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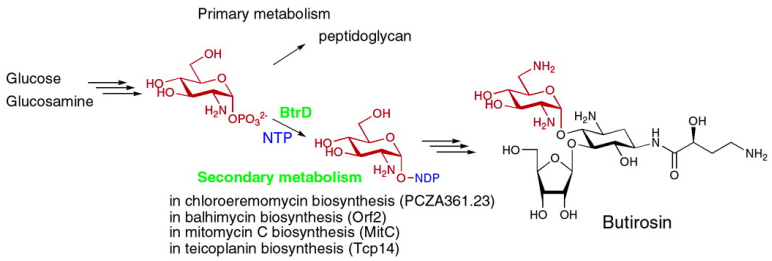

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A New Family of Glucose-1-phosphate/ Glucosamine-1-phosphate Nucleotidylyltransferase in the Biosynthetic Pathways for Antibiotics

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Abstract: Aminoglycoside antibiotics are composed of aminosugars and a unique aminocyclitol aglycon including 2-deoxystreptamine (DOS), streptidine, actinamine, etc., and nucleotidylyltransferases, sugar modifying enzymes, and glycosyltransferases appear to be essential for their biosynthesis. However, the genes encoding those enzymes were unable to be identified by a standard homology search in the butirosin biosynthetic *btr* gene cluster, except that the *btr*M gene appeared to be a glycosyltransfease. Disruption studies of the *btr*D gene indicated that BtrD was involved in the supply of a glycosyl donor immediately prior to the glycosylation of DOS giving paromamine. As anticipated, BtrD expressed in *Escherichia coli* was able to catalyze UDP-D-glucosamine formation from D-glucosamine-1-phosphate and UTP. Both dTTP and UTP were good NTP substrates, and D-glucose-1-phosphate and D-glucosamine-1-phosphate were good sugar phosphates for the enzyme reaction. This finding is the first to identify an enzyme which activates a sugar donor in the DOS-containing antibiotics. Interestingly, BtrD homologues have been reported as functionally unknown open reading frames (ORFs) in the biosynthetic gene clusters for several antibiotics including teicoplanin, balhimycin, chloroeremomycin, and mitomycin C. It appears therefore that gene clusters for antibiotic biosynthesis provide their own nucleotidylyltransferases, and the BtrD homologues are among the secondary metabolism specific enzymes.

Glycosyl transfer reaction by glycosyltransferase (transglycosylase) is among key transformations involved in both primary and secondary metabolic pathways. Particularly, those in the latter pathway are significant in providing chemical diversities and biological activities for secondary metabolites including clinically important antibiotics, immunosupressants, and cardiac glycosides.[1,2] Structurally diverse NDP-sugars (nucleotide diphosphate sugars) serve as glycosyl donors in the glycosyltransferase reactions, and the biosynthesis of NDP-sugars starts from ubiquitous primary metabolites, sugar-1-phosphates, and NTP (nucleotide triphosphates), by the catalysis of nucleotidylyltransferase, followed by subsequent structural modifications in the sugar moiety. It appears therefore that the initial formation of NDP-sugar starter is a crucial branching point of the secondary metabolic pathway from the primary. In the primary metabolism, for example, *N*-acetylglucosamine-1-phosphate uridyltransferase (UDP-*N*-acetylglucosamine pyrophosphorylase) synthesizes UDP-*N*-acetylglucosamine from *N*-acetylglucosamine-1-phosphate and UTP as a precursor to the bacterial cell wall.[3−6] Glucose-1-phosphate thymidylyltransferase is responsible for the biosynthesis of L-rhamnose, a component of both Gram-positive and Gram-negative bacteria.[7−9] The same is true in the secondary metabolisms and varieties of glycosylated metabolites are produced as beneficial bioactive compounds, particularly in microorganisms. In most cases, the glycosyl moieties of these secondary metabolites are indispensable for exerting the bioactivities.

In the biosynthesis of these bioactive glycosylated secondary metabolites, a specific NDP-sugar is specifically transferred to a target aglycon, and such an NDP-sugar in the secondary metabolism is usually biosynthesized from NDP-D-glucose through chemical transformations by modifying enzymes to the proper ultimate structures as exemplified in the biosynthesis of classical macrolide antibiotics.[10] Indeed, a well-related family of the genes for NDP-glucose synthases, mostly TDP-glucose synthases, together with those of the modifying enzymes are

† Department of Chemistry.
‡ Department of Chemistry and Materials Science.

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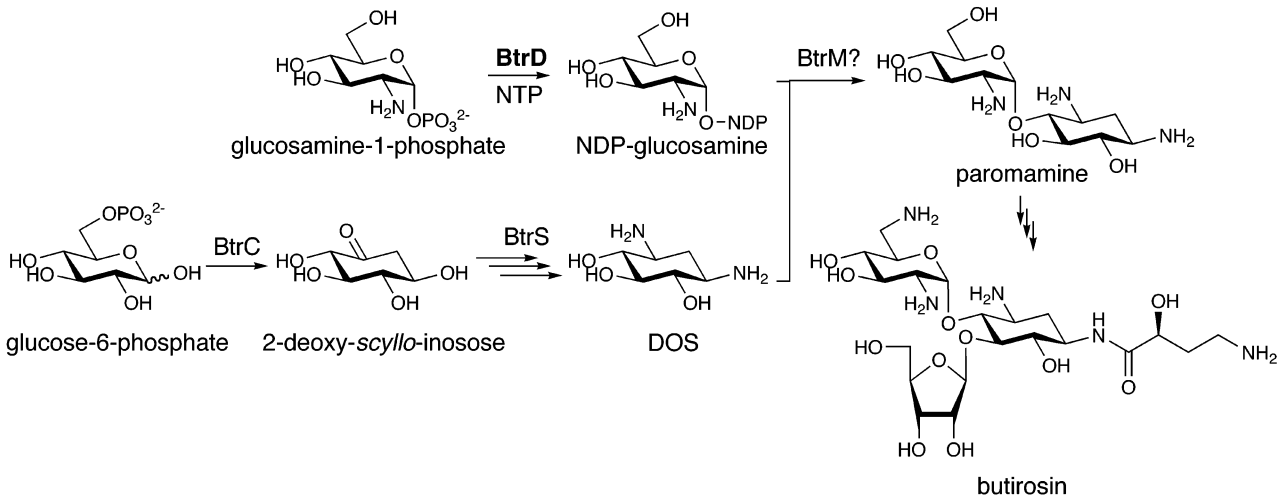
(10) He, X. M.; Liu, H. W. *Annu. Rev. Biochem.* **2002**, *71*, 701−754.

Scheme 1. Proposed Biosynthetic Pathway Leading to Butirosin



known in the secondary metabolic gene clusters.[11] However, the number of biosynthetic gene clusters so far clarified is limited, and the possibility of other types of sugar activating nucleotidylyltransferases in the secondary metabolic pathways remains to be addressed.

Clinically important aminoglycoside antibiotics are basically composed of aminosugars and an unique aminocyclitol aglycon, and 2-deoxystreptamine (DOS) is the key aglycon of a major class of these antibiotics. For the biosynthesis of DOS-containing antibiotics, those enzymes of nucleotidylyl-transferases, sugar modifying enzymes, and glycosyltransferases appear to be essential, in addition to those involved in an assembly line for an aminocyclitol aglycon, to produce an ultimate aminoglycoside. It appears that the biosynthetic machinery for aminoglycosides should have great potential to be explored toward the extension of biochemical combinatorial glycosylation. We were the first to identify the biosynthetic gene cluster of the DOS-containing aminoglycosides butirosin (Scheme 1).[12,13] Several other gene clusters for this class of antibiotics have been reported very recently.[14−16]

It should be pointed out here that, in the biosynthetic gene cluster of butirosin, most of the open reading frames (ORFs) could not been functionally identified by means of simple homology search. The gene *btrM* was an exception, since its corresponding amino acid sequence suggested its function to be a hexosyltransferase, which appeared to be involved in the first glycosylation of DOS. However, the actual chemical species of glycosyl donor for the first glycosylation and precursor thereof as well as the enzymes involved in these key transformations remained unsolved. Information for these events in the biosynthetic pathway of DOS-containing aminoglycosides appears to be invaluable for better understanding and application of the glycosylation systems in the microbial secondary metabolism.

The gene *btrD* locates at the next most downstream of *btrC*, which encodes the key 2-deoxy-*scyllo*-inosose synthase in the biosynthesis of DOS.[13] The deduced protein from the gene *btrD* showed certain homology to several reported genes in the biosynthetic clusters of several glycopeptide antibiotics, teicoplanin,[17−19] balhimycin,[20] and chloroeremomycin,[21] and to the one in the biosynthesis of mitomycin C[22,23] (Figure 1). Although all of these genes have not been functionally identified, an obvious common feature in these antibiotic is the involvement of aminohexopyranose. We, therefore, envisioned that BtrD might be involved in the aminohexopyranose-related reaction in the butirosin biosynthesis and launched the functional analysis of BtrD. As a result, BtrD has now been identified as a novel class of nucleotidylyltransferase to specifically produce thymidylyl α-D-glucopyranoside/α-D-glucosaminide. The function of the above-mentioned homologous genes involved in the antibiotic biosynthetic gene clusters has also been discussed.

Results and Discussion

Tetracycline resistance gene (Tc^r) was inserted into the specific *ScaI* site of *btrD* in the *B. circulans* chromosome using a plasmid pHBCTc^{r}E to disrupt the gene by conventional homologous recombination. The resulting *btrD* mutant showed no production of any antibiotics under the standard fermentation conditions, while the complementation of this disruption by transformation with pHBrbsbtrD recovered the antibiotic production (Table 1). These results clearly confirmed that the *btrD* gene was involved in the butirosin biosynthesis. To determine which chemical step was blocked in the pathway with *btrD* disruption, complementation with biosynthetic intermediates was examined. Feeding of paromamine to the culture recovered the antibiotic production, but neither with 2-deoxy-*scyllo*-inosamine

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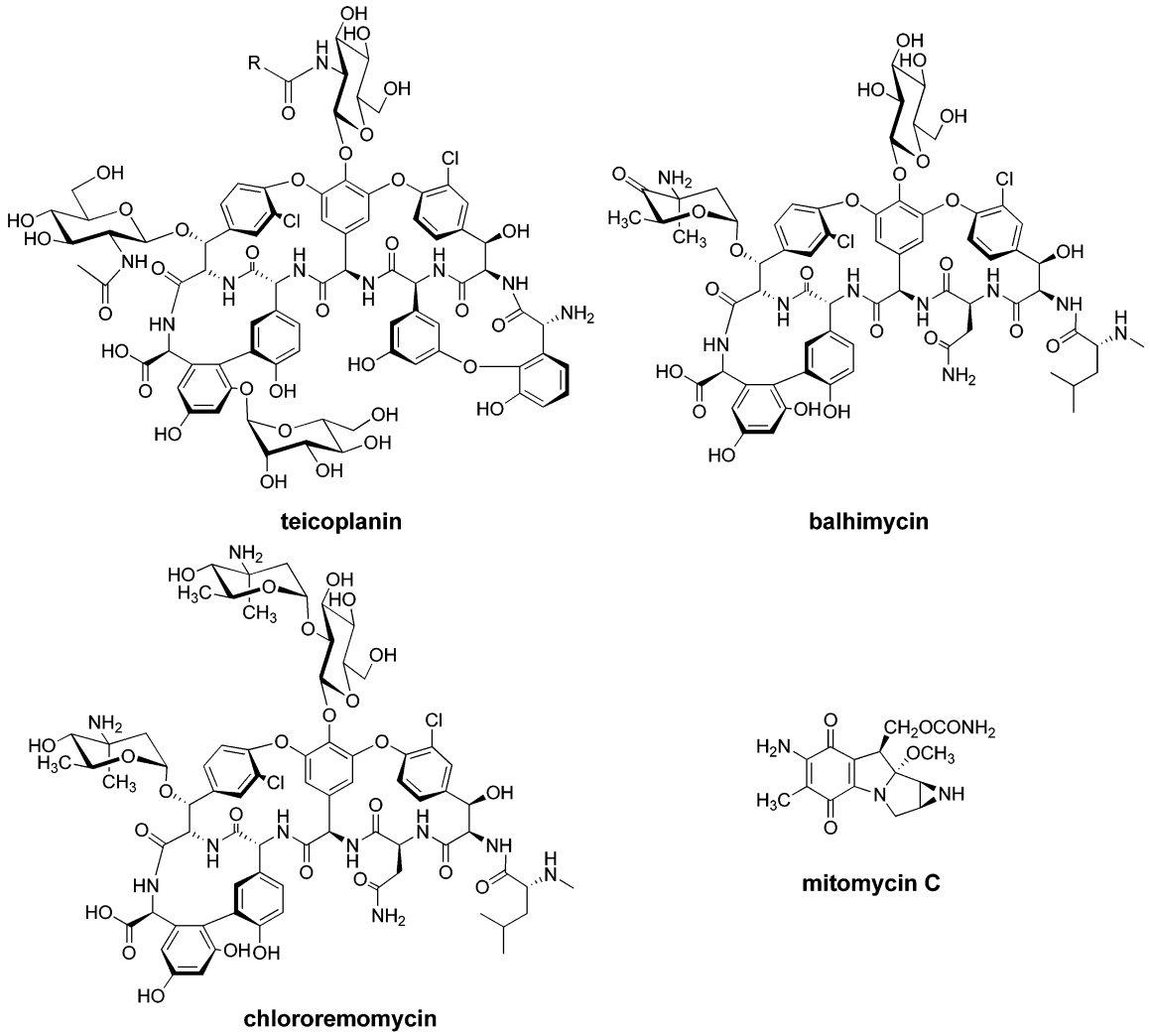

Figure 1. Antibiotics containing *btr*D homologue in the biosynthetic gene clusters

Table 1. Complementation of *btrD* Muant with Biosynthetic Intermediates

B. circulans strain + additive	antibiotic production[a]
wild type	+
Δ*btrD*	−
Δ*btrD* with pHBrbsbtrD	+
Δ*btrD* + 2-deoxy-*scyllo*-inosamine	−
Δ*btrD* + 2-deoxystreptamine	−
Δ*btrD* + paromamine	+

[a] +, production; -, no production.

nor DOS. These results suggested that the *btrD* gene product could be involved in the glycosylation pathway between an activated D-glucosamine and DOS giving paromamine, or in the preparation of glycosyl donor immediately prior to the glycosylation. Since a potential glycosyltransferase *btrM* was already proposed in the butirosin gene cluster, *btrD* was envisioned to have the latter function.

BtrD was overexpressed in *E. coli* to elucidate its function in vitro. The successfully expressed protein was straightforwardly purified to an electrophoretically homogeneous state. The purified BtrD was analyzed by ESI-MS to confirm the molecular weight 31 813 Da, which showed good agreement with the estimated mass 31 822 Da from the deduced amino acids.

Since it is generally accepted that, in the glycosylation reactions, a glycosyl donor is a nucleotidylyl-sugar as mentioned above, NDP-D-glucosamine was proposed as the corresponding donor in the glycosylation yielding to paromamine (Scheme 1). Because UDP-*N*-acetyl-D-glucosamine should exist in a wide varieties of bacterial cells as a building block for their cell walls, deacetylation of UDP-*N*-acetyl-D-glucosamine was hypothesized as one possibility for the supply of UDP-D-glucosamine. On the other hand, direct activation of D-glucosamine molecule with NTP should be an alternative, though little has been known for such D-glucosamine-1-phosphate specific nucleotidylyltransferase to date. Therefore, we pursued two sets of enzyme assay with BtrD.

The former possibility was first examined and turned out not to be the case, since UDP-*N*-acetyl-D-glucosamine was not hydrolyzed at all with BtrD under various conditions tested (data not shown). Surprisingly, however, an incubation of UTP and D-glucosamine-1-phosphate with BtrD clearly afforded a nucleotidylated product, UDP-D-glucosamine, which was unambiguously identified by comparison with a chemically synthesized standard[24,25] as shown in Figure 2. Further, BtrD was able to catalyze thymidylation of D-glucosamine-1-phosphate with dTTP

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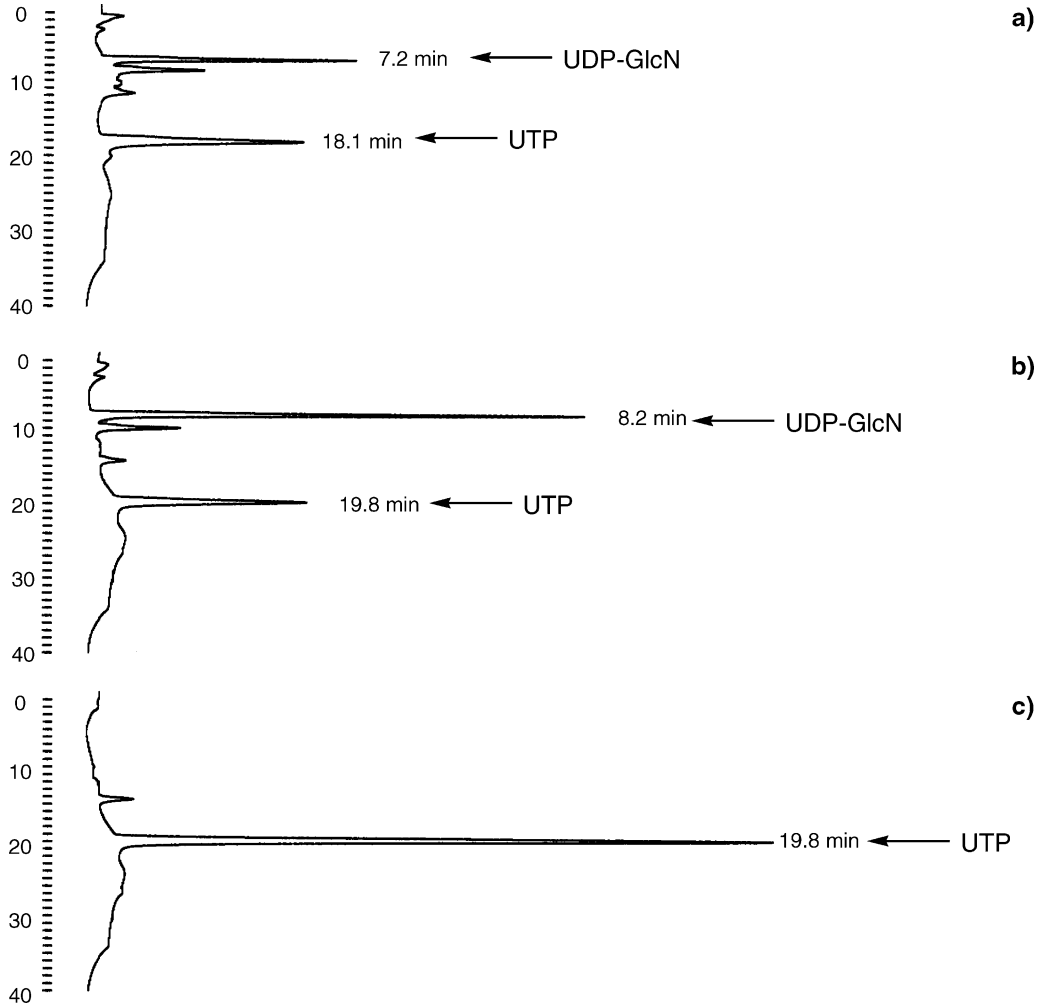

Figure 2. BtrD reaction with UTP and glucosamine-1-phosphate. (a) BtrD reaction with UTP and D-glucosamine-1-phosphate. (b) Part a plus authentic UDP-D-glucosamine. (c) Control reaction using heat-treated BtrD.

more efficiently. BtrD was inactive with ATP, GTP, or CTP, and the observed trend of nucleotide specificity appeared to be similar to the known pyrimidinylyltransferase Ep derived from *Salmonella*.[26] Most of D-glucose-1-phosphate thymidylyltransferases are known to utilize both uridine and thymidine triphosphate with similar efficiency. It is noteworthy that such a D-glucosamine-1-phosphate specific nucleotidylyltransferase was found in the secondary metabolic pathway. Since D-glucosamine-1-phosphate appears to be formed by a series of housekeeping enzymes in the biosynthesis of the bacterial peptidoglycan monomer unit,[27] BtrD-catalyzed UDP-D-glucosamine/dTDP-D-glucosamine formation is proposed as a crucial branching point of the secondary metabolic pathway from the primary carbohydrate metabolism.

In general, nucleotidylyltransferase requires a divalent metal for activity. Thus, requirement of divalent metal for the BtrD reaction was examined, and the results were shown in Table 2. Interestingly, various transition metals such as Mn^{2+}, Co^{2+}, and Ni^{2+} were able to accelerate the enzyme reaction in addition to Mg^{2+}. Requirement of Mg^{2+} is just the same as that of well-studied TDP-D-glucose synthase. In any case, such a divalent metal cation appears to be involved in the coordination of nucleotidylyl triphosphate and sugar phosphate substrates to an optimal orientation for catalysis.

Table 2. Effect of Divalent Metal Ions on BtrD Reaction[a,b]

metal ion (5.5 mM)	activity (%)
Mg^{2+}	100
Co^{2+}	124
Mn^{2+}	98.5
Ni^{2+}	97.1
Zn^{2+}	17.9
Ca^{2+}	2.1
Cu^{2+}	0.6
Fe^{2+}	0.3
none	0
EDTA	0

[a] Initial velocity relative to Mg^{2+}. [b] UTP and D-glucosamine-1-phosphate were used as substrates.

Combinatorial biosynthesis is a field attracting extensive efforts. To pursue combinatorial biosynthesis of glycosylated compounds, preparation of varieties of NDP-sugars is important for the ultimate glycosylation to obtain designed molecules, since certain glycosyltransferases having broad specificity, such as GtfE,[28] are known and glycorandomization approach with various NDP-sugars may be a method of choice for the

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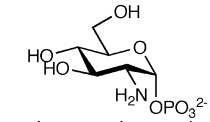
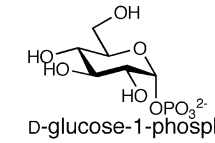
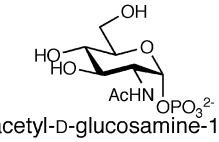
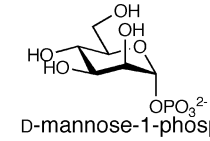
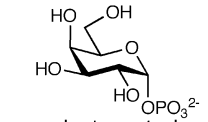
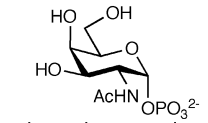
Table 3. BtrD Catalyzed Conversion of Substrates[b]

Substrate	dTTP Conv. (%)	UTP Conv. (%)
 D-glucosamine-1-phosphate	72 ± 6	5.9 ± 0.6
 D-glucose-1-phosphate	91 ± 3	45 ± 1
 *N*-acetyl-D-glucosamine-1-phosphate	n.d.[a]	1.6 ± 0.1
 D-mannose-1-phosphate	n.d.[a]	n.d.[a]
 D-galactose-1-phosphate	n.d.[a]	n.d.[a]
 *N*-acetyl-D-galactosamine-1-phosphate	n.d.[a]	n.d.[a]

[a] No product was detected. [b] The values are the conversion ratio in 1 h reaction period.

construction of a library of bioactive glycosides.[29−31] In their pioneering work, Thorson et al. explored TDP-glucose synthase derived from *Salmonella enterica* LT2 (Ep), showing its broad substrate specificity toward different kinds of α-D-hexose-1-phosphates.[32−34] In addition, subsequent structure-based protein engineering has allowed the enzyme to acquire altered substrate specificity.[29,35,36] It appears, therefore, different kinds of sugar activating enzymes are highly desirable for the extension of glycorandomization and for the ultimate development of useful bioactive compounds.

Thus, specificity of BtrD toward sugar-1-phosphate was next investigated (Table 3). Free forms of D-glucose and D-glucosamine were absolutely inactive. Therefore, the 1-phosphate group attached to the sugar is essential for the enzyme reaction. Interestingly, BtrD was able to take D-glucose-1-phosphate as a better substrate rather than D-glucosamine-1-phosphate. On the other hand, *N*-acetyl-D-glucosamine-1-phosphate was nucleotidylated to a lesser extent by BtrD. Any other tested hexose-1-phosphates were not nucleotidylated under the same conditions at all. Thus, it is clear that only α-D-*gluco*-hexopyranosyl phosphates are a good substrate for BtrD. The best catalytic activity of BtrD was found as D-glucose-1-phosphate thymidylyltransferase (40 nmol of TDP-D-glucose production/min/mg). Apparently, the stereochemistry of hexose is crucial for the BtrD recognition. On the other hand, the C-2 functionality is not much recognized, since a hydroxy, amino, or *N*-acetamide group at C-2 of the hexose-1-phosphate precursor was accepted by BtrD. These results suggest that the functional group at C-2 may be recognized by putative carboxylate functions of acidic residues through hydrogen bonding. It may thus be envisioned that precise structural studies of BtrD are helpful to open a new way of engineering the protein to be more promiscuous to a wide variety of sugar phosphates.

Multialignment of BtrD homologues is shown in Table 4. All of them are functionally unknown and were mostly reported as a gene in the biosynthetic gene clusters including those of several glycopeptides, mitomycin C, and kanamycin. It should

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Table 4. Amino Acid Sequence Alignment of BtrD Homologs[a]

```
BtrD          1:MNQDKRAFMF-----ISPHFDDVILSCASTLMELMNQGHTCKVLTVFGGC--PSVRFQPGEIARQYAAE-DLGLFEDEIEG 73
spr0138       1:MVMSDNTKYIF----LSPHLDDAIFSCGDYISKLTSEGEIVLVITIFSGY--PLSQQLQPSAKQFHKLCNLGK-YPIE--- 71
SP0139        1:MVMSNNTKYIF----LSPHLDDAIFSCGDYISKLTSEGEIVLVITIFSGY--PLSQQLQPSAKQFHKLCNLGK-YPIE--- 71
Dbv21         1:MLQDADRTRILA---ISPHLDDAVLSVGASLAQAEQDGGKVTVFTVFAGSAAP---P-YSPAAERFHARWGLSPTEDA--- 71
orientalis    1:MSQNLGAGRLLA---ISPHLDDAVLSFGAGLARAAQDGAKVTVYTVFAGTATP---P-YSPAAERLHGIWGLSPDQDA--- 71
Orf2          1:MPQDLDADRILA---ISPHLDDAVLSFGAGLARAAQAGAKVTVHTVFAGTAAP---P-YSPAAERLHAIWELSPDQDA--- 71
plu0451       1:MI-----H-LF----LSPHLDDAALSAGGLIHKLVSENQKVIILTFFTEYDKDLTSHYSHSAHNDN-IYSFIKLYSK---- 66
mitC          1:MSGTPATAPYGP-VVLSPHADDAVWSLGGRL------ARWA--A--EGP--RPTVVTVFAGPAAGKPE-SW-RSAA-D--- 62
tcp14         1:MPHDPGATRLLA---ISPHLDDAVLSFGAGLAQAAQDGANVLVYTVFAGAAQP---P-YSPAAQRMHTIWGLAPDDDA--- 71
Orf15         1:VR----------VLLVSPHPDDIALSFGGWVA---AHA-RGLAA--KGW--RFDLLTVFGTTLYAP-HSP--RAVTKE--- 57
                .              .***.**. .* .  .      .  . . . . .   .        ..
                                •••••••••

BtrD         74:DHLSILVARRLQEDQQAFRHLPGVQVEVLSFPDAIYRENK---GQPYYRTEAD-L-FGI----PDKQDE-DIFLPKI--ET 142
spr0138      72:-----E--RKKEDRLA-CERLQCD-FR-HLSY-YECLYRKDRNGN--FLYRH---IYS-----ELKNE--D--TL-KNDII 126
SP0139       72:-----E--RKKEDRLA-CERLQCD-FR-HLSY-YECLYRKDRNGN--FLYRH---IYS-----ELKNE--D--TL-KNDII 126
Dbv21        72:P----L-RRRNEDIAA-LDQLGAGHRHGRFLD-AIY--RRSPDGQWLLHHNEGS--MVRQQS-PANNH--DLVAA-IREDI 137
orientalis   72:S----LHR-RNEDIAA-LDHLGVDYRHGRFLD-AIY--RTLPDGRWLADNVPGRQKLAISRLSPQTDP--DLFAA-VRDDI 140
Orf2         72:S----LRR-RDEDIAA-LDHLGVDYRHGRFLD-AIY--RKLPDGRWLADNVPGRQKLAIGRQSPQGDP--ELFSA-VRADI 140
plu0451      67:--------RANEDIAF-CNKLSVIPI--HGKI-LDCIYRTDQYGE--PMYKNSAMIYT-----GQIHKSDDASDM-AQELI 127
mitC         63:P----A--VRRAEDRA-ACAELGVRHV-PLGF-TDAALRTASGAY--LYASPRR-LFG-----PWHPADLPLLEE-VRAAL 125
tcp14        72:V----LYR-RKEDIAA-LDHLRVAHRHGRFLD-SIY--RKLPDGRWLTAHVEGRQKLAVNDHSPDSDH--DLVGE-VADDI 140
Orf15        58:-----A--ISTLRERE-DR-DYARRH----GL-RLTSLRQEDCSC--LGMDDEEELIA-----P-EATDPRRA-A-VRQL- 113
                         . ..  .    .                 .    .                   .      .         .

BtrD        143:YLQSCDLARKYTWVFPAIS--KHVDHRLLTKAGLRLMSQGY-PVLFYSEFPYWQQHNEFLQDGWRQ-LELRN-SVYTPVKR 218
spr0138     127:KELLMHLDDKCVVYC-PLSLGDHVDHVFVNSIGRALEFMR-YKVIYYEDFPYVSDS-SMVSYMGKTK-EL----KMYQEE- 198
SP0139      127:KELLMHLDDKCVVYC-PLSLGDHVAHVFVNSIGRALEFMR-YKVIYYEDFPYVSDS-SMVSYMGKTK-EL----KMYQEE- 198
Dbv21       138:ESMIAECDPTLVLTCVAI--GKHPDHKATRDATLLAARERGIPLRLWQDLPYAAYSQD------LAE--LPDGLR------ 202
orientalis  141:KSVVEECDPTLILTCAAG--NGHIDNEITRDAALLVAHEKDLPVRLWEDLPHAMFGAG------PAE--LPEGFD------ 205
Orf2        141:ESIVEEYAPALILTCAAG--NGHVDNEIARDAALFVAYEKGIRVRLWEDLPHAMFAEG------AAE--LPDGFR------ 205
plu0451     128:DKILLNYQPDYI-YA-PLGIGRHVDHIIINNLVPNIKGSRKFKILLYEDFPYVLGEYPIIN-PDSLESALLRNNK-FDKRA 204
mitC        126:L-P-LCAGASS-VHV-PLAAGRHVDHRLVRGAVEP-LSPARTVF--YEDFPYRLRERDHTNL--RPRTERLPSEAVDRWLT 197
tcp14       141:RSIIDEFDPTLVVTCAAI--GEHPDHEATRDAALFATHEKNVPVRLWEDLPYAVFKSG------AVE--LPQGFR------ 205
Orf15       114:IAAALA-GA-DLV-VAPLAVGGHVDHRIVRTAVRQSLGATPCLW--YEDLPYALESPVEVPSDHRPWLVDIRGHEAAKRAD 189
                              .     . *...   . .               .. *.                 .
                                      •••••                   ━━━━━━

BtrD        219:AAV--LEYKT--QLL-GLFGEEAET----K-INNGGVLSEAE-L--F--WIQETDT--Q---AWRVFRSLSPEPLQT     275
spr0138     199:--------LDEKHYIDR-IS-SILCYKSQILIIWKSVEKLLNNIKELYLRNGAAYSI-----------RFWIKK---     251
SP0139      199:--------LDEKNYIDR-IS-SILCYKSQILIIWKSVEKLLNNIKELYLRNGAAYSI-----------RFWIKK---     251
Dbv21       203:LGSPELSFVDEEARTRK--FQAMKHYATQLSVLDGPNKNLFAKLDE-HARNAAPDGGYNET-TWP-VIRYAAE----     270
orientalis  206:LGTGDFGSVTTDMRDRK--FEALRLYPSQMLMLHGPGKDFFAQLDE-HARKNSPQGGYGET-TWP-VVSREDNS---     274
Orf2        206:LGPPDFGSVEPEARARK--FEALRLYSSQMLMLHGPEKDFFAQLDG-HARKSAPGGGYGET-TWP-VVSREDNG---     274
plu0451     205:-------ILVDIDLKEK-AQ-NILFYESQLEPLFDNKSNILISLEK-YHRTINEIKVQE---------RFWLIR---     259
mitC        198:AAGHYSSQASAHFGGAA-ALREAL-FARARAHGGPGRPGHADRHWVPVG--QDDRGEARP---A----PVERGP---     260
tcp14       206:LGSADVSSVKPEMRSQK--FQAVERYSSQMVLLNGSENNLFDRLDE-HARQNAPHGGYGET-TWP-VVRSDDS----     273
Orf15       190:LAL-YRSQMTAA--DTS----EVLSY---RPDGASV-PC--ERLWSSAGFPQ-DLAERMALATLAAVTP-DKESL--     249
                                         . ..              .     .
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[a] BtrD: BAC41211 *Bacillus circulans*, butirosin producer; spr0138: NP_357732 *Streptococcus pneumoniae* R6; SP0139: NP_344682 *Streptococcus pneumoniae* TIGR4; Dvb21: CAD91216; *Nonomuraea* sp. ATCC 39727, glycopeptide A40926 producer; orientalis: T30588 *Amycolatopsis orientalis*, chloroeremomycin producer; Orf2: CAC48365 *Amycolatopsis balhimycina*, balhimycin producer; plu0451: NP_927804 *Photorhabdus luminescens* subsp. Laumondii TTO1; mitC: AAD27813 *Streptomyces lavendulae*, mitomycin C producer; tcp14: CAE53355 *Actinoplanes teichomyceticus*, teicoplanin producer. Orf15: BAD20763 *Streptomyces kanamyceticus*, kanamycin producer. The conserved amino acid residues are marked with an asterisk, and similar amino acid residues are marked with a period. The putative metal binding motifs are shown as a dotted line. The proposed pyrimidine recognition motifs are shown as a solid line.

be pointed out that the *btrD* homologue exists in the biosynthetic gene cluster of a closely related aminoglycoside kanamycin.[15] Corresponding proteins of these genes have a similar molecular size comprising ca. 260 amino acids. It may be emphasized that the *N*-terminus region is well conserved, particularly, a motif SPHxDDxxxS is completely conserved in all of these functionally unknown proteins. A motif of HxDH(N) is also conserved in the middle of the polypeptides. Further, a motif (Y/W)x(E/D)(L/F)P(Y/H) close to the C-terminus was found to be highly conserved in BtrD homologues.

Despite the fact that much studies have been concentrated on glycosyltransferases such as GtfE and GtfD in the biosynthesis of vancomycin,[28] information on how an activated glycosyl donor is supplied in the natural biosynthetic system is scanty except for the well-characterized TDP-glucose synthase. As we have successfully shown in the present study the function of BtrD as a novel nucleotidylyltransferase, the above-mentioned BtrD homologues may have a similar function of the activation of sugar, probably of D-glucose or D-glucosamine. NDP sugars synthesized by these BtrD homologues may further be modified by several enzymes for the ultimate glycosyl donors, and then the corresponding glycosyltransferases can take over to attach each sugar onto the corresponding aglycon. This scenario can be reasonable because a certain mechanism for sugar activation

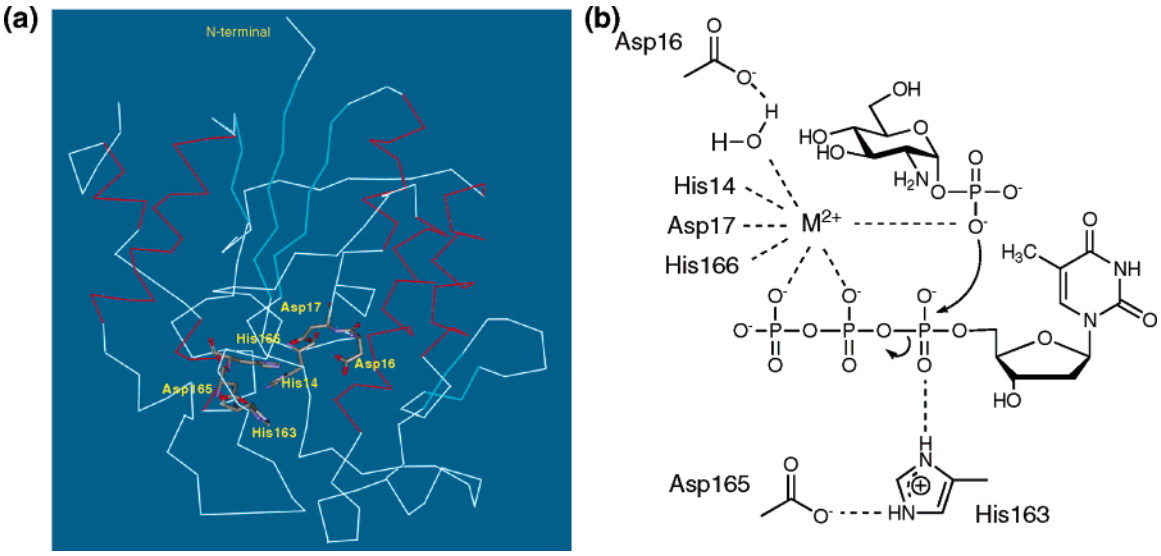

Figure 3. A model of BtrD by 3D-PSSM.[37,38] (a) A BtrD model structure was generated by 3D-PSSM based on MshB crystal structure (pdb 1q74). The image was constructed by WebLab Viewer. The α-helices and the β-sheets are colored in red and in light blue, respectively. The amino acid residues of 1−4, 183−199, and 242−275 are omitted for clarity. Probable active site amino acid residues are labeled. (b) A proposed mechanism of BtrD reaction.

must be involved, and in fact, in the gene clusters containing the *btrD* homologues, any possible gene for the well-known TDP-glucose synthase does not exist at all. Our finding, therefore, strongly suggests that the BtrD homologues are secondary metabolism specific sugar-activating enzymes. In other words, functionally unknown hypothetical proteins in the secondary metabolic gene clusters could have a similar function to that of well-known primary metabolic enzymes, even with little sequence homology. In certain cases, novel function of secondary metabolic proteins may be independently acquired during evolutionally processes.

Interestingly, the structural search for BtrD by 3D-PSSM[37,38] hit a quite similar protein with a low *E*-value of $8.78e^{-17}$, which is 1-D-*myo*-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside deacetylase (MshB) from *Mycobacterium tubeculosis*.[39,40] MshB catalyzes deacetylation of 1-D-*myo*-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside in the mycothiol biosynthesis. Based on its crystal structure (pdb code 1q74), a model of BtrD was constructed also by 3D-PSSM (Figure 3). The resulting BtrD model implied that the N-terminal His14, Asp17, and His166 in BtrD could bind a divalent metal ion. The carboxylate of Asp16 would interact with the metal ion through a water molecule. His163 and Asp165 could function as a catalytic dyad at the active site. These putative catalytic residues are all conserved in other BtrD homologues as well. Thus, these two motifs could be critical to bind a divalent metal ion in the active site and catalyze the nucleophilic attack of a sugar phosphate anion to an a-phosphorus of nucleotidyl triphopsphate. These two motifs are well-conserved in the related hypothetical proteins. Therefore, we suggest that these motifs could universally exist in combination as a divalent metal binding motif which stimulates an attack of an appropriate nucleophile in all of these related hypothetical proteins.

Further, it should also be pointed out that the C-terminus region motif (Y/W)x(E/D)(L/F)P(Y/H) found in BtrD homologues is not found in MshB. Thus, this motif may reflect the functional difference between them and may be involved in the interaction with the nucleotide moiety of NTP. Further precise structural studies of BtrD is currently underway in our laboratory.

In conclusion, our detailed studies of the gene *btrD* in the biosynthetic gene cluster for butirosin have lead to the elucidation of its function as a novel nucleotidyltransferase, although previous sequence analysis failed to suggest any function. Putative functions of homologous genes found in the biosynthetic gene cluster of various antibiotics has also been postulated. Therefore, the present approach on the protein level is quite significant to identify the function of metabolic enzymes. We currently continue to tackle the unknown processes in the aminoglycoside biosynthesis with a hope of creating combinatorial methodology for antibiotic production by the use of these structurally diverse aminoglycosides biosynthetic machineries.

Experimental Section

Materials. *Bacillus circulans* SANK 72073 was the source of butirosin biosynthetic genes and was used for the construction of gene disruption mutants. *E. coli* JM 109 was used as a host strain for *btrD* gene cloning and a test strain for antibiotic activity. *E. coli* BL21-(DE3) was used for *btrD* gene expression. *Bacillus subtilis* PCI219 was used for antibacterial testing. An insertionally modified plasmid for gene disruption was constructed with an *E. coli*−*Bacillus* shuttle vector pHB201 (Tanaka et al., unpublished). pUC 119 was routinely used as a vector for subcloning and sequencing. pET30b(+) was used for *btrD* overexpression. D-Glucosamine-1-phosphate, UDP-D-glucosamine, dTDP-D-glucosamine, ADP-D-glucosamine, GDP-D-glucosamine, and CDP-D-glucosamine were chemically synthesized according to literature procedures.[24,25] D-Glucose-1-phosphate, *N*-acetyl-D-glucosamine-1-phosphate, D-mannose-1-phosphate, D-galactose-1-

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phosphate, *N*-acetyl-D-galactosamine-1-phosphate, UDP-*N*-acetyl-D-glucosamine, and UDP-D-glucose were purchased from Sigma. Other chemicals were of the highest grade commercially available.

Disruption of *btrD*. Standard in vitro techniques were used for DNA manipulation.[41] The plasmid pDS2 containing *btrD* was digested with *EcoRI* and *SacI*, and an appropriate fragment was inserted into the corresponding restriction enzyme site of pHB201.[12] The resulting modified plasmid pHB201 containing *btrD* was digested with *ScaI*, and the tetracycline-resistance gene (Tc^r) cassette derived from pBEST309 [42] digested with *SmaI* was inserted into the *ScaI* site of *btrD*. The reverse direction of Tc^r against *btrD* was confirmed by *EcoRI* digestion. The resulting plasmid pHBCTc^{r}E was introduced into *B. circulans* SANK 72073 by electroporation 1600−2000 V/0.2 cm (EasyjecT Optima, EquiBio, UK), and a *btrD* gene disruptant was constructed by homologous recombination according to the previously described method.[12] Disruption of *btrD* was confirmed by PCR with amplification primers for the whole *btrD* (btrD-*f*′5′-GGCGATG-TATAACCAAACGC-3′ and btrD-r′5′-TTTCCATGGAAAGCACTCCT-3′) and by Southern blotting with the DIG labeled Tc^r gene as a probe. PCR conditions were 95 °C, 5 min for denature, 30 cycles of 95 °C, 1 min, 40 °C, 1 min, 72 °C, 3 min for extension of DNA.

The *btrD* disruptant was cultured in a glycerol-supplemented nutrient broth medium,[43] and an aliquot of the culture was collected everyday for 5 days. The supernatant of each culture was tested for antibiotic activity by a paper disk diffusion method against *Bacillus subtilis* PCI219.[44]

To complement the *btrD* mutation, a whole *btrD* gene was amplified by PCR with primers btrD-f 5′-GGCTGTACATATGAACCAG-GATAAG-3′, btrD-r 5′-CTCGAATTCTTTGTGTCAGGTTTGA-3′ on the template pDS2. PCR conditions were 95 °C, 1 min for denature, 30 cycles of 95 °C, 30 s, 60 °C, 45 s, 72 °C, 30 s for extension of DNA. The amplified DNA fragment was phosphorylated and then subcloned into a *SmaI* site of pUC119. After confirmation of the inserted sequence (Long Readir 4200, Li-Cor), the *NdeI-EcoRI* fragment derived from the PCR fragment was inserted into pET30b (+). The resulting plasmid named as pETbtrD was digested with *XbaI-EcoRI*, and the fragment containing Shine−Dalgarno sequence derived from the pET vector was subcloned into pUC119. The resulting plasmid was further digested with *SphI-EcoRI* and was cloned into the corresponding restriction enzyme site of pHB201 to yield pHBrbsbtrD. The resulting expression plasmid pHBrbsbtrD was introduced into a *btrD* disruptant by electroporation for complementation. The complemented strain was cultured in a fermentation medium supplemented with 1 mg/mL of erythromycin. An aliquot of the culture was collected everyday. The supernatant of each specimen was tested for antibiotic activity by a paper disk diffusion method against *E. coli* JM109.

Antibiotic production by the *btrD* disruptant was examined by supplementation (final 100 μg/mL) of butirosin biosynthetic intermediates, DOS, 2-deoxy-*scyllo*-inosamine[45] and paromamine, to the fermentation after 1 day. Each culture was tested for antibiotic production as described above.

Expression of BtrD. The *btrD* expression vector pETbtrD was introduced into *E. coli* BL21(DE3). The transformant precultured at 37 °C for 12 h was inoculated to an LB medium containing 30 μg/mL of kanamycin and was cultured at 28 °C. The expression was induced at OD_{600} 0.6 by adding final 0.05 mM IPTG, and the culture continued for additional 5 h. The cells were collected by centrifugation and were suspended in a 50 mM phosphate buffer (pH 7.5) and then disrupted by sonication (Sonifier Type-250, Branson) for 30 s 5 times. After removal of cell debris by centrifugation (13 000 rpm × 10 min), the supernatant was loaded onto a column of DEAE Sepharose Fast Flow, previously equilibrated with a 50 mM phosphate buffer (pH 7.5). BtrD was eluted with a linear gradient of 0−0.5 M of NaCl in the same buffer. The fractions containing BtrD were collected and concentrated by ultrafiltration (Vivaspin 20, Vivascience, Germany) according to the manufacturer's protocol. The concentrated solution was further purified with a Superdex 200 gel filtration column using the same buffer containing 0.1 M NaCl. Purified BtrD was concentrated and stored at 4 °C until use. The molecular weight of the expressed BtrD was analyzed by SDS-PAGE and LC-ESI-MS (LCQ, Finnigan).

Enzyme Assay. The BtrD reaction was examined in a mixture containing 2.5 mM NTP, 5.0 mM hexose-1-phosphate, and 5.5 mM $MgCl_2$ in a total 50 μL of a 50 mM sodium phosphate buffer (pH 7.5) and was initiated by the addition of BtrD. The reaction was carried out with slow agitation at 37 °C for 1 h and was then quenched by adding 50 μL of methanol. After removing the precipitates by centrifugation, a 5 μL aliquot of the supernatant was injected into an HPLC system (L-7000 series, Hitachi) with a Senshu-Pak SAX-1251-N column 4.6 ø × 250 mm (Senshu Scientific), the eluant being a linear gradient 0−300 mM phosphate buffer (pH 5.0) for 15 min and a 300 mM phosphate buffer (pH 5.0) for 15 min, flow rate 1.5 mL/min. The elution was monitored spectrophotometrically at the wavelength 260 nm. The UDP-D-glucosamine product was confirmed by comparing with a synthetic standard. The other products, dTDP-D-glucosamine, UDP-D-glucose, and UDP-*N*-acetyl-D-glucosamine, were also compared with authentic samples. The unreacted residual NTP in the enzyme reaction were simultaneously analyzed under the same HPLC conditions. The amounts of NDP-hexose and NTP were estimated from the absorption at λ_{260} using ϵ_{9520} for normalization.

Divalent metal requirement was examined with supplementation of a 5.5 mM concentration of $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, and $ZnCl_2$. The control was without metal chloride in the presence of additional 1.0 mM EDTA.

Specificity for NTP was analyzed with 2.5 mM ATP, CTP, GTP, UTP, and dTTP, and specificity for sugar phosphate was studied with 5.0 mM glucosamine-1-phopsphate, glucose-1-phosphate, *N*-acetylglucosamine-1-phosphate, mannose-1-phosphate, galactose-1-phosphate, and *N*-acetylgalactosamine-1-phosphate in the presence of cosubstrate UTP and dTTP for 1 h (triplicate). The conversion ratio was calculated as (NDP-sugar production)/[(remaining NTP) + (NDP-sugar production)] × 100.

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