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Expression, purification and biochemical characterization of GumI, a monotopic membrane GDP-mannose:glycolipid 4-β-D-mannosyltransferase from *Xanthomonas campestris* pv. *campestris*

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We describe the first biochemical characterization of the *gumI* gene product, an essential protein for xanthan polysaccharide synthesis. Cellular fractionation experiments reveal the presence of a protein associated with the membrane fraction, even in the absence of the other proteins responsible for the synthesis of glycolipid intermediates and the proteins involved in the polymerization and transport of the xanthan chains. By alkaline buffer extraction and detergent phase partitioning, GumI was categorized as a monotopic membrane protein. GumI was overexpressed in *Escherichia coli*, solubilized and purified in an active and stable form using a simple and reproducible two-step procedure. The purified recombinant GumI is a nonprocessive β-mannosyltransferase that uses GDP-Man as a donor substrate and glucuronic acid-β-1,2-mannose-α-1,3-glucose-β-1,4-glucose-PP-polyisoprenyl as an acceptor. We also established the optimal biochemical conditions for GumI enzymatic activity. Sequence analysis revealed the presence of a conserved domain for glycosyltransferases (GTs) of the GT-B superfamily and homologous proteins in several prokaryote organisms. On the basis of this biochemical characterization, GumI may represent the founding member of a new GT family in the Carbohydrate-Active EnZymes classification.

Keywords: glycosyltransferase / mannosyltransferase / membrane-associated proteins / xanthan / *Xanthomonas campestris*

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Introduction

Bacterial glycosyltransferases (GTs) are key enzymes involved in the biosynthesis of glycolipids, peptidoglycans, lipopolysaccharides, capsular polysaccharides and exopolysaccharides, showing a wide range of biological functions and substrates. Much effort has been devoted to identifying genes that encode GTs, their enzymatic functions and the structures of these enzymes. GTs are classified as inverting or retaining enzymes according to the anomeric configuration of the reaction product with respect to the sugar donor (Sinnott 1990). Two main structural superfamilies have been described for nucleotide-diphospho-sugar-dependent GTs, named GT-A and GT-B. These topologies are variations of a Rossman-like domain (Lairson et al. 2008). Despite the relatively low structural variety, there is high sequence variability, and it is not yet possible to reproducibly predict the reactions catalyzed by a given GT. Currently, there are more than 90 GT families in the Carbohydrate-Active EnZymes (CAZy) database (http://www.cazy.org) (Campbell et al. 1997; Coutinho et al. 2003; Cantarel et al. 2009). In December 2007, less than 10% of all proteins in CAZy were enzymatically characterized (Cantarel et al. 2009); only some of them are membrane-associated. Membrane proteins are essential for numerous fundamental biological processes and for information transfer between intracellular compartments and between the cell and its environment (Forneris and Mattevi 2008). It is difficult to characterize membrane proteins and to determine their structures because of the challenge in protein production, purification and stabilization.

In this report, we describe the biochemical characterization of a *gum* gene product that is involved in the biosynthesis of xanthan (Ielpi et al. 1993). Xanthan is a branched acidic heteropolysaccharide comprised polymerized pentasaccharide repeating units, consisting of glucose (Glc), mannose (Man) and glucuronic acid (GlcA) (Scheme 1).

This acidic exopolysaccharide is produced by the phytopathogen Gram-negative bacterium *Xanthomonas campestris*. Xanthan acts as a virulence factor for a disease known as "black rot" or "blight", which causes enormous agricultural losses (Hayward 1993; Büttner and Bonas 2010), and is also one of the most important industrial biopolymers (Garcia-Ochoa et al. 2000).

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-4)-Glc-β-1,4-Glc-β-(1,-
3
|
Man-β-1,4-GlcA-β-1,2-Man-α-1

Scheme 1. Xanthan repeating unit.

The synthesis of xanthan proceeds by a Wzy-dependent mechanism. Current data indicate (Katzen et al. 1998; Barreras et al. 2004) that individual pentasaccharide repeating units are synthesized on the cytoplasmic face of the inner membrane by the stepwise addition of monosaccharides to a polyisoprenyl (Pol) phosphate lipid carrier molecule. The *gum* gene locus of *X. campestris* pv. *campestris* (*Xcc*) has been characterized and consists of 12 open reading frames (ORFs). This locus includes the *gumD*, *gumM*, *gumH*, *gumK* and *gumI* genes, which encode the set of GTs that are responsible for the ordered synthesis of a lipid-linked pentasaccharide (Capage et al. 1987; Katzen et al. 1998).

Although genetics and biosynthetic processes of xanthan are well documented, the enzymatic components remain poorly characterized. Our main aim is to understand how proteins involved in xanthan biosynthesis are organized in the bacterial cell to support a very efficient process. To comprehend the functional association of GTs, it is necessary to examine the functions and structures of individual GTs. Recently, recombinant GumK has been purified, and its localization, enzymatic activity and substrate specificity were determined (Barreras et al. 2004). In addition, the crystal structure of apo-GumK and of GumK complexed with its donor substrate together with site-directed mutagenesis provided the first insights into the active site of this enzyme and its transfer mode of action (Barreras et al. 2006, 2008). The purpose of this study was to expand this analysis to the *gumI* gene product. We previously found that the mutational interruption of the *gumI* gene leads to synthesis of GlcA-β-1,2-Man-α-1,3-Glc-β-1,4-Glc-PP-polyisoprenyl (GlcA-Man-Glc_2-PP-Pol), suggesting that GumI catalyzes the last step in the biosynthesis of Man-β-1,4-GlcA-Man-Glc_2-PP-Pol (Man-GlcA-Man-Glc_2-PP-Pol) (Katzen et al. 1998). Thus, GumI might be a link between GTs and the polymerization/secretion apparatus in xanthan production. On the basis of its amino acid sequence, GumI is considered to be an unclassified GT in the CAZy database, but it contains a domain that is typical for enzymes of the GT-B superfamily.

In this work, we first prove that the expression of GumI in trans restores normal xanthan production to an *Xcc gumI*-defective mutant. We investigate the subcellular localization of GumI and the dependence of this localization on the remaining Gum proteins. We also describe a simple and reproducible procedure for the heterologous overexpression, solubilization and purification of GumI in an active and stable form. We use an appropriate enzymatic assay and characterize the transferase activity of the pure recombinant enzyme preparations. Finally, we describe in vitro conditions for lipid-linked xanthan repeating unit synthesis using Man-Glc_2-PP-Pol as an acceptor, sugar nucleotides and purified GumK and GumI enzymes.

Results

Analysis of the gumI *gene by functional complementation*

XcI (an *Xcc gumI* mutant) displays a nonmucoid phenotype, indicating that it has a reduced abundance of polysaccharides relative to the wild-type XcFC2 strain (Katzen et al. 1998) (Figure 1A). We have previously reported that in XcFC2, the

Fig. 1. Functional complementation of the XcI mutant by the *gumI* gene. (**A**) Rescue of mucoid phenotype. Counterclockwise from the upper left: XcFC2 (wt), XcI, XcI/pBGI supplemented with 0.05% arabinose, XcI/pBBAD22K. (**B**) Restoration of Man-GlcA-Man-Glc_2-PP-Pol synthesis. The oligosaccharides from the glycolipids produced after incubating permeabilized cells of XcI (lane 1), XcI/pBGI (lane 2) and XcFC2 (lane 3) with UDP-Glc, GDP-Man and UDP-[^{14}C]GlcA were released from the diphosphate and polyisoprenol and analyzed by PC. The migration of standards [^{14}C] GlcA-labeled GlcA-Man-Glc_2 (T) and Man-GlcA-Man-Glc_2 (P) is indicated. (**C**) Restoration of polysaccharide production. The polymer produced in vivo by *Xcc* strains under the indicated conditions was quantified by the cetylpyridinium chloride method (see *Materials and Methods* for further details) and expressed relative to that produced by XcFC2. Error bars denote the standard deviation from three independent experiments. (**D**) The expression of GumI-His_6 in total extracts of XcI/pBGI induced with L-arabinose was analyzed by SDS–PAGE. The arrowhead points to the GumI protein, and the positions of the molecular weight markers are indicated.

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complete assembled lipid-linked pentasaccharide Man-GlcA-Man-Glc_2-PP-Pol is synthesized, whereas in the XcI mutant, the lipid-linked tetrasaccharide GlcA-Man-Glc_2-PP-Pol dominates (Katzen et al. 1998). This result is confirmed in Figure 1B (lanes 1 and 3). Thus, GumI appears to be a non-processive β-mannosyltransferase that uses GDP-Man as a sugar donor and GlcA-Man-Glc2-PP-Pol as an acceptor. To examine whether the proposed GumI protein was active, we performed in vivo complementation assays with the XcI mutant and the pBGI plasmid. The recombinant plasmid contains the postulated *gumI* ORF linked to a hexahistidine coding sequence inserted into pBBAD22K under the control of the araBAD promoter (Sukchawalit et al. 1999). The colony phenotypes were screened, and the resulting glycolipid intermediate profiles were analyzed. GumI-His_6 was able to rescue the mucoid phenotype and transfer a [^{14}C]Man residue from GDP-Man to the GlcA-Man-Glc_2-PP-Pol in vitro to generate a product that comigrates with the wild-type Man-GlcA-Man-Glc_2-PP-Pol, as shown in Figure 1A and B. Polymers were isolated and quantified from stationary-phase cultures of XcFC2, XcFC2/pBBAD22K, XcI, XcI/pBBAD22K and XcI/pBGI (Figure 1C). The addition of the inductor to the culture media or vector alone in XcFC2 had no significant effect on xanthan production. We observed a decrease in xanthan production of more than 95% in XcI and XcI/pBBAD22K compared with XcFC2. In the presence of 0.05–0.5% L-arabinose, XcI/pBGI transformants produced xanthan at levels similar to XcFC2. In the absence of an inductor, XcI/pBGI produced approximately a 50% less xanthan than XcFC2, suggesting that the expression of GumI was leaky (Figure 1C). Finally, expression of GumI driven by the araBAD promoter was analyzed. The amount of the GumI protein is proportional to the L-arabinose concentration (Figure 1D), suggesting that expression of GumI is not affected by the presence of glucose in the culture medium.

Subcellular localization of the GumI-His$_6$ protein

The lipid-linked tetrasaccharide acceptor of GumI is membrane-associated, suggesting that membrane association might be necessary for GumI activity. To determine the localization of GumI, a cellular fractionation was performed. The *gumI* ORF is predicted to encode a protein of 349 residues that is 38.972 kDa in size. The GumI protein was not detected in the wild-type strain XcFC2 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or immunoblotting with a GumI-specific antiserum (data not shown). Subcellular localization assays were performed by expressing GumI-His_6 (357 amino acids, 40.038 kDa) from pBGI in XcFC2, XcI and Xc1231 (Figure 2A). In each case, a protein of approximately 36 kDa was detected exclusively in the membrane fraction. This value corresponds closely to the predicted recombinant translation product of the *gumI* gene. Aberrant migration patterns are common for membrane proteins (Rath et al. 2009). Thus, GumI is attached to or associated with the *Xcc* membrane fraction.

Membrane-binding properties of GumI

We next asked which types of interactions mediate the association of GumI with the membrane. First, we employed several

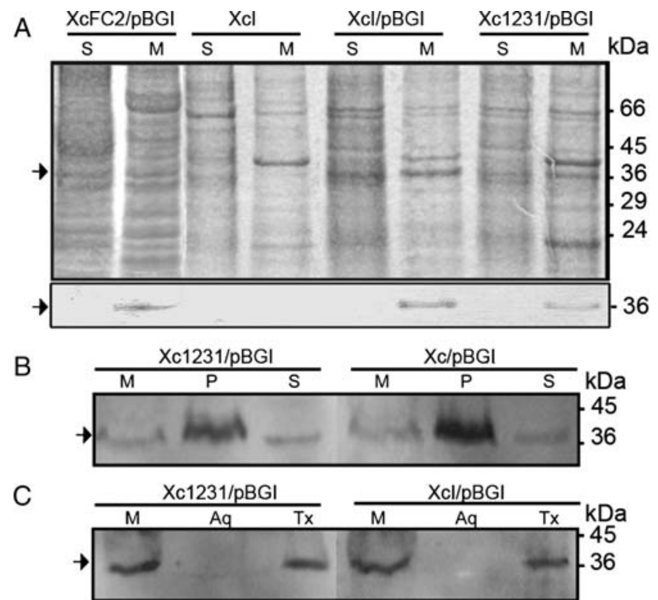

Fig. 2. GumI subcellular localization and membrane association. (**A**) Subcellular localization of GumI. SDS–PAGE and immunoblot of proteins from soluble (S) and membrane (M) fractions of Xcc. (**B**) Solubilization of proteins from cell membranes of XcI/pBGI or Xc1231/pBGI with Na_2CO_3. After treatment, total cell membranes were collected by ultracentrifugation, and samples corresponding to the starting cell membranes (M), insoluble (P) and soluble (S) fractions were subjected to immunoblotting. (**C**) Total cell membranes derived from XcI/pBGI or Xc1231/pBGI were extracted and subjected to phase separation with Triton X-114. Samples corresponding to the starting cell membranes (M), the aqueous (Aq) and detergent phases (Tx) were subjected to immunoblotting. Positions of the molecular weight markers are indicated on the right. The arrows point to the GumI protein.

computational prediction methods to analyze the GumI amino acid sequence. Transmembrane helices or membrane-anchored regions were not identified. GT-B proteins have distinct N- and C-terminal domains that each contain a typical Rossmann fold (Lairson et al. 2008). Analyses of the electrostatic surface potential for the solved structure of GTs belonging to the GT-B superfamily (Hu et al. 2003; Guerin et al. 2009) have indicated that these proteins are polar with positively charged N-domains. By sequence, GumI has a predicted p*I* of 8.4 for the entire protein, 10.3 for only the N-terminal half and 5.4 for the C-terminal half. Thus, basic and hydrophobic amino acids may be involved in mediating membrane association. We investigated whether GumI is associated with the membrane fraction through electrostatic interactions. Total cell membranes obtained from XcI/pBGI and Xc1231/pBGI were incubated in the presence of 0.1 M Na_2CO_3 at pH 11.4. Soluble and insoluble proteins were separated by ultracentrifugation. Immunoblots of the resulting fractions showed that GumI was recovered almost completely in the membrane fraction (Figure 2B), suggesting that the interaction between GumI and the membranes is not purely due to electrostatic binding to phospholipid head groups.

To further understand the anchoring of GumI to the membrane, we separated hydrophilic peripheral membrane proteins from integral proteins in the membranes of XcI/pBGI and Xc1231/pBGI with a Triton X-114 extraction (Brusca and

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Radolf 1994). Proteins in each fraction were analyzed with immunoblots. After phase separation, we observed a complete segregation of GumI in the detergent phase (Figure 2C). Cumulatively, these results show that GumI is an integral monotopic protein.

Expression, purification and function of recombinant GumI-His

To facilitate the purification of GumI and, ultimately, to confirm its enzymatic activity in a cell-free assay, we cloned the polymerase chain reaction (PCR) product corresponding to the *gumI* gene into the pET-22b expression vector. GumI-His$_6$ was expressed in *Escherichia coli* under mild induction conditions to prevent the formation of inclusion bodies. Triton X-100 and high ionic strength was required to extract and solubilize GumI from the membranes. Glycerol was used to keep the protein stable and homogenous and to prevent protein aggregation. GumI was purified in two steps using affinity and size-exclusion chromatography. With SDS–PAGE and immunoblotting, recombinant GumI appears as a unique protein band with an estimated molecular mass of about 36 kDa and no degradation products (Figure 3A). A protein of similar size could also be observed when GumI was tagged at the N terminus (data not shown). With matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), we obtained a molecular mass that is very close to the predicted value, confirming the identity of the purified protein (Supplementary data, Figure S1). The purified protein is conformationally homogeneous, as judged by gel-filtration chromatography and static light scattering (Supplementary data, Figure S2). With this method, 1 L of induced *E. coli* cells yields about 5 mg of purified recombinant protein. GumI-His$_6$ at 5 mg/mL was found to be stable for at least 1 month at 4°C and 5 months at −20°C with no significant loss of activity.

We investigated the enzymatic activity of recombinant GumI-His6 in a cell-free system by incubating it with its predicted substrates. We used an XcI glycolipid extract enriched in GlcA-Man-Glc$_2$-PP-Pol as an acceptor and GDP-[^{14}C]Man as a sugar donor. Under standard conditions, more than 50 nmol of [^{14}C]Man per min per mg of protein were incorporated into the organic phase. The [^{14}C]oligosaccharides were released from the lipid anchor into the aqueous phase, and soluble compounds were analyzed by paper chromatography (PC). A major radioactive product comigrating with standard Man-GlcA-Man-Glc$_2$ was detected (Figure 3B), indicating that the lipid-linked carbohydrate is a pentasaccharide containing Man. To confirm that GlcA-Man-Glc$_2$-PP-Pol is the acceptor of the Man residue, we prepared [^{14}C]GlcA-Man-Glc2-PP-Pol from XcI-permeabilized cells. The labeled acceptor was incubated in the presence of unlabeled

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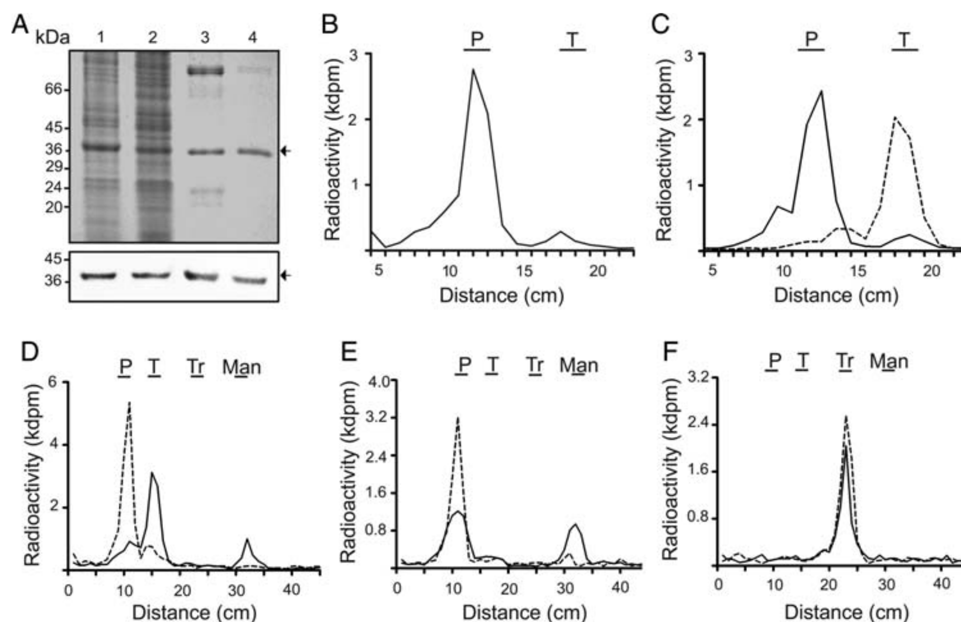


Fig. 3. SDS–PAGE during the course of purification and functional analyses of the GumI-His$_6$ protein. (**A**) The GumI-His$_6$ protein was overproduced in *E. coli*. The two-step purification procedure involved nickel chelating and size-exclusion chromatography, as described in *Materials and Methods*. Aliquots of the sample after each purification step were analyzed by SDS–PAGE and immunoblots. Lane 1, crude extract from BL21(DE3)/pETHisIC after IPTG induction; lane 2, 100,000 × *g* supernatant of BL21(DE3)/pETHisIC disrupted cells; lane 3, 200 mM imidazole-containing fraction; lane 4, purified GumI-His$_6$ eluted from the Superdex 200 column. Positions of the molecular weight markers are indicated on the left. Arrows point to the GumI protein. Purified recombinant GumI-His$_6$ activity was measured by incubating (**B**) GlcA-Man-Glc$_2$-PP-Pol, GDP-[^{14}C]Man and GumI or (**C**) [^{14}C]GlcA-Man-Glc$_2$-PP-Pol and GDP-Man, with (full line) or without GumI (dashed line). Lipid-linked oligosaccharides were extracted and separated by PC. For the anomeric configuration analysis, [^{14}C]Man oligosaccharides obtained from the incubation of XcFC2-permeabilized cells with UDP-Glc, GDP-[^{14}C]Man and UDP-GlcA (**D**) or without UDP-GlcA (**F**) were used as positive and negative controls, respectively. (**E**) The controls and the GumI reaction product obtained in (B) were subjected (full lines) or not (dashed lines) to β-mannosidase treatment and separated by PC. The migrations of the standards Man-GlcA-Man-Glc$_2$ (P), GlcA-Man-Glc$_2$ (T), Man-Glc$_2$ (Tr) and Man are indicated.

GDP-Man and GumI-His_6. Under these conditions, a newly formed radioactive compound comigrating with standard Man-GlcA-Man-Glc2 was observed (Figure 3D). In contrast, in the absence of purified enzyme, only unmodified [^{14}C]GlcA-Man-Glc_2 was observed (Figure 3C).

Because we used a crude acceptor fraction and only small amounts of the product were formed, we could not perform a chemical analysis of the pentasaccharide. Thus, we used *Helix pomatia* β-mannosidase to determine the stereochemistry of the Man linkage formed by GumI. The pentasaccharide obtained using a radioactive donor was digested with β-mannosidase. The release of a [^{14}C]Man residue was observed in the digested GumI product (Figure 3E). [^{14}C]Man-labeled Man-GlcA-Man-Glc2 obtained from XcFC2 released [^{14}C]Man-tetrasaccharide and [^{14}C]Man after digestion (Figure 3D). The absence of α-mannosidase activity was proved using Man-Glc_2 as a substrate (Figure 3F), which contains an α-mannosyl linkage. These results are consistent with a β-mannosyl-glycosidic linkage.

These results suggest that GumI is a nonprocessive β-mannosyltransferase catalyzing the transfer of a mannose residue from GDP-Man to GlcA-Man-Glc_2-PP-Pol to form the lipid-linked pentasaccharide. The formation of the β-mannosidic bond catalyzed by GumI is shown in Scheme 2.

Biochemical conditions for optimal enzyme activity

After obtaining active recombinant GumI, we investigated the reaction conditions for the optimal transfer of Man. The studied reaction parameters included time, pH, cation, detergent and temperature. The incubation of GumI-His_6 with [^{14}C]GlcA-Man-Glc_2-PP-Pol as an acceptor and unlabeled GDP-Man as a glycosyl donor led to rapid and time-dependent elongation of the acceptor to Man-[^{14}C]GlcA-Man-Glc_2-PP-Pol (not shown). GumI-His_6 exhibited maximal transfer around pH 8. Activity decreased below pH 7.5 and above pH 9.5 (Figure 4A). Above pH 10, both substrates would be expected to be chemically instable. The reaction was enhanced by ethylenediaminetetraacetic acid (EDTA), and the addition of divalent cations, such as Mn^{2+} or Mg^{2+}, alone or with EDTA had no significant effect. The addition of Zn^{2+} ions was slightly inhibitory (Figure 4B). The concentration of detergent proved to be an important factor for the transfer of the Man residue. We observed maximal activity in the presence of dodecylmaltoside at a concentration of 33.3 critical micelle concentration (CMC; 6 mM or 0.3%). Maximal GumI activity in the presence of Triton X-100 [133 CMC (32 mM or 2%)] represented about 45% of that observed with dodecylmaltoside (Figure 4C). We also investigated the temperature dependence of the transferase reaction. We found that maximal activity occurs around 35°C with a 20% drop at 20°C and a near-complete inhibition at 60°C, probably due to enzyme denaturation (Figure 4D).

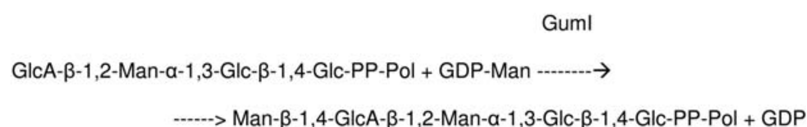
We investigated the donor and acceptor substrate specificity of purified GumI (Supplementary data, Figure S3). Two sugar nucleotides were evaluated as alternative sugar donors for GumI by using standard incubations with [^{14}C]GlcA-Man-Glc_2-PP-Pol (Supplementary data, Figure S3A). ADP-Man can act as a mannose donor, yielding around 35% of the reaction product obtained by using GDP-Man. Interestingly, GDP-Glc showed to be a sugar donor, although yielding an enzyme activity of less than 15%. These results revealed the flexible donor substrate specificity of GumI. We found no indication of mannose transfer when incubating GumI with GDP-Man and [^{14}C]GlcA-Man-Glc_2 (Supplementary data, Figure S3B), suggesting that the diphosphate group and the lipid portion of the acceptor substrate are strictly required for the mannose transfer reaction.

The product of GumK is the acceptor of GumI

In the biosynthesis of the xanthan repeating unit, GumK catalyzes the transfer of a GlcA residue from UDP-GlcA to Man-Glc_2-PP-Pol, forming GlcA-Man-Glc_2-PP-Pol (Barreras et al. 2004), which is the acceptor for the GumI enzyme. The availability of recombinant pure GumK and GumI allowed us to carry out the enzymatic synthesis of Man-GlcA-Man-Glc_2-PP-Pol starting from Man-Glc_2-PP-Pol. GumI and GumK have different optimal temperatures (as well as different substrates): 20°C for GumK and 35°C for GumI. Assays were performed at 25°C for 1 h in a final volume of 100 µL containing one radioactively labeled sugar donor, Man-Glc_2-PP-Pol as the acceptor, and the purified enzymes. β-1,2 glucuronyltransferase (GumK) was assayed using UDP-[^{14}C]GlcA as a sugar donor (Figure 5A). Man-GlcA-Man-Glc_2-PP-Pol was formed only when GumI and GDP-Man were also present (Figure 5B and C). Similar results were obtained with unlabeled UDP-GlcA and GDP-[^{14}C]Man as the radiolabeled sugar donor (Figure 5D). The GumI reaction had a >95% yield.

GumI homologs

The psi-blast algorithm (Altschul et al. 1997) was used to search the nonredundant NCBI database for similarities to the GumI amino acid sequence. After two psi-blast iterations (expected threshold set to 1×10^{-5}), we identified 2217 reliable homolog proteins. These homologs belong to different organisms, mainly Gram-negative and G + C rich Gram-positive bacteria. As expected, the top hits were from *Xanthomonas* species, with almost identical sequences in *X. campestris* pv. *vasculorum*, *X. campestris* pv. *musacearum*, *X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri*, *X. fuscans* sp. *aurantifolii*, *X. oryzae* pv. *oryzicola* and *X. campestris* pv.



Scheme 2. Schematic representation of the mannosyl transfer reaction catalyzed by the nonprocessive-inverting GT GumI.

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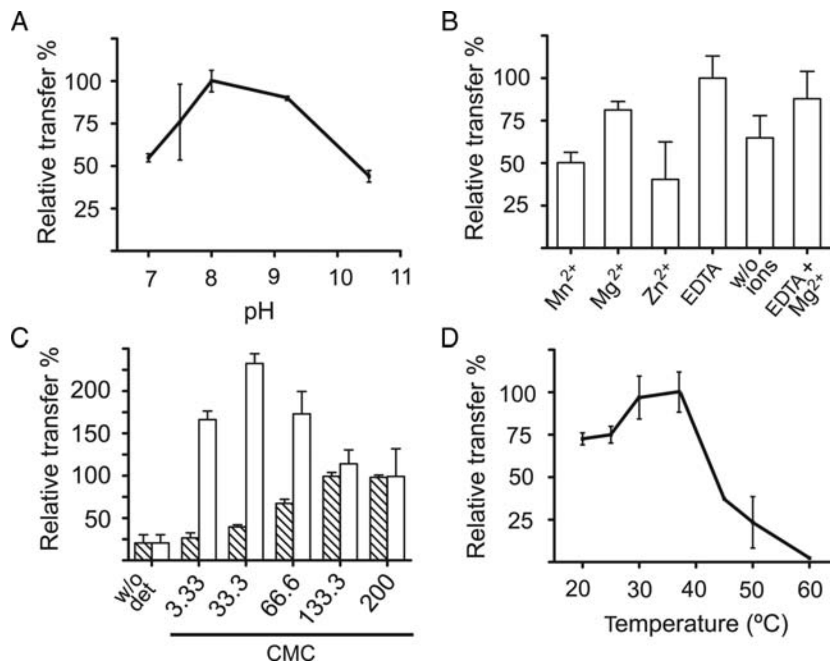

Fig. 4. Analysis of the mannosyltransferase activity of purified GumI. GumI-His$_6$ activity was measured while varying different reaction parameters. (**A**) pH dependence. Tris–HCl buffer was used for pH 7.0–8.0, and glycine-NaOH was used for pH 9.2 and 10.5. (**B**) The effect of metal ions and EDTA. The enzyme was incubated in the presence of 10 mM ions or 10 mM EDTA. (**C**) The effect of detergent. The activity was analyzed in the presence of Triton X-100 (lined bars) and dodecylmaltoside (empty bars). The results are expressed relative to the amount of activity in the presence of Triton X-100. (**D**) Temperature dependence. Each datapoint represents the mean of three independent experiments.

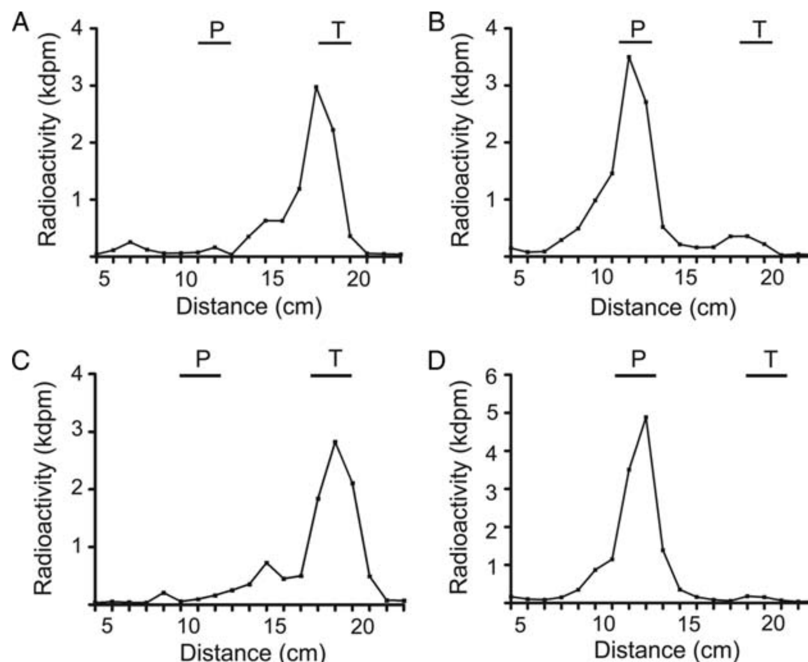

Fig. 5. Enzymatic synthesis of Man-GlcA-Man-Glc$_2$-PP-Pol using recombinant GumK and GumI. The acceptor substrates Man-Glc$_2$-PP-Pol and GumK were incubated for 1 h at 25°C in the presence of (**A**) UDP-[^{14}C]GlcA; (**B**) UDP-[^{14}C]GlcA, GDP-Man, GumI; (**C**) UDP-[^{14}C]GlcA, GumI, without GDP-Man as a negative control; (**D**) UDP-GlcA, GDP-[^{14}C]Man, GumI. Lipid-linked oligosaccharides were extracted, and the oligosaccharide moieties were released. Aliquots were analyzed by PC. The migrations of the standard [^{14}C]GlcA-labeled GlcA-Man-Glc$_2$ (T) and Man-GlcA-Man-Glc$_2$ (P) are indicated.

vesicatoria (84–86% amino acid identities; Supplementary data, Table I). These species produce or are predicted to produce an exopolysaccharide that is identical or similar to xanthan. Proteins from the high G + C Gram-positive *Clavibacter michiganensis*, *Nakamurella multipartite* and *Cellulomonas flavigena* are more than 40% identical to GumI,

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and sequences from *Cellvibrio japonicus* (γ-proteobacteria), *Trichodesmium erythraeum* (cyanobacteria) and *Rhodospirillum centenum* (α-proteobacteria) have an identity of about 30%. The proteins in Table I contain a typical motif for GTs belonging to GT-B superfamily (conserved domain search). The psi-blast searches identified only two proteins with an established function. Both are retaining GTs, and their structures have been solved. They are the membrane-associated PimA, GDP-Man: phosphatidylinositol mannosyltransferase (Guerin et al. 2009) (pdb 2GEJ) (coverage 81%, identities 20%; E, 5×10^{-9}) from *Mycobacterium smegmatis* and the nonmembrane-associated MshA, UDP-GlcNAc: inositol-P *N*-acetylglucosaminyltransferase (Vetting et al. 2008) (pdb 3C48) (coverage 95%, identities 16%; E-value, 3×10^{-8}) from *Corynebacterium glutamicum*. The BLAST searches also identified proteins in Green-nonsulfur bacteria, firmicutes, Cytophaga-Flavobacter-Bacteroides group, verrucomicrobia and Archaea (data not shown). Hence, the *Xcc* mannosyltransferase GumI has homologs in several prokaryote organisms.

Discussion

The results presented in this paper demonstrate that the *Xcc* ORF *gumI* encodes a functional inverting mannosyltransferase that catalyzes the transfer of one β-mannose residue onto GlcA-Man-Glc_2-PP-Pol to form the xanthan glycolipid repeating unit (Figure 6).

GumI is also present in *X. campestris* pv. *vesicatoria*, *X. oryzae* pv. *oryzae*, *X. oryzae* pv *oryzicola*, *X. axonopodis* pv. *citri*, but it is absent in the closely related *Xylella fastidiosa*, according to the published genomic sequences (Simpson et al. 2000). Psi-blast searches also identified homologs in Gram-negative bacteria, Gram-positive bacteria and Archaea. Among the identified homologs, we found that PimA

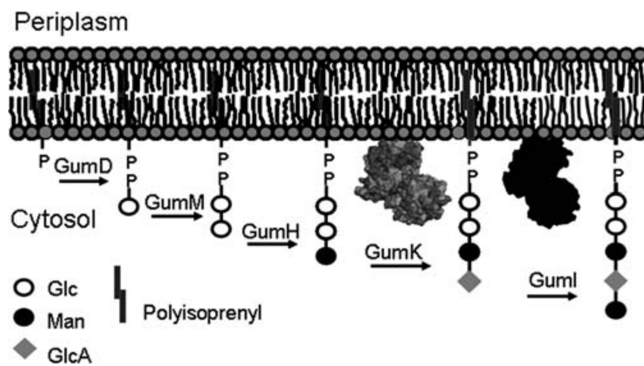

Fig. 6. Model for the formation of lipid-linked pentasaccharide on the cytoplasmic side of the inner membrane of *X. campestris* pv. *campestris*. Biosynthesis starts with the transfer of a Glc-phosphate to Pol-phosphate with the formation of the diphosphate bond, catalyzed by GumD. The second and third steps are catalyzed by GumM and GumH, respectively. Following the addition of the β-1,2-linked glucuronic acid by GumK, the final mannose is transferred by GumI, as demonstrated in this work. The pentasaccharide is then translocated to the periplasm and polymerized. All sugar residues are donated by sugar nucleotides. The membrane and GumK (Barreras et al. 2008) are drawn to scale. GumI is represented as a black shape for illustrative purpose only. The glycolipids have been enlarged several times for clarity.

is the only mannosyltransferase with solved structure. Inspection of GumI and PimA sequences with psi-blast shows that the alignment covers the C-terminal and a part of the N-terminal domains of PimA, but it excludes the proposed membrane association region (Supplementary data, Figure S4). Despite the sequence similarity, the alignment detects only two invariant residues among the residues involved in PimA GDP-Man binding (Guerin et al. 2007). The predicted differences between the GDP-Man binding sites may be related to the mechanistic strategy employed by both enzymes (inverting vs retaining). Further structural studies of GumI are necessary to understand the structural basis for substrate specificity.

Here, we studied the membrane association of GumI, which is essential for the organization and functionality of the polysaccharide biosynthetic apparatus. Computational analysis indicated that the sequence of the GumI protein contains no structural features that can account for its membrane association. We found that GumI segregates in the membrane fraction after cell disruption and centrifugal fractionation. Some bacterial GTs are associated with one layer of the membrane bilayer by ionic and hydrophobic interactions. Integral monotopic proteins are stably associated with the membrane from one side via an amphipathic helix that partially penetrates into the hydrophobic region of the membrane and through electrostatic interactions with the major part of the protein embedded into the soluble fraction (Blobel 1980). Recently, crystal structures for monotopic GTs of the GT-B superfamily have been solved, including MurG (GT-28), WaaG and PimA (GT-4), WaaC and WaaF (GT-9) and GumK (GT-70). In these proteins, the membrane-binding site is located in the N-terminal domain (Guerin et al. 2009). These N-terminal domains contain a patch of basic and hydrophobic amino acids, contributing to basic isoelectric points for these domains. The basic isoelectric point of the N-terminal half of GumI and its association with the membrane fraction suggest that both basic and exposed hydrophobic amino acids of the N-terminal half of the protein may contribute to membrane association and acceptor binding. Alkaline carbonate and detergent extraction showed that GumI behaves as a monotopic membrane protein. The original work of Bordier (1981) established that integral membrane proteins partition preferentially into the detergent phase after thermal condensation of aqueous solutions of Triton X-114. We found that GumI is efficiently extracted into the detergent phase in Triton X-114 partitioning experiments and is almost quantitatively resistant to Na_2CO_3 extraction. In addition, the association of GumI with the membrane does not seem to require the presence of the rest of the *gum* operon, as it is located to the membranes in the *gum* operon-deleted strain Xc1231 expressing only the GumI protein. The same is also true for GumK (Barreras et al. 2008), the other analyzed GT of the xanthan biosynthetic machinery. The functional importance of the presence of substrates, glycolipids, membrane potential, intrinsic curvature strain and critical membrane thickness for catalysis of GTs is now being identified. Guerin et al. (2009) recently reported experimental data supporting a model wherein substrate-induced conformational changes of the GT PimA are important for activity. Ge et al. (2011) propose a model

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where unique bilayer interface segments containing tryptophan residues are crucial for diglycosyldiacylglycerol synthase catalysis and for membrane association. In spite of that, there is much to uncover concerning structure–function relationships of GTs at the protein–lipid interface.

In this study, we used a number of different substrates to analyze the specificity of the enzyme. We found that although GumI prefers GDP-Man, it can also utilize ADP-Man and even GDP-Glc, two nonphysiological sugar nucleotides in *Xcc*. This flexible donor substrate specificity was also shown in other GTs from the GTB superfamily (Losey et al. 2001; Albermann et al. 2003; Kanipes et al. 2003; Chen et al. 2009). In contrast, we observed a strict specificity of GumK for UDP-GlcA as the donor substrate (our work, unpublished). We also observed that GumI is unable to transfer a Man residue to the free-tetrasaccharide used as an acceptor. This result indicates that the lipid moiety in the acceptor is of major importance for acceptor binding and catalysis, although we have not analyzed variations in the structure of the hydrophobic group. In general, a hydrophobic group is required for catalysis of GTs, although the structure of the lipid portion may be flexible (Zhang et al. 2006; Perlstein et al. 2010; Xu et al. 2011).

We purified recombinant GumI and identified the optimal reaction conditions for maximal activity. We found that the addition of EDTA does not affect activity, indicating that divalent cations do not contribute to the reaction. GumI activity requires a hydrophobic environment, as reducing the detergent concentration results in an 80% reduction in activity. This requirement likely reflects the need to protect lipid–protein interaction surfaces and maintains local stability. Unfortunately, the kinetics of the reaction could not be measured because the acceptor was not available in sufficient quantities. Based on the biochemical characterization reported in this work and the fact that GumI is an unclassified GT in the CAZy's classification, we propose that GumI should be the founding member of a new GT family.

The purification protocol presented here is reproducible and capable of producing milligram quantities of stable protein, which will be useful in future experiments to characterize the molecular properties and physiological functions of this membrane-associated protein. Resolving the structure and mechanism of action of GumI will extend our understanding of how precisely this GT functions and how certain structural features contribute to efficient synthesis.

Materials and methods

Materials and general procedures

GDP-Man, UDP-Glc and UDP-GlcA were purchased from Sigma-Aldrich. GDP-[U-^{14}C]Man (280 Ci/mol) and UDP-[U-^{14}C]GlcA (310 Ci/mol) were prepared by the Sugar-Nucleotide Facility, Institute Leloir. Proteins were separated by 10% SDS–PAGE followed either by Coomassie blue staining or immunoblotting after transfer to a polyvinylidene fluoride membrane. Immunoblotting was performed with a monoclonal antipolyhistidine mouse (Sigma-Aldrich, Buenos Aires, Argentina) primary antibody and an antimouse IgG alkaline phosphatase-conjugated (Sigma-Aldrich) secondary antibody. Nitro blue tetrazolium (Promega, Madison, WI, USA) and 5-bromo-4-chloro-3-indolylphosphate (Promega) were used as substrates to develop the immunoblots. Xanthan production in *Xcc* wild-type and mutant strains (grown on XOL-modified medium for 72 h) were quantified by the cetylpyridinium chloride polysaccharide precipitation method (Barreras et al. 2004). Protein concentration was determined by the Bradford method (Bradford 1976).

Bacterial strains, growth conditions and plasmids

E. coli DH5α was used for DNA cloning, and *E. coli* BL21 (DE3) was used for protein overexpression. *E. coli* cells were grown in Luria-Bertani (LB) broth (Sambrook et al. 1989) at 37°C (unless otherwise indicated) with 200 rpm shaking. *X. campestris* XcFC2 (Katzen et al. 1998) is a Rif derivative of the wild-type strain NRRL-B1459. XcI (*gumI::lacZ-aacC1*) and XcK (*gumK::lacZ-aacC1*) are nonpolar *gum* mutant derivatives of XcFC2 and have been described previously (Katzen et al. 1998). Xc1231 is a complete *gum*-deletion mutant derived from NRRL-B1459 (Capage et al. 1987). *Xcc* cells were grown in YM medium (Harding et al. 1993) or in modified XOL medium (Barreras et al. 2004) at 28°C with 200 rpm shaking. Antibiotics were used at the following concentrations (mg/L): for *E. coli*, ampicillin, 200; for *Xcc*, kanamycin, 50; gentamycin, 30; tetracycline, 10. When necessary, L-arabinose was added to the cultures or plates at the indicated concentrations.

To overexpress *gumI* and to purify the enzyme with affinity chromatography, the *gumI* gene (Accession No. U22511, nucleotides 9838–10884) was amplified with PCR from *Xcc* genomic DNA using primers with *Nde*I and *Xho*I sites at each end. This product was then cloned into the same restriction sites of pET22b(+) (Novagen, Madison, WI, USA), yielding the plasmid pETHisIC. To express the *gumI* gene in *Xcc*, the hexahistidine-tagged *gumI* gene was amplified using pETHisIC as template and primers with *Nco*I and *Hind*III sites at each end. This product was then cloned into the same sites of pBBAD22K (Sukchawalit et al. 1999) to yield plasmid pBGI. The primers used for constructing pETHisIC were 5′-GAGGCCGTCATATGAGCGCGTC-3′ and 5′-CCGCTCGAGCAGCGCGGCGTCTTCATC-3′; those used to construct pBGI were 5′-CATGCCATGGGCGTGTCGCTTCGCTG-3′ and 5′-CCCAAGCTTTCAGTGGTGGTGGTGGTGGTG-3′. The restriction sites are underlined. DNA sequencing was performed to ensure that all cloned fragments were correct (DNA Sequencing Service, Instituto Leloir).

Cell fractionation and membrane solubilization

For subcellular fractionation, cells were grown to the stationary phase in YM medium supplemented with 0.5% L-arabinose to induce the expression of the GumI-His$_6$ fusion protein. Cells were collected by centrifugation, washed twice with water and resuspended in resuspension buffer (50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$). Cells were broken by three passages through a French pressure cell at 20,000 psi. Unbroken cells and cellular debris were removed by centrifugation at 12,000 × *g* for 10 min at 4°C. The cell-free lysate was centrifuged at 100,000 × *g* for 1 h at 4°C to separate the total membrane fraction from the soluble fraction. The total

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membrane fraction was solubilized in resuspension buffer in an equivalent volume as the soluble fraction. Both fractions were subjected to SDS–PAGE, and GumI-His_6 was detected with immunoblotting.

To solubilize proteins with a high pH buffer, samples of the membrane fraction were suspended in 0.1 M Na_2CO_3, pH 11.4, at 20°C, at a final protein concentration of 1.5 mg/mL. The suspensions were incubated for 60 min at 4°C with agitation. The samples were centrifuged for 60 min at 100,000 × *g* at 4°C. The supernatants were removed, and the pellets were washed with phosphate-buffered saline (PBS). The supernatant and the wash fractions were pooled, the pH was adjusted with 0.1 volume of 1 M Tris–HCl, pH 7.0, and proteins were recovered by precipitation with 10 volumes of acetone at −20°C. Samples of the resulting supernatants and pellets, which contained peripheral and integral membrane proteins, respectively, were subjected to immunoblotting.

For Triton X-114 phase partitioning of the membrane fraction, we followed the protocol of Brusca and Radolf (1994) to identify integral membrane proteins. A sample of the membrane fraction was incubated with 2% Triton X-114 in PBS buffer at a protein–detergent ratio of 1:10 for 2 h at 4°C. Insoluble detergent material was removed by centrifugation (13,000 × *g*, 10 min, at 4°C). The supernatant was incubated for 10 min at 37°C to induce phase separation. Aqueous and detergent phases were separated by centrifugation at 13,000 × *g* for 10 min. The aqueous phase, which contained solubilized protein and monomeric detergent, was washed with detergent solution. The detergent phase, which contained integral membrane proteins and detergent micelles, was washed with PBS. Each phase was precipitated with 10 volumes of acetone at −20°C for 1 h, followed by centrifugation at 13,000 × *g* for 10 min. Samples were subjected to SDS–PAGE, and GumI-His_6 was detected by immunoblotting.

Heterologous overexpression and purification of GumI-His

E. coli BL21(DE3)/pETHisIC cells were grown at 37°C in LB to mid-log phase. After the culture was shifted to 20°C for 30 min, protein expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 18–20 h at 20°C. Cells were harvested, washed twice in 50 mM Tris–HCl, pH 8.0, and stored at −20°C until use. The frozen cell pellet was thawed, resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 2% Triton X-100, 0.5 M NaCl, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride as protease inhibitor) and disrupted by three passages through a French pressure cell at 20,000 psi. Disrupted cells were diluted with lysis buffer to a protein concentration of 10 mg/mL, incubated at 4°C for 1 h, and centrifuged at 13,000 × *g* for 10 min. The cell-free supernatant was ultracentrifuged at 100,000 × *g* for 1 h. The supernatant was loaded onto a HiTrap Chelatin HP (GE-Healthcare, Buenos Aires, Argentina) column preequilibrated with buffer A (50 mM Tris–HCl, pH 8.0, 0.05% Triton X-100, 0.5 M NaCl, 20% glycerol, 3 mM imidazole) and unbound proteins were removed by washing with buffer A. Bound proteins were eluted with a continuous gradient of 3–350 mM imidazole in buffer A over 12 column volumes. Fractions with >25% of the GumI-His_6 protein were pooled, concentrated 7- to 15-fold to 3–5 mg/mL by ultrafiltration (10,000 MWCO Amicon Ultra, Millipore, Billerica, MA, USA) and applied to a Superdex 200 (GE-Healthcare) size-exclusion chromatography column preequilibrated with buffer B (50 mM Tris–HCl, pH 8.0, 0.05% Triton X-100, 0.15 M NaCl, 20% glycerol). Eluted proteins were analyzed by SDS–PAGE, and GumI-His_6 was detected in immunoblots. GumI-His_6 protein was stored in buffer B containing 40% glycerol at 4°C for immediate use or at −20°C for long periods.

The molecular mass of GumI-His_6 protein was confirmed by MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA). Samples were acidified by adding 0.1% trifluoroacetic acid, spotted with sinapinic acid solution (6 mg/mL sinapinic acid, 0.1% trifluoroacetic acid, 70% acetonitrile) and dried. To assess the conformational homogeneity, the purified protein was analyzed on a Precision Detectors PD2010 light scattering instrument tandemly connected to an LKB 2248 high-performance liquid chromatography and to Waters 486 UV detector. In general, 450 µL of GumI-His_6 (0.8 mg/mL) was loaded on a Superdex 200 (GE-Healthcare) column and eluted with buffer B. The 90° light scattering, refractive index and UV signals of the eluting material were recorded and analyzed with the Discovery32 software supplied by Precision Detectors.

Preparation of glycolipid acceptors

The GumI glycolipid acceptor GlcA-Man-Glc_2-PP-Pol was prepared by incubating permeabilized XcI cells with UDP-Glc, GDP-Man and UDP-GlcA (or UDP-[^{14}C]GlcA for radiolabeled acceptor) as previously reported (Ielpi et al. 1993; Katzen et al. 1998). The GumK glycolipid acceptor Man-Glc_2-PP-Pol was prepared by incubating permeabilized XcFC2 cells with UDP-Glc and GDP-Man (Barreras et al. 2004). The extracts containing the glycolipid acceptor were reduced to an appropriate volume under a stream of nitrogen, solubilized in 0.01% Triton X-100 and used in enzyme activity assays. The concentration of the glycolipid GlcA-Man-Glc_2-PP-Pol was estimated based on parallel incubations employing UDP-[^{14}C]GlcA of low specific radioactivity (0.44 mCi/mol).

For acceptor specificity assays, [^{14}C]GlcA-Man-Glc_2 was added to the reaction mixture as the sole acceptor. The Pol-PP-free acceptor was obtained by mild-acid hydrolysis (in 100 µL of 0.01 N HCl acid for 10 min at 100°C). The GumI activity was assayed in standard reaction mixtures for 10 min, and the aqueous phase was analyzed by PC.

Determination of the GumI-His_6 activity

The activity of GumI is based on the transfer of the [^{14}C]Man residue from the donor GDP-[^{14}C]Man to the acceptor GlcA-Man-Glc_2-PP-Pol. The standard reaction mixture contained 10 µM glycolipid acceptor, 5 mM $MgCl_2$, 2% Triton X-100, 50 mM Tris–HCl, pH 8.0, 0.15 µCi GDP-[^{14}C]Man and 0.2 µg of GumI-His_6, in a volume of 100 µL. The reaction was started by adding GumI-His_6 and incubated at 25°C for 2 min unless otherwise indicated. The reaction was stopped by adding 200 µL of chloroform/methanol (1:1) and 120 µL of water. Glycolipids were then extracted and washed, and the radioactivity in the organic fraction was measured in one-tenth of aliquots. Oligosaccharide moieties were released

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from the diphosphate and polyisoprenol by mild-acid hydrolysis, and aliquots were analyzed by PC in isopropanol/acetic acid/water (27:4:9) performed on no. 1 chromatography paper from Whatman (Maidstone, UK). Paper chromatograms were analyzed with scintillation counting of 1-cm paper fragments or visualized with the ImageQuant software and a Storm 840 scanner (Molecular Dynamics, Sunnyvale, CA, USA). To analyze the effect of time, temperature, pH, metal ions and detergent, all parameters were kept constant except the parameter under study. The CMC values for Triton X-100 (0.25 mM) and dodecylmaltoside (180 µM) were taken from le Maire et al. (2000). Additional details concerning the PC analysis and the preparation of the standards Man-GlcA-Man-Glc_2 and GlcA-Man-Glc_2 have been described previously (Ielpi et al. 1993; Katzen et al. 1998). The specificity of GumI with regard to nucleotide sugars was studied by incubating the enzyme and [^{14}C] GlcA-Man-Glc_2-PP-Pol with 1 mM of ADP-Man, GDP-Glc or GDP-Man (as positive control), in standard reaction mixtures. The reaction was carried out for 10 min and the products in the organic fraction were analyzed as, described for the standard reaction mixture.

β-Mannosidase treatments

The enzyme reaction mixture contained 50 mM sodium citrate, pH 4.4, 11 nCi of [^{14}C]Man-labeled oligosaccharides, and 0.2 U of β-mannosidase from *H. pomatia* (Sigma), in a final volume of 65 µL. Incubations were carried out for 16 h at 25°C and stopped by adding 1 vol of ethanol. Aliquots were analyzed with PC as described above.

Bioinformatic analyses

Amino acid sequence analysis was performed using the publicly available GumI sequence (GenBank® accession number AAA86377.1) and public domain software. To analyze the primary structure, we used the software PROTPARAM (Wilkins et al. 1999). The sequence similarity search was performed with two iterations of psi-blast (Altschul et al. 1997), setting the expected value threshold to 1×10^{-5}. The predictions of transmembrane regions and protein orientations were made using the following software: HMMTOP (Tusnady and Simon 1998), DAS (Cserzo et al. 1997), SOSUI (Hirokawa et al. 1998), TMHMM (Sonnhammer et al. 1998; Krogh et al. 2001) and AmphipaSeeK (Sapay et al. 2006).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

None declared.

Abbreviations

CMC, critical micelle concentration; EDTA, ethylenediaminetetraacetic acid; Glc, glucose; GlcA, glucuronic acid; GlcA-Man-Glc_2, glucuronic acid-β-1,2-mannose-α-1,3-glucose-β-1,4-glucose; GT, glycosyltransferase; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; Man, mannose; Man-Glc_2, mannose-α-1,3-glucose-β-1,4-glucose; Man-GlcA-Man-Glc_2, mannose-β-1,4-glucuronic acid-β-1,2-mannose-α-1,3-glucose-β-1,4-glucose; ORF, open reading frame; PC, paper chromatography; PCR, polymerase chain reaction; Pol, polyisoprenyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Xcc*, *X. campestris* pv. *campestris*.

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