A Bifunctional Glycosyltransferase from *Agrobacterium tumefaciens* synthesizes Monoglucosyl and Glucuronosyl diacylglycerol under Phosphate Deprivation* 

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*Running title: Glucosyl-Glucuronosyl-Transferase in Agrobacterium

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**Background:** Despite of the high diversity of glycolipids, found in many organisms, only a few glycosyltransferases have been isolated.

**Results:** A bifunctional glycosyltransferase, synthesizing glucuronosyl or monoglucosyl diacylglycerol was isolated from *Agrobacterium*. 

**Conclusion:** Glycolipids and other non-phospholipids can mutually replace each other, enhancing the ability to adapt to changing environments.

**Significance:** First report on the isolation of a glucuronosyl diacylglycerol synthase.

**ABSTRACT** 

Glycolipids are mainly found in phototrophic organisms (like plants and cyanobacteria), in Gram-positive bacteria and a few other bacterial phyla. Besides of the function as bulk membrane lipids they often play a role under phosphate deprivation as surrogates for phospholipids. The Gram-negative *Agrobacterium tumefaciens* accumulates four different glycolipids under phosphate deficiency including digalactosyl diacylglycerol and glucosylgalactosyl diacylglycerol synthesized by a processive glycosyltransferase. The other two glycolipids have now been identified by mass spectrometry and nuclear magnetic resonance spectroscopy as monogluosyl diacylglycerol and glucuronosyl diacylglycerol. These two lipids are synthesized by a single promiscuous glycosyltransferase encoded by the ORF *atu2297*, with UDP-glucose or UDP-glucuronic acid as sugar donors. The transfer of sugars differing in their chemistry is a novel feature not observed before for lipid glycosyltransferases. Further, this enzyme is the first glucuronosyl diacylglycerol synthase isolated. Deletion mutants of *Agrobacterium* lacking monoglucosyl diacylglycerol and glucuronosyl diacylglycerol or all glycolipids are not impaired in growth or virulence during infection of tobacco leaf discs. Our data suggest that the four glycolipids and the non-phospholipid diacylglycerol trimethylhomoserine can mutually replace each other during phosphate deprivation. This redundancy of different non-phospholipids may represent an adaptation mechanism to enhance the competitiveness in nature.

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*While phospholipids are widespread constituents in biological membranes, the occurrence of glycolipids is mainly restricted to plants, cyanobacteria, Gram-positive bacteria and a few other bacterial phyla (1). Glycolipids are...*
characterized by a high head group diversity, which is determined by the number and type of sugars (glucose, galactose, mannose, or charged sugars like glucuronic acid or sulfoquinovose) with different anomeric configurations ($\alpha$, $\beta$) and linkages to each other ($1\rightarrow2$, $1\rightarrow3$, $1\rightarrow4$, $1\rightarrow6$). In Gram-positive bacteria glycolipids represent building blocks for membranes or serve as membrane anchors for lipoteichoic acids (1, 2). Glycolipids also play an important role in several members of Gram-negative bacteria (Proteobacteria) under phosphate deprivation, similar to plants and cyanobacteria, where they replace phospholipids to save phosphate required for the synthesis of phosphate containing metabolites (1, 3, 4). Besides of glycolipids, further phosphate-free lipids are often involved in this physiological stress response in bacteria (5). Two representatives of such non-phospholipids are diacylglyceryl trimethyl-homoserine (DGTS) or the glycerol-free ornithine lipid (OL) (6).

These two lipids accumulate in different Proteobacteria, like in the nodule-forming bacterium Sinorhizobium meliloti (Rhizobiaceae, Rhizobiales) when grown under phosphate deprivation. Sinorhizobium further contains the charged glycolipid sulfoquinovosyl diacylglycerol (SQD), which is also involved under these conditions (6). The plant pathogen Agrobacterium tumefaciens (Rhizobiaceae) or the nodule-forming bacterium Mesorhizobium loti (Phyllobacteriaceae, Rhizobiales) were recently shown to synthesize a series of further glycolipids grown under phosphate starvation, i.e. digalactosyl diacylglycerol (DGD), glucosylgalactosyl diacylglycerol (GGD), different triglycosyl diacylglycerols, with all sugars bound in the anomeric configuration of the sugar (9, 10). The two glycosyltransferases synthesizing the agrobacterial or mesorhizobial glycolipids DGD, GGD and triglycosyl diacylglycerols were characterized as processive glycosyltransferases, designated Pgt (3, 4). They transfer both glucose and galactose, with diacylglycerol (DAG) as primary acceptor. The two enzymes with high sequence similarity are members of GT21 (11).

The identification and characterization of the enzyme(s) responsible for the synthesis of the two unknown glycolipids U1 and U2 in Agrobacterium and the elucidation of the glycolipid head group structures are part of this study.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids, and growth conditions** – Agrobacterium tumefaciens strain C58C1 (pGV2260) was grown at 28 °C in YEP medium in the presence of rifampicin (60 mg/L) (4). Gentamicin (25 mg/L) was used for selection of the single mutant $\Delta$agt; gentamicin and kanamycin (50 mg/L) were used for the selection of the double mutant $\Delta$agt $\Delta$ptgt. E. coli strains ElectroSHOX (Bioline) and BL21 (DE3) (Novagen) were used as expression hosts for atu2297. Growth curve experiments of Agrobacterium cells were performed as described (4) starting with an initial OD$_{600}$ of 0.05 in AB minimal medium (12) with high (25 mM) or low (20 µM) phosphate. The OD$_{600}$ was determined for 96 h.

**Virulence tests with tobacco leaf disc transformation** – Leaf disc transformation was performed with Agrobacterium wild type, $\Delta$agt or $\Delta$agt $\Delta$ptgt with each strain in a separate experiment, as described (4).

**Cloning of the ORF atu2297 from A. tumefaciens** – The ORF atu2297 was amplified using the primers bn799 (CCTAGGTATGACGAGAATCAGATTGTGTC) and bn800 (GGATCTCATGCAAGCCGAGCGC) containing XmaI and BamHI restriction sites (as underlined). The PCR product was subcloned in
the pGEM-T Easy vector (Promega) and released with XmaI/BamHI. The vector pTnVagro (11) was used as an expression vector, linearized with XmaI and BamHI, and ligated with the released ORF \textit{atu2297} (pTnV-atu2297). The empty vector (pTnV) was created by blunting the linearized pTnVagro vector (with the Klenow fragment, Thermo Scientific) and subsequent circularization.

Construction of \textit{A. tumefaciens} deletion mutants \textit{Δagt} and \textit{Δagt Δpgt} by disruption of the \textit{atu2297} locus by homologous recombination using gentamycin and kanamycin resistance cassettes –

The mutant line \textit{Δagt} was generated using the \textit{Agrobacterium} strain C58C1 (pGV2260). The primers used for the amplification of the homologous sequences were \textit{bn934} (TCATCGGCCATCATGGCGC) and \textit{bn935} (TATACCATGTTGCCCGCCATTTGGAAACC) to generate the 5'-flanking sequence with an 3'-NcoI restriction site, while \textit{bn936} (ATATACGCGTTCATTGAAGCGCTGGCCAG) and \textit{bn937} (CGTATTACATCTGCCATCACG) were used for the amplification of the 3'-flanking sequence with a 5'-MluI restriction site. All PCR products were subcloned in pGEM-T easy vectors. MluI, NcoI, and other restriction sites on the cloning vectors were used for further cloning. The two flanking sequences and the gentamicin resistance cassette were cloned in one step in pGEM-T Easy with the gentamicin resistance cassette inserted in antisense orientation relative to the flanking sequences. The double knock out mutant \textit{Δagt Δpgt} was double mutated using the primer pairs \textit{bn938} (TTGCCCGTTACGCACCGGA) and \textit{bn939} (GCCTCAAATACAGGTGCAGAT); \textit{bn249} (AGTGCGCTCTCTATACAAAGTTG) and \textit{bn938} (TTGCCCGTTACGCACCGGA); \textit{bn250} (TTCCGTCAAGGTTCGGACC) and \textit{bn939} (GCCTCAAATACAGGTGCAGAT).

Lipid isolation, separation and analysis by GC-MS and quadrupole-time-of-flight mass spectrometry (Q-TOF MS) –

Lipids and fatty acids were analyzed as described (3, 4). The fragmentation energy for MGlcD/U1 and GlcAD/U2 analyzed with Q-TOF MS/MS was 12 V and 20 V, respectively. The solvent used for one-dimensional TLC was acetone/toluene/water (91:30:8). For two-dimensional TLC, chloroform/methanol/water (65:25:4) was used for the first dimension and chloroform/methanol/acetic acid/water (90:15:10:4) for the second dimension. For NMR spectroscopy glycolipids from \textit{Agrobacterium} and \textit{E. coli} expressing \textit{atu2297} were separated by preparative one-dimensional TLC. Purification of U2 from \textit{Agrobacterium} required two steps because of co-migrating lipids. In the first step total lipid extract from \textit{Agrobacterium} were separated on TLC plates pre-treated with ammonium sulfate (0.15 M) and activated by heat (120 °C, 2.5 h) prior to use. In this system U2 is protonated and migrates clearly above MGD (4). U2 and co-migrating lipids were scraped off and extracted from the silica material with chloroform/methanol (2:1) in an ultrasonic bath for 30 min. The extracted lipid mixture was subjected to a second TLC step using non-treated plates to obtain pure U2 lipid, which migrates similar to DGD in this TLC system (this study).

Enzyme assays –

For enzyme assays \textit{E. coli} (ElectroSHOX) cells containing pTnV-atu2297 were grown at 37 °C to an OD\textsubscript{600} of 0.6, induced with 500 µM IPTG, and incubated for 24 h at 16 °C. The culture was harvested by centrifugation and the pellet was resuspended in 1 mL of buffer (11) and disrupted with glass beads with the Precellys homogenizer (Peqlab). Cell debris was removed by centrifugation at 70 x g for 1 min. The assays were performed in a final volume of 205 µL.
with 100 μL of buffer 2 (15 mM Tricine-KOH/pH 7.2, 30 mM MgCl₂, 3 mM DTT), 50 μL UDP-glucuronic acid (UDP-GlcA) or UDP-glucose (UDP-Glc) (40 pmol/μL), 50 μL E. coli protein extract and 5 μL DAG-14:0/14:0 (10 nmol/μL in ethanol). After incubation for 60 min at 28 °C, the assays were terminated by the addition of 3 mL chloroform/methanol (2:1) and 0.5 mL NaCl solution (0.9%). The lipids were extracted as described (3, 4) and separated by TLC. MGD and DGD were used as reference lipids to identify the positions of MGlcD and GlcAD on the TLC plate. Corresponding bands were scraped off from the silica plate and extracted for 30 min with chloroform/methanol (2:1) in an ultrasonic bath. The extracted reaction products were analyzed with the Q-TOF mass spectrometer (Agilent) by direct nanospray infusion in the positive mode as described (3, 4) using fragmentation energies of 12 V for MGlcD and 20 V for GlcAD.

**Compositional analyses** – In order to confirm the nature of hexose and hexuronic acid samples were hydrolysed with 2 M HCl/MeOH at 85°C for 2 h, followed by acetylation (85°C, 10 min.) and detection by GC-MS [Hewlett-Packard HP 5890 (series II) gas chromatograph equipped with a fused-silica SPB-5 column (Supelco, 30 m × 0.25 mm × 0.25 μm film thickness), FID and MS 5989A mass spectrometer with vacuum gauge controller 59827A]. The temperature program was 150°C for 3 min, then 5°C/min to 330°C. The sugars were identified by comparison with the authentic standards. The determination of the absolute configuration of the constituents was performed as described (13).

**NMR spectroscopy** - NMR spectroscopy experiments were carried out in CDCl₃ with tetramethylsilan (δ_H 0.00, δ_C 0.00) as an internal standard. ¹H,¹³C, and 2D homonuclear (¹H,¹H) COSY, TOCSY, and ROESY, as well as (¹H,¹³C) HSQC-DEPT, coupled HSQC and HMBC experiments were recorded at 27°C with a Bruker DRX Avance 700 MHz spectrometer (operating frequencies 700.75 MHz for ¹H NMR, 176.2 MHz for ¹³C NMR), equipped with a 5 mm CPQCI multinuclear-inverse cryo-probehead with a z gradient, and applying standard Bruker software. COSY, TOCSY, and ROESY experiments were recorded using data sets (t1 by t2) of 4096 by 512 points, COSY with 1 and TOCSY, ROESY with 8 scans. The TOCSY experiment was carried out in the phase-sensitive mode with mixing times of 60 ms and ROESY of 300 ms. Additionally, NMR spectra of U2 were carried out on a Bruker Avance AVIII 500 spectrometer equipped with a TCI-cryoprobe. All spectra were measured in MeOD/CDCl₃ (16.66%/83.37%) at 27°C. Double-quantum filtered (dqf) COSY, TOCSY, and ROESY experiments were recorded using data sets (t1 by t2) of 2048 by 512 points, with 16 scans. The TOCSY experiment was carried out in the phase-sensitive mode with mixing times of 120 ms and ROESY of 300 ms.

**RESULTS**

Expression of an agrobacterial glycosyltransferase (atu2297) led to the accumulation of MGlcD and monohexuronosyl diacylglycerol in E. coli – The presence of the unknown glycolipid U2 both in A. tumefaciens and M. loti (3, 4) suggests the existence of glycosyltransferases with homologous sequences in the two organisms. For the identification we first searched for annotated glycosyltransferase sequences in A. tumefaciens in the CAZy data base (http://www.cazy.org/) (8). To restrict the number of genes we selected only candidates with homologous sequences in Agrobacterium and Mesorhizobium. This search revealed about 14 sequences, which were all cloned from Agrobacterium and expressed in E. coli ElectroSHOX or BL21 (DE3). Only one of the open reading frames (atu2297; GT4, containing α-glycosyltransferases) led to the accumulation of two new glycolipids in E. coli BL21 (DE3) migrating slightly above MGD or below DGD, respectively (Fig. 1 A). Expression in E. coli ElectroSHOX led to the accumulation of only one glycolipid migrating slightly above MGD (data not shown). One explanation for the absence of the second glycolipid may be a reduced availability of the respective sugar donor in this E. coli strain. Compositional and structural analysis with NMR spectroscopy confirmed one of the lipids as MGlcD with α-d-configuration of the glucose (Fig. 1 B). The α-configuration was confirmed by the 3J(1,2) value of 3.6 Hz measured from the ¹H spectrum. Therefore, the ORF atu2297 codes for an α-glycosyltransferase (Agt) synthesizing MGlcD. The second glycolipid expressed in E. coli migrates similar to U2 from Agrobacterium and Mesorhizobium on TLC plates (3, 4). It was further analyzed with Q-TOF MS/MS where it was detected as an ammonium adduct (in the positive
ion mode), with one out of several main molecular species (Fig. 1 C). The calculated m/z of the parental ion was 774.5727. The neutral loss of m/z 211.0660 of the fragmented lipid indicates the loss of hexuronic acid as ammonia adduct representing the lipid head group. The different fragment ions in the spectrum are derived from DAG-16:0/17:0c (m/z 563.4999) and from monoacyl glyceryl-16:0 (m/z 313.2718) and -17:0c (m/z 325.2716). Therefore, this second glycolipid isolated from *E. coli* represents monohexuronosyl diacylglycerol.

**U1 and U2 from Agrobacterium contain glucose or glucuronic acid in their head groups, respectively** – To reveal the structural details of the agrobacterial glycolipids U1 and U2 we separated them via two-dimensional TLC and analyzed the isolated lipids with Q-TOF MS/MS. This analysis does not allow distinguishing between different epimeric and anomeric configurations of the sugars. The fragmentation spectra of a main molecular species of each agrobacterial lipid are shown in Fig. 2. The neutral losses of m/z 197.0905 and m/z 211.0748 are derived from hexose and hexuronic acid, respectively, as ammonia adducts (Fig. 2). The fragment ions m/z 617.5498 (or 617.5448) are derived from DAG-18:1/19:0c, while the fragment ions 339.2873 (or 339.2854) and 353.3052 (or 353.3032) are derived from monoacylglycerol-18:1 and -19:0c, respectively. Therefore, U1 and U2 represent two glycerolipids containing hexose or hexuronic acid in their head groups, respectively (Fig. 2). These results were confirmed further by GC analyses together with NMR spectroscopy that allowed determining the nature as well as absolute and anomeric configuration of the head groups of the two lipids. U1 could be identified as MGlcD (Table 1) with α-D-GlcP, and U2 as monoglucuronosyl diacylglycerol (GlcAD) with α-D-GlcPα linked to DAG (Table 2). The $^1$H/C1 value of 172.26 Hz proved the α-configuration of U2.

**Deletion of atu2297 resulted in the loss of the two glycolipids MGlcD and GlcAD by Agt** – The accumulation of MGlcD and monohexuronosyl diacylglycerol in *E. coli* after expression of Agt, together with the absence of MGlcD and GlcAD in the *Agrobacterium* Δagt mutant suggests that Agt is able to transfer both glucose and glucuronic acid onto diacylglycerol using the respective UDP-sugars. However, it may be also conceivable that GlcAD is formed from MGlcD in a second step by an unknown enzyme with glucose-6-dehydrogenase activity. To reveal the mechanism for the formation of MGlcD and GlcAD and to exclude the possibility that a further enzyme is involved in the formation of GlcAD by modification of MGlcD, further experiments were performed. To this end, we included in addition to Agt from *Agrobacterium* the glucosyltransferase αMGS from *Acholeplasma* (7) in our studies, which is known synthesize only MGlcD but not GlcAD. To express the two enzymes in a glycolipid free background, the *Agrobacterium* double knock out mutant Δagt Δpgt was produced. This mutant was created by insertion of a kanamycin resistance cassette into the atu2297 locus of cells with Δpgt background (4). The Δpgt mutants are characterized by the disruption of the
processive glycosyltransferase Pgt and by the lack of GGD and DGD. The purpose of creation of this double knock out was to abolish Agt activity and to prevent any activities resulting from Pgt, such as the synthesis of β-MGD or β-MGlcD (11), which cannot be distinguished in mass spectrometry (by their m/z and fragmentation pattern) from α-MGlcD synthesized by Agt or aMGS from Acholeplasma. Furthermore, the lack of DGD/GGD facilitates the detection and isolation of GlcAD, which migrates similar to DGD during TLC.

Agt or aMGS were then introduced into the Agrobacterium Δagt Δpgt double mutant. The two transformed strains are expected to produce MGlcD, and the strain expressing Agt is expected to contain in addition GlcAD. If the MGlcD lipid was the precursor for the synthesis of GlcAD by a second enzyme, then the aMGS expressing strain should also contain GlcAD. On the other hand, if Agt itself is responsible for the synthesis of GlcAD then GlcAD should be absent from cells expressing aMGS.

The cell lines were grown under phosphate starvation, because the glycolipid accumulation of expression cultures is higher compared to growth under phosphate replete conditions (4); the lipids were analyzed via TLC and Q-TOF MS/MS. In the cells expressing Agt we could detect both MGlcD and GlcAD as shown in Fig. 4 and supplemental Fig. 3. The empty vector control is free of any glycolipids. The line expressing aMGS from Acholeplasma accumulates MGlcD but lacks GlcAD (Fig. 4, supplemental Fig. 3). Therefore, these results indicate that Agt synthesizes both MGlcD and GlcAD; the involvement of further MGlcD modifying enzymes involved in GlcAD synthesis in Agrobacterium can be excluded.

Enzyme assays with recombinant Agt protein confirm the synthesis of MGlcD and GlcAD by Agt – For the confirmation of these results and further analysis of the sugar specificities of Agt we performed enzyme assays with protein extracts prepared from E. coli cells expressing Agt or the empty vector as control. UDP-Glc or UDP-GlcA were used as sugar donors, and DAG-14:0/14:0 as sugar acceptor. The formation of the respective glycolipids was analyzed via Q-TOF MS/MS. We detected MGlcD (14:0/14:0) or GlcAD (14:0/14:0) in the lipid extracts of enzyme assays of Agt, using UDP-Glc or UDP-GlcA as substrates, respectively (Fig. 5). Protein extracts from E. coli harboring an empty vector were free of these two activities. These results demonstrate that GlcAD is not formed by modification of MGlcD, but that the two lipids MGlcD and GlcAD are synthesized by Agt, exhibiting two different substrate specificities for UDP-Glc and UDP-GlcA.

MGlcD and GlcAD are replaced by DGD/GGD in the Δagt single mutant, while all glycolipids are replaced by DGTS in the Δagt Δpgt double mutant – For investigation of the role of glycolipids in Agrobacterium, we analyzed the lipid composition of wild type, Δagt, and of the glycolipid free double knock out mutant Δagt Δpgt. For this purpose we grew the three lines under phosphate replete and depleted conditions and quantified the lipids by measuring their fatty acid methyl esters via GC-MS. The different cell lines grown under phosphate replete conditions were not distinguishable in their lipid compositions (Fig. 6). Differences could be observed under phosphate starvation in the different mutant lines compared to the wild type (Fig. 6). The single knock out mutant Δagt compensates the loss of MGlcD (1.5% in wild type) and GlcAD (5% in wild type) by a strong accumulation of the Pgt dependent glycolipids GGD and DGD (from 10 in wild type to more than 20% in Δagt). This change in glycolipid composition is accompanied by a strong reduction of the phospholipid phosphatidyl choline (PC). All the other lipids are hardly affected. The double knock out mutant compensates the loss of all glycolipids by a strong accumulation of DGTS. Again, PC is strongly reduced.

Glycolipids are not required for growth or virulence of Agrobacterium under phosphate deprivation – The accumulation the different glycolipids DGD, GGD, MGlcD and GlcAD suggest that they are important for growth under phosphate deprivation. As shown in a previous study, the loss of DGD and GGD in the Δpgt mutant does not affect growth or virulence under phosphate starvation presumably because these two glycolipids are compensated for by DGTS and the remaining glycolipids MGlcD and GlcAD. In this study we analyzed the growth and virulence of Δagt and Δagt Δpgt to reveal any effect of the lack of MGlcD/GlcAD or the complete loss of glycolipids. For growth curve experiments wild type and the two mutant lines were grown in minimal medium with high (25 mM) or low (20 μM) phosphate. Growth of all lines is reduced
under phosphate deprivation, but there is no difference between wild type, ∆agt and ∆agt ∆pgt (Fig. 7). The growth experiments were repeated four times. The virulence tests were performed three times with tobacco leaf discs inoculated separately with wild type, ∆agt or ∆agt ∆pgt under conditions with high or low phosphate. The number of calli formed on the leaf discs should give an indication for the virulence of the respective strains. Again, no differences could be observed between wild type and the two mutant lines (data not shown). These data suggest that glycolipids are not required for growth or virulence of Agrobacterium under normal and phosphate deficient conditions.

**DISCUSSION**

*A. tumefaciens* accumulates four different glycolipids under phosphate starvation, which are GGD, DGD and two unknown glycolipids U1 and U2 (4). In the present study we determined the structures of U1 (MGlcD) and U2 (GlcAD) and identified and characterized the responsible glycosyltransferase encoded by the ORF *atu2297*. This study is the first report on a lipid glycosyltransferase acting as bifunctional enzyme by synthesizing neutral and acidic glycolipids. Furthermore, Agt is the first GlcAD synthase isolated.

In general, glycosyltransferases exhibit a high sugar donor specificity. The best studied glycosyltransferases include the MGD/MGlcD and DGD synthases from plants and cyanobacteria (15-18), the MGlcD and DGlC synthases from *Acholeplasma* (7, 19), and the processive glycosyltransferases from the Gram-positive bacteria *Staphylococcus* or *Bacillus* (20, 21). There are only a few glycosyltransferases characterized as promiscuous enzymes, such as Pgt from *Agrobacterium* or *Mesorhizobium*, or the processive glycosyltransferases from the human pathogens *Mycoplasma pneumoniae* and *M. genitalium* using UDP-Glc and UDP-galactose (UDP-Gal) as sugar donors (11, 22, 23). All these promiscuous glycosyltransferases transfer sugars with similar chemistry, while there are no reports on glycosyltransferases transferring sugars with different chemistry as shown for Agt.

The presence of MGlcD in representatives of the *Rhizobiales* has never been described before. Therefore, *Agrobacterium* is the first member of the *Rhizobiales* synthesizing MGlcD. With respect to other Gram-negative bacteria, MGlcD is mainly restricted to the cauliform bacteria and relatives (*α-Proteobacteria*), and to the *Spirochaetes* (1). It represents a bulk lipid in these organisms. MGlcD (with α- or β-anomeric configuration) is also found in many Gram-positive bacteria (1), often building the precursor for the synthesis of DGlC D (2).

There are only a few bacteria synthesizing the acid glycolipid GlcAD. With respect to *Rhizobiales*, this lipid was described for the first time for the anoxygenic phototrophic bacterium *Blastochloris viridis* (24), but it has never been found before in non-phototrophic *Rhizobiales*. Therefore, *Agrobacterium* is the first representative of non-phototrophic *Rhizobiales* synthesizing GlcAD. It is further found in the cauliform bacteria and relatives (25-28). In most of these bacteria GlcAD represents a bulk lipid, and it is synthesized independently from phosphate supply (25, 29). There are only a few reports on GlcAD accumulating under phosphate deprivation as surrogate for phospholipids (30, 31). Outside of Gram-negative bacteria GlcAD or higher glycosylated forms exist only in a few other bacterial species (1, 32-34). The responsible GlcAD synthase in all these organisms have never been isolated. Enzyme assays revealed UDP-GlcA as sugar donor (29). GlcAD was recently also detected in plants, where it is probably synthesized by the SQD synthase SQD2 (35).

SQD, another acidic glycolipid, is widespread in phototrophic and non-phototrophic organisms. It is generally accepted that SQD especially replaces the acidic phospholipid phosphatidyl glycerol under phosphate limiting conditions (1, 6, 36, 37). Different species of *Rhizobiales* contain either SQD (*Sinorhizobium, Rhizobium*) or GlcAD (*Blastochloris*), but there are no reports on the presence of the two lipids in one species of *Rhizobiales* (1, 3). Thus, GlcAD and SQD might be counterpart lipids with similar functions in the different organisms.

The deletion of *atu2297* with loss of MGlcD/GlcAD in ∆agt or the complete loss of all glycolipids in ∆agt ∆pgt had no influence on growth or virulence of *Agrobacterium*. This may be explained by a mutual replacement of glycolipids and DGTS. A triple mutant of *Sinorhizobium* lacking SQD, OL and DGTS was not impaired in its ability to form nodules on its host alfalfa (38). However, the loss of OL and DGTS or of all non-phosphorus lipids (OL, DGTS,
SQD) resulted in a decreased growth under phosphate starvation, suggesting that these lipids serve as bulk lipids. This may also be true for the glycolipids and DGTS in *Agrobacterium*. Therefore, a triple knock out mutant of *Agrobacterium* lacking glycolipids and DGTS may be impaired in growth under phosphate limited conditions. OL or hydroxy-OL may not be as surrogate for glycolipids in *Agrobacterium* (4) (Fig 7).

In contrast to growth and virulence, the deletion of *atu2297* had a strong effect on lipid composition under phosphate deprivation. Surprisingly, PC was strongly reduced in cells lacking MGlcD/GlcAD, while the amount of DGTS in ∆agt was not changed. In contrast to *Agrobacterium*, the content of PC is negatively correlated to that of DGTS in *Sinorhizobium* (38, 39). The reduction of PC in ∆agt or ∆agt ∆pgt may have different reasons. (i) PC reduction may result from its degradation by a phospholipase C active under phosphate deprivation to provide DAG for the increased synthesis of GGD/DGD in ∆agt or DGTS in ∆agt ∆pgt (4, 40). (ii) The reduction of PC may be necessary to sustain special membrane functions in a changed lipid environment caused by the lack of the acidic glycolipid GlcAD. Therefore, the differences in the lipid compositions in the different cell lines may reflect the ability and adaptability of *Agrobacterium* to compensate for the loss of the different glycolipids. With respect to this extended lipid redistribution caused by the loss of relatively small amounts of MGlcD/GlcAD a special but unknown function especially of GlcAD may not be excluded.

A common but important function of membrane lipids is the maintenance of membrane structure and fluidity. A certain ratio of bilayer stabilizing and destabilizing lipids is crucial for optimal membrane functions. The bilayer forming properties of membrane lipids can be regulated on different levels (41, 42). In *Acholeplasma* the ratio of non-bilayer forming to bilayer forming lipids is regulated by glucosylation of the non-bilayer lipid MGlcD to form the bilayer forming DGlcD with the DGlcD synthase as key enzyme (43-45). A similar mechanism was described for *Mycoplasma*. In this case the regulative enzyme is a processive glycosyltransferase adjusting the ratio of MGlcD and DGlcD (22). A novel and alternative regulation mechanism would be the introduction of different head groups into the glycolipid by a bifunctional glycosyltransferase as shown for Agt (this study), with GlcAD probably as a bilayer forming glycolipid (46). Therefore, Agt may be an interesting candidate for a new type of glycosyltransferases playing a role for the membrane bilayer formation and function under phosphate deprivation.

Gene expression regulated by phosphate availability is often mediated via the two component PhoB-PhoR regulatory system, with the PhoB protein binding in its phosphorylated form to a highly conserved sequence motif in the promoter region of the regulated gene. Such a PhoB regulon may activate Pgt and Agt in *Agrobacterium* (4, 47). Accumulation of glycolipids in *Agrobacterium* was suggested to depend further on the availability of DAG as the sugar acceptor (4). The regulatory role of the sugar donors UDP-Glc, UDP-Gal and UDP-GlcA in glycolipid accumulation has never been tested. Sugar nucleotides play a central role in the carbohydrate metabolism with participation in many different pathways in *Agrobacterium*, such as the formation of lipopolysaccharides in the outer membrane (48). The bulk of UDP-Glc is required for the synthesis of exo-polysaccharides (cellulose, succinoglycan, cyclic β-glucans) found in biofilms (49-52). Biofilm production is increased under phosphate deprivation and regulated via the PhoB regulon (53). A key enzyme in the carbohydrate metabolism is UDP-Glc pyrophosphorylase which produces UDP-Glc. UDP-Glc-4-epimerase and UDP-Glc dehydrogenase convert UDP-Glc to UDP-Gal or UDP-GlcA, respectively. *Agrobacterium* contains ORFs (*atu3778, atu3315, atu4149*) with sequence similarity to the respective enzymes in *Sinorhizobium* (54-57). Earlier results indicate that sugar nucleotides may have a regulatory role in the glycolipid accumulation in *Agrobacterium* during phosphate deprivation (11). While Pgt has a higher preference for UDP-Gal compared to UDP-Glc, this preference is not reflected in the ratio of glucose- to galactose-containing glycolipids produced in *Agrobacterium*. The composition of these different glycolipids rather depends on the host employed for heterologous expression of Pgt and on the growth conditions (4, 11), indicating that the ratio of GGD to DGD in *Agrobacterium* might be regulated via the changing availability of UDP-Glc and UDP-Gal. Similarly, expression of Agt in different *E. coli* strains results in the production of glycolipids...
with different ratios of MGlcD and GlcAD (Fig. 1A, data not shown). Therefore, the incorporation of glucose or glucuronic acid into glycolipids by Agt might depend on the availability of both substrates, UDP-Glc and UDP-GlcA. The total amount of glycolipids synthesized may be influenced by upregulation of UDP-Glc pyrophosphorylase, UDP-Glc-4-epimerase and UDP-Glc dehydrogenase under phosphate deprivation.
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FOOTNOTES

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5The abbreviations used are: DGTS, diacylglyceryl trimethyl-homoserine; OL, ornithine lipid; SQD, sulfoquinovosyl diacylglycerol; DGD, digalactosyl diacylglycerol; GGD, glucosylgalactosyl diacylglycerol; MGlcD, monoglucosyl diacylglycerol; DGlcD, diglucosyl diacylglycerol; aMGs, MGlcD synthase from A. laidlawii; GT4, glycosyltransferase family 4; MGD, monogalactosyl diacylglycerol; DAG, diacylglycerol; UDP-GlcA, UDP-glucuronic acid; UDP-Glc, UDP-glucose; Q-TOF MS, quadrupole-time-of-flight mass spectrometry; GlcAD, glucuronosyl diacylglycerol; PC, phosphatidyl choline; UDP-Gal, UDP-galactose

FIGURE LEGENDS

FIGURE 1. Accumulation of two new glycolipids in E. coli expressing Agt from Agrobacterium. (A) TLC of lipid extracts from E. coli BL21 (DE3) expressing Agt or the empty vector as control. The new glycolipids were identified as MGlcD and monohexuronosyl diacylglycerol. (B) Overlay of 1H,HSQCdeint and HMBC spectra of MGlcD. Spectra were recorded in CDCl3 at 27°C utilizing the Bruker DRX Avance 700 MHz spectrometer. Important intra residual scalar correlations are marked in the box. R1 and R2 stands for: 14:0; 16:1; 16:0; 17:0c (omega 9,10); 18:1; 19:0c (omega 9,10). TMS, tetramethylsilan; FA, fatty acids. (C) Q-TOF MS/MS spectrum of monohexuronosyl diacylglycerol. Parental ions were selected as ammonium adducts and fragmented. The figure shows the spectrum of one main species with m/z 774.5727. The fragment with m/z 563.4999 represents DAG-16:0/17:0c (as protonated form with loss of H2O). The neutral loss of 211.0660 (m/z 774.5655 minus 563.4999) is derived from hexuronic acid (as ammonium adduct). The ions m/z 313.2718 and 325.2716 represent monoacylglycerol-16:0 and monoacylglycerol-17:0c, respectively, each as protonated form with loss of H2O.

FIGURE 2. Q-TOF MS/MS spectra of monohexosyl diacylglycerol (U1) and monohexuronosyl diacylglycerol (U2). Parental ions (as ammonium adducts) of one main molecular species of U1 (calculated m/z 814.6403) and U2 (calculated m/z 828.6196) were selected in the positive mode (with a detection window of m/z 1.2) and fragmented. The fragments with m/z 617.5498 (or 617.5448) represent
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DAG-18:1/19:0c (as protonated form with loss of H₂O). The neutral loss of 197.0905 (m/z 814.6403 minus 617.5498) is derived from hexose (as ammonia adduct); the neutral loss of 211.0748 (m/z 828.6196 minus 617.5448) is derived from hexuronic acid (as ammonia adduct). The ions m/z 339.28 and 353.30 represent monoacylglycerol-18:1 and monoacylglycerol-19:0c, respectively, (each as protonated form with loss of H₂O). COO-R1 and COO-R2 represent two different fatty acyl residues (18:1 or 19:0c) bound to the glycerol back bone. The head group structures of U1 (MGlcD) and U2 (GlcAD) were further determined by NMR containing α-glucose or α-glucuronic acid, respectively, as illustrated in the figure.

FIGURE 3. Two-dimensional TLC of lipid extracts from Agrobacterium wild type (A) or Δagt (B) grown under phosphate deprivation, with MGlcD and GlcAD being absent from the mutant. PE, phosphatidyl ethanolamine; MMPE, monomethyl-PE; PG, phosphatidyl glycerol; CL, cardiolipin; OL-OH, hydroxylated form of OL.

FIGURE 4. Accumulation of MGlcD and GlcAD in Agrobacterium double knock out mutant Δagt Δpgt transformed with the empty vector, alMGS from Acholeplasma, or Agt from Agrobacterium. (A) TLC of lipid extracts from the three lines with expression of alMGS leading to the synthesis of MGlcD, and Agt to the synthesis of both MGlcD and GlcAD. (B) Q-TOF MS/MS spectra of MGlcD and GlcAD. Parental ions with a calculated m/z 828.6196 (with a detection window m/z 1.2) were selected in the positive mode. Due to similarity of their m/z values, ions representing ammonium adducts of both GlcAD-18:1/19:0c (calculated m/z 828.6196) and MGlcD-19:0c/19:0c (calculated m/z 828.6560) were selected together for fragmentation in this experiment. The fragments with m/z 617.6 and 631.6 represent DAG-18:1/19:0c and DAG-19:0c/19:0c (as protonated form with loss of OH), respectively. The neutral loss of 197.0 (m/z 828.6 minus 631.6) is derived from glucose (as ammonium adduct); the neutral loss of 211.0 (m/z 828.6 minus 617.6) is derived from glucuronic acid (as ammonium adduct). Therefore, DAG-18:1/19:0c is derived from GlcAD, while DAG-19:0c/19:0c is derived from MGlcD. The peaks at m/z 353.3877/353.4034 or 339.3745 represent protonated monoacylglycerol (MAG) species containing a 19:0c or 18:1 fatty acid, respectively, with loss of H₂O. The molecular species of MGlcD and GlcAD shown in the spectra represent one example each of different molecular species of the two glycolipids detected in Agrobacterium with all showing the same result that GlcAD is only present in cells expressing Agt and absent in cells expressing alMGS from Acholeplasma.

FIGURE 5. Q-TOF MS/MS spectra of MGlcD and GlcAD synthesized in enzyme assays with protein extracts from E. coli cells expressing Agt from Agrobacterium or harboring the empty vector as control. The assays were supplemented with DAG-14:0/14:0 as sugar acceptor and UDP-Glc or UDP-GlcA as sugar donors. Parental ions with calculated m/z 692.5308 or 706.5101 representing ammonium adducts of MGlcD or GlcAD, respectively, were selected in the positive mode (with a detection window of m/z 1.2). The fragment ions with m/z 495.4432 (or 495.4488) represent the protonated form of DAG-14:0/14:0 (with loss of H₂O), and m/z 285.2469 (or 285.4491) the protonated form of monoacylglycerol-14:0 (MAG-14:0) (with loss of H₂O). The respective fragments were absent in the spectra of the empty vector control assays. The neutral losses of 197.0876 (692.5308 minus 495.4432) or 211.0613 (706.5101 minus 495.4488) represent the ammonia adducts of glucose or glucuronic acid, respectively. These spectra prove the formation of both MGlcD and GlcAD (by supplementation of UDP-Glc or UDP-GlcA, respectively) by Agt. The detection of unspecific fragments (m/z 551.5 and 565.5) may result from the fragmentation of ions with similar m/z to the selected ions with m/z 692.5308 or 706.5101, which were also observed in the control assays.

FIGURE 6. Lipid composition of Agrobacterium wild type, Δagt, and Δagt Δpgt grown under phosphate replete (+P) or depleted (-P) conditions. The bars represent mean values in mol% ± SD of three measurements. PE, phosphatidyl ethanolamine; MMPE, monomethyl-PE; PG, phosphatidyl glycerol; CL,
cardiolipin; OL-OH, hydroxylated form of OL (4) Lipids were quantified after isolation from the TLC plate, transmethylation and measurements of fatty acid methyl esters by GC-MS.

**FIGURE 7.** Growth curves of *Agrobacterium* cells grown with 25 mM (+P) or 20 µM (-P) phosphate show no differences between wild type (WT) and deletion mutants (Δagt, Δagt Δpgt) under the respective conditions. Mean values ± SD were calculated from three independent measurements.
Table 1. \(^1\)H and \(^{13}\)C chemical shifts of U1 identified as 1,2-diacyl-3-\(\alpha\)-Glc\(p\)-sn-Gro reported [internal standard: tetramethylsilan]. Spectra were recorded in CDCl\(_3\) at 27°C utilizing a Bruker DRX Avance 700 spectrometer. Gro stands for glycerol.

|          | 1a  | 1b  | 2   | 3a  | 3b  | 4   | 5   | 6a  | 6b  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **\(\alpha\)-Glc** |     |     |     |     |     |     |     |     |     |
| H (\(\delta\)) | 4.85 | 3.50 | 3.72 | 3.58 | 3.59 | 3.81 | 3.83 |     |     |
| (\(^3\)J1,2 2.6 Hz) |     |     |     |     |     |     |     |     |     |
| C (\(\delta\)) | 99.13 | 72.16 | 74.30 | 70.05 | 71.85 | 61.84 |     |     |     |
| **Gro** |     |     |     |     |     |     |     |     |     |
| H (\(\delta\)) | 4.39 | 4.15 | 5.24 | 3.81 | 3.60 |     |     |     |     |
| C (\(\delta\)) | 62.41 | 69.85 | 66.35 |     |     |     |     |     |     |
Table 2. $^1$H and $^{13}$C chemical shifts of 1,2-diacyl-3-$\alpha$-D-Glc$\alpha$A-$sn$-Gro (U2) reported (internal standard: tetramethylsilan, $\delta_H$ 0.00, $\delta_C$ 0.00). Spectra were recorded in MeOD/CDCl$_3$ (16.66%/83.37%) at 27°C utilizing Bruker Avance AVIII 500 and DRX Avance 700 spectrometers.

|        | 1a  | 1b  | 2   | 3a  | 3b  | 4   | 5   | 6a  | 6b  |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| $\alpha$-D-Glc$\alpha$A |     |     |     |     |     |     |     |     |     |
| H      | 4.90| 3.51| 3.69| 3.59| 4.10| -   | -   |     |     |
| C      | 99.50| 71.61| 73.31| 71.89| 70.95| 172.26 |     |     |     |
| $\delta$ |     | 173 Hz |     |     |     |     |     |     |     |
| Gro    |     |     |     |     |     |     |     |     |     |
| H      | 4.43| 4.18| 5.25| 3.88| 3.68| -   | -   | -   | -   |
| C      | 62.47| 70.03| 66.78| -   | -   | -   | -   | -   | -   |
Figure 1

A

MGD

DGD

MGlcD

monohexuronosyl diaclylglycerol

C

monoheururonosyl diaclylglycerol

16:1/17:0
C_{42}H_{76}O_{11}

M
756.5388

[M+NH$_4$]$^+$
774.5655

m/z

200 300 400 500 600 700 800

relative number of counts in %

313.2718
325.2716
563.4999

B

2 Gro

1a Gro 1b Gro 6 Glc

3a Gro 4 Glc 2 Glc

Glc

anomeric

3aH Gro/ 3bH Gro/
1C Glc 1C Glc

CH=CH

2H Gro/COOR1 1H Gro/COOR2

13C [ppm]

0 50 100 150

1H [ppm]
Figure 2

A. Glucosyl-Glucuronosyl-Transferase in Agrobacterium

B. Glucosyl-Glucuronosyl-Transferase in Agrobacterium
Figure 3
Figure 4

A

empty vector  aiMGS  Agt

MGlcD

GlcAD

B

empty vector

aiMGS

Agt

relative number of counts in %

828.4515
353.4034  631.6739  828.4431
339.3745  353.3877  617.6568  631.6866  828.6368

m/z

200  300  400  500  600  700  800  900
Figure 5

Glucosyl-Glucuronosyl-Transferase in Agrobacterium
Figure 6

[Graph showing lipid content (mol%) for wild type, Δagt, and Δagt Δpgt under +P and -P conditions.]
Figure 7

Growth (OD₆₅₀) vs. Time (h) for different conditions.
A bifunctional glycosyltransferase from Agrobacterium tumefaciens synthesizes monoglucosyl and glucuronosyl diacylglycerol under phosphate deprivation
Adrian Semeniuk, Christian Sohlenkamp, Katarzyna Duda and Georg Hölzl

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