

## Identification of Essential Amino Acids in the Bacterial $\alpha$ -Mannosyltransferase AceA\*

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The  $\alpha$ -mannosyltransferase AceA from *Acetobacter xylinum* belongs to the CaZY family 4 of retaining glycosyltransferases. We have identified a series of either highly conserved or invariant residues that are found in all family 4 enzymes as well as other retaining glycosyltransferases. These residues included Glu-287 and Glu-295, which comprise an EX<sub>7</sub>E motif and have been proposed to be involved in catalysis. Alanine replacements of each conserved residue were constructed by site-directed mutagenesis. The mannosyltransferase activity of each mutant was examined by both an *in vitro* transferase assay using recombinant mutant AceA expressed in *Escherichia coli* and by an *in vivo* rescue assay by expressing the mutant AceA in a *Xanthomonas campestris gumH*<sup>-</sup> strain. We found that only mutants K211A and E287A lost all detectable activity both *in vitro* and *in vivo*, whereas E295A retained residual activity in the more sensitive *in vivo* assay. H127A and S162A each retained reduced but significant activities both *in vitro* and *in vivo*. Secondary structure predictions of AceA and subsequent comparison with the crystal structures of the T4  $\beta$ -glucosyltransferase and MurG suggest that AceA Lys-211 and Glu-295 are involved in nucleotide sugar donor binding, leaving Glu-287 of the EX<sub>7</sub>E as a potential catalytic residue.

polysaccharides. AceA is a non-processive  $\alpha$ -1,3 mannosyltransferase from *Acetobacter xylinum*, which transfers mannose from GDP-mannose (GDP-Man)<sup>1</sup> to polyprenyl-pyrophosphate-linked cellobiose (Glc<sub>2</sub>-PP-Lip) during the assembly of the heptasaccharide repeat unit of the exopolysaccharide acetan (2, 3). Acetan is structurally related to the widely used exopolysaccharide xanthan gum from *Xanthomonas campestris* (see Fig. 1). AceA is homologous to the *X. campestris*  $\alpha$ -1,3 mannosyltransferase GumH, which catalyzes the analogous reaction in the biosynthesis of the repeat unit of xanthan gum. Indeed, the *aceA* gene was cloned by functional complementation of a *X. campestris gumH* mutant strain (2). AceA and GumH are both retaining glycosyltransferases as they catalyze the formation of a glycosidic linkage with retention of stereochemistry about the donor sugar anomeric carbon. It was first reported that AceA and GumH belong to a relatively small family of mostly prokaryotic  $\alpha$ -mannosyltransferases. This family includes the eukaryotic *N*-acetylglucosaminyltransferase PigA, as well as the mannosyltransferases WbdA, WbdB, and WbdC (3), which are required for the assembly of the *Escherichia coli* 09 serotype lipopolysaccharide O-antigen. Moreover, a number of the amino acids, His-127, Ser-162, Lys-211, Leu-270, Glu-287, and Glu-295, were found to be highly conserved in this family and proposed to play a functional role (4).

Bacterial polysaccharides assembled from polyprenol pyrophosphate-linked repeat units form a large group of polysaccharides possessing an extreme structural diversity. The repeat unit is the structural and biosynthetic building block and is present in many important cell surface-related polysaccharides such as the O-antigen portion of lipopolysaccharides and capsular polysaccharides, as well as in exopolysaccharides (1). A large number of genes thought to encode glycosyltransferases involved in the assembly of repeat units have been identified, reflecting the extreme structural diversity of these bacterial

More recent and more wide reaching glycosyltransferase classification schemes have placed AceA in larger families again comprising mostly retaining glycosyltransferases. For example, AceA is found in family 4 of Campbell's scheme (CaZY) (5, 6), in glycosyltransferase 1 family (GT1F) of the Pfam data base (7), and in family NRD1 defined by Kapitonov and Yu (8). CaZY family 4 comprises more than 300 proteins. Pfam GT1F embodies 397 proteins, including all glycosyltransferases of CaZY family 4 and a limited number of glycosyltransferases from families 3 and 5. GT1F enzymes share a 150-amino acid domain called the GT1 domain (GT1D). In AceA, this domain is found toward the COOH terminus and corresponds to the region in which we had previously identified the highly conserved residues Lys-211, Leu-270, Glu-287, and Glu-295 (Fig. 2). Three of these residues (excluding Leu-270) are in fact strictly conserved among all members of CaZY family 4. The two Glu residues, 287 and 295, correspond to the EX<sub>7</sub>E motif. This motif is also found in other important retaining

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<sup>1</sup> The abbreviations used are: GDP-Man, GDP-mannose; Glc<sub>2</sub>-PP-Lip, Glc $\beta$ (1-4)Glc $\alpha$ -P-P-Lip; GT1F, glycosyltransferase 1 family; GT1D, GT1 domain; XcH, *X. campestris gumH*<sup>-</sup>; T4 $\beta$ GlcT, phage T4 $\beta$ -glucosyltransferase; EPS, exopolysaccharide; HCA, hydrophobic cluster analysis; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside; HMGS, human muscle glycogen synthase; .

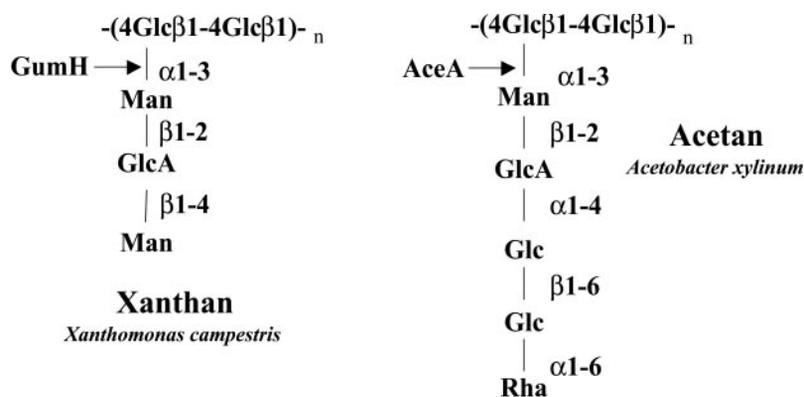


FIG. 1. Structure of the repeat unit of xanthan and acetan.

glycosyltransferases such as yeast and mammalian glycogen synthases, which belong to CaZY family 3 but not to Pfam GT1F. It has been suggested that the Glu residues of the EX<sub>7</sub>E motif may be the catalytic residues of a proposed double displacement mechanism for glycosyltransferases that function with retention of the anomeric configuration (4, 8). However, to date there are little biochemical and no structural data of EX<sub>7</sub>E-containing glycosyltransferases to support this hypothesis.

Using recombinant AceA expressed in *E. coli*, we have recently shown that AceA behaves as a soluble protein and is capable of transferring mannose from GDP-Man to Glc<sub>2</sub>-PP-Lip with formation of an  $\alpha$  linkage *in vitro* and *in vivo*. Here we have extended our studies by using site-directed mutagenesis to create a series of AceA mutants in which each of the highly conserved amino acids identified were replaced by alanine. The functional importance of each mutant was subsequently determined both *in vivo*, by complementation of XcH mutant strain, and *in vitro* with the recombinant form of AceA expressed in *E. coli*. We have found that only mutations at positions Lys-211 and Glu-287, the first Glu of the EX<sub>7</sub>E motif, completely inactivated the enzyme both *in vitro* and *in vivo*. Mutation of Glu-295, the second Glu of the EX<sub>7</sub>E motif, resulted in an enzyme with very low, but reproducible residual activity *in vivo*. Comparison of the primary sequence of AceA with the recent crystal structure of the *N*-acetylglucosaminyltransferase MurG suggests that Lys-211 and Glu-295 may be involved in nucleotide sugar binding, leaving Glu-287 as a potential catalytic residue. Finally, while assessing the integrity of the recombinant form of AceA in *X. campestris*, we have also found that AceA is associated with the membrane fraction, suggesting that AceA is recruited by a membrane receptor.

#### EXPERIMENTAL PROCEDURES

**Strains and Growth Conditions**—The *X. campestris* strains used were FC2 (a derivative of the wild type strain NRRL B-1459) and two mutants defective in xanthan production: XcH (FC2 carrying plasmid pGum52-18S integrated into the  $\alpha$ -mannosyltransferase coding sequence (Ref. 9)) and Xc1231 (a *X. campestris* strain carrying a complete deletion of the *gum* region (Ref. 10)). These strains were grown in YM (11) or in minimal medium (12) at 28 °C. The *E. coli* strains used were JM109, S17-1 (13), XL1-Blue, and BL21 (DE3) (Novagen). They were all grown in Luria-Bertani medium at 37 °C, except BL21 (DE3) that was grown in TB medium (14) for the preparation of recombinant enzyme. Antibiotics were supplemented as required at the following concentrations: tetracycline, 12.5  $\mu\text{g/ml}$ ; kanamycin, 50  $\mu\text{g/ml}$ ; and ampicillin, 100  $\mu\text{g/ml}$ .

**General Procedures**—Protein determinations were done by the method of Lowry, except in membrane preparations, in which a modification of the method was used (15). Quantification of partially purified AceA was done by densitometry of Coomassie Blue-stained gels using Gelworks 1D Analysis software (NonLinear Dynamics Ltd.). SDS-PAGE, Western blot, cloning, and transformation procedures were done according to established protocols.

**Bioinformatic Methods**—The secondary structure predictions were

created by the Jpred<sup>2</sup> server (available on the World Wide Web) using AceA as seed (16). We have chosen this method because it uses multiple sequence alignments obtained by BLAST, after removal of redundant sequences. The classification of glycosyltransferases by Campbell *et al.* (5, 6) is accessible at the CaZY data base on the World Wide Web. Threading studies were performed using Procyon (17). Pfam GT1F can be accessed at the Sanger protein families (Pfam) data base on the World Wide Web.

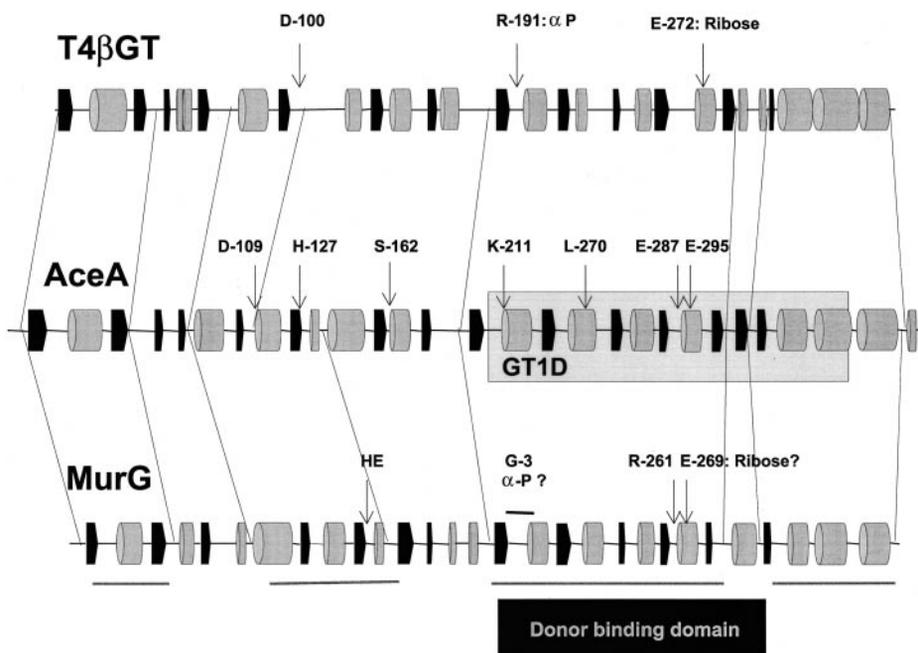
**Molecular Biology and Genetic Procedures**—Site directed mutants were obtained using the USE mutagenesis kit and the Toggle selection primer *SspI/StuI* (Amersham Pharmacia Biotech) on pDGC2 (3), using appropriate mutagenic primers. The fidelity of the mutation reaction was confirmed by single strand sequencing of the complete open reading frame. The cloning of wild type and mutant forms of *aceA* into the expression vector pET29a was done as described previously (3). Positive clones were identified by restriction analysis and termed pCrGC2 s. Subsequently, they were introduced into BL21 (DE3). pBBR-AceA and the corresponding mutants were constructed by cloning a 1,259-base pair fragment comprising the *aceA* gene from pDGC2, into the *KpnI/PstI* site of the broad host range vector pBBR1 MCS3 (tet<sup>R</sup>). The ligation products were used to transform *E. coli* JM109. Clones harboring the desired construct were confirmed by *KpnI/PstI* digestion of plasmid DNA. The plasmids were then introduced into *E. coli* S17-1 and transferred to *X. campestris* by biparental conjugation.

**Overexpression of Wild Type and Mutant AceA S-tag**—The cloning of the AceA open reading frame into the plasmid pET29 to obtain pCrGC2 was done as described previously (3). The S-tag peptide was fused to the NH<sub>2</sub>-terminal end of the AceA protein. *E. coli* BL21 (DE3)/pCrGC2 cells were grown as indicated above. When the cultures reached an A<sub>600</sub> value of 0.8, protein expression was induced by adding IPTG at a final concentration of 1 mM. After 2 h, cells were harvested by centrifugation and washed with 70 mM Tris-HCl, pH 8.0. Aliquots of induced and uninduced samples were resuspended in denaturing buffer and submitted to SDS-PAGE. Proteins were detected by Coomassie Blue staining. The fusion AceA-S-tag was confirmed by Western blot using the S-tag Western blot kit (Novagen) according to the manufacturer's instructions.

**Antibody Preparation**—The rabbit polyclonal antiserum to the AceA protein was obtained from renatured inclusion bodies. The inclusion bodies were obtained by overexpression of wild type AceA in *E. coli* BL21 (DE3)/pCrGC2. Cells were collected by centrifugation and disrupted as described below. The total cell extracts were centrifuged at 3,000  $\times g$  for 5 min at 4 °C to remove aggregates. The inclusion bodies were recovered from the 3,000  $\times g$  supernatant by centrifugation at 13,000  $\times g$  for 15 min at 4 °C. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, and washed twice with the same buffer supplemented with 2 M urea and 0.5% Triton X-100. The washed inclusion bodies were resuspended in 50 mM Tris-HCl, pH 7.5, 8 M urea, and agitated at 4 °C during 24 h for solubilization. Afterward they were centrifuged at 12,000  $\times g$  for 10 min at 4 °C. The supernatant was diluted to 0.3 M urea. Urea was removed by ultrafiltration through an Amicon PM 10 membrane. The remaining protein was lyophilized and used to immunize rabbits (18). For the purification of the antiserum, an affinity resin was obtained by linking an induced extract of BL21 (DE3)/pET29a to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). The binding reaction was performed in the batch mode at 4 °C in a shaker at 30 rpm overnight. The obtained supernatant was enriched in antibodies specific to AceA.

**Subcellular Fractionation and Protein Purification**—Fractionation of *E. coli* or *X. campestris* cultures was performed as follows. Cells were

**FIG. 2. Secondary structure alignment of AceA, T4 $\beta$ GlcT, and MurG.** Secondary structure of T4 $\beta$ GlcT was obtained from the PDB entry 1C3J and MurG from the entry 1F0K. The prediction of the secondary structure of AceA was obtained as described under "Experimental Procedures." This alignment was also derived from an alignment of the HCA plots (available upon request). The gray barrels correspond to  $\alpha$ -helices and the dark arrow  $\beta$ -sheets. Gray horizontal lines indicate the regions of similar secondary structure (also joined by vertical lines). The GT1D is indicated by a gray box in AceA. Location of mutated amino acids in AceA, and the equivalent amino acid or regions in T4 $\beta$ GlcT and MurG are shown by a vertical arrow. The thick black box indicates that this domain is mostly involved in the binding of the nucleotide sugar in both MurG and T4 $\beta$ GlcT.



grown as described above and collected by centrifugation. After washing with 70 mM Tris-HCl, 10 mM EDTA, pH 8.0, they were resuspended in an appropriate volume of the same buffer and disrupted by three passages through a French pressure cell (18,000 p.s.i.). The cell extract was successively centrifuged at  $2,000 \times g$ ,  $15,000 \times g$ , and finally at  $100,000 \times g$  during 1 h. Aliquots of each fraction were then analyzed by SDS-PAGE and/or Western blot. Partial purification of AceA was accomplished from the  $100,000 \times g$  supernatants of *E. coli* BL21 (DE3)/pCrGC2, using the S-tag thrombin purification kit (Novagen). AceA was eluted with biotinylated thrombin according to the manufacturer's instructions. Thrombin was subsequently removed with streptavidin-agarose (Novagen). AceA represented 40–60% of total protein in the purified fraction, as judged by densitometry of Coomassie Blue-stained gels.

**In Vitro Activity of Recombinant AceA**—The *in vitro* activity assays were carried out as described previously (3). The acceptor (Glc<sub>2</sub>-PP-Lip) was freshly prepared using *X. campestris* permeabilized cells. The acceptor was immediately used in the mannosyltransferase assays. The reaction mix contained: 0.4  $\mu$ g of AceA, 1% Triton X-100, 6 mM MgCl<sub>2</sub>, 15  $\mu$ l of cold acceptor, and 0.25  $\mu$ Ci of GDP-[<sup>14</sup>C]Man in a final volume of 100  $\mu$ l. After incubation at 37  $^{\circ}$ C for 8 min, the reaction was stopped by addition of 200  $\mu$ l of chloroform:methanol (1:1). Glycolipids were isolated from the organic phase and the protein pellet. Oligosaccharides were released by mild acid hydrolysis and treated with alkaline phosphatase as described (3). Aliquots of the remaining aqueous phase were counted in a liquid scintillation counter, and the rest of the samples concentrated and submitted to TLC.

**Assay of Mannose Incorporation in Permeabilized XcH**—XcH cells expressing the different forms of AceA were permeabilized by EDTA treatment as described previously (19). The reaction mixtures contained: 70 mM Tris-HCl, pH 8.2, 8 mM MgCl<sub>2</sub>, permeabilized cells (0.6–0.8 mg of protein), 4 mM UDP-glucose, and 0.25  $\mu$ Ci of GDP-[<sup>14</sup>C]Man (277  $\mu$ Ci/ $\mu$ mol) in a final volume of 70  $\mu$ l. Reactions were carried out at 20  $^{\circ}$ C for 30 min and stopped by adding 200  $\mu$ l of 70 mM Tris-HCl, 10 mM EDTA buffer, pH 8.2. The reaction mixtures were centrifuged and the cells washed with the same buffer. Glycolipids were obtained from the cell pellet by extraction with chloroform:methanol:water (1:2:0.3). Oligosaccharides were obtained and processed as described above.

**Quantification of Secreted Polysaccharides**—Cultures of XcH harboring either the wild type or a mutant *aceA* gene were grown in minimal medium (12) during 48 h. Cells were separated by centrifugation, and 500- $\mu$ l aliquots of the supernatants were added with two volumes of ethanol. The precipitated xanthan was resuspended in water, and quantification was done by the meta-hydroxybiphenyl method (20).

**Spectroscopic Studies of Secreted Polysaccharides**—The relevant *X. campestris* strains were cultured in minimal medium (12) for 48 h. Cells from 1-liter cultures were removed by centrifugation at  $3,000 \times g$ . Polymers were precipitated by adding two volumes of ethanol and 1 M

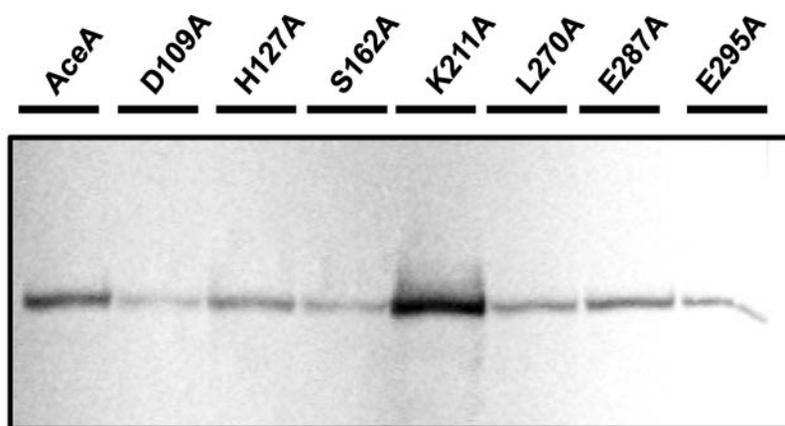
NaCl to the culture supernatants. The precipitates were recovered by centrifugation, and subsequently washed with ethanol at 70%, 75%, 80%, 85%, 90%, and 95%. The samples were resuspended in water and sonicated to reduce viscosity. Finally they were lyophilized and resuspended in D<sub>2</sub>O. The <sup>1</sup>H NMR spectra were recorded with a Bruker 300-MHz spectrometer and referenced to residual H<sub>2</sub>O.

## RESULTS

**Secondary Structure Prediction and Location of the Conserved Amino Acids**—AceA residues His-127, Ser-162, Lys-211, Leu-270, Glu-287, and Glu-295 were initially identified as conserved residues as a result of sequence comparison studies performed on a group of glycosyltransferases, which comprises mostly retaining mannosyltransferases (4). Three of these residues, Lys-211, Glu-287, and Glu-295, are strictly conserved within all members of CaZY family 4, which comprises retaining glycosyltransferase with a wide variety of donor and acceptor specificities. In order to gain insight as to how these conserved residues may be situated with respect to the structure of AceA, we performed secondary structure predictions using the Jpred<sup>2</sup> server and AceA as seed (see "Experimental Procedures"). The strength of this method is that the prediction is made using proteins similar to the seed (AceA). As shown in Fig. 2, AceA and other proteins that belong to the GT1F are predicted to consist of alternating  $\alpha$ -helices and  $\beta$ -sheets (data not shown). This prediction is compatible with a variant of the Rossman fold. A common structural feature of the few available crystal structures of glycosyltransferases resolved to date is the presence of variants of the Rossman fold. This  $\alpha/\beta$  fold consists of an  $\alpha$ - $\beta$ - $\alpha$  sandwich and is involved in the binding of the nucleotide moiety of the nucleotide-diphosphosugar (21–25). In the case of AceA, each of the conserved residues except Glu-295 are found in regions of the protein predicted to be close to the beginning of  $\alpha$ -helices or the ends of  $\beta$ -sheets, which is consistent with the idea that these residues may play some functional role.

To search for further ideas about the structure of AceA, and the putative function of conserved amino acids, we have performed threading studies using AceA and other family 4 protein sequences as seed. The best and most significant scores were obtained with the structure of the *E. coli* N-acetylglucosaminyltransferase MurG and T4 $\beta$ GlcT. MurG transfers GlcNAc from UDP-GlcNAc to undecaprenol pyrophosphate-linked

**FIG. 3. Subcellular localization of AceA in *E. coli* BL21 (DE3).** Production of recombinant proteins and fractionation of cell extracts were carried out as described in "Experimental Procedures." 100,000  $\times$  g supernatants containing 15  $\mu$ g of total proteins were submitted to 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The different forms of the AceA-S-tag fusion proteins were detected with the S-protein alkaline phosphatase conjugate.



MurNac during the biosynthesis of the peptidoglycan building block, while T4 $\beta$ GlcT glucosylates DNA at 5-hydroxymethylcytosine residues. Both of these enzymes act with inversion of the configuration of the reaction center. MurG belongs to CaZY family 28, which is thought to be related to families 3, 4, and 5. T4 $\beta$ GlcT remains unclassified. Secondary structure predictions of AceA and the three-dimensional structures of MurG and T4 $\beta$ GlcT, as well as a comparative HCA analysis, led to a secondary structure alignment of these three glycosyltransferases (see Fig. 2). Although neither MurG nor T4 $\beta$ GlcT possess an EX<sub>7</sub>E motif, both possess a Glu residue in the middle of an  $\alpha$ -helix at the equivalent position as AceA Glu-295. In both the T4 $\beta$ GlcT and MurG crystal structures, the glutamate side chain makes a direct hydrogen bond to the ribose-OH group of the nucleotide sugar donor (21, 22, 25). In the structure of T4 $\beta$ GlcT bound to UDP, Arg-191 interacts with the  $\alpha$ -phosphate of UDP (21, 22). Secondary structure alignments based on the threading results suggested that the conserved residue Lys-211 of AceA is in the same position as Arg-191 of T4 $\beta$ GlcT. Similarly, the model of MurG bound to UDP-GlcNAc predicts contacts between the  $\alpha$ -phosphate of the nucleotide sugar donor and of the G3 loop (25), which is located in the same position as the loop of AceA containing Lys-211. Finally, the model of UDP-Glc bound to the nucleotide binding site of T4 $\beta$ GlcT revealed that Asp-100 of T4 $\beta$ GlcT is in the best position to act as catalytic amino acid (22). Secondary structure alignments, based on the threading results, suggested that nonconserved Asp-109 of AceA is in the same position as Asp-100 of T4 $\beta$ GlcT. With these clues in mind, we have created a series of site-directed mutants of each of the residues of interest within AceA, *i.e.* Asp-109, His-127, Ser-162, Lys-211, Leu-270, Glu-287, and Glu-295, in which each targeted residue was replaced by Ala.

**Characterization of Mutant Forms of AceA Expressed in *E. coli***—In order to carry out biochemical studies to elucidate the function of conserved amino acids, it was necessary to overexpress wild type and mutant forms of AceA in *E. coli*. The site directed mutants were expressed as described under "Experimental Procedures." Total cell extracts obtained before and after induction with IPTG were submitted to SDS-PAGE. A major band of about 46 kDa, corresponding to AceA, appeared upon induction with IPTG (data not shown). It was previously reported that AceA behaves as a soluble protein when expressed in *E. coli* (3). None of the mutations affects the localization of AceA in *E. coli*, since all of them are found in the cytosol (data not shown). The expression levels of the different forms of AceA in the 100,000  $\times$  g supernatants differ considerably, probably due to differences in stability (Fig. 3).

Wild type and mutant forms of AceA were purified from the 100,000  $\times$  g supernatants by affinity chromatography using

S-protein agarose to bind the S-tag fusion epitope. The purified fraction contained AceA and a 66-kDa protein. Amino-terminal microsequencing revealed that the 66-kDa protein corresponds to the *E. coli* chaperone GroEL (data not shown). For the *in vivo* activity assay, the different forms of AceA were quantified by densitometry of a Coomassie Blue-stained acrylamide gel.

To evaluate the effect of amino acid replacement on enzymatic activity, we have determined the ability of the different forms of AceA to transfer [<sup>14</sup>C]Man from GDP-[<sup>14</sup>C]Man to the native Glc<sub>2</sub>-PP-Lip acceptor isolated from *X. campestris* as described previously (3). Since the Glc<sub>2</sub>-PP-Lip can only be isolated in minute quantities, it was not possible to assess either its purity or precise concentration. To overcome these limitations, we have used the same stock of freshly prepared acceptor for each series of experiments, allowing us to determine the  $V_i$  of the wild type and mutant proteins for a given concentration of substrate (Fig. 4). Replacement of Asp-109, His-127, or Ser-162 by Ala resulted in a strong decrease of  $V_i$  (97–98% of wild type AceA). By contrast, Ala replacement of either of the highly conserved amino acids present in GT1D (Lys-211, Glu-287, and Glu-295) led to complete inactivation of the protein as judged by this assay. The mutant AceA-L270A retained 40% of AceA activity. Release of the radioactive oligosaccharides from the lipid anchor and TLC analysis confirmed that AceA, and its enzymatically active variants D109A, H127A, S162A, and L270A (data not shown) produced the expected trisaccharide.

**Effect of the Substitution of Conserved Amino Acids in AceA on the Complementation of the XcH Strain**—To further assess the importance of the conserved amino acids to AceA activity, the wild type and mutant forms of the enzyme were introduced into the XcH mutant strain that does not produce xanthan gum (*gum*<sup>-</sup> phenotype) as a result of an insertion in the GumH coding sequence (9). Quantification of the xanthan accumulated by the XcH strain expressing AceA mutant forms provides a powerful means to detect minor levels of AceA activity that may not be detected by the *in vitro* assay. Differences in colony morphologies were observed among the transconjugants (data not shown). The mucoid phenotype, a distinctive feature of *gum*<sup>+</sup> strains, was partially restored in XcH complemented with wild type AceA as well as D109A, H127A, S162A, and L270A, while complementation with K211A, E287A, and E295A resulted in a non-mucoid phenotype. Xanthan produced by each of the complemented strains was isolated and quantified as described under "Experimental Procedures" (Fig. 5). Since the quantity of xanthan produced by *X. campestris* XcH (*gumH*<sup>-</sup>) complemented with wild type AceA was 60% less than that normally produced by the wild type strain *X. campestris* FC2, the amount of xanthan produced in the site-directed mutants was compared with that of XcH complemented with AceA

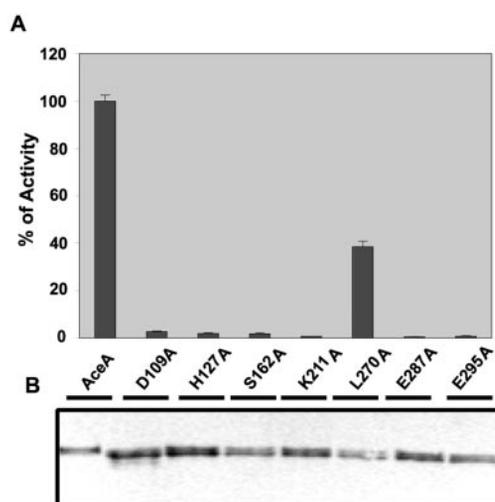


FIG. 4. *In vitro* activity of AceA-S-tag. The activities of partially purified fusion proteins were detected as described under "Experimental Procedures." *A*, relative activities of the mutant forms compared with wild type AceA. Values are the mean of two determinations. *B*, normalized amount of protein used for the *in vitro* assay, detected by Western blot with the S-protein alkaline phosphatase conjugate.

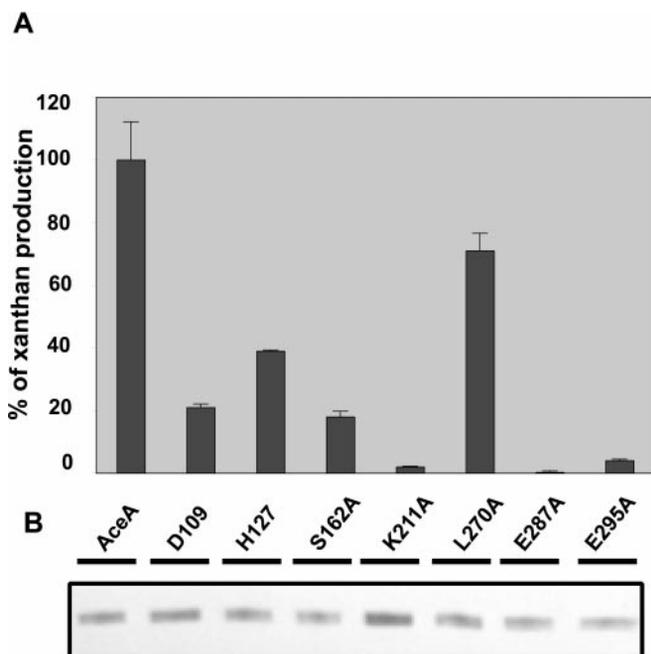


FIG. 5. Xanthan production in XcH complemented strains. Xanthan produced by XcH strains harboring different forms of AceA in 48-h cultures was isolated and quantified as described under "Experimental Procedures." *A*, relative amounts of xanthan produced by the mutant forms compared with wild type AceA. The percentages shown are the mean values of three independent determinations. *B*, normalization of protein quantities shown by Western blot with the polyclonal antiserum raised against AceA. D109 and H127 stands for D109A and H127A, respectively.

(100%). XcH AceA-L270 accumulated 70% as much xanthan as XcH AceA did. Surprisingly, the XcH strains expressing the mutants that are marginally active *in vitro*, D109A, H127A, and S162A accumulated between 20 and 40% of AceA *in vivo*. The levels of polysaccharide in the XcH strains harboring K211A and E287A were not distinguishable from the non-complemented XcH, indicating again that these two amino acids are strictly essential for biochemical activity. Interestingly, the XcH strain expressing AceA-E295A produced a low, but detectable level of EPS (4%). These results also show that

XcH AceA-K211A and -E287A are completely incapable of restoring EPS synthesis.

To confirm that the EPS produced by XcH strains expressing mutant forms of AceA is actually xanthan, we have purified the polysaccharides and submitted them to structural analysis. Since E295A produced very little amounts of polysaccharide, we have used the strain XcH E295G that produces slightly more EPS than XcH E295A.<sup>2</sup> The EPSs produced by strains D109A, H127A, S162A, L270A, and E295G were viscous, indicating the presence of a high molecular weight polysaccharide. The <sup>1</sup>H NMR spectra of D109A, H127A, S162A, and L270A display the characteristic signals of xanthan gum, in particular at  $\delta$  1.8, 2.1, and 5.2, which correspond to the acetyl, ketal pyruvate and  $\alpha$ (1-3)-Man moieties (Fig. 6). The same peaks were also found in the <sup>1</sup>H NMR spectrum of the EPS isolated from E295G, however, other peaks are also present and they may correspond to yet uncharacterized polysaccharides.<sup>3</sup> We have concluded then that D109A, H127A, S162A, L270A, and E295A produce xanthan gum. On the other hand, the ethanol-insoluble material from XcH strains expressing either K211A or E287A did not show a viscous appearance and was of insufficient quantity for NMR analysis.

*[<sup>14</sup>C]Mannose Incorporation in the XcH Strains Expressing Wild Type and Mutant AceA Forms*—Since it is possible that AceA mutant forms K211A and E287A block the production of xanthan due to uncontrolled transfer to other substrates, we have analyzed the lipid-linked oligosaccharides in the different complemented XcH strains. Permeabilized XcH cells complemented with the different forms of AceA, were incubated in presence of GDP-[<sup>14</sup>C]Man. The glycolipid fraction was isolated, and oligosaccharides were released and analyzed by TLC (see "Experimental Procedures"). Although the rapid transformation of Man-Glc<sub>2</sub>-PP-Lip into the higher intermediates of xanthan gum biosynthesis prevented its accurate quantification, we found that the trisaccharide [<sup>14</sup>C]Man-Glc<sub>2</sub> was produced by all the strains except XcH AceA-K211A and XcH AceA-E287A (data not shown). These two strains did not produce any other labeled oligosaccharide. These results indicate that the failure of the XcH AceA-K211A and E287A to produce xanthan gum is due to inactivation of AceA.

*Subcellular Localization of AceA in the XcH Strain*—Since either the lack and/or reduction of activity could be due to low levels of the recombinant AceA protein when expressed in XcH, we have immunolocalized AceA in Western blots of total extracts from the different XcH strains as described under "Experimental Procedures." AceA was found to be present in these extracts (data not shown). Nevertheless, lack of activity may be due to a different subcellular location of the mutant AceA forms. We then performed a subcellular fractionation of total extracts, and localized AceA by Western blot. Although in *E. coli* AceA is present in the soluble fraction, unexpectedly in the XcH strains, AceA was found to be associated with the membrane fraction. As shown in Fig. 7, all the mutant forms of AceA were also present in the membrane fraction and are expressed at least at the level of wild-type AceA. In all cases more than 90% of AceA was found associated to the membrane fraction. It has been suggested that polysaccharide biosynthesis enzymes are organized in a multiprotein complex associated with membranes (1, 26). Knowing that AceA is expressed as a soluble protein in *E. coli*, we reasoned that another Gum protein may recruit AceA to the membrane. To test this hypothesis, we introduced AceA into *X. campestris* Xc1231, a strain lacking the *gum* region, and looked for the subcellular localization of AceA

<sup>2</sup> P. L. Abdian, unpublished results.

<sup>3</sup> A. Heyraud, unpublished results.

FIG. 6.  $^1\text{H}$  NMR spectra of exopolysaccharide produced by XcH strain expressing different forms of AceA. Exopolysaccharides were purified as described under "Experimental Procedures." The vertical arrow shows the peak at  $\delta$  5.2, characteristic of the  $\alpha(1\text{--}3)\text{Man}$ , P stands for pyruvate, A for acetate, and the asterisk shows a peak corresponding to an uncharacterized polysaccharide.

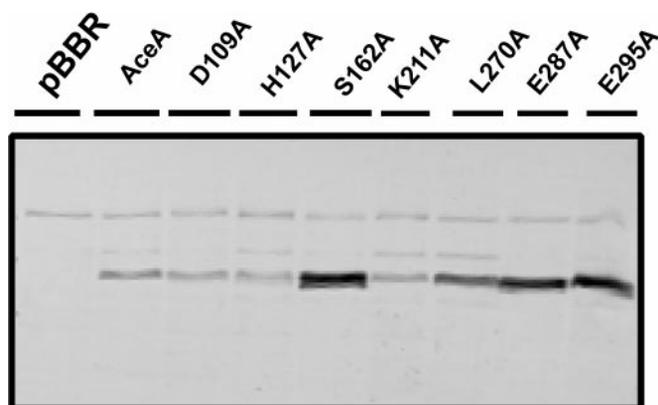
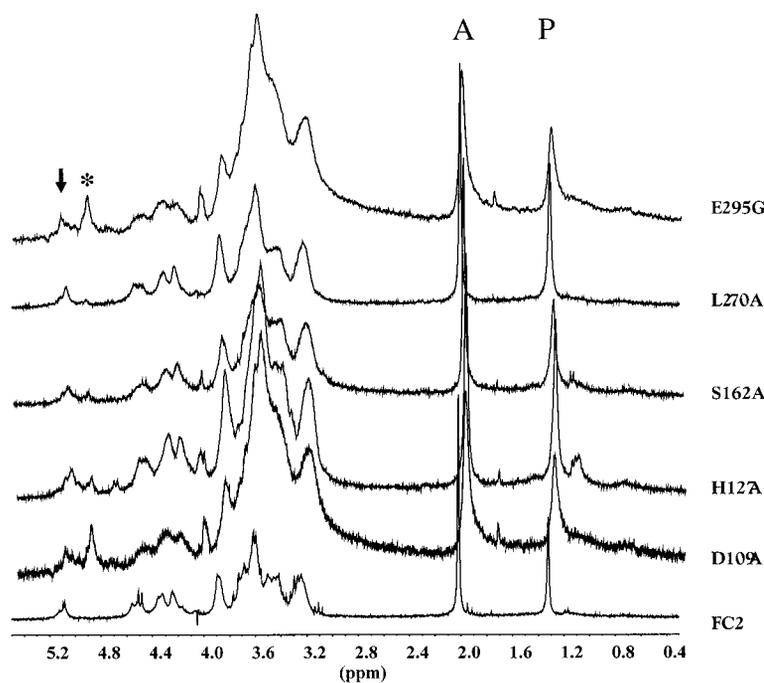


FIG. 7. Subcellular localization of mutant forms of AceA in XcH complemented strains. XcH cell cultures harboring wild type or mutant forms of AceA were disrupted and subsequently fractionated into membrane and soluble fractions. Samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. AceA was detected in membrane fractions ( $100,000 \times g$  pellets) using a partially purified polyclonal antiserum against wild type AceA (see "Experimental Procedures").

by Western blot. In most of the experiments, AceA was not detected in total extracts, preventing cell fractionation experiments. However, in one single experiment, we found that AceA was expressed in small quantities and that it was associated with the membrane fraction (data not shown). This result shows that AceA is associated with membranes even in the absence of other proteins of the *gum* region, although is perhaps less stably expressed. This suggests that other protein partners for AceA may exist that differ between *E. coli* and *X. campestris*.

#### DISCUSSION

Here we report the results of the mutational analysis of a number of amino acids of the  $\alpha$ -1,3 mannosyltransferase AceA that are highly conserved among CaZY family 4 glycosyltransferases. These residues include His-127, Ser-162, Lys-211, Glu-287, Glu-295, and Leu-270 (4). Of these six residues, three (Lys-211, Glu-287, and Glu-295) are strictly conserved within the family. In addition, we have analyzed a mutant of residue

Asp-109. This residue was identified as a potential catalytic residue after threading experiments against the known three-dimensional structure of T4 $\beta$ GlcT.

The targeted amino acids can be classed into four groups on the basis of the phenotype of the corresponding Ala substitution in AceA. First, mutation of residues Lys-211 and Glu-287 resulted in complete loss of AceA activity, as judged by both the *in vitro* mannosyltransferase assay and by the *in vivo* complementation assay. In contrast, mutation of Glu-295 resulted in a slightly different phenotype where AceA E295A was completely inactive *in vitro* but was capable of restoring xanthan production in the *in vivo* assay to a very low but reproducible level ( $\sim 4\%$  of wt AceA). Residues Asp-109, His-127, and Ser-162 constitute a third group in which replacement of Ala resulted in a loss of 97–98% of activity *in vitro*; however, these mutants retained a significant ability to produce xanthan in the *in vivo* assay (20–40%). Finally, despite the fact that the residue Leu-270 is highly (although not strictly) conserved, AceA L270A retained 40% of wild type activity *in vitro* and 71% of the activity *in vivo*, strongly suggesting that this residue does not play a critical role in the reaction mechanism. These results confirm that the three highly conserved residues play an important role in AceA activity both *in vitro* and *in vivo*, although only Lys-211 and the first glutamate residue of the EX<sub>7</sub>E motif, Glu-287, are indispensable for activity.

By analogy with the well defined mechanism of retaining glycosylhydrolases, it has been proposed that retention of the configuration of the reaction center during glycosyltransfer would be achieved by a double displacement mechanism involving two catalytic residues with the formation of a glycosyl-protein intermediate (27). Acidic residues such as Glu or Asp would be the prime candidates to act as catalytic residues. In the case of proteins harboring the GT1 domain, it has been proposed that the two Glu residues of the EX<sub>7</sub>E motif are the catalytic amino acids (4, 8, 28). A role for catalysis of the first Glu of the EX<sub>7</sub>E motif, Glu-287, is supported by our mutagenesis results but is neither supported nor contradicted by the structural comparisons as neither MurG nor T4 $\beta$ GlcT possess this residue. Interestingly, all known MurG proteins cloned from different Gram-negative bacteria have a strictly conserved Arg residue (Arg-261) in place of Glu-287. The func-

tional importance of the EX<sub>7</sub>E motif (residues Glu-510 and Glu-518) of the human muscle glycogen synthase (HMGS) was recently evaluated by replacement of either residue by Ala (28). HMGS is a retaining processive glycosyltransferase that belongs to CaZY family 3. The results obtained are essentially the same as those reported here, where mutant E510A of HMGS (equivalent to AceA E287) is essentially inactive, while E518A (corresponding to AceA E295) retains residual activity. It was also found that both HMGS E510A and E518A mutant proteins retained their ability to translocate and bind glycogen, strongly suggesting that the EX<sub>7</sub>E motif is not critical for acceptor substrate binding but rather forms the active site of the glycogen synthase. Recent studies on the retaining processive glycosyltransferase starch synthase II, which belongs to CaZY family 5, again revealed that the replacement of the first residue of the motif Glu-391 (equivalent to AceA Glu-287 and HMGS Glu-510) by Gln inactivates the enzyme (29). The mutagenic results of the second Glu of the EX<sub>7</sub>E motif, AceA Glu-295 and HMGS 518, indicate that this residue plays an important role in the transferase activity, and the threading analysis as well as secondary structure comparison studies with MurG and T4 $\beta$ GlcT suggest that this residue is involved in binding the ribose moiety of the nucleotide sugar donor. It has also been reported that a variant of the EX<sub>7</sub>E motif (EX<sub>7</sub>(E,Y,H)) is present in members of CaZY family 5 (28). If the role of the second Glu of the EX<sub>7</sub>E motif is indeed to bind a hydroxyl group of ribose, it is conceivable that this function could be undertaken by protic amino acids such as Tyr or His.

The secondary structure alignment based on the threading studies with the T4 $\beta$ GlcT suggested that AceA Asp-109 is analogous to T4 $\beta$ GlcT Asp-100 and therefore might be a catalytic amino acid. However, the AceA mutant D109A retained significant activity, especially in the *in vivo* assay, indicating that this non-conserved residue may serve some role in the activity, but is most likely not one of the residues directly involved in catalyzing the transferase reaction. In the T4 $\beta$ GlcT structure, Asp-100 contacts Arg-191 by interdomain salt bridges and also binds the  $\beta$ -phosphate of UDP through a water molecule (22). Therefore, by analogy, reduction in AceA activity upon mutation of Asp-109 may reflect a disruption of the nucleotide sugar binding pocket. Finally, functional roles for residues His-127 and Ser-162, mutation of which resulted in only a partial inactivation of AceA, could not be proposed from the structural comparisons with T4 $\beta$ GlcT and MurG. We note, however, that AceA His-127 is located at a position equivalent to MurG His-125 (25), which in turn is strictly conserved among all MurG proteins cloned to date, and proposed to be located in the acceptor binding site.

All members of CaZY family 4 possess a strictly conserved Lys residue. In AceA this residue is Lys 211. The secondary structure predictions place this residue in a loop early in the COOH-terminal domain between a short  $\beta$ -sheet and an  $\alpha$ -helix. The UDP-binding contacts observed in the T4 $\beta$ GlcT-UDP complex include Arg-191, which is found in an analogous structural position as that predicted for AceA Lys-211 (see Fig. 2). The Arg-191 side chain makes a direct contact with the  $\alpha$ -phosphate of UDP (21, 22). In the model of MurG bound to its substrate UDP-GlcNAc, again, the binding of the nucleotide sugar  $\alpha$ -phosphate is attributed to a loop (G3 loop) (25) in the analogous position as those containing AceA Lys-211 and T4 $\beta$ GlcT Arg-191. These structural observations, taken together with the fact that replacement of AceA Lys-211 with Ala results in a complete loss of activity, lead us to propose that AceA Lys-211 binds one of the phosphate residues of the GDP-Man donor. Interestingly, despite the fact that both MurG and T4 $\beta$ GlcT have been reported to require a divalent cation to

function, no cation has been described in the crystal structures (22, 25). In the group of glycosyltransferases described here, it appears that nucleotide binding involves a direct protein interaction with a positively charged element of the protein. This is in contrast to the "DXD"-containing glycosyltransferases (30), in which the available structures show (24) or suggest (23) that a Mn<sup>2+</sup> ion bind the nucleotide-sugar phosphate groups forming a bridge with the conserved Asp residues in the binding site.<sup>4</sup> Our preliminary results suggest that AceA does not have a strict functional requirement for Mn<sup>2+</sup> or Mg<sup>2+</sup>, as this enzyme is active in the absence of added divalent cation, and retains >70% activity in the presence of 1 mM EDTA or EGTA. Neither glycogen synthase nor starch synthase have requirement for a divalent cation either. It is possible that enzymes such as MurG and T4 $\beta$ GlcT require a divalent cation for effective acceptor substrate binding rather than nucleotide sugar binding, since both their substrates, lipid I and DNA, respectively, also contain pyrophosphates groups. A crystal structure of a retaining glycosyltransferase belonging to CaZY family 4 as well as more sophisticated biochemical studies of AceA and mutant proteins will begin to shed more light on the catalytic mechanisms of this exciting group of glycosyltransferases.

Based on the very low (<3%) *in vitro* activity of recombinant AceA D109A, H127A, or S162A, we expected a low production of xanthan gum in the respective XcH strains. However, the xanthan accumulated by these strain accounts for 21%, 39%, and 18% of the quantities accumulated by XcH expressing wild-type AceA. A similar phenomenon was observed by Garinot-Schneider *et al.* (31) when expressing low active forms of the glucosyltransferase ExoM in the *Sinorhizobium meliloti* *exoM* mutant strain. These results suggest that the bottle neck for exopolysaccharide production is not the assembly of the repeat unit oligosaccharide, but possibly the supply of either enzyme substrates (nucleotide-diphosphosugar or polyprenol), or downstream steps (translocation or polymerization). For example, it has been reported that introduction of a DNA fragment-containing phosphomannose isomerase (first enzyme in the pathway to GDP-Man) into *X. campestris* results in a 10–20% increase in xanthan production (32). Identification of the hierarchy of bottlenecks in the biosynthetic pathway will be important for future biotechnological applications such as polysaccharide engineering.

In our earlier work, AceA expressed in *E. coli* was located in the 100,000  $\times$  *g* supernatant, despite the presence of a predicted transmembrane helix (3). During this work, in verifying the correct expression of AceA in *X. campestris*, it was discovered that AceA is found in the membrane fraction rather than the soluble fraction. This finding suggests that AceA might be retained at the membrane via interactions with other members of the xanthan biosynthetic pathway as would be predicted if a multi-enzyme biosynthetic complex existed. To determine whether the membrane-retaining partner of AceA is part of the xanthan biosynthetic pathway, we expressed AceA in *X. campestris* strain Xc 1231 in which the entire *gum* region has been deleted. Surprisingly, we found that AceA remained associated to the membrane fraction, suggesting that some other feature is necessary for membrane retention in *X. campestris*. Since it was difficult to obtain stable expression of AceA in the *gum* deletion strain, it is possible that this protein is less stable in the absence of the other Gum proteins. As AceA is not native to *X. campestris*, nor does *X. campestris* possess the acetan biosynthetic mechanism that AceA would normally be associated with, these results neither contradict nor confirm the

<sup>4</sup> An Mn<sup>2+</sup> ion was also found in the structure of human glucuronosyltransferase 1 (33).

existence of a multi-enzyme complex being involved in xanthan biosynthesis.

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