subjected to a second amplification using the abridged anchor primer with the second nested primer RACE3 or RACES (Table S1). The PCR product was gel-purified and a portion sequenced directly using the oligonucleotide RACE3 or RACE5 as primer.

**Gus assays.** The DNA fragments to be assessed for promoter activity were cloned individually as XbaI-KpnI PCR fragments in pGUS (9) and cleaved with the same two restriction enzymes, and the resulting constructs were introduced into *S. coelicolor* M1152, M145, and M571 (*ΔrelA* mutant of M145; see reference 25) by conjugation, whereupon they integrated at the chromosomal ϕC31 attB sites of each strain. The ability of the cloned fragments to direct transcription of the uidA gene encoding β-glucuronidase (Gus) was determined by plating the exconjugants on RS and SMM5 agar medium (24) containing 0.16 mg/ml X-gluc (5-bromo-4-chloro-3-indol-3-yl beta-o-glucopyranosiduronic acid) (cyclohexylammonium salt; Gold Biotechnology).

**Construction of deletion mutants.** To construct the *tunACDEGHJKLMN* mutants, the 12.9-kb SalI fragment of pU12003a, a derivative of pRT802 (21), was first subcloned into pBlueScript II KS to give pBDW7. Gene deletions were made using the method of Gust et al. (2003) (14) by targeting pBDW7 with apramycin resistance gene (apr) replacement cassettes generated using the PCR primers listed in Table S1 and pIJ773 as the template DNA, and the cassettes were subsequently deleted using FLP recombinase to give in-frame deletion mutants; in all cases, the ribosome-binding site of the downstream gene was retained in the mutant construct. The SalI fragments from the resulting plasmids were subcloned into pRT802, and those with the fragment inserted in the same orientation as in pU12003a (determined by restriction enzyme digestion) were selected for further study. To construct the *tunF* mutant, pU12003a was targeted in the same manner (see Table S1 for the primers used) to yield the mutant derivative pIJ12542. Each of the mutated plasmids was introduced into *E. coli* ET15267/pR906 (17; David Figurski, personal communication) by transformation and then into *S. coelicolor* M1152 by conjugation.

**Complementation of deletion mutants.** A PCR fragment was generated for selected deleted *tun* genes using the primers in Table S1, pIJ12003a DNA as the template, and Phusion High Fidelity DNA polymerase (New England BioLabs Inc.). The PCR products for genes using the primers in Table S1, pIJ12003a DNA as the template, and Phusion High Fidelity DNA polymerase were ligated into pSET152 (32) that had been similarly treated to give pSET152 (22) to yield the mutant derivative pIJ12544. All of the individual complementation constructs, which were confirmed by DNA sequencing, were introduced into *E. coli* ET15267/pUZ8002 (16) by transformation and then into the appropriate *S. coelicolor* deletion strain by conjugation.

**Bioassays for tunicamycin production.** Lawns of the strains to be assayed were made by spreading 105 spores in 100 μl of water on RS agar plates, followed by incubation at 30°C for 48 h. Soft nutrient agar (SNA) was melted, cooled to 55°C, and inoculated with a one-tenth volume of a mid-logarithmic growth culture of *B. subtilis* EC1524. Cylindrical plugs (approximately 8 mm in diameter) were cut from the Streptomyces lawns using a cork borer and were either set into or laid on top of 40 ml of the SNA inoculated with *B. subtilis* EC1524 in a 10-cm2 plastic petri dish, which was then incubated overnight at 30°C.

**Analysis of immunity to tunicamycin.** Lawns of the strains to be assayed were made by spreading 105 spores in 100 μl of water onto RS plates, which were allowed to dry for 20 min. Filter paper discs with various amounts of tunicamycin (dissolved in DMSO) were laid onto the lawns, and the plates were incubated at 30°C for 48 h.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00130-18.

**SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.**

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