Production of hybrid glycopeptide antibiotics *in vitro* and in *Streptomyces toyocaensis*

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Background: The glycopeptide antibiotics vancomycin and teicoplanin are currently the last line of defence against some microorganisms that are resistant to many drugs. The emergence of vancomycin-resistant and teicoplanin-resistant enterococci underscores the need for more potent antibiotics. The glycosylation patterns of glycopeptides and chemical modifications of the glycosyl moieties have been shown to greatly influence their antibiotic activity, and certain combinations have resulted in highly active new compounds. To explore further the production of more potent glycopeptide antibiotics, we assessed whether glycosyltransferases could be used to produce hybrid compounds that contain various combinations of sugars and peptide cores.

Results: We cloned five glycosyltransferase genes from *Amycolatopsis orientalis* strains that produce vancomycin or a related glycopeptide, A82846. The *gtfB* and *gtfE'* genes from *A. orientalis* strains expressed in *Escherichia coli* produced glucosyltransferase activities that added glucose or xylose to the vancomycin heptapeptide. The GtfE' protein added glucose efficiently to two other heptapeptides related to teicoplanin to produce hybrid glycopeptide antibiotics. The cloned *gtfE'* gene, driven by the strong constitutive promoter *ermEp**, was introduced into *Streptomyces toyocaensis*, which produces the antibiotic A47934, a heptapeptide related to teicoplanin; recombinant organisms produced glucosyl A47934, a hybrid glycopeptide antibiotic.

Conclusions: Cloned glycosyltransferases from glycopeptide antibiotic producers can be used to produce novel hybrid antibiotics, both *in vitro* and *in vivo*. Because similar enzymes have differing degrees of substrate specificity, it is advantageous to characterize the substrate specificity with enzymes expressed in *E. coli* prior to constructing recombinant actinomycetes for production.

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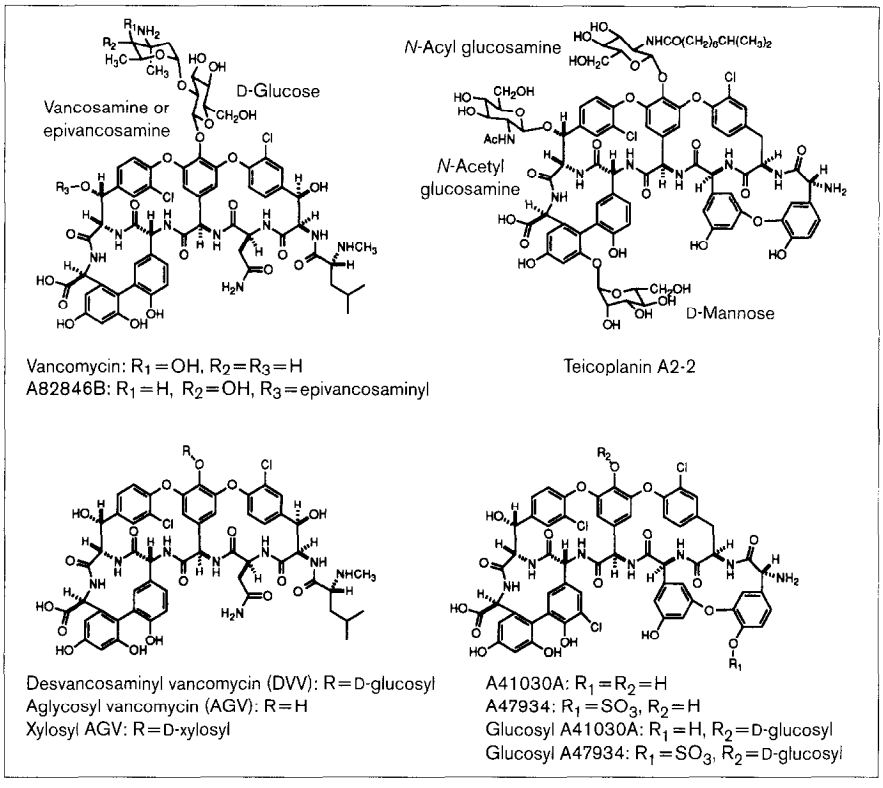
Introduction

The glycopeptide antibiotics vancomycin and teicoplanin are important for the treatment of infections caused by Gram-positive bacteria. The glycopeptide vancomycin is the drug of choice for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* [1]. The glycopeptide antibiotic A82846B [2], which shares the heptapeptide core of vancomycin but contains three sugars rather than two as found in vancomycin (Fig. 1), has been chemically modified at the amino position of the epivancosamine in the disaccharide moiety to generate novel glycopeptides that are active against vancomycin-resistant enterococci [3,4]. One or more of these compounds may be clinically useful in treating these otherwise often fatal infections.

There are a number of glycopeptide antibiotic structures that differ in the heptapeptide or sugar residues [5]. Because antibiotics of the vancomycin/A82846B and teicoplanin types differ substantially in their heptapeptide cores and glycosylation patterns (Fig. 1) [5,6], it would be interesting to explore the production of hybrid glycopeptides containing different combinations of heptapeptide and sugars to provide novel starting materials for chemical modification. In fact, two 'glycopeptides' of the teicoplanin heptapeptide class that lack sugar residues have been identified, namely A47934 [7] and A41030A [8]. Interestingly, both molecules have very good antibacterial activities *in vitro* against Gram-positive pathogens, including *S. aureus*, *Streptococcus pyogenes* and *Enterococus faecalis* [5]. The microorganisms that produce these antibiotics, *Streptomyces virginiae* (A41030) and *Streptomyces toyocaensis* (A47934) are therefore potential hosts for the production of novel glycopeptides related to teicoplanin. We have previously shown that *S. toyocaensis* is a suitable host for gene cloning [9]. We report here the cloning of two glycosyltransferase genes from the vancomycin producer *Amycolatopsis orientalis* C329.4, and three glycosyltransferase genes from the A82846B producer, *A. orientalis* A82846, and describe the use of these genes to produce hybrid monoglycosylated

Figure 1



Glycopeptide structures.

glycopeptides derived from A41030A, A47934 and the vancomycin core, both *in vitro* and *in vivo* in a recombinant *S. toyocaensis* strain.

Results and discussion

Cloning and sequence analysis of five glycosyltransferase genes from microorganisms that produce glycopeptide antibiotics

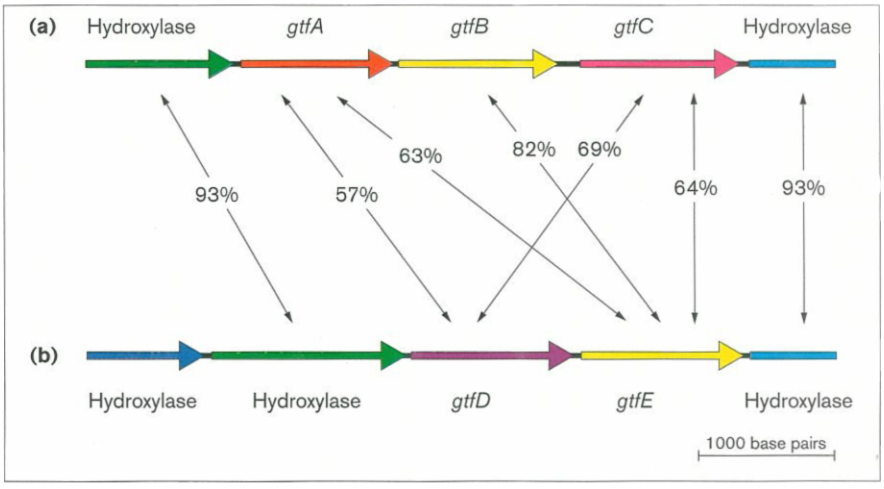
A series of polymerase chain reactions (PCR) was used to isolate DNA fragments containing glycosyltransferase genes from *A. orientalis* C329.4 and *A. orientalis* A82846 (see the Materials and methods section). DNA analysis for open reading frames (ORFs), analysis of the GC content of the codons [10], and comparison of the predicted genes to the nonredundant GENEMBL database, suggested that a 5.7 kilobase (kb) *Bgl*II restriction fragment isolated from *A. orientalis* A82846 contained three glycosyltransferase genes, *gtfA*, *gtfB*, and *gtfC*, and the 5.7 kb *Bgl*II restriction fragment isolated from *A. orientalis* C329.4 contained two glycosyltransferase genes, *gtfD* and *gtfE* (Fig. 2). The glycosyltransferases had amino-acid identities that ranged from 57% to 82% (Figs 2,3) and were flanked by ORFs homologous to hydroxylases that had about 93% amino-acid identities (Fig. 2). The glycosyltransferase encoded by the *gtfE* gene appeared to be the TDP-glucose:aglycosyl vancomycin glucosyltransferase on the basis of its identity to the amino-terminal amino-acid sequence of this enzyme (M. Zmijewski and B. Briggs, personal communication). The *gtfB* gene from *A. orientalis* A82846 appeared to encode an enzyme with the same function because GtfE had a higher percentage amino-acid sequence identity to GtfB (82%) than to GtfA (63%) or GtfC (64%; Fig. 2). In addition, the higher percentage amino-acid sequence identity of GtfC to GtfD (69%) than GtfA to GtfD (57%; Fig. 2) suggested that the GtfC and GtfD proteins may have similar functions, namely the addition of epivancosamine or vancosamine to glucose in the biosynthesis of A82846 or vancomycin. Because GtfA is the least similar to the other glycosyltransferases, it may add the third sugar (epivancosamine) to A82846 (Fig. 1).

Enzymatic glycosylation of glycopeptide aglycones *in vitro*

The *in vitro* glycosylation reactions are summarized in Figure 4. The GtfB and GtfE′ enzymes expressed in *Escherichia coli* added glucose to aglycosyl vancomycin (AGV) [11] in the presence of TDP-glucose *in vitro* to produce desvancosaminyl vancomycin (DVV) [11], establishing that these enzymes are glucosyltransferases (Fig 5a,e). (GtfE′ is a variant of GtfE with a single serine-for-proline substitution (Fig. 3) produced by a mutation introduced by PCR amplification of the gene.) GtfA and GtfC did not produce a glucosylated product in similar reactions. UDP-glucose appeared to function as well as TDP-glucose as a sugar donor for both GtfB and GtfE′ (data not shown). GtfE′ also added glucose relatively efficiently from TDP-glucose or UDP-glucose to alternative

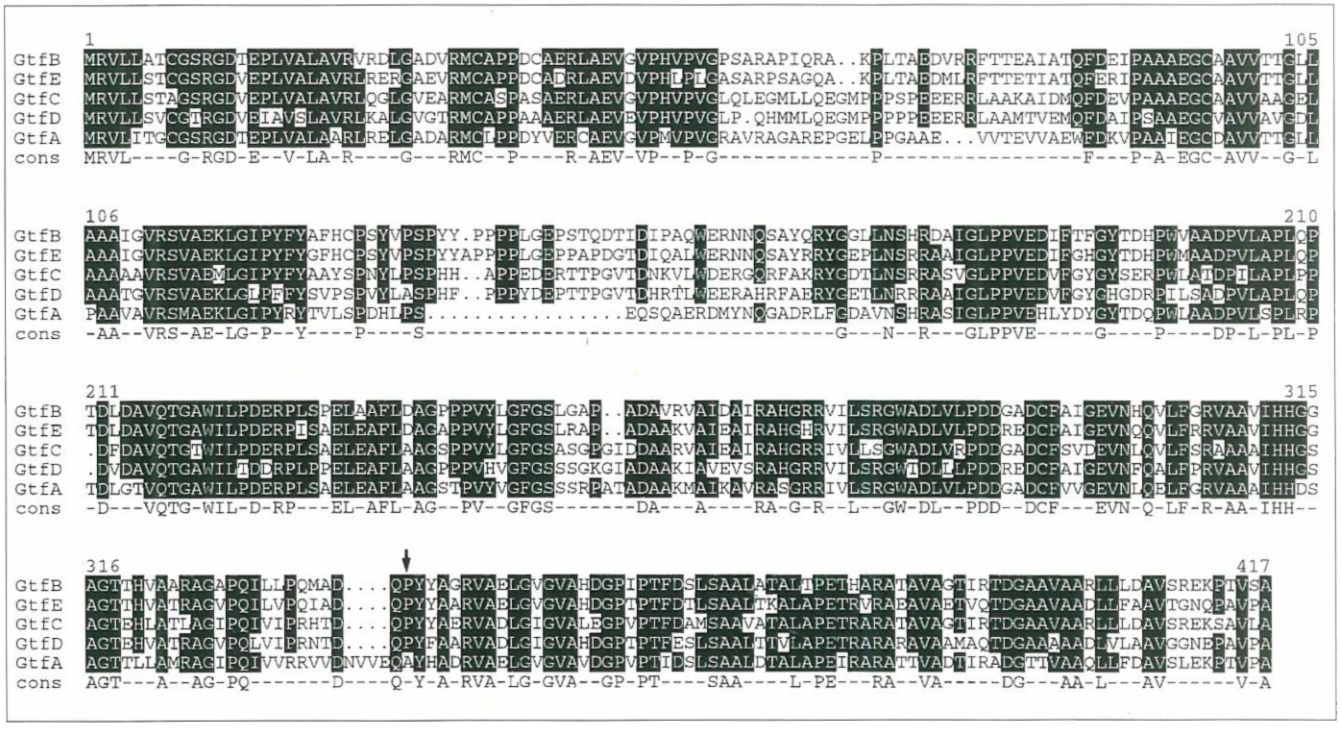
Figure 2

The percentage of identical amino acids in the glycosyltransferases and proteins encoded by the flanking genes. **(a)** The 5.7 kb *Bgl*II fragment from *A. orientalis* A82846*UV37B*. **(b)** The 5.7 kb *Bgl*II fragment from *A. orientalis* C329.4 (see text for details). The nucleotide sequences have been assigned Genbank accession numbers U84349 and U84350.



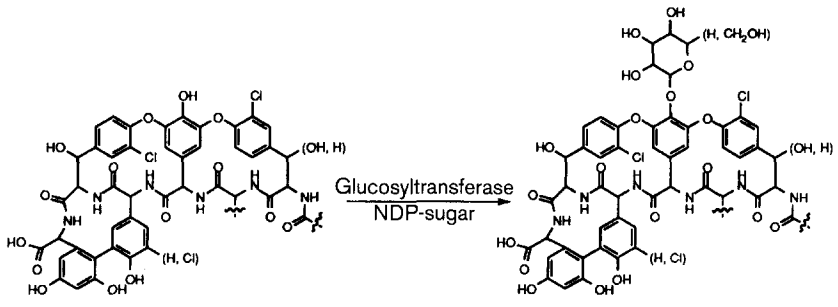
heptapeptide cores, A47934 and A41030A, as determined by high performance liquid chromatography (HPLC; Fig. 5b,c,f,g) and mass spectroscopy (data not shown). The purified glucosyl A41030A and glucosyl A47934 had antibacterial activity against *Micrococcus luteus*. A reaction containing GtfB, A41030 and TDP-D-glucose resulted in a small peak when analyzed by HPLC, which appeared to be glucosyl A41030. GtfB did not appear to glucosylate A47934. It was surprising that GtfE′ much more readily glucosylated an alternative heptapeptide substrate than did

Figure 3



PILEUP of glycosyltransferase amino-acid sequences. Amino acids occurring in at least four of five sequences are highlighted. Amino acids occurring in five of five sequences are listed on the consensus (cons) line. An arrow (↓) indicates the position of the proline-to-serine mutation in GtfE′. Numbering refers to the number of characters per line and does not take into account the gaps.

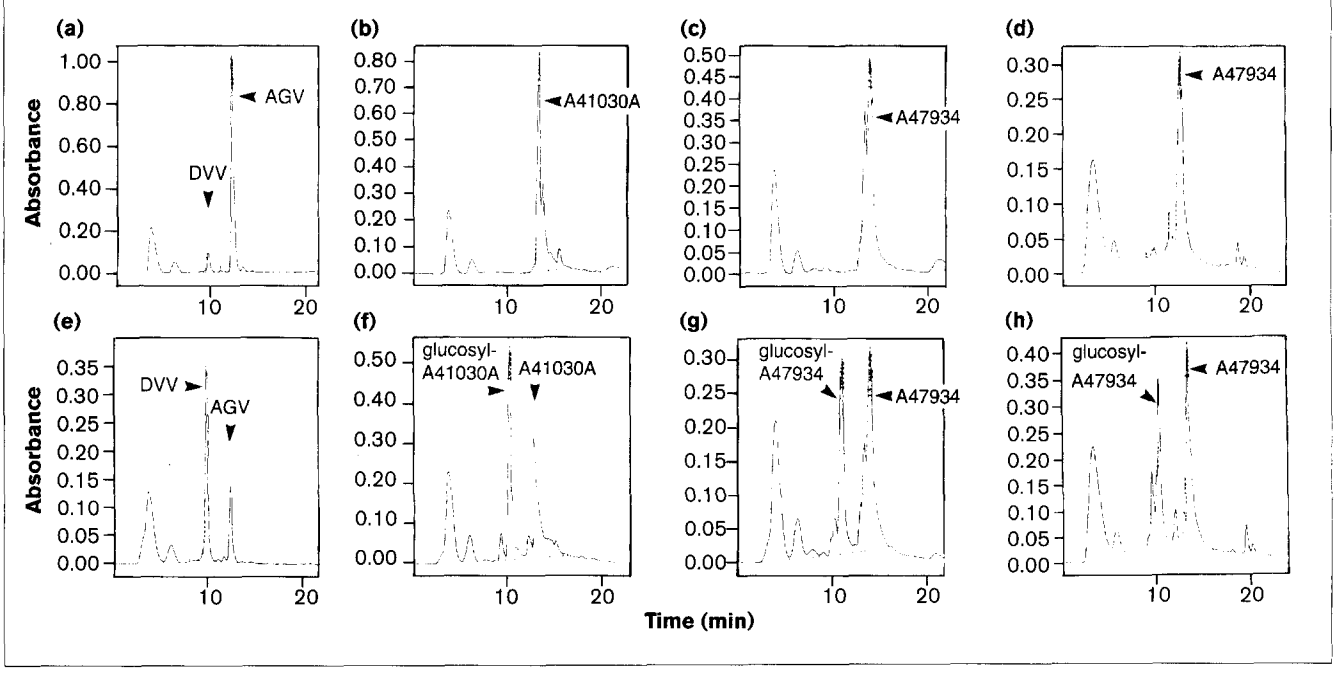
Figure 4



Heptapeptide	NDP-sugar	GtfE′	GtfB
AGV	TDP-D-glucose	+	+
	UDP-D-glucose	+	+
	UDP-D-xylose	+	+
	UDP-D-galactose	–	–
	UDP-D-mannose	–	–
	UDP-*N*-acetylglucosamine	–	–
	UDP-galacturonic acid	–	–
A41030A	TDP-D-glucose	+	(+)
	UDP-D-glucose	+	NT
A47934	TDP-D-glucose	+	–
	UDP-D-glucose	+	NT

Summary of *in vitro* glycosylation reactions using GtfE′ or GtfB. Symbols: +, 25–75% conversion to glycosylated product; (+), <2% conversion to glycosylated product; –, no conversion observed. NT, not tested; AGV, aglycosyl vancomycin.

Figure 5



HPLC profiles of glycosylation reactions. **(a–c)** *In vitro* reactions containing aglycosyl vancomycin (AGV), A41030A or A47934 and TDP-glucose without *E. coli* lysate. **(d)** *S. toyocaensis* A47934 fermentation. **(e–g)** *In vitro* reactions containing AGV, A41030A or A47934 and TDP-glucose with GtfE′ expressed in *E. coli*. **(h)** *S. toyocaensis* A47934::pCZA345 fermentation. The AGV standard used in (a) and (e) contained a small amount of desvancosaminyl vancomycin (DVV).

Table 1

Strains and plasmids.

Strains and plasmids	Description	Source/reference
E. coli DH5alpha	F⁻ *endA1 hsdR17* (r_k^-) *supE44 thi-1* λ⁻ *recA1 deoR gyrA96 relA1* Δ (*argF-lacZYA*) U169 ϕ80d*lacZ*Δ*M15 Nal*R	Gibco BRL
E. coli XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* Tet^r)]	Stratagene
E. coli S17-1	C600::RP4 2-Tc::Mu-Km::Tn7*hsdR hsdM*⁺*recA*	[25]
E. coli BL21(DE3)	F⁻ *ompT* r_B^- m_B^- DE3 lysogen	Novagen, [26]
A. orientalis A82846	Produces glycopeptide A82846	NRRL 18098
A. orientalis A82846*UV37B*	Enhanced producer of A82846B	Lilly culture collection
A. orientalis C329	Produces vancomycin	NRRL 2452
A. orientalis C329.4	Produces elevated quantity of vancomycin	Lilly culture collection
S. toyocaensis A80934	Produces glycopeptide A47934	[7]
S. virginiae A41030	Produces glycopeptide A41030A	[8]
pCR-Script SK(+)	PCR fragment cloning vector	Stratagene
pET-11a	*E. coli* expression vector	Novagen
pET-16b	*E. coli* expression vector, adds purification handle	Novagen
pIJ2926	pUC19 derivative containing *Bgl*II sites flanking the multiple cloning site	[24]
pIJ4070	pIJ2926 derivative containing *ermEp** promoter	M.J. Bibb
pCZA299	pIJ2926 derivative containing *A. orientalis* A82846*UV37B* 5.7 kb *Bgl*II fragment with *gtfA*, *gtfB* and *gtfC*	This work
pCZA301	pOJ436 derivative containing *A. orientalis* A82846*UV37B gtfA*, *gtfB* and *gtfC*	This work
pCZA318	pET-11a containing *gtfB*	This work
pCZA319	pZErO-1 containing *A. orientalis* C329.4 *gtfA* and *gtfB*	This work
pCZA333	pSET152 derivative containing Φ(*ermEp*-gtfB*) (*pLac* reading against *gtfB*)	This work
pCZA334	pSET152 derivative containing Φ(*ermEp*-gtfB*) (*pLac* reading with *gtfB*)	This work
pCZA338	pET-11a containing *gtfE′*	This work
pCZA339	pSET152 derivative containing *A. orientalis* C329.4 *gtfD* and *gtfE*	This work
pCZA344	pSET152 containing Φ(*ermEp*-gtfE′*) (*pLac* reading with *gtfE′*)	This work
pCZA345	pSET152 containing Φ(*ermEp*-gtfE′*) (*pLac* reading against *gtfE′*)	This work
pCZA361	Cosmid containing glycosyltransferase genes on 38 kb *A. orientalis* A82846*UV37B* genomic fragment	This work
pCZA362	Cosmid containing glycosyltransferase genes on 33 kb *A. orientalis* A82846*UV37B* genomic fragment	This work
pCZA363	Cosmid containing glycosyltransferase genes on 41 kb *A. orientalis* A82846*UV37B* genomic fragment	This work
pOJ436	Cosmid cloning vector, ϕC31 *attP*, *oriT*	[19]
pSET152	integrating plasmid, ϕC31 *attP*, *oriT*	[19]
pZErO-1	*E. coli* cloning vector	Invitrogen

GtfB, because these enzymes have a high degree of amino-acid identity and have the same natural substrates. The difference in activity between GtfB and GtfE′ cannot be attributed to the single amino-acid difference in GtfE′ because native GtfE from *A. orientalis* C329.4 glucosylated A47934 and A41030 relatively efficiently [12].

Both GtfB and GtfE′ converted ~25% of AGV to xylosyl AGV as confirmed by HPLC and mass-spectrometry analysis when UDP-D-xylose was substituted for TDP-glucose in the assay (data not shown). The xylosyl AGV product had antibacterial activity against *M. luteus*. Glycosylated products of AGV were not observed when UDP-D-galactose, UDP-D-mannose, UDP-D-galacturonic acid or UDP-*N*-acetylglucosamine were used as sugar donors. Interestingly, mannose and galactose differ from glucose only in the stereochemistry of one hydroxyl group, whereas xylose has the same stereochemistry as glucose but lacks a hydroxymethyl substituent.

Production of a hybrid glycopeptide antibiotic by a genetically engineered strain of *S. toyocaensis*

We recently developed a gene-cloning system for *S. toyocaensis* [9] that uses conjugal vectors that integrate site-specifically into the bacteriophage ϕC31 *attB* site without disrupting A47934 production. *A. orientalis* C329.4 and A82846*UV37B* DNA that contained glycosyltransferase genes was introduced into *S. toyocaensis* to determine whether glucosylation of A47934 would occur *in vivo*. *S. toyocaensis* A80934 containing cosmids pCZA361, pCZA362 and pCZA363 (Table 1 and the Materials and methods section) integrated into the chromosome did not appear to produce a new compound as determined by HPLC analysis. In addition, new compounds were not seen from *S. toyocaensis* clones containing the *gtfA*, *gtfB*, and *gtfC* genes from *A. orientalis* A82846 on the integrating plasmid pCZA301 or the *gtfD* and *gtfE* genes from *A. orientalis* C329.4 on the integrating plasmid pCZA339. When the strong constitutive *ermEp** promoter was inserted in front of *gtfE′* on

plasmids pCZA344 and pCZA345 and these plasmids were inserted into the ϕC31 *attB* site of *S. toyocaensis*, however, a new peak with antibacterial activity was observed after fermentation that had the same HPLC retention time as glucosyl A47934 (Fig. 5d,h). A new peak was not observed using similar constructs containing the *gtfB* gene (plasmids pCZA333 and pCZA334).

These results suggest that a promoter is not present on the DNA fragments used for expression of the glycosyltransferases, or that the natural *A. orientalis* promoter(s) are not recognized by *S. toyocaensis*, since *ermEp** was needed to obtain a glycosylated product. The *in vivo* results also agree with the *in vitro* results indicating that the GtfE' enzyme more readily accepts the A47934 substrate than the GtfB enzyme. Because GtfB and GtfE have 82% amino-acid identity, these glucosyltransferases should be useful in exploring the amino-acid sequences involved in determining the specificity of glycopeptide substrate recognition. Furthermore, the five cloned glycosyltransferase genes can be used to determine the regions involved in substrate and cofactor specificity, and will provide the starting materials for further biosynthesis of novel glycopeptides. By the production of glucosyl A47934, glucosyl A41030A, and xylosyl AGV, we have shown that hybrid glycopeptide antibiotics can be produced *in vitro* and *in vivo* using cloned glycosyltransferase genes. Use of these and possibly other glycosyltransferase genes may allow the production of a wide array of novel glycopeptide antibiotics which can serve as starting materials for further chemical modification.

Significance

This work describes the first use of cloned and expressed glycosyltransferase genes to produce hybrid glycopeptide antibiotics. Enzymatic glycosylation allows us to produce specific glycopeptides, a process that is difficult to perform by chemical synthesis. Glycosyltransferases have some flexibility in their substrate specificity, however, making it possible to synthesize hybrid glycopeptide antibiotics that differ in their sugar content or peptide cores.

The expression of the glycosyltransferase in *E. coli* and the use of an active enzyme extract to glycosylate *in vitro* allows sugar or peptide core substrates that do not occur naturally in a particular host organism to be used. The expression of a glycosyltransferase in a recombinant host that produces the core peptide makes possible the production of the hybrid antibiotic during growth of the organism, and there is potential for production on a larger scale. The use of additional glycosyltransferases and modification of these enzymes by manipulating the domains that contribute to substrate specificity may make possible the enzymatic synthesis of a wide variety of glycosylated antibiotics.

Materials and methods

Bacterial strains, culture conditions and compounds

Bacterial strains used in this study are listed in Table 1. The actinomycete and *E. coli* cultures were grown in TSB and TY broth respectively [13]. Compounds A47934, A41030A, AGV and apramycin were obtained from Eli Lilly and Co. Nucleotide-sugars were purchased from Sigma Chemical Co. The isolated compounds were tested for antibacterial activity by resuspending a small amount of the compound in water and spotting on TY agar [13] seeded with *M. luteus*. After incubation overnight at 37°C the plates were checked for a zone of growth inhibition.

DNA cloning, amplification and sequencing

DNA cloning and *E. coli* transformations were performed as described [14]. Plasmids were introduced by transformation or by conjugation from *E. coli* S17-1 to *S. toyocaensis* as described [9]. Plasmid DNA was isolated from *E. coli* using Qiagen columns (Qiagen, Inc.) according to the manufacturer's protocol. Genomic DNA was isolated from actinomycetes as described [15]. DNA fragments were purified from gels using Gene Clean II (Bio 101, Inc.) except for the 84 base-pair (bp) fragment which was purified using Mermaid (Bio 101, Inc.). Southern hybridizations were as described [16]. Restriction enzymes, alkaline phosphatase and DNA polymerase were used according to the suppliers specifications; 3% Nusieve 3:1 agarose gels (FMC) were used for the analysis of DNA fragments <1 kb in size. The thermocycling procedure for PCR reactions consisted of 99°C for 1–5 min, 80°C for sufficient time to add the DNA polymerase [17] followed by 30 cycles of amplification (95° or 96°C for 30s, annealing temperatures ranging from 62°–68° for 30s, followed by 72°C for 1–2 min). Touchdown PCR [18] was used for heterologous or degenerate primers. Stoffel DNA polymerase (Perkin Elmer) was used for PCR amplification unless otherwise indicated. Broth culture (2 μl) and Gene Releaser™ (BioVentures, Inc.) was used for PCR amplification from the cosmid library. PCR and sequencing primers were synthesized using an Applied Biosystems 380B oligonucleotide synthesizer (PE-ABI) or obtained from Genosys Biotechnologies, Inc. DNA fragments were either cloned and sequenced or sequenced directly after gel isolation by Dye Terminator chemistry using a PRISM cycle sequencing Ready Reaction Kit (PE-ABI). Reactions were cycled using 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Gel preparation, electrophoresis, and data collection using a Model 377 DNA Sequencer (PE-ABI) were according to the manufacturer's protocol. The 5.7 kb *Bgl*II fragment from *A. orientalis* A82846 was sequenced by Beksan Ltd. Sequence comparison and analysis was performed using the Wisconsin Package (Genetics Computer Group).

Preparation of the cosmid library

A cosmid library of *A. orientalis* A82846*UV37B* was prepared essentially as described for a two-cos system [13]. Cosmid vector pOJ436 [19] was digested with *Hpa*I, treated with calf alkaline phosphatase, and then digested with *Bam*HI. A82846*UV37B* DNA embedded in soft agarose [20] was partially digested with *Sau*3A to yield the maximum amount of 38–50 kb fragments. The *Sau*3A fragments were ligated with the *Bam*HI-digested pOJ436 and packaged with Gigapack II-XL (Stratagene) lambda extracts according to the supplier's protocol. After infection, 1152 *E. coli* XL-1 Blue colonies containing cosmids were inoculated into individual wells in 12 96-well microtiter plates and into wells of a deep 96-well plate so that each deep well was inoculated with 12 colonies from one row of the microtiter plate. The plates containing two-times concentrated TY broth [13] with 100 μg ml^{-1} apramycin were sealed and incubated overnight at 37°C with gentle shaking. Glycerol was added to 20% final concentration and the library was stored at −70°C.

Isolation of glycosyltransferase genes from A. orientalis *A82846*UV37B *and* A. orientalis *C329.4*

PCR primers PJS49 (5′ACITGYGGITCICGIGGIGACGTIGARCC3′) and PJS50 (5′CATCATICGIACYTCIGCICCRCAYTC3′) were designed by back-translation of the 33 amino-acid amino-terminal sequence

(M. Zmijewski and B. Briggs, personal communication) of the TDP-glucose:aglycosyl vancomycin glucosyltransferase isolated from *A. orientalis* C329 [21]. Primers contained deoxyinosine or were degenerate at several of the ambiguous codon positions [22]. PCR reactions containing *A. orientalis* C329.4 genomic DNA, but not A82846*UV37B* genomic DNA, successfully amplified a 84 bp PCR product using primers PJS49 and PJS50 and *Taq* DNA polymerase (BRL). The 84 bp product was sequenced, and the 28 bp amplified between the primer sequences encoded amino acids that agreed with the amino-terminal amino-acid sequence (data not shown). A new primer, PJS72 (5′GARCCACTGGTGGCGTTGGCGGT3′) was designed for amplification of the downstream region of the glucosyltransferase based on the 28 bp sequence. In addition, a search of a nonredundant Genbank and EMBL databases as of February 8, 1994 using TBLASTN [23] with the 33 amino-acid amino-terminal sequence found two sequences that had similarity at their amino-terminal ends. These sequences encoded a rhamnosyltransferase from *Pseudomonas aeruginosa* (accession number L28170) and a hypothetical protein from *Mycobacterium leprae* (accession number U00023). Conserved amino acids in these two sequences, which had 25% identity to each other, were used to design a series of 10 downstream primer sequences (not shown). PCR amplification using primers PJS49 and PJS57 (5′GGIACTCCGGCCCTIAGGCTIGCIGCIGTIGTICCIGCGCCICCRTG3′), and reamplification two times using primers PJS72 and PJS57, resulted in a 950 bp fragment amplified from *A. orientalis* C329.4 which encoded an amino-acid sequence with about 27% identity to the *P. aeruginosa* and *M. leprae* sequences. Several primers (not shown) were designed based on amino acids conserved between the three sequences. Of these, primers PJS86 (5′TTGCGGGAGCGCGGCGCCGAGGT3′) and PJS92 (5′CCGGCGTCCAGGAACGCCTCCAGCTC3′) amplified a band from *A. orientalis* A82846*UV37B* genomic DNA. Using these primers, PCR amplification of a *A. orientalis* A82846*UV37B* cosmid library (pools of clones then individual clones) was used to identify three cosmids, pCZA361, pCZA362 and pCZA363, containing glycosyltransferase genes. Cosmid pCZA361 contained ~38 kb of genomic DNA and extended downstream of the glycosyltransferase genes. Cosmid pCZA363 contained ~41 kb of genomic DNA and extended upstream of the glycosyltransferase genes. Cosmid pCZA362 contained ~33 kb and is a subset of cosmid pCZA363. Cosmids pCZA363 and pCZA361 had ~6.5 kb of genomic DNA in common and together represent coverage of ~72 kb of the genome.

The 950 bp fragment amplified from *A. orientalis* C329.4 hybridized to 5.7 kb *Bgl*II fragments from cosmid pCZA361 and both *A. orientalis* A82846*UV37B* and *A. orientalis* C329.4 genomic DNA. Cosmids pCZA362 and pCZA363 contained larger hybridizing fragments. The 5.7 kb *Bgl*II-fragment from pCZA361 was cloned into the *Bam*HI site of pIJ2926 [24] resulting in plasmid pCZA299 and was sequenced by Beksan Ltd.

C329.4 genomic DNA (20 μg) was digested with *Bgl*II and the resulting DNA fragments were separated on a 0.75% agarose gel. The gel, containing DNA fragments ranging in size from ~5.0–6.5 kb, was sliced into four pieces containing pools of similar sized DNA fragments. DNA from the four pools was used as a template in PCR reactions containing primers PJS86 and PJS92. The pool of DNA which gave a PCR-amplified band at the expected size of 650 bp was cloned into *Bam*HI-digested pZErO-1 (Invitrogen), according to the manufacturer's protocol. PCR using the same primers and ~25% of a transformed colony as a source of template was used to identify one clone, pCZA319, of the 50 tested, which contained a 5.7 kb fragment and gave an amplified fragment of 650 bp.

Construction of plasmids

PCZA299 was digested with *Acs*I and *Nar*I and the 1.2 kb fragment containing *gtfB* was subcloned into *EcoR*I- and *Nar*I-digested pUC19 resulting in pCZA305; *gtfB* was amplified by PCR using primers PJS115 (5′GGGAAGCTT*CATATG*CGTGTGCTGTTGGCGACGTGTG3′ and PJS118 (5′GGG*AGATCTT*TACGCGGAAACAGTCGGCTTTTCC3′) and UlTma DNA polymerase (Perkin Elmer) to add an *Nde*I site at the 5′ end and a *Bgl*II site (italics) at the 3′ end of the gene. The blunt PCR product was cloned into pCR-Script SK(+) vector resulting in plasmid pCZA308. The *Nde*I to *Bam*HI fragment of pCZA308 encoding *gtfB* was subcloned into pET-16b resulting in pCZA312. An *Rsr*II to *Aat*II fragment of pCZA312 (PCR-derived clone) was replaced with an *Rsr*II to *Aat*II fragment of pCZA305 (genomically derived clone) resulting in plasmid pCZA315. This corrected three nucleotide errors that encoded amino-acid changes in the PCR-derived *gtfB* sequence that resulted in an inactive glycosyltransferase (data not shown). PCZA318 was constructed by cloning the 1.2 kb *Nde*I fragment from pCZA315 into the *Nde*I site of pET-11a so that the T7 promoter on the plasmid could drive expression of the *gtf*B gene.

PCZA319 was digested with *Nco*I and the 3.4 kb fragment containing *gtfE* was isolated and amplified by PCR in 10 separate reactions using primers PJS142 (5′GGGAAGCTT*CATATG*CGTGTGTTGTTGTCGACC3′) and PJS143 (5′GGG*AGATCTT*CAGGCGGGAACGGCGGGCTGGT3′) and Vent$_R$® DNA polymerase (NEB) to add an *Nde*I site at the 5′ end and a *Bgl*II site (italics) at the 3′ end of the gene. Nine of the ten reactions had fragments of ~1250 bp. Fragments from five reactions were cloned into pCR-Script SK(+) and then subcloned on an *Nde*I- to *Bgl*II-fragment into *Nde*I- and *Bgl*II-digested pET-11a. *In vitro* glycosylation assays using AGV and TDP-glucose showed that one of these clones, containing plasmid pCZA338, expressed glucosyltransferase activity.

PCZA338 was digested with *Xba*I and *Hin*dIII, and the ~1.5 kb fragment containing *gtfE′* was cloned into *Xba*I- and *Hin*dIII-digested pIJ4070 resulting in plasmid pCZA340. (The *gtfE′* gene contained two PCR-induced mutations resulting in one amino-acid difference between GtfE and GtfE′). The 1.8 kb *Bgl*II fragment of pCZA340 containing *gtfE′* downstream of *ermEp** was cloned in both orientations into *Bam*HI-digested pSET152 resulting in pCZA344 and pCZA345. Similarly, pCZA318 was digested with *Xba*I and *Hin*dIII and the ~1.5 fragment containing *gtfB* was cloned into *Xba*I- and *Hin*dIII-digested pIJ4070, resulting in plasmid pCZA329. The 1.8 kb *Bgl*II fragment of pCZA329 containing *gtfB* downstream of *ermEp** was cloned in both orientations into *Bam*HI-digested pSET152 resulting in pCZA333 and pCZA334.

PCZA299 was digested with *Bgl*II and the fragment containing *gtfA*, *gtfB* and *gtfC* was cloned into *Bam*HI-digested pOJ436, resulting in plasmid pCZA301. This plasmid was digested with *Pvu*II and the ~4.6 kb fragment containing *gtfD* and *gtfE* and the ORF upstream was cloned into *Eco*RV-digested pSET152, resulting in pCZA339.

In vitro *glycosylation reactions*

An overnight culture of *E. coli* BL21(DE3) cells containing expression plasmids pCZA318 or pCZA338 (5 ml) was diluted into TY broth containing carbenicillin at 50 μg ml^{-1} and grown with shaking at 27°C to an A_{600} of 0.4–1.0. The cells were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubated for 2.5–3 h. The cells were pelleted and resuspended in 0.1 vol of 50 mM TRIS (pH 9.0). Lysozyme was added to 0.5 mg ml^{-1} and incubated for 10 min at room temperature. The cells were broken by sonication or by forcing them through a 23-gauge needle with a syringe several times. Aliquots of the lysate (2 ml) were pelleted for 1 min in a microfuge. The glycosylation reactions based on reactions previously described [21] contained 1–2 mg of AGV, A41030A or A47934 dissolved in 50 mM TRIS (pH 9.0), 3–5 mg of a nucleotide-sugar in 100 μl H_2O, 5 μl 1.0 M dithiothreitol (DTT), 10 μl 100 mg ml^{-1} bovine serum albumin (BSA) and about 585 μl of the cell supernatant. Reactions (1 ml) were incubated at 37°C with slow tumbling overnight. Reactions were filtered through an Acrodisc 13 CR PTFE 0.45 mM filter (Gelman) and then analyzed by HPLC and mass spectrometry.

Fermentation of recombinant S. toyocaensis *strains*

Recombinant *S. toyocaensis* strains were fermented on the glucose-containing medium SGC2 for 7 days at 30°C. The fermentation medium

at pH 6.8–7.0 contained (per liter): glucose, 15 g; potato dextrin, 30 g; blackstrap molasses, 3.0 g; nutrisoy flour, 15 g; casein, 5.0 g; $CaCO_3$, 5.0 g. After fermentation the cells were pelleted and the supernatant was analyzed by HPLC.

HPLC analysis and isolation of glycosylated compounds

The *in vivo* and *in vitro* glycosylations were monitored by analytical HPLC (reversed-phase) using a Waters μBondapak C18 column (3.9×300 mm) at a flow rate of 2 ml min^{-1} with UV detection at 280 nm. Elution was accomplished with a linear gradient of 5% CH_3CN–95% buffer to 80% CH_3CN–20% buffer over 30 min. The buffer used was 0.5% triethylamine in water, adjusted to pH 3 with H_3PO_4. Purification of the glycosylated products was accomplished by semi-preparative HPLC using a Zorbax SB-C18 column (21.2×250 mm) at a flow rate of 15 ml min^{-1} and UV detection at 280 nm. Elution was accomplished with the same gradient/solvent system as described above. The desired fraction was desalted with a Waters Sep-Pak (12 cc) then lyophilized to afford the product as a white powder.

Electrospray LC/MS analyses

Mobile phases consisted of 0.1% (v/v) trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid (v/v) in acetonitrile (Solvent B). A linear gradient from 5–80% Solvent B over 45 min at a flow rate of 1 ml min^{-1} was used. Peaks were detected at a wavelength of 280 nm. A 20 μl min^{-1} post-column split flow rate was delivered to the pneumatically assisted electrospray source of a PE-Sciex API III triple quadrupole mass spectrometer. Mass spectral analyses were performed in the positive ion detection mode over a range of 500–2000 u in 0.35 u intervals for a dwell time of 0.75 msec per interval.

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