Identification, genetic and biochemical analysis of genes involved in synthesis of sugar nucleotide precursors of xanthan gum

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A genetic and biochemical analysis of Xanthomonas campestris chromosomal functions required for xanthan polysaccharide synthesis (xps) was undertaken. Seven xps DNA regions were isolated after conjugation of chemically induced non-mucoid mutants with a genomic library of X. campestris DNA. No overlapping segments between regions were detected, based on physical mapping, indicating the unlinked character of these regions. Clones complementing several different mutants belonging to the same region contained overlapping segments of X. campestris chromosomal DNA. Complementation and biochemical analysis, and DNA mapping were used to identify and characterize xpsIII, IV and VI DNA regions. Mutants in these three regions were able to synthesize both lipid intermediates and xanthan gum in vitro when sugar nucleotides were provided as substrates. HPLC analysis of the intracellular sugar nucleotide content showed that the XpsIII group comprises two different classes of mutants: XpsIIIA, defective in UDP-glucose, UDP-glucuronic acid and GDP-mannose, and XpsIIIB, defective in GDP-mannose. XpsIV mutants were defective in UDP-glucose and UDP-glucuronic acid, and XpsVI mutants were defective only in UDP-glucuronic acid. Analysis of enzyme activities involved in the synthesis of UDP-glucose, GDP-mannose and UDP-glucuronic acid indicated that the xpsIIIA region affects the activity of the phosphoglucomutase/phosphomannomutase enzyme, and the xpsIIIB region affects the mannoisomerase/phosphomannoisomerase activities. The xpsIV mutations affect the activity of the UDPG-pyrophosphorylase enzyme, and the xpsVI mutations affect the activity of the UDPG-dehydrogenase enzyme.

Introduction

Xanthan gum is an acidic heteroexopolysaccharide produced by Xanthomonas campestris; it consists of polymerized pentasaccharide repeating units, composed of glucose, mannose, and glucuronic acid in a molar ratio of 2:2:1 (Jansson et al., 1975). Biosynthesis of xanthan gum requires: (a) synthesis of the precursors UDP-glucose, UDP-glucuronic acid and GDP-mannose; (b) sequential transfer of these sugar units to a lipid phosphate to form the repeating pentasaccharide unit diphosphate lipid; (c) addition of acetyl and pyruvate groups (Ielpi et al., 1981a, b, 1983). The subsequent polymerization of the repeating units produces the xanthan gum.

The application of recombinant DNA technology to study the biosynthesis and genetic regulation of biopolymers is being investigated using X. campestris. Recently, a large DNA region xpsI (xanthan polysaccharide synthesis), essential for xanthan polysaccharide synthesis, has been described (Barrere et al., 1986; Harding et al., 1987; Thorne et al., 1987). Plasmid pCHC3 containing a portion of this DNA region is able to increase pyruvylation and xanthan gum production (Harding et al., 1987). In the present work, by chemical
mutagenesis, complementation and biochemical analysis, we have characterized three new DNA regions essential for xanthan gum synthesis, and have shown that they are involved in the synthesis of sugar nucleotides required for ps-P-P-lipid assembly.

Methods

Bacterial strains, plasmids, media, and genetic procedures. The bacterial strains and plasmids used in this study are listed in Table 1. Culture conditions, genetic and molecular genetic procedures were as described by Harding et al. (1987). Mutants deficient in xanthan gum synthesis were derived from X. campestris strain 0100 or a derivative of strain 0100 containing plasmid pCHC3. These were isolated by non-selective plating of 0100 on MM medium containing 200 mg/ml ampicillin and 10 mg/ml chloramphenicol. Rifampin-resistant derivatives of these mutants were isolated.

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Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<td>E. coli IZ279</td>
<td>recA56 lacY galK2 galT22 metB1 trpR55 supE44 supF38 hisdR14 MC4100galU59</td>
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<td>FF4001</td>
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<td>0543</td>
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<td>Tc' Km' Mob+ Tra-</td>
<td>D. R. Helinski*</td>
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<td>pAB8</td>
<td>Tc' algC</td>
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Analytical procedures. Sugar nucleotides were extracted from 200 ml of cells with formic acid (Payne & Ames, 1982) as described by Betlach et al. (1987). Briefly, bacteria were grown in defined medium to a density of approximately $2 \times 10^8$ (OD$_{600} = 1.0$). Cells were collected by centrifugation, washed twice with defined medium salts and suspended in 2 ml of the defined medium, but using 20 mM glucose. This suspension was incubated for 10 min in a rotary shaker at 300 r.p.m., transferred to a tube containing 0.2 ml cold formic acid, vortexed and stored at $-20^\circ$C over night. Following centrifugation to remove cell debris, the supernatant was lyophilized to eliminate formic acid. The residue was then resuspended in 300 µl of water (cell extracts) and stored at $-20^\circ$C.

A Waters HPLC system, consisting of two M6000A solvent delivery systems, a model 660 solvent programmer, a Jasco UV spectrophotometer model Uvitec-100-IV detector and a Jasco RC-150 recorder, were used to separate compounds within the cell extracts. Columns used were Ultrasphere 5 µm ODS (4.6 x 250 mm) (Beckman) and in more recent studies, a Supelco 5 µm ODS (4.6 x 250 mm). The compounds were eluted in the isocratic mode with 40 mM-triethylamine-phosphate buffer, pH 6.5, with a flow rate of 1.0 ml min$^{-1}$ at room temperature. Absorbance at 260 nm was used to detect compounds.

Xanthan gum biochemistry. Preparation of EDTA-treated cells and the assay for in vitro synthesis of xanthan gum were done as previously described (Ielpi et al., 1981a), but using UDP-$[^{14}C]$GlcA as radio-labelled sugar, and unlabelled UDP-Glc and GDP-Man as sugar donors (Marzocca et al., 1991). Under these conditions, in incubations done with EDTA-treated wild-type cells, the main product extracted in the lipid fraction is $[^{14}C]$-GlcA-ps-P-P-lipid, but small amounts of a $[^{14}C]$-GalA-lipid-bound galacturonic acid, which is also formed, may be present (Baldessari et al., 1990). The amount of radioactive label incorporated in a portion of the lipid fractions was determined, and the remainder of these fractions were analysed by ascending chromatography in solvent D. This system, which is equivalent to mild alkali treatment, converts ps-P-P-lipid into its cyclic phosphate ester (Cousso et al., 1982) with a mobility ($R_p = 0.23$-0.25) clearly different from lipid-bound galacturonic acid ($R_p = 0.84$). To confirm the identity of the radioactive compounds with $R_p$ values of 0.23-0.25, they were eluted from the paper strip with water and subjected to paper electrophoresis in solvent B.
this system the cyclic phosphate ester of the pentasaccharide migrates with a mobility of R\text{umd} = 1 (Marzocca et al., 1991). Radioactive xanthan gum produced in vitro was isolated by gel-filtration on a Bio-Gel A 5m column (Bio-Rad) (Ielpi et al., 1981a). Values for incorporation of radioactivity are means from at least two independent experiments.

**Enzyme determinations.** All enzyme assays were done at 37 °C for 1 h, using EDTA-treated cells. The protein concentration of extracts, estimated by the Lowry method, was adjusted to 15–30 mg ml\textsuperscript{−1}. The activity was expressed relative to the activity obtained for the NRRL B-1459 strain, and values are means of at least two independent determinations.

**PGM.** The PGM reverse reaction, in a total volume of 50 µl, contained 2.5 µmol Tris/HCl buffer (pH 7.8), 0.5 µmol MgCl\textsubscript{2}, 20 nmol unlabelled α-D-glucose 1-P, 25000 c.p.m. [\textsuperscript{14}C]glucose 1-P, 0.1 nmol glucose 1,6-diP, 0.2 µmol mannose and 15 µl of EDTA-treated cells. The reactions were stopped by addition of 0.1 vol. 0.1 M-HCl. This solution was heated at 100 °C for 10 min. Under these conditions hexose 6-P is stable, but hexose 1-P is hydrolysed to hexose and phosphate. After removal of HCl by evaporation under a stream of nitrogen, the samples were subjected to paper chromatography with solvent A. Radioactivity on chromatograms was located with a scanner. Compounds with mobilities similar to glucose 6-P were eluted from the paper strip with water and electrophoresed in solvent B. Compounds with migration similar to glucose 6-P were eluted and counted for radioactivity.

**UDPG-PP.** The UDPG-PP forward reaction, in a total volume of 50 µl, contained 5 µmol Tris/HCl buffer (pH 7.8), 0.5 µmol MgCl\textsubscript{2}, 1 µmol UTP, 20 nmol unlabelled α-D-glucose 1-P, 25000 c.p.m. [\textsuperscript{14}C]glucose 1-P and 5 µl of EDTA-treated cells. The reactions were stopped by addition of 1 vol. ethanol, followed by paper chromatography in solvent A. Compounds with mobilities similar to that of UDP-glucose were eluted, and counted for radioactivity.

**UDPG-deH.** The UDPG-deH forward reaction, in a total volume of 100 µl, contained 10 µmol glycyglycine buffer (pH 8.5), 0.6 µmol NAD\textsuperscript{+}, 20 nmol unlabelled UDPG-Glc, 25000 c.p.m. UDPG-[\textsuperscript{14}C]Glc and 15 µl of EDTA-treated cells. The reactions were stopped by the addition of 1 vol. ethanol, followed by paper chromatography in solvent A. Compounds with mobilities similar to that of UDP-glucuronic acid were eluted, and counted for radioactivity.

**PGI.** The PGI forward reaction, in a total volume of 50 µl, contained 2.5 µmol Tris/HCl buffer (pH 7.8), 50 nmol CoCl\textsubscript{2}, 20 nmol unlabelled glucose 6-P, 78000 c.p.m. [\textsuperscript{14}C]glucose 6-P and 20 µl of EDTA-treated cells. The reactions were stopped by addition of 1 vol. ethanol, the phosphate group was removed by treatment with bacterial alkaline phosphatase, followed by paper electrophoresis in solvent C. Unlabelled glucose, fructose, and mannose were added as internal standards. Compounds with mobility similar to that of fructose were eluted and counted for radioactivity.

**PMI.** The PMI reverse reaction, in a total volume of 50 µl, contained 2.5 µmol Tris/HCl buffer (pH 7.8), 0.5 µmol MgCl\textsubscript{2}, 0.5 µmol GTP, 0.1 nmol glucose 1,6-diP, 20 nmol unlabelled mannose 6-P, 44000 c.p.m. [\textsuperscript{14}C]mannose 6-P and 15 µl of EDTA-treated cells. The reactions were stopped by addition of 1 vol. ethanol, followed by paper chromatography in solvent A. Compounds with mobilities similar to that of GDP-mannose were eluted and counted for radioactivity.

**GDPM-PP.** The GDPM-PP forward reaction, in a total volume of 50 µl, contained 2.5 µmol Tris/HCl buffer (pH 7.8), 0.5 µmol MgCl\textsubscript{2}, 0.5 µmol GTP, 20 nmol unlabelled mannose 1-P, 85000 c.p.m. [\textsuperscript{14}C]mannose 1-P and 5 µl of EDTA-treated cells. The reactions were stopped by addition of 1 vol. ethanol, followed by paper chromatography in solvent A. Compounds with mobilities similar to that of GDP-mannose were eluted and counted for radioactivity.

**Chromatography and electrophoresis.** Chromatography and electrophoresis on Whatman no. 1 paper were done as described by Cosso et al. (1982), with the following solvents: solvent A, ethanol/1 M-ammonium acetate, pH 3.8; solvent B, pyridine/acetic acid/water (1:0.04:9, by vol.; pH 6.5); solvent C, 0.05 M-sodium borate, pH 9.2; solvent D, ethanol (96%)/ammonium hydroxide (7:3, v/v). Radioactive areas were located by using a model 7201 scanner (Packard), reducing substances were located with alkaline silver reagent (Trevelyan et al., 1950), and standard sugar nucleotides with a UV lamp.

**Radiochemicals and biochemicals.** α-D-[\textsuperscript{14}C]Glucose 1-phosphate, specific activity 3130 Ci mol\textsuperscript{−1} (1 Ci = 37 GBq), α-D-[\textsuperscript{14}C]glucose 6-phosphate, specific activity 603 Ci mol\textsuperscript{−1}, and α-D-[\textsuperscript{14}C]mannose, specific activity 216.5 Ci mol\textsuperscript{−1}, were purchased from Dupont–NEN. α-D-[\textsuperscript{14}C]Mannose 1-phosphate, specific activity 216.5 Ci mol\textsuperscript{−1} was prepared by enzymic digestion of GDP-[\textsuperscript{14}C]Man (Cosso et al., 1980) with nucleotide pyrophosphatase. α-D-[\textsuperscript{14}C]Mannose 6-phosphate, specific activity 216.5 Ci mol\textsuperscript{−1} was prepared by enzymic phosphorylation of α-D-[\textsuperscript{14}C]mannose with hexokinase and ATP. UDP-[\textsuperscript{14}C]glucose, specific activity 285 Ci mol\textsuperscript{−1}, and UDP-[\textsuperscript{14}C]glucuronic acid, specific activity 2850 Ci mol\textsuperscript{−1}, were prepared as described previously (Ielpi et al., 1981a; Marzocca et al., 1991). ATP, GTP, UTP, UDP-glucose, UDP-glucuronic acid, GDP-mannose, hexokinase, and nucleotide pyrophosphatase were purchased from Sigma. All other reagents were of analytical grade.

**Results**

**Isolation of non-mucoid mutants**

Mutants non-mucoid by appearance on YM plates were isolated after mutagenesis with ethylmethane sulphonate. These formed dark-yellow, flat to slightly mucoid colonies, smaller than those of the wild-type. To select for mutations in regions other than the xps\textsubscript{I} region described previously (Harding et al., 1987), some non-mucoid mutants were derived by mutagenesis of a strain harbouring plasmid pCHC3, which contains most of the xps\textsubscript{I} region. These mutants were then cured of pCHC3 by displacement with plasmid pRK252 (Ditta et al., 1985), which is unstable in X. campestris. Spontaneous rifampin-resistant derivatives were then isolated.

**Identification and isolation of xanthan polysaccharide synthesis DNA regions**

A genomic library of X. campestris DNA in pRK293 stored in E. coli JZ279 (Harding et al., 1987) was transferred to selected rifampin-resistant nonmucoid mutants by conjugation on solid media, selecting for resistance to rifampin and kanamycin and screening for mucoid phenotype on YM agar as described previously (Harding et al., 1987). Plasmids were purified from mucoid transconjugants and characterized as to SalI
Fig. 1. Physical maps of cloned xpsIII, IV, and VI regions. Genetic data pertaining to complementation of XpsIII, XpsIV and XpsVI mutants are also shown. (a), (g), (i), inserts in plasmids isolated from a X. campestris gene library by complementation of non-mucoid Xps mutants. (a), SalI inserts in pJC433. (b)-(e), Cloned DNAs representing various portions of the xpsIII region. (f) Size (kb) of EcoRI fragments in pJC433. (g), SalI inserts in pJC440. (h)-(k), Cloned DNAs representing various portions of xpsIV region. (l), 9.2 kb SalI insert in pJC461. (m)-(n), Cloned DNA representing portions of xpsVI region. The results of complementation assays for mucoid phenotype with the indicated Xps mutant strains are shown to the right of each DNA. Restriction sites are shown for SalI (S), BamHI (B), EcoRI (E) and XhoI (X).

Restriction analysis of the xpsIII, IV and VI DNA regions

Plasmids from the gene library with the smallest insert allowing complementation of all mutants in classes III, IV and VI were digested with restriction enzymes BamHI, EcoRI, SalI and XhoI to derive the physical maps shown in Fig. 1. Based on the physical maps, appropriate smaller fragments were cloned into pRK293 to further define the location of the mutations. Most XpsIII mutants (17 of 18) were complemented by a 2.1 kb XhoI fragment. One mutant, 3332 (xpsIII-475), was not complemented by this fragment, but was complemented by the partially overlapping 6.0 kb SalI fragment, indicating that at least two genes are located in this region. Ten Xps mutants were complemented by plasmid pJC440 (defining xps region IV) and eleven Xps mutants were complemented by pJC461 (defining xps region VI). All XpsIV mutants were complemented by subclones of pJC440 containing a 5.8 kb SalI fragment and adjacent small SalI fragments (Fig. 1h, i, but not by the 5.8 kb.


\[\text{SalI fragment alone (Fig. 1k).} \]

\[\text{XpsVI mutants were mapped to a 3-9 kb SalI–XhoI fragment as shown in Fig. 1m.}\]

None of the XpsIII, IV or VI mutants were complemented by the DNA from the other regions, and no overlapping fragments were detected by restriction enzyme mapping, indicating that as far as can be determined by this analysis, these three regions are in different parts of the \textit{Xanthomonas} chromosome.

\textbf{In vitro assay for the synthesis of ps-P-P-lipid intermediate and polysaccharide}

We have previously described an \textit{in vitro} system consisting of permeabilized cells, which when incubated in the presence of UDP-glucose, GDP-mannose and UDP-glucuronic acid, produce both ps-P-P-lipid and polysaccharide (Ielpi \textit{et al.}, 1981a). In addition, a lipid-linked galacturonide, that does not participate in the biosynthesis of xanthan gum, was observed when the label was in UDP-[\(^{14}\)C]GlcA (Baldessari \textit{et al.}, 1990). EDTA-treated cells of several mutants in each region were prepared, incubated and analysed to determine if these Xps mutants were able to produce the ps-P-P-lipid as well as the polysaccharide. As shown in Table 2, all the XpsIII, IV and VI mutants analysed in this study produced both the lipid-linked repeating unit and polysaccharide, although in different amounts. No significant differences could be detected in the radioactive pattern of polysaccharide between Xps mutants and the wild-type strain when analysed by Bio-Gel A-5m column chromatography. Thus, these non-mucoid mutants were \textit{in vitro} deficient but, when the precursor sugar nucleotides were provided, \textit{in vitro} producers of xanthan gum.

\textbf{Determination of the intracellular level of sugar nucleotides}

Since sugar nucleotides were added in the \textit{in vitro} assay for the synthesis of ps-P-P-lipid and polysaccharide, we decided to study whether the lack of formation of xanthan gum \textit{in vivo} by XpsIII, IV and VI mutants was due to a deficiency of some of the required sugar nucleotides. Formic acid extracts of cells grown in minimal medium containing glucose were analysed by HPLC using a reversed-phase, ion-pair column and a UV detector. Due to some variations in the elution time, a sample of authentic sugar nucleotides was run just before each sample of cell extracts and the positions of these standards are shown by arrows on the chromatograms.

Analysis of the internal level of sugar nucleotides of the wild-type strain showed, as expected, the presence of UDP-glucose, GDP-mannose and UDP-glucuronic acid, required for xanthan gum synthesis (Fig. 2a). In addition, UDP-N-acetylgalactosamine and UDP-galacturonic acid were detected; the former is a precursor of peptidoglycan and the latter is a precursor of lipid-linked galacturonide. Analysis of mutant 3188 (\textit{xpsIII-40I}) revealed the absence of UDP-glucose, GDP-mannose and UDP-glucuronic acid (Fig. 2b). However, analysis of mutant 3332 (\textit{xpsIII-475}) of the same \textit{xps} region showed the presence of UDP-glucose and UDP-glucuronic acid and the absence only of GDP-mannose (Fig. 2c). The phenotype of mutant 3188 (\textit{xpsIII-40I}) is unlikely to be due to a double mutation, since three mutants tested, 3188 (\textit{xpsIII-40I}), 3269 (\textit{xpsIII-427}) and 3293 (\textit{xpsIII-436}), all lack these three sugar nucleotides, and one of these mutants was derived from a separate

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Strain & Mutation & Lipid fraction & Polymer & \textsuperscript{[\textsuperscript{14}\text{C}]GlcA incorporation*} & Structure of lipid-linked oligosaccharide† \\
\hline
B-1459 & None & 90 & 80 & - & ps \\
3188 & \textit{xpsIII-40I} & 114 & 122 & - & ps \\
3269 & \textit{xpsIII-427} & 42 & 24 & - & ps \\
3332 & \textit{xpsIII-475} & 74 & 56 & - & ps \\
3192 & \textit{xpsIV-405} & 86 & 76 & - & ps, GalA \\
3258 & \textit{xpsIV-415} & 26 & 14 & - & ps \\
3322 & \textit{xpsIV-465} & 53 & - & - & - \\
3193 & \textit{xpsVI-407} & 130 & 123 & - & ps, GalA \\
3262 & \textit{xpsVI-419} & 67 & 44 & - & ps, GalA \\
3294 & \textit{xpsVI-437} & 132 & 41 & - & ps, GalA \\
3319 & \textit{xpsVI-462} & 122 & 38 & - & ps, GalA \\
\hline
\end{tabular}
\caption{\textit{In vitro synthesis of ps-P-P-lipid and polysaccharide in wild-type and XpsIII, IV, and VI mutant strains}}
\end{table}

\textit{* Values for incorporation of radioactivity are expressed as pmol mg\textsuperscript{-1}, and are means from at least two independent experiments. Not tested.}

\textit{† ps, \textbeta{}-mannose-(1,4)-\textbeta{}-glucuronic acid-(1,2)-\textalpha{}-mannose-(1,3)-\textalpha{}-glucose-(1,4)-\textalpha{}-glucose; GalA, galacturonic acid.}
mutagensis and is thus not a sibling. These mutants, but not 3332 (xpsIII-475), were complemented by a 2.1 kb XhoI fragment (Fig. 1b). Thus, the XpsIII group is composed of two different classes of mutants: one class, IIIA, showing absence of UDP-glucose, UDP-glucuronic acid and GDP-mannose, and a second class, IIIB, showing absence of GDP-mannose.

The three XpsIV mutants analysed showed a similar pattern, with GDP-mannose present and UDP-glucose and UDP-glucuronic acid absent. The two XpsVI mutants analysed showed absence of UDP-glucuronic acid and presence of UDP-glucose and GDP-mannose. The patterns obtained with strains 3322 (xpsIV-465) and 3294 (xpsVI-437) are shown in Fig. 2(d, e) respectively.

In vitro activity of enzymes involved in sugar nucleotide synthesis.

Analysis of the internal level of sugar nucleotides of the Xps mutants suggests that a deficiency in some of the
Table 3. Activities of UDP-glucose, UDP-glucuronic acid and GDP-mannose biosynthetic enzymes in XpsIII, XpsIV and XpsVI strains of X. campestris

Activities were analysed as described in Methods, and are expressed as percentages of wild-type activities. Enzyme activities of the wild-type (nmol h⁻¹ mg⁻¹; means of three independent experiments) were as follows: PGM, 54.6; UDPG-PP, 30.0; UDPG-deH, 23.4; PMM, 5.4; GDPM-PP, 21.6; PGI, 4.8; PMI, 12.0. –, Not tested.

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Fig. 3. Paper electrophoresis analysis of reaction products from the mannose isomerase assay. The MI reaction mixture, total volume 100 μl, contained 5 μmol Tris/HCl buffer (pH 7.8), 0.5 μmol MgCl₂, 30000 c.p.m. [¹⁴C]mannose and 2 mg dialysed EDTA-treated cells. The reactions were stopped by the addition of 1 vol. ethanol, followed by paper electrophoresis in solvent C. Unlabelled glucose (Glc), fructose (Fru) and mannose (Man) were added as internal standards. The assay in (a) was done with cells from B-1459; (b) with cells from 3332 (xpsIII-475); (c) with cells from 4005 [xpsIII-475(pJC433)]. The positions of the unlabelled compounds are indicated (—).
graphic analysis of the endogenous pool of sugar nucleotides. As can be seen in Table 3, extracts of strain 3269 was not sufficient to support detectable carbon source, because it enables mannose 6-P to be differences were observed in the levels of the other enzymes tested, compared to the wild-type (Table 3).

PMI activity in strain 3332 (xpsIII-475), was reduced to only 23% of that of the wild-type, while no discernible differences were observed in the levels of the other enzymes tested, compared to the wild-type (Table 3). In E. coli, PMI is essential for growth on mannose as sole carbon source, because it enables mannose 6-P to be catabolized via the glycolytic pathway. However, de Crecy-Lagard et al. (1991a) suggested that in X. campestris mannose is isomerized into fructose, which is subsequently phosphorylated by fructokinase. This assumption was confirmed in two ways. First, no mannose 6-P was found when EDTA-treated wild-type cells were incubated in the presence of ATP (data not shown); second, MI activity was detected in the wild-type strain as shown in Fig. 3a. Interestingly, mutation xpsIII-475 decreases growth on minimal medium with mannose as carbon source (data not shown), and MI activity (Fig. 3b).

The UDPG-PP activity was significantly lower (less than 5% of that of the wild-type) in XpsIV mutants 3192 (xpsIV-405), 3258 (xpsIV-415) and 3322 (xpsIV-465), and in turn the NADP*-dependent UDP-formation of UDP-glucuronic acid by the wild-type strain could no longer be detected in the XpsVI mutants 3193 (xpsVI-407), 3262 (xpsVI-419), 3266 (xpsVI-424) and 3294 (xpsVI-437) (Table 3).

Table 4 shows that the defective enzyme activities were recovered after appropriate recombinant plasmids were introduced into these Xps mutants. When present in strain 3188, plasmid pJC433 led to the recovery of PGM and PMM, and the recovery of PMI and MI (Fig. 3c) activities when present in strain 3332. Plasmids pJC440 and pJC461 each complemented representative XpsIV and VI mutants respectively. Heterologous complementation data also supports the characterization of the xpsIII-A gene. Mutants 3188 (xpsIII-401), 3269 (xpsIII-427) and 14 other group III mutants, but not mutant 3332 (xpsIII-475) were complemented for colony morphology by plasmid pAB8 encoding the PMM structural gene of Pseudomonas aeruginosa (Berry et al., 1988; Zielinski et al., 1991). Similarly, pJC440 was introduced into E. coli strain FF4001, carrying a mutation in galU, the structural gene for UDPG-PP. E. coli galU mutants are unable to grow on galactose as carbon source (Gläver et al., 1988). FF4001 cells transformed with pJC440 were able to grow on minimal media containing galactose as carbon source.

### Discussion

In this work, we identified and studied three DNA regions of X. campestris xps genes involved in the synthesis of sugar nucleotide precursors of xanthan gum, using a combination of genetic and biochemical analysis. Several research groups have identified a cluster of genes for xanthan synthesis (Barrere et al., 1986; Thorne et al., 1987; Vanderslice et al., 1989), which we have designated xpsI (Harding et al., 1987). This cluster of genes encodes functions required for assembly of the lipid-bound repeating unit (Vanderslice et al., 1989; Marzocca et al., 1991; authors' unpublished data). It would be expected that other genes would be required for xanthan synthesis, in particular for synthesis of the sugar nucleotide precursors. By complementation of a large number of chemically induced non-mucoid mutants with plasmids from a gene library of X. campestris, we have identified additional regions required for xanthan synthesis. Chemically induced mutants in the regions described in this study were negative for xanthan production in vivo, but capable of xanthan synthesis in vitro when sugar nucleotides were provided. By physical

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation (plasmid)</th>
<th>PGM</th>
<th>PMM</th>
<th>PMI</th>
<th>UDPG-PP</th>
<th>UDPG-deH</th>
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<tbody>
<tr>
<td>4004</td>
<td>xpsIII-401(pJC433)</td>
<td>101</td>
<td>85</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4005</td>
<td>xpsIII-475(pJC433)</td>
<td>-</td>
<td>-</td>
<td>115</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4006</td>
<td>xpsIV-465(pJC440)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>174</td>
<td>-</td>
</tr>
<tr>
<td>4007</td>
<td>xpsVI-437(pJC461)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>85</td>
</tr>
</tbody>
</table>
mapping of complementing plasmids, cloning of smaller fragments and complementation analysis, we have shown that these mutations map to three unlinked regions, one of which (xpsIII) contains at least two genes.

Regions of DNA other than the xpsI gene cluster, which complement Xps− mutants, have been described by other authors. Hötte et al. (1990) isolated cosmids clones complementing transposon-induced xanthan-deficient mutants. Some of their mutants mapped to 1, 0.5 and 3.8 kb EcoRI fragments. Based on a comparison of restriction enzyme sites for EcoRI, XhoI and BamHI, we have found that this portion of the clone reported by Hötte et al. (1990) corresponds to our clone pJC433 (Fig. 1). Köplin et al. (1992), from biochemical and complementation experiments, and sequence data for this region, suggested the presence of two genes: one encodes an enzyme which has PGM and PMM activities, and the second encodes a bifunctional enzyme with PMI and GDPM-PP activities. Betlach et al. (1990) communicated the isolation and characterization by HPLC of similar mutants affecting sugar nucleotide synthesis.

In this study, the biosynthetic enzymes PGM, UDPG-PP, UDPG-deH, PGI, PMI, PMM and GMP were detected in the X. campestris wild-type strain. These results are analogous to known enzymic interconversions of sugar phosphates and nucleotide diphosphate glycoses (Gabriel, 1987).

HPLC analysis of the cell content of sugar nucleotides confirms that the xps mutations studied in this work affect the biosynthesis of UDP-glucose, UDP-glucuronic acid or GDP-mannose, all of which are intermediates in the biosynthesis of xanthan gum (Ielpi et al., 1981a). Four class of mutants were identified: XpsIII-A mutants were defective in UDP-glucose, UDP-glucuronic acid and GDP-mannose; XpsIII-B consisted of a mutant defective in GDP-mannose; XpsIV mutants were defective in UDP-glucose and UDP-glucuronic acid and GDP-mannose; XpsIII-B consisted of a mutant defective in GDP-mannose; XpsIV mutants were defective in UDP-glucose and UDP-glucuronic acid; and Xps VI mutants were defective in UDP-glucuronic acid. In addition, these xps mutations have no apparent effect on the synthesis of lipid-phosphate carrier, the activity of glycosyl transferases involved in ps-P-P-lipid formation, and the polymerase enzyme(s).
Table 5. Summary of the properties of XpsIII, IV and VI mutants of X. campestris*
+ Wild-type levels; ± slight activity (less than 5% of wild-type level); -, no activity. Extracellular polysaccharide production was determined after precipitation of 50 ml of culture supernatant with 2 vols ethanol and drying. Glc, glucose; Man, mannose; GlcA, glucuronic acid.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Polymer in vivo</th>
<th>Polymer in vitro</th>
<th>Cell content of: UDP-Glc</th>
<th>GDP-Man</th>
<th>UDP-GlcA</th>
<th>Defective activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL B-1459</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>3188, 3269, xpsIII</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>PGM-PMM</td>
</tr>
<tr>
<td>3293</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MI, PMI</td>
</tr>
<tr>
<td>3332</td>
<td>xpsIII B</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>UDPG-PP</td>
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<td>3192, 3258, xpsIV</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>UDPG-deH</td>
</tr>
<tr>
<td>3322</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>UDPG-deH</td>
</tr>
<tr>
<td>3193, 3262, 3266, 3319 xpsVI</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>UDPG-deH</td>
</tr>
</tbody>
</table>

Analysis of the activity of the enzymes involved in the biosynthesis of UDP-glucose, UDP-glucuronic acid and GDP-mannose, correlates with HPLC analysis. XpsIII-A mutants, strains 3188 (xpsIII-401) and 3269 (xpsIII-427), were found to be defective in both PGM and PMM activities. Nine extra independent XpsIII-A mutants were found to be defective in both phosphomutase activities (data not shown), suggesting that similar to many others systems studied (Ray & Peck, 1972), the PGM and PMM activities of X. campestris are catalysed by the same enzyme. Phosphomutase catalyses the phosphate transfer between the 1 and 6 positions of many α-D-hexoses, or the 2 and 3 positions of glyceric acid.

The XpsIII-B mutant, strain 3332, was found to have lowered activities of both MI and PMI. Mannose isomerization was first described in Pseudomonas saccharophila (Palleroni & Doudoroff, 1956), and is constitutive and widely distributed in Xanthomonas species (Takasaki & Tanabe, 1964). Strain 3332 grows poorly on minimal medium containing mannose as a carbon source, has similar wild-type activities of PMM and GDPM-PP, and does not produce GDP-mannose. Recently, de Crécy-Lagard et al. (1991b) have studied the pathway for fructose phosphorylation in X. campestris. They have characterized a fructokinase, which is able to phosphorylate fructose producing fructose 6-phosphate. Taken together, these results suggest that in X. campestris, mannose is isomerized to fructose, which in turn is phosphorylated to fructose 6-phosphate (Fig. 4).

Enzymic analysis of XpsIV mutants has indicated that they are defective in UDPG-PP activity. An E. coli strain deficient in the structural gene for UDPG-PP was complemented by the xpsIV DNA region, suggesting that xpsIV mutations are located in the corresponding structural gene of X. campestris. The enzyme is present in nearly all cells, generally with a multimeric structure of identical subunits. In Salmonella typhimurium, the gene galU codes for the structural protein, and the gene galF modifies the polypeptide into different forms (Turnquist & Gaurth Hansen, 1973). Additional studies are required to establish whether a similar system is present in X. campestris.

UDPG-deH was totally abolished in the XpsVI mutants. Thus, the inability of XpsVI mutants to produce xanthan gum is probably due to a defective UDPG-deH. In E. coli, the UDPG-deH comprises two subunits that appear to be identical with M, values of 47000 (Gabriel, 1987).

The properties of the four class of mutants analysed in this work are summarized in Table 5. Only the XpsVI mutants produce a slight amount of polymer (less than 5% of the wild-type level). Structural analysis of the polysaccharide produced by mutant 3294 (xpsVI-437) revealed the absence of glucuronic acid, and a glucose/mannose ratio close to two (not shown). A polymer with this structure was previously described, studied and named polytrimer by Betlach et al. (1987).

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Sugar nucleotide synthesis in Xanthomonas campestris


