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RESEARCH ARTICLE

Characterization of the tunicamycin gene cluster unveiling unique steps involved in its biosynthesis

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ABSTRACT

Tunicamycin, a potent reversible translocase I inhibitor, is produced by several *Actinomycetes* species. The tunicamycin structure is highly unusual, and contains an 11-carbon dialdose sugar and an *α, β*-1″,11′-glycosidic linkage. Here we report the identification of a gene cluster essential for tunicamycin biosynthesis by high-throughput heterologous expression (HHE) strategy combined with a bioassay. Introduction of the genes into heterologous non-producing *Streptomyces* hosts results in production of tunicamycin by these strains, demonstrating the role of the genes for the biosynthesis of tunicamycins. Gene disruption experiments coupled with bioinformatic analysis revealed that the tunicamycin gene cluster is minimally composed of 12 genes (*tunA*–*tunL*). Amongst these is a putative radical SAM enzyme (Tun B) with a potentially unique role in biosynthetic carbon-carbon bond formation. Hence, a seven-step novel pathway is proposed for tunicamycin biosynthesis. Moreover, two gene clusters for the potential biosynthesis of tunicamycin-like antibiotics were also identified in *Streptomyces clavuligerus* ATCC 27064 and *Actinosynnema mirums* DSM 43827. These data provide clarification of the novel mechanisms for tunicamycin biosynthesis, and for the generation of new-designer tunicamycin analogs with selective/enhanced bioactivity via combinatorial biosynthesis strategies.

KEYWORDS tunicamycin, biosynthetic gene cluster, high-throughput heterologous expression, bioassay, combinatorial biosynthesis

INTRODUCTION

Tunicamycins (Fig. 1) are a group of structurally related nucleoside antibiotics produced by several *Actinomycetes* species (Tsvetanova and Price, 2001; Tsvetanova et al., 2002; Price and Tsvetanova, 2007). The structure of tunicamycins consists of uracil, *N*-acetylglucosamine (GlcNAc), a unique 11-carbon 2-aminodiadose sugar (tunicamine), and an *N*-acyl chain with variable lengths (Tsvetanova and Price, 2001; Price and Tsvetanova, 2007). Structurally, the naturally occurring tunicamycins family antibiotics, including mycospocidins (Eckardt et al., 1975), streptovirudins (Eckardt et al., 1975; Keenan et al., 1981), MM 19290 (Kenig and Reading, 1979) and corynetoxins (Vogel et al., 1981) are all similar except the difference in the *N*-acyl chain or/and replacement of 5,6-dihydrouracil for the uracil moiety (Fig. S1) (Eckardt, 1983; Price and Tsvetanova, 2007).

As a reversible, competitive translocase I inhibitor, tunicamycin targets bacterial cell wall biosynthesis by inhibition of phospho-UDP-N-acetylmuramoyl-pentapeptide translocase (MraY, Translocase I) which catalyzes an early stage of peptideglycan biosynthesis (Kimura and Bugg, 2003; Xu et al., 2004; Price and Momany, 2005; Winn et al., 2010). Unlike many other nucleoside antibiotics, tunicamycin

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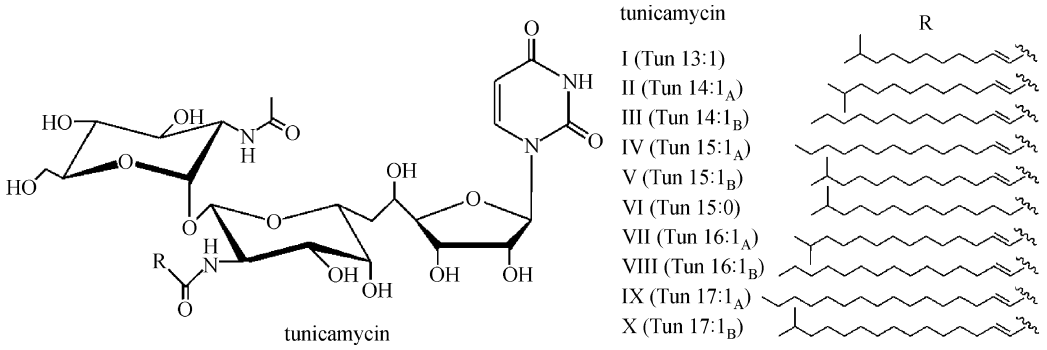

Figure 1. Chemical structures of tunicamycins. Different components are designated as tunicamycin I–X, which correspond to components of Tun13:1–17:1$_B$ as indicated in parentheses, and the latter nomination was adopted in this paper.

displayed high toxicity to mammalian, which precludes its use as antibacterial agent (Kimura and Bugg, 2003; Winn et al., 2010). This is because in eukaryotes the tunicamycins mimic the transition state analog (GlcNAc-1-P) of UDP-*N*-acetylglucosamine dolichol phosphate GlcNAc-1-P transferase (GPT), that catalyzes the first step in *N*-linked protein glycosylation (Winn et al., 2010). The capabilities of inhibiting the post-translational glycosylation of proteins make tunicamycin a useful tool in the study of protein glycosylation (Winn et al., 2010). Moreover, tunicamycins have also been shown to block *N*-palmitoylation of acyl-proteins by inhibiting palmitoyltransferase (Patterson and Skene, 1994; Liang et al., 2002; Price and Tsvetanova, 2007).

Recent investigations performed with isotope feeding experiments have proposed a tentative pathway for tunicamycin biosynthesis (Tsvetanova et al., 2002). For all that, little is known on the precise molecular mechanism for the tunicamycin biosynthesis which must account for the biosynthesis of the unusual 11-carbon sugar, tunicamine, and the *α, β*-1″,11′-glycosidic linkage (Tsvetanova et al., 2002). These labeling experiments showed that tunicamine is formed from 6- and 5-carbon carbohydrate precursors, rather than by sequential addition of 2-carbon units.

Here we describe the cloning of the tunicamycins biosynthetic gene cluster by utilization of high-throughput heterologous expression (HHE) strategy combined with bioassay. The production of tunicamycins in heterologous non-producing *Streptomyces* strains demonstrated the role of 12 genes essential for tunicamycin biosynthesis, thereof leading to a proposed pathway for tunicamycin biosynthesis. Two potential gene clusters related to the biosynthesis of the (potential) tunicamycin-like antibiotics were also identified from *Streptomyces clavligerus* ATCC 27074 (*S. clavligerus* hereafter) and *Actinosynnema mirum* DSM 43827 (*A. mirum* hereafter). The availability of the tunicamycin biosynthetic gene cluster will provide a foundation for mechanistic investigations into tunicamycin biosynthesis, and pave the way for rational generation of novel tunicamycin derivatives via the strategies of combinatorial biosynthesis and pathway engineering.

RESULTS

Identification of the tunicamycin biosynthetic gene cluster from *S. chartreusis*

The unique structural features within tunicamycin render direct cloning of its gene cluster both challenging and unfeasible. Unlike classical natural products including polyketides and nonribosomal peptides, there were no distinctive conserved genes that could be used as optimal probes to screen the genomic library of *S. chartreusis*.

The genome information of *S. clavuligerus* ATCC27064, a previously reported tunicamycin-like antibiotic (MM 19290) producer (Kenig and Reading, 1979), was released online in 2009. It seems to present us with the potential hopes for the identification of the tunicamycin gene cluster; however, efforts with bioinformatic and genetic analysis of the genome data were unsuccessful because of the paucity of the knowledge on tunicamycin biosynthesis (Price and Tsvetanova, 2007; Medema et al., 2010).

We therefore developed an alternative approach. A HHE strategy combined with bioassay was utilized to screen pJTU2463-derived genomic library (see Experimental Procedures). Three of the *S. lividans* TK24 recombinants (11C3, 11D8 and 12G1) were identified as showing obvious inhibition against the indicator strain *Bacillus subtilis* (Fig. S2), indicating the conferred capability of producing bioactive metabolites. Furthermore, the three positive cosmids digested by *Bam*HI were revealed to show significant overlap (Fig. S2). Bioinformatic analysis of the sequenced double ends of the *Bam*HI-derived fragments (cloned in counterpart site of pIJ2925) of 11C3 showed that the polypeptides encoded by related open reading frames exhibit high homologies to those in the genome of *S. clavuligerus* (data not shown). These data implied that the three positive cosmids probably possess the entire tunicamycin biosynthetic gene cluster which conferred the TK24 strains with the

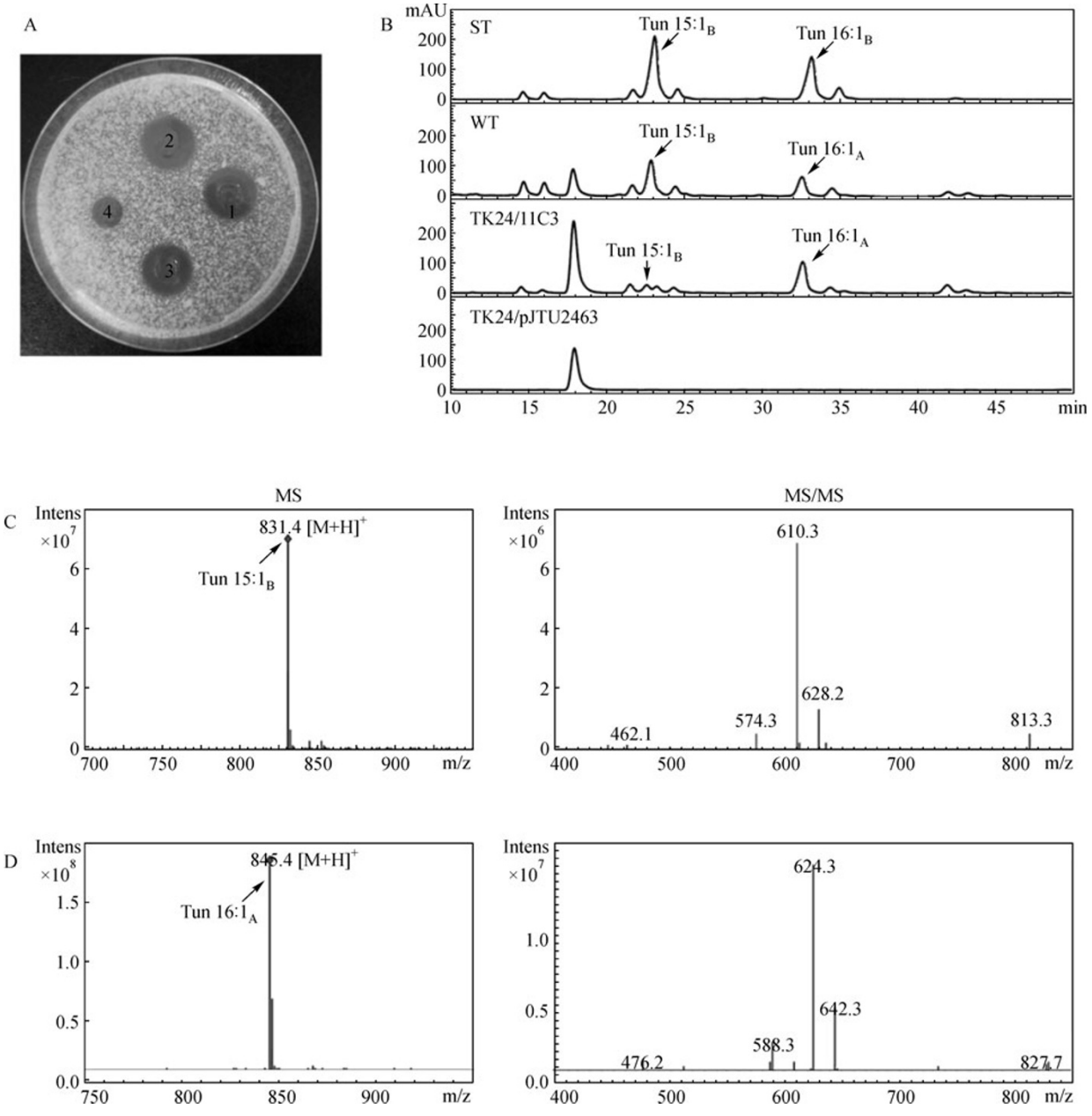

Figure 2. Heterologous production of tunicamycin in *S. lividans* TK24. (A) Bioassay of the metabolites produced by the TK24 recombinant. *Bacillus subtilis* was used as indicator strain, and a volume of 40 μL samples (except that 20 μL 1.0 mg/mL of tunicamyicn authentic standard was used in this assay) solved in methanol were used for each assay in this study. Recombinants (*S. lividans* TK24 carrying 11C3) spotted in (3) was compared with wild-type *S. chartreusis*(2) and tunicamycin authentic standard (1) as a positive controls (1) and *S. lividans* TK24/pJTU2463 as a negative control (4). (B) HPLC analysis of the metabolites produced by the engineered TK24 recombinant. Tunicamycin authentic standard (ST), and samples from wild-type of *S. chartreusis* (WT), *S. lividans* TK24/11C3 (TK24/11C3), and *S. lividans* TK24/pJTU2463 (TK24/pJTU2463), were compared. (C) MS and MS/MS analysis of the Tun 15:1_B component from the sample of TK24/11C3. The $[M + H]^+$ ion at *m/z* 831.4 of Tun 15:1_B component was fragmented into 610.3, 628.2, 574.3, *etc*. (D) MS and MS/MS analysis of the Tun 16:1_A component from the sample of TK24/11C3. The $[M + H]^+$ ion at *m/z* 845.4 of Tun 16:1_A component was fragmented into 624.3, 642.3, 588.3, *etc*. The MS and MS/MS profiles and fragmentation pattern of Tun 15:1_B and Tun 16:1_A of the tunicamycin authentic standard were indicated in Fig. S4.

capabilities of producing tunicamycin.

Heterologous production of tunicamycin in non-producer *S. lividans* TK24

To demonstrate that the TK24 recombinants (individually containing 11C3, 11D8 and 12G1) could make tunicamycins, they were inoculated in TSBY liquid medium for fermentation. Samples of the TK24 recombinants were prepared and subjected for the bioassay. These results indicated that all three of the TK24 recombinants display obvious bioactivity against the indicator strain *Bacillus subtilis*, in full agreement with the previous bioassay experiments. Moreover, the sample of the TK24/11C3 showed similar bioactivity against the indicator stain to that of wild type *S. chartreusis* (Fig. 2A).

For further identification of the bioactive metabolites produced by the TK24 recombinants (independently containing 11C3, 11D8 and 12G1) as tunicamycin, extracts of the TK24 recombinants were subjected for HPLC analysis. The results confirmed that the TK24 recombinants gave rise to characteristic tunicamycin peaks with retention times of 22.6 min (Tun 15:1$_B$) and 32.6 min (Tun 16:1$_A$) (Fig. 2B and Fig. S3). These corresponded to HPLC peaks produced by the tunicamycin authentic standard, and the sample from wild type *S. chartreusis*, while the negative control, *S. lividans* TK24/pJTU2463 lacked these characteristic peaks (Fig. 2B). Furthermore, the ratio of the major components of Tun 15:1$_B$ and Tun 16:1$_A$ is ca. 3:2, whereas the major component produced by the TK24/11C3 recombinant was shown to be Tun 16:1$_A$, whose production is 17.0 μg/mL (crude broth). Hence this is 1.56 fold more than that produced by the wild type strain.

To confirm the identities of the tunicamycin components, the two peaks of the TK24/11C3 sample and tunicamycin authentic standard were independently subjected for MS and MS/MS assessment. The results indicated that the peak of Tun 15:1$_B$ give a $[M + H]^+$ ion at *m/z* 831.4, which was fragmented into the ions at *m/z* 610.3, 628.2, 574.3, and 462.1 (Fig. 2C). Likewise, the Tun 16:1$_A$ peak generated a $[M + H]^+$ ion at *m/z* 845.4, and MS/MS analysis showed that the main fragment ions were at 624.3, 642.3, 588.3, and 476.2 (Fig. 2D). The 14-Da mass difference between these two sets of ions is due to the -CH_2- difference between $C_{16:1}$ and $C_{15:1}$ *N*-acyl chains. Moreover, all of these ions were fully consistent with the MS and MS/MS pattern of the two peaks of the tunicamycin authentic standard (Fig. S4), unambiguously demonstrating the identities of the two components produced by TK24/11C3 recombinants as Tun 15:1$_B$ and Tun 16:1$_A$.

LC/MS and Q-TOF MS analysis of the tunicamycin profiles of related *Streptomcyes* recombinants

To see if 11C3 could confer other *Streptomyces* (*S. avermitilis* and *S. albus*) with the capability of producing variable tunicamycin profiles, extracts of the two recombinants were subjected for bioassay and HPLC analysis. These results showed that the extracts of the recombinants were able to display similar bioactivities against the indicator strain, *Bacillus subtilis*, to that of TK24 recombinant. Moreover, HPLC results also showed somewhat variable tunicamycin profiles among the three extracts (Fig. S5).

MS/MS fragmentation of tunicamycins has been detailed previously (Tsvetanova and Price, 2001; Tsvetanova et al., 2002). Fragmentation of molecular adduct ions give rise to $[M-221]^+$ ions by neutral loss of the *N*-acetylglucosaminyl group (Fig. S4). Because these retain the *N*-acyl substituent, these are characteristic of individual tunicamycins. Further fragmentation involves a neutral loss of the uracil motif and cross-ring fragmentation of the tunicamine sugar backbone, to generate an *N*-acyloxy ion that is characteristic of the tunicamycin *N*-acyl group (Tsvetanova et al., 2002). Hence, *m/z* 638, 624, 610, and 596 are characteristic of Tun17:1, Tun16:1, Tun15:1, and Tun14:1, respectively, and give rise to corresponding *N*-acyloxy ions at *m/z* 251, 237, 223, and 209. These masses correspond to unsaturated $C_{17:1}$–$C_{14:1}$ acyl chains, as shown in Fig. 1. Selective ion monitoring of these ions established the tunicamycin profiles for the transconjugate strains (Fig. S6).

Sequence analysis of the tunicamycin gene cluster

A contiguous 37,699-bp region arising from sequencing the positive cosmid 11C3 (GenBank accession number HQ111437), harbors G + C content of 67.81% (while a SacI fragment containing the minimal tunicamycin gene cluster contains G + C content of 64.92%) (Fig. S7), somewhat lower than that of the typical *Streptomyces* genomes exemplified by *Streptomyces coelicolor* A3(2) (Bentley et al., 2002) and *Streptomycs avermitilis* (Omura et al., 2001), implying the probable non-actinomycial origin of the tunicamycin gene cluster. Frameplot 3.0 Beta online program analysis revealed 39 complete Open Reading Frames (orfs) in sequenced region (cosmid 11C3) as shown in Table 1, Fig. 3 and Fig S7. Of them, 12 *orfs* (designated *tunA*–*tunL*) in one transcriptional unit constitute the gene cluster involved in tunicamycin biosynthesis (Fig. 3).

As proposed, the tunicamycin biosynthetic gene cluster would start with *tunA*, which encodes a probable NAD-dependent epimerase/dehydratase. This gene shows high homology (42% identity, 58% positives) to SSAG-01084 of *Streptomyces* sp. Mg1. The second gene, *tunB*, encodes a protein exhibiting homology (32% identity, 48% positives) to Htur_3864, a radical SAM domain protein of *Haloterrigena turkmenica* DSM 5511. Furthermore, TunB also shows homology with 33% identity to coenzyme PQQ synthesis protein E (PqqE) of *Mycobacterium abscessus* ATCC 19977. This family of enzymes typically uses a radical-catalyzed

Table 1 Deduced functions of the open reading frames in the *tun* gene cluster

protein	aa	proposed function	homolog, origin	identity, similarity (%)	accession no.
A	321	NAD-dependent epimerase/dehydratase	SSAG_01084, *Streptomyces* sp. Mg1	42,58	ZP_04996782.1
B	338	radical SAM protein	Htur_3864, *Haloterrigena turkmenica* DSM 5511	32,48	YP_003405396.1
C	318	GCN5-related N-acetyltransferase	Fnod_1042, *Fervidobacterium nodosum* Rt17-B1	33,50	YP_001410548.1
D	472	glycosyl transferase	Mmc1_1923, *Magnetococcus* sp. MC-1	26,42	YP_865837.1
E	234	de-N-acetylase	CRC_02908, *Cylindrospermopsis raciborskii* CS-505	43,57	ZP_06309433.1
F	327	UDPglucose 4-epimerase	POTG_01647, *Paenibacillus* sp. oral taxon 786 str. D14	46,63	ZP_04852226.1
G	203	phosphoglycerate mutase	Francci3_2350, Frankia sp. Ccl3	29,47	YP_481446.1
H	515	pyrophosphatase	Bcep18194_A6493, *Burkholderia* sp. 383	35,50	YP_370731.1
I	304	ABC transporter	SBI_04506, *S. bingchenggensis* BCW-1	61,75	ADI07626.1
J	262	ABC-2 transporter	Tter_0475, *Thermobaculum terrenum* ATCC BAA-798	32,51	YP_003322218.1
K	81	acyl carrier protein	Tbis_2371, *Thermobispora bispora* DSM 43833	39,58	YP_003652969.1
L	229	PA-phosphatase related protein	MicauDRAFT_2890, *Micromonospora aurantiaca* ATCC 27029	33,42	ZP_06217896.1

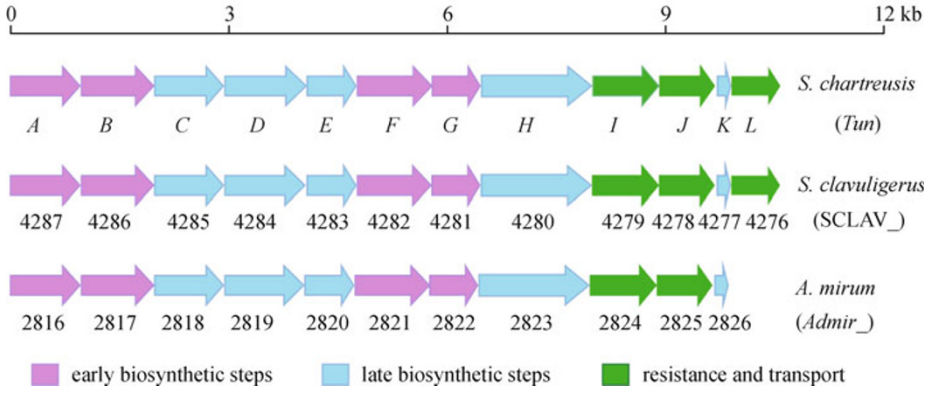

Figure 3. Genetic organization of the complete/partial tunicamycin biosynthetic gene clusters. The proposed functions of the *tun* genes were described in Table 1. The gene loci located in genomes of *S. clavuligerus* and *A. mirum* were utilized to nominate the target genes for (potential) tunicamycin biosynthesis.

mechanism to generate *de novo* carbon-carbon bonds. The third gene in the cluster, *tunC* encodes an enzyme showing 33% identity to Fond_1042, a GCN5-related N-acetyltransfrase of *Fervidobacterium nodosum* Rt17-B1 with broad substrates specificity. TunD shows weak homology (26% identity) to Mmc1_1923 of *Magnetococcus* sp. MC-1, which probably functions as a glyosyltransferase. Based on homology, it seems to be a unique class of glycosyltransferase with as yet uncharacterized catalytical mechanism. The gene *tunE* encodes a protein homologous to *N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase (LmbE) family protein of *Cylindrospermopsis raciborskii* CS-505. TunE

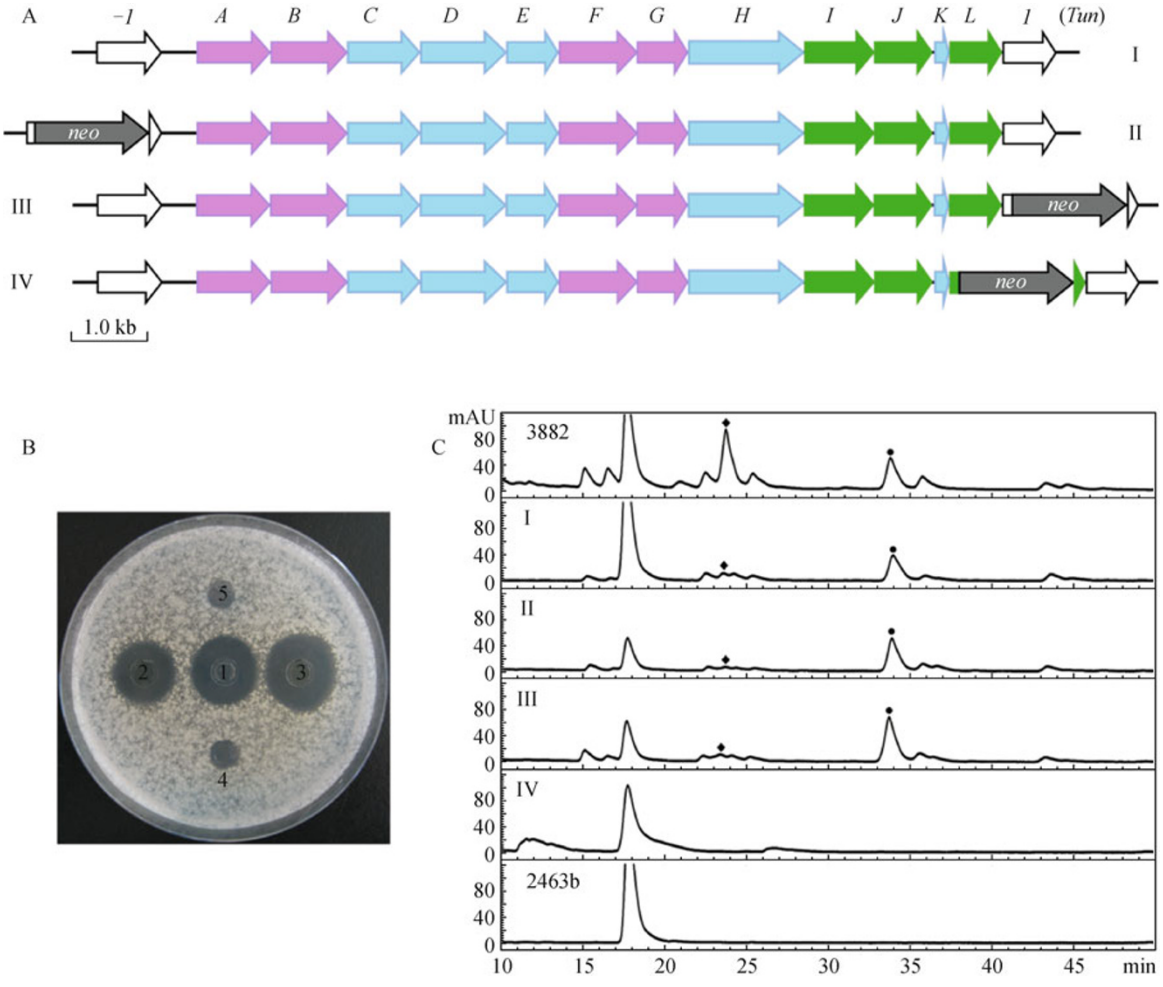

Figure 4. Determination of the minimal gene cluster of tunicamycin. (A) Schematic representation for the mutation of related gene so as to determine the boundaries of tunicamycin gene cluster. I: pJTU5751 wild type, II: pJTU5751 derivative with *orf-1* disrupted by *neo*, III: pJTU5751 derivative with *orf1* disrupted by *neo*, IV: pJTU5751 derivative with *tunL* disrupted by *neo*. (B) Bioassay of the metabolites produced by relevant TK24 recombinants carrying pJTU5751 with corresponding mutation in target genes. TK24 recombinant containing intact pJTU5751 was used as positive control (spot 1), and 2463b as negative control (spot 5). TK24 recombinants individually containing pJTU5751 with counterpart mutation of *orf-1, tunL* and *orf1* were indicated as spots 2, 4, and 3, respectively. (C) HPLC analysis of the metabolites produced by related TK24 recombinants. Wild-type of *S. chartreusis* (3882) and TK24 recombinant carrying pJTU5751 (I) were used as positive controls, and samples (II), (III) and (IV) correspond to those indicated by Fig. 4A. Sample of TK24 recombinant bearing pJTU2463b (2463b) was used as negative control. (◆): Tun 15:1$_B$, (●): Tun 16:1$_A$.

protein is therefore deduced to be responsible for removal of the tunicamine-uracil *N*-acetyl group, prior to addition of the *N*-acylsubstituent. TunF displays significant homology (46% identity, 63% positives) with POTG_01647, a UDPglucose 4-epimerase, of *Frankia*. The next gene, *tunG*, encodes a 203-aa protein that harbors 29% identity with the Francci3_2350 of *Frankia* sp. CcI3, which is likely to be a CobC-type alpha-ribazole-5′-phosphate phosphatase. The putative TunH enzyme also exhibits certain homology (35% identity) to Bcep18194_A6439 of *Burkholderia* sp. 383, which has been proposed to be a Type I phosphodiesterase/ nucleotidepyrophosphatase. As a result, TunH probably functions as a pyrophosphosphatase in the tunicamycin biosynthetic pathway.

Four genes *tunI-tunL* are sequentially located downstream of *tunH* (Fig. 3). Of these, *tunI* encodes a 304-aa protein with 61% identity to an ABC transporter (SBI_04506) of *Streptomyces bingchenggensis*. The protein encoded by *tunJ* presents 32% identity of homology to Tter_0475, an ABC-2 transporter of *Thermobaculum terrenum* ATCC BAA-798. More interestingly, the two genes (*tunI* and *tunJ*) correspondingly show homologies matched to the N terminus and C terminus of the protein encoded by *mtrA* (mithramycin resistance gene). As a result, we propose that these two

genes are involved in the self-resistance and transport of tunicamycin. According to bioinformatic analysis, *tunK* encodes an 81-aa distinctive protein, which exhibits homology (39% identity, 58% positives) to an acyl carrier protein (ACP) of *Thermobispora bispora* DSM 43833, implicating its unique role in the biosynthesis of tunicamycins. The gene *tunL* situated in the terminal of the tunicamycin gene cluster encodes a protein resembling a phosphoesterase PA-phosphatase-related protein (MicauDRAFT_2890) of *Micromonospora aurantiaca* ATCC 27029 with 33% identity. This protein is a super-family of phosphatases and haloperoxidases, which may act as a membrane-associated lipid phosphatase.

Determination of the minimal gene cluster essential for tunicamycin biosynthesis

According to bioinformatic analysis, *tunA-tunL* constitutes one operon, suggesting that *orf-1* is probably the left boundary of the tunicamycin gene cluster. For further confirmation, *orf-1* was directly disrupted in pJTU5751 by utilization of PCR-targeting technology (Gust et al., 2003), and the resultant pJTU5751 derivative (*orf-1* mutated) (Fig. 4A) was introduced into *S. lividans* TK24 for heterologous expression. After fermentation, extract of the recombinant was subjected for bioassay and LC/MS analysis, and the results showed that the extract of the TK24 recombinant was capable of both showing obvious bioactivity against *Bacillus subtilis*, and generating the distinctive peaks corresponding to those of the authentic standards of Tun $15{:}1_B$ and Tun $16{:}1_A$ (Fig. 4B and 4C), undoubtedly demonstrating that *orf-1* is not essential for tunicamycin biosynthesis. Moreover, based on the methods described as above, *tunL* and *orf1* were individually disrupted (Fig. 4A), and the generated phenotypes of resultant TK24 recombinants (bearing pJTU5751 derivatives with *orf1* or *tunL* disrupted) were investigated. The results indicated that mutation of *orf1* results in no influence on tunicamycin production (Fig. 4B and 4C), however, target inactivation of *tunL* directly leads to abolishment of tunicamycin production as indicated by bioassay and HPLC analysis (Fig. 4B and 4C). These data unambiguously define that *tunA-tunL* constitutes the minimal gene cluster of tunicamycin.

Comparative analysis of the (potential) gene clusters for tunicamycin-like family antibiotics biosynthesis in actinomycetes

From Blast searches and genome mining, two potential tunicamycin-like gene clusters were individually identified in the published genomes of *S. clavuligerus* 27064 and *A. mirum* DSM 43827 (Fig. 2). *S. clavuligerus* was previously reported to be capable of making a tunicamycin-like antibiotic (MM 19290) (Kenig and Reading, 1979) which harbors the same backbone as tunicamycin except for the attachment of different *N*-linked acyl chains, while the strain *A. mirum* DSM 43827 was not previously identified as a producer of tunicamycin-like antibiotics. Initially, several *A. mirum* strains including the DSM43827 were fermented to isolate the potential tunicamycin-like antibiotics; however, these efforts were subsequently demonstrated to be negative (data not shown).

Bioinformatic analysis revealed that the three gene clusters have identical genetic organization as indicated in Fig. 3. Interestingly, the *tunL* homolog was missing in the potential gene cluster of *A. mirum* DSM 43827, which may partly account for the lack of tunicamycin production by this strain. In addition, the sequence homologies among the three gene clusters were relatively low (Table S3) compared with the gene clusters of validamycin, which was produced by several *Streptomyces*, for example (Yu et al., 2005; Bai et al., 2006; Jian et al., 2006; Singh et al., 2006). Remarkably, there are no discernable nucleotide homologies in two tunicamycin gene clusters of *S. chartreusis* and *S. clavuligerus* (Table S3).

In-frame deletion of *tunA* directly results in abolishment of tunicamycin production

The gene *tunA* represents the start of the tunicamycin gene cluster. To obtain genetic evidence that *tunA* was indispensible for tunicamycin biosynthesis, this gene in pJTU5751 was mutated by in-frame deletion with PCR targeting technology (Gust et al., 2003) (Fig. 5A). After that, the resultant pJTU5751/Δ*tunA* was conjugated into *S. lividans* TK24, and the transconjugants validated by PCR were inoculated for further fermentation tests.

As the bioassay results showed, the extracts of the TK24 recombinants containing the pJTU5751/Δ*tunA* and the negative control (2463b) have lost bioactivity against *Bacillus subtilis*, whereas the positive control (TK24/pJTU5751) displays distinct inhibition against the indicator strain (Fig. 5C). Additionally, HPLC analysis results showed that the recombinant TK24/ pJTU5751/Δ*tunA* did not yield the distinctive peaks of Tun $15{:}1_B$ and Tun $16{:}1_A$ when compared with positive controls (Fig. 5D). Hence, these data clearly establish that *tunA* is essential for tunicamycin biosynthesis.

DISCUSSION

The use of tunicamycin to inhibit protein glycosylation or bacterial peptidoglycan biosynthesis is well documented in over 5000 citations. The chemical structure and potent bioactivities are known (Tsvetanova et al., 2002; Price and Tsvetanova, 2007), but the precise molecular mechanism for tunicamycin biosynthesis has remained obscure for decades. Indeed, cloning and characterization of the tunicamycin gene cluster not only provides insights into the tunicamycin

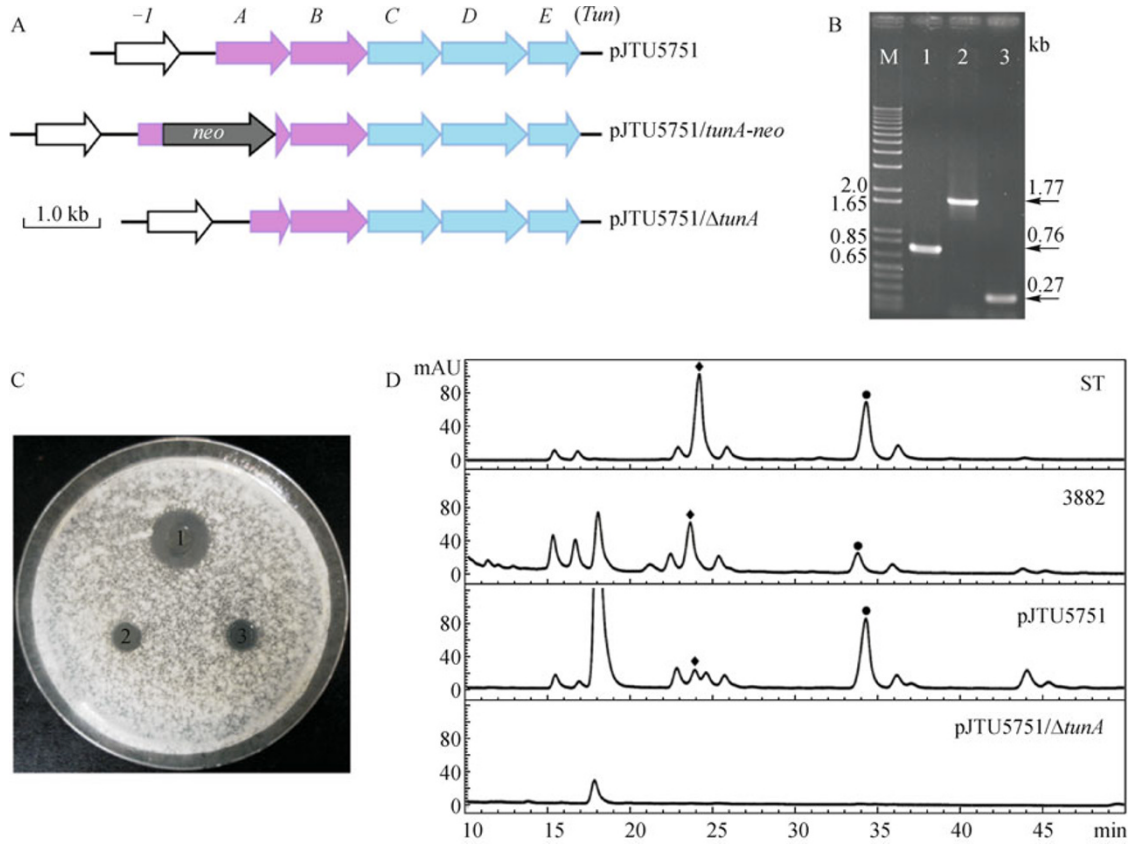

Figure 5. In-frame deletion of *tunA*. (A) Schematic representation for the in-frame deletion of *tunA* in pJTU5751. pJTU5751/*tunA-neo* means that the *neo* cassette replaces 489-bp region of *tunA* in pJTU5751, pJTU5751/Δ*tunA* indicated that the *neo* cassette was removed by XbaI-SpeI double digestion with leaving of an n-frame deletion scar. (B) PCR validation of the *tunA* mutation in pJTU5751, as the *neo* cassette was recombined into *tunA*, pJTU5751/*tunA-neo* would give a ca. 1.77-kb PCR product, while the pJTU5751 shows 0.76-kb PCR product; and the PCR product of pJTU5751/Δ*tunA* is 0.27-kb in size. (C) Bioassay of the metabolites produced TK24 recombinants carrying pJTU5751/Δ*tunA*. (D) The authentic tunicamycin standard (ST), samples of *S. chartreusis*wild-type (3882) as well as TK24 recombinants independently containing pJTU5751 and pJTU5751/Δ*tunA* were compared. (◆): Tun 15:1$_B$, (●): Tun 16:1$_A$.

biosynthetic pathway, but may also provide the basis for combinatorial biosynthesis of novel tunicamycin analogs with improved/selective bioactivity.

Earlier metabolic feeding experiments have established that tunicamycin biosynthesis is initiated by utilization of uridine and UDP-GlcNAc as starting substrates. The latter is sequentially converted into the sugar nucleotide UDP-GalNAc-5,6-ene, while uridine was deduced to be oxidized to form uridine-5′-aldehyde as the acceptor for ligation to the UDP-GalNAc-5,6-ene (Tsvetanova et al., 2002; Price and Tsvetanova, 2007).

In the present paper, this mechanism is shown to be essentially correct. Four enzymes, including TunA (dehydratase), TunB (radical SAM protein), TunF (epimerase) and TunG (mutase) are shown to be involved in early biosynthetic steps of tunicamycin. TunA was deduced to utilize UDP-GlcNAc to form UDP-4-keto-GlcNAc-5,6-ene, and then this intermediate is further modified to UDP-GlcNAc-5,6-ene under control of TunF (Fig. 6A). Similar studies have previously reported that a biofunctional UDP-GlcNAc C-6 dehydratase/C4 epimerase, FlaA1, from *Helicobacter pylori* catalyzes sequential conversion of UDP-GlcNAc to UDP-4-keto-6-methyl-GlcNAc, which is stereospecifically reduced to UDP-QuiNAc (Creuzenet et al., 2000). Similarly, a well-characterized dTDP-glucose 4,6-dehydratase (RffG) from *E. coli* uses dTDP-glucose to generate dTDP-4-keto-6-deoxyglucose. More importantly, this enzyme generates an intermediate, dTDP-4-keto-glucose-5,6-ene (Gross et al., 2000), which is analogous to the corresponding intermediate currently proposed in the tunicamycin biosynthetic pathway.

It is tempting to propose that the radical SAM protein homolog TunB enzyme catalyzes the formation of 5′-uridine radical from uridine, which then reacts with UDP-4-keto-6-deoxy-GlcNAc to form a carbon-carbon bond at the C-6’

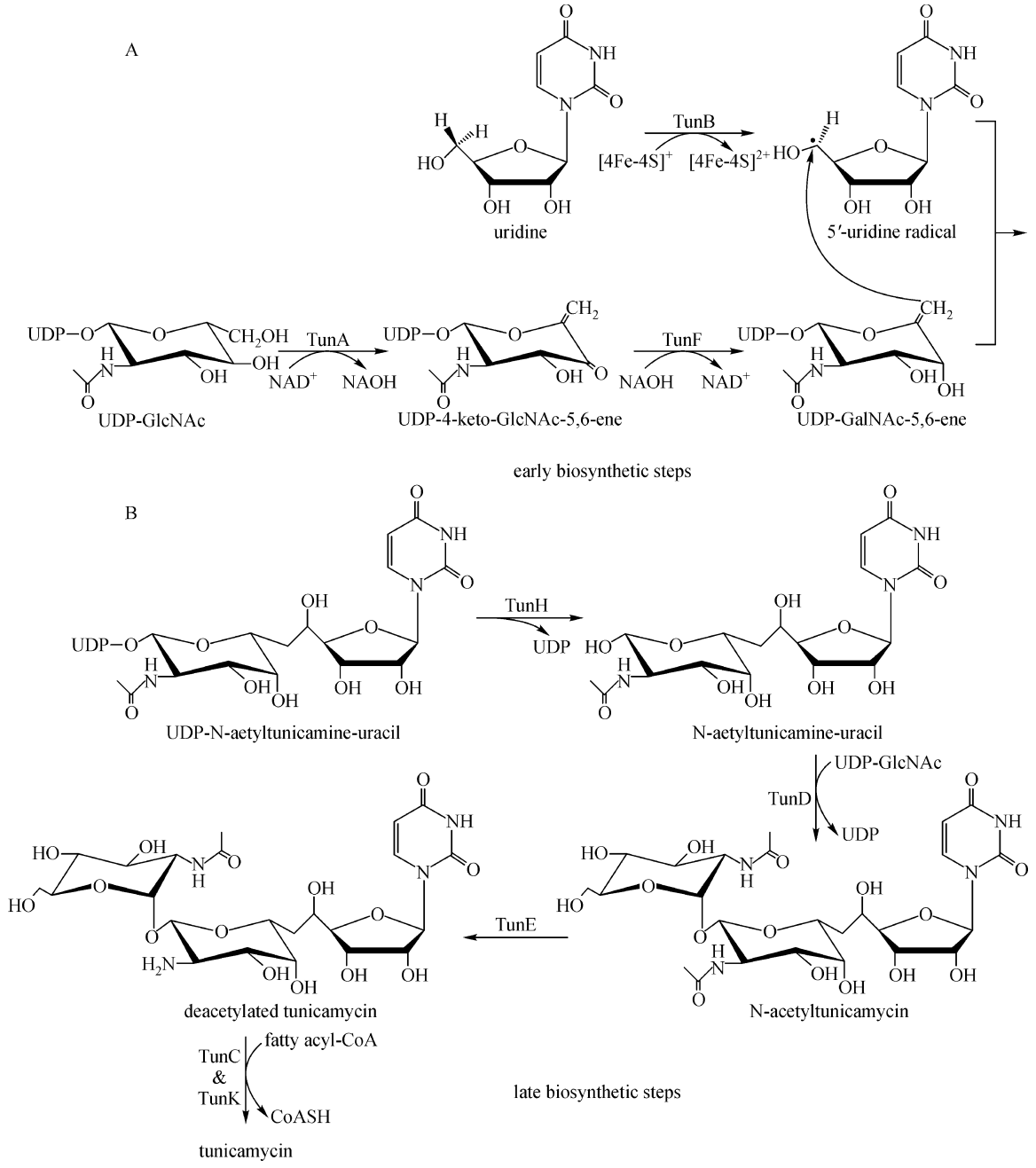

Figure 6. Proposed pathway for tunicamycin biosynthesis. (A) Early biosynthetic steps for tunicamycin biosynthesis, and the late steps for tunicamycin biosynthesis are indicted as (B).

position (Fig. 6A). A radical SAM protein (PqqE) homologous to TunB from *Klebsiella pneumoniae* is capable of catalyzing carbon-carbon bond formation in the biosynthesis of coenzyme PQQ (Wecksler et al., 2009). However, to the best of our knowledge this mechanism has not been previously observed for carbohydrate metabolism. Related to this, TunG, a putative phosphoglycerate mutase with homology to Francci3_2350, is potentially a CobC-type alpha-ribazole-5′-phosphate phosphatase. CobC is involved in the biosynthesis of cobalamin (vitamin B12), wherein the dephosphorylation of adenosylcobalamin-5′-phosphate by the CobC phosphatase is the last step of the pathway (Zayas and Escalante-Semerena, 2007). Since cobalamin is a co-factor for the radical SAM proteins (Tun B), it is quite possible that the TunG promotes the production of cobalamin for its use during the biosynthesis of tumicamycin.

The later steps for tunicamycin biosynthesis are also proposed based on the feeding experiments with

isotope-labeled substrates (Tsvetanova et al., 2002; Price and Tsvetanova, 2007). The initial reaction involves the hydrolysis of UDP from UDP-*N*-acetyltuniamine uracil to give *N*-acetyltunicamine uracil. This then acts as the acceptor substrate for addition of the *α, β*-1″,1′-GlcNAc residue, after which tunicamyin is bio-synthesized by stepwise reactions of deacetylation and transacylation (Price and Tsvetanova, 2007). Our analysis of the tunicamycin biosynthetic gene cluster identified five putative proteins: TunC (acetyltransferase), TunD (glycosyltransferase), TunE (deacetylase), TunH (nucleotide pyrophosphatase) and TunK (ACP), as candidate enzymes responsible for the late steps for tunicamycin biosynthesis. These late biosynthetic steps are initiated by TunH catalyzing the conversion of UDP-*N*-acetyltunicamine uracil to *N*-acetyltunicamine uracil. This subsequently acts as the glycosidic acceptor for addition of an *α, β*-1″,1′-linked GlcNAc residue from the donor molecule UDP-GlcNAc, as catalyzed by TunD (Fig. 6B). Structurally, the *α, β*-1″,11′-glycosidic-glycosidic linkage is highly unusual and, additionally, TunD displays only marginal homology to known glycosyl transferase family protein, suggesting that this enzyme harbors an unprecedented catalytical mechanism. The intermediate *N*-acetyltunicamycin is subsequently deacetylated by TunE to deacetylated tunicamycin (Fig. 6B). TunC and TunK are proposed as the targeted enzymes essential for acyl chains transfer and *N*-acylation. We further propose that TunC is responsible for independently selecting different fatty acyl CoA and transferring them to TunK (Fig. 6B). The naturally-occurring tunicamycins can exhibit various *N*-linked acyl chains, suggesting that the *N*-acyl transferase, TunC, has similarly broad substrate flexibility. Despite gaining insight into the tunicamycin biosynthetic gene cluster, we were unable to identify an obvious candidate gene responsible for the amide bond formation by homology alone, but it may be distributed in different loci of the genome as reported for several previous examples of biosynthesis (Yu et al., 2002; Ostash et al., 2007; Chen et al., 2009). However, the location of the the small protein encoded by *tunK* in the tunicamycin gene cluster suggests a potential role for this enzyme for the amide bond formation (Fig. 6B). Moreover, the genes governing biosynthesis of the fatty acyl chains were missing from the tunicamycin gene cluster, as reported for the caprazamycin and liposidomycin biosynthetic pathways (Kaysser et al., 2009, 2010). Accordingly, it is reasonable to believe the fatty acyl chains are derived from primary metabolic pathways, and manipulation of the fatty acid biosynthetic pathway would be of great potential to generate novel tunicamycin derivatives.

Investigation of the tunicamycin biosynthetic gene cluster also identified three proteins with a potential role in a mechanism of self-resistance. TunI (ABC transporter, ATP binding protein), TunJ (ABC-2 transporter) and TunL (phosphatase) were assigned as the functions responsible for the resistance and transport of tunicamycin. Recent research on the crystal structure of the TmrD tunicamycin resistance protein revealed that it is more likely a kinase (Kapp et al., 2008) rather than an ATP-dependent transporter (TmrB) as previously described by Noda et al. (1992, 1995). Accordingly, it is proposed that TunI functions as ATPase that inactivates tunicamycin via phosphorylation, which is subsequently transported and dephosphorylated by TunJ and TunL.

In summary, we report the finding and functional analysis of the tunicamycin biosynthetic gene cluster. Twelve contiguous genes, *tunA-L*, have been identified from a known tunicamycin producer, *S. chartreusis*. Nine of these encode for functions implicated in a novel seven step biosynthetic pathway. These include a radical SAM protein (TunB) with a unique role in the formation of the 5′-6’ carbon-carbon bond in the 11-carbon tunicamine sugar backbone. Another putative protein (TunD) is an unusual glycosyltransferase that forms an anomeric-to-anomeric *α, β*-1″,1′-glycosidic bond. Three other genes (*tunJ, K, L*) are implicated in tunicamycin transport and self-resistance. We also identified homologs of this gene cluster in two other organism; *S. clavuligerus*, a known producer of tunicamycin-like antibiotics, and a non-producing strain of *A. mirum*. We anticipate that manipulation of the pathway would be of great potential to generate novel tunicamycin-based inhibitors with modified activity and selectivity.

MATERIALS AND METHODS

Strains, plasmids (cosmids), general methods and culture conditions

Strains, plasmids (cosmids) used are described in Supplemental Data (Table S1), and PCR primers were listed in the Table S2. *Streptomyces* and its derivatives were grown at 30°C on MS agar (Kieser et al., 2000), or in TSBY liquid medium (3% Difco tryptic soy broth powder, 10.3% sucrose, 1% Difco yeast extract, pH 7.2). General methods for *E. coli* or *Streptomyces* manipulation were based on protocol of Sambrook et al. (1989) or Kieser et al. (2000). The final antibiotic concentrations used for selection of *E. coli* or *Streptomyces* were as follows: ampicillin 100 μg/mL, apramycin 30 μg/mL, and kanamycin 50 μg/mL.

Genomic library construction for *S. chartreusis*

Standard methods were adopted for the construction of the pJTU2463 (see Table S1) derived genomic library for *S. chartreusis* (Sambrook et al., 1989; Kieser et al., 2000) and *E. coli* XLUZ (Table S1, Tao et al., unpublished) was selected as suitable host cells.

High-throughput screening of the positive cosmids from genomic library of *S. chartreusis*

Cosmids of the genomic library of *S. chartreusis* were transferred into *S. lividans* TK24 with adoption of the method described previously by Martinez et al. (2004). *Bacillus subtilis* was used as an indicator strain for bioassay screening of the *S. lividans* TK24 recombinants bearing

positive cosmids that contained the complete tunicamycin gene cluster (Tsvetanova and Price, 2001).

Nucleotide sequence accession number

The nucleotide sequence reported in this paper is available in the GenBank database under accession No. HQ111437.

DNA sequencing and sequence analysis

DNA sequencing was accomplished at Shanghai Major BioTech Co. Ltd using an Applied Biosystems model 3730 automated DNA sequencer, and sequence analysis was carried out with FramePlot 3.0beta online program (http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl) (Ishikawa and Hotta, 1999). DNA and protein homology searches were carried out with PSI-BlastP online software.

Construction of pJTU5751 for mutational analysis of the target tun genes

For construction of pJTU571, 11C3 cosmid (containing XbaI and SpeI sites which were correspondingly located at both sides of foreign insertion) was initially digested by XbaI, and the resultant cohesive ends of linear fragment were blunted by Klenow Fragment (Fermentas). After that, the blunted linear fragment was self-ligated to result in pJTU5751a, in which the SpeI site was subsequently blocked with the method described as above.

Direct mutation of *tunL* (target gene) in the positive cosmid pJTU5751

For direct mutation of *tunL (target gene)* in cosmid pJTU5751, Primers TunLtgtF and TunLtgtR (Table S2) were designed to amplify the *neo* cassette (kanamycin resistance gene from SuperCos 1) so as to disrupt *tunL* via PCR-targeting technology (Gust et al., 2003), and the PCR primers tunLexf and tunLexr (Table S2) were used to validate the *tunL* mutation. The same method described as above was performed for direct mutation of other target genes in pJTU5751.

In-frame deletion of *tunA* in pJTU5751

For in-frame deletion of *tunA*, a *neo* cassette from SuperCos 1 was amplified with primers tunAtgtF and tunAtgtR (Table S2), and subsequently recombined into the *tunA* gene in pJTU5751 to produce pJTU5751/*tunA-neo* by PCR-targeting technology (Gust et al., 2003). For in-frame deletion of *tunA*, the *neo* cassette was removed from pJTU5751/*tunA-neo* by XbaI-SpeI double digestion, and an in-frame deletion scar was left to give rise to pJTU5751/Δ*tunA*. The PCR primers tunAexf and tunAexr were designed to identify the *tunA* mutant.

Tunicamycin production, purification and assay

S. chartreusis and related *Streptomyces* recombinants were cultivated in TSBY liquid medium for tunicamycin production. After fermentation at 30°C for 5 d, samples were purified according to the method of Tsvetanova and Price (2001). A bioassay and LC/MS (Agilent 1100 series LC/MSD Trap system) were used for detection of tunicamycin. For the bioassay, *Bacillus subtilis* was selected as indicator strain, which was grown on LA medium (1.0% agar). LC/MS analysis was performed on a ZORBA SB-C18 Column (Agilent, 3.5 μm, 2.1 × 150 mm) with an elution gradient of 35%–45% acetonitrile: 0.1% aqueous acetic acid (HPLC grade) over 50 min at 0.2 mL/min. The elution was monitored at 260 nm with a DAD detector, and the data were analyzed offline with Agilent data software.

Quantification of specific components of tunicamycin

For the quantification of tunicamycin produced by related strains, commercial tunicamycin (Sigma-Aldrich, St. Louis, IL) was used to generate a standard curve, and the quantity of specific tunicamycin component was calculated based on this curve.

Conditions for LC/MS and MS/MS analysis

An Agilent 1100 series LC/MSD Trap system with an electrospray ionization source was used for MS analysis of tunicamycin in positive ion detection mode. The parameters for MS analysis are as follows: drying gas flow: 10 L/mL, nebulizer pressure: 30 psi, and drying gas temperature 325°C. MS/MS fragmentations were obtained using a quadrupole orthogonal time-of-flight (Q-oTOF) mass spectrometer (Applied Biosystems/MDS Sciex QSTAR Elite, Toronto, Canada) with turbospray ionization. Samples were introduced by online RP-HPLC as described with a split flow rate of 10 μL/min. Data was processed using Analyst QS 2.0. MALDI-TOF mass spectra were recorded on a Bruker-Daltonic (Billerica, MA) Omniflex instrument operating in reflecton mode using 2,5-dihydrobenzoic acid matrix. Laser excitation was at 337.1 nm, typically at 75% of 150 μJ maximum output, and 60 shots were accumulated.

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ABBREVIATIONS

aa, amino acid; *aac(3)IV*, apramycin resistance gene; ABC-transporter, ATP binding cassette transporter; ACP, acyl carrier protein; AT, acyl transferase; GalNAc, N-acetylgalactose; GlcNAc, N-acetylglucosamine; GPT, GlcNAc-1-P transferase; HHE, high-throughput heterologous expression; kDa, kilo-dalton; MraY, phospho-MurNAc-pentapeptide translocase; *neo*, neomycin/kanamycin resistance

gene; *oriT*, origin of transfer; SAM, S-adenosyl methionine; TmrB (TmrD), tunicamycin resistance protein

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