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Genetics of Xanthan Production in *Xanthomonas campestris*: the *xanA* and *xanB* Genes Are Involved in UDP-Glucose and GDP-Mannose Biosynthesis

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The nucleotide sequence of a 3.4-kb *Eco*RI-*Pst*I DNA fragment of *Xanthomonas campestris* pv. *campestris* revealed two open reading frames, which were designated *xanA* and *xanB*. The genes *xanA* and *xanB* encode proteins of 448 amino acids (molecular weight of 48,919) and 466 amino acids (molecular weight of 50,873), respectively. These genes were identified by analyzing insertion mutants which were known to be involved in xanthan production. Specific tests for the activities of enzymes involved in the biosynthesis of UDP-glucose and GDP-mannose indicated that the *xanA* gene product was involved in the biosynthesis of both glucose 1-phosphate and mannose 1-phosphate. The deduced amino acid sequence of *xanB* showed a significant degree of homology (59%) to the phosphomannose isomerase of *Pseudomonas aeruginosa*, a key enzyme in the biosynthesis of alginate. Moreover, biochemical analysis and complementation experiments with the *Escherichia coli manA* fragment revealed that *xanB* encoded a bifunctional enzyme, phosphomannose isomerase–GDP-mannose pyrophosphorylase.

Xanthomonas campestris is a gram-negative bacterium which is phytopathogenic for cruciferous plants (15). The mechanism of the pathogen-plant interaction remains unclear, but surface components such as lipopolysaccharides (LPS) and exopolysaccharides (EPS) are considered determining factors in the interaction (13). A direct link between the ability to synthesize xanthan, the EPS of *X. campestris*, and plant pathogenicity has not yet been established (18, 32), although it is purported to be involved in the initial stage of infection (57). Because of its unique rheological properties such as high viscosity and pseudoelasticity (43), xanthan has a potential application in industrial production processes (4, 47). Detailed knowledge of the chemistry and biosynthesis of xanthan has accumulated over the years (26, 28, 29, 40). The chemical structure of this acidic heteropolysaccharide is well known. It consists of an (1→4)-linked β-D-glucan (cellulose) backbone substituted at O-3 of every second glucose residue by the trisaccharide β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→. The mannose sugars are acetylated and pyruvylated at specific sites, but to various degrees (10). In addition, during biochemical and physiological studies, steps in xanthan biosynthesis have been identified which are very similar to steps in the EPS biosynthesis of other gram-negative bacteria (56). Detailed in vitro studies have shown that the assembly of the pentasaccharide repeating unit from the precursers UDP-glucose, UDP-glucuronic acid, and GDP-mannose occurs on a lipid carrier (28). The pyruvic and acetyl residues are obtained from phosphoenolpyruvate and acetyl-coenzyme A, respectively (29, 30). The pentasaccharide units are eventually secreted and polymerized. To date, a number of genes involved in xanthan biosynthesis have been characterized, and evidence suggests that they are clustered together (5, 22, 58).

Recently, we reported the cloning and mapping of a 35.3-kb DNA region involved in xanthan biosynthesis (27). This DNA region, which was organized into 12 complementation groups, complemented a variety of EPS-deficient (EPS^-) mutants. In this report, the results of a more detailed analysis of a 3.4-kb *Eco*RI-*Pst*I fragment of this DNA region are presented. Two genes involved in xanthan biosynthesis encode enzymes involved in the biosynthesis of the sugar nucleotides UDP-glucose and GDP-mannose.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are described in Table 1.

Media and growth conditions. *X. campestris* strains were grown in modified M9 medium (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4Cl in 1 liter of H_2O) supplemented with 3% glucose and 0.05% Casamino Acids. The antibiotics streptomycin and kanamycin were used at concentrations of 800 and 80 μg/ml, respectively.

Biochemicals. The chemicals used were obtained as follows: mannose 1-phosphate, glucose 1,6-diphosphate, and mannose 6-phosphate, Sigma Chemical Co., St. Louis, Mo.; dithiothreitol (DTT), sugar nucleotides, NAD, NADH, NADP, and all enzymes used in coupling reactions, Boehringer, Mannheim, Germany; protein assay reagents, Pierce, Rockford, Ill.; and morpholine propanesulfonic acid (MOPS), Serva, Heidelberg, Germany.

Recombinant DNA methods. Plasmid DNA was obtained from *Escherichia coli* by the rapid isolation method (lysis by boiling) described elsewhere (23). Restriction endonucleases and T4 ligase were purchased from Boehringer and used in accordance with the manufacturer's recommendations. Agarose gel electrophoresis was carried out as described by Maniatis et al. (37).

Complementation experiments in *X. campestris*. The EPS^- mutants B100-14, B100-15, and B100-13 were complemented by plasmid pAD3, which contains the *E. coli manA* gene. Filter conjugations (50) employing the broad-host-range mo-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli S17.1	*E. coli* 294, RP4-2-Tc::Mu-Km::Tn7	52
X. campestris		27
B100	DSM1526, Sm^r	27
B100-152	Spontaneous EPS^- mutant	27
B100-13	Spontaneous EPS^- mutant	27
B100-14	Tn*5-lac*-induced EPS^- mutant	27
B100-15	Tn*5-lac*-induced EPS^- mutant	27
Plasmids		
pSUP205	pBR325-based cosmid, Mob^+ Cm^r Tc^r	52
pXCB1002	pSUP205 carrying a 35.5-kb insert of the DSM1526 chromosome	27
pAD3	pCP13 (Tc^r) *manA*$^+$	16

bilizing strain *E. coli* S17-1 (52) enabled plasmids to be transferred into *X. campesteris*.

Nucleotide sequence analysis. Subclones for DNA sequencing were obtained by cloning defined restriction fragments into pSVB sequencing vectors (2) and by nested deletions, using exonuclease III and S1 nuclease (nested deletions kit; Pharmacia). Their DNA sequence was determined by the chain termination method (48), using the double-stranded DNA technique (3). To verify the DNA sequencing data, the complementary DNA strand sequence was obtained for all subclones by the chemical degradation method of Maxam and Gilbert (39) as modified by Arnold et al. (3).

The DNA sequence was analyzed with the aid of the sequence analysis programs of Staden (53). The deduced amino acid sequence was compared with sequences in the most up-to-date version of the NBRF protein data bank, using the FASTA programs (36).

Preparation of *X. campestris* cell extracts. Bacterial cultures grown to early stationary phase were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C. To prepare cell extracts, 2 g of pelleted cells was suspended in 8 ml of sonication buffer, and the cells were disrupted by sonic vibration (three 10-s pulses, 120 W) with a Labsonic 1510 (B. Braun, Melsungen, Germany). The sonication buffer consisted of 50 mM MOPS, 1 mM DTT, 3 mM EDTA, and 1% Triton X-114 (pH 7.0). DTT and EDTA were omitted when cell extracts were prepared for the phosphomannose isomerase (PMI) assay. The cell debris was removed by centrifugation at 40,000 × *g* for 15 min at 4°C. The supernatant was centrifuged at 100,000 × *g* for 60 min, and an aliquot thereof was passed through a prepacked Sephadex G-25 column (PD10; Pharmacia). The column was equilibrated as instructed by the manufacturer with sonication buffer lacking Triton X-114.

Determination of protein concentrations. The protein concentration of gel-filtered extracts was determined by the Micro BCA protein assay reagent (Pierce) as instructed by the manufacturer. Bovine serum albumin was used as the protein reference substance.

Enzyme assays. Freshly prepared extracts were used for the enzyme assays. In each experiment, two extracts were prepared independently and their enzymatic activities were ascertained. The activities of glucokinase (GK), phosphoglucose isomerase (PGI), UDP-glucose pyrophosphorylase (UGP), phosphoglucomutase (PGM), phosphomannomutase (PMM), and GDP-mannose pyrophosphorylase (GMP) were ascertained by determining the increase in optical density (for GK, PGI, UGP, PGM, and PMM) and the decrease in optical density (for GMP). All values were measured at 340 nm and 30°C with a Uvicon 820 spectrophotometer (Kontron). PMI activity was determined by a discontinuous assay. Since the PMI of brewer's yeast was shown to be inhibited in a time-dependent process by, among others, DTT and EDTA (19), these chemicals were omitted from the buffers used. Control assays lacking the extract, substrate, or cofactor were also carried out. The enzyme activities were calculated by subtracting the values of the endogenous activities.

GK activity. GK (EC 2.7.1.2) activity was measured as described by Bergmeyer et al. (6). The 1-ml reaction mixture contained 100 mM MOPS buffer (pH 7.0), 0.9 mM NADP, 8 mM $MgCl_2$, 0.6 mM ATP, 0.5 U of glucose 6-phosphate dehydrogenase (G6P-DH), 5 μl of cell extract, and 0.22 mmol of glucose, which started the reaction.

PGI activity. PGI (EC 5.3.1.9) activity was assayed as described elsewhere (7). The 1-ml reaction mixture contained 100 mM MOPS buffer (pH 7.0), 1.4 mM NADP, 6.8 mM $MgCl_2$, 0.5 U of G6P-DH, 1 to 2 μl of cell extract, and 1.4 μmol of fructose 6-phosphate, which started the reaction.

UGP activity. UGP (EC 2.7.7.9) activity was assayed as described elsewhere (8). The 1-ml reaction mixture contained 100 mM MOPS buffer (pH 7.6), 1.5 mM $MgCl_2$, 0.38 mM NADP, 20 μM glucose 1,6-diphosphate, 0.9 U of G6P-DH, 6.6 U of PGM, 1 μmol of UDP-glucose, and 10 to 20 μl of cell extract. The reaction was started by adding 1.5 μmol of PP_i.

PMI activity. PMI (EC 5.3.1.8) activity was measured by a discontinuous assay. The 0.9-ml reaction mixture contained 0.1 M MOPS buffer (pH 7.0) and 100 μl of cell extract. The reaction was started by adding 100 μl of 0.1 M mannose 6-phosphate, and samples of 200 μl were taken after 0, 10, 20, and 30 min. The samples were deproteinized with 200 μl of ice-cold perchloric acid, and after centrifugation, the supernatant was neutralized with the appropriate amount of $KHCO_3$. The amounts of glucose 6-phosphate and fructose 6-phosphate produced were assayed with PGI and G6P-DH by the endpoint method described by Michal (41).

PGM activity. PGM (EC 5.4.2.2.) activity was assayed according to the method of Bergmeyer et al. (9). The 1-ml reaction mixture contained 100 mM MOPS buffer (pH 7.6), 0.075 mM glucose 1,6-diphosphate, 1.7 mM $MgCl_2$, 0.2 mM NADP, 0.5 U of G6P-DH, and 5 to 20 μl of cell extract. The addition of 3 μmol of glucose 1-phosphate started the reaction.

PMM activity. The method of Sá-Correia et al. (46) was used to ascertain the PMM (EC 5.4.2.8.) activity. The 1-ml reaction mixture contained 100 mM MOPS buffer (pH 7.6), 10 mM $MgCl_2$, 0.075 mM glucose 1,6-diphosphate, 1 mM NADP, 1 mM mannose 1-phosphate, 0.7 U of G6P-DH, 1.1 U of PGI, and 0.9 U of PMI. The reaction was started by the addition of 2 to 10 μl of cell extract.

GMP activity. GMP (EC 2.7.7.13) activity was measured according to the method of Verachtert et al. (59). The 1-ml reaction mixture contained 0.1 M MOPS buffer (pH 7.6), 1 mM $MgCl_2$, 0.6 mM 3-phosphoglycerate, 0.125 mM NADH, 0.2 mM GDP-mannose, 18 U of phosphoglycerate kinase, 16 U of glycerin aldehyde 3-phosphate dehydrogenase, 0 to 20 μl of cell extract, and 1 μmol of PP_i, which started the reaction.

Nucleotide sequence accession number. The DNA sequence containing the *X. campestris xanA* and *xanB* genes has been assigned GenBank no. M83231.

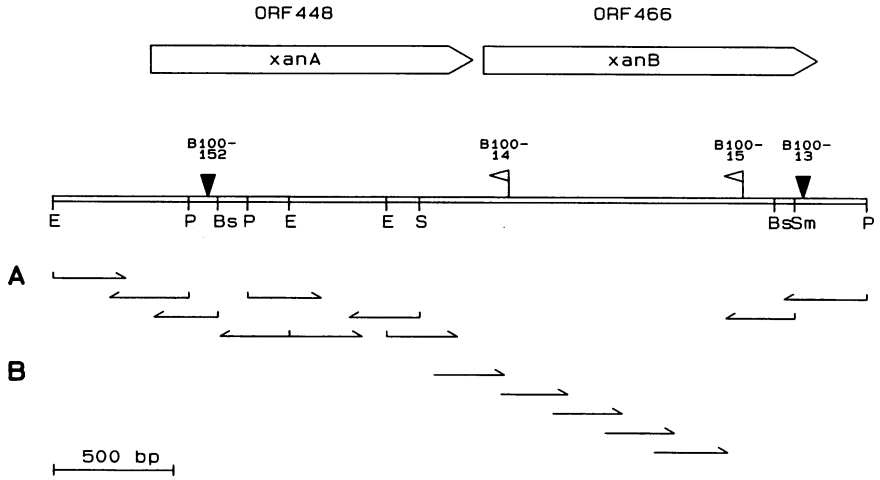

FIG. 1. Coding region of an *X. campestris* DNA fragment involved in xanthan production. The restriction endonuclease map of the sequenced *X. campestris* 3.4-kb *Eco*RI-*Pst*I fragment is presented. The locations of ORF 448 and ORF 466 are indicated by open arrows. (A) Strategy for sequencing defined restriction fragments; (B) direction of sequencing and the extent of each fragment that was generated by nested deletions, using exonuclease III and S1 nuclease. The insertion site of the insertion sequence element in mutant B100-13 was corrected with respect to the location previously published (27). Symbols: ⊲, Tn*5-lac* insertion indicating the orientation of the *lacZ* gene; ▼, putative insertion sequence element. Abbreviations: E, *Eco*RI; Bs, *Bst*EII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

RESULTS

Identification of two ORFs of *X. campestris* located on a 3.4-kb *Eco*RI-*Pst*I fragment involved in xanthan biosynthesis. In a recent report (27), we described the recombinant cosmid pXCB1002, which carried a 35.3-kb *X. campestris* chromosomal DNA fragment. Several mutations which resulted in an EPS$^-$ phenotype were located on a 3.4-kb *Eco*RI-*Pst*I subfragment of cosmid pXCB1002 (27) (Fig. 1). The restriction map and strategy for sequencing this DNA fragment are presented in Fig. 1. Various restriction fragments were subcloned into pSVB sequencing vectors (2), resulting in a series of overlapping clones. Both DNA strands of the 3.4-kb fragment were sequenced. The DNA sequence of this 3,410-bp DNA fragment is given in Fig. 2. By using the computer programs of Staden (53), a coding region analysis was carried out. The locations of start and termination codons in the different reading frames permitted the identification of two ORFs that had a high coding probability on one DNA strand. No convincing coding sequence could be detected on the opposite strand. These two ORFs were called ORF 448 and ORF 466. The locations of the ORFs and the extents and directions of their coding regions are shown in Fig. 1. ORF 448 is located between nucleotide positions 411 and 1757 (Fig. 2). A putative 5′-AGGA-3′ ribosome binding site (54) was found 7 bp in front of the ATG initiation codon. No promoter consensus sequences were detected upstream of the ribosome binding site. The translation product of ORF 448 deduced from the DNA sequence is a protein of 448 amino acids with a molecular weight of 48,919. The deduced amino acid sequence of this ORF showed no significant homology to any protein sequence of the NBRF protein data base.

ORF 466, which is located 45 bp downstream of ORF 448, spans 1,401 bp. There is a putative 5′-GGAG-3′ ribosome binding site (54) 12 bp in front of the ATG initiation codon. A search for the −10 and −35 promoter regions by using the known consensus sequences of gram-positive and gram-negative bacteria (20) did not reveal any promoter sequences upstream of ORF 466. The translation product predicted for this ORF is a protein of 466 amino acids with a molecular weight of 50,873. An amino acid sequence comparison revealed 59% homology to the bifunctional enzyme of *P. aeruginosa*, PMI-GMP (Fig. 3) (49). An inverted repetitive sequence which resembled a bidirectional termination structure (11) was identified 26 bp downstream of the translational stop codon of ORF 466. The inverted sequences, which were separated by 4 bp, consisted of 18 repetitive nucleotides that corresponded exactly (indicated in Fig. 2). The formation of a stem-loop structure in the mRNA corresponds with a free-energy change of −38.7 kcal (ca. −162 kJ)/mol.

The *X. campestris* genes *xanA* and *xanB* are both involved in xanthan biosynthesis. Cosmid pXCB1002 was found to contain at least 12 complementation groups (27). The 3.4-kb *Eco*RI-*Pst*I fragment carries the DNA sequences belonging to both complementation groups A and B. Mutant B100-152 belongs to group A, while mutants B100-14, B100-15, and B100-13 belong to complementation group B (27). As found by restriction analysis, the mutation responsible for the B100-152 phenotype was located in the coding region of ORF 448 (Fig. 1) and consisted of an insertion of approximately 0.3 kb within the 0.9-kb *Eco*RI fragment (27). The colonies of strain B100-152 were smooth in appearance, and they produced small quantities of a low-viscosity xanthan gum (data not shown). Since ORF 448 was clearly involved in xanthan production, this gene was designated *xanA*.

Two other mutants belonged to complementation group B. B100-14 and B100-15 were Tn*5-lac*-induced mutants, whereas B100-13 resulted from an insertion of the *X. campestris*-specific insertion element Xc*B100-2. This insertion sequence was 1.7 kb in size and contained an additional *Eco*RI site (51). Hybridization experiments confirmed that the elongation of the *Bst*EII-*Pst*I fragment located within the coding region of ORF 466 was due to an Xc*B100-2 insertion (data not shown).

The location of the Tn*5-lac* insertion in mutants B100-14 and B100-15 was determined by dideoxy-chain termination DNA sequencing using a Tn*5*-specific primer (17). As shown in Fig. 2, Tn*5-lac* inserted at bp 1905, which was 103 bp

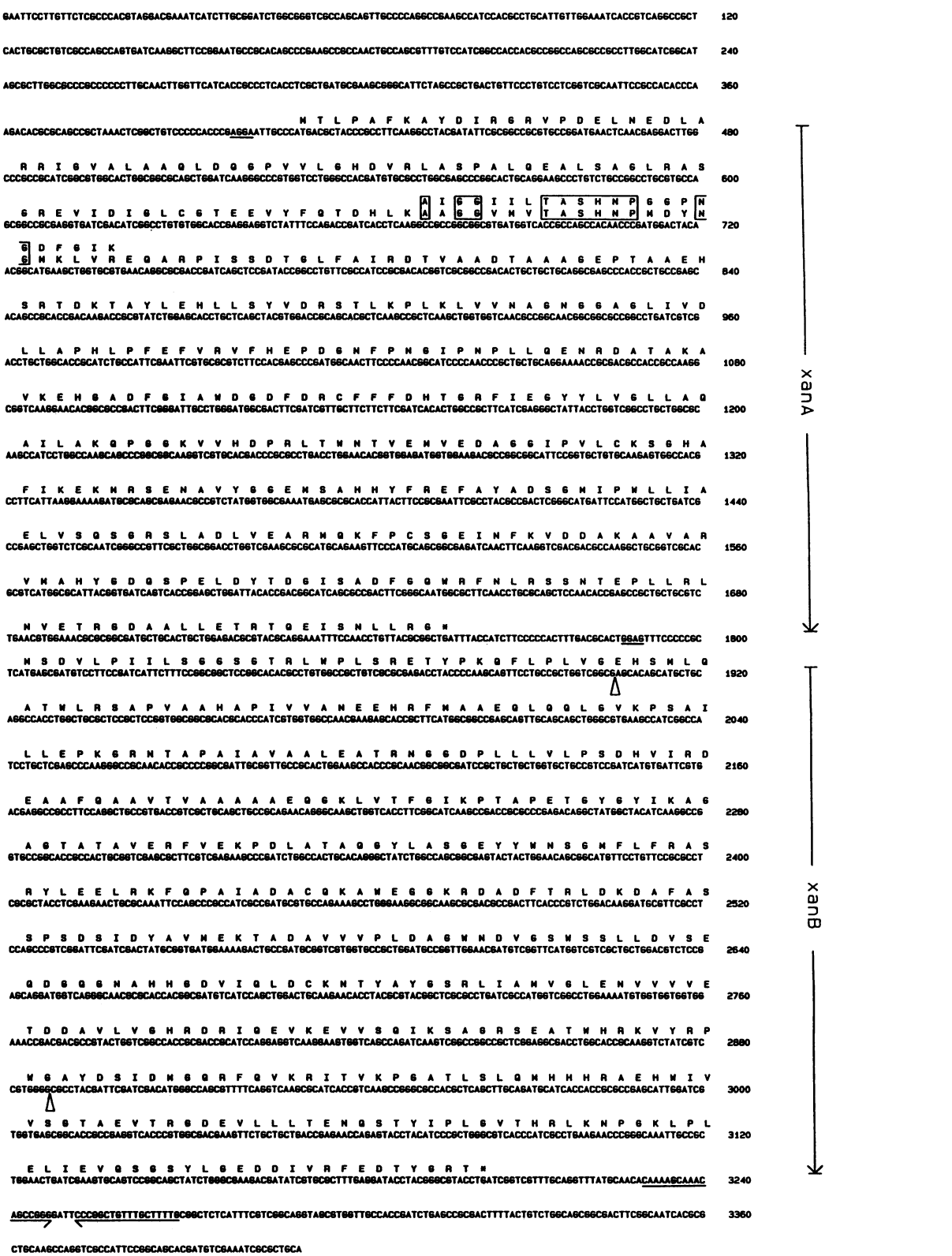

FIG. 2. Nucleotide sequence of an *X. campestris* DNA fragment involved in xanthan production. The nucleotide sequence of one strand of the 3.4-kb *Eco*RI-*Pst*I DNA fragment is presented in the 5′→3′ direction. The two ORFs that were identified are indicated by their deduced amino acid sequences, which are given in one-letter code above the nucleotide sequence. The extents and directions of the ORFs are indicated by arrows. The inverted repetitive DNA sequences downstream of *xanB* are shown by arrows below the nucleotide sequence. Putative ribosome binding sites are underlined. The insertion sites of Tn*5-lac* are indicated by open triangles. The region representing the active-center peptide of rabbit muscle PGM (45) is given above the amino acid sequence of *xanA*. Identical residues are boxed.

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                10        20        30        40        50        60
XcxanB  MSDVLPIILSGGSGTRLWPLSRETYPKQFLPLVGEHSMLQATWLRSAPVAAHAPIVVANE
        ..|.|||||||.|||||||. ||||...|.|.....|.|  | |  . .||..| |.
PaalgA     MIPV!LSGGSGSRLWPLSRKQYPKQLVALTGDDTLFQQTIKRLAFDGMQAPLLVCNK
                  10        20        30        40        50

                70        80        90       100       110       120
XcxanB  EHRFMAAEQLQQLGVKPSAILLEPKGRNTAPAIAVAALEATRNGGDPLLLVLPSDHVIRD
        ||||...|||.. .. . |||||| |||||||.|.||.. . .| | |||.||.|||. |
PaalgA  EHRFIVQEQLEAQNLASQAILLEPFGRNTAPAVAIAAMKLVAEGRDELLLILPADHVLED
         60        70        80        90       100       110

               130       140       150       160           170
XcxanB  EAAFQAAVTVAAAAAEQGKLVTFGIKPTAPETGYGYIKAGAGT-----ATAVERFVEKPD
        . |||.|...|..||||.|..| ||| .. |||||||.|.|..      .. |..|||||||
PaalgA  QRAFQQALALATNAAEKGEMVLFGIPASRPETGYGYIRATADAQLPEGVSRVQSFVEKPD
        120       130       140       150       160       170

          180       190       200       210       220       230
XcxanB  LATAQGYLASGEYYWNSGMFLFRASRYLEELRKFQPAIADACQKAWEGGKRDADFTRLDK
         | |....|.|.|||||||||||||||||.| ...| |.|  | | ...|.|....|
PaalgA  EARAREFVAAGGYYWNSGMFLFRASRYLEELKKHDADIYDTCLLALERSQHDGDLVNIDA
        180       190       200       210       220       230

          240       250       260       270       280       290
XcxanB  DAFASSPSDSIDYAVMEKTADAVVVPLDAGWNDVGSWSSLLDVSEQDGQGNAHHGDVIQL
        ..|...|..||||||||||. | ||||.|||||||||||. || ..|..||. .|||.
PaalgA  ATFECCPDNSIDYAVMEKTSRACVVPLSAGWNDVGSWSSIWDVHAKDANGNVTKGDVLVH
        240       250       260       270       280       290

          300       310       320       330       340       350
XcxanB  DCKNTYAYG-SRLIAMVGLENVVVVETDDAVLVGHRDRIQEVKEVVSQIKSAGRSEATWH
        |..|  ..| ..|....|||..|||||.||....|.||.|.||.||......||||.  |
PaalgA  DSHNCLVHGNGKLVSVIGLEDIVVVETKDAMMIAHKDRVQDVKHVVKDLDAQGRSETQNH
        300       310       320       330       340       350

             360       370       380       390       400       410
XcxanB  RKVYRPWGAYDSIDMGQRFQVKRITVKPGATLSLQMHHHRAEHWIVVSGTAEVTRGDEVL
        ..|||||.|||.||| |||||.|||||| ||||||||||||||||||||.|| .|...
PaalgA  CDLYRPWGSYDSVDMGGRFQVKHITVKPGARLSLQMHHHRAEHWIVVSGTAQVTCDDKTF
        360       370       380       390       400       410

             420       430       440       450       460
XcxanB  LLTENQSTYIPLGVTHRLKNPGKLPLELIEVQSGSYLGEDDIVRFEDTYGRT
        ||||||||||.. .||| |||||.|||.|||||||||||||| |.||.||||
PaalgA  LLTENQSTYIPIASVHRLANPGKIPLEIIEVQSGSYLGEDDIERLEDVYGRTAEPALQVV
        420       430       440       450       460       470


XcxanB

PaalgA  AGSR
        480
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FIG. 3. Comparison between the deduced amino acid sequences of *xanB* of *X. campestris* and *algA* of *P. aeruginosa*. The deduced amino acid sequences of the *X. campestris xanB* gene (XcxanB) and the *P. aeruginosa algA* gene (PaalgA) were compared with each other by means of the FASTA computer program (36). Identical residues are marked by vertical lines; similar residues are marked by dots.

downstream of the ATG start codon of ORF 466 of mutant B100-14. The Tn*5-lac* insertion of mutant B100-15 interrupted ORF 466 at bp 2887. All mutations of complementation group B were localized within the coding region of ORF 466. All of these mutants were unable to produce xanthan, and hence ORF 466 was designated *xanB*. Additional phenotypical alterations such as rough colony appearance, autoagglutination in TY liquid medium, lack of motility on TY soft agar plates, and an altered pattern in silver-stained sodium dodecyl sulfate-polyacrylamide gels of LPS were also observed (27). It is known that in the case of other gram-negative bacteria, rough colony appearance, autoagglutination, and loss of motility are correlated to defective LPS production (12, 33). We therefore presumed that *xanB* participated in both EPS and LPS biosynthesis.

Determination of the enzymatic activities of enzymes involved in the synthesis of UDP-glucose and GDP-mannose. Glucose and mannose are common components of *X. campestris* LPS (60) and EPS (31). Sugar nucleotides are considered the immediate precursors of the repeating units of EPS (56). The extensive homology between the amino acid sequence deduced from the *xanB* gene and that of the *Pseudomonas aeruginosa* PMI, which is involved in the synthesis of GDP-mannose, as well as clustering of genes

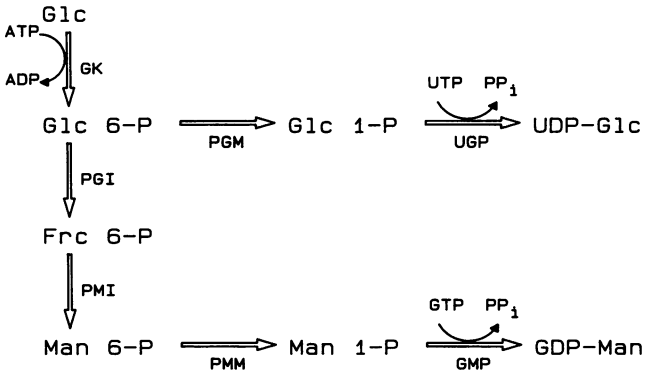

FIG. 4. Biosynthetic pathway of the formation of UDP-glucose and GDP-mannose. Part of the biosynthetic pathway proposed for the formation of UDP-glucose and GDP-mannose is taken from Sutherland (55). Abbreviations not given in the text: Glc, glucose; Glc 6-P, glucose 6-phosphate; Frc 6-P, fructose 6-phosphate; Man 6-P, mannose 6-phosphate; Glc 1-P, glucose 1-phosphate; Man 1-P, mannose 1-phosphate; UDP-Glc, UDP-glucose; GDP-Man, GDP-mannose.

involved in xanthan biosynthesis (5, 22, 58), suggested that *xanA* and *xanB* were involved in the synthesis of the precursors UDP-glucose and GDP-mannose.

The biosynthetic pathway proposed for the formation of UDP-glucose and GDP-mannose in *X. campestris* is shown in Fig. 4. In the biosynthetic pathway, the synthesis of glucose 6-phosphate leads to that of glucose 1-phosphate, which results in UDP-glucose; the enzymes required are GK, PGM, and UGP, respectively.

For the formation of GDP-mannose, glucose 6-phosphate is isomerized to fructose 6-phosphate, catalyzed by PGI, an enzyme of the Embden-Meyerhof pathway. This pathway is shown to be ineffective in members of the *Xanthomonades* because of a lack of phosphofructokinase (63). The intermediates mannose 6-phosphate and mannose 1-phosphate precede the formation of GDP-mannose; the enzymes required are PMI, PMM, and GMP, respectively. These three enzymes are known to be involved in the biosynthesis of GDP-mannuronic acid, a nucleotide sugar precursor of alginic acid in *P. aeruginosa* and *Azotobacter vinelandii* (44, 46).

The sequence of reactions leading to the formation of UDP-glucose and GDP-mannose from glucose in *X. campestris* was determined by studying the individual enzymes of the proposed biosynthetic pathway (55). The specific activities of all of these enzymes excepting PMI were determined in coupled spectrophotometric assays of cell extracts of *X. campestris* as described in Materials and Methods. The specific activity of PMI was assayed by a discontinuous procedure. The results are summarized in Table 2. The activities of all enzymes of the proposed biosynthetic pathway (Fig. 4) were detected in the *X. campestris* wild-type strain, which was cultured in minimal medium with glucose as a carbon source. PGI exhibited the highest specific activity, 1,075 nmol per min per mg of protein. The activities of the enzymes PGM, PMM, and GMP, which are barely detectable in the mucoid alginate-producing *P. aeruginosa* (46) or *A. vinelandii* (25, 44), were easily detected in *X. campestris*. The next-highest specific activities were detected for PMM (593 mU/mg) and PGM (388 mU/mg). Again, the activities of these enzymes in *X. campestris* were several times higher than those reported for *P. aeruginosa* and *A.*

TABLE 2. Specific activities of enzymes involved in UDP-glucose and GDP-mannose biosynthesis in wild-type and mutant strains of *X. campestris*

Strain	Mutation	Sp act (mU/mg)[a]						
		GK	PGI	PGM	PMM	PMI	UGP	GMP
B100		54	1,075	388	593	31	5	96
B100-152	*xanA*	29	1,069	1	1	12	13	33
B100-14	*xanB*	46	934	449	665	0	3	0
B100-15	*xanB*	30	790	338	460	0	3	1
B100-13	*xanB*	39	1,184	479	711	0	6	39

[a] One milliunit is defined as the conversion of 1 nmol of substrate per min.

vinelandii. Control experiments lacking the cofactor glucose 1,6-diphosphate showed no detectable activity, since PGM and PMM have an absolute requirement for their sugar diphosphate.

The *xanA* gene is involved in the biosynthesis of glucose 1-phosphate and mannose 1-phosphate. The function of *xanA* was investigated by biochemical analysis. The *xanA* mutant B100-152 was grown in minimal medium with glucose as a carbon source. The specific activities of the enzymes of the proposed biosynthetic pathway (Fig. 4) were measured in cell extracts and compared with those of the wild-type strain grown under the same conditions (Table 2). The specific activities of GK, PMI, and GMP were reduced, whereas the UGP activity was increased. Both the PGM and PMM activities were drastically reduced, to approximately 0.2% of the wild-type activity. It was presumed, therefore, that *xanA* encoded an enzyme or regulatory factor necessary for the biosynthesis of mannose 1-phosphate and glucose 1-phosphate.

Assuming that the *X. campestris xanA* gene coded for a PGM, the amino acid sequence deduced from *xanA* was compared with that of rabbit muscle PGM, the only PGM in the data base. The 23 amino acids located around the active-site phosphoserine (Ser-116) within the active center of the rabbit muscle PGM (residues 107 to 129) (45) showed about 48% homology to a similar region within the amino acid sequence of the *xanA* gene product (indicated in Fig. 2). The motif GXGXXG, which is commonly found in protein kinases (21) and nucleotide-binding proteins (61), was also found in both sequences.

The *xanB* gene codes for the bifunctional enzyme which has the activity of PMI and GMP. The specific activities of the enzymes involved in the synthesis of UDP-glucose and GDP-mannose were also determined for the three mutants B100-14, B100-15, and B100-13, all containing mutations within *xanB* (Table 2).

Mutant B100-14, which contained Tn*5-lac* 103 bp downstream of the ATG initiation codon, lacked both PMI and GMP activities. Mutant B100-15, which was characterized by a Tn*5-lac* insertion 1,085 bp downstream of the start codon, also lacked PMI activity but, in contrast to B100-14, showed a detectable GMP activity of <2% of the wild-type level. Mutant B100-13 was of particular interest since it clearly demonstrated the presence of one gene product which had two different enzyme activities. This mutant lacked PMI activity but showed nearly 40% of the wild-type GMP activity (Table 2). These results suggested that *xanB* encoded a bifunctional enzyme which was active as a PMI and GMP.

Further evidence was obtained from complementation experiments. The introduction of plasmid pXCB1002 into mutants B100-14, B100-15, and B100-13 restored the wild-type phenotype (data not shown). However, when the mobilizable broad-host-range plasmid pAD3, constructed by Darzins et al. (16), was introduced, very different results were observed. Plasmid pAD3 contains a 6.2-kb fragment carrying the *E. coli* AC80 origin of replication. This fragment also contains the *E. coli manA* gene, which encodes PMI but not GMP. The plasmid was mobilized from *E. coli* S17.1 into the three different *xanB* mutants (Fig. 5). From the colony morphology, it was deduced that plasmid pAD3 complemented the *xanB* mutations in mutants B100-13 and B100-15 but not in mutant B100-14. Evidently, the *E. coli manA* gene was expressed in *X. campestris* and its product complemented the missing PMI activity of mutants B100-15 and B100-13. In contrast to this, the mutation of B100-14 could not be complemented, since this mutant lacked GMP as well as PMI activity. These data therefore confirmed that *xanB* contained the structural gene of PMI and GMP, two enzymes involved in the biosynthesis of GDP-mannose, which is one of the immediate precursors of xanthan gum.

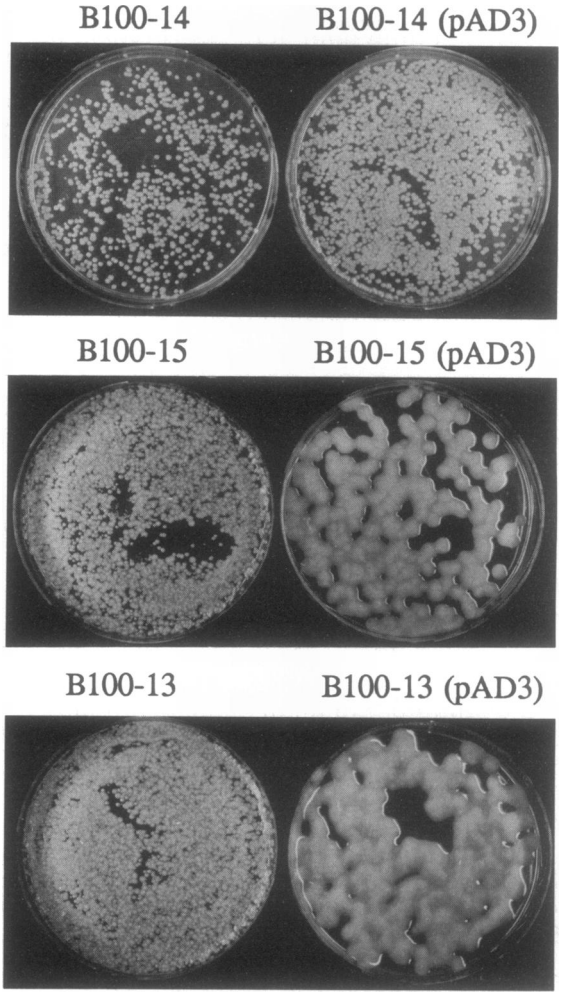

FIG. 5. Complementation of the *xanB* mutants B100-14, B100-15, and B100-13 by a plasmid containing the *manA* gene of *E. coli*. B100-13 and the Tn*5-lac*-derived mutants B100-14 and B100-15 were tested for complementation by plasmid pAD3, which contains the *manA* gene of *E. coli*. Colonies were grown in minimal medium with glucose as the carbon source.

DISCUSSION

In this report, we have described the sequencing and functional analysis of a 3.4-kb *Eco*RI-*Pst*I DNA fragment of cosmid pXCB1002, which carries genes involved in xanthan biosynthesis. DNA sequence analyses of this 3.4-kb *Eco*RI-*Pst*I fragment revealed two ORFs, ORF 448 and ORF 466. It was ascertained by mutation analysis that the ORFs represented the coding region of the genes *xanA* and *xanB*. These two genes corresponded well with the identified complementation groups A and B, which were obtained by cross-complementation (27). The *xanA* mutant resulted from the spontaneous insertion of an approximately 0.3-kb insertion sequence element into the 0.9-kb *Eco*RI fragment. It remains unclear whether this mutation had a polar effect, since data obtained from cross-complementation suggested the presence of two different transcriptional units. The *xanA* mutant colonies were smooth in appearance and produced small amounts of an acidic EPS. Whitfield et al. (62) described mutants of *X. campestris* which produced an EPS with an unusual composition, containing sugars normally found in LPS. To exclude this possibility, we analyzed the sugar composition of the EPS produced by mutant B100-152. It consisted of glucose, mannose, and glucuronic acid in a ratio expected for xanthan gum (data not shown). Hence, the EPS produced was confirmed as being xanthan. The mutation in *xanA*, derived from an insertion of an insertion sequence element, led to a drastic decrease in but not loss of the enzyme functions. Hence, it was expected that the cells were able to produce small quantities of xanthan. *X. campestris xanB* mutants showed pleiotropic effects. All mutants showed no xanthan production, autoagglutination in liquid medium, and no motility.

Biochemical analysis proved that *xanA* was involved in the conversion of glucose 6-phosphate and mannose 6-phosphate and their corresponding 1-phosphates. A single mutation in *xanA* led to a drastic decrease of both PGM and PMM activities, although low levels of the enzymes remained detectable. There are various possible explanations for the observation that a single mutation influenced the activities of two enzymes. One enzyme or its reaction product could be responsible for the induction of the other enzyme; hence, a reduced activity of the first enzyme would depress the activity of the second. Another possibility is that *xanA* is a regulatory gene which, when mutated, would reduce but not abolish the presence of PGM and PMM. Furthermore, the presence of two copies of the gene in the mutant could not be excluded. The remaining enzyme activities of <1% which were detectable could, however, be due to the nonspecific reaction of other mutases. PGM mutants of *E. coli* that also showed a reduced metabolism of glucose 1-phosphate were described by Adhya and Schwartz (1). We propose that *xanA* encodes an enzyme which has PGM and PMM activities. This assertion was supported by comparison of the deduced amino acid sequence of *xanA* with that of the rabbit muscle PGM. Although no significant overall homology existed, there was 48% homology to the active region around the active-site phosphoserine (Ser-116) (45). The low levels of PGM and PMM activities detectable in the mutant could account for the phenotype observed and could be sufficient for LPS synthesis, although not for the production of large amounts of xanthan gum.

The amino acid sequence deduced from *xanB* showed a significant degree of homology (59%) to the recently characterized bifunctional enzyme PMI-GMP of *P. aeruginosa* (49). In contrast to this, no significant homology was found to *manA*, the PMI gene of *E. coli* (42). The extensive homology between *xanB* and the *P. aeruginosa algA* gene strongly enhanced the probability that the gene that we identified encoded a similar, bifunctional enzyme. The function of *xanB* was further confirmed by enzymatic assays in cell extracts. The differences in the enzyme activities of PMI-GMP in the three *xanB* mutants and the results of complementation experiments (Fig. 5) suggested that two domain structures, which probably are independent of each other, existed for the two enzymatic functions. The colonies of the *xanB* mutants had rough phenotypes. The loss of a mucoid phenotype due to undetectable levels of PMI, GMP, and GDP-mannose dehydrogenase was also reported for *A. vinelandii* (24) and *E. coli* (35, 38). In the case of *P. aeruginosa*, PMI activity was proved for alginate synthesis (16). Mannose was found to be a component of LPS and EPS in *X. campestris* (31, 60). Although the hexose content of the LPS of the *xanB* mutants remains to be analyzed, the phenotypic alterations observed, i.e., rough colony appearance, autoagglutination, and lack of motility, indicated that in addition to their EPS^- phenotype, these mutants produce a surface polysaccharide with modified structure. The inability to synthesize GDP-mannose because of a lack of PMI and/or GMP fully accounts for the surface polysaccharide defects of *xanB* mutants. The biosynthetic pathway leading to GDP-mannose seems closely related to that described for alginate-producing *P. aeruginosa*, with the exception that the low to undetectable activities of PMI, PMM, and GMP in *P. aeruginosa* (46) were easily detectable in *X. campestris*. The reason for these relative high activities in *X. campestris* remains unclear, since less than 2% of the wild-type GMP activity is sufficient to restore all phenotypic alterations (Table 2 and Fig. 5).

E. coli mutants unable to synthesize PMI could not grow on mannose as the sole carbon source (38). *X. campestris xanB* mutants grew well on minimal medium containing mannose as the sole carbon source. No PMI activity was found in the cell extracts of B100-13 grown on mannose. Furthermore, only a slight increase in PMI activity was detected in the wild type. This finding suggests that the mannose utilization occurs through a different pathway, one not significantly involving PMI activity (34). It is speculated, therefore, that *xanB* is involved exclusively in the synthesis of bacterial surface polysaccharides and is not required for the catabolism of mannose. The enzymes involved in sugar nucleotide synthesis were shown to be physically separated from those involved in the assembly and polymerization of the pentasaccharide repeating unit (14). This seems logical, since the biosynthesis of sugar nucleotides provides key intermediates of cyclic glucans, EPS, and LPS. A regulatory control mechanism that enables the microorganisms to channel intermediates to one polymer or another might exist at this level. Analysis of the functions of the adjacent DNA regions and characterization of the regulation of the genes described are in progress.

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ADDENDUM

After the submission of this report, the sequences of the genes encoding enzymes involved in two steps of the synthesis of GDP-mannose were published (53a). These two genes, *cpsB* and *cpsG*, which were thought to be part of the gene cluster for the M-antigen capsular polysaccharide, showed a high degree of homology to our genes *xanA* and *xanB*, respectively. The amino acid sequence deduced from *cpsG*, which codes for PMM, revealed 60% homology to the amino acid sequence of *xanA*. Furthermore, the amino acid sequence deduced from *cpsB*, which codes for GMP, showed 60% homology to that from *xanB*.

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