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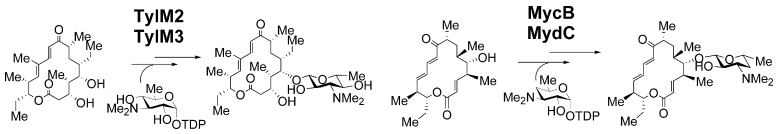

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Characterization of *tyl*M3/*tyl*M2 and *myd*C/*myc*B Pairs Required for Efficient Glycosyltransfer in Macrolide Antibiotic Biosynthesis

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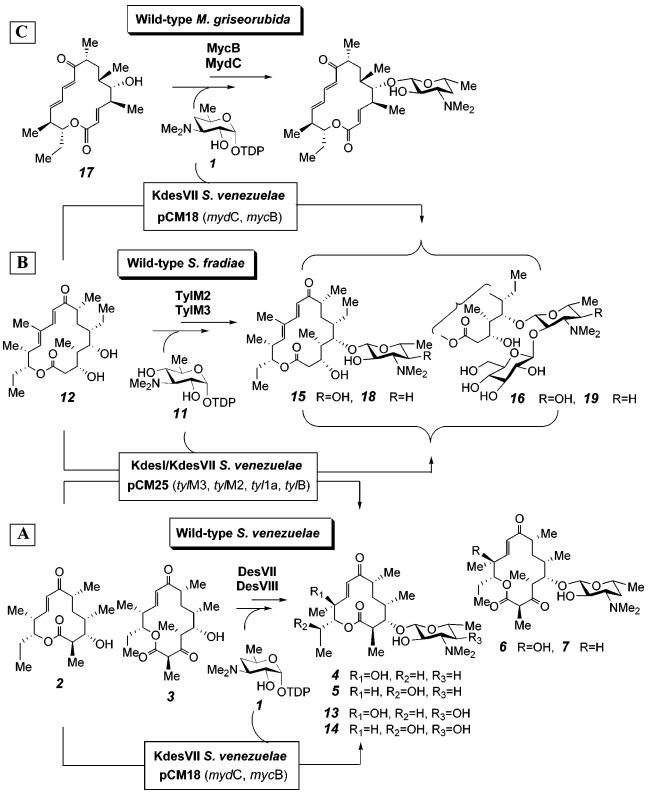
The presence of deoxy- and aminosugars in the structures of many secondary metabolites, including many antibiotics, is essential for their biological activities.[1] It is well-known that changing the sugar appendage(s) of these compounds has a profound impact on the spectrum and/or potency of their biological activities. Since the sugar components are attached to the parent aglycones by the action of glycosyltransferases (GTs), an understanding of the factors controlling the activity and specificity of these GTs is critical for the generation of new glycosylated natural products having improved or novel biological properties with potential clinical applications.

Although many putative GT genes have been identified in gene clusters responsible for the biosynthesis of secondary metabolites, only a handful of the corresponding gene products have been purified and their catalytic functions verified. However, we have recently demonstrated the in vitro activity of a macrolide GT, DesVII, which catalyzes the attachment of TDP-D-desosamine (**1**) to either the 12- or the 14-membered macrolactone ring (**2** or **3**) to make methymycin/neomethymycin (**4**/**5**) and narbomycin/pikromycin (**6**/**7**) in *Streptomyces venezuelae* (Scheme 1A). This enzyme is almost entirely dependent on an auxiliary protein, DesVIII, for activity.[2] To our knowledge, this is the only reported example of a GT having such a requirement.

A BLAST search using the DesVIII sequence as the query identified a total of 15 DesVIII homologues in the databank, all but one of whose encoding genes are found directly upstream of GT-encoding genes in macrolide and anthracycline antibiotic biosynthetic gene clusters.[3] The presence of these DesVIII homologues in other antibiotic biosynthesis pathways suggests that each may function as an auxiliary protein to its respective GT. However, the activity of a second macrolide GT, EryCIII, in the erythromycin pathway of *Saccharopolyspora erythraea*, was recently demonstrated in vitro without use of the DesVIII homologue EryCII, whose encoding gene is directly upstream of that of EryCIII.[4] Clearly, with only DesVII and EryCIII characterized, the generality of the macrolide GT requirement for its auxiliary protein remained to be established. Herein, we report two new examples of macrolide GTs, TylM2 from the tylosin pathway of *Streptomyces fradiae* (Scheme 1B)[5] and MycB from the mycinamicin pathway of *Micromonospora griseorubida* (Scheme 1C),[6] whose activities are significantly enhanced by the coexpression of the DesVIII homologue, TylM3 and MydC, respectively, from each pathway. It was also found that TylM2 and MycB show relaxed substrate specificity as their substitution for DesVII in *S. venezuelae* led to the isolation of several new glycosylated macrolides. These results have profound implications for future strategies of glyco-diversification through a combinatorial biosynthetic approach.

To establish a genetic system suitable for in vivo testing of the activity of the mycaminosyltransferase TylM2 from *S. fradiae*, an *S. venezuelae* double mutant (KdesI/KdesVII) was created using

Scheme 1



the previously reported KdesI mutant as the template.[7] With *des*I and *des*VII disrupted, the desosamine pathway intermediate, TDP-4-keto-6-deoxy-D-glucose (**8**) (Scheme 2), and the aglycones (**2**/**3**) (Scheme 1A) accumulate and serve as substrates for heterologously expressed enzymes. A pair of expression plasmids, pCM25, which contains *tyl*M3, *tyl*M2, *tyl*1a, and *tyl*B, and pCM26 which contains *tyl*M2, *tyl*1a, and *tyl*B, were also constructed. The *tyl*1a gene encodes an isomerase which converts **8** to **9**. The *tyl*B gene encodes a previously characterized aminotransferase catalyzing the conversion of the Tyl1a product **9** to the corresponding 3-amino sugar **10**.[8] When these genes are introduced into the KdesI/KdesVII mutant, the normal D-desosamine biosynthetic pathway, which is halted at **8**, should be diverted to form the TylB product **10**. The latter will be converted to TDP-D-mycaminose (**11**) by the action of DesVI, a 3-amino-*N*,*N*-dimethyltransferase, which uses **10** (Scheme 2).[9] The mycaminose produced in the mutant strain may then be processed by the coexpressed mycaminosyltransferase, encoded by *tyl*M2, to glycosylate either **2**/**3** or exogenously supplied tylactone (**12**), its natural substrate. Since pCM25 contains both *tyl*M2 and the proposed auxiliary gene, *tyl*M3, while pCM26

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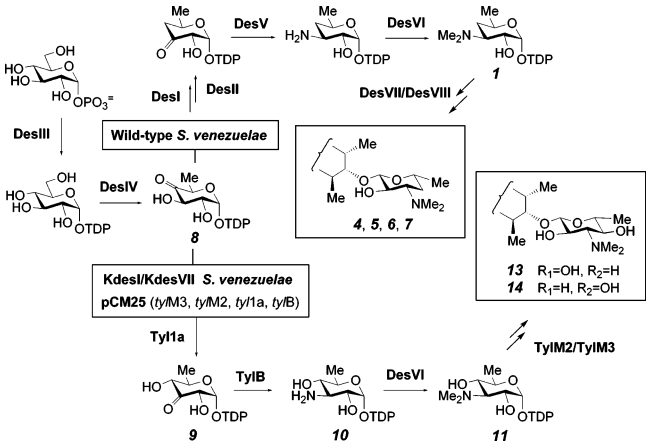
Scheme 2



contains only *tyl*M2, these two plasmids are designed to test the effect of TylM3 on the function of TylM2.

Each of the aforementioned plasmids was introduced separately into the KdesI/KdesVII strain by conjugal transfer.[10] After growth of these mutants in parallel for 60 h, followed by chloroform extraction of metabolites from the culture broth and analysis of the macrolide derivatives produced,[11] it was found that the KdesI/KdesVII/pCM26 strain produced no detectable glycosylated macrolides, while the KdesI/KdesVII/pCM25 strain, which contains *tyl*M3, converted ~4% of the endogenous aglycone **2** to its mycaminosyl derivatives (Scheme 1A). The formation of 3-*O*-mycaminosylmethynolide (**13**) and 3-*O*-mycaminosylneomethynolide (**14**) was verified by high-resolution CI−MS, and the structure of **13** was further confirmed by NMR spectroscopy.[11] These results provided initial evidence suggesting that TylM3 is important for TylM2 activity.

In a separate experiment, cultures of these two mutants were grown in parallel for 14 h after being fed 10 mg of tylactone (**12**). When the metabolites were extracted from the culture broth and analyzed by HPLC, a 40-fold enhancement (61 vs 1.5% conversion) in the production of 5-*O*-mycaminosyltylactone (**15**) and its 2′-glucosylated derivative (**16**) by KdesI/KdesVII/pCM25 over KdesI/KdesVII/pCM26 was observed (Scheme 1B). The 2′-glucosyl-5-*O*-mycaminosyltylactone (**16**) is presumably formed by action of an endogenous resistance GT which is present in *S. venezuelae*.[12] Clearly, the presence of TylM3 significantly enhances the ability of TylM2 to glycosylate acceptor substrates. Our results also show that TylM2 is flexible toward the acceptor substrate, glycosylating 16-membered tylactone (**12**) and 12-membered ring macrolide (**2**) produced by *S. venezuelae*. This flexibility afforded the new compounds **15** and **16**.[13]

To verify the function of MycB and to test the effect of the proposed auxiliary protein MydC on MycB activity, two other plasmids, pCM17 and pCM18, were constructed and introduced into *S. venezuelae* mutant (KdesVII) in which *des*VII alone was inactivated.[11] Plasmid pCM17 contains *myc*B alone, while pCM18 contains *myd*C and *myc*B. The *myc*B gene encodes a GT that catalyzes the desosaminylation of protomycinolide IV (**17**), a 16-membered aglycone (Scheme 1C).[14] Extraction and HPLC analysis showed that KdesVII/pCM18 produces methymycin (**4**) and neomethymycin (**5**) at wild-type levels (99% conversion of **2**), while KdesVII/pCM17 produces these compounds in significantly lower yield (5% conversion) after growth for 60 h (Scheme 1A). Additionally, these mutants were grown in parallel for 60 h after feeding of tylactone (**12**). It was found that KdesVII/pCM18 produces small amounts of 5-*O*-desosaminyltylactone (**18**)[15] and 2′-glucosyl-5-*O*-desosaminyltylactone (**19**) (Scheme 1B), but KdesVII/pCM17 fails to produce any discernible glycosylated tylactone derivatives.[11] Again, the auxiliary protein MydC significantly enhances the glycosyltransfer activity of MycB in vivo. Also, MycB was shown to be flexible toward its acceptor substrate, glycosylating two aglycones that differ in structure from its natural substrate.[13]

This work shows that the requirement of an auxiliary protein by GTs involved in macrolide production is not unique to the DesVII case, but rather occurs in at least two other macrolide antibiotic biosynthetic pathways. Because of the significant level of sequence identity among DesVIII, TylM3, MydC, and their 13 other homologues, and the similarity of their locations relative to GTs in their respective gene clusters, it is likely that all of these are auxiliary proteins that are critical to the proper functioning of their partner GTs. These findings will facilitate the use of those auxiliary-requiring GTs for combinatorial generation of new antibiotics carrying rationally designed sugar moieties. Several such new glycosylated macrolide compounds were generated as a result of this study (**13**−**16** and **19**). While the function of DesVIII and its homologues in the mechanism of glycosyltransfer is not known, these results set the stage for an in-depth investigation. Future work will be aimed toward examining the specific role of DesVIII and its homologues in the glycosyltransfe reaction, as well as using these GTs, their auxiliary genes/proteins, and various sugar biosynthetic genes/enzymes to combinatorially generate novel macrolide antibiotics in vivo and in vitro.

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Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) While growth under conditions favoring the production of narbonolide (**4**) was not attempted, it is expected that this 14-membered ring aglycone is also a substrate for TylM2/TylM3 and MycB/MydC pairs.

(14) The *myd*C gene was originally proposed to encode a TDP-sugar 3,4-isomerase (ref 6).

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