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Xanthomonas campestris pv. campestris *gum* Mutants: Effects on Xanthan Biosynthesis and Plant Virulence

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Xanthan is an industrially important exopolysaccharide produced by the phytopathogenic, gram-negative bacterium *Xanthomonas campestris* pv. campestris. It is composed of polymerized pentasaccharide repeating units which are assembled by the sequential addition of glucose-1-phosphate, glucose, mannose, glucuronic acid, and mannose on a polyprenol phosphate carrier (L. Ielpi, R. O. Couso, and M. A. Dankert, J. Bacteriol. 175:2490–2500, 1993). A cluster of 12 genes in a region designated *xpsI* or *gum* has been suggested to encode proteins involved in the synthesis and polymerization of the lipid intermediate. However, no experimental evidence supporting this suggestion has been published. In this work, from the biochemical analysis of a defined set of *X. campestris gum* mutants, we report experimental data for assigning functions to the products of the *gum* genes. We also show that the first step in the assembly of the lipid-linked intermediate is severely affected by the combination of certain *gum* and non-*gum* mutations. In addition, we provide evidence that the C-terminal domain of the *gumD* gene product is sufficient for its glucosyl-1-phosphate transferase activity. Finally, we found that alterations in the later stages of xanthan biosynthesis reduce the aggressiveness of *X. campestris* against the plant.

Xanthomonas campestris pv. campestris is a gram-negative bacterium which is a pathogen of cruciferous plants (15). One of the products of *X. campestris* is an extracellular polysaccharide named xanthan gum. Because of its rheological properties, xanthan is a useful polymer for a growing list of commercial applications (3). The structure of xanthan consists of a β-1,4-linked D-glucose backbone with trisaccharide side chains composed of mannose-(β-1,4)-glucuronic acid-(β-1,2)-mannose attached to alternate glucose residues in the backbone by α-1,3 linkages (33). The mannose residues are acetylated and pyruvylated at specific sites but to various degrees (10, 50) (Fig. 1).

The biosynthetic pathway of xanthan comprises five stages: (i) conversion of simple sugars to nucleotidyl derivative precursors, (ii) assembly of pentasaccharide subunits attached to the inner membrane polyprenol phosphate carrier, (iii) addition of acetyl and pyruvate groups, (iv) polymerization of pentasaccharide repeat units, and (v) secretion of the polymer (24, 29, 31, 32).

Chromosomal regions *xpsIII*, *xpsIV*, and *xpsVI* and a 35.3-kb gene cluster are required for the first stage of xanthan biosynthesis (25, 36). These regions comprise gene functions involved in the biosynthesis of the sugar nucleotide precursors. Proteins related to the subsequent stages of xanthan biosynthesis have been proposed to be encoded by the *xpsI* or *gum* region (11, 23, 54).

The *gum* region encompasses 16 kb of the *X. campestris* genome. Nucleotide sequence analysis predicted the presence of 12 open reading frames (*gumB* to *gumM*) (GenBank accession no. U22511) (Fig. 2).

Functions for the products of these genes have been proposed (11, 13, 26, 51, 54), but strong experimental support has not been presented. The only *gum* gene characterized by genetic studies and biochemical analysis is the *kpt* or *gumL* gene encoding the ketal pyruvate transferase enzyme (37).

Although it has been suggested that extracellular enzymes and xanthan are collectively essential for the pathogenicity of *X. campestris*, the roles of the individual factors are not fully understood (5). Although a correlation between a *gum* gene mutation and reduced plant virulence has been recently shown (12), it is not clear whether any specific step in the assembly, decoration, or polymerization of pentasaccharide repeat units is required for plant infection.

In this report, we describe experimental data for the assignment of a biochemical function to every *gum* gene product. Our results are based on the ability of a defined set of *X. campestris gum* mutants to synthesize lipid sugar intermediates and a polymer by using previously developed in vitro assays (31). We show that the first glycosyltransferase activity within the assembly pathway of the pentasaccharide subunit is severely affected by the inactivation of other genes, and we provide evidence that this catalytic activity is located in the C-terminal domain of the *gumD* product. Furthermore, we analyzed the pathogenicity of several *gum* and non-*gum* mutant strains to determine how the inactivation of different *gum* genes may affect plant virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. *X. campestris* strains were grown in TY (5 g of tryptone, 3 g of yeast extract, and 0.7 g of $CaCl_2$ per liter of H_2O), in modified M9 medium (36), or in YM medium (25) at 28°C. Antibiotics from Sigma (St. Louis, Mo.) were supplemented as required at the following concentrations (in micrograms per milliliter): for *X. campestris*, gentamicin, 30; kanamycin, 50; rifampin, 100; and tetracycline, 10; for *E. coli*, gentamicin, 5; kanamycin, 30; ampicillin, 100; and tetracycline, 10.

DNA biochemistry. Plasmid DNA from *E. coli* was prepared as described by Priefer (42). DNA restriction, agarose gel electrophoresis, cloning procedures, and Southern hybridizations were carried out in accordance with established

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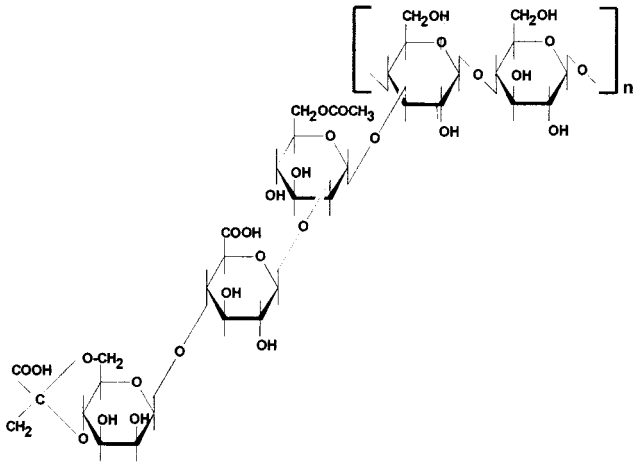

FIG. 1. Organization of the repeating unit of xanthan. The structure of the xanthan repeating unit is according to Jansson et al. (33). The amount of substituents is variable. Some external mannoses contain a second *O*-acetyl instead of a pyruvyl substituent (50).

protocols (45). Total DNA from *X. campestris* was isolated as described by Meade et al. (38). Transformation of *E. coli* cells was performed by the method of Morrison (39). Plasmid DNA was introduced into *X. campestris* cells by electroporation as instructed by Bio-Rad (Richmond, Calif.) or by conjugation as described by Simon (48).

Permeabilized cells, reaction mixtures, and assay procedures. Permeabilized cells were prepared by EDTA treatment of *X. campestris* cells as described previously (31). The standard reaction mixtures for the synthesis of lipid-linked intermediates contained 70 mM Tris-HCl buffer (pH 8.2), 8 mM $MgCl_2$, permeabilized cells (0.6 to 0.8 mg of protein), UDP-glucose, GDP-mannose, and UDP-glucuronic acid, either labeled or not (UDP-[^{14}C]glucose, 15.7 μM; UDP-[^{14}C]glucuronic acid, 17.1 μM; GDP-[^{14}C]mannose, 15.7 μM; UDP-glucose, 285 μM; UDP-glucuronic acid, 285 μM; GDP-mannose, 142 μM). Acetyl coenzyme A (Ac-CoA), 0.7 mM, or phosphoenolpyruvate (PEP), 4.3 mM, was added as indicated later in the text. Reactions were performed in a total volume of 70 μl at 20°C for 30 min and stopped by adding 0.2 ml of 70 mM Tris-HCl–10 mM EDTA buffer (pH 8.2). The mixtures were vortexed and centrifuged at 6,000 × *g* for 5 min, and the pellets were resuspended and washed two times with the same buffer. The combined supernatants, which contain the in vitro polymerization products, were analyzed by gel filtration chromatography on a Bio-Gel A-5m column (30 by 0.9 cm) in 0.1 M pyridinium acetate (pH 5). Fractions of 1 ml were collected, and the amount of radioactivity was determined by liquid scintillation. Data were confirmed by changing the labeled donor or doing duplicate experiments. The washed cell pellets were each extracted three times with 150 μl of chloroform-methanol-water (1:2:0.3, vol/vol), referred to as the 1203 extract, and the organic phase was subjected to paper chromatography in a solvent system of ethanol (96%)-ammonium hydroxide (7:3) (solvent A). Alternatively, the glycolipids present in the 1203 extract were mild-acid hydrolyzed (0.01 N HCl, 100°C, 10 min) and subjected to paper chromatography in a solvent system of either 2-propanol–acetic acid–water (27:4:9) (solvent B) or butanol-pyridine-water (6:4:3) (solvent C). Alternatively, samples were analyzed by paper electrophoresis in a solvent system of pyridine-acetic acid-water (1:0.04:9) as previously described (37, 41). Under the mild-acid hydrolysis conditions used, only the phosphate linkages are split, releasing the labeled oligosaccharide from the unlabeled lipid. Radioactivity was detected with a Packard 7201 radiochromatogram scanner (Packard Instrument Co., Rockville, Md.). Deacetylation of oligosaccharides was performed with 60 mM NaOH as previously described (32).

Radiochemicals and biochemicals. UDP-[^{14}C]glucose (300 Ci/mol), UDP-[^{14}C]glucuronic acid (300 Ci/mol), and GDP-[^{14}C]mannose (300 Ci/mol) were prepared as described previously (31). UDP-glucose, GDP-mannose, UDP-glucuronic acid, PEP (monopotassium salt), and Ac-CoA were purchased from Sigma.

Virulence tests. *X. campestris* was grown in modified M9 medium until early log phase and washed with 0.9% NaCl. Cabbage (*Brassica oleracea* cv. Braunschweiger) was grown in a growth chamber at 25°C and 75% humidity for 4 weeks. Bacteria (10^8 CFU) were injected into the petioles of mature leaves, and the symptoms were rated 10 days after injection on the basis of the following factor scale: 0, no visible effects; 1, chlorosis around the infection site; 2, chlorosis extending from the infection site; 3, blackened leaf veins; 4, chlorosis of leaf tissue; 5, death and drying of tissue; 6, complete rotting of the entire leaf. The virulence index was calculated for each strain as a weight ratio of percentages of plants having the same pathogenicity factor. Each strain was inoculated into at least 10 independent plants. Mock infection was performed by inoculation of modified M9 medium into 10 independent plants.

Analysis of nucleotide and protein sequences. The nucleotide and amino acid sequences were analyzed by using the MacVector Sequence Analysis Software (Oxford Molecular Limited). The amino acid sequences deduced from the nucleotide sequences were compared to those in the GenBank database by using the BLAST algorithm (1).

RESULTS

Construction of a defined set of *X. campestris gum* mutants. Since *gum* genes were shown to be encoded by a single transcriptional unit (34), nonpolar *gum* mutants were constructed by transcriptional fusion or plasmid integration (Table 1). Transcriptional fusion mutants were constructed by marker exchange mutagenesis by using the nonpolar, promoterless *lacZ-aacC1* interposon inserted in the same direction as the proposed *gum* open reading frames (34). Plasmid integration *gum* mutants were constructed by cloning fragments of *gum* genes into suicide vector pK18mob (46). In this case, *gum* sequences were cloned in the same direction as vector promoters, allowing transcription downstream of the integration site. Hybrid plasmids were transferred to *X. campestris* strains. *X. campestris* strains with hybrid plasmids integrated into the genome by single crossover events were selected by use of the vector-encoded antibiotic resistance, and the changes were verified by Southern hybridization.

We were unable to mutate the *gumB*, *gumC*, *gumE*, *gumJ*, and *gumM* genes in the FC2 strain (Table 1) without further chromosomal rearrangements. This lethality might be due to accumulation of certain toxic, lipid-linked intermediates. However, all of these genes could be inactivated in strain 3192, which is deficient in UDP-glucose pyrophosphorylase (25). Due to its failure to synthesize UDP-glucose, mutant 3192 lacks, in vivo, all of the xanthan biosynthetic lipid intermediates. Figure 2 shows the insertion and integration sites for all of the mutants.

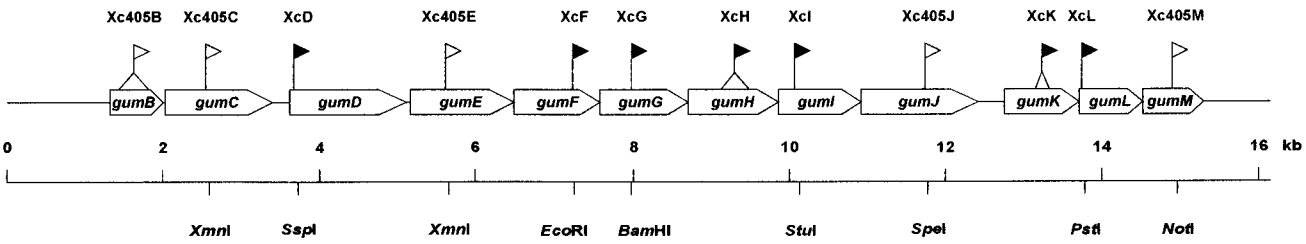

FIG. 2. *X. campestris* mutant strains generated for *gum* gene function analysis. Structural map of the *X. campestris gum* operon showing the organization of the genes as determined by Capage et al. (11), including the modification reported by Becker et al. (8). Transcriptional fusion mutants are represented by single-foot flags; plasmid integration mutants are denoted by double-foot flags. Black flags represent *lacZ-aacC1* insertions or plasmid integrations in *X. campestris* FC2; white flags represent *lacZ-aacC1* insertions or plasmid integrations in *X. campestris* 3192. Relevant restriction sites used for construction of mutant strains are also shown.

TABLE 1. Bacterial strains and plasmids used in this work

Plasmid or strain	Relevant characteristics	Source or reference
Plasmids		
pK18mob	pUC18 derivative; *lacZ*α (Km^r); *mob* site	46
pGum36-18S	pK18mob containing 231-bp fragment of *gum* gene cluster (nucleotides 13129–13360)[a]	This work
pGum52-18S	pK18mob containing 401-bp fragment of *gum* gene cluster (nucleotides 9054–9455)[a]	This work
pGum57-18S	pK18mob containing 437-bp fragment of *gum* gene cluster (nucleotides 1372–1809)[a]	This work
pJC440	Plasmid based on pRK293 carrying *xpsIV* region of *X. campestris*	25
pRK404	*oriV*(RK2) Tet^r *oriT*(*mob*$^+$) *tra*	18
pCD2	pRK404 containing PCR-amplified fragment encoding last 198 amino acids of GumD and first 22 amino acids of GumE (nucleotides 4500–5244)[a]	D. Ferreiro
Strains		
Escherichia coli		
DH5α	*recA1* Δ*lacU169* φ80d*lacZ*Δ*M15*	Bethesda Research Laboratories
S17-1	*E. coli* 294 *thi* RP4-2-Tc::Mu-Km::Tn*7* integrated into chromosome	49
Xanthomonas campestris		
NRRL B-1459	Wild type	35
FC2	Rif^r derivative of NRRL B-1459	L. Ielpi
3192	0100 *ugp* Rif^r	25
3332	0100 *pmi* Rif^r	25
Xc405C	3192 Φ(*gumC-lacZ-aacC1*) insertion site: *Xmn*I (nucleotide 2556)[a]	34
XcD	FC2 Φ(*gumD-lacZ-aacC1*) insertion site: *Ssp*I (nucleotide 3705)[a]	34
Xc405E	3192 Φ(*gumE-lacZ-aacC1*) insertion site: *Xmn*I (nucleotide 5633)[a]	34
XcF	FC2 Φ(*gumF-lacZ-aacC1*) insertion site: *Eco*RI (nucleotide 7247)[a]	34
XcG	FC2 Φ(*gumG-lacZ-aacC1*) insertion site: *Bam*HI (nucleotide 7963)[a]	34
XcI	FC2 Φ(*gumI-lacZ-aacC1*) insertion site: *Stu*I (nucleotide 10097)[a]	34
Xc405J	3192 Φ(*gumJ-lacZ-aacC1*) insertion site: *Spe*I (nucleotide 11716)[a]	34
XcL	FC2 Φ(*gumL-lacZ-aacC1*) insertion site: *Pst*I (nucleotide 13708)[a]	34
Xc405M	3192 Φ(*gumM-lacZ-aacC1*) insertion site: *Not*I (nucleotide 14871)[a]	34
Xc405B	3192 carrying plasmid pGum57-18S integrated into genome	This work
XcH	FC2 carrying plasmid pGum52-18S integrated into genome	This work
XcK	FC2 carrying plasmid pGum36-18S integrated into genome	This work
Xc405K	3192 carrying plasmid pGum36-18S integrated into genome	This work
Xc475K	3332 carrying plasmid pGum36-18S integrated into genome	This work

[a] Numbers correspond to positions in the nucleotide sequence of the *gum* region (GenBank accession no. U22511).

Biochemical characterization of lipid-linked intermediates from *gum* mutant strains generated in a wild-type background. Permeabilized cells were incubated with UDP-glucose, GDP-mannose, and UDP-glucuronic acid, one of them labeled in the sugar moiety, and processed as described in Materials and Methods. Lipid-linked xanthan intermediates were extracted, and oligosaccharides released from the lipid fraction were characterized by descending chromatography on solvent B (see Materials and Methods). Results are shown in Fig. 3. Previous studies have shown that labeled oligosaccharides released from the glycolipid fraction obtained from permeabilized FC2 cells consisted mainly of pentasaccharide repeating units and their pyruvylated or acetylated derivatives (Fig. 3A). The pentasaccharide repeating unit corresponds to the peak migrating between maltopentaose and maltohexaose (31). The acetylated pentasaccharide and the pyruvylated pentasaccharide migrate like maltotetraose. The existence of endogenous acetyl and pyruvyl donors was previously reported (31, 32). The fastest-migrating peak was characterized as glucose. It could arise from either glucose diphosphate polyprenol or lipid-bound glucose (see below), which does not seem to participate in xanthan biosynthesis.

The *gumD* mutant XcD was the unique mutant strain, generated in a wild-type background, that showed no released labeled oligosaccharides when permeabilized cells were labeled with UDP-[^{14}C]glucose (Fig. 3B). Cells labeled with UDP-[^{14}C]glucuronic acid or GDP-[^{14}C]mannose showed similar results (data not shown). The predicted GumD protein is similar to a large group of glycosyl-1-phosphate transferases. Among others, the *gumD* product is similar to galactosyl-1-phosphate transferases WbaP (55) and ExoY (40, 43). WbaP catalyzes the transfer of galactosyl-1-phosphate from UDP-galactose to undecaprenyl phosphate, the first reaction of O-antigen synthesis of *Salmonella enterica* serovar typhimurium. In turn, ExoY of *Rhizobium meliloti* catalyzes the first reaction in the synthesis of the exopolysaccharide succinoglycan. Taken together, these data suggest that GumD catalyzes the transfer of glucosyl-1-phosphate from UDP-glucose to polyprenol phosphate, which is the first step in the biosynthesis of lipid intermediates involved in the synthesis of xanthan (31). This function was previously termed glycosyltransferase I (54).

Oligosaccharides obtained from *gumH* strain XcH could be resolved into two components: a compound with the same mobility as the disaccharide cellobiose and glucose (Fig. 3D). XcH cells labeled with UDP-[^{14}C]glucuronic acid or GDP-[^{14}C]mannose did not incorporate radioactivity into the 1203 extract (data not shown). These results suggest that *gumH* encodes glycosyltransferase III, catalyzing the addition of an

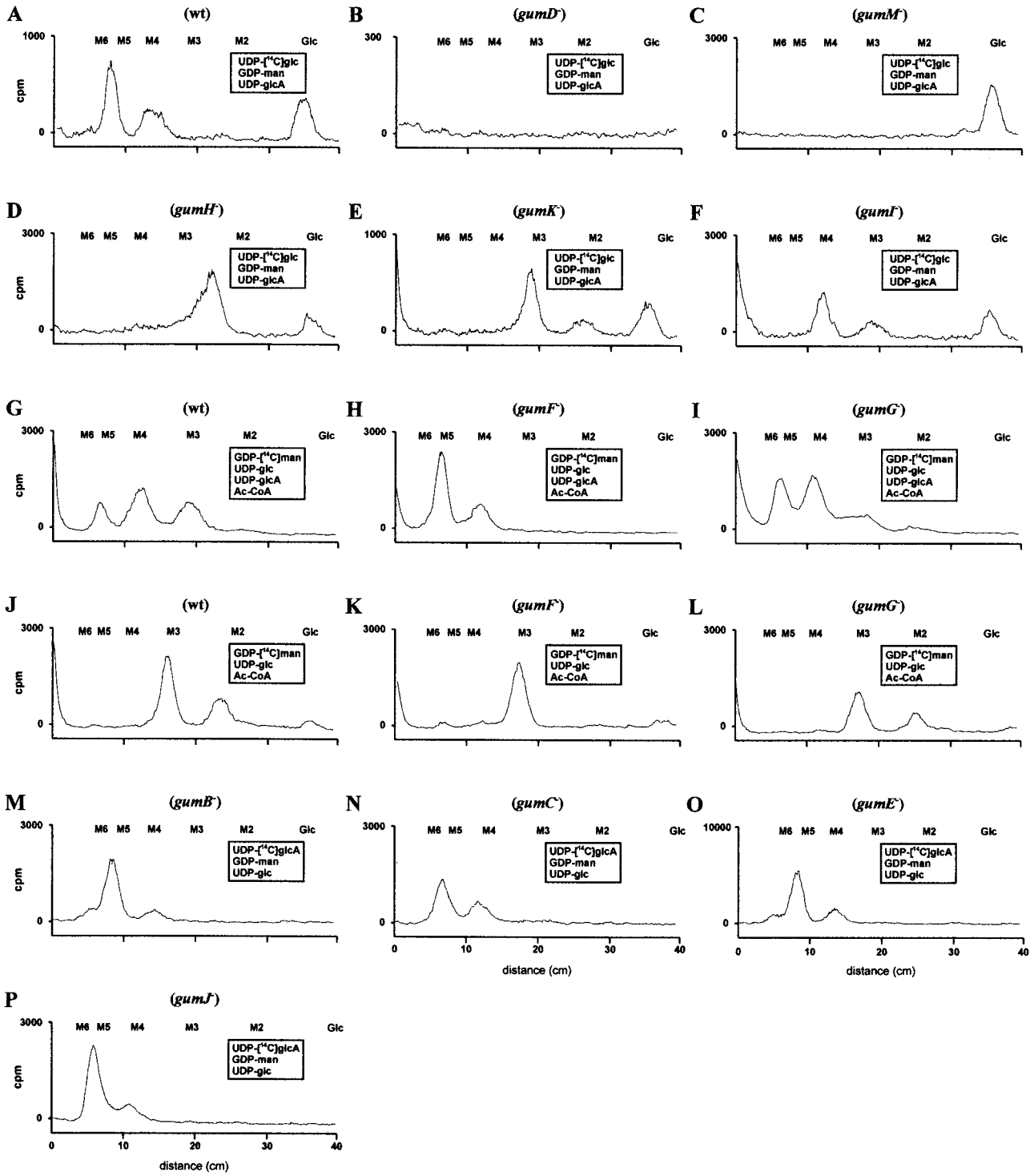

FIG. 3. Lipid-linked intermediates in *gum* mutant strains. Standard incubations were carried out in the presence of the substrates indicated in each panel. The *gum* genotype of each strain is indicated. 1203 extracts were treated by mild-acid hydrolysis and submitted to paper chromatography on solvent B as indicated in Materials and Methods. The *X. campestris* strains used were FC2 (A, G, and J), XcD (B), Xc405M (C), XcH (D), XcK (E), XcI (F), XcF (H and K), XcG (I and L), Xc405B/pCD2 (M), Xc405C/pCD2 (N), Xc405E/pCD2 (O), and Xc405J/pCD2 (P). Glucose (Glc) and maltooligosaccharides (M_2 to M_6) were run as standards. UDP-glc, UDP-glucose; GDP-man, GDP-mannose; UDP-glcA, UDP-glucuronic acid; wt, wild type.

α-mannosyl residue from GDP-mannose to cellobiose diphosphopolyprenol to produce mannosyl-(α-1,3)-cellobiose diphosphopolyprenol.

Oligosaccharides released from the lipid fraction of *gumK* strain XcK could be resolved into three species when permeabilized cells were labeled with UDP-[^{14}C]glucose (Fig. 3E). The component with a mobility slightly slower than that of the maltotriose corresponds to mannosyl-cellobiose. The compo-

nent having a mobility similar to that of maltose corresponds to acetylated mannosyl-cellobiose (32). After deesterification with 60 mM NaOH, this compound yielded mannosyl-cellobiose, confirming the presence of the acetyl group (data not shown). Peaks similar in mobility appeared upon the labeling of these cells with GDP-[^{14}C]mannose, but no radioactivity was detected in 1203 extracts after the labeling of XcK cells with UDP-[^{14}C]glucuronic acid (data not shown). The fastest-migrating component was characterized as glucose. Hence, the *gumK* product appears to be glycosyltransferase IV, which is responsible for the addition of a glucuronic acid residue from UDP-glucuronic acid to mannosyl-(α-1,3)-cellobiose diphosphopolyprenol to produce glucuronyl-(β-1,2)-mannosyl-(α-1,3)-cellobiose diphosphopolyprenol.

As shown in Fig. 3F, three compounds were obtained upon the labeling of *gumI* mutant XcI with UDP-[^{14}C]glucose. The major compound released from the resulting lipid fraction showed a mobility similar to that of maltotetraose. This is the same mobility as that of the already described tetrasaccharide (31). As pointed out above, this mobility is also similar to that of acetylpentasaccharide. After deesterification with 60 mM NaOH, acetylpentasaccharide produced pentasaccharide, with a shift from near maltooligosaccharide M_4 to M_5-M_6, while the compound from strain XcI remained unaltered. The minor compound corresponds to acetylated tetrasaccharide. The presence of the acetyl group was confirmed by deacetylation. After paper chromatography on solvent B, the mobility of the product was similar to that of the tetrasaccharide. Both peaks could be reproduced by labeling of permeabilized cells with either GDP-[^{14}C]mannose or UDP-[^{14}C]glucuronic acid (data not shown). The fastest-migrating component was characterized as glucose. Thus, it seems likely that *gumI* encodes glycosyltransferase V, which is involved in the transference of a β-mannosyl residue from GDP-mannose to glucuronyl-(β-1,2)-mannosyl-(α-1,3)-cellobiose diphosphopolyprenol to render mannosyl-(β-1,4)-glucuronyl-(β-1,2)-mannosyl-(α-1,3)-cellobiose diphosphopolyprenol. Although the assignment of glycosyltransferase V to GumI is in agreement with earlier reports, accumulation of a lipid-linked tetrasaccharide was not detected previously (51, 54).

The radioactive oligosaccharides obtained from *gumF* and *gumG* mutants (XcF and XcG, respectively) labeled with GDP-[^{14}C]mannose in the presence of Ac-CoA could be resolved into two major species (Fig. 3H and I, respectively) with the same mobilities of the pentasaccharide and acetylated pentasaccharide obtained from FC2 cells (Fig. 3A and G). In contrast, oligosaccharides released from FC2 cells labeled under identical conditions could be separated into three compounds (Fig. 3G). Deacetylation of oligosaccharides showed that these components represent, from left to right, pentasaccharide, monoacetylated pentasaccharide, and diacetylated pentasaccharide (data not shown). No clear diacetylated pentasaccharide unit was observed among the compounds released from XcF and XcG 1203 extracts (compare panels G, H, and I in Fig. 3). These data indicated that strains XcF and XcG are unable to simultaneously acetylate both of the mannoses present in the repeating unit, suggesting that *gumF* and *gumG* encode specific *O*-acetyltransferases. Since acetylation of the inner mannose could also occur at the trisaccharide-diphosphate-prenol level (32) we decided to use this trisaccharide acceptor to distinguish which gene product, GumF or GumG, is responsible for acetylation of the inner mannose. Permeabilized FC2 cells incubated in the presence of UDP-glucose, GDP-[^{14}C]mannose, and Ac-CoA released two major components from the lipid fraction, identified as mannosyl-cellobiose and acetylated mannosyl-cellobiose (32) (Fig. 3J). Permeabilized XcG cells similarly incubated showed the same pattern of oligosaccharides (Fig. 3L), indicating that the inner mannose is partially acetylated. On the other hand, permeabilized XcF cells incubated under the same conditions released only mannosyl-cellobiose from the lipid fraction (Fig. 3K). In each case, deacetylation of putative acetylmannosyl-cellobiose gave compounds with mobility identical to that of mannosyl-cellobiose (data not shown). These results indicate that *gumF* encodes an acetyltransferase whose acceptor is the innermost mannose of the lipid-linked pentasaccharide intermediate (acetyltransferase I), while *gumG* appears to encode a related enzyme in which the acceptor is the external mannose of the same intermediate (acetyltransferase II).

Two radioactive products appeared in paper electrophoresis upon mild-acid hydrolysis of 1203 extracts of permeabilized FC2 cells labeled with UDP-[^{14}C]glucuronic acid and with the addition of PEP. These compounds were characterized as pentasaccharide and pyruvylated pentasaccharide (Fig. 4A) (30). The analysis of oligosaccharides released from *gumL* mutant strain XcL labeled in the presence of PEP revealed that this strain is unable to synthesize the pyruvylated intermediate in vitro, as judged by paper electrophoresis (Fig. 4B). Therefore, the simplest hypothesis is that GumL is the ketal pyruvate transferase.

Analysis of the lipid-linked compounds produced in vitro by *gum* mutants generated in a UDP-glucose-deficient background: glucosyl-1-phosphate transferase is severely affected under certain conditions. Permeabilized *ugp-gumM* cells (Xc405M) labeled with UDP-[^{14}C]glucose incorporated radioactivity into the glycolipid fraction. The single radioactive peak was characterized as glucose by paper chromatography on solvent B (Fig. 3C) and solvent C. The radioactive material incorporated into the glycolipid fraction was further analyzed by paper chromatography on solvent A (data not shown) (31). In this alkaline solvent, glucose diphosphate polyprenol is converted to give the 1,2-cyclic phosphate ester of glucose, with a mobility (R_f) of 0.5. Two compounds were observed in about similar amounts: peak 1, with an R_f of 0.5, and peak 2, with an R_f of 0.9. Peak 2 was partially characterized as a glucose-bound lipid (see below). Peak 1 had the same mobility in paper electrophoresis as the standard 1,2-cyclic phosphate ester of glucose. After hydrolysis at pH 1 and incubation with alkaline phosphatase, this compound released free glucose. From these results, it was assumed that Xc405M cells synthesized glucose diphosphate polyprenol but were unable to produce cellobiose diphosphate polyprenol. A likely explanation for this is that *gumM* encodes glycosyltransferase II, which is involved in the addition of a β-glucosyl residue from UDP-glucose to glucose diphosphate polyprenol to produce cellobiose diphosphate polyprenol.

Surprisingly, permeabilized *ugp-gumB* (Xc405B), *ugp-gumC* (Xc405C), *ugp-gumE* (Xc405E), and *ugp-gumJ* (Xc405J) cells incorporated no radioactivity into the 1203 extract when labeled with UDP-[^{14}C]glucuronic acid or GDP-[^{14}C]mannose (data not shown). Upon the labeling of these cells with UDP-[^{14}C]glucose, radioactivity was detected in the glycolipid fraction. This material was labile to mild-acid treatment, releasing free glucose when analyzed by paper chromatography or high-pressure liquid chromatography on an Aminex HPX-87H ion exclusion column as previously described (41). A single compound with an R_f of 0.9 was detected by paper chromatography in alkaline solvent A. As pointed out above, an R_f of 0.5 was expected for glucose diphosphate polyprenol. The absence of a diphosphate bridge between the glucose and the lipid moiety was reinforced by DEAE-cellulose column chromatography in 99% methanol performed as previously reported (4). The com-

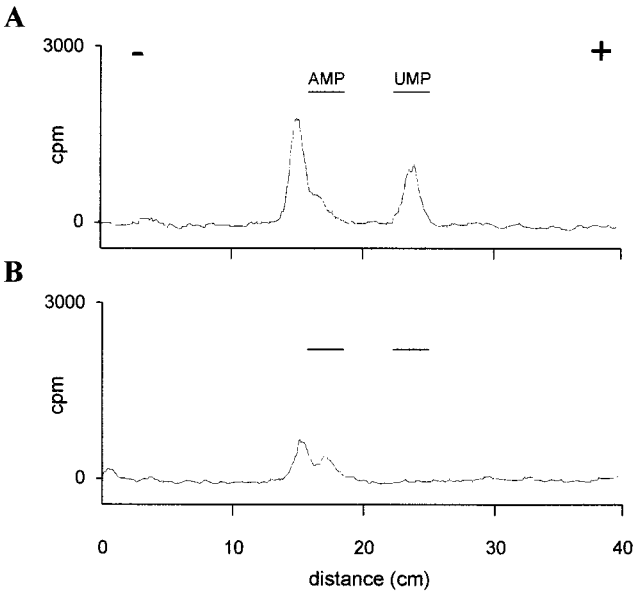

FIG. 4. Pyruvylation of the lipid-linked intermediate in *gumL* and wild-type strains. Incubations were performed in the presence of UDP-[^{14}C]glucuronic acid, GDP-mannose, UDP-glucose, and PEP. Radioactive material released from 1203 extracts by mild-acid hydrolysis was submitted to paper electrophoresis. *X. campestris* FC2 (A) and XcL (B) were employed. AMP and UMP were added as internal standards.

pound eluted at 0.14 M ammonium acetate. Under the same conditions, galactose monophosphate polyprenol is eluted at 0.11 M and glucose diphosphate polyprenol is eluted at 0.23 M. These results suggest that the radioactive compound formed could be a lipid-bound glucose with only one negative charge. Its function is unknown, and it does not seem to participate in xanthan biosynthesis. Taken together, these results suggest that the glucosyl-1-phosphate transferase activity was absent in strains Xc405B, Xc405C, Xc405E, and Xc405J. One possible explanation is that inactivation of certain *gum* genes within a strain containing a *ugp* mutation alters the normal *gum* biosynthetic pathway.

To test whether a combination of a *gum* mutation with a sugar nucleotide deficiency would lead to arrest of the normal *gum* biosynthetic pathway, we decided to generate a known glycosyltransferase *gum* mutation in a background with altered sugar nucleotide levels. Strain 3192 does not produce UDP-glucose (25) but preserves the ability to produce xanthan and its lipid-linked intermediates in vitro if sugar nucleotides are exogenously provided (25). Thus, we disrupted the *gumK* gene in strain 3192, generating strain Xc405K. In this case, disruption of *gumK* was carried out with the same construction used to obtain *gumK* strain XcK. Permeabilized Xc405K cells showed no released labeled trisaccharide when labeled with either UDP-[^{14}C]glucose or GDP-[^{14}C]mannose, while strain XcK was able to synthesize trisaccharide diphosphate polyprenol in vitro (data not shown). Similar results were obtained upon the generation of an identical *gumK* mutation in strain 3332, producing strain Xc475K. Strain 3332 is deficient in phosphomannose isomerase; therefore, it does not produce GDP-mannose (25). These results suggest that deficiencies which lead to a lack of UDP-glucose or GDP-mannose, in combination with certain *gum* mutations, are likely to abolish the assembly of the lipid-linked intermediates in vitro. The simplest hypothesis is that the first step in the assembly of pentasaccharide subunits is being affected. The exception appeared when the combination involved the *gumM* gene.

Restoration of the *gum* biosynthetic pathway in a UDP-glucose-defective background: glucosyl-1-phosphate transferase activity seems to be located in the C-terminal domain of *gumD*. If the first reaction is a limiting step in the biosynthesis of xanthan, it could be reversed by increasing levels of glucosyl-1-phosphate transferase (the *gumD* product). Whereas some glycosyl-1-phosphate transferases display homology to GumD along the whole sequence, others appear to be homologous only to its C-terminal portion (40, 43, 55). These data let us hypothesize that the glucosyl-1-phosphate domain of GumD resides in its C-terminal half. The coding sequence for the C-terminal portion of GumD was cloned into broad-host-range vector pRK404 (18), producing plasmid pCD2 (Table 1). This plasmid complemented the xanthan defect in the XcD strain, rendering mucoid colonies. These results suggest that the glucosyl-1-phosphate transferase activity of GumD relies on its C-terminal half. The ability of Xc405K and Xc475K to synthesize trisaccharide diphosphate polyprenol in vitro was restored by pCD2 (data not shown). The plasmid also restored the biosynthesis of cellobiose diphosphate polyprenol in an additional *gumH* mutant generated in the strain containing a *ugp* mutation (data not shown).

Characterization of lipid-linked intermediates in *gum* mutant strains generated in a UDP-glucose-deficient background in the presence of plasmid pCD2. Plasmid pCD2 was introduced into Xc405B, Xc405C, Xc405E, and Xc405J by conjugation. Permeabilized cells were labeled with UDP-[^{14}C]glucuronic acid, and the oligosaccharides released from the 1203 extract upon mild-acid hydrolysis were analyzed. The radioactive oligosaccharides showed a pattern similar to that of those obtained from permeabilized FC2 cells (Fig. 3A, M, N, O, and P). Similar results were obtained upon the labeling of these cells with UDP-[^{14}C]glucose or GDP-[^{14}C]mannose. These data indicate that these strains are capable of both synthesizing and decorating the lipid-linked pentasaccharide repeating unit intermediate. These data suggest that the *gumB*, *gumC*, *gumE*, and *gumJ* products are not related to the biosynthesis of the lipid-linked intermediates of xanthan.

Plasmid pCD2 was also introduced into Xc405M. A biochemical analysis similar to that described for Xc405M revealed that Xc405M/pCD2 behaves exactly the same as parental strain Xc405M, indicating that the presence of pCD2 does not modify the xanthan biosynthetic pathway in this strain (data not shown).

Assessment of polysaccharide production in *gum* mutant strains. Previous studies have shown that a radiolabeled polymer obtained by in vitro incubations, upon gel filtration through a Bio-Gel A-5m column, coeluted with a sample of authentic xanthan gum obtained in vivo. This result indicated similar degrees of polymerization since both products had the same apparent molecular weight (about 4×10^6) (29, 31). Permeabilized cells from all of the strains depicted in Fig. 3 were labeled with UDP-[^{14}C]glucose, and their aqueous supernatants were filtered through a Bio-Gel A-5m column.

While inactivation of *gumD*, *gumM*, and *gumH* (encoding the first three glycosyltransferases) completely abolished in vitro polymer formation (data not shown), mutations in genes *gumK* and *gumI* (encoding the fourth and fifth glycosyltransferases, respectively) had less effect on the amount of in vitro-produced polysaccharide (Fig. 5B and C). Therefore, the lipid-linked trisaccharide and the lipid-linked tetrasaccharide may fulfill the requirements of substrates for polymerization. It should be noted that introduction of plasmid pCD2 into Xc405M does not allow the strain to synthesize a polymer in

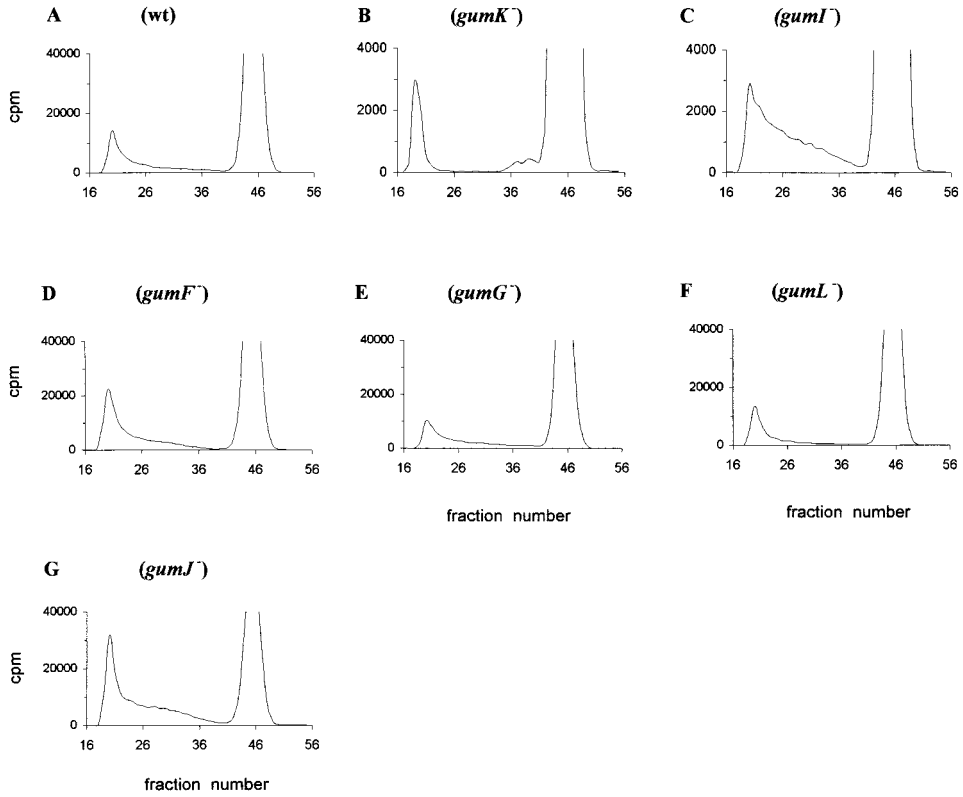

FIG. 5. Gel filtration of in vitro-synthesized polymer. Incubations were performed and processed as indicated in Materials and Methods, by using UDP-[^{14}C]glucose as the labeled donor in the presence of UDP-glucuronic acid and GDP-mannose. The *gum* genotype of each strain is indicated. The aqueous incubation supernatants were filtered through a Bio-Gel A-5m column at a rate of 0.25 ml min^{-1}. Fractions of 1 ml were collected, and the amount of radioactivity was determined by liquid scintillation. *X. campestris* FC2 (A), XcK (B), XcI (C), XcF (D), XcG (E), XcL (F), and Xc405J/pCD2 (G) are shown. Void and inclusion volumes were 20 and 45 ml, respectively. These results could be reproduced by using UDP-[^{14}C]glucuronic acid or GDP-[^{14}C]mannose as the labeled donor. wt, wild type.

vitro (data not shown). Neither pyruvylation nor acetylation is essential for polysaccharide production, since inactivation of *gumF*, *gumG*, or *gumL* does not affect polymerization (Fig. 5A, D, E, and F).

Mutant strains with deficiencies in the expression of gene *gumB*, *gumC*, or *gumE* are the only strains that accumulate lipid-linked pentasaccharide in vitro without producing polymer (data not shown). Consequently, the functions of these genes are probably related to processes involved in polymerization. Permeabilized Xc405J/pCD2 cells were able to produce polymer in vitro (Fig. 5G). Similar results were obtained upon the labeling of cells with UDP-[^{14}C]glucuronic acid or GDP-[^{14}C]mannose. Considering that this strain has no defect in the assembly of the lipid-linked pentasaccharide and the polysaccharide produced is large enough to be excluded by Bio-Gel A5m, the function of the *gumJ* product cannot be associated with a particular xanthan biosynthetic step. It should be noted that our in vitro system does not allow us to detect defects in the xanthan secretion pathway. Therefore, a secretion role for GumJ cannot be ruled out.

Virulence of *X. campestris* stable mutant strains altered in different steps of xanthan biosynthesis. Phytopathogenicity-related genes in *X. campestris* have different effects on polysaccharide production. While mutations within the *rpf* cluster (5, 52) or in the *clp* gene (16) reduce polysaccharide production, *hrp* mutants showed no alteration in polymer biosynthesis (2). However, it has not been established whether any specific stage in xanthan biosynthesis is required for plant virulence. To address this question, we analyzed the virulence of several stable *gum* mutants, comparing them to pleiotropic strain 3192 and to strain FC2. Strains containing episomes or chromosomally integrated plasmids were not analyzed, since they are unstable without selective pressure.

Compared to strain FC2, xanthan nonproducer strains 3192 and XcD exhibited a 50% virulence index reduction (Fig. 6). Whereas the absence of a pyruvylated lipid-linked pentasaccharide does not have any influence on *X. campestris* pathogenicity, mutants unable to simultaneously diacetylate the subunit showed slightly reduced virulence. A similar result was obtained with the *gumI* strain. Although this strain is unable to add the second mannose to the lipid-linked tetrasaccharide, its virulence index is not severely affected. These results indicate that the polysaccharide participates in, but appears not to be essential for, plant infection.

DISCUSSION

Exploration of the genetics of xanthan gum led to the identification of several genetic loci involved in its biosynthesis (6, 23, 25, 27, 36, 53). Among these gene clusters, the *gum* region was proposed as being responsible for directing the final assembly of the polymer (11, 13, 24, 51, 54). However, data unequivocally ascribing functions to *gum* genes are not readily available in the public domain. In this report, we present, for the first time, direct experimental evidence which allows us to assign biochemical functions to the *X. campestris gum* region.

XcD was the only mutant which did not accumulate any xanthan lipid intermediate. This phenotype would be expected

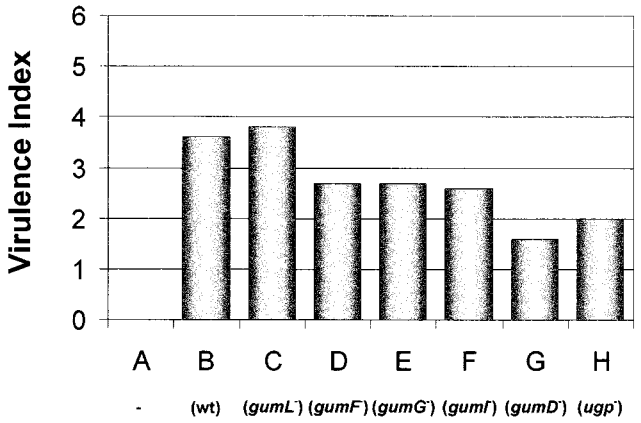

FIG. 6. Virulence of *X. campestris* stable *gum* mutants. The virulence indexes of *X. campestris* FC2 (B), XcL (C), XcF (D), XcG (E), XcI (F), XcD (G), and 3192 (H) were determined. The relevant genotype of each strain is indicated. Column A shows the results obtained with mock-infected plants. The standard error was less than 20% in all cases. wt, wild type.

for the gene encoding glycosyltransferase I, as well as any gene product involved in the regulatory control of the expression of the *gum* genes. Since absence of the *gumD* product does not abolish transcription from the main promoter of the *gum* region (34), GumD may not be responsible for transcription of the *gum* operon and probably corresponds to the first step of xanthan biosynthesis. The predicted GumD protein is similar to a large group of bacterial glucosyl- and galactosyl-1-phosphate transferases, suggesting that they constitute a new family of proteins (55).

XcD deficiency could be complemented by the C-terminal half of *gumD*, suggesting that the glucosyl-1-phosphate transferase activity of GumD is located in its C-terminal domain. Similar results were obtained with the *wbaP* product, which catalyzes the first step of O-antigen synthesis (55).

Analysis of the intermediates accumulated in *gumM*, *gumH*, *gumK*, and *gumI* mutants showed that these genes encode glycosyltransferases II, III, IV, and V, respectively, in agreement with previous reports (11, 54). The predicted GumM product revealed homology to a variety of proteins probably involved in the transference of a glycosyl residue to growing lipid-linked oligosaccharides, such as AceB of *Acetobacter xylinum* (GenBank accession no. X94217), which is presumably involved in the second step of acetan biosynthesis. It should be noted that acetan and xanthan have similar structures, and the first four reactions in the assembly of each repeating unit should be catalyzed by a set of similar glycosyltransferases (22, 41). While the predicted GumH protein is similar to a large family of prokaryotic α-mannosyltransferases (19, 41), the GumI and GumK proteins do not display homology to any published sequence.

With the analysis of the in vitro acetylation of the lipid-linked intermediates, we were able to ascertain that GumF is an *O*-acetyltransferase that modifies the inner mannose while GumG directs acetylation of the outer mannose. The predicted GumF and GumG proteins are 39% identical, and they might be membrane-associated proteins, as judged by their hydrophobicity plots. Homologous sequences can be found in different gram-negative and gram-positive bacteria, such as *S. enterica* (GenBank accession no. X60666), *Bacillus subtilis* (GenBank accession no. Z99111), and *R. loti* (GenBank accession no. U22899). Similarities among bacterial *O*-acetyltransferases, including NodX of *R. leguminosarum*, NolL of *R. loti*, and GumF of *X. campestris*, were reported previously (47).

The gene for ketal pyruvate transferase was previously located on a 1.4-kb *Bam*HI fragment of the *gum* region (37). Since the *gumL-lacZ* fusion in XcL corresponds to the same complementation group as the 31313 mutant (37) and both strains share the same phenotype, it seems very likely that the mutation in strain 31313 is located in the *gumL* gene. GumL is 23.7% identical to ketal pyruvate transferase ExoV of *R. meliloti* (20), which is involved in the addition of a pyruvate substituent to the terminal glucose of the side chain of succinoglycan (44). Although, insertions in *exoV* seem to be deleterious, *gumL* mutants are not affected in growth and produce normal nonpyruvylated polymer levels (data not shown).

Under our conditions, we have found that functional *gumD*, *gumM*, and *gumH* genes are required for polymerization of lipid-linked subunits while *gumK*, *gumI*, *gumF*, *gumG*, and *gumL* mutants are able to produce a labeled polysaccharide. Whereas *gumF*, *gumG*, and *gumL* strains produced amounts of polymer similar to those produced by the wild type, the *gumK* strain produced a very low amount of polymer in vivo. This product was described and named polytrimer (9). The *gumI* strain produced about 10% of the polymer amount produced by the wild-type strain (data not shown).

We have observed that it is not possible to mutate *gumB*, *gumC*, *gumE*, *gumJ*, and *gumM* genes within a wild-type background. Deleterious mutations in genes required for polysaccharide biosynthesis were also described in *R. meliloti* (20). Inactivation of these *gum* genes could be performed only in strains which also contained a second mutation. However, most of the double mutants obtained presented alterations in the *gum* biosynthetic pathway. Xc405M arose as an exception, and further experiments are needed to explain this contradictory behavior. This effect could not be reversed by introduction of plasmid pJC440. Plasmid pJC440 complements the *ugp* mutation in strain 3192 (25) but was unable to reestablish the production of lipid-linked intermediates in vitro in double mutants (data not shown). On the other hand, introducing addi-

TABLE 2. Similarities between some *gum* gene products and proteins with verified polysaccharide secretion roles

Gene in *X. campestris* *gum* region[a]	Homologous gene	Bacterium	Polysaccharide	Identity (%)[b]	Gene product activity	Reference
gumB (213)	*kpsD*	*E. coli*	Polysialic acid	25 (558)	Translocates polymer to cell surface	57
gumC (449)	*exoP*	*R. meliloti*	Succinoglycan	25 (789)	Influences high-molecular-weight to low-molecular-weight polysaccharide ratio	8
gumJ (499)	*exoT*	*R. meliloti*	Succinoglycan	13 (520)	Influences production of certain low-molecular-weight oligosaccharides	21

[a] The gene product size in amino acids is in parentheses.
[b] Percent identity at the amino acid level. The number of amino acids of the longest gene product is in parentheses.

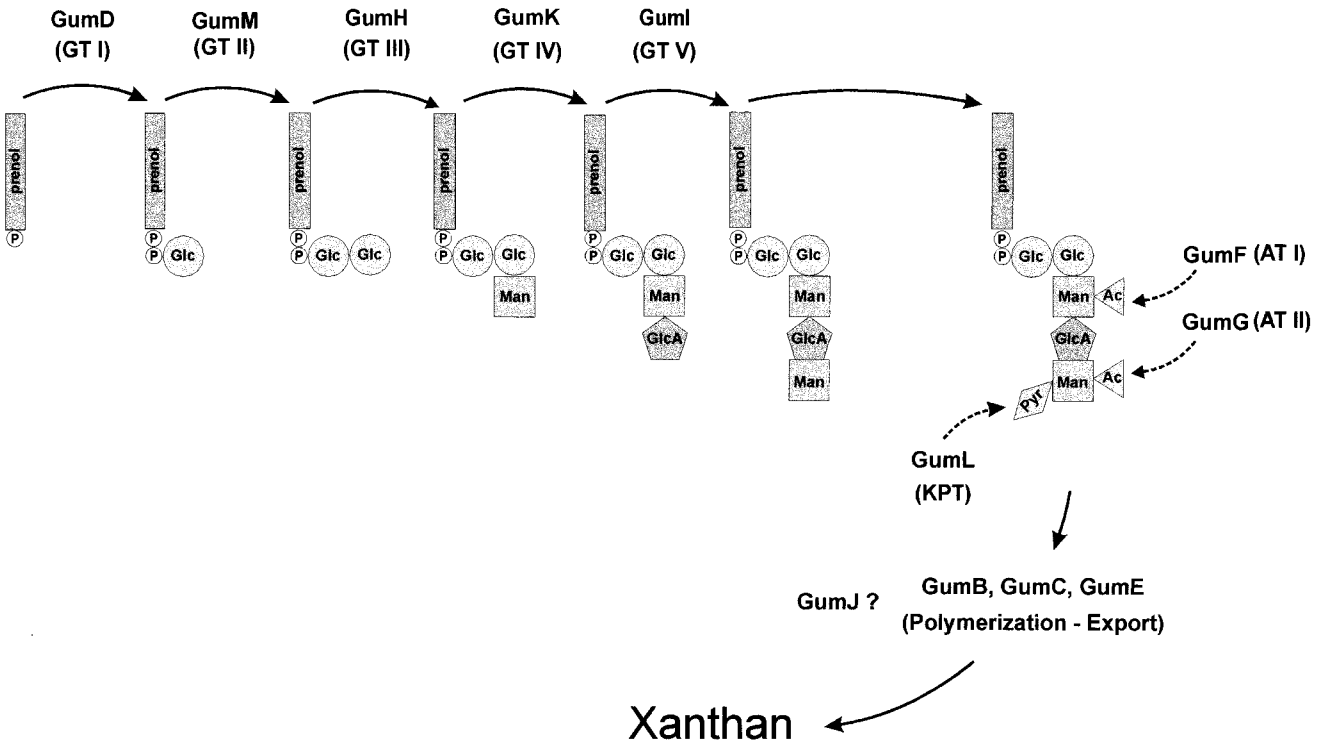

FIG. 7. Scheme of the proposed *gum* gene functions for the biosynthesis of the exopolysaccharide xanthan in *X. campestris*. The components of the lipid-linked intermediates are represented as follows: Glc, glucose; Man, mannose; GlcA, glucuronic acid; Ac, acetyl group; Pyr, pyruvyl group. The designation of each protein is followed by its proposed function as follows: GT, glycosyltransferase; AT, acetyltransferase; KPT, ketal pyruvate transferase. Dashed arrows indicate that repeating units are variably decorated. GumJ could not be associated with any particular *gum* biosynthetic step, although the possibility of its participation in pre- or postpolymerization processes cannot be eliminated (see text).

tional copies of sequences encoding the C-terminal domain of GumD allowed us to detect in vitro lipid-linked intermediates of xanthan. Something comparable occurred in *R. meliloti*, since inactivation of the negative regulator ExoR within a specific background increased the efficiency of labeling of intermediates (44).

Combined genetic and biochemical approaches resulted in detailed models of the biosynthesis of polymer subunits of many bacterial polysaccharides. In contrast, much less is known about the polymerization and export of these macromolecules. Most of the proposed strategies for secretion of polysaccharides from different bacteria were only suggested by sequence similarities between essential gene products. Linkage between polymerization and export processes was reported in either polysaccharide or O-antigen biosynthesis (44, 56).

Since *gumB*, *gumC*, and *gumE* strains appear to accumulate complete xanthan subunits in vitro but are unable to synthesize polymer, the products of these genes may be needed for polymerization or polymer export. GumB and GumC display homology to only two proteins with known functions (Table 2). These sequence similarities, together with our results, let us hypothesize that GumB might be involved in polysaccharide translocation, whereas GumC might be involved in determination of the degree of polymerization. GumE displays a hydropathic profile very similar to that of *R. meliloti* protein ExoQ (40), which was proposed to be involved in the polymerization of high-molecular-weight succinoglycan (21). Taking into account these considerations, together with our results, we can speculate that the *gumE* product might be directly related to the polymerization of xanthan. These three gene products seem to be membrane-associated proteins, as judged by their hydrophobicity plots. Inactivation of any of these three genes in the wild-type strain was lethal. This lethality can be explained if we presume that these gene products are part of a membrane complex. Absence or deficiency of one of them would disrupt the polymerization process, impairing accumulation of lipid-linked intermediates. Elevated amounts of these compounds might be toxic for the cell. Work is in progress to further characterize polymerization intermediates in *gumB*, *gumC*, and *gumE* strains (28).

In contrast, our *gumJ* mutant produces lipid-linked pentasaccharide subunits in vitro but is able to polymerize them into xanthan. As can be seen in Table 2, GumJ is homologous to the *R. meliloti* ExoT protein. An *R. meliloti exoT* mutant produces high-molecular-weight succinoglycan and octasaccharide subunits in vitro but fails to synthesize trimers and tetramers of the octasaccharide subunits (21) which are normally produced in the wild-type strain (7). It should be noted that the amount of high-molecular-weight succinoglycan produced by the *exoT* mutant was very low compared to that of the polymer synthesized by the wild-type strain, suggesting that absence of the ExoT protein also disturbs polysaccharide production. However, reduction of in vitro pentasaccharide subunit or polysaccharide production was not observed in strain Xc405J/pCD2 compared to the wild type (Fig. 3P and 5G). Low-molecular-weight xanthan has not been detected in *X. campestris* cultures. Providing that polymerization and export of xanthan are coupled events, GumJ might be involved in a parallel or subsequent stage of these processes. Considering that *gumJ* disruption within a wild-type background was lethal, *gumJ* might be necessary to prevent accumulation of a harmful product or for recycling of essential substrates. Figure 7 sum-

marizes the proposed Gum function in the xanthan biosynthetic pathway.

All of the mutants described in this report were similar in color. In addition, 1203 extracts exhibited identical absorption spectrum patterns (not shown), indicating that the ratio of different xanthomonadins had not changed. These results stand in contrast to those of a recently published report which proposed that the *X. campestris gumD* gene is involved not only in xanthan biosynthesis but also in plant virulence and normal cell pigmentation (12).

Since a correlation between xanthan production and plant virulence was established by employing pleiotropic mutants, the participation of xanthan in bacterial pathogenesis must be interpreted cautiously (17). Unless total loss of pathogenicity takes place (rather than variance in the aggressiveness of the pathogen), differences in virulence between strains could be very subtle (14). Therefore, we decided to express bacterial virulence as an index, which involves the inspection of a statistically significant number of infected plants. Xanthan biosynthesis can be blocked at different steps of the biosynthetic pathway (24). Inactivation of enzymes involved in the synthesis of sugar nucleotides abolished xanthan production and has pleiotropic effects, since these enzymes are also involved in the biosynthesis of cyclic glucans and lipopolysaccharides (36). In contrast, gene disruption within the *gum* region is likely to abolish enzyme activity restricted to the polysaccharide biosynthetic pathway.

Figure 6 shows that pyruvylation was the only change in the lipid-linked intermediate, which is absolutely irrelevant to virulence. However, slight variations in xanthan intermediates, like shortening of the subunit to four sugars or inactivation of acetyltransferases, reduced the virulence index by approximately 25%. In particular, the virulence of two xanthan-deficient strains mutated at different stages of xanthan biosynthesis was 50% lower than that of the wild-type strain. Taken together, our data show that although xanthan gum is not essential for plant virulence, specific changes in the last stages of xanthan biosynthesis point to a reduction of the aggressiveness of *X. campestris* against the host.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Arlat, M., C. L. Gough, C. E. Barber, C. Boucher, and M. J. Daniels.** 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. **4:**593–601.
3. **Baird, J., P. Sandford, and I. Cottrell.** 1983. Industrial applications of some new microbial polysaccharides. Bio/Technology **1:**778–783.
4. **Baldessari, A., L. Ielpi, and M. A. Dankert.** 1990. A novel galacturonide from *Xanthomonas campestris*. J. Gen. Microbiol. **136:**1501–1507.
5. **Barber, C. E., J. L. Tang, J. X. Feng, M. Q. Pan, T. J. Wilson, H. Slater, J. M. Dow, P. Williams, and M. J. Daniels.** 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. Mol. Microbiol. **24:**555–566.
6. **Barrère, G. C., C. E. Barber, and M. J. Daniels.** 1986. Molecular cloning of genes involved in the production of the extracellular polysaccharide xanthan by *Xanthomonas campestris* pv. campestris. Int. J. Biol. Macromol. **8:**372–374.
7. **Battisti, L., J. C. Lara, and J. A. Leigh.** 1992. Specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa. Proc. Natl. Acad. Sci. USA **89:**5625–5629.
8. **Becker, A., K. Niehaus, and A. Pühler.** 1995. Low-molecular-weight succinoglycan is predominantly produced by *Rhizobium meliloti* strains carrying a mutated ExoP protein characterized by a periplasmic N-terminal domain and a missing C-terminal domain. Mol. Microbiol. **16:**191–203.
9. **Betlach, M. R., M. A. Capage, D. H. Doherty, R. A. Hassler, N. M. Henderson, R. W. Vanderslice, J. D. Marrelli, and M. B. Ward.** 1987. Genetically engineered polymers: manipulation of xanthan biosynthesis, p. 35–50. *In* M. Yalpani (ed.), Industrial polysaccharides: genetic engineering, structure/property relations and applications. Elsevier, Amsterdam, The Netherlands.
10. **Cadmus, M. C., S. P. Rogovin, K. A. Burton, J. E. Pittsley, C. A. Knutson, and A. Jeanes.** 1976. Colonial variation in *Xanthomonas campestris* NRRL B-1459 and characterization of the polysaccharide from a variant strain. Can. J. Microbiol. **22:**942–948.
11. **Capage, M. R., D. H. Doherty, M. R. Betlach, and R. W. Vanderslice.** March 1987. Recombinant-DNA mediated production of xanthan gum. International patent WO87/05938.
12. **Chou, F. L., H. C. Chou, Y. S. Lin, B. Y. Yang, N. T. Lin, S. F. Weng, and Y. H. Tseng.** 1997. The *Xanthomonas campestris gumD* gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. Biochem. Biophys. Res. Commun. **233:**265–269.
13. **Coplin, D. L., and D. Cook.** 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. **3:**271–279.
14. **Daniels, M. J.** 1993. Genetics of *Xanthomonas*, p. 301–339. *In* J. G. Swings and E. L. Civerolo (ed.), Xanthomonas. Chapman & Hall, London, England.
15. **Daniels, M. J., D. B. Collinge, J. M. Dow, A. E. Osbourn, and I. N. Roberts.** 1987. Molecular biology of the interaction of *Xanthomonas campestris* with plants. Plant Physiol. Biochem. **25:**353–359.
16. **de Crécy-Lagard, V., P. Glaser, P. Lejeune, O. Sismeiro, C. E. Barber, M. J. Daniels, and A. Danchin.** 1990. A *Xanthomonas campestris* pv. *campestris* protein similar to catabolite activation factor is involved in regulation of phytopathogenicity. J. Bacteriol. **172:**5877–5883.
17. **Denny, T. P.** 1995. Involvement of bacterial polysaccharides in plant pathogenesis. Annu. Rev. Phytopathol. **33:**173–197.
18. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X. W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
19. **Geremia, R. A., E. A. Petroni, L. Ielpi, and B. Henrissat.** 1996. Towards a classification of glycosyltransferases based on amino acid sequence similarities: prokaryotic alpha-mannosyltransferases. Biochem. J. **318:**133–138.
20. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J. Bacteriol. **175:**7045–7055.
21. **Gonzalez, J. E., C. E. Semino, and G. C. Walker.** Submitted for publication.
22. **Griffin, A. M., V. J. Morris, and M. J. Gasson.** 1996. Identification, cloning and sequencing the *aceA* gene involved in acetan biosynthesis in *Acetobacter xylinum*. FEMS Microbiol. Lett. **137:**115–121.
23. **Harding, N. E., J. M. Cleary, D. K. Cabañas, I. G. Rosen, and K. S. Kang.** 1987. Genetic and physical analyses of a cluster of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. J. Bacteriol. **169:**2854–2861.
24. **Harding, N. E., J. M. Cleary, and L. Ielpi.** 1995. Genetics and biochemistry of xanthan gum production by *Xanthomonas campestris*, p. 495–514. *In* Y. H. Hui and G. Khachatourians (ed.), Food biotechnology microorganism. VCH Publishers, Inc., New York, N.Y.
25. **Harding, N. E., S. Raffo, A. Raimondi, J. M. Cleary, and L. Ielpi.** 1993. Identification, genetic and biochemical analysis of genes involved in synthesis sugar nucleotide precursors of xanthan gum. J. Gen. Microbiol. **139:**447–457.
26. **Hassler, R. A., and D. H. Doherty.** 1990. Genetic engineering of polysaccharide structure: production of variants of xanthan gum in *Xanthomonas campestris*. Biotechnol. Prog. **6:**182–187.
27. **Hötte, B., I. Rath-Arnold, A. Pühler, and R. Simon.** 1990. Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production by *Xanthomonas campestris* pv. campestris. J. Bacteriol. **172:**2804–2807.
28. **Ielmini, M. V., and L. Ielpi.** Unpublished data.
29. **Ielpi, L., R. Couso, and M. Dankert.** 1981. Lipid-linked intermediates in the biosynthesis of xanthan gum. FEBS Lett. **130:**253–256.
30. **Ielpi, L., R. O. Couso, and M. A. Dankert.** 1981. Pyruvic acid acetal residues are transferred from phosphoenolpyruvate to the pentasaccharide-P-P-lipid. Biochem. Biophys. Res. Commun. **102:**1400–1408.
31. **Ielpi, L., R. O. Couso, and M. A. Dankert.** 1993. Sequential assembly and

polymerization of the polyprenol-linked pentasaccharide repeating unit of the xanthan polysaccharide in *Xanthomonas campestris*. J. Bacteriol. **175:** 2490–2500.
32. **Ielpi, L., R. O. Couso, and M. A. Dankert.** 1983. Xanthan gum biosynthesis: acetylation occurs at the prenyl-phospho-sugar stage. Biochem. Int. **6:**323–333.
33. **Jansson, P. E., L. Kenne, and B. Lindberg.** 1975. Structure of extracellular polysaccharide from *Xanthomonas campestris*. Carbohydr. Res. **45:**275–282.
34. **Katzen, F., A. Becker, A. Zorreguieta, A. Pühler, and L. Ielpi.** 1996. Promoter analysis of the *Xanthomonas campestris* pv. campestris *gum* operon directing biosynthesis of the xanthan polysaccharide. J. Bacteriol. **178:**4313–4318.
35. **Kidby, D., P. Sandford, A. Herman, and M. Cadmus.** 1977. Maintenance procedures for the curtailment of genetic instability: *Xanthomonas campestris* NRRL B-1459. Appl. Environ. Microbiol. **33:**840–845.
36. **Köplin, R., W. Arnold, B. Hötte, R. Simon, G. Wang, and A. Pühler.** 1992. Genetics of xanthan production in *Xanthomonas campestris*: the *xanA* and *xanB* genes are involved in UDP-glucose and GDP-mannose biosynthesis. J. Bacteriol. **174:**191–199.
37. **Marzocca, M. P., N. E. Harding, E. A. Petroni, J. M. Cleary, and L. Ielpi.** 1991. Location and cloning of the ketal pyruvate transferase gene of *Xanthomonas campestris*. J. Bacteriol. **173:**7519–7524.
38. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
39. **Morrison, D. A.** 1977. Transformation in *Escherichia coli*: cryogenic preservation of competent cells. J. Bacteriol. **132:**349–351.
40. **Müller, P., M. Keller, W. M. Weng, J. Quandt, W. Arnold, and A. Pühler.** 1993. Genetic analysis of the *Rhizobium meliloti exoYFQ* operon: ExoY is homologous to sugar transferases and ExoQ represents a transmembrane protein. Mol. Plant-Microbe Interact. **6:**55–65.
41. **Petroni, E. A., and L. Ielpi.** 1996. Isolation and nucleotide sequence of the GDP-mannose:cellobiosyl-diphosphopolyprenol α-mannosyltransferase gene from *Acetobacter xylinum*. J. Bacteriol. **178:**4814–4821.
42. **Priefer, U.** 1984. Isolation of plasmid DNA, p. 14–25. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics. Springer-Verlag KG, Berlin, Germany.
43. **Reed, J. W., M. Capage, and G. C. Walker.** 1991. *Rhizobium meliloti exoG* and *exoJ* mutations affect the ExoX-ExoY system for modulation of exopolysaccharide production. J. Bacteriol. **173:**3776–3788.
44. **Reuber, T. L., and G. C. Walker.** 1993. Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. Cell **74:** 269–280.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
47. **Scott, D. B., C. A. Young, J. M. Collins-Emerson, E. A. Terzaghi, E. S. Rockman, P. E. Lewis, and C. E. Pankhurst.** 1996. Novel and complex chromosomal arrangement of *Rhizobium loti* nodulation genes. Mol. Plant-Microbe Interact. **9:**187–197.
48. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the *in vitro* constructed Tn*5*-*Mob* transposon. Mol. Gen. Genet. **196:**413–420.
49. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
50. **Stankowski, J., B. Mueller, and S. Zeller.** 1993. Location of a second *O*-acetyl group in xanthan gum by the reductive-cleavage method. Carbohydr. Res. **241:**321–326.
51. **Sutherland, I. W.** 1993. Xanthan, p. 363–388. *In* J. G. Swings and E. L. Civerolo (ed.), Xanthomonas. Chapman & Hall, London, England.
52. **Tang, J. L., Y. N. Liu, C. E. Barber, J. M. Dow, J. C. Wootton, and M. J. Daniels.** 1991. Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar campestris. Mol. Gen. Genet. **226:**409–417.
53. **Thorne, L., L. Tansey, and T. J. Pollock.** 1987. Clustering of mutations blocking synthesis of xanthan gum by *Xanthomonas campestris*. J. Bacteriol. **169:**3593–3600.
54. **Vanderslice, R. W., D. H. Doherty, M. A. Capage, M. R. Betlach, R. A. Hassler, N. M. Henderson, J. Ryan-Graniero, and M. Tecklenburg.** 1988. Genetic engineering of polysaccharide structure in *Xanthomonas campestris*, p. 145–157. *In* V. Crescenzi, I. C. M. Dea, S. Paoletti, S. S. Stivala, and I. W. Sutherland (ed.), Biomedical and biotechnological advances in industrial polysaccharides. Gordon and Breach Science Publishers, New York, N.Y.
55. **Wang, L., D. Liu, and P. R. Reeves.** 1996. C-terminal half of *Salmonella enterica* WbaP (RfbP) is the galactosyl-1-phosphate transferase domain catalyzing the first step of O-antigen synthesis. J. Bacteriol. **178:**2598–2604.
56. **Whitfield, C., P. A. Amor, and R. Köplin.** 1997. Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. Mol. Microbiol. **23:**629–638.
57. **Wunder, D. E., W. Aaronson, S. F. Hayes, J. M. Bliss, and R. P. Silver.** 1994. Nucleotide sequence and mutational analysis of the gene encoding KpsD, a periplasmic protein involved in transport of polysialic acid in *Escherichia coli* K1. J. Bacteriol. **176:**4025–4033.