Processive lipid galactosyl/glucosyltransferases from Agrobacterium tumefaciens and Mesorhizobium loti display multiple specificities

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The glycosyltransferase family 21 (GT21) includes both enzymes of eukaryotic and prokaryotic organisms. Many of the eukaryotic enzymes from animal, plant, and fungal origin have been characterized as uridine diphosphoglucose (UDP-Glc) ceramide glucosyltransferases (glucosylceramidases [Gcs], EC 2.4.1.80). As the acceptor molecule ceramide is not present in most bacteria, the enzymatic specificities and functions of the corresponding bacterial glycosyltransferases remain elusive. In this study, we investigated the homologous enzymes of eukaryotic and prokaryotic organisms. Many of these hypothetical proteins have been characterized as Gcs by automatic open reading frame (ORF) annotation, although none of them has been characterized experimentally up to now. Bacterial representatives containing a putative gcs gene are Agrobacterium tumefaciens and Mesorhizobium loti. The plant parasite A. tumefaciens belongs to the family of Rhizobiaceae with members able for fixing nitrogen and maintaining symbiosis with plants. The nitrogen-fixing plant symbiont M. loti belongs to the closely related family of Phyllobacteriaceae (Young et al., 2001). Interestingly, the occurrence of glucosylceramides or other glycosphingolipids has never been reported for members of Rhizobiaceae and for M. loti (Wilkinson, 1988). With the exception of Rhizobium (Orgambide et al., 1992), not even glycosylglucosylceramides, which predominantly occur in Gram-positive bacteria and cyanobacteria, have been found in these organisms. Because of the lack of such glycolipids, it is surprising that both A. tumefaciens and M. loti contain putative gcs genes. In view of these facts, the actual enzymatic activity of bacterial representatives of GT21 remains obscure.

The aim of this study was to determine the enzymatic function of the hypothetical Gcs enzymes from A. tumefaciens and M. loti. For this purpose, we analyzed the lipid extract.

Key words: galactosyl diacylglycerol/galactosyltransferase/GCS/glucosylceramide/glycosyltransferase family 21

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of *A. tumefaciens* for the occurrence of glycosylceramides and glycosyglycerolipids. But because these glycolipids could not be detected, both bacterial Gcs sequences were cloned and expressed in different host organisms with subsequent glycolipid analyses and enzyme assays. In the following, we will demonstrate that the Gcs from *A. tumefaciens* and *M. loti* differ from the eukaryotic members of GT21 in several ways: they favour uridine diphosphogalactose (UDP-Gal) over UDP-Glc as sugar donor and diacylglycerol (DAG) over ceramide as acceptor. In addition, the bacterial Gcs consecutively transferred up to three glycosyl residues to an accordingly changed lipid acceptor.

**Results**

**Cloning of glycosyltransferases from Agrobacterium tumefaciens and Mesorhizobium loti**

Our efforts to find the ORFs encoding putative glycosyltransferases in the two bacteria made use of the BLAST search (Altschul et al., 1990) based on the amino acid sequence of the Gcs from *Homo sapiens* (Ichikawa et al., 1996). Two promising candidates were recognized in the fully sequenced genomes of *A. tumefaciens* (GenBank accession number, NP_354792; locus tag, AGR_C_3323) and *M. loti* (GenBank accession number: NP_106273; locus tag, mlr5650). The two corresponding polypeptides shared 60% identity, whereas their similarity to the query sequence was significantly lower (about 23%). The recently characterized Gcs glycosyltransferase activity and its products are not lethal for *A. tumefaciens*.

Due to sequence similarity, the encoded enzymes from *A. tumefaciens* and *M. loti* fall into the glycosyltransferase family GT21 (Campbell et al., 1997; Coutinho et al., 2003; Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/CAZY/) (Figure 1A). Like all other members of the family, they contain a putative N-terminal transmembrane domain and the widely spaced D₁,D₂,D₃(Q/R)XXRW motif (Marks et al., 2001) (Figure 1B). This motif was previously shown to be characteristic for processive β-glycosyltransferases of GT2 (Saxena et al., 1995). Although D₁,D₂,D₃(Q/R)XXRW is present in all Gcs sequences (Leipelt et al., 2001; Marks et al., 2001), none of the GT21 family members which have been characterized so far, showed processivity.

On the basis of the sequence similarity to the other Gcs enzymes of GT21, the sequences from *A. tumefaciens* and *M. loti* had been automatically annotated as putative ceramide glycosyltransferases. However, enzymatic evidence for their functions has not been provided so far, and neither the actual sugar donors nor the sugar acceptors were known. Therefore, we tried to identify the enzymatic activity of the two enzymes by various approaches based on the assumption that the glycosyltransferases may contribute to the biosynthesis of glycolipids.

**Agrobacterium tumefaciens lacks detectable proportions of “conventional” glycolipids**

As a first approach to identify the function of the glycosyltransferase from *A. tumefaciens*, we prepared lipid extracts from this bacterium and looked for the presence of conventional glycolipids extractable by chloroform/methanol. Thin layer chromatography (TLC) of the total lipid extract did not show the presence of any glycolipids. To exclude that very low proportions of glycolipids escaped detection, we fractionated the total lipid extract by preparative column chromatography into neutral lipids, glycolipids, and phospholipids. All three fractions were redissolved in very small volumes of solvent for subsequent spotting onto TLC plates, but still we could not detect any conventional glycolipids in any of the three fractions (Figure 2A). To find out, whether the glycosyltransferase gene of *A. tumefaciens* exerts any effect on the lipid pattern of this bacterium, we deleted this gene by homologous recombination with an antibiotic resistance cassette (Figure 2C). The lipid extract of the transformed bacteria was subjected to the procedure described above, but no change compared to wild type cells, and again no glycolipids could be detected (Figure 2A). From these results, we conclude that the absence of the putative glycosyltransferase activity and its products are not lethal for *A. tumefaciens*.

Finally, we checked the possibility that a product of the Gcs glycosyltransferase activity was not detectable, because of promoter repression under the growth conditions used. For this purpose, the ORF of the gcs⁺ was replaced by a heterologous glucosyltransferase from *Staphylococcus aureus* (Ugt106B1), serving as a reporter sequence to determine the activity of the gcs⁺ promoter (Figure 2D). We had shown before (Jorasch et al., 2000) that the enzyme encoded by igt106B1 on expression in various hosts resulted in the formation of the diglucosyldiacylglycerol βGlcβGlcD (abbreviations and structures are given in Table I). The new ORF sequence, together with a downstream selection marker, was inserted exactly at the original start codon of the replaced gcs⁺. The homologous recombination event was confirmed by appropriate polymerase chain reaction (PCR) experiments (data not shown). This genetic engineering led to the expression of the heterologous glucosyltransferase under the control of the genuine gcs⁺ promoter. The glycolipid fraction of the mutant cells was analyzed by TLC showing a new glycolipid, which comigrated with authentic βGlcβGlcD (Figure 2A). The appearance of βGlcβGlcD in the transformed cells showed that the gcs⁺ promoter was active. Therefore, the gcs⁺ gene can be expected to be transcribed under these experimental conditions in wild type *A. tumefaciens*. On the other hand, in wild type cells we could not detect any glycolipids (beyond a threshold value of 0.5%) resembling in structure and extractability conventional glycolipids comprising glycosylated derivatives of DAG, ceramides, and sterols.

**Expression of the Gcs from Agrobacterium tumefaciens in Pichia pastoris led to the synthesis of new glycolipids of very different structures**

After these negative results concerning the identification of the enzymatic activity of the Gcs enzyme from *A. tumefaciens*, we switched to heterologous expression of the Gcs ORF in previously successful expression hosts. One of the hosts used before in our laboratory for functional expression of different glycosyltransferases was the yeast *Pichia pastoris*. This organism does not contain glyco-
but it constitutively synthesizes ceramide glucosides and sterol glucosides as well as DAG used as intermediate for phospholipid biosynthesis. Therefore, *P. pastoris* represents an appropriate host for the expression of glycosyltransferases requiring DAG, sterols, or ceramides as glycosyl acceptors. We used a glycolipid-free double null mutant of *P. pastoris* (∆gcs/gtc) as an appropriate host for the expression of glycosyltransferases, requiring DAG, sterols, or ceramides as glycosyl acceptors. This strain was transformed to express the Gcs from *A. tumefaciens*. The transformed cells were subjected to extraction of lipids which were fractionated and analyzed as described above.

TLC of the glycolipid fraction resolved numerous new glycolipids comigrating with glycosylceramide and various glycosylglycerolipid standards available from this and previous work (Jorasch et al., 1998; Leipelt et al., 2001) (Figure 3). A satisfactory separation of the various glycolipids by TLC required the use of different solvent mixtures. These are described in the legend of each figure and listed in Table I of the supplementary data. For structural identification glycolipids were isolated, acetylated, and subjected to compositional and structural analysis by combined gas–liquid chromatography/mass spectrometry (GLC–MS), by nuclear magnetic resonance (NMR) spectroscopy and in most cases also by electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS). By this, we isolated and identified nine different glycolipids which were fractionated and analyzed as described above.

Fig. 1. (A) Dendrogram showing similarities between glycosyltransferase family 21 (GT21) polypeptides from animals, fungi, plants, and bacteria. Enzymes whose function has been confirmed experimentally are framed (Ichikawa et al., 1996; Ichikawa and Hirabayashi, 1998; Wu et al., 1999; Leipelt et al., 2001; Kohyama-Koganeya et al., 2004), and those studied in this work are marked by asterisks. The other sequences represent hypothetical polypeptides deduced from genomic sequences of the respective organisms. The dendrogram has been constructed from pair wise similarities of amino acid sequences using ClustalX (Thompson et al., 1997). The sequences used for the alignment are present at the Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/CAZY/ except for the sequences of the rat Gcs is given. Only three amino acids essential for the activity of the rat Gcs are different in the Gcs from *A. tumefaciens* (Thompson et al., 2001; Kohyama-Koganeya et al., 2004), which is devoid of both DAG, sterols, or ceramides as glycosyl acceptors. The other sequences were from *H. sapiens* ([predicted gene id at http://www.wormbase.org/], Ce2 = F20B4.6, Ce3 = T06C12.10), *K. lactis* (Ta, CAC11705), *C. albicans* (Ca, protein CaO19.4592, EAL0327). The other sequences were from *Agrobacterium tumefaciens* (Atum), *Arabidopsis thaliana* (At), *Caenorhabditis elegans* (Ce1 = F59G1.1 [predicted gene id at http://www.wormbase.org/]), Ce2 = F20B4.6, Ce3 = T06C12.10), *D. melanogaster* (Dm), *G. arboresum* (Ga), *H. sapiens* (Hs), *K. lactis* (Kl), *M. loti* (Ml), *N. crassa* (Syn), *P. pastoris* (Pp), *R. norvegicus* (Rn), *S. cerevisiae* (Sk), *S. pombe* (Sp), *S. quadripartita* (Sq), *S. wingei* (Swe). The putative N-terminal transmembrane domain and the hydrophobic domains at the C-terminus are underlined. Amino acids in bold lettering including those of the D1, D2, D3, Q/R motif indicate that the corresponding amino acids of the rat Gcs are essential for enzyme activity as demonstrated by site-directed mutagenesis (Wu et al., 1999; Marks et al., 2001). The position of the corresponding amino acids from the rat Gcs is given. Only three amino acids essential for the activity of the rat Gcs are different in the Gcs from *A. tumefaciens*.
glycolipids with a DAG backbone: βGlcD, βGalD, βGlcβGlcD, βGlcβGalD, Glc-(1→3)-βGlcD and βGalβGalD (abbreviations for glycolipids used throughout the text are given in Table I). The remaining three glycolipids turned out to be glycosphingolipids. Their synthesis represents at least partial support and confirmation of the sequence-based Ges-
glycosyl headgroups of these new glycolipids, the Gcs of *A. tumefaciens*, expressed in *P. pastoris*, can use galactose as well as glucose donors in a promiscuous manner to form β-linked glycosides. These glycosyl residues are transferred to a variety of lipophilic acceptors such as DAG, ceramide, βGalD and βGlcD, from which only DAG and ceramides are present in untransformed cells.

The acceptance of both DAG and glycosyldiacylglycerols results in the formation of diglycosyldiacylglycerols starting from DAG. The predominant products of these two glycosylation steps carry the terminal glycosyl residue in β-1→6-linkage. The possibility that these diglycosyldiacylglycerols result from glucosylation of the corresponding monoglycosyldiacylglycerols by native glycosyltransferase activities of *P. pastoris* can be excluded, because expression of the human Gcs protein in *P. pastoris* resulted in the synthesis of βGlcD, but not of further glycosylation products (Leipelt et al., 2001). An exception is the structure of the βGlc(1→3)βGlcD. If really attributable to the activity of the Gcs from *A. tumefaciens*, it would remarkably extend the relaxed specificity of this enzyme. However, at present there is no unequivocal evidence that the βGlc(1→3)βGlcD is a product of the *A. tumefaciens* Gcs.

Table II. Properties of glucosylceramide synthase (Gcs) enzymes from different kingdoms

<table>
<thead>
<tr>
<th>Property</th>
<th>Bacteria (<em>Agrobacterium tumefaciens</em>)</th>
<th>Plants (<em>Gossypium arboreum</em>)</th>
<th>Fungi (<em>Candida albicans</em>)</th>
<th>Animals (Mammals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar acceptor</td>
<td>DAG &gt; ceramide</td>
<td>Ceramide &gt; sterola</td>
<td>Ceramide</td>
<td>Ceramide &gt; DAGb</td>
</tr>
<tr>
<td>Sugar donor</td>
<td>UDP-Gal &gt; UDP-Glc</td>
<td>UDP-Glc</td>
<td>UDP-Glc &gt; UDP-Gal</td>
<td>UDP-Glc &gt;&gt; UDP-Galf</td>
</tr>
<tr>
<td>&quot;Processivity&quot;</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Intracellular localization</td>
<td>?</td>
<td>ERd</td>
<td>?</td>
<td>Golgi apparatusc</td>
</tr>
<tr>
<td>D1,D2,D3(Q/R)XXRW</td>
<td>Present</td>
<td>Not present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Sensitivity to inhibitor EtDo-P4</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

DAG, diacylglycerol; UDP-Gal, uridine diphosphogalactose; UDP-Glc, uridine diphosphoglucose.

aHillig et al., 2003.
bGcs from *Homo sapiens* (Leipelt et al., 2001).
cGcs from *Homo sapiens* and *Rattus norvegicus* (Sprong et al., 1998; Wu et al., 1999).
dO. Kusmakov and E. Heinz, unpublished results. ER, endoplasmic reticulum.
eGcs from *Rattus norvegicus* (Futerman and Pagano, 1991; Marks et al., 1999).
fI. Hillig and E. Heinz, unpublished results. EtDO-P4 = D-threo-1-(3',4'-ethylenedioxy) phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (Lee et al., 1999)

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Overexpression of the two bacterial Gcs sequences in Agrobacterium tumefaciens resulted in the synthesis of di- and triglycosyldiacylglycerols

Our experiments had shown a remarkably wide specificity of the Gcs sequence from *A. tumefaciens* when expressed in *P. pastoris*. But despite of this finding, Gcs function in its natural hosts is still not clear. To specify the activity of the Gcs in *A. tumefaciens*, the Gcs sequences were inserted into a vector for expression in *A. tumefaciens*. This vector was constructed by insertion of the pVS1 sequence of the origin of replication (from pCambia2200) into an *Escherichia coli* expression vector for stable maintenance in *A. tumefaciens*. Gene expression is controlled by a strong, inducible promoter. Cells of *A. tumefaciens* were transformed with these
and analyzed by GLC-MS, ESI FT-ICR-MS, and 1H-NMR spectroscopy as described above. The experiments on the overexpression of the Gcs sequence from A. tumefaciens under the control of a strong promoter in this bacterium suggest that the formation of βGalβGlcD and βGlcβGalD may represent the natural activity of this Gcs in Agrobacterium.

Vectors containing in addition the ORF of the Gcs either from A. tumefaciens or from M. loti. Lipid extracts prepared from the transformed cells were analyzed by TLC, and all glycolipid components were isolated, per-O-acetylated, and analyzed by GLC-MS, ESI FT-ICR-MS, and 1H-NMR spectroscopy as described above.

The lipid patterns of the two transformants differed from each other and from the mixture found in P. pastoris (Figure 5, Table I). As expected, glycosylceramides were not present in the bacterial glycolipid fractions. Moreover, no monoglycosylacylglycerols could be detected, which represented a prominent fraction in the lipid extract from the transformed P. pastoris cells. On the other hand, the expression of both Gcs sequences resulted in the formation of βGalβGalD and βGlcβGalD, whereas βGlcβGlcD could not be detected. The lipid extract from cells expressing the Gcs from M. loti contained additional and predominating triglycosyldiacylglycerols. The major species was identified as βGalβGalβGalD (Table IIb of supplementary data). The band with slightly higher mobility represents a mixture which contains βGlcβGlcβGalD (Jorasch et al., 1998) and other triglycosylacylglycerols having a terminal glucose, and both galactose and glucose as sugar moieties in the two inner positions (Table I).

Interestingly, the two bacterial Gcs enzymes expressed in A. tumefaciens showed a preference for UDP-Gal, because lipids with only galactosyl residues (βGalβGalD and βGalβGalβGalD) predominated, whereas βGlcβGlcD was not detected. These findings are in contrast to the observations made by Gcs expression in P. pastoris (Figure 3), where the glucolipids were the predominant components, although the ratio of gluco- to galactolipids seems to depend to some extent on variations in culture conditions (data not shown).

The experiments on the overexpression of the Gcs sequences from A. tumefaciens resulted in additional triglycosyldiacylglycerols which were separated into two major bands. The upper one (triglycosyl diacylglycerol [TGD]) contained glucose and galactose as sugar components. The lower band represents the pure βGalβGlcβGalD.

In vitro enzymatic assays of Gcs activity support the preference for UDP-Gal
To identify the actual sugar donors and to confirm their promiscuous use by the Gcs enzyme of A. tumefaciens, we carried out in vitro enzyme assays. As enzyme source, we used membrane fractions which were prepared from both host lines.
P. pastoris and E. coli cells expressing the Gcs from A. tumefaciens. The Gcs activity expressed in P. pastoris was characterized by assays with radiolabelled UDP-sugars (Figure 6A). The membrane fractions were incubated with UDP-[\(^{14}\)C]Glc or UDP-[\(^{14}\)C]Gal without addition of a lipophilic acceptor. After incubation, the lipids were extracted and separated by TLC followed by radioscanning. In control assays with membranes prepared from untransformed P. pastoris, no radioactivity was detected in lipids. In the assay performed with membranes from transformed cells and UDP-[\(^{14}\)C]Glc, five components were labelled. On the basis of reference compounds, they were tentatively identified as \(\beta\)GlcD, \(\beta\)GalD, two different species of \(\beta\)GlcCer and \(\beta\)Glc\(\beta\)GalD (Figure 6). The appearance of \(\beta\)GalD may be explained by the presence of residual epimerase activity in the membrane fraction by which part of the UDP-[\(^{14}\)C]Glc was converted to UDP-[\(^{14}\)C]Gal. In the corresponding assay carried out with UDP-[\(^{14}\)C]Gal, significantly more radioactivity was incorporated into glycolipids, but a similar set of compounds was labelled. This time galactolipids were clearly dominating, whereas glucolipids were hardly labelled. On the basis of their chromatographic behaviour the major components labelled with UDP-[\(^{14}\)C]Gal were tentatively identified as \(\beta\)GalD, \(\beta\)GalCer, \(\beta\)Glc\(\beta\)GalD, and \(\beta\)Gal\(\beta\)GalD.

A second series of assays were performed using the membrane fraction prepared from E. coli, which were incubated with the fluorescent ceramide (\(\delta\)-erythro-[\(\beta\]-amino-\(N\)-4´ (7-nitrobenzo-2-oxa-1,3-diazolo)-hexanoyl]-ceramide) (NBD-Cer) and either unlabelled UDP-Glc or UDP-Gal (Figure 6B). After incubation, the lipophilic compounds were separated by TLC and their fluorescence was determined. Only two reaction products were detected which were tentatively identified as glucosyl- and galactosyl-NBD-Cer.

The preferred in vitro-labelling of galactolipids by the Gcs of A. tumefaciens is in line with the observation that in lipid extracts from A. tumefaciens expressing this sequence more galacto- than the corresponding glucolipids were found (Figure 5). Therefore, in vivo and in vitro data suggest that the Gcs from A. tumefaciens can use both sugar nucleotides, but UDP-Gal is significantly preferred.

For a confirmation of these conclusions, we performed kinetic measurements with the Gcs sequence from A. tumefaciens expressed in E. coli to determine the affinity of the enzyme for the two sugar nucleotides. Normally, the simplified determination of the apparent affinity for UDP-Glc and UDP-Gal is carried out in the presence of excess acceptor substrate. Because membrane preparations were used as enzyme source and neither DAG nor ceramide can be introduced into the assay mixture without the use of detergents, we used a further simplified approach by just varying the concentration of the sugar nucleotide in the presence of constant quantities of membrane protein and thus also constant, but most likely not saturating lipophilic acceptor. This approach resulted in apparent \(K_M\) and \(V_{max}\) values which can only be used for comparative purposes. The membrane fraction was prepared from cells of E. coli C41(DE3) expressing the Gcs from A. tumefaciens. It was used for in vitro enzyme assays in the presence of different concentrations of either UDP-[\(^{14}\)C]Gal or UDP-[\(^{14}\)C]Glc. First, analysis of the reaction products by TLC showed that galactolipids were formed (data not shown), which were identical to the products of the assay performed with A. tumefaciens cells overexpressing the Gcs sequence (Figure 5). Subsequently, the incorporated total radioactivity from additional assays was measured by scintillation counting. In the experiments with UDP-Gal, sufficient radioactivity was incorporated into lipids, whereas in the assays with UDP-Glc, performed under the same conditions, the measured values were too low to be used for a reliable evaluation. With
UDP-Gal typical Michaelis-Menten-like kinetics were observed (data not shown). Substrate concentrations at \(V_{\text{max}}/2\) between 40 and 60 \(\mu\text{M}\) UDP-Gal were calculated with \(V_{\text{max}}\)-values ranging between 30 and 40 pmol/min/mg. These experiments show that the Gcs enzyme expressed in \textit{E. coli} has a significantly higher affinity for UDP-Gal.

**Discussion**

The Gcs enzymes from \textit{A. tumefaciens} and \textit{M. loti} are the first bacterial representatives of the GT21 family which have been characterized experimentally. Despite a significant sequence similarity to other members in GT21, both Gcs enzymes from \textit{A. tumefaciens} and \textit{M. loti} differ from known GlcCer synthases in three features. These are sugar acceptor specificity, sugar donor specificity, and “processivity,” which will be discussed in the following. The different glycosylation alternatives and the resulting products are summarized in Figure 4.

Gcs enzymes from animals, fungi, and plants transfer a sugar moiety to ceramide, whereas the Gcs enzymes from the two bacteria use DAG as acceptor molecule. This finding may be explained by the fact that DAG and ceramide are structurally similar (Jorasch et al., 2000), that \textit{A. tumefaciens} and \textit{M. loti} apparently do not contain ceramides and that broad sugar acceptor specificity regarding DAG and ceramide is a common feature of many other glycosyltransferases (Jorasch et al., 1998, 2000). The overexpression of the human and the bacterial Gcs enzymes in \textit{P. pastoris}, which contains both DAG and ceramide, revealed this broad specificity concerning the glycosyl acceptor. While expression of the human Gcs resulted in the glycosylation of mainly ceramides with lower proportions of glycosylated DAG (Leipelt et al., 2001), we here could demonstrate that the Gcs from \textit{A. tumefaciens} synthesized predominantly glycosydiaicylglycerols and lower proportions of glycosylceramides. A further erosion of acceptor specificity is typical for another Gcs, cloned from cotton, which on overexpression produces glycosylceramides and sterol glycosides (Hillig et al., 2003). In conclusion, many Gcs enzymes from different species exhibit broad acceptor specificity, but the Gcs from \textit{A. tumefaciens} and \textit{M. loti} differ from Gcs enzymes of animals, fungi, and plants by their preference of DAG over ceramide (Table II).

The second differing feature is the sugar-donor specificity. While all eukaryotic Gcs use UDP-Glc as sugar donor, the two bacterial glycosyltransferases favour UDP-Gal over UDP-Glc (Table II). In vitro enzyme assays have shown that the \(K_m\) of mammalian Gcs for UDP-Glc is at least 200 times lower than for UDP-Gal (Sprong et al., 1998; Wu et al., 1999). Because eukaryotic Gcs activity always results in the synthesis of GlcCer but not of galactosylceramide in vivo, these enzymes are referred to as glucosyltransferases (Ichikawa et al., 1996; Sprong et al., 1998; Wu et al., 1999; Leipelt et al., 2001). Although we were not able to determine the \(K_m\) of the bacterial Gcs for UDP-Glc, we demonstrated by in vitro assays that the enzymes transfer both galactosyl and glucosyl moieties with a pronounced preference for galactose. These in vitro data are reflected by the isolation of both galactosylated and glucosylated products from hosts expressing the bacterial Gcs enzymes. The synthesis of, for example, \(\beta\text{Glc\betaGalID}\) by the \textit{A. tumefaciens} Gcs in its natural host justifies that this Gcs is referred to as a galactosyl/glucosyltransferase.

Relaxed substrate specificity of an enzyme concerning donor and acceptor is an interesting phenomenon in terms of the reaction mechanism and the structure of its reaction centre which is not the subject of our present work. In addition, this characteristic may have consequences for the biological functions of the enzyme. For most prokaryotic organisms, inaccurate recognition of the sugar acceptor by the Gcs enzyme may not be relevant, because they contain only DAG. In contrast, eukaryotic organisms contain both DAG and ceramide, which not only serve as backbone for membrane lipid biosynthesis, but each also being involved in different signalling cascades. Therefore, eukaryotes would be expected to have Gcs enzymes with strict acceptor specificity, or there should be precautions for a spatial separation between the enzyme and “unwanted” substrates. This assumption is generally applicable to other enzymes acting on DAG and ceramide. Because many of these eukaryotic enzymes apparently do not show strict specificity (Jorasch et al., 1998; Leipelt et al., 2001; Tadano-Aritomi and Ishizuka, 2003), it seems likely that in most of the cases the enzymes are specifically targeted to intracellular membrane systems to confine their contact to the “desired” substrates.

In contrast to relaxed specificity for the sugar acceptor, most glycosyltransferases show a strict specificity for the sugar donor. Eukaryotes, for example, use two different enzymes to synthesize glucosyl- and galactosylceramide, respectively (Schulte and Stoffel, 1993; Ichikawa et al., 1996). Thus, the transfer of alternative sugar moieties to the acceptor, in particular the transfer of glucosyl or galactosyl residues by bacterial Gcs enzymes as demonstrated by our data, is one of the few exceptions to this rule. Another exception is the \(\alpha\text{-N-acetyllactosaminyltransferase}\) of animal tissues catalyzing the transfer of both \(N\)-acetylgalcosamine and \(N\)-acetylgalactosamine (Lind et al., 1998; Pedersen et al., 2003). An even more interesting representative of these exceptions is the glycosyltransferase LpsB of \textit{Sinorhizobium mellotii}—whose natural activity may be the transfer of glucose—with the ability to complement a mutant of \textit{Rhizobium leguminosarum} defective in the orthologous and highly selective mannosyltransferase LpeC (Kaniajes et al., 2003). In this case, the unspecificity in substrate acceptance does not only involve two epimeric sugar moieties (glucose and mannose), but most likely also different nucleotide portions (UDP and GDP).

It is generally assumed that given glycosyl moieties play specific roles in glycolipids, glycoproteins and polysaccharides which cannot be fulfilled by different sugar moieties. Blood group factors are one of the many examples which confirm this assumption. However, concerning simple glycosphingolipids and other glycolipids there are only very few data regarding the specific functions of particular sugar moieties. It is, for example, still unclear whether the functions of common glycolipids such as \(\beta\text{-GlcCer}, \text{sterol-}\beta\text{-glucoside}, \beta\text{-galactosylceramide}, \beta\text{-galactosyldiacylglycerol, and } \alpha\text{-galactosyl-(1\rightarrow6)}\beta\text{-galactosyldiacylglycerol could be fulfilled by corresponding lipids with a different glycosyl...
moiety or with an identical sugar moiety of different anomic structure. This in turn would provide evolutionary pressure on maintaining or widening substrate specificities including the chance to develop new characteristics.

The third differing feature is “processivity.” In this context, “processivity” means the successive transfer of glycosyl residues to a lipid acceptor in the first step and to glycolipids with a growing glycan chain in the following steps. We could however not demonstrate that the successive addition of sugar residues occurred without dissociation of the enzyme-acceptor complex. In all cases which lack evidence for such a mechanism we therefore marked the term “processive” by inverted commas. While eukaryotic members of GT21 exclusively synthesize monoglycosylated lipids, the bacterial Gcs successively transfer one or two additional sugar moieties in β-1→6-linkage to the monoglycosyl diacylglycerol. In agreement with the “processivity” of bacterial Gcs enzymes, their sequences contain a D1,D2,D3(Q/R)XXRW motif, which was previously identified as a characteristic feature of processive β-glycosyltransferases of GT2 such as cellulose synthase or chitin synthase (Saxena et al., 1995). Interestingly, the nonprocessive eukaryotic members of GT21 exhibit a more or less complete D1,D2,D3(Q/R)XXRW motif as well (Table II) (Leipelt et al., 2001), which is essential for enzymatic activity (Marks et al., 2001). Therefore, it can be assumed that this motif is required but not sufficient for “processivity” of the bacterial GT21 glycosyltransferases.

“Processivity” is a common feature of other lipid glycosyltransferases, for example from Bacillus subtilis and S. aureus. Their GT28 glycosyltransferases (YfpP) synthesize βGlcpD, βGlcpβGlcpD, and βGlcpβGlcpβGlcpD which serve as membrane lipids and glycolipid anchors of lipoteichoic acids (Jorash et al., 1998, 2000; Kiriukhin et al., 2001). Plants contain a hypothetical “processive” galactosyltransferase activity which may form the series of polygalactolipids up to a penta-galactosyl diacylglycerol (Heinz, 1996; Kelly et al., 2003; Xu et al., 2003). Several higher glycosylated derivatives of ceramide, DAG, and sterols occur in plants and fungi, which may suggest the existence of additional, but so far unknown “processive” lipid glycosyltransferases (Heinz, 1996; Sterling et al., 2004). Despite the ubiquitous occurrence of products of “processive” lipid glycosyltransferases, the function of their products are mainly unknown except for the hypothesis that sitosterol cellodextrins serve as precursors for cellulose biosynthesis in plants (Peng et al., 2002).

In this work we have characterized in vivo and in vitro activities of the bacterial Gcs, but the biological functions of these glycosyltransferases in A. tumefaciens and M. loti are still not clear. Deletion of the gcs+ gene in A. tumefaciens was not lethal, and the mutant cells grew like the wild type under the given laboratory conditions. Although the gcs+ promoter is active, the corresponding glycosyltransferase products of wild type cells could not be detected. Therefore, we conclude that only trace amounts of Gcs products are synthesized in A. tumefaciens, which are below the limits of our detection system. Such proportions, however, may be sufficient to fulfill biological functions, for example in signalling chains. Another possibility is, that expression of the gcs+ gene is very low only under laboratory conditions, but may increase under changed conditions (e.g., stress, plant-infection). This could lead to the synthesis of considerable amounts of glycolipids which may fulfill their function as membrane components or serve as precursors, for example, cell wall polymers.

In this context it should be mentioned that some Rhizobiales and related species change their lipid composition under low oxygen stress which occurs in culture or in symbiosis with plants. For example, Bradyrhizobium accumulates phosphatidyl inositol (Tang and Hollingsworth, 1998), whereas S. mellioti and Rhodobacter sphaeroides respond to phosphate limiting conditions by the synthesis of phosphate-free membrane lipids (Benning et al., 1995; Geiger et al., 1999). During plant–microbe interaction, R. leguminosarum accumulates diglycosyl diacylglycerol which in turn elicits various symbiosis-relevant morphological changes of the host plant (Orgambide et al., 1994). In view of these data, the identification of the Gcs activities of A. tumefaciens and M. loti provides a basis for further studies on glycolipid functions in parasitic or symbiotic bacteria.

Materials and methods

Bacterial and yeast strains, growth and recombinant DNA techniques

Escherichia coli strains XL1-Blue (MR+) (Stratagene, La Jolla, CA) and C41(DE3) (Mioux and Walker, 1996) were routinely grown aerobically at 37°C in Luria-Bertani medium (Sambrook et al., 1989). Ampicillin (100 mg·L⁻¹), kanamycin (30 mg·L⁻¹), or chloramphenicol (30 mg·L⁻¹) were included for growth of plasmid-bearing cells. Agrobacterium tumefaciens strain ATHVC58C1, a derivative from the strain EHA 101 (Hood et al., 1986), which was kindly provided by Dr. J. Dettendorfer (KWS, Einbeck, Germany) was grown at 28°C in YEP (10 g·L⁻¹ peptone, 10 g·L⁻¹ yeast extract, 5 g·L⁻¹ NaCl, pH 7.2) in the presence of rifampicin (80 mg·L⁻¹). Kanamycin (50 mg·L⁻¹) or chloramphenicol (50 mg·L⁻¹) was included for growth of plasmid-bearing cells. Streptomycin (300 mg·L⁻¹) and spectinomycin (100 mg·L⁻¹) or kanamycin (50 mg·L⁻¹) were included to select A. tumefaciens after homologous recombination. The yeast strain used in this study was P. pastoris JC 308 Δgcs/Δgus51BI (Hillig et al., 2003), grown at 30°C in YPG medium (10 g·L⁻¹ yeast extract, 20 g·L⁻¹ peptone, 10 mL·L⁻¹ glycerol). For gene expression driven by the AOX1 promoter, 0.5% methanol was added to the growth medium. The vectors pET24d(+) (Novagen, Madison, WI), pUC18 (Yanish-Perron et al., 1989), pBluescript (Stratagene), pTrcHis2C (Invitrogen, Carlsbad, CA), pCamb2200 (Cambia, Canberra, Australia), pLH7000 (Hausmann and Toepfer, 1999), pFP1-3 (Götze et al., 1999), pEsay24 (Jorash et al., 2000), and pPIC3.5 (Invitrogen) were used for cloning. Standard methods were followed for DNA isolation, restriction endonuclease analysis, and ligation (Sambrook et al., 1989).

Cloning of the Gcs from Agrobacterium tumefaciens and Mesorhizobium loti

The ORF sequences of the gcs+ genes from A. tumefaciens and M. loti were amplified from genomic DNA by PCR.
using the specific oligonucleotide primer pairs 1F/1R or 2F/2R, respectively (Table III of supplementary data which also lists all other primers). Pfu polymerase (Stratagene) was used for the amplification of the 1164 bp and 1152 bp products containing the entire Gcs ORF sequences of \textit{A. tumefaciens} and \textit{M. loti}. These amplicons were inserted into a SmaI-linearized pUC18 vector resulting in pUCAGRO and pUCMESO which were used for transformation of \textit{E. coli} XL1Blue cells. Exact in-frame cloning and identity of the PCR-cloned fragments were confirmed by sequencing. The PCR fragment corresponding to the gcs\textsuperscript{+} gene of \textit{A. tumefaciens} was also cloned into pPIC3.5/SmaBI resulting in pPICAGRO to be used as expression vector in \textit{P. pastoris} JC 308 Δgcs/Δugt51B1.

For expression in \textit{E. coli} C41(DE3) the \textit{A. tumefaciens} gcs\textsuperscript{+} ORF sequence was amplified with the primers 3F and 3R using pUCAGRO as template. After subcloning into pUC18 the insert was released with NcoI/BamHI and ligated with the NcoI/BamHI-linearised expression vector pET24d.

To provide the Gcs nucleotide sequences of \textit{A. tumefaciens} and \textit{M. loti} with the restriction sites AvrII and BamHI, they were amplified with the specific oligonucleotide primers 8F and 8R using pUC18 as template. After subcloning into pUC18 the insert was released with NcoI/BamHI and ligated with the NcoI/BamHI-linearised expression vector pET24d. The vector pBa1saySSa2 was used to disrupt the gcs\textsuperscript{+} gene from \textit{A. tumefaciens} with simultaneous insertion of the heterologous glycosyltransferase from \textit{S. aureus} (ugt106B1) behind the native gcs\textsuperscript{+} promoter of \textit{A. tumefaciens} (Figure 2D). For this purpose, about 600 bp at the 5'-end of the gcs\textsuperscript{+} gene sequence were replaced by the replacement of the heterologous glycosyltransferase from \textit{S. aureus} together with a streptomycin/spectinomycin resistance (Sm\textsuperscript{R}/Sp\textsuperscript{R}) cassette. The \textit{S. aureus} glycosyltransferase was inserted exactly at the locus of the replaced gcs\textsuperscript{+} ORF.

The vector pBa1saySSa2 was constructed in several steps with successive integration of four fragments beginning with the linearised vector pBluescript (KpnI/EcoRV). These fragments were released from p18agro1 (KpnI/EcoRV; left flanking sequence), p18Staph (EcoRV/SmaI; heterologous glycosyltransferase), p18SS (SmaI/NotI; Sm\textsuperscript{R}/Sp\textsuperscript{R}-cassette), and p18agro2 (NotI/SacI; right flanking sequence). The vector pBa1saySSa2 resulted from the ligation of pUC18 (SmaI) with the 2100 bp flanking sequence upstream of the gcs\textsuperscript{+} gene of \textit{A. tumefaciens} which was amplified from genomic DNA with the primers 4F and 4R. A 600 bp sequence corresponding to the 3'-end of the gcs\textsuperscript{+} gene was amplified with the primers 5F and 5R and ligated with pUC18 (SmaI) resulting in the vector p18agro2. The “processive” glycosyltransferase from \textit{S. aureus} was amplified with the primers 6F and 6R using the vector pEsay2 as template and ligated with pUC18 (SmaI) resulting in the vector p18Staph. The Sm\textsuperscript{R}/Sp\textsuperscript{R}-cassette was amplified from pLH7000 with the primers 7F and 7R and inserted into pUC18 (SmaI) giving p18SS. For transformation, the vectors pBa1na5 and pBa1saySSa2 were digested with KpnI/SacI to release the linearised transformation constructs. They were used for transformation of competent \textit{A. tumefaciens} cells by electroporation. Kanamycin- or streptomycin/spectinomycin-resistant transformants were selected by growth on YEP plates containing the appropriate antibiotics.

Replacement of the wild type gcs\textsuperscript{+} gene sequence was monitored by PCR with Taq DNA Polymerase (NEB) or Herculase\textsuperscript{R} Enhanced DNA Polymerase (Stratagene) (data not shown). To check the deletion of the gcs\textsuperscript{+} gene and its replacement by the Kan\textsuperscript{R}-cassette the following primer pairs were used (Figure 2B–D, Table III): 12F/16R— to check the correct insertion at the 5'-end; 15F/15R— to check the correct insertion at the 3'-end; 12F/15R— to check the complete replacement comprising the region from the 5'- to 3'-end. The replacement by the ugt106B1 sequence from \textit{S. aureus} plus the Sm\textsuperscript{R}/Sp\textsuperscript{R}-cassette was examined by the following primer pairs: 12F/12R (5'-end); 13R/13F (3'-end); 14F/14R (complete replacement, 5'- to 3'-end).
Heterologous expression of the Gcs ORF sequences from Agrobacterium tumefaciens and Mesorhizobium loti in different hosts

Escherichia coli XL1-Blue (MRF') was used as expression host for the vectors pTrcHis2 C, pTnagro and pTnmeso, whereas E. coli C41(DE3) was used as expression host for the vectors pET24d and pETagro. Cultures of E. coli were grown overnight at 37°C. Expression cultures were started at OD600 = 0.05 and grown to an optical density of 0.8. Induction was performed by adding 0.4 mM isopropyl thio-

β-D-galactoside and further incubation for 4 h at 37°C. Cells were collected by centrifugation (15 min, 5000 g). Pichia pastoris JC 308 Δgcs/ΔgtsIB1 cells were grown at 30°C in YPG medium to an OD600 between 1 and 2. Expression was driven by the strong AOX1 promoter and induced by addition of 0.5% methanol to the medium. Cells were harvested by centrifugation (10 min, 5000 g) 20 h after induction. A. tumefaciens cells were used as expression host for the vectors pTnVagro and pTnVmeso. The cultures were grown at 28°C to an OD600 = 1.0 and induced by adding 0.4 mM isopropyl-thio-

β-D-galactoside and further incubation for 15 h at 28°C. Cultures of A. tumefaciens wild type and knock out mutants, including mutants containing the gene of the heterologous glucosyltransferase of S. aureus, were grown at 28°C to an OD600 = 2.0 without induction. The A. tumefaciens cells were harvested by centrifugation (30 min, 8000 g).

Enzymatic assays of glycosyltransferase activities

For enzymatic assays, the cell pellets recovered from 25 mL of the E. coli, A. tumefaciens, or P. pastoris expression cultures were resuspended in 0.5 mL of buffer 1 (50 mM Tris–HCl, pH 8.0; 7%, v/v, glycerol). All subsequent steps were carried out at 4°C. The E. coli or A. tumefaciens cells were disrupted by ultrasonication (eight times for 10 s). The P. pastoris cells were disrupted by adding 2–3 g of glass beads (0.4 mm diameter) and vortexing for 30 s followed by cooling on ice for 30 s. This procedure was repeated 10 times. Cell debris were removed by centrifugation (1 min, 2800 g). Supernatant fractions were centrifuged at 100,000 g for 30 min, and the sedimented membrane fraction was resuspended in 400 μL (E. coli or A. tumefaciens) or 800 μL (P. pastoris) of buffer 2 (50 mM Tris–HCl, pH 7.6; 7%, v/v, glycerol).

The actual assays were performed in a final volume of 100 μL of buffer 2. Radioactive assays were supplied with UDP-[14C]Glc (150,000 dpm, specific activity 10 GBq/
mol, final concentration 2.5 μM) or UDP-[14C]Gal (150,000 dpm, specific activity 10.9 GBq/mmol, final concentration 2.3 μM). Assays with NBD-Cer (Matreya, Pleasant Gap, PA; final concentration 0.01 μg/μL) were supplied with unlabelled UDP-Glc or UDP-Gal (each in a final concentration of 500 μM). Each assay was supplied with 50 μL of the resuspended membrane fraction and incubated for 30 min at 30°C. The reaction was terminated by the addition of 3 mL chloroform/methanol (2:1, v/v) and 0.75 mL of 0.45% NaCl solution (w/v). The extracted lipids were separated by TLC. Radioactivity on TLC plates was detected by radioscanning with a BAS-1000 BioImaging Analyzer (Raytest, Straubenhardt, Germany). NBD-Cer fluorescence on TLC plates was scanned using an AlphaDigiDoc™ Gel Documentation & Image Analysis System (Alpha Innotech Corporation, San Leandro, CA).

Lipid extraction and analysis

Expression cultures of E. coli, P. pastoris, and A. tumefaciens were grown and harvested as described above. The sedimented cells were boiled for 10 min in water and centrifuged again. Lipid extraction was performed with chloroform/methanol 1:2 (v/v) and chloroform/methanol 2:1 (v/v). The lipid extract was washed by Folch partitioning (Folch et al., 1957) (CHCl₃:methanol:0.45% NaCl solution, 2:1:0.75), and the organic phase was evaporated. The residue was dissolved in CHCl₃ and fractionated by chromatography on SPE SI-1 columns (Phenomenex, Torrance, CA). Neutral lipids were eluted with chloroform, the glycolipid fraction was obtained with acetone/isopropanol 9:1 (v/v), and phospholipids were eluted with methanol. These fractions were subjected to analytical and preparative TLC separations using CAMAG Automatic TLC Sampler 4 (CAMAG, Muttenz, Schweiz) for spotting. Glycolipids were visualized by spraying with α-naphthol/sulphuric acid and subsequent heating to 160°C. Preparative separations were performed by TLC on silica gel 60 (Merck, Darmstadt, Germany). The solvents used to separate the different glycolipids are given in Table I of supplementary data. Lipids were visualized under UV light after spraying with ANS solution (8-anilino-1-naphthalenesulfonic acid ammonium salt, 0.2%, w/v in methanol). For NMR spectroscopy and mass spectrometry (MS), the purified glycolipids were acetylated (with acetic anhydride in pyridine, 1:1) overnight at room temperature and subjected to repurification by preparative TLC in diethyl ether.

Structural analysis

Compositional analysis. Fatty acids in the glycolipids were methanolyzed with 0.5 M HCl in methanol at 85°C for 45 min. After removal of the solvent, the products were per-acetylated with acetic anhydride in pyridine (1:1.5, v/v, 85°C, 20 min). For analysis of the sugar components glycolipids were methanolyzed under stronger conditions (2 M HCl in methanol at 85°C for 16 h), then hydrolyzed with aqueous 4 M CF₃CO₂H at 100°C for 2 h, conventionally reduced with borohydride and peracetylated (Sawardeker et al., 1965).

Methylation analysis. Purified glycolipids were methylated with CH₃I in dimethyl sulfoxide in the presence of solid NaOH (Ciucanu and Ke rek, 1984) and subsequently hydrolyzed with 2 M CF₃CO₂H (100°C, 2 h). The partially methylated monosaccharides were reduced with borohydride, peracylated, and analyzed by GLC and GLC-MS as described below.

GLC-MS analysis. The sugar and fatty acid derivatives were analyzed by GLC on a Hewlett-Packard HP 5890 Series II chromatograph, equipped with a 30-m fused-silica SPB-5 column (Supelco, St. Louis, MO) using a temperature gradient of 150°C (3 min) →320°C at 5°C/min, and GLC-MS on a Hewlett-Packard HP 5989A instrument equipped with a 30-m HP-5MS column (Hewlett-Packard, Palo Alto, CA) and operated under the same conditions (Zähringer et al., 1997).
**ESI FT-ICR MS**. High resolution FT-MS was performed in the positive ion modes using an APEX II–instrument (Bruker Daltonics, Billerica, MA) equipped with an actively shielded 7 Tesla magnet and an (nano) ESI ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. Samples were dissolved in a 50:50:0.03 (v/v/v) mixture of 2-propanol, water, 30 mM ammonium acetate adjusted with acetic acid to pH 4.5 at a concentration of 10 ng·µL⁻¹. The samples were sprayed at a flow rate of 2 µL·min⁻¹, and the drying gas temperature was set to 150°C. Each spectrum represents an average of at least 20 transients composed of 1 M data points.

Proton (¹H) nuclear magnetic resonance spectroscopy. All one- (1D) and two- (2D) dimensional homonuclear ¹H-NMR spectra were recorded at 600 MHz (Bruker Avance DRX 600, Bruker Instruments, Billerica, MA). Before the measurements, the purified per-O-acetylated glycolipids (25–500 µg) were exchanged twice from ²HClCl₃ (99.8%D, Aldrich, St. Louis, MO). All 1D and 2D ¹H-NMR spectra (COSY, RELAY, TOCSY) were recorded in 3 mm microtubes (Kontes, Vineland, NJ) in ²HClCl₃ (99.96%, Eurisotop, Gil-surr-Yvette, Saint-Aubin France) at 300 K. Chemical shifts were referenced to internal chloroform (δH = 7.260 ppm). Bruker standard software XWINNMR 3.5 was used.

Supplementary data are available at Glycobiology online (http://glycob.oupjournals.org/).

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**Abbreviations**

DAG, diacylglycerol(s); ESI FT-ICR MS, electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry; Gcs, glucosylceramide synthase; GLC-MS, combined gas–liquid chromatography/mass spectrometry analysis; GT21, glycosyltransferase family 21; MS, mass spectrometry; NBD-Cer, d-erythro-β-[6-amino-β-(7-nitrobenzo-2-oxaizin-1-3-diazolo)]-hexanoyl-ceramide; NMR, nuclear magnetic resonance; ORF, open reading frame; PCR, polymerase chain reaction; TGD, triglycosyl diacylglycerol; THF, tetrahydrofuran; TLC, thin layer chromatography; UDP-Gal, uridine diphosphogalactose; UDP-Glc, uridine diphosphoglucose.

**References**


