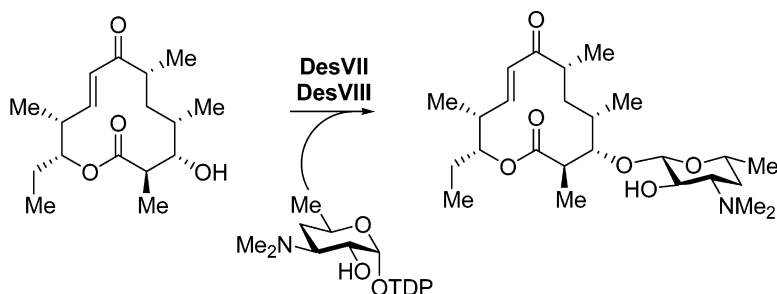Communication

Characterization of the Glycosyltransferase Activity of DesVII: Analysis of and Implications for the Biosynthesis of Macrolide Antibiotics

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Characterization of the Glycosyltransferase Activity of DesVII: Analysis of and Implications for the Biosynthesis of Macrolide Antibiotics

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Deoxy and amino sugars are often constituents of secondary metabolites, many of which are of clinical importance.[1] These unusual sugars are attached through O-, N-, or C- linkages to their respective aglycons and commonly play essential roles in determining the biological activities of the parent compounds.[2] Altering the composition of sugar appendages in glycoconjugates holds promise for creating new products with novel biological activities. Hence, efforts to develop effective glycosylation strategies have been vigorously pursued by scientists for decades. Significant progress has been made recently using genetic and biochemical methods to perform glycosylation in vivo. For example, the feasibility of re-engineering sugar biosynthetic machineries to generate diverse sugar structures and to incorporate foreign glycosyltransferase genes into appropriate strains to facilitate coupling between various sugar donors and aglycon acceptors in a combinatorial manner has been demonstrated.[1,3] However, yields are generally low, and product analysis is tedious. Moreover, the production of any antimicrobial agents limits the general applicability of such an in vivo approach because of inhibited cell growth or death. One potential solution to overcome these hurdles is to carry out the glycosylation step in vitro using the isolated glycosyltransferase. The in vitro approach would also enable the generation of mutant glycosyltransferase with the desired substrate specificity.

Although many putative glycosyltransferase genes have been tentatively identified in gene clusters responsible for the biosynthesis of glycosylated secondary metabolites, few of the corresponding gene products have been purified and their catalytic roles verified. Moreover, examples of characterized glycosyltransferases involved in the biosynthesis of antibiotics are scarce and are limited to those participating in the formation of nonribosomal peptide antibiotics[4] and aromatic polyketide antibiotics.[5] The lack of any in vitro characterization of glycosyltransferases involved in the biosynthesis of macrolide antibiotics is particularly startling despite the pressing need of catalysts capable of glycosylating new macrolide aglycons generated via manipulation of the polyketide biosynthetic pathways. Herein we describe, for the first time, the biochemical verification of a glycosyltransferase, DesVII, which catalyzes the attachment of TDP-D-desosamine (1) onto 12- and 14-membered macrolactone rings (2 and 3) to make methymycin/neomethymycin (6/7) and narbomycin/pikromycin (5/8), respectively, in *Streptomyces venezuelae* (Scheme 1). To our surprise, the purified DesVII is active only in the presence of another protein, DesVIII, at high pH. These unusual criteria for activity make DesVII unique among known glycosyltransferases and may account for the fact that its activity has not been previously verified by conventional assays. This discovery will significantly impact the future characterization and application of this group of glycosyltransferases.

The *desVII* gene product was first assigned as the desosaminyltransferase in the methymycin/pikromycin pathway on the basis of its sequence similarity to other glycosyltransferases.[6,7] To verify

Scheme 1. Glycosylation by DesVII as a Part of Methymycin/Pikromycin Biosynthesis
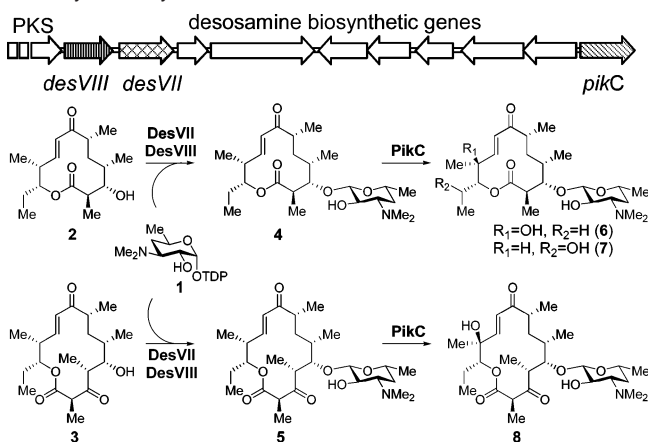
its assigned function, a *S. venezuelae* KdesVII mutant in which *desVII* gene is replaced with the thiostrepton resistance gene was constructed according to a literature procedure.[8] As expected, no glycosylated macrolides were produced by the KdesVII mutant,[9] consistent with the above assignment. To confirm its catalytic function, the *desVII* gene was cloned into pET24b(+) vector and expressed in *Escherichia coli* BL21 under the induction by 0.5 mM IPTG. The C-terminal His$_6$-tagged DesVII protein (47.5 kDa) was purified to greater than 90% purity using Ni−NTA chromatography. In parallel, the sugar donor, TDP-D-desosamine (1), the aglycon acceptor, 10-deoxymethynolide (2), and one of the predicted products, 10-deoxymethymycin (4, also known as YC-17), were also prepared.[10]

With all the necessary components in hand, we tested the glycosyltransferase activity of DesVII. To our disappointment, no product was found after 2−4 h incubation of 1 and 2 in a buffer of pH 6−8 at 29 °C.[10] A variety of conditions were explored, such as changing the substrate concentration and incubation time, using alternative organic solvents to prepare the stock solution of 2, replacing Mg^{2+} with different metal ions and adding various common cofactors, with no apparent effect on DesVII activity. Having eliminated the obvious explanations for the absence of activity, the need of a second protein component emerged as an appealing option. Unfortunately, the incorporation of crude fermentation broth or the cell-free extract of the wild-type *S. venezuelae* in the assay mixture failed to produce the activity.

In view of these results, we redirected our search for the missing partner of DesVII at the gene level. The *desVIII* gene stood out as a likely candidate because it is the only unassigned gene in the desosamine biosynthetic gene set. While early studies on the formation of 5−8 had ruled out the direct involvement of *desVIII* in the desosamine biosynthetic pathway,[11] replacing the *desVIII* with the kanamycin resistance gene rendered the resulting KdesVIII
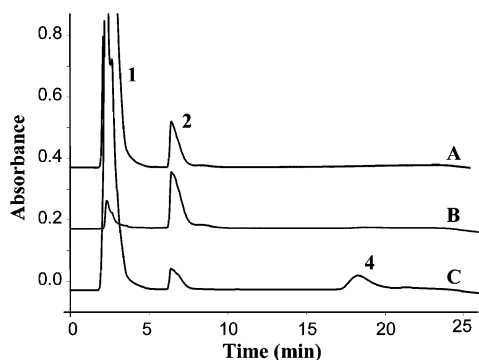
Figure 1. Typical HPLC traces for DesVII/DesVIII glycosylation reactions. (A) In the presence of DesVIII but no DesVII (0% conversion). (B) In the presence of DesVII but no DesVIII (2.9%). (C) In the presence of both DesVII and DesVIII (55.8%).

mutant incapable of producing any glycosylated compounds. This result provided initial evidence implicating an active role, either catalytic or regulatory, of *desVIII* in glycosylation during the biosynthesis of **5−8**. To determine the function of DesVIII, the *desVIII* gene was cloned into the pET24b(+) vector and expressed in *E. coli* BL21 cells as a C-terminal His$_6$-tagged fusion protein (43.6 kDa). Although the recombinant DesVIII was produced in inclusion bodies, a small portion (3 mg/4 L of culture) could be recovered in soluble form via a denaturation/renaturation sequence. Isolated DesVIII was 60−70% pure as assessed by SDS-PAGE. Further purification was unsuccessful because of DesVIII precipitation during Mono Q and Superdex S-200 chromatography. Thus, the partially purified DesVIII was used as a supplement to the assay mixture.[10] However, no new product was discernible after incubation at 29 °C for 19 h in the presence of both DesVII and DesVIII.

Again, different reaction conditions were explored. To our delight, enzymatic turnover was eventually achieved by performing the assay at pH 9. When HPLC was used to analyze the reaction progress, a new peak coeluted with the YC-17 standard was observed (Figure 1).[10] The percent conversion, calculated from the integration of the substrate (**2**) and product (**4**) peaks on the HPLC chromatogram, was found to be in a range of 56−92%, depending on the DesVIII batch. The identity of the product isolated by HPLC from a large-scale incubation was confirmed to be YC-17 by ^{1}H NMR and CI-MS analysis ([M + H] C$_{25}$H$_{44}$NO$_6$ found 454.3161, calcd 454.3169). Desosaminylation of narbonolide (**3**) by DesVII and DesVIII to give narbomycin (**5**)[12] was also detected under the same conditions.

The above results unequivocally establish DesVII as the glycosyltransferase responsible for the attachment of TDP-desosamine (**1**) to two macrolactones (**2** and **3**) of varied ring size. This is the first report of demonstrated in vitro activity of a glycosyltransferase involved in the biosynthesis of macrolide antibiotics.[13] The discovery of DesVIII as a necessary component and the high pH dependence for DesVII/DesVIII activity is important for the development of in vitro glycosylation of macrolides and, perhaps, other classes of natural products. High pH requirement was also reported for the glycosyltransferases involved in chloroeremomycin and vancomycin biosyntheses.[4] While the participation of DesVIII in glycosylation catalyzed by DesVII is essential, its actual role in catalysis remains unclear. It is important to note that *desVIII* homologues exist in a number of amino sugar-containing antibiotic

biosynthetic gene clusters.[14] Their gene products show end-to-end sequence similarity to cytochrome P450 enzymes, but lack the rigorously conserved cysteine residue that serves as a ligand to the heme iron. Whether these DesVIII homologues play a similar role in their respective pathways remains to be demonstrated. Such experiments will also indicate whether a second protein component is a general requirement for glycosylation of macrolides or only a special condition for efficient coupling with amino sugar. In either case, the findings presented herein will have a significant impact on the design of novel macrolide antibiotics using a combinatorial approach.

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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(7) DesVII belongs to the GT-B superfamily of glycosyltransferases based on the sequence comparison to representative glycosyltransferases with known crystal structures (Hu, Y. N.; Walker, S. *Chem. Biol.* **2002**, *9*, 1287−1296). It shares high sequence homology to glycosyltransferases involved in the biosynthesis of macrolide antibiotics, such as OleG1 and OleG2 of the oleandomycin pathway in *Streptomyces antibioticus* (50.5% and 53.5% identity, respectively), TylM2 of the tylosin pathway in *Streptomyces fradiae* (51.8%), and EryCIII of the erythromycin pathway in *Saccharopolyspora erythraea* (50.7%). DesVII also exhibits varied sequence similarity to GtfB, C, D, E (18.8−20.9%), and NovM (32.6%).

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(13) A few glycosyltransferases responsible for resistance and self-resistance to macrolide antibiotics had been characterized, such as OleD and OleI from the oleandomycin producer *S. antibioticus*: (a) Quirós, L. M.; Carbajo, R. J.; Braña, A. F.; Salas, J. A. *J. Biol. Chem.* **2000**, *275*, 11713−11720. (b) Quirós, L. M.; Salas, J. A. *J. Biol. Chem.* **1995**, *270*, 18234−18239. However, in these cases the enzymes catalyze the attachment of glucose to the sugar appendage of macrolide, not onto aglycon directly.

(14) A few examples of DesVIII homologues include EryCII of the erythromycin pathway in *S. erythraea* (36% identity), TylMIII of the tylosin pathway in *S. fradiae* (42%), and DnrQ of the daunorubicin pathway in *Streptomyces peucetius* (42%).

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