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

FEDERICO KATZEN,1,2 DIEGO U. FERREIRO,1 CRISTIAN G. ODDO,1 M. VERÓNICA IELMINI,1 ANKE BECKER,2 ALFRED PÜHLER,2 AND LUIS IELPI1**

Instituto de Investigaciones Bioquímicas Fundación Campomar, Facultad de Ciencias Exactas y Naturales, UBA, and CONICET, (1405) Buenos Aires, Argentina,1 and Lehrstuhl für Genetik, Universität Bielefeld, D-33501 Bielefeld, Germany2

Received 17 October 1997/Accepted 24 January 1998

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 xspI 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.

Materials and Methods

**Biochemistry.** Plasmid DNA from E. coli has been suggested to encode a 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.
glucuronic acid, 17.1

The amount of substituents is variable. Some external mannoses contain a second -O-acetyl instead of a pyruvyl substituent (50).

Data were confirmed by changing the labeled donor or doing duplicate experiments. Figure 2 shows the insertion and integration sites for all mutants. 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 phosphate linkages are split, releasing the labeled oligosaccharide from the unlabeled

The washed cell pellets were each extracted three times with 150 µl of 2 M; UDP-glucuronic acid, 285 µM; GDP-mannose, 142 µM; UDP-glucose, 285 µM; GDP-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. Deacetylation of oligosaccharides was performed with 60 mM NaOH as previously described (32).

Radiochemicals and biochemicals. UDP-[14C]glucose (300 Ci/mol), UDP-[14C]glucuronic acid (300 Ci/mol), and GDP-[14C]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. Braun-schweiger) was grown in a growth chamber at 25°C and 75% humidity for 4 weeks. Bacteria (10⁶ 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.
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 chondrose repeating unit corresponds to the peak migrating between maltopentaose and maltohexaose (31). The acetylated or pyruvylated derivatives (Fig. 3A). The pentasaccharide repeating unit corresponds to the peak migrating between maltopentaose and maltohexaose (31). The acetylated chondrose repeating unit corresponds to the peak migrating between maltopentaose and maltohexaose (31).

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-[14C]glucose (Fig. 3B). Cells labeled with UDP-[14C]glucuronic acid or GDP-[14C]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 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 (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-[14C]glucose (Fig. 3B). Cells labeled with UDP-[14C]glucuronic acid or GDP-[14C]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 (see below), which does not seem to participate in xanthan biosynthesis.
α-mannosyl residue from GDP-mannose to cellobiose diphosphopolypropenol to produce mannosyl-(α-1,3)-cellobiose diphosphopolypropenol.

Oligosaccharides released from the lipid fraction of gumK strain XcK could be resolved into three species when permeabilized cells were labeled with UDP-[14C]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-[14C]mannose, but no radioactivity was detected in 1203 extracts after the labeling of XcK cells with UDP-[14C]glucuronic acid (data not shown). The fastest-migrating component was characterized as glucose. Hence, the gumph product appears to be glucosyltransferase 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 labeling of guml mutant XcI with UDP-[14C]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 maltotetraose to M₄-M₆, while the compound from strain XcI remained unaltered. The minor compound corresponds to acetylated tetrasaccharide. The presence of the acetyl group was confirmed by deacylation.

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-[14C]mannose or UDP-[14C]glucuronic acid (data not shown). The fastest-migrating component was characterized as glucose. Thus, it seems likely that gumph encodes glucosyltransferase V, which is involved in the transferral of a β-mannosyl residue from GDP-mannose to glucuronol-(β-1,2)-mannosyl-(α-1,3)-cellobiose diphosphopolyprenol to render mannosyl-(β-1,4)-glucuronol-(β-1,2)-mannosyl-(α-1,3)-cellobiose diphosphopolyprenol. Although the assignment of glucosyltransferase V to GumI was in agreement with earlier reports, accumulation of a lipid-linked tetrasaccharide was not detected previously (51, 54).

The radioactive oligosaccharides obtained from gumph and gumg mutants (XcF and XcG, respectively) labeled with GDP-[14C]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). Deacylation 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 gumph 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-[14C]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 gumph encodes an acetyltransferase whose acceptor is the innermost mannose of the lipid-linked pentasaccharide intermediate (acyetyltransferase I), while gumg appears to encode a related enzyme in which the acceptor is the external mannose of the same intermediate (acyetyltransferase II).

Two radioactive products appeared in paper electrophoresis upon mild-acid hydrolysis of 1203 extracts of permeabilized FC2 cells labeled with UDP-[14C]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 XCf 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-[14C]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 polyol is converted to give the 1,2-cyclic phosphate ester of glucose, with a mobility (Rf) of 0.5. Two compounds were observed in about similar amounts: peak 1, with an Rf of 0.5, and peak 2, with an Rf 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 polyol but were unable to produce cellobiose diphosphate polyol. 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 polyol to produce cellobiose diphosphate polyol.

Surprisingly, permeabilized ugp-gumB (Xc405B), ugp-gumC (Xc405C), ugp-gumE (Xc405E), and ugp-gumI (Xc405I) cells incorporated no radioactivity into the 1203 extract when labeled with UDP-[14C]glucuronic acid or GDP-[14C]mannose (data not shown). Upon the labeling of these cells with UDP-[14C]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 Rf of 0.9 was detected by paper chromatography in alkaline solvent A. As pointed out above, an Rf of 0.5 was expected for glucose diphosphate polyol. 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).
pound eluted at 0.14 M ammonium acetate. Under the same conditions, galactose monophosphate polypreol 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 1-phosphate transferase (the gumD protein). Whereas some glucosyl-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 cellubiose 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-[14C]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-[14C]glucose or GDP-[14C]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 gumI 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 \times 10^6) (29, 31). Permeabilized cells from all of the strains depicted in Fig. 3 were labeled with UDP-[14C]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

![Figure 4](http://jb.asm.org/Downloaded.on October 19, 2018 by guest)
vivo (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-[14C]glucuronic acid or GDP-[14C]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, lrp 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
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, 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 BamHI 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, gumG, 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, 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. GumL 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).

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

| Gene in X. campestris gum region | Homologous gene | Bacterium | Polysaccharide | Identity (%) | 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        |
| gumI (499)                      | exoT           | R. meliloti| Succinoglycan | 13 (520)     | Influences production of certain low-molecular-weight oligosaccharides | 21       |

* 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.
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 hydrophobicity 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-

---

**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).
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 employed by 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 biosynthesis of cyclic glucans and lipopolysaccharides (36). In particular, the virulence of two xanthan-deficient strains carrying a mutant ExoP protein characterized by a periplasmic N-terminal domain and a missing C-terminal domain. Mol. Microbiol. 16:191–209.

14. Beijers, H. R. M., M. A. Capano, 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.

15. Cadmus, M. C., S. P. Rogovin, K. A. Burton, J. E. Pitsillies, C. A. Knutson, and A. Jeannes. 1976. Colonial variation in Xanthomonas campestris NRRL B-1459 and characterization of the polysaccharide from a variant strain. Can. J. Microbiol. 22:942–948.

16. Capage, M. R., M. R. Betlach, and R. W. Vanderslice. March 1987. Recombinant-DNA mediated production of xanthan gum. Internat. patent WO87/00308.

17. 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 gumE gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. Biochem. Biophys. Res. Commun. 233:263–269.

18. Cooper, D. L., and D. Cook. 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. Mol. Plant-Microbe Interact. 3:271–279.

19. Daniels, M. J. 1993. Genes of Xanthomonas, p. 301–339. In J. G. Swings and E. L. Civerolo (ed.), Xanthomonas. Chapman & Hall, London, England.

20. 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.

21. de Crécy-Lagard, V., P. Glaser, P. Lejeune, O. Simeone, 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.

22. Denny, T. P. 1995. Involvement of bacterial polysaccharides in plant pathogenesis. Annu. Rev. Phytopathol. 33:173–197.

23. Ditta, G. T., Schmidhauser, E. Yakobson, P. Lu, W. Y. 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.

24. 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.

25. Glucksmann, M. A., T. L. Reuber, and G. C. Walker. 1990. Cloning and isolation of a cluster of xanthan sugar nucleotide precursors of xanthan gum. J. Gen. Microbiol. 136:293–303.

26. Griffin, A. M., V. J. Morris, and M. J. Gasson. 1996. Identification, cloning and sequencing the modification, polymerization, export, and processing of succinoglycan by X. campestris pv. campestris. Int. J. Biol. Macromol. 19:2804–2854.

27. Harding, N. E., J. M. Cleary, D. K. Caban˜as, I. G. Rosen, and K. S. Kang. 1995. Genetics and biochemistry of xanthan gum biosynthesis and a missing C-terminal domain. Mol. Microbiol. 17:2081–2092.

28. Hoppin, R. C., C. E. Barber, and M. J. Daniels. 1993. Identification, genetic and biochemical analysis of genes involved in synthesis of host polysaccharide structure: production of variants of xanthan gum in Xanthomonas campestris. Biotechnol. Prog. 9:182–187.

29. Hötting, B., I. Rath-Arnold, A. Pühler, and R. Simon. 1990. Cloning and analysis of a 53.5-kilobase DNA region involved in xopolysaccharide production by Xanthomonas campestris pv. campestris. J. Bacteriol. 172:2804–2807.

30. Ielpi, L. R. O. Couso, and M. A. Dankert. 1991. Linker-mediated intermediates in the biosynthesis of xanthan gum. FEBS Lett. 230:253–256.

31. Ielpi, L. R., O. Couso, and M. A. Dankert. 1981. Pyruvic acid acetal residues are transferred from phosphoenoxypruvate to the pentasaccharide-P-P-lipid. Biochem. Biophys. Res. Commun. 101:402–406.

32. 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. Kidly, D., P. Sandford, A. Herman, and M. Cadmus. 1977. Maintenance procedures for the curtailment of genetic instability. Xanthomonas campestris. Appl. Environ. Microbiol. 33:840–845.

36. Kühni, 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. 1992. Physical and genetic characterization of symbiotic and auxotrophic mutants of Rhizobium meliloti induced by transposon Tn5 mutagenesis. I. Bacteriol. 149:111–122.

38. Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel. 1993. Genetic analysis of the Rhizobium meliloti exoYFQ operon: ExoY is homologous to sugar transferases and ExoQ represents a transmembrane periplasmic protein involved in transport of polysialic acid in Escherichia coli. J. Bacteriol. 175:7519–7524.

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. Quandi, 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 exo mutations affect the ExoX-ExoY system for modulation of exopoly saccharide 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 Tn5-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. J. Bacteriol. 170:3593–3600.

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. Köpflin. 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.