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Engineered Biosynthesis of Disaccharide-Modified Polyene Macrolides

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Recent work has uncovered genes for two glycosyltransferases that are thought to catalyze mannosylation of mycosaminyl sugars of polyene macrolides. These two genes are *nypY* from *Pseudonocardia* sp. strain P1 and *pegA* from *Actinoplanes caeruleus*. Here we analyze these genes by heterologous expression in various strains of *Streptomyces nodosus*, producer of amphotericins, and in *Streptomyces albidoflavus*, which produces candicidins. The NypY glycosyltransferase converted amphotericins A and B and 7-oxo-amphotericin B to disaccharide-modified forms *in vivo*. The enzyme did not act on amphotericin analogs lacking exocyclic carboxyl or mycosamine amino groups. Both NypY and PegA acted on candicidins. This work confirms the functions of these glycosyltransferases and provides insights into their acceptor substrate tolerance. Disaccharide-modified polyenes may have potential as less toxic antibiotics.

Amphotericin B (Fig. 1) is used to treat life-threatening systemic mycoses and diseases caused by *Leishmania* parasites. It is also active against enveloped viruses and pathogenic prion proteins (1, 2). As an antifungal antibiotic, it is important because few alternatives are available and because resistance has been slow to emerge. However, the use of amphotericin B is restricted by severe side effects. Lipid formulations have reduced toxicity but are expensive (3). Chemical modification of the natural product can improve pharmacological properties, but this approach has not yet delivered a second-generation derivative that has advanced into clinical use (4). Several new analogs have been biosynthesized by genetic manipulation of the amphotericin B-producing microorganism *Streptomyces nodosus*. These new analogs have been obtained by reprogramming the polyketide synthase and by engineering late genes required for modification of the macrolactone core (5, 6, 7). Biosynthetic engineering has generated analogs of other important polyene macrolides, such as nystatin, candicidin, pimaricin, and rimocidin (8, 9, 10, 11).

Chemical studies have shown that modifying the mycosamine sugar of amphotericin B can have beneficial effects. Alkylation of the amine with two 3-aminopropyl groups increases potency and reduces hemolytic activity (12). Removal of the C-2′ hydroxyl group improves selective toxicity (13). Conjugation with arabinogalactan polysaccharides converts amphotericin B to a water-soluble form (14). MFAME (*N*-methyl-*N*-D-fructosyl amphotericin B methyl ester), a semisynthetic derivative with a single additional sugar residue, has greater water solubility and reduced toxicity (15). Recent findings suggest that it may be possible to engineer bacteria to produce disaccharide-modified amphotericins and analogs. This would be important because biological production of these potentially superior compounds could be scalable and cost-effective.

Pseudonocardia autotrophica has been found to synthesize tetraene NPP (nystatin-like *Pseudonocardia* polyene), a nystatin with an *N*-acetyl-glucosaminyl residue linked to the mycosaminyl sugar. This compound is 300 times more water soluble than nystatin A1 but only half as active. Engineering of the NPP polyketide synthase gave a heptaene analog that has improved activity (16). The extending glycosyltransferase gene was not present in the main NPP biosynthesis cluster but may be located nearby (17). Another new nystatin obtained from *Pseudonocardia* sp. strain P1 was found to contain a hexosyl residue attached to the mycosaminyl sugar. The NypY glycosyltransferase that adds the extra sugar was identified after genome sequencing (18).

Actinoplanes caeruleus synthesizes 67-121C, an aromatic heptaene modified with a mannosyl residue β-1,4 linked to the mycosaminyl sugar (19). Recently, we carried out draft genome sequencing of this organism and identified 67-121 late biosynthetic genes. The *pegA* gene for the extending glycosyltransferase was expressed in *Streptomyces nodosus*, but only trace amounts of mannosyl-amphotericins were produced (20). It was not possible to determine whether this was due to inefficient recognition of amphotericins by PegA, low intracellular levels of GDP–D-mannose, rapid export of amphotericins before mannosylation could occur, or other unknown limiting factors.

PegA and NypY show 51% sequence identity (20), suggesting that both enzymes use GDP–α-D-mannose as the activated sugar donor. In this study, we investigated whether NypY could recognize amphotericin B or its analogs *in vivo*. The activities of PegA and NypY toward aromatic heptaene acceptors were also assessed by expressing the genes in a candicidin producer.

MATERIALS AND METHODS

Streptomyces nodosus, *S. nodosus* NM (Δ*amphNM*), *S. nodosus* DII (Δ*amphDII*), *S. nodosus* DII-NM (Δ*amphDII-NM*), and *S. nodosus* KR16 were from our laboratory collection. *Streptomyces albidoflavus* DSM40624 (formerly *Streptomyces griseus* IMRU 3570) was obtained from DSMZ, Braunschweig, Germany.

The pIJ10257-*nypY* plasmid was a kind gift of Ryan Seipke and Matt Hutchings, University of East Anglia, United Kingdom. The *nypY* gene was amplified from this template using primers NypF2 5′-CTAGAGATC TTCTAGAACAGGAGGCCCCATATG-3′ and NypR2 5′-GATCAAGCT TCAGGGGGTGGCCGGGTGACGGTTC-3′. PCR was carried out using

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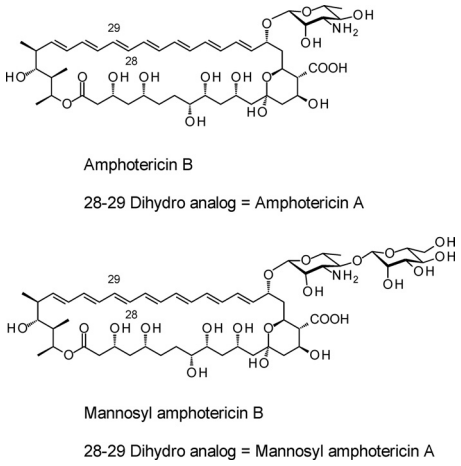

FIG 1 Structures of amphotericins. Amphotericins B and A are cometabolites.

Phusion high fidelity DNA polymerase. Cloning of the gene into the pIAGO plasmid (21) and transformation of *Streptomyces* protoplasts were conducted as described previously (5).

For polyene production, transformants were grown on fructose-dextrin-soya medium containing thiostrepton (50 μg/ml), and extractions were performed as detailed previously (5, 7). High-performance liquid chromatography (HPLC) was conducted using a reverse-phase C_{18} column (4.6 by 150 mm). Solvent A was 0.1% (vol/vol) formic acid in water, and solvent B was methanol containing 0.1% (vol/vol) formic acid. Polyenes were separated using a gradient of 50 to 100% solvent B over 30 min at a flow rate of 1 ml/min. Heptaenes and tetraenes were detected by monitoring A_{405} and A_{315}, respectively.

Liquid chromatography coupled to mass spectrometry (LC-MS) was carried out using a Xevo QTof mass spectrometer coupled to an Acquity LC system with an Acquity UPLC BEH C_{18} column (2.1 by 50 mm) (Waters Corporation, Massachusetts, USA). Solvent A was 0.1% (vol/vol) formic acid in water, solvent B was 0.1% (vol/vol) formic acid in acetonitrile. The flow rate was 0.6 ml/min, and the gradient was as follows: 95% solvent A with solvent 5% B for 0.5 min, followed by a linear gradient to 100% solvent B over the next 2.1 min. After 1 min at 100% solvent B, the gradient was returned to 95% solvent A and 5% solvent B over 0.2 min. Polyene separation was comparable to that obtained by HPLC. The electrospray ionization (ESI) capillary voltage was 3 kV, the cone voltage was 30 V, and the collision energy was 4 eV. The MS acquisition rate was 10 spectra per second and *m/z* data ranging from 50 to 2,000 Da was collected. Mass accuracy was achieved using a reference lock mass scan, once every 10 s.

RESULTS

The *nypY* gene was amplified from plasmid pIJ10257-*nypY* using primers NypF2 and NypR2. The PCR product was digested with BglII and HindIII and cloned between the BamHI and HindIII sites of the expression vector pIAGO. The resulting plasmid pIAGO-*nypY* was transformed into *S. nodosus*, and polyenes were extracted and analyzed. HPLC analysis revealed new tetraene and heptaene species in addition to the more abundant amphotericins B and A (Fig. 2). Further analysis by LC-MS revealed that the new compounds were mannosyl-amphotericins B ($[M + H]^+$ = 1,086.6) and A ($[M + H]^+$ = 1,088.6) (Fig. 1). High-resolution mass spectrometry revealed exact masses of 1,086.5464 and 1,088.5640 for protonated forms of mannosyl-amphotericin B (calculated for $C_{53}H_{84}NO_{22}$ = 1,086.5485) and mannosyl-amphotericin A (calculated for $C_{53}H_{86}NO_{22}$ = 1,088.5641) (see Fig.

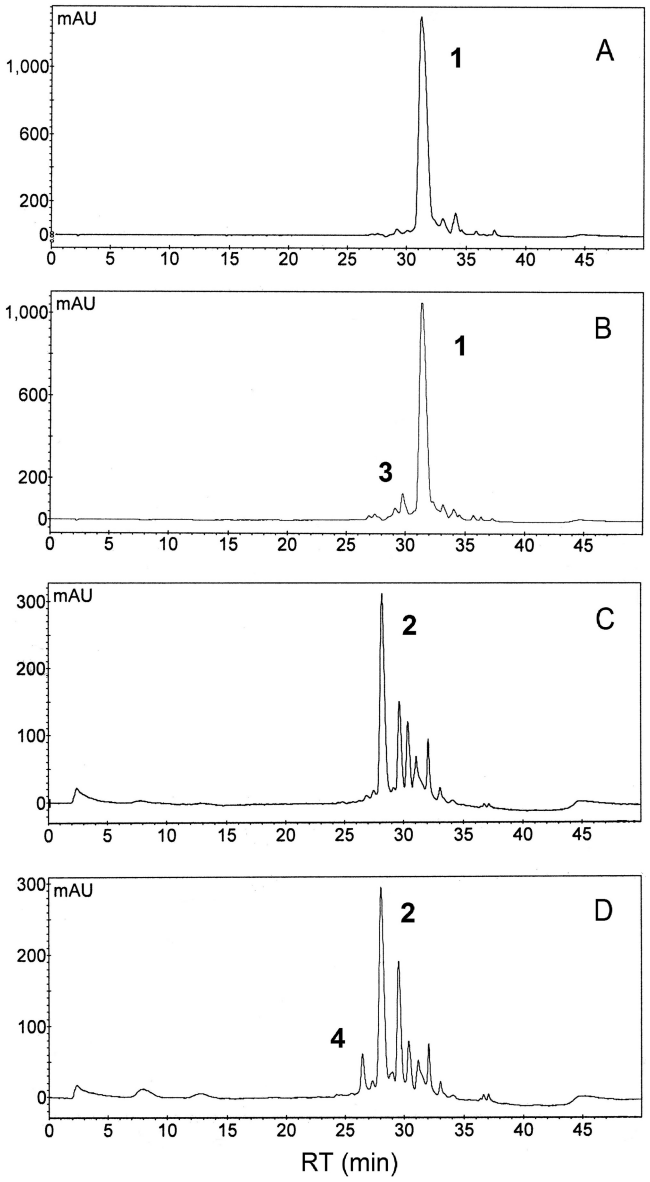

FIG 2 Detection of mannosyl-amphotericins A and B by HPLC. Heptaenes and tetraenes were detected by monitoring at A_{405} and A_{315}, respectively. (A and C) Heptaenes (A) and tetraenes (C) from *S. nodosus* pIAGO. (B and D) Heptaenes (B) and tetraenes (D) from *S. nodosus* carrying pIAGO-*nypY*. The peaks are numbered as follows: 1, amphotericin B; 2, amphotericin A; 3, mannosyl-amphotericin B; 4, mannosyl-amphotericin A. Abbreviations: mAU, milli absorbance units; RT, retention time.

S1 to S4 in the supplemental material). Only about 5% of the total polyene was converted to disaccharide-modified forms.

We were keen to assess the prospects for enzymatic addition of mannose residues to other monoglycosylated amphotericin analogs. A number of mutants that synthesize these compounds are available. These strains were used as hosts to assess the acceptor substrate tolerance of NypY *in vivo*. Analogs lacking exocyclic carboxyl groups are of interest because of their reduced toxicity (22). *S. nodosus* strain NM synthesizes 16-descarboxyl-16-methyl-amphotericin B and 8-deoxy-16-descarboxyl-16-methyl-amphotericin A (22). Strain DII lacks the AmphDII GDP–D-mycosamine

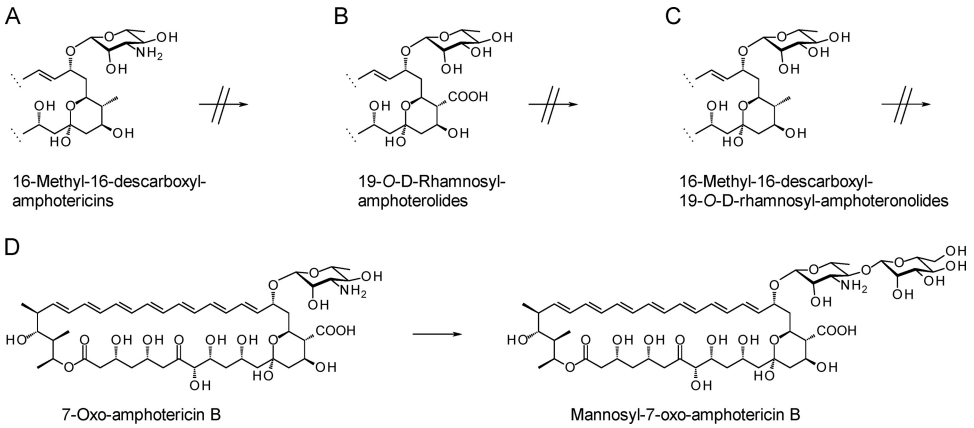

FIG 3 Activity of NypY against amphotericin analogs produced by *S. nodosus* NM (A), *S. nodosus* DII (B), *S. nodosus* DII-NM (C), and *S. nodosus* KR16 (D). 7-Oxo-amphotericin A was not detected in extracts from KR16 transformants.

synthase and synthesizes analogs modified with a neutral sugar, 19-*O*-D-rhamnosyl-amphoteronolides A and B (5). A double mutant, strain DII-NM, synthesizes analogs lacking the exocyclic carboxyl and sugar amino groups (19-*O*-D-rhamnosyl-16-descarboxyl-16-methyl-amphoteronolides A and B) (5). The pIAGO-*nypY* plasmid was transformed into each strain. HPLC and LC-MS analyses revealed no new polyenes. In each case, the range of products was identical to that synthesized by control strains containing the empty vector (not shown). The exocyclic carboxyl and sugar amino groups appear to be essential for efficient recognition by NypY (Fig. 3).

Strain KR16 synthesizes 7-oxo-amphotericins A and B, which contain an additional ketone group at C-7 (6). In this case, the structural alteration in the macrolactone ring is more distant from the glycosylation site. In *S. nodosus* KR16, NypY mannosylated about 5% of the 7-oxo-amphotericin B. The exact mass of the mannosyl-7-oxo-amphotericin B analog ($[M + H]^+$) was 1,100.5271 (calculated for $C_{53}H_{82}NO_{23}$ = 1,100.5278) (Fig. 3; see Fig. S5 to S7 in the supplemental material). No form of the 7-oxo-amphotericin A tetraene analog was detected in extracts of *S. nodosus* KR16 carrying pIAGO-*nypY*.

We also investigated whether the NypY and PegA polyene-extending glycosyltransferases could act on aromatic heptaene acceptors. The candicidin producer *S. albidoflavus* DSM40624 was transformed with the pIAGO-*pegA1* (20) and pIAGO-*nypY* plasmids. The transformants were cultured, and polyenes were extracted into methanol by the same procedures used for amphotericins. The *S. albidoflavus* pIAGO control strain produced heptaenes with masses appropriate for the components of the candicidin complex (9). These heptaenes were candicidin I ($[M + H]^+$ = 1,111.5912), candicidin III ($[M + H]^+$ = 1,109.5789), and candicidin IV ($[M + H]^+$ = 1093.5835) (Fig. 4; see Fig. S8 to S11 in the supplemental material). The *S. albidoflavus* pIAGO-*pegA1* transformant yielded these heptaenes and mannosylated forms ($[M + H]^+$ = 1,273.6360, $[M + H]^+$ = 1,271.6313, and $[M + H]^+$ = 1,255.6376) (see Fig. S10). The *S. albidoflavus* pIAGO-*nypY* transformant gave the same mannosylated forms (see Fig. S11). HPLC separation of mannosylated and unmodified candicidins was poor, and further work will be required to isolate the new compounds.

DISCUSSION

Enzymatic methods to extend polyene macrolide glycosylation may eventually have biotechnological importance. NypY recognizes amphotericins A and B, 7-oxo-amphotericin B, and all components of the candicidin complex. PegA shows very little activity toward amphotericins A and B (20) but did mannosylate candicidins, analogs of its natural substrate, 67-121A. Unlike 67-121 aromatic heptaenes, the various candicidins differ in the polyol chain region as a result of variable β-ketone processing during polyketide synthesis (9). PegA is able to tolerate this structural variation in acceptor substrates. With both NypY and PegA, the yields of disaccharide-modified polyenes from heterologous hosts were low. Protein engineering should increase yields and allow

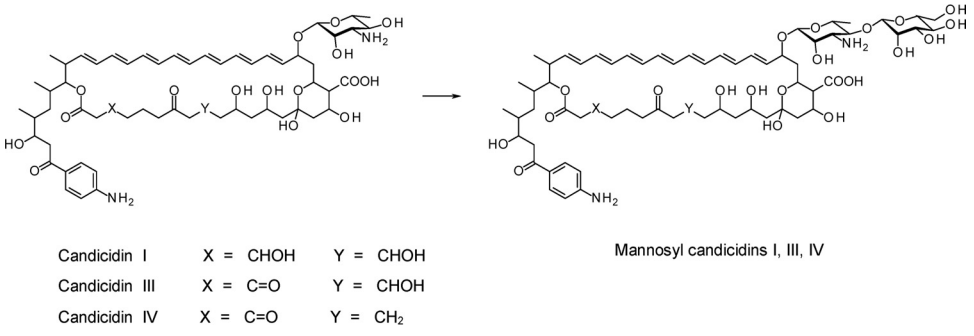

FIG 4 Activity of NypY and PegA toward candicidins. Candicidin II is an isomer of III in which the hemiketal is opened.

mannosylation of analogs lacking exocyclic carboxyl groups or sugar amino groups.

Since no dimycosaminylated polyenes were detected, NypY and PegA appear to discriminate between GDP–D-mycosamine and GDP–D-mannose. Future work will attempt to engineer the extending glycosyltransferases to add mycosaminyl rather than mannosyl sugars and to assess and further redesign sugar substrate specificity.

Polyene macrolide glycosyltransferases are not closely related to enzymes that glycosylate other natural products, some of which have been crystallized and redesigned to use alternative substrates (23, 24). However, Lei and coworkers have already identified amino acid residues in the FscMI mycosaminyltransferase that are critical for donor and acceptor substrate specificity (25). This should assist future efforts to improve yields of disaccharide-modified polyenes, which will then allow purification, complete structural characterization, and assessment of biological activities.

In the biosynthesis of landomycins, regulatory mechanisms ensure that a hexasaccharide side chain is fully assembled before the antibiotic is exported from the producing cell. The transport proteins are induced only at a late stage by partially glycosylated forms; this prevents premature secretion of intermediates (26). Another challenge will be to investigate whether similar mechanisms are important in biosynthesis of disaccharide-modified polyenes. The identification of NypY by Barke and coworkers (18) will help several groups to extend their work (8, 9, 10, 11, 16) on engineered biosynthesis of new polyene macrolides.

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