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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 (glucosylceramide synthases [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 and heterologous expression of GT21 enzymes from *Agrobacterium tumefaciens* and *Mesorhizobium loti* in *A. tumefaciens*, *Escherichia coli*, and the yeast *Pichia pastoris*. Glycolipid analyses of the transgenic organisms revealed that the bacterial glycosyltransferases are involved in the synthesis of mono-, di- and even tri-glycosylated glycolipids. As products resulting from their activity, we identified 1,2-diacyl-3-(*O*-β-D-*galacto*-pyranosyl)-*sn*-glycerol, 1,2-diacyl-3-(*O*-β-D-*gluco*-pyranosyl)-*sn*-glycerol as well as higher glycosylated lipids such as 1,2-diacyl-3-[*O*-β-D-*galacto*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol, 1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol, 1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→6)-*O*-β-D-*gluco*-pyranosyl]-*sn*-glycerol, and the deviatingly linked diglycosyldiacylglycerol 1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→3)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol. From a mixture of triglycosyldiacylglycerols, 1,2-diacyl-3-[*O*-β-D-*galacto*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol could be separated in a pure form. In vitro enzyme assays showed that the glycosyltransferase from *A. tumefaciens* favours uridine diphosphogalactose (UDP-Gal) over UDP-Glc. In conclusion, the bacterial GT21 enzymes differ from the eukaryotic ceramide glucosyltransferases by the successive transfer of up to three galactosyl and glucosyl moieties to diacylglycerol.

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

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Introduction

Glycoconjugates, in the plant and animal kingdom as well as in prokaryotic cells, are formed by the action of a multiplicity of different glycosyltransferases. At present, several thousands of these glycosyltransferases are classified into 77 distinct sequence-based families (Campbell *et al*., 1997; Coutinho *et al*., 2003; Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/CAZY/). The value of this classification continuously increases by the biochemical characterization of additional members of each of these glycosyltransferase families. Such characterization has been performed with many eukaryotic representatives of the glycosyltransferase family 21 (GT21) (Ichikawa *et al*., 1996; Ichikawa and Hirabayashi, 1998; Wu *et al*., 1999; Leipelt *et al*., 2001; Hillig *et al*., 2003; Kohyama-Koganeya *et al*., 2004). All GT21 genes studied so far from animal, fungal, and plant origin have been found to code for uridine diphosphoglucose (UDP-Glc):ceramide glucosyltransferase (synonymous with glucosylceramide synthase [Gcs], EC 2.4.1.80). The formation of glucosylceramide (GlcCer) represents the first glycosylation step in the biosynthesis of more complex glycosphingolipids which occur ubiquitously in cell membranes of eukaryotic organisms (Warnecke and Heinz, 2003).

Glycosphingolipids are also found in a few bacterial genera such as *Sphingobacterium* and *Sphingomonas* (Kawahara *et al*., 1991, 2000; Olsen and Jantzen, 2001). Therefore, it does not seem surprising that GT21 includes also members of bacterial origin. Most of these hypothetical proteins had been referred to 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 glycosylceramides 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 glycoglycerolipids, 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

of *A. tumefaciens* for the occurrence of glycosylceramides and glycoglycerolipids. 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 enzymes from fungi and plants showed similarities even as low as 9–21 % to the human Gcs (Leipelt *et al*., 2001).

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 D_1,D_2,D_3(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 D_1,D_2,D_3(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 glucosyltransferases. 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.

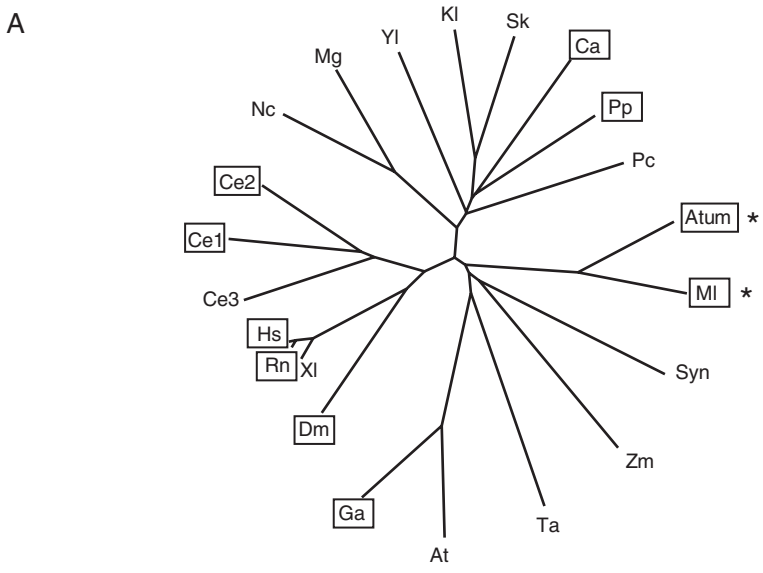
Agrobacterium tumefaciens *lacks detectable proportions of "conventional" glycolipids*

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 *ugt106B1* 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 βGlcβ GlcD (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 glycoglycerolipids,



B

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MGVELASMETVFALCAIMLLAFNLLGIVLAGWRLTRRDAENDLVRQKPPVSLVVPLRGVENFTPLTLSRAFGLDW
                D1                                    K124                    D2 G146
PDYELLFCVADEFDPVIAEVRKAAAAFPSVSMQLLIGDDRVSANPKLNNCVKGWRAAKYEWVILADSNVLMPKSY
                                                H193 R195          C207 G210          G224 G225
IATMMSAWRPDSGLVCSPPLGSRPVGFWANVECAFLNGSQGRWQYAAEAIGIGFAQGKSMLWNKPFLEDRGGIRA
          D3                                    (R/Q)XXRW
LAAEIAEDAASTKLVRSAGRKVHLVSAPFEQPLGQRHAGEIWSRQTRWARLRRVTFPQYFAPEILTGALPPLLFA
LAATAMMDVNLAATASVVLALMFGPELALAALNGWPVSLYTLPAMIARDLIMPVVWARSWIGSAVAWRGNVMTIG
TAESTLAGPPAE
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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 *Thermoplasma acidophilum* (Ta, CAC11705) and *Candida albicans* (Ca, protein CaO19.4592, EAL03927). 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), *Drosophila melanogaster* (Dm), *Gossypium arboreum* (Ga), *Homo sapiens* (Hs), *Kluyveromyces lactis* (Kl), *Magnaporthe grisea* (Mg), *Mesorhizobium loti* (Ml), *Neurospora crassa* (Nc), *Pichia pastoris* (Pp), *Pneumocystis carinii* (Pc), *Rattus norvegicus* (Rn), *Saccharomyces kluyveri* (Sk), *Synechocystis* (Syn), *Xenopus laevis* (Xl), *Yarrowia lipolytica* (Yl), *Zymomonas mobilis* (Zm). (**B**) Deduced amino acid sequence of the Gcs from *A. tumefaciens*. 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 D_1,D_2,D_3,Q/R*XX*RW 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*.

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* (Δ*gcs*/Δ*ugt51B1*; Hillig *et al.*, 2003), which is devoid of both sterol and ceramide glucosyltransferase activity. 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 (Figure 3). Six out of these had been described by us before (Jorasch *et al*., 1998, 2000; Leipelt *et al*., 2001; Sakaki *et al*., 2001) and, therefore, only data for the compounds not previously studied in our laboratory are given (Table IIa and IIb of the supplementary data). Aiming to identify the sugar specificities of the glycosyltransferases, we focussed on the glycosyl part structures, and only a few data for the aglycon lipid portions (DAG and ceramide) have been included (Table I). The expression of the Gcs from *A. tumefaciens* in *P. pastoris* induced the accumulation of six

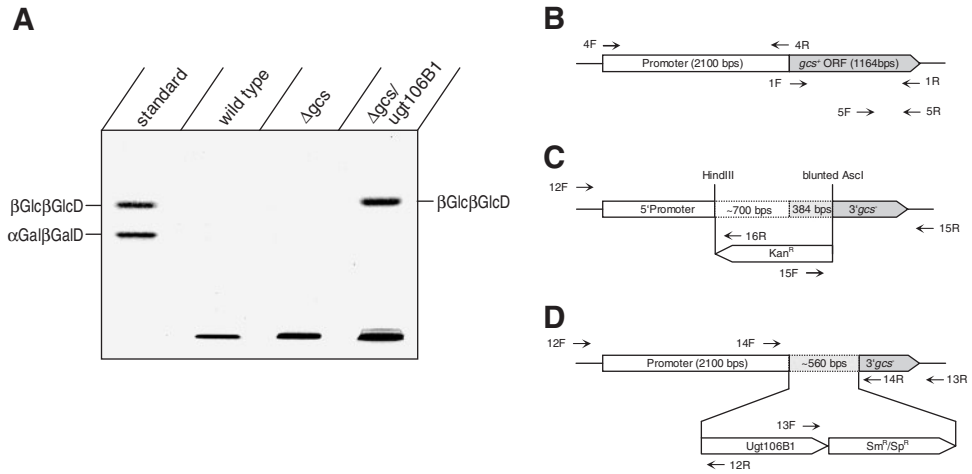

Fig. 2. (**A**) Glycolipid fractions from the wild type and from two knock-out mutants of *Agrobacterium tumefaciens*. The extracts of total lipids obtained from the strains were fractionated on Sep pak columns to yield the glycolipid fractions which were separated by thin layer chromatography (TLC) in hexane/tetrahydrofuran /isopropanol/H_2O 40:0.4:50:8. The wild type strain as well as the knock-out mutant with deleted gcs^+ sequence (Δgcs) did not contain any detectable glycolipids. Heterologous expression of a glucosyltransferase from *Staphylococcus aureus* (Δgcs/ugt106B1) under control of the gcs^+ promoter led to the synthesis of βGlcβGlcD. (**B**–**D**) Disruption of the gcs^+ gene in *A. tumefaciens* and expression of the heterologous glucosyltransferase from *S. aureus*. (**B**) Arrangement of the putative promoter region followed by the gcs^+ ORF found in the wild type. (**C**) The gene deletion was performed by insertion of a Kan^R-cassette at the locus of the gcs^+ gene by homologous recombination. The 5′ sequence of the promoter and the 3′ sequence of the gcs^+ ORF were used as flanking sequences to introduce the Kan^R-cassette leading to the deletion of 384 bps of the gcs^+ ORF and of about 700 bps of its adjacent promoter sequence. (**D**) The expression construct for the heterologous glucosyltransferase contained the Ugt106B1 sequence from *S. aureus* plus a Sm^R/Sp^R-cassette. The promoter sequence and the 3′ end of the gcs^+ ORF served as flanking sequences for the integration of the construct. The positions of primers are given, which were used to amplify genomic DNA fragments or to verify the correct insertion of the constructs as described in the material and methods section.

Table I. Glycolipids isolated from transformed hosts overexpressing the "processive" galactosyl/glucosyltransferases from *Agrobacterium tumefaciens* and *Mesorhizobium loti*

Abbreviation	Structure	Recent analytical data
βGlcD	1,2-diacyl-3-(*O*-β-D-*gluco*-pyranosyl)-*sn*-glycerol	Jorasch *et al*. (2000)
βGalD	1,2-diacyl-3-(*O*-β-D-*galacto*-pyranosyl)-*sn*-glycerol	Jorasch *et al*. (2000)
βGlcβGlcD	1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→6)-*O*-β-D-*gluco*-pyranosyl]-*sn*-glycerol	Jorasch *et al*. (1998, 2000)
βGlcβGalD	1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol	This study
βGalβGalD	1,2-diacyl-3-[*O*-β-D-*galacto*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol	This study
βGlcβGlcβGlcD	1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→6)-*O*-β-D-*gluco*-pyranosyl-(1→6)-*O*-β-D-*gluco*-pyranosyl]-*sn*-glycerol	Jorasch *et al*. (1998)
βGalβGalβGalD	1,2-diacyl-3-[*O*-β-D-*galacto*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl-(1→6)-*O*-β-D-*galacto*-pyranosyl]-*sn*-glycerol	This study, Kojima *et al*. (1990)
βGlc-(1→3)-βGlcD	1,2-diacyl-3-[*O*-β-D-*gluco*-pyranosyl-(1→3)-*O*-β-D-*gluco*-pyranosyl]-*sn*-glycerol	This study
βGlcCer-1	1-*O*-β-D-*gluco*-pyranosyl-ceramide	Sakaki *et al*. (2001)
βGlcCer-2	1-*O*-β-D-*gluco*-pyranosyl-ceramide	Toledo *et al*. (1999)
βGalCer	1-*O*-β-D-*galacto*-pyranosyl-ceramide	Toledo *et al*. (1999)

All the compounds listed have been isolated and structurally identified in the present work. But only data for those compounds are listed in Table II of supplementary data that have not been included in our previous studies of recombinant glycosyltransferases. Nearly all of the compounds listed above have been identified before in various organisms, but in the present context reexamination was required as structural proof going beyond chromatographic behaviour. The hydrophobic moieties (diacylglycerol and ceramide) are not detailed in the present context (Leipelt *et al*., 2001).

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 Gcs-annotation. The interpretation of the structural data resulted in the identification of two glucosyl- and one galactosylceramide (Figure 3, Table I).

The structures of the isolated glycolipids demonstrate that the expressed enzyme has an exceptionally broad substrate specificity regarding both glycosyl donor and lipophilic acceptor (Figure 4). As obvious from the different

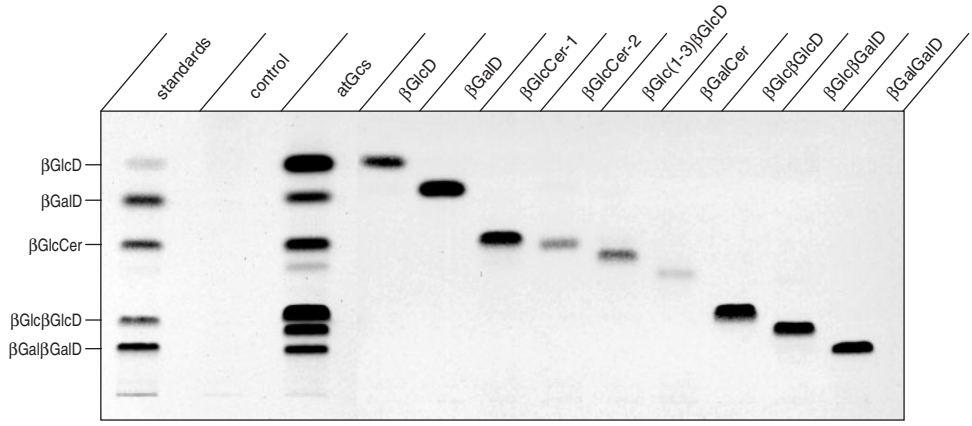

Fig. 3. Formation of glycosyldiacylglycerols and glycosylceramides in *Pichia pastoris* by expression of the Gcs sequence from *Agrobacterium tumefaciens*. The glycolipid fraction of a glycolipid-free double mutant of *P. pastoris* expressing the Gcs sequence from *A. tumefaciens* was analyzed by thin layer chromatography (TLC) in chloroform/hexane/tetrahydrofuran (THF)/isopropanol/methanol/H_2O, 35:35:0.35:40:5:4. In contrast to the control, the glycolipid fraction of the cells expressing the *A. tumefaciens* Gcs sequence (atGcs) contained a diversity of new glycolipids. The other lanes show the various glycolipids after purification by preparative TLC. All the components were subjected to detailed structural analysis.

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

Property	Bacteria (*Agrobacterium tumefaciens*)	Plants (*Gossypium arboreum*)	Fungi (*Candida albicans*)	Animals (Mammals)
Sugar acceptor	DAG > ceramide	Ceramide > sterol[a]	Ceramide	Ceramide > DAG[b]
Sugar donor	UDP-Gal > UDP-Glc	UDP-Glc	UDP-Glc	UDP-Glc >> UDP-Gal[c]
"Processivity"	Yes	No	No	No
Intracellular localization	?	ER[d]	?	Golgi apparatus[e]
$D1,D_2,D_3$(Q/R)XXRW	Present	Not present	Present	Present
Sensitivity to inhibitor EtDo-P4[f]	Resistant	Resistant	Resistant	Sensitive

DAG, diacylglycerol; UDP-Gal, uridine diphosphogalactose; UDP-Glc, uridine diphosphoglucose.
[a]Hillig *et al*., 2003.
[b]Gcs from *Homo sapiens* (Leipelt *et al*., 2001).
[c]Gcs from *Homo sapiens* and *Rattus norvegicus* (Sprong *et al*., 1998; Wu *et al*., 1999).
[d]O. Kusmakov and E. Heinz, unpublished results. ER, endoplasmic reticulum.
[e]Gcs from *Rattus norvegicus* (Futerman and Pagano, 1991; Marks *et al*., 1999).
[f]I. 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)

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, βGlcD and βGalD, 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 glucosyltransferase 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.

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

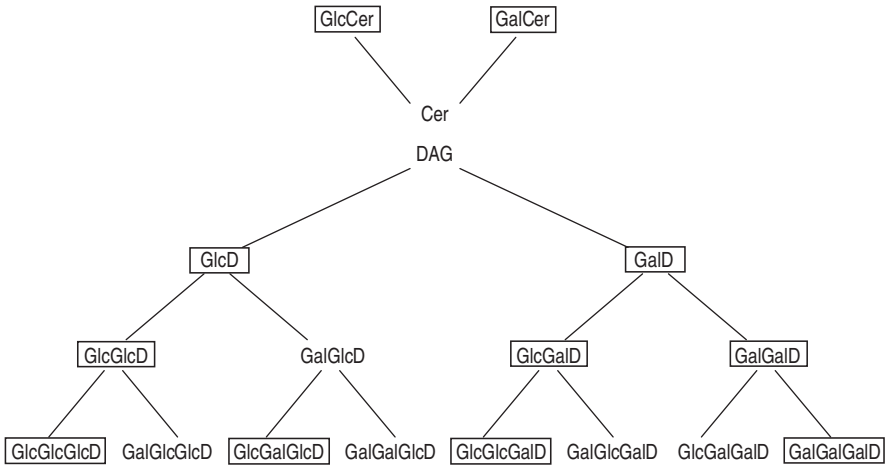

Fig. 4. Glycolipids that were formed by expression of the Gcs sequences from *Agrobacterium tumefaciens* and *Mesorhizobium loti* in pro- and eukaryotic hosts. Depending on the host used (*A. tumefaciens* or *Pichia pastoris*), diacylglycerol (DAG), and ceramide (Cer) can function as primary acceptors. Both Gcs enzymes operate with "processivity" and C4-epimeric unspecificity, but β-anomeric selectivity. Accordingly, β-linked glucosyl and galactosyl residues are transferred resulting in mono-, di- and triglycosyl headgroups in which glycosyl residues are interconnected by β(1→6)-glycosidic linkages. The compounds isolated and structurally identified in this work are framed. Not included is the βGlc(1→3)βGlcD which cannot be ascribed unequivocally to the expressed activity of the Gcs enzymes.

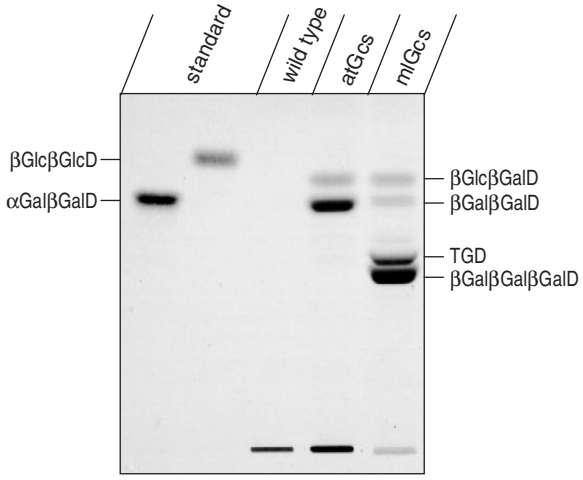

Fig. 5. Overexpression of the Gcs sequence from either *Agrobacterium tumefaciens* or *Mesorhizobium loti* in *A. tumefaciens* led to the synthesis of glycosyldiacylglycerols. Glycolipid fractions of *A. tumefaciens* cells were separated by thin layer chromatography (TLC) in hexane/tetrahydrofuran (THF)/isopropanol/H_2O, 40:0.4:50:10. Wild type cells did not contain detectable quantities of any glycolipids migrating in the solvent used. The expression of the Gcs sequence from *A. tumefaciens* (atGcs) led to the synthesis of βGlcβGalD and βGalβGalD, whereas the expression of the *M. loti* Gcs (mlGcs) 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 βGalβGalβGalD.

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 ^{1}H-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 monoglycosyldiacylglycerols 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 βGlcβGlcβGlcD (Jorasch *et al*., 1998) and other triglycosyldiacylglycerols 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 sequence from *A. tumefaciens* under the control of a strong promoter in this bacterium suggest that the formation of βGalβGalD and βGlcβGalD may represent the natural activity of this Gcs in *Agrobacterium*.

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

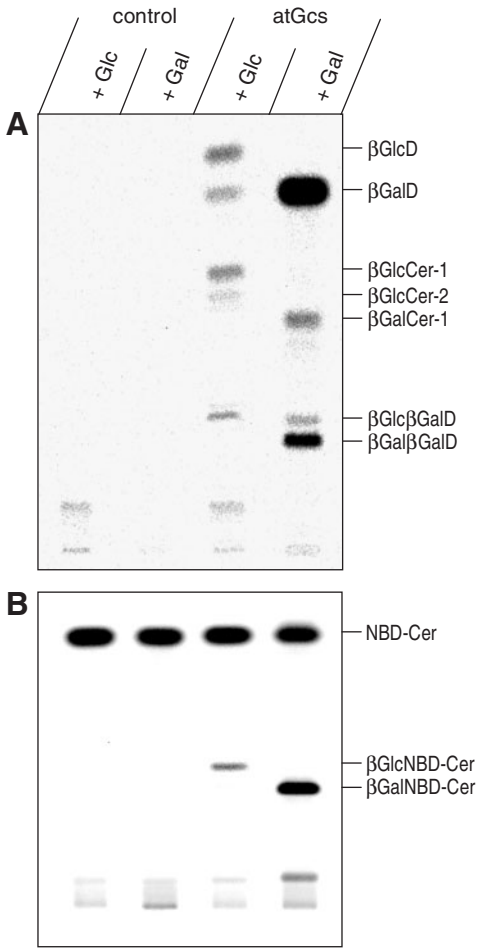

Fig. 6. In vitro synthesis of glycosyldiacylglycerols and glycosylceramides by membranes obtained from *Pichia pastoris* or *Escherichia coli* expressing the Gcs sequence of *Agrobacterium tumefaciens* (atGcs). (**A**) For Gcs expression a double null mutant of *P. pastoris* was used which lacks sterol glucosides and glucosylceramides. Membrane fractions were prepared and supplied with either UDP-[^{14}C]Glc or UDP-[^{14}C]Gal. On the basis of cochromatography with unlabelled standards, the glycolipids labelled by glucose (+ Glc) were tentatively identified as βGlcD, two species of βGlcCer (GlcCer-1, GlcCer-2), βGalD and βGlcβGalD. The appearance of glycolipids containing radioactive galactose can be ascribed to residual epimerase activity which converts uridine diphosphoglucose (UDP-Glc) to uridine diphosphogalactose (UDP-Gal). Supply of radioactive UDP-Gal (+ Gal) resulted in the formation of GalD, βGlcβGalD, βGalβGalD, and GalCer-1. The lipophilic compounds were separated by thin layer chromatography (TLC) in chloroform/hexane/tetrahydrofuran (THF)/isopropanol/methanol/H_2O, 35:35:0.35:40:5:4. The radioactivity was detected by radioscanning. (**B**) Membrane fractions of *E. coli* expressing the Gcs from *A. tumefaciens* were incubated with NBD-Cer and either UDP-Glc or UDP-Gal. The products glucosyl- and galactosyl-NBD-Cer were visualized under UV-light.

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 βGlcD, βGalD, two different species of βGlcCer and βGlcβGalD (Figure 6). The appearance of βGalD 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 βGalD, βGalCer, βGlcβGalD, and βGalβGalD.

A second series of assays were performed using the membrane fraction prepared from *E. coli*, which were incubated with the fluorescent ceramide (D-*erythro*-*N*[6-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 glycolipids 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 $V_{max}/2$ between 40 and 60 μM UDP-Gal were calculated with V_{max}-values ranging between 30 and 40 pmol/min/mg. These experiments show that the Gcs enzyme expressed in *E. coli* has a significantly higher affinity for UDP-Gal.

Discussion

The Gcs enzymes from *A. tumefaciens* and *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 *A. tumefaciens* and *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 *A. tumefaciens* and *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 *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 *A. tumefaciens* synthesized predominantly glycosyldiacylglycerols 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 *A. tumefaciens* and *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, βGlcβGalD by the *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 α-*N*-acetylhexosaminyltransferase of animal tissues catalyzing the transfer of both *N*-acetylglucosamine and *N*-acetylgalactosamine (Lind *et al*., 1998; Pedersen *et al*., 2003). An even more interesting representative of these exceptions is the glycosyltransferase LpsB of *Sinorhizobium meliloti*—whose natural activity may be the transfer of glucose—with the ability to complement a mutant of *Rhizobium leguminosarum* defective in the orthologous and highly selective mannosyltransferase LpcC (Kanipes *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 β-GlcCer, sterol-β-glucoside, β-galactosylceramide, β-galactosyldiacylglycerol, and α-galactosyl-(1→6)-β-galactosyldiacylglycerol could be fulfilled by corresponding lipids with a different glycosyl

moiety or with an identical sugar moiety of different anomeric 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 β1→6-linkage to the monoglycosyldiacylglycerol. In agreement with the "processivity" of bacterial Gcs enzymes, their sequences contain a D_1,D_2,D_3(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 D_1,D_2,D_3(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 glucosyltransferases (YpfP) synthesize βGlcD, βGlcβGlcD, and βGlcβGlcβGlcD which serve as membrane lipids and glycolipid anchors of lipoteichoic acids (Jorasch *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 pentagalactosyldiacylglycerol (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; Sperling *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 was active, the corresponding glycosyltransferase products of wild type cells could not been 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 fulfil 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 fulfil their function as membrane components or serve as precursors, for example, cell wall polymers.

In this context it should be mentioned that some Rhizobiaceae 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. meliloti* 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 diglycosyldiacylglycerol 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 symbiontic bacteria.

Materials and methods

Bacterial and yeast strains, growth and recombinant DNA techniques

Escherichia coli strains XL1-Blue (MRF′) (Stratagene, La Jolla, CA) and C41(DE3) (Miroux and Walker, 1996) were routinely grown aerobically at 37°C in Luria-Bertani medium (Sambrook *et al*., 1989). Ampicillin (100 $mg \cdot L^{-1}$), kanamycin (30 $mg \cdot L^{-1}$), or chloramphenicol (30 $mg \cdot L^{-1}$) 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 $g \cdot L^{-1}$ peptone, 10 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ NaCl, pH 7.2) in the presence of rifampicin (80 $mg \cdot L^{-1}$). Kanamycin (50 $mg \cdot L^{-1}$) or chloramphenicol (50 $mg \cdot L^{-1}$) was included for growth of plasmid-bearing cells. Streptomycin (300 $mg \cdot L^{-1}$) and spectinomycin (100 $mg \cdot L^{-1}$) or kanamycin (50 $mg \cdot L^{-1}$) were included to select *A. tumefaciens* after homologous recombination. The yeast strain used in this study was *P. pastoris* JC 308 Δ*gcs*/Δ*ugt51B1* (Hillig *et al*., 2003), grown at 30°C in YPG medium (10 $g \cdot L^{-1}$ yeast extract, 20 $g \cdot L^{-1}$ peptone, 10 $mL \cdot L^{-1}$ 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), pTrcHis2 C (Invitrogen, Carlsbad, CA), pCambia2200 (Cambia, Canberra, Australia), pLH7000 (Hausmann and Toepfer, 1999), pFP1-3 (Götz *et al*., 1999), pEsay24 (Jorasch *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 *A. tumefaciens* and *M. loti*. These amplicons were inserted into a SmaI-linearized pUC18 vector resulting in pUCAGRO and pUCMESO which were used for transformation of *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^+ gene of *A. tumefaciens* was also cloned into pPIC3.5/SnaBI resulting in pPICAGRO to be used as expression vector in *P. pastoris* JC 308 Δ*gcs*/Δ*ugt51B1*.

For expression in *E. coli* C41(DE3) the *A. tumefaciens* gcs^+ 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 *A. tumefaciens* and *M. loti* with the restriction sites AvrII and BamHI, they were amplified with the specific oligonucleotide primers 8F and 8R using pUCAGRO as template and 9F and 9R with pUCMESO as template. The purified PCR products were inserted into the SmaI-linearized pUC18 vector resulting in p18agro and p18meso. The inserts from the AvrII/BamHI-digested vectors p18agro and p18meso were ligated with the AvrII/BamHI-linearised expression vector pTnsyn3, resulting in pTnagro and pTnmeso. pTnsyn3, pTnagro and pTnmeso were originally used for the expression of different glycosyltransferases in cyanobacteria (unpublished data), but in this study pTnagro and pTnmeso were used for the Gcs expression in *E. coli* XL1-Blue. pTnsyn3 was created by the ligation of pTrcHis2 C (NcoI/HindIII) with the inserts from the vectors p18Nsyn3 released with NcoI/BamHI and p18BnptIIH released with BamHI/HindIII. p18Nsyn3 resulted from the ligation of pUC18 (SmaI) with the PCR product of a synthetic sequence that provided the restriction sites NcoI, AvrII and BamHI, the corresponding primers were 11F and 11R. p18BnptIIH bears a kanamycin resistance (Kan^R)-cassette which was amplified from pFP1-3 (Götz *et al*., 1999) with the primers 10F and 10R.

pTnVagro and pTnVmeso were used as expression vectors in *A. tumefaciens*. These vectors resulted from pTnagro and pTnmeso linearised with BstZ17I and ligated with the pVS1 rep sequence from pCambia2200 (BstZ17I/HincII).

Deletion of the genomic gcs^+ sequence from Agrobacterium tumefaciens *and expression of a heterologous glucosyltransferase in this mutant*

The vector pBa1na5 was used to disrupt the gcs^+ gene of *A. tumefaciens* by homologous recombination. The disruption was performed by replacement of the 5′-end of the gcs^+ gene sequence comprising about 385 bp plus the flanking sequence of about 700 bp upstream of the gcs^+ ORF sequence by a Kan^R -cassette (Figure 2C). The vector pBa1na5 is the ligation product of the vector pBa1n (EcoRV/SpeI) and the 5′-truncated ORF of the gcs^+ from *A. tumefaciens* (blunted at 5′-end and SpeI-digested at 3′-end). This fragment was generated by the digestion of the Gcs ORF in p18agro with AscI to cut off the 5′-end followed by blunting this restriction site by a fill-in reaction. The fragment was released with SpeI. pBa1 is the ligation product of pBluescript (KpnI/SalI) with two inserts which were the 3′-truncated flanking sequence released from p18agro1 (KpnI/HindIII) and the Kan^R-cassette from p18BnptIIH (HindIII/SalI).

The vector pBa1saySSa2 was used to disrupt the gcs^+ gene from *A. tumefaciens* with simultaneous insertion of the heterologous glucosyltransferase from *S. aureus* (*ugt106B1*) behind the native gcs^+ promoter of *A. tumefaciens* (Figure 2D). For this purpose, about 600 bp at the 5′-end of the gcs^+ gene sequence were replaced by the sequence of the heterologous glucosyltransferase from *S. aureus* together with a streptomycin/spectinomycin resistance (Sm^R/Sp^R)-cassette. The *S. aureus* glucosyltransferase was inserted exactly at the locus of the replaced gcs^+ 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^R/Sp^R-cassette), and p18agro2 (NotI/SacI; right flanking sequence). The vector p18agro1 resulted from the ligation of pUC18 (SmaI) with the 2100 bp flanking sequence upstream of the gcs^+ gene of *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^+ gene of *A. tumefaciens* was amplified with the primers 5F and 5R and ligated with pUC18 (SmaI) resulting in the vector p18agro2. The "processive" glucosyltransferase from *S. aureus* was amplified with the primers 6F and 6R using the vector pEsay24 as template and ligated with pUC18 (SmaI) resulting in the vector p18Staph. The Sm^R/Sp^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 *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^+ gene sequence was monitored by PCR with Taq DNA Polymerase (NEB) or Herculase^R Enhanced DNA Polymerase (Stratagene) (data not shown). To check the deletion of the gcs^+ gene and its replacement by the Kan^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 *S. aureus* plus the Sm^R/Sp^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*/*Δugt51B1* 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-[^{14}C]Glc (150,000 dpm, specific activity 10 GBq/mmol, final concentration 2.5 μM) or UDP-[^{14}C]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 90 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) ($CHCl_3$:methanol:0.45% NaCl solution, 2:1:0.75), and the organic phase was evaporated. The residue was redissolved in $CHCl_3$ 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 peracetylated 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 CF_3CO_2H at 100°C for 2 h, conventionally reduced with borohydride and peracetylated (Sawardeker *et al.*, 1965).
Methylation analysis. Purified glycolipids were methylated with CH_3I in dimethyl sulfoxide in the presence of solid NaOH (Ciucanu and Kerek, 1984) and subsequently hydrolyzed with 2 M CF_3CO_2H (100°C, 2 h). The partially methylated monosaccharides were reduced with borohydride, peracetylated, 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°/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 ^{1}H-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 $^2HCCl_3$ (99.8%D, Aldrich, St. Louis, MO). All 1D and 2D ^{1}H-NMR spectra (COSY, RELAY, TOCSY) were recorded in 3 mm microtubes (Kontes, Vineland, NJ) in $^2HCCl_3$ (99.96%, Eurisotop, Gif-sur-Yvette, Saint-Aubin France) at 300 K. Chemical shifts were referenced to internal chloroform (δ_H = 7.260 ppm). Bruker standard software XWINNMR 3.5 was used to acquire and process all 1D and 2D spectra. A selection of analytically relevant data of new compounds is given in Table II of supplementary data.

Supplementary data

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*-*N*[6-amino-*N*-4′(7-nitrobenzo-2-oxa-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.

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