

Genetic dissection of the biosynthetic route to gentamicin A₂ by heterologous expression of its minimal gene set

Je Won Park[†], Jay Sung Joong Hong[‡], Niranjan Parajuli[†], Won Seok Jung[‡], Sung Ryeol Park[†], Si-Kyu Lim[§], Jae Kyung Sohng[¶], and Yeo Joon Yoon^{†||}

[†]Division of Nano Sciences, Ewha Womans University, 11-1 Daehyung-dong, Seodaemun-gu, Seoul 120-750, Republic of Korea; [‡]Interdisciplinary Program of Biochemical Engineering and Biotechnology, Seoul National University, San 56-1, Shilim-dong, Gwanak-gu, Seoul 151-742, Republic of Korea; [§]Genotech Corporations, 59-5 Jang-dong, Usung-gu, Daejeon 305-343, Republic of Korea; and [¶]Department of Pharmaceutical Engineering, Institute of Biomolecule Reconstruction, Sun Moon University, 100 Kalsanri, Tangeonmyun, Asansi, Chungnam 336-708, Republic of Korea

Communicated by Arnold L. Demain, Drew University, Madison, NJ, April 10, 2008 (received for review November 1, 2007)

Since the first use of streptomycin as an effective antibiotic drug in the treatment of tuberculosis, aminoglycoside antibiotics have been widely used against a variety of bacterial infections for over six decades. However, the pathways for aminoglycoside biosynthesis still remain unclear, mainly because of difficulty in genetic manipulation of actinomycetes producing this class of antibiotics. Gentamicin belongs to the group of 4,6-disubstituted aminoglycosides containing a characteristic core aminocyclitol moiety, 2-deoxystreptamine (2-DOS), and the recent discovery of its biosynthetic gene cluster in *Micromonospora echinospora* has enabled us to decipher its biosynthetic pathway. To determine the minimal set of genes and their functions for the generation of gentamicin A₂, the first pseudotrisaccharide intermediate in the biosynthetic pathway for the gentamicin complex, various sets of candidate genes from *M. echinospora* and other related aminoglycoside-producing strains were introduced into a nonaminoglycoside producing strain of *Streptomyces venezuelae*. Heterologous expression of different combinations of putative 2-DOS biosynthetic genes revealed that a subset, *gtmB-gtmA-gach*, is responsible for the biosynthesis of this core aminocyclitol moiety of gentamicin. Expression of *gtmG* together with *gtmB-gtmA-gach* led to production of 2'-*N*-acetylparomamine, demonstrating that GtmG acts as a glycosyltransferase that adds *N*-acetyl- β -glucosamine (GLcNA) to 2-DOS. Expression of *gtmM* in a 2'-*N*-acetylparomamine-producing recombinant *S. venezuelae* strain generated paromamine. Expression of *gtmE* in an engineered paromamine-producing strain of *S. venezuelae* successfully generated gentamicin A₂, indicating that GtmE is another glycosyltransferase that attaches β -xylose to paromamine. These results represent *in vivo* evidence elucidating the complete biosynthetic pathway of the pseudotrisaccharide aminoglycoside.

aminoglycoside biosynthesis | *Micromonospora echinospora* | *Streptomyces venezuelae* | pathway engineering

Gentamicin is an aminoglycoside antibiotic complex produced by various *Micromonospora* species (1). Aminoglycosides act by binding to the bacterial ribosome inhibiting protein synthesis and generating errors in the translation of the genetic code (2). This class of antibiotics has been used clinically for the treatment of severe bacterial infections for >60 years, beginning with the use of streptomycin to treat tuberculosis (3). However, the genetics underlying the biosynthesis of aminoglycosides, including gentamicin, still remains unclear. Structurally, gentamicin is a 4,6-disubstituted aminocyclitol composed of the core aminocyclitol moiety, 2-deoxystreptamine (2-DOS, **1a**), which is decorated by purpurosamine and garosamine aminosugars at positions C-4 and C-6, respectively. In its therapeutic form, gentamicin comprises a complex of gentamicin C₁, C_{1a}, and C₂, which differ only in the degree of methylation of the C-6' position of the sugar attached at C-4 of 2-DOS (Fig. 1). Other

minor components of gentamicin are also produced by *Micromonospora echinospora*, including gentamicin A₂ (**4a**), the precursor from which other forms of gentamicin are derived (4).

The recent discovery of gene clusters for biosynthesis of butirosin and neomycin in *Bacillus circulans* and *Streptomyces fradiae*, respectively, has facilitated genetic and biochemical studies on the biosynthesis of 4,5-disubstituted aminoglycosides (5–9). Concurrently, the biosynthetic gene clusters relating to the 4,6-disubstituted aminoglycosides kanamycin, tobramycin, and gentamicin have been isolated from *Streptomyces kanamyceticus*, *Streptomyces tenebrarius*, and *M. echinospora*, respectively (10–13). However, little progress has been made to evaluate the proposed biosynthetic routes to these antibiotics, mainly because of difficulty in genetic manipulation of the producing microorganisms. Gentamicin-producing *M. echinospora* has innate resistance to most antibiotics precluding the use of standard *Streptomyces* cloning vectors displays low transformation efficiency (<10⁻⁷ transformants per microgram of DNA) (14). Current knowledge regarding the biosynthesis of 2-DOS containing aminoglycosides was recently reviewed, and most of cited works relied on *in vitro* studies of individual gene products (15). When the biosynthetic route to the gentamicin complex was first predicted, it was based on isolation of metabolites from cell culture extracts and precursor feeding studies of blocked mutants (16, 17). However, functional studies of gentamicin biosynthetic genes have not been possible, so that assignment of the roles of gene products has hitherto been based purely on amino acid sequence comparisons pending verification by direct experimental evidence.

We show here that heterologous expression of different combinations of genes from the gentamicin biosynthetic gene cluster (refs. 11 and 12: GenBank accession nos. AJ575934 and AY524043) in a nonaminoglycoside producing strain of *S. venezuelae* caused production of intermediates from the gentamicin biosynthetic pathway including 2-DOS (**1a**), 2'-*N*-acetylparomamine (**2a**), paromamine (**3a**), and the first pseudotrisaccharide, gentamicin A₂ (**4a**). This provided experimental evidence for assigning the functions of individual gene products, allowing us to recognize a complete biosynthetic pathway for generation

Author contributions: J.W.P. and J.S.J.H. contributed equally to this work; J.W.P., N.P., S.-K.L., J.K.S., and Y.J.Y. designed research; J.W.P., J.S.J.H., N.P., W.S.J., and S.R.P. performed research; J.W.P. and J.K.S. contributed new reagents/analytic tools; J.W.P., J.S.J.H., N.P., S.-K.L., J.K.S., and Y.J.Y. analyzed data; and J.W.P., J.S.J.H., and Y.J.Y. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AM946392).

||To whom correspondence should be addressed. E-mail: joonyoon@ewha.ac.kr.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803164105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

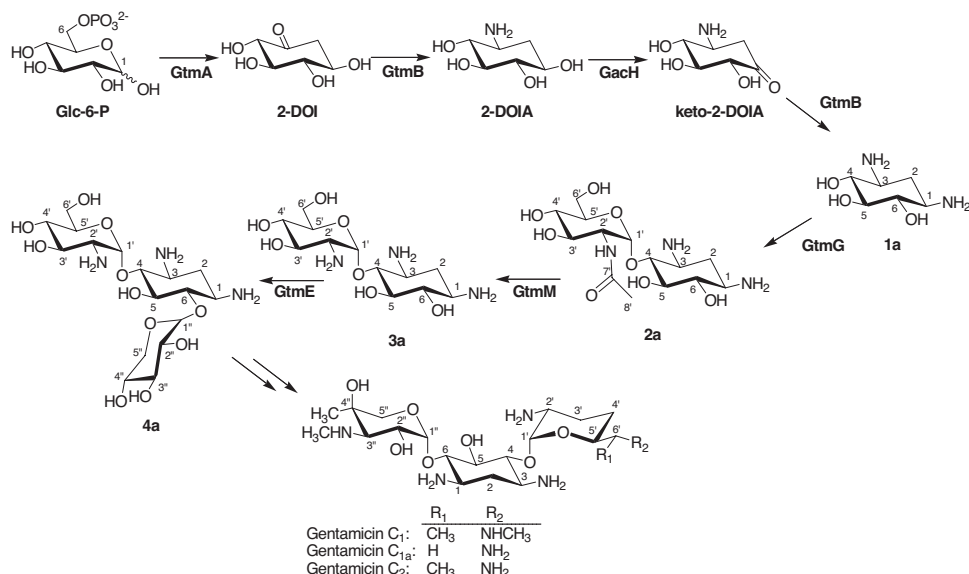


Fig. 1. Proposed biosynthetic pathway of gentamicin A₂ (**4a**) and its biosynthetic intermediates 2-deoxystreptamine (**1a**), 2'-N-acetylparomamine (**2a**), and paromamine (**3a**).

of gentamicin A₂. This work has established an efficient heterologous expression platform based on a genetically amenable streptomycete to dissect the biosynthetic pathways of other aminoglycoside antibiotics produced by less tractable actinomycetes.

Results and Discussion

Putative gentamicin biosynthetic genes, suggested by previous studies to encode the 2-DOS biosynthetic enzymes, two glycosyltransferases and a deacetylase, were amplified by PCR (8, 10, 18) and cloned into a thiostrepton-resistance vector, pSE34, under control of the *ermE** promoter (19). Plasmids harboring different sets of genes necessary for the biosynthesis of gentamicin and its intermediates were heterologously expressed in a mutant strain of *S. venezuelae* YJ003 that lacks the ability to biosynthesize its native deoxysugar (TDP-D-desosamine) (20). The culture extracts from the resulting strains were analyzed by using HPLC-ESI-MS/MS to detect gentamicin-related compounds, the structures of which were then further identified by comparison of the retention times, MS/MS fragmentation patterns, and NMR spectra with those obtained from authentic reference compounds.

A Minimal Set of Genes Necessary and Sufficient for 2-Deoxystreptamine Biosynthesis. Gentamicin biosynthesis begins with D-glucose-6-phosphate (Glc-6-P), which is modified to 2-deoxy-scyllo-inosose (2-DOI) by carbocyclization through the action of GtmA, as described in ref. 12. Two aminotransferase reactions and a dehydrogenase step are then required for the conversion of 2-DOI via 2-deoxy-scyllo-inosamine (2-DOIA) and 1-keto-2-deoxy-scyllo-inosamine (keto-2-DOIA) to produce 2-DOS (**1a**) (21–24) (Fig. 1). Based on an earlier study isolating the gentamicin biosynthetic gene cluster (11, 12), we cloned the *gtmD-gtmB-gtmA-gtmC* genes, which encode the inosose synthase (GtmA) and putative aminotransferases (GtmB and GtmD), plus the deduced dehydrogenase (GtmC), and expressed them in the heterologous host *S. venezuelae* YJ003 using pYJ494 in what proved to be an unsuccessful attempt to biosynthesize 2-DOS (**1a**). This negative result prompted us to replace *gtmC* with *gacH* (otherwise known as *gntP*), which locates between *gtmK* and *gacI* within the gentamicin biosynthetic gene cluster, following the prediction (15) that GacH might be the dehydrogenase involved

in 2-DOS biosynthesis, based on its sequence homology to the 2-DOIA dehydrogenase Neo5 [CAH58688, otherwise known as NeoA (BAD95818)] involved in neomycin biosynthesis. When the resultant plasmid pYJ495 harboring *gtmD-gtmB-gtmA-gacH* was expressed in the heterologous host, 2-DOS (**1a**) was indeed produced (Fig. 24). The two transamination reactions required for biosynthesis of 2-DOS during butirosin production by *B. circulans* are catalyzed by a single enzyme, BtrS [BAE07061, otherwise known as BtrR (CAD41947)] (23). To ascertain whether the biosynthesis of 2-DOS during gentamicin production likewise involves dual activity of a single aminotransferase, two separate constructs pYJ496 and pYJ497 expressing *gtmB-gtmA-gacH* and *gtmD-gtmA-gacH*, respectively, were tested. The expression of *gtmB*, *gtmA*, and *gacH* produced ≈120 μg per liter of 2-DOS (**1a**) (corrected for ≈40% mean recoveries of **1a**), whereas, *gtmD*, *gtmA*, and *gacH* did not do so (Fig. 2 B and C), indicating that GtmB catalyzes both transamination steps in the synthesis of 2-DOS, whereas GtmD may act in a later step during gentamicin production. The lower recoveries (<50%) of both 2-DOS (**1a**) and 2'-N-acetylparomamine (**2a**) were observed compared with those of other biosynthetic intermediates of gentamicin, probably because of their weak adsorption on the solid-phase extraction (SPE) cleanup column during analysis (25). The predicted products of *gtmA*, *gtmB*, and *gacH* are similar to those of other 2-DOS (**1a**) biosynthetic genes from several 4,5- and 4,6-disubstituted aminoglycoside producers [see [supporting information \(SI\) Figs. S1–S3 and SI Text](#)]. Interestingly, the deduced product of *gacH*, which contains two zinc-binding motifs found in the NAD(P)-dependent dehydrogenase NeoA (Neo5) (22), does not show sequence similarity to the recently characterized AdoMet-dependent dehydrogenase BtrN (24), suggesting that GacH belongs to the NAD(P)-dependent dehydrogenase family (see [Fig. S3](#)).

Conferring Gentamicin Resistance in the Heterologous Host. A prerequisite for the biosynthesis of gentamicin in a heterologous host is self-protection of the host strain. To confer resistance to gentamicin in *S. venezuelae*, a plasmid, pYJ489, harboring three putative resistance genes (*gtmF-gtmL-gtmK*), was introduced into this mutant strain. The resistance genes *gtmF* and *gtmL* were chosen based on their ability to confer gentamicin resistance via specific methylation of the bacterial ribosomes without modify-

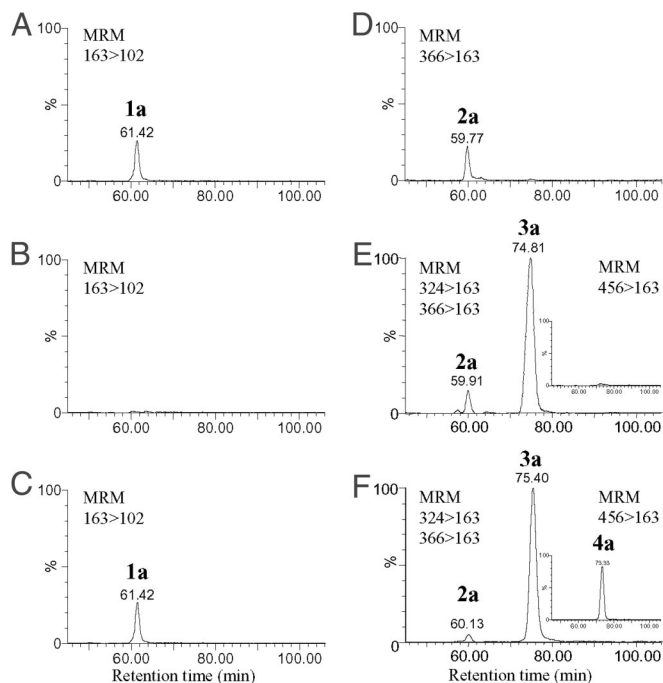


Fig. 2. HPLC-ESI-MS/MS analysis of extracts from *S. venezuelae* strains harboring the plasmids (A) pYJ495 expressing *gtmD-gtmB-gtmA-gach*. (B) pYJ497 expressing *gtmD-gtmA-gach*. (C) pYJ496 expressing *gtmB-gtmA-gach*. (D) pYJ498 expressing *gtmB-gtmA-gach-gtmG*. (E) pYJ503 expressing *gtmB-gtmA-gach-gtmG-gtmM*. (F) pYJ505 expressing *gtmB-gtmA-gach-gtmG-gtmE-gtmM*. pYJ503 and pYJ505 coexpress the resistance genes *gtmF-gtmL-gtmK*. Monitoring of gentamicin A₂ (4a) (456 > 163) is separately illustrated as an *Inset* to each chromatogram E and F.

ing the antibiotic itself (11). The *gtmK* gene presumably encodes a transport protein involved in the export of aminoglycosides (11). Transformants harboring pYJ489 were resistant to 100 μg/ml of the commercially available gentamicin sulfate compared with ≈1 μg/ml in control strains. The cell culture was extracted and analyzed for gentamicin components, and any of their derivatives (but only unmodified gentamicins) were recovered. These resistance determinants were expressed together with the gentamicin biosynthetic genes in the heterologous host in subsequent experiments.

GtmG Acts as an UDP-GlcNAc Glycosyltransferase to Generate 2'-N-Acetylparomamine (2a). Two glycosylation steps each involving NDP sugar cosubstrates are proposed to be required for gentamicin biosynthesis: the addition of D-glucosamine (GlcN) to form the pseudodisaccharide intermediate paromamine (3a), and the subsequent addition of D-xylose to form gentamicin A₂ (4a) (Fig. 1). Two glycosyltransferases genes (*gtmE* and *gtmG*) identified within the gentamicin gene cluster (12) were expressed separately in the heterologous host to determine their roles. First, pYJ498 was constructed containing *gtmG* together with the 2-DOS biosynthetic genes (*gtmB-gtmA-gach*) plus the resistance genes (*gtmF-gtmK-gtmL*). However, transformants containing pYJ498 did not synthesize paromamine (3a) but instead produced ≈105 μg/liter of 2'-N-acetylparomamine (2a) (corrected for ≈40% mean recoveries of 2a) (Fig. 2D). In contrast, transformants harboring pYJ499, derived from pYJ498 by replacement of *gtmG* by *gtmE*, produced neither 2'-N-acetylparomamine (2a) nor paromamine (3a). It therefore seemed plausible that GtmG might be accepting NDP-GlcNA rather than NDP-GlcN as the cosubstrate for attachment to 2-DOS (1a). This was confirmed by using cell-free extracts from the recom-

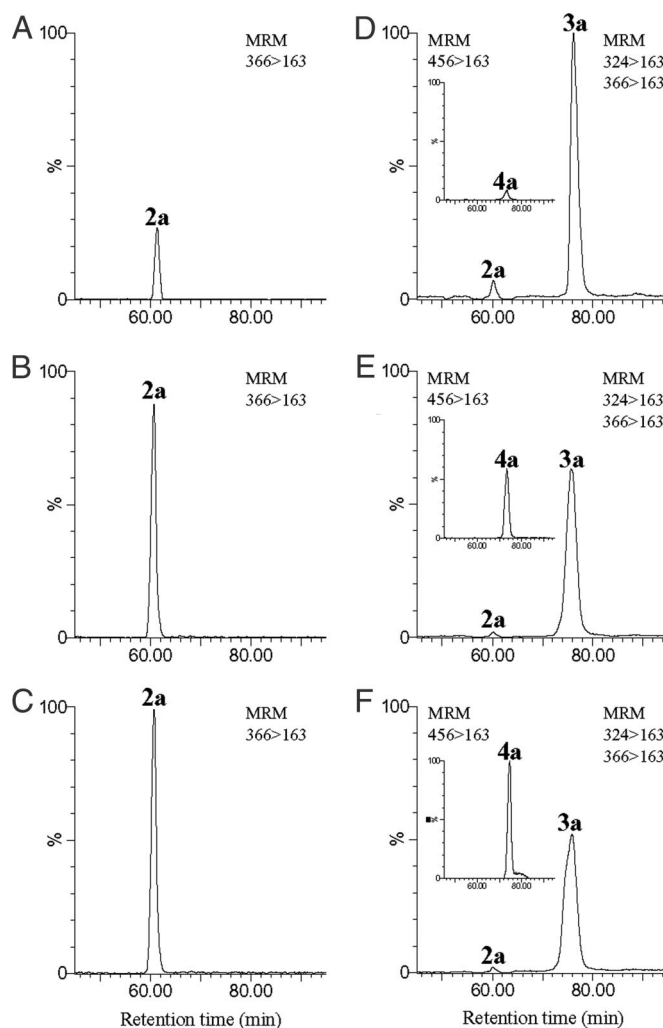


Fig. 3. HPLC-ESI-MS/MS chromatograms for the glycosyltransferase-catalyzed production of 2'-N-acetylparomamine (2a) and gentamicin A₂ (4a). (A–C) GtmG assay using cell-free extracts from *S. venezuelae* strain harboring pYJ498 (A) without exogenous UDP-N-acetyl-D-glucosamine (UDP-GlcNA), (B) with addition of 0.11 mM UDP-GlcNA, and (C) with addition of 0.55 mM UDP-GlcNA. (D–F) GtmE assay using cell-free extracts from *S. venezuelae* strain harboring pYJ504 (D) without exogenous UDP-D-xylose (UDP-Xyl), (E) with addition of 0.12 mM UDP-Xyl, and (F) with addition of 0.60 mM UDP-Xyl. Monitoring of gentamicin A₂ (4a) (456 > 163) is separately illustrated as an *Inset* to each chromatogram (D, E, and F).

binant *S. venezuelae* because of difficulties in obtaining soluble GtmG protein by using standard expression systems. The crude cell-free extracts of the strain harboring pYJ498 were prepared and incubated with different concentrations of exogenous UDP-GlcNA. The amount of 2'-N-acetylparomamine (2a) formed was elevated up to 4-fold as the input of UDP-GlcNA increased (Fig. 3 A–C). As expected, *in vitro* experiment using the same cell-free extracts supplemented with UDP-GlcN at the respective concentrations did not result in the production of paromamine (data not shown). It has been suggested by others (15) that a putative UDP-GlcNA glycosyltransferase gene, *btmM* (from the butirosin gene cluster) and its homologues (*neod8*, *kanF*, and *gtmG* from neomycin, kanamycin, and gentamicin biosynthetic gene clusters, respectively; sequence alignment of the predicted amino acid sequences of these genes is illustrated in Fig. S4) encode enzymes that catalyze the transfer of UDP-GlcNA to 2-DOS (1a) to give 2'-N-acetylparomamine (2a). Although those other enzymes have yet to be functionally

characterized using purified proteins, our present results strongly suggest that GtmG is indeed a UDP-GLcNA glycosyltransferase, which is a previously uncharacterized enzyme involved in the formation of pseudodisaccharide intermediate during aminoglycoside biosynthesis.

GtmM Acts as a 2'-N-Acetylparomamine Deacetylase to Generate Paromamine (3a). During the biosynthesis of butirosin, a paromamine deacetylase, BtrD, acts on 2'-N-acetylparomamine (2a) to produce paromamine (3a) (18), and a similar activity is required during the biosynthesis of gentamicin. A homologue of *btrD* is found in the region flanking either side of *genD* from the extended gentamicin biosynthetic gene cluster (GenBank accession no. AJ628149). Detailed sequence analysis revealed a possible start codon 142 nt upstream of *genD* gene. Further DNA sequencing of the PCR product of the ORF-corrected *genD* found the insertion of single cytosine residue at the 51th nucleotide position in *genD*, causing the frame shift in the coding region. A new ORF (*gtmM*, GenBank accession no. AM946392) encoding putative deacetylase is located 11,096 nt downstream of *gacH*. When the deduced product of *gtmM* was aligned with those of the well characterized deacetylases, two highly conserved regions (HXDD and HXDH) (18) were evident (see Fig. S5). We imported the deacetylase genes *btrD*, *kacA*, *neo16*, and *gtmM* from the gene clusters for butirosin, kanamycin, neomycin, and gentamicin biosynthesis, respectively (5, 6, 10). The four deacetylase genes were separately cloned into constructs containing the genes for 2-DOS biosynthesis, glycosyltransfer, and resistance (*gtmB-gtmA-gacH-gtmF-gtmG-gtmK-gtmL*) to produce plasmids pYJ500, pYJ501, pYJ502, and pYJ503, respectively. Expression of all four plasmids in the heterologous host led to production of paromamine (3a) along with its precursor 2'-N-acetylparomamine (2a) (Fig. 2E). Strains expressing the *Streptomyces* genes *kacA*, *neo16*, and *gtmM* biosynthesized similar amounts of paromamine (3a) (150 μ g per liter, corrected for \approx 90% mean recoveries of 3a), whereas slightly lower amounts were obtained with *btrD*, which is of *Bacillus* origin and might have been expressed less efficiently in the heterologous host, *S. venezuelae*. These results verify *KacA*, *Neo16*, and *GtmM* as deacetylases involved in the biosynthesis of paromamine (3a) and are in agreement with the previous report that paromamine (3a) is produced via the intermediate 2'-N-acetylparomamine (2a) using His₆-tagged BtrD (18). Here, we produced both 2'-N-acetylparomamine (2a) and paromamine (3a) *in vivo*, which reveals that 2'-N-acetylparomamine (2a) is a true biosynthetic intermediate in the formation of gentamicin and other aminoglycosides.

GtmE Acts as an UDP-Xylose Glycosyltransferase to Generate Gentamicin A₂ (4a). A second glycosylation, using NDP-D-xylose (NDP-Xyl) as cosubstrate, is required for the formation of gentamicin A₂ (4a) from paromamine (3a). Expression of *gtmE* with the genes necessary for biosynthesis of paromamine (*gtmB-gtmA-gacH-gtmF-gtmG-kacA-gtmM-gtmK-gtmL*) led to production of gentamicin A₂ (4a) together with its precursors 2'-N-acetylparomamine (2a) and paromamine (3a) (Fig. 2F). Therefore, GtmE appears to be the glycosyltransferase required for the transfer of NDP-Xyl to paromamine (3a). Sequence alignment of the predicted product of *gtmE* with other putative glycosyltransferases, which are found in the biosynthetic gene clusters of other 4,6-disubstituted aminoglycosides, is shown in Fig. S6. The yield of gentamicin A₂ (4a) obtained from the culture extract was only 4.5 μ g/liter (corrected for \approx 95% mean recoveries of 4a), and a substantial amount of paromamine (3a) remained unaltered, suggesting that NDP-Xyl might be present in *S. venezuelae* in limiting amounts. To determine whether D-xylose is attached directly to paromamine or whether D-xylose is generated from glucose or ribose, whereas they are bound to paromamine (26),

crude cell-free extracts from the strain harboring pYJ504 were prepared and incubated with different concentrations of exogenous UDP-Xyl and UDP-glucose (UDP-Glc). Increased input of UDP-Xyl resulted in enhanced production of gentamicin A₂ up to 8-fold (4a), whereas in reciprocal fashion the amount of residual paromamine (3a) dropped significantly (Fig. 3 D-F). However, UDP-Glc did not support biosynthesis of gentamicin A₂. Although NDP-ribose could not be tested because of its unavailability, these results provide evidence that UDP-D-xylose (UDP-Xyl) is a cofactor for gentamicin A₂ biosynthesis, and that its transfer to paromamine is catalyzed by GtmE. It was reported (27) that an UDP-glucuronic acid (UDP-GlcA) decarboxylase AviE2 was involved in the formation of UDP-Xyl in avilamycin-producing *Streptomyces viridochromogenes* Tu57 (27). CalS8 of calicheamicin-producing *M. echinospora* was reported as an UDP-Glc dehydrogenase involved in deoxypentose biosynthesis. Furthermore, several natural products such as maduropeptin from *Actinomadura madurea*, esperamicin from *Actinomadura verrucosospora*, and evernimicin from *Micromonospora carbonacea* bear deoxypentose(s) (28). UDP-Glc dehydrogenase and UDP-GlcA decarboxylase may be the key enzymes necessary to synthesize nucleotide-activated deoxypentoses such as UDP-Xyl in actinomycetes in a similar way to plants and fungi (29, 30), and it seems likely that such metabolic pathways are present in *S. venezuelae* to provide NDP-Xyl.

In conclusion we have elucidated the minimal complement of genes required for the formation of gentamicin A₂ and identified its biosynthetic intermediates. 2-DOS (1a) is synthesized by the products of *gtmB*, *gtmA*, and *gacH*. The *gtmG* gene encodes a glycosyltransferase that transfers GLcNA to produce 2'-N-acetylparomamine (2a). A deacetylase GtmM required to remove the acetyl group to form paromamine (3a) was found in *M. echinospora*, and GtmM and its homologues BtrD, KacA, and Neo16 were each able to carry out this function. GtmE attaches D-xylose to paromamine (3a) to produce gentamicin A₂ (4a). The constructed gene combinations and the obtained biosynthetic intermediates are shown in Table 1. The assigned functions of these genes are summarized in Table 2. This report represents an *in vivo* study regarding the expression of biosynthetic enzymes for complete synthesis of the pseudotrisaccharide form of an aminoglycoside in a heterologous host. Direct evidence is provided for the biosynthetic route to this 4,6-disubstituted aminoglycoside allowing the unequivocal assignment of specific gene products to each biosynthetic step. Further studies regarding the *in vitro* characterization of both glycosyltransferases, GtmG and GtmE, toward their substrates are impending. A detailed understanding of the complete biosynthetic pathway of gentamicin C components would assist in comprehending the biosynthesis of other 4,6-disubstituted aminoglycosides and form the basis for evolution of pathway engineering toward the generation of aminoglycosides active against rapidly emerging aminoglycoside-resistant pathogens. A continuing challenge of this heterologous expression approach is the relatively low productivity of the target compounds. However, understanding the limited intracellular availability of one co-substrate (NDP-D-xylose) provided a strategy for enhancing the production level.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. *Escherichia coli* DH10B and plasmid Litmus28 (New England Biolabs) were used for routine subcloning. High-copy number *E. coli-Streptomyces* shuttle vector pSE34 containing the strong *ermE** promoter plus a thiostrepton resistance marker was used as an expression plasmid (19). The gentamicin biosynthetic genes from *M. echinospora* are located on the cosmid pGEN01 (12). *S. venezuelae* YJ003, which is deficient in biosynthesis of the endogenous deoxysugar TDP-D-desosamine, was used as a heterologous host (20). Protoplast formation and transformation procedures of *S. venezuelae* were performed as described (31). *S. venezuelae* YJ003 and derived strains were maintained on SPA agar (20) and

Table 1. Constructed gene combinations and the products obtained

Plasmid	Gene combination*	Product†			
		1a	2a	3a	4a
pYJ489	<i>gtmF-gtmK-gtmL</i>				
			Resistance genes		
pYJ494	<i>gtmD-gtmB-gtmA-gtmC</i>	×	×	×	×
pYJ495	<i>gtmD-gtmB-gtmA-gacH</i>	○	×	×	×
pYJ496	<i>gtmB-gtmA-gacH</i>	○	×	×	×
pYJ497	<i>gtmD-gtmA-gacH</i>	×	×	×	×
pYJ498	<i>gtmB-gtmA-gacH-gtmF-gtmG-gtmK-gtmL</i>	○	○	×	×
pYJ499	<i>gtmB-gtmA-gacH-gtmF-gtmE-gtmK-gtmL</i>	○	×	×	×
pYJ500	<i>gtmB-gtmA-gacH-gtmF-gtmG-btrD-gtmK-gtmL</i>	○	○	○	×
pYJ501	<i>gtmB-gtmA-gacH-gtmF-gtmG-kacA-gtmK-gtmL</i>	○	○	○	×
pYJ502	<i>gtmB-gtmA-gacH-gtmF-gtmG-neo16-gtmK-gtmL</i>	○	○	○	×
pYJ503	<i>gtmB-gtmA-gacH-gtmF-gtmG-gtmM-gtmK-gtmL</i>	○	○	○	×
pYJ504	<i>gtmB-gtmA-gacH-gtmF-gtmG-gtmE-kacA-gtmK-gtmL</i>	○	○	○	○
pYJ505	<i>gtmB-gtmA-gacH-gtmF-gtmG-gtmE-gtmM-gtmK-gtmL</i>	○	○	○	○

*Using pSE34 *E. coli-Streptomyces* shuttle vectors (19).

†1a, 2a, 3a, and 4a representing 2-deoxystreptomine, 2'-N-acetylparomamine, paromamine, and gentamicin A₂, respectively.

grown in liquid R2YE (31) at 30°C for preparation of protoplasts, which were regenerated on R2YE agar medium supplemented with thiostrepton (30 µg/ml). The *E. coli* strains were grown in LB medium supplemented with ampicillin (50 µg/ml) to select for plasmid vectors. For production of gentamicin intermediates, *S. venezuelae* strains expressing gentamicin biosynthetic genes were cultivated at 30°C for 4 days in one liter of baffled Erlenmeyer flasks containing 300 ml of R2YE medium supplemented with thiostrepton (25 µg/ml). To obtain authentic gentamicin A₂, *M. echinospora* ATCC 15385 was grown for 7 days at 28°C in 50 ml of N-Z amine medium (1% glucose, 2% soluble starch, 0.5% yeast extract, 0.5% N-Z amine and 0.2% calcium carbonate) in 500-ml baffled flasks on a rotary shaker (25).

PCR Amplification of Gentamicin Biosynthetic Genes. DNA fragments containing various gentamicin biosynthetic genes were amplified by PCR (Table S1). Each pair of deoxyoligonucleotide primers contained two restriction sites to facilitate subcloning of each DNA fragment. PCR was performed by using *Pfu* polymerase (Fermentas) under the manufacturer's recommended conditions. The DNA fragments containing each of *gtmF*, *gtmK*, *gtmL*, *gtmD*, *gtmB*, *gtmA*, *gtmC*, *gacH*, *gtmG*, and *gtmE* were amplified by using pGEN01 as a template (12). The fragments encoding GtmM, BtrD, KacA, and Neo16 were amplified by using genomic DNA from *M. echinospora* ATCC 15385, *Bacillus circulans* ATCC 21557, *S. kanamyceticus* ATCC 12853, and *S. fradiae* ATCC 10745, respectively, due to template. All PCR products were cloned into Litmus28 and sequenced to confirm their authenticity.

Construction of Expression Plasmids. This was done as summarized in Table 1. Amplified products of the putative resistance genes *gtmF-gtmK-gtmL* were ligated in series into pSE34 by using standard protocols, and the resultant plasmid, pYJ489, was used to confer gentamicin resistance in *S. venezuelae*.

For expression of a combination of putative 2-DOS (1a) biosynthetic genes, PCR-amplified fragments containing *gtmD*, *gtmB*, *gtmA*, *gtmC*, or *gacH* were ligated in series into Litmus28 and then transplanted into pSE34 to generate a set of plasmids containing *gtmD-gtmB-gtmA-gtmC*, *gtmD-gtmB-gtmA-*

gacH, *gtmB-gtmA-gacH*, and *gtmD-gtmA-gacH*. These were designated pYJ494, pYJ495, pYJ496, and pYJ497, respectively. In a further set of constructions, the resistance genes *gtmF-gtmK-gtmL* from pYJ489 were introduced into pYJ496 together with either of the glycosyltransferase genes *gtmG* or *gtmE* to generate pYJ498 or pYJ499, respectively. The latter two vectors allowed expression of 2-DOS biosynthetic genes and gentamicin resistance determinants in addition to the respective glycosyltransferase genes. The next set of expression vectors, pYJ500, pYJ501, pYJ502, and pYJ503, were equivalent to pYJ498 supplemented with deacetylase genes *btrD*, *kacA*, *neo16*, and *gtmM*, respectively, and finally introduction of *gtmE* into *gtmG*-containing pYJ501 and pYJ503 generated a plasmid, pYJ504 and pYJ505, respectively, capable of expressing the entire complement of resistance determinants plus the minimal gene set for gentamicin A₂ production.

Analyses of Gentamicin Biosynthetic Intermediates. Gentamicin biosynthetic intermediates produced by *S. venezuelae* strains were extracted by using the OASIS MCX (Waters) SPE cleanup procedure was done as described (25) and analyzed by HPLC-ESI-MS/MS. Analytical HPLC-ESI-MS/MS was performed on a Waters/Micromass Quattro micro/MS interface using two XTerra MS C₁₈ (3.5 µm, Waters) columns, 150 × 2.1 and 250 × 2.1 mm, connected in tandem. The analytes were eluted at a flow rate of 60 µl/min with 30% (vol/vol) acetonitrile in water with 10 mM heptafluorobutyric acid (HFBA, Fluka) and monitored by ESI in the positive mode. Quantification of the analytes was conducted by using MS/MS in the multiple reactions monitoring mode. This was done by selecting the two mass ions set to detect a transition of the parent ion to the product ion specific to the selected analytes (26): 2-DOS (1a), 163 > 102; 2'-N-acetylparomamine (2a), 366 > 163; paromamine (3a), 324 > 163; gentamicin A₂ (4a), and 456 > 163. Five separate cultivations and extractions were performed.

Isolation and Identification of Gentamicin Biosynthetic Intermediates as Authentic Standards. 2-DOS (1a) produced by *S. venezuelae* strains was identified by using commercially available 2-DOS (1a) (GeneChem) as a standard. 2'-N-acetylparomamine (2a) was purified directly from an *S. venezuelae* strain harboring the plasmid pYJ498, and its spectral data were authenticated by comparison with previous data (18). Paromamine (3a) from *S. venezuelae* expressing plasmid pYJ501 was identified by using standard material obtained from hydrolysis of paromomycin (Sigma). Gentamicin A₂ (4a) produced by *S. venezuelae* harboring pYJ504 was compared with its purified form from wild-type *M. echinospora*. Details for the preparation and analysis of both authentic standards and gentamicin biosynthetic intermediates produced by *S. venezuelae* strains harboring the respective gene sets are given in *SI Text*; see also *Figs. S7–S14* *Tables S2–S5*. Preparative HPLC was performed with a Spherisorb S5 ODS2 (250 × 20 mm, Waters) semiprep column eluted with the same mobile phase used in the analytical HPLC system, at a flow rate of 12 ml/min over a period of 100 min. This eluent was fractionated into 5-ml portions that were monitored by analytical HPLC-ESI-MS/MS to detect and characterize the presence of gentamicin biosynthetic intermediates. Fractions containing products of interest were pooled and extracted by using OASIS MCX SPE cleanup (25) followed by freeze-drying. ¹H, ¹³C, and 2D ¹H-¹H COSY

Table 2. Functional assignment of genes associate with the biosynthesis gentamicin A₂

Gene	Proposed role of the gene product	Ref(s)
<i>gtmA</i>	2-DOI synthase	12
<i>gtmB</i>	2-DOI and keto-2-DOIA aminotransferase	This work
<i>gacH</i>	2-DOIA dehydrogenase	This work
<i>gtmG</i>	NDP-N-acetyl-D-glucosaminyl transferase	This work
<i>gtmM</i>	2'-N-Acetylparomamine deacetylase	This work
<i>gtmE</i>	NDP-D-xylosyl transferase	This work
<i>BtrD</i>	2'-N-Acetylparomamine deacetylase	15
<i>kacA</i>	2'-N-Acetylparomamine deacetylase	This work
<i>neo16</i>	2'-N-Acetylparomamine deacetylase	This work

NMR spectra were acquired by using a Varian INOVA 500 spectrometer at 298 K. Chemical shifts were reported in ppm by using trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (TSP) as an internal reference. The assignment of each compound was carried out by comparison with previously assigned ^1H NMR spectra (18, 32–34) and by a combination of 1D and 2D NMR experiments. All NMR data processing was done by using MESTREC (Magnetic Resonance Companion) software.

In Vitro Glycosyltransfer Reactions Using Cell-Free Extracts. Cell-free extracts of *S. venezuelae* strains harboring pYJ498 or pYJ504 were prepared by glass-bead homogenization (31). The mycelium (6-g wet weight) of each strain had been cultivated in R2YE medium for 3 days at 30°C, harvested by centrifugation, washed twice with 0.1 M Tris-HCl (pH 7.6), and then resuspended in 20 ml of extraction buffer (0.1 M Tris-HCl, 10 mM MgCl_2 , 6 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.6) at 4°C. After mixing with 10 g of precooled glass beads (150 to 212 μm in diameter, Sigma), the mycelium was disrupted by vigorous agitation by using a vortex mixer. This involved 10 bursts of 30 sec each, with intermittent cooling in ice. Next, the glass beads were eliminated by low-speed centrifugation, and cellular debris was removed by centrifugation at $18,000 \times g$ for 20 min. The entire process was carried out at 4°C and generated 15 ml of resulting supernatant. As one of proposed substrates for GtmG reaction, UDP-GlcN was prepared by enzymatic reaction of UDP-D-glucose pyrophosphorylase (E.C. 2.7.7.9, Sigma) with glucosamine-1-phosphate

(Sigma) and UTP (Sigma), as described in ref. 35. Glycosyltransfer reactions were initiated by adding activated sugar cosubstrates to the cell-free extracts as follows: for GtmG, UDP-GlcNA (GeneChem) or UDP-GlcN was used at final concentrations between 0 and 0.55 mM; for GtmE, UDP-D-xylose (CarboSource Services, University of Georgia, Griffin, GA) or UDP-Glc (Sigma) was used at final concentrations between 0 and 0.60 mM. Reaction mixtures were incubated at 30°C for 10 h before quenching with 15 ml of ice-cold phenol/chloroform/isoamyl alcohol (25:24:1, Sigma). After centrifugation, gentamicin-related compounds were extracted by using OASIS MCX SPE cleanup as previously described and then reconstituted to 200 μl with water. A portion of each extract was subjected to HPLC-ESI-MS analysis (see conditions described above). Independent experiments were performed in duplicate.

ACKNOWLEDGMENTS. We thank Prof. Eric Cundliffe (University of Leicester, Leicester, U.K.) for helpful suggestions and critical reading of this manuscript. This work was supported by Ministry of Commerce, Industry, and Energy (MOCIE); the Marine and Extreme Genome Research Center Program of the Ministry of Land, Transportation and Maritime Affairs; Korea Science and Engineering Foundation (KOSEF), funded by the Korea government (MEST) (Grant M10749000201-07N4900-20110, R11-2005-008-00000-0) and the National Research Laboratory (NRL) program grant (ROA-2008-000-20030-0). J.W.P. gratefully acknowledges a Grant 20080401034038 from the BioGreen 21 program, Rural Development Administration, and we thank the Ministry of Education for the Brain Korea 21 Fellowship.

- Weinstein MJ, Luedemann GM, Oden EM, Wagman GH (1963) Gentamicin, a new antibiotic complex from *Micromonospora*. *Antimicrob Agents Chemother* 122:463–464.
- Carter AP, et al. (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 407:340–348.
- Schatz A, Bugie E, Waksman SA (1944) Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. *Proc Soc Exp Med Biol* 55:66–69.
- Testa RT, Tilley BC (1976) Biotransformation, a new approach to aminoglycoside biosynthesis: II. Gentamicin. *J Antibiot* 29:140–146.
- Ota Y, et al. (2000) Butirosin-biosynthetic gene cluster from *Bacillus circulans*. *J Antibiot* 53:1158–1167.
- Huang F, et al. (2005) The neomycin biosynthetic gene cluster of *Streptomyces fradiae* NCIMB 8233: Characterisation of an aminotransferase involved in the formation of 2-deoxystreptamine. *Org Biomol Chem* 3:1410–1418.
- Huang F, et al. (2007) Elaboration of neosamine rings in the biosynthesis of neomycin and butirosin. *ChemBioChem* 8:283–288.
- Llewellyn NM, Li Y, Spencer JB (2007) Biosynthesis of butirosin: transfer and deprotection of the unique amino acid side chain. *Chem Biol* 14:379–386.
- Kudo F, Fujii T, Kinoshita S, Eguchi T (2007) Unique O-ribosylation in the biosynthesis of butirosin. *Bioorg Med Chem* 15:4360–4368.
- Kharel MK, et al. (2004) A gene cluster for biosynthesis of kanamycin from *Streptomyces kanamyceticus*: Comparison with gentamicin biosynthetic gene cluster. *Arch Biochem Biophys* 429:204–214.
- Unwin J, et al. (2004) Gene cluster in *Micromonospora echinospora* ATCC 15835 for the biosynthesis of the gentamicin C complex. *J Antibiot* 57:436–445.
- Kharel MK, et al. (2004) Molecular cloning and characterization of a 2-deoxystreptamine biosynthetic gene cluster in gentamicin-producing *Micromonospora echinospora* ATCC 15835. *Mol Cells* 18:71–78.
- Kharel MK, et al. (2004) Isolation and characterization of the tobramycin biosynthetic gene cluster from *Streptomyces tenebrarius*. *FEMS Microbiol Lett* 230:185–190.
- Love SF, Maiese WM, Rothstein DM (1992) Conditions for protoplasting, regenerating, and transforming the calicheamicin producer, *Micromonospora echinospora*. *Appl Environ Microbiol* 58:1376–1378.
- Llewellyn NM, Spencer JB (2006) Biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics. *Nat Prod Rep* 23:864–874.
- Testa RT, Tilley BC (1979) Biosynthesis of sisomicin and gentamicin. *Jpn J Antibiot* 32 Suppl:547–559.
- Kase H, Odakura Y, Nakayama K (1982) Sagamicin and the related aminoglycosides: fermentation and biosynthesis. I. Biosynthetic studies with the blocked mutants of *Micromonospora sagamiensis*. *J Antibiot* 35:1–9.
- Truman AW, Huang F, Llewellyn NM, Spencer JB (2007) Characterization of the enzyme BtrD from *Bacillus circulans* and revision of its functional assignment in the biosynthesis of butirosin. *Angew Chem Int Ed* 46:1462–1464.
- Smirnova N, Reynolds KA (2001) Engineered fatty acid biosynthesis in *Streptomyces* by altered catalytic function of beta-ketoacyl-acyl carrier protein synthase III. *J Bacteriol* 183:2335–2342.
- Hong JSJ, et al. (2004) New olivoyl derivatives of methymycin/pikromycin from an engineered strain of *Streptomyces venezuelae*. *FEMS Microbiol Lett* 238:391–399.
- Huang F, Li Y, Yu J, Spencer JB (2002) Biosynthesis of aminoglycoside antibiotics: Cloning, expression and characterisation of an aminotransferase involved in the pathway to 2-deoxystreptamine. *Chem Commun* 7:2860–2861.
- Kudo F, et al. (2005) Biosynthesis of 2-deoxystreptamine by three crucial enzymes in *Streptomyces fradiae* NBRC 12773. *J Antibiot* 58:766–774.
- Yokoyama K, et al. (2005) Stereochemical recognition of doubly functional aminotransferase in 2-deoxystreptamine biosynthesis. *J Am Chem Soc* 127:5869–5874.
- Yokoyama K, et al. (2007) Characterization and mechanistic study of a radical SAM dehydrogenase in the biosynthesis of butirosin. *J Am Chem Soc* 129:15147–15155.
- Park JW, et al. (2007) Analytical profiling of biosynthetic intermediates involved in the gentamicin pathway of *Micromonospora echinospora* by high-performance liquid chromatography using electrospray ionization mass spectrometric detection. *Anal Chem* 79:4860–4869.
- Rinehart KL, Jr (1979) Biosynthesis and mutasynthesis of aminocyclitol antibiotics. *Jpn J Antibiot* 32 Suppl:532–546.
- Hofmann C, et al. (2005) Genes encoding enzymes responsible for biosynthesis of L-lyxose and attachment of eurekaanate during avilamycin biosynthesis. *Chem Biol* 12:1137–1143.
- Billign T, Shepard EM, Ahlert J, Thorson JS (2002) On the origin of deoxypentoses: Evidence to support a glucose progenitor in the biosynthesis of calicheamicin. *Chem-BioChem* 3:1143–1146.
- Harper AD, Bar-Peled M (2002) Biosynthesis of UDP-xylose. Cloning and characterization of a novel *Arabidopsis* gene family, *UXS*, encoding soluble and putative membrane-bound UDP-glucuronic acid decarboxylase isoforms. *Plant Physiol* 130:2188–2198.
- Bar-Peled M, Griffith CL, Doering TL (2001) Functional cloning and characterization of a UDP-glucuronic acid decarboxylase: The pathogenic fungus *Cryptococcus neoformans* elucidates UDP-xylose synthesis. *Proc Natl Acad Sci USA* 98:12003–12008.
- Kieser T, et al. (2000) in *Practical Streptomyces Genetics* (John Innes Foundation, Norwich, UK), pp 75–97.
- Nagabhushan TL, Turner WN, Daniels PJL, Morton JB (1975) The gentamicin antibiotics. 7. Structures of the gentamicin antibiotics A₁, A₃, and A₄. *J Org Chem* 40:2830–2834.
- Nagabhushan TL, Daniels PJL, Jaret RS, Morton JB (1975) The gentamicin antibiotics. 8. Structure of gentamicin A₂. *J Org Chem* 40:2835–2836.
- Haskell TH, French JC, Bartz QR (1959) Paromomycin. I. Paromamine, a glycoside of D-glucosamine. *J Am Chem Soc* 81:3480–3481.
- Lee HC, Lee SD, Sohng JK, Liou K (2004) One-pot enzymatic synthesis of UDP-D-glucose from UMP and glucose-1-phosphate using an ATP regeneration system. *J Biochem Mol Biol* 37:503–506.