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Isolation and Nucleotide Sequence of the GDP-Mannose:Cellobiosyl-Diphosphopolyprenol α-Mannosyltransferase Gene from *Acetobacter xylinum*

E. ALEJANDRO PETRONI AND LUIS IELPI*

Instituto de Investigaciones Bioquímicas Fundación Campomar, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and CONICET, 1405 Buenos Aires, Argentina

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A genetic locus from *Acetobacter xylinum* involved in acetan polysaccharide synthesis has been characterized. The chromosomal region was identified by screening a genomic library of *A. xylinum* in a *Xanthomonas campestris* mutant defective in xanthan polysaccharide synthesis. The *A. xylinum* cosmid clone can functionally complement a xanthan-negative mutant. The polymer produced by the recombinant strain was found to be indistinguishable from xanthan. Insertion mutagenesis and subcloning of the cosmid clone combined with complementation studies allowed the identification of a 2.3-kb fragment of *A. xylinum* chromosomal DNA. The nucleotide sequence of this fragment was analyzed and found to contain an open reading frame (*aceA*) of 1,182 bp encoding a protein of 43.2 kDa. Results from biochemical and genetic analyses strongly suggest that the *aceA* gene encodes the GDP-mannose:cellobiosyl-diphosphopolyprenol α-mannosyltransferase enzyme, which is responsible for the transfer of an α-mannosyl residue from GDP-Man to cellobiosyl-diphosphopolyprenol. A search for similarities with other known mannosyltransferases revealed that all bacterial α-mannosyltransferases have a short COOH-terminal amino acid sequence in common.

Complex extracellular polysaccharides (EPS) are an important component of many bacteria. They consist of polymerized repeating units. The synthesis of EPS has been studied intensively. Each repeating unit is assembled as polyprenyl glycosyl disphosphate in a sequential series of reactions catalyzed by specific glycosyltransferases. The repeating units are polymerized, and the resulting EPS may be attached to the cell surface as a capsule or it may be freely released into the medium. There is considerable interest in the molecular biology of bacterial polysaccharides, since they are involved in human and plant pathogenesis and in plant-bacterium interactions and because of their innumerable industrial applications (2, 25, 32, 33, 48). Although in several cases the biosynthetic pathway in which glycosyltransferases participate has been studied in considerable detail, major questions remain concerning the biochemical functions of individual gene products.

Acetobacter xylinum is a gram-negative bacterium which characteristically synthesizes cellulose. In addition, *A. xylinum* B42 elaborates a second EPS, termed acetan, a polymer of heptasaccharide subunits that contains four glucoses, one mannose, one glucuronic acid, and one rhamnose (7). Cellulose biosynthesis genes have been identified (44, 52). However, the genetic aspects of acetan biosynthesis are still unknown. The isolation of structural glycosyltransferase genes involved in the biosynthesis of acetan by the ability of a B42 genomic library to complement the deficiencies in acetan synthesis mutants is impaired by the unsuccessful introduction of DNA into *A. xylinum* B42 and its cellulose-deficient derivative PEA-1 (41), possibly by the presence of a DNA modification-restriction system (40). We were not able to overcome this problem despite the use of different transformation and electroporation conditions and the attempts to isolate *A. xylinum* clones or to construct high-competence mutants (14). Therefore, we considered alternative ways to identify genes involved in acetan biosynthesis.

For a number of years, we have studied xanthan, an EPS well known for its extensive industrial applications (19) produced by the plant pathogen *Xanthomonas campestris*. Acetan and xanthan contain a common structure consisting of a cellulose backbone with side chains α1-3-linked to alternate glucose residues. In acetan, the side chain is the pentasaccharide Rhaα1-6Glcβ1-6Glcα1-4GlcUAβ1-2Manα1-, while in xanthan, the side chain is the trisaccharide Manβ1-4GlcUAβ1-2Manα1-. In addition, acetyl groups are present in both EPS (7, 26, 27, 47). Pyruvyl groups which are present in xanthan have not been found in acetan (7, 27).

The early steps involved in the synthesis of acetan and xanthan have been established in considerable detail. A lipid-linked tetrasaccharide intermediate, GlcUAβ1-2Manα1-3Glcβ1-4Glc-P-P-polyprenol, is assembled in a sequential series of reactions that are identical for both pathways (see Fig. 1). Subsequent steps utilize the tetrasaccharide glycolipid as a substrate to complete the lipid-linked heptasaccharide X_7 or pentasaccharide X_5 repeating units, which are further polymerized to produce acetan and xanthan, respectively (8, 23, 45).

The present study describes the isolation of an *A. xylinum* cosmid clone which complements the xanthan production of an *X. campestris* mutant. Insertion mutagenesis and subcloning of the cosmid clone combined with complementation studies, sequencing, and biochemical analysis have allowed the identification of an *A. xylinum* gene which codes for the GDP-Man: cellobiosyl-diphosphopolyprenol α-mannosyltransferase enzyme. The enzyme catalyzes the transference of an α-mannosyl residue from GDP-Man to cellobiosyl-P-P-polyprenol to produce mannosylα1-3-cellobiosyl-P-P-polyprenol. By comparison with other known mannosyltransferases, a common amino acid conserved sequence for bacterial α-mannosyltransferases was found.

* Corresponding author. Mailing address: Instituto de Investigaciones Bioquímicas Fundación Campomar, Patricias Argentinas 435, 1405 Buenos Aires, Argentina. Phone: (54-1) 863-4011. Fax: (54-1) 865-2246. Electronic mail address: lielpi@iris.iib.uba.ar.

MATERIALS AND METHODS

Abbreviations. The abbreviations used are as follows: X_7, Rhaα1-6Glcβ1-6Glcα1-4GlcUAβ1-2Manα1-3Glcβ1-4Glc; X_5, Manβ1-4GlcUAβ1-2Manα1-3Glcβ1-4Glc; X_4, GlcUAβ1-2Manα1-3Glcβ1-4Glc; Glcβ1-4Glc, cellobiose; mMu-*tac*, miniMu-*tac*; dTDP-L-Rha, thymidine diphospho-L-rhamnose; HPLC, high-performance liquid chromatography; P_{lac}, promoter of lactose operon; ORF, open reading frame.

Bacterial strains and cultivations. The *A. xylinum* strain used was NRRL B42, which produces cellulose and acetan (7). The *X. campestris* strains used were NRRL B-1459 (23) and its xanthan-defective derivative 2444 (*xps-108*; a gift of Nancy Harding, Kelco, Unit of Monsanto Co., San Diego, Calif.) (18). The *Escherichia coli* strains used for cloning and sequencing techniques were HB101 and DH5α (37). *E. coli* VGR2 is a VGR1 (17) mMu-*tac* lysogenic derivative and was constructed as described previously (10). *A. xylinum* B42 was grown at 28°C in static conditions, and cells were freed from cellulose and harvested as described previously (7). *X. campestris* cells were grown at 28°C in yeast-maltose medium (20) with kanamycin, rifampin, or tetracycline when required (50, 100, or 10 μg/ml, respectively). *E. coli* strains were grown at 37°C in Luria-Bertani medium (37) with kanamycin or tetracycline when required (50 or 15 μg/ml, respectively).

DNA manipulations. High-molecular-weight chromosomal DNA of *A. xylinum* B42 was isolated by the method of Hull et al. (21). *A. xylinum* and *X. campestris* plasmid DNA were isolated as reported previously (18), with minor modifications. *E. coli* plasmid isolations, general cloning procedures, and Southern analysis have been described elsewhere (43). Southern blots were probed and visualized with a digoxigenin DNA labeling and detection kit (Genius; Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

The gene library of *A. xylinum* B42 was constructed in the broad-host-range cosmid vector pVK102 (29) by cloning the total DNA, partially digested with *Hin*dIII. This vector confers tetracycline and kanamycin resistance and contains a unique *Hin*dIII site in the gene for kanamycin resistance. Recombinant phage DNA was packaged in vitro by using Gigapack Plus (Stratagene, La Jolla, Calif.). Transductions of lambda phage-like extracts into *E. coli* HB101 were performed as described previously (50). The library was screened for kanamycin sensitivity.

To construct pAP1 and pAP3, a 10-kb *Pst*I fragment (see Fig. 4C) was isolated from pGB108.1 and ligated into the *Pst*I site of the pRK404 expression vector (12). Clones showing both orientations were identified by restriction analysis. The 4.4-kb-long *Eco*RI-*Hin*dIII DNA fragment of pGB108.2 was subcloned into pBluescript II KS(+) (Stratagene), giving rise to plasmid pTAX1. Construct pAP7 was prepared by digestion of pTAX1 with *Hin*dIII and *Sma*I, and the resulting 3.5-kb fragment was ligated into the *Hin*dIII and filled-in *Pst*I sites of the pRK404 polylinker. Plasmid pTAX1 was digested with *Eco*RI plus *Pst*I, and a series of 5′ deletions were produced by unidirectional digestions with exonuclease III (Erase-a-Base system; Promega, Madison, Wis.). Plasmids harboring inserts of 2.3, 2.1, and 1.6 kb, termed pTAX13, pTAX16, and pTAX10, respectively, were obtained. This series of deletion constructs was moved into pRK404 by digestion of pTAX with *Hin*dIII and *Sma*I and ligation of the resulting fragments into the *Hin*dIII and filled-in *Pst*I sites in pRK404. The plasmids obtained were named pAP8, pAP9, and pAP10. pGB108.2 containing mMu-*tac* insertion 240 was used to construct plasmids pAP240.1 and pAP240.2. A 2.3-kb fragment from the *Bam*HI site, next to the right end of the *tax* region, to an internal *Hin*dIII site of mMu-*tac* (located at 1.0 kb from the left end) was cloned in both directions within the polylinker region of pRK404.

Genetic procedures. *E. coli* strains were electrotransformed with plasmid DNA by the method of Bio-Rad (Richmond, Calif.) with a Gene Pulser Transfection apparatus. Recombinant IncP group plasmids were transferred from *E. coli* to *X. campestris* by triparental matings with the helper plasmid pRK2073 (11), as described previously (18). Two-step mMu-*tac* (17) transposon mutagenesis was performed on strain VGR2(pGB108.2), by using phage λ *imm21 c*I *b538 red3* (36), by the method described by de Mendoza and Rosa (10).

DNA sequencing. Nucleotide sequencing was performed by the dideoxy chain termination method with the Sequenase 2.0 kit (United States Biochemical, Cleveland, Ohio) and [^{35}S]dATP. Exonuclease III deletion derivative plasmids pTAX13 and pTAX10 were used as templates. Both strands were sequenced, with initial direction provided by the M13 primers and subsequent direction by a series of 2.3-kb-fragment sequence-determined primers (National Biosciences, Plymouth, Minn.). Analysis of the DNA sequence and the derived protein sequences was carried out with MacVector software (Eastman Kodak, Rochester, N.Y.). Hydropathy plots were obtained by use of the Kyte-Doolittle algorithm (30).

Permeabilized cells, reaction mixtures, and assay procedures. Permeabilized cells were prepared by EDTA treatment of *X. campestris* cells as described previously (23). The standard reaction mixtures for the synthesis of lipid-linked intermediates contained 70 mM Tris-HCl buffer (pH 8.2), 8 mM $MgCl_2$, EDTA-treated cells (300 to 400 μg of protein), and sugar nucleotides either labeled or not (UDP-[U-^{14}C]Glc, 22 μM; UDP-[U-^{14}C]GlcUA, 20 μM; UDP-Glc, 285 μM; GDP-Man, 142 μM; UDP-GlcUA, 285 μM) as indicated in each case. The reactions were performed with a total volume of 70 μl at 20°C for 30 min and stopped by adding 0.3 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). Fractions of 0.5 ml were collected, and the amount of radioactivity was determined by liquid scintillation. The washed pellets were each extracted three times with 150 μl of chloroform-methanol-water (1:2:0.3). This extract, which contains the glycolipids, will be referred to as 1203 extract. The reaction mixture for the formation of X_7-P-P-polyprenol contained 5.8 μM dTDP-L-[^{14}C]Rha, unlabeled UDP-Glc (357 μM), GDP-Man, and UDP-GlcUA. Two-step incubations were also carried out. The first step was a standard incubation, performed with unlabeled UDP-Glc and GDP-Man. Cells were washed as described above and resuspended in 70 mM Tris-HCl buffer (pH 8.2)–8 mM $MgCl_2$ and reincubated at 20°C for 30 min, in the presence of UDP-[^{14}C]GlcUA, alone or with GDP-Man as indicated. The reactions were stopped and processed as described for the standard assay.

The amount of radioactive label incorporated in a portion of the 1203 extract was determined. The remainder of this fraction was reduced to about a third of its original volume at room temperature under a stream of nitrogen, water was added, and the procedure was repeated once again. The resulting emulsion was hydrolyzed in 0.01 N HCl at 100°C for 10 min and treated with alkaline phosphatase (1 h at 30°C). Under these mild hydrolysis conditions, only the phosphate linkages are split, releasing the labeled oligosaccharide from the unlabeled lipid. The treated fraction was analyzed by descending chromatography on a solvent system of 2-propanol–acetic acid–water (27:4:9). [^{14}C-GlcUA]X_7, [^{14}C-GlcUA]X_5, [^{14}C-GlcUA]X_4, and [^{14}C-Glc]Manα1-3cellobiose were run as standards. Radioactive compounds on chromatograms were located by cutting the paper strip into 1-cm sections and analyzed by liquid scintillation (23).

Purification and analysis of in vivo-produced EPS. EPS produced in vivo by *X. campestris* or *A. xylinum* strains were isolated from stationary-phase culture supernatants and purified as described previously (7). For the hexose content, 2 mg of purified EPS was hydrolyzed in 1 N HCl at 100°C for 4 or 24 h (partial and total hydrolysis, respectively). The hydrolysate was lyophilized and dissolved in 200 μl of distilled water. The hexose composition was determined either by paper chromatography on a solvent system of 2-propanol–acetic acid–water (27:4:9), or by HPLC on an Aminex HPX-87H ion-exclusion column (Bio-Rad) on an eluent solvent of 4 mM H_2SO_4, with a flow rate of 0.4 ml/min at 40°C. Detection was performed with a model 2142 refractive index detector (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). The pyruvate content was determined by HPLC on an Aminex HPX-87H ion-exclusion column after acid hydrolysis (0.25 M H_2SO_4, 96°C, 4 h) as described by Tait et al. (49).

Radiochemical and biochemicals. [^{35}S]dATP was purchased from DuPont NEN (Wilmington, Del.). UDP-D-[U-^{14}C]Glc (specific activity, 300 Ci/mol), UDP-D-[U-^{14}C]GlcUA (specific activity, 300 Ci/mol), and dTDP-L-[U-^{14}C]Rha (specific activity, 261 Ci/mol) were synthesized enzymatically as described previously (8). UDP-Glc, GDP-Man, and UDP-GlcUA were purchased from Sigma Chemical Co. (St. Louis, Mo.). [^{14}C-GlcUA]X_7, [^{14}C-GlcUA]X_5, [^{14}C-GlcUA]X_4, and [^{14}C-Glc]Manα1-3cellobiose were prepared by mild acid treatment of the corresponding prenyl-phospho-oligosaccharides obtained with B-1459 or B42 enzymatic preparations as described previously (8, 23). Restriction enzymes, T4 DNA ligase, and DNA polymerase I (Klenow enzyme) were obtained from New England BioLabs, Inc. (Beverly, Mass.).

Nucleotide sequence accession number. The full *aceA* locus sequence has been deposited in the GenBank database under accession number U37258.

RESULTS

Restoration of EPS production in *X. campestris* 2444 mutant by the *A. xylinum* genomic library. As shown in Fig. 1, acetan and xanthan have similar structures. In addition, the first four reactions of each repeating unit assembly should be catalyzed by a set of similar glycosyltransferase enzymes. We decided to study whether an EPS$^-$ mutant of *X. campestris*, deficient in one of the first four steps of the repeating unit biosynthesis, could be complemented by the genomic library of *A. xylinum*. A number of xanthan polysaccharide synthesis-defective mutants have been described by Harding et al. (18). By biochemical analysis of strain 2444, we have determined that this mutant is deficient in the α-mannosyltransferase enzyme (see below), which encodes the third enzyme of the biosynthetic pathway (Fig. 1). The *A. xylinum* gene bank was conjugatively transferred to strain 2444. Mucoid colony morphologies, a typical phenotypic behavior of EPS$^+$ strains (Fig. 2), were observed in 1.4% of the transconjugants. Mucoid transconjugants showed a similar amount of EPS production in vivo compared with that of the wild-type strain B-1459.

By clone analysis of 12 *X. campestris* 2444 mucoid transconjugants, three different plasmid clones were identified and

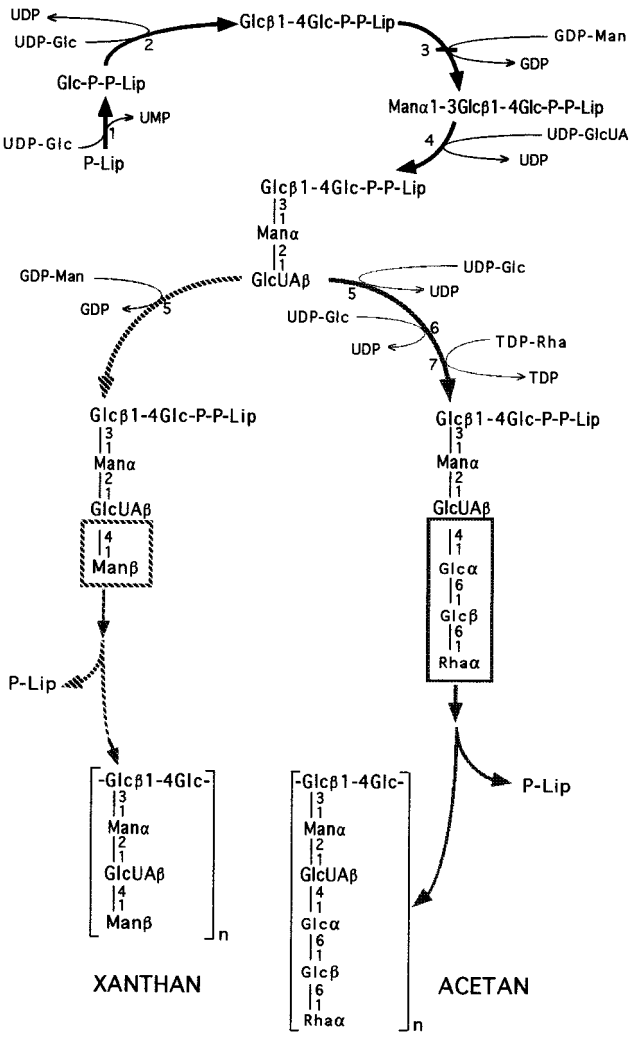

FIG. 1. Proposed pathways for xanthan and acetan biosynthesis (8, 19, 23, 45). Equivalent reactions in both pathways are represented by thick black arrows. Reactions not common to both pathways are represented by dashed (for xanthan) or thick grey (for acetan) arrows. A small thick horizontal line represents the absence of GDP-Man:cellobiosyl-diphosphopolyprenol α-mannosyltransferase activity (reaction 3) in *X. campestris* 2444. Lip indicates polyprenyl carrier. Boxes indicate structures not common to both lipid-linked repeating units.

named pGB108.1, pGB108.2, and pGB108.3. The smallest insert of these plasmids (pGB108.2) was about 22 kb long and was fully contained in the other two (see below). This DNA region was named *tax*. Southern blot analysis indicated that *Hin*dIII fragments contained in pGB108.2 and pGB108.3 were colinear on the *A. xylinum* genome. The additional *Hin*dIII fragment present in pGB108.1 was found to be noncontiguous with the remaining region. A probe constructed with a 5.6-kb *Hin*dIII fragment on the left side of pGB108.2 showed no hybridization with *A. xylinum* plasmid DNA, indicating that the *tax* region is chromosomally located (data not shown).

Biochemical characterization of *X. campestris* 2444 mucoid transconjugants. Since plasmid pGB108.2 contained the smallest insert able to restore the EPS production of mutant 2444, we analyzed the EPS produced in vivo by the transconjugant 2444(pGB108.2). The chromatography patterns after partial acid hydrolysis were indistinguishable compared with those of xanthan. Cellobiose and glucuronic acid-mannose in addition to Glc, Man, and GlcUA were detected. Gentiobiose, a characteristic disaccharide of acetan (7), was not found. In addition, the HPLC pattern after total acid hydrolysis of the EPS produced by the mutant 2444(pGB108.2) was similar to that of xanthan. Essentially equal ratios of glucose to mannose were observed [1.2:1 and 1.4:1 for mutant 2444(pGB108.2) EPS and xanthan, respectively], and the presence of rhamnose was not detected. Both HPLC patterns were different from that of acetan. In addition, the pyruvate content of the EPS produced in vivo by 2444(pGB108.2) was 60% of that of xanthan produced by wild-type strain B-1459 (data not shown). Acetan produced by *A. xylinum* B42 contains no pyruvyl side groups (7, 27).

Biochemical analysis was also carried out by using previously developed in vitro assays consisting of EDTA-permeabilized cells (23). Incubation of EDTA-treated cells from strain B-1459 with UDP-[^{14}C]Glc resulted in the incorporation of radioactivity into cellobiosyl-diphosphopolyprenol (Fig. 3A). When GDP-Man was added to the incubation mixture, radioactive mannosylα1-3cellobiosyl-diphosphopolyprenol in addition to cellobiosyl-diphosphopolyprenol was recovered from 1203 extracts (Fig. 3B). These two compounds have been characterized previously by partial acid hydrolysis, column chromatography, permethylation assays, and enzymatic degradation (23). Parallel experiments with EDTA-permeabilized cells from strain 2444 revealed the incorporation of [^{14}C]Glc in cellobiosyl-diphosphopolyprenol (Fig. 3C). No incorporation of [^{14}C]Glc into mannosylα1-3cellobiosyl-diphosphopolyprenol was detected when GDP-Man was added to the incubation mixture (Fig. 3D). Analysis of standard incubations resulted in the appearance of α-mannosyltransferase activity when EDTA-permeabilized cells of 2444(pGB108.2) were used (Fig. 3E and F).

We tested whether the polyprenol-bound trisaccharide synthesized by 2444(pGB108.2) was able to function as an acceptor of UDP-GlcA and GDP-Man to form X_5-P-P-polyprenol by using two-step incubations. The first incubation was carried out in the presence of UDP-[^{14}C]Glc and GDP-Man to accumulate the trisaccharide glycolipid. When UDP-GlcUA and GDP-Man were present in the second incubation, a radioactive compound with the properties of the X_5-P-P-polyprenol was

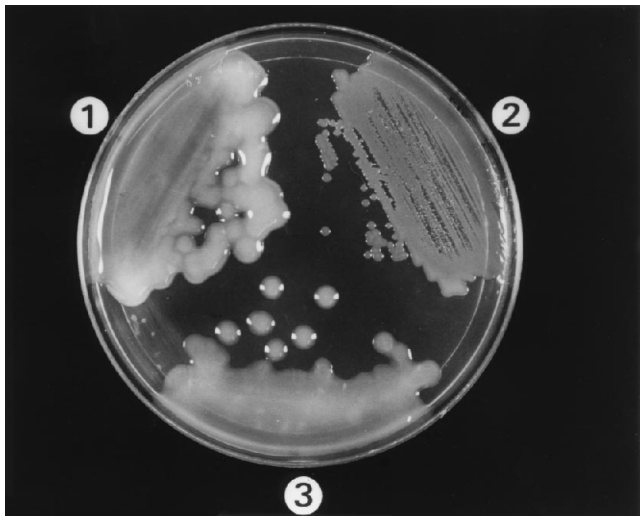

FIG. 2. Colony phenotype of *X. campestris* B-1459 (labeled 1), 2444 (labeled 2), and 2444(pGB108.2) (labeled 3). Strains were grown on yeast-maltose agar plates for 3 days.

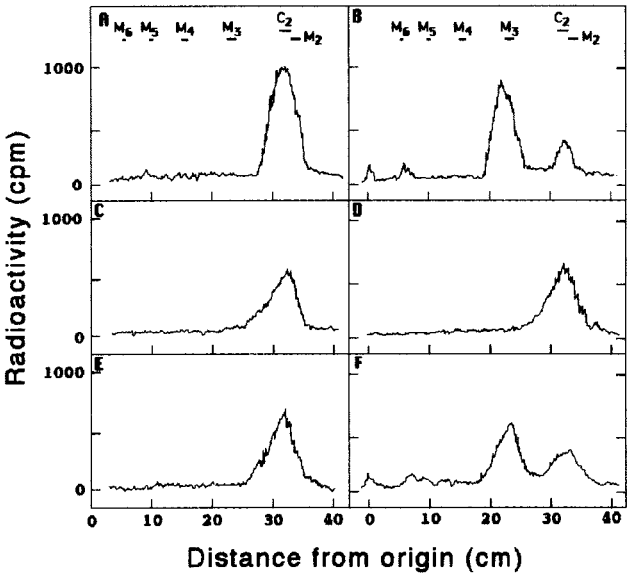

FIG. 3. Heterologous complementation of *X. campestris* 2444. Standard incubations were carried out in the presence of UDP-[^{14}C]Glc, and GDP-Man where indicated, by employing EDTA-permeabilized cells of the following strains: B-1459 (A), B-1459 and GDP-Man (B), 2444 (C), 2444 and GDP-Man (D), 2444(pGB108.2) (E), 2444(pGB108.2) and GDP-Man (F). 1203 extracts were submitted to mild acid hydrolysis followed by paper chromatography as described in Materials and Methods. Cellobiose (C_2) and maltooligosaccharides (M_2 to M_6) were run as standards.

detected. When the second incubation was carried out in the presence of UDP-GlcUA alone, a compound with the properties of the X_4-P-P-polyprenol was formed. In vitro synthesis of X_5-P-P-polyprenol by 2444(pGB108.2) was also accompanied by the incorporation of radioactivity into a polymeric product, although in reduced amounts (approximately 15% of the polysaccharide produced by the wild-type strain). When analyzed by a Bio-Gel A-5m column chromatography, this material coeluted with a sample of authentic xanthan gum (data not shown). Taken together, these results indicate that 2444 harboring the *tax* region has a restored ability to synthesize xanthan.

Since X_7, the acetan repeating unit, contains a rhamnose residue, we performed an incubation with dTDP-L-[^{14}C]Rha in addition to UDP-Glc, GDP-Man, and UDP-GlcUA. No incorporation of radioactivity into the 1203 extract was detected, confirming the in vivo analysis. Therefore, this region harbors an *A. xylinum* function that complements, in a heterologous way, the defective α-mannosyltransferase activity of *X. campestris.*

Determination of the minimum *A. xylinum* DNA region that complements *xps-108* mutation. The 22-kb insert of the plasmid pGB108.2 (Fig. 4A) was subjected to transposon mutagenesis. We used a two-step system: mMu-*tac* insertion into pGB108.2 followed by in vivo packaging into phage lambda heads and transduction to a new host. Restriction analysis of 70 transductant colonies detected 40 insertions and 10 insertions plus deletions, both of which were randomly located in the 22-kb insert of pGB108.2 (Fig. 4B). Eight insertions were located on the vector pVK102, and 10 were similar to others already tested.

As shown in Fig. 4C, a 9-kb *Hin*dIII-*Pst*I fragment retained the ability to complement strain 2444, regardless of its orientation with respect to the P_{lac} vector (plasmids pAP1 and pAP3). Therefore, the pGB108.2::mMu-*tac* isolates, whose insertions mapped within this region, were tested for their ability to complement EPS production of the 2444 mutant. Insertions in a single 1.2-kb fragment failed to restore the EPS$^+$ phenotype to strain 2444 (Fig. 4B). The 1.2-kb fragment was subcloned from the pGB108.2 containing mMu-*tac* insertion 240 into pRK404, producing pAP240.1 and pAP240.2. Complementation analysis carried out with the two P_{lac} orientation derivative constructions showed that only plasmid pAP240.1 was able to restore EPS production to strain 2444 (Fig. 4C).

In addition, a number of pAP7 subclones were constructed by exonuclease III digestion. Plasmids pAP8, pAP9, and pAP10, harboring 2.3-, 2.1-, and 1.6-kb inserts, respectively, were obtained. As shown in Fig. 4C, the 2.1-kb insert of pAP9 resulted in the smallest *A. xylinum* DNA region that was able to restore EPS production of strain 2444. This result agrees with those of transposon mutagenesis and subcloning.

Sequence of the *A. xylinum* DNA fragment that complements the *xps-108* mutation. The entire nucleotide sequence of the 2.3-kb insert in recombinant pAP8 was obtained from both strands by employing universal sequencing primers and subsequently a series of custom-made primers (GenBank accession number U37258). The nucleotide sequence of the insert in pAP10 was also obtained. Computer analysis identified an ORF, termed *aceA*, spanning base pairs 866 to 2047. The sequence of the *aceA* gene consists of a single ORF of 1,182 bp that encodes a protein of 393 amino acids with an estimated M_r of 43,219 and a isoelectric point of 9.7. A putative ribosomal binding site (GAG) was identified 8 nucleotides upstream from the proposed start codon. A putative promoter sequence similar to the *E. coli* consensus sequence or *A. xylinum* proposed sequence (3, 6, 24) was not found. The codon usage of α-mannosyltransferase is similar to that of other genes from *A. xylinum* which have been sequenced (data not shown).

A truncated ORF, named δ*aceB*, and ORF3 were found flanking the *aceA* ORF. The physical location of the δ*aceB* and ORF3 genes in relation to *aceA* is indicated in Fig. 4D. The putative δAceB protein product has an ABC binding motif (13) and was found to be 28% identical at the amino acid level to the ExoP protein of *Rhizobium meliloti*, a putative succinoglycan export protein (16) (data not shown). The hydrophobicity profile for δAceB protein predicts a transmembrane domain, probably anchoring the protein to the cytoplasmic membrane. No significant homology was found between ORF3 and any other protein in the database.

Comparison of AceA with other α-mannosyltransferases. A computer search of the derived amino acid sequence of AceA at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Md.) revealed a significant degree of homology with the α-mannosyltransferase GumH of *X. campestris* (obtained from the GenBank file server under the accession number U22511 [submitted by T. J. Pollock, 1995]). The polypeptides are similar in size, with primary sequences that are 42% identical. Both proteins exhibit two regions of strong similarities: R1 in the amino terminus, from amino acids 97 to 134, and R2 in the carboxy terminus, from amino acids 282 to 314 of AceA (Fig. 5A). AceA and GumH are relatively hydrophilic molecules (Fig. 5B), and analysis of the membrane-spanning regions predicts that both proteins are probably attached to the cytoplasmic membrane at the carboxy terminus.

No significant homology was found between AceA and any other protein in the database. We also examined whether small regions of AceA were homologous to other mannosyltransferases, all of which are known or thought to transfer mannose residues from a nucleotide diphosphate-mannose to a growing

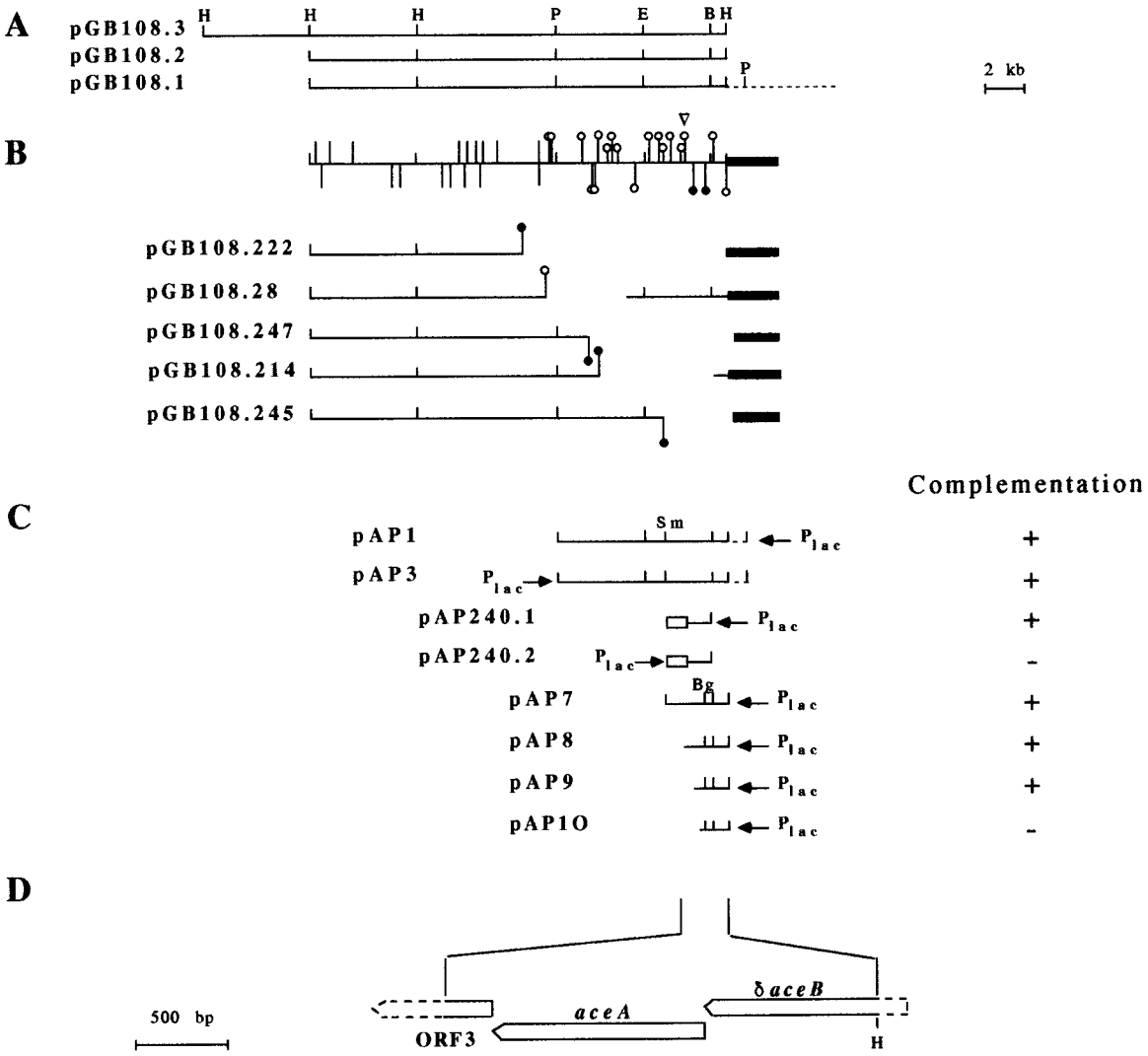

FIG. 4. Recombinant cosmids containing the *tax* region of *A. xylinum*. Genetic data concerning recombinant cloned inserts, transposon mutagenesis, heterologous complementation of *X. campestris* 2444 mutant, and the positions of *aceA*, δ*aceB*, and ORF3 genes are also shown. (A) Structures of cosmids containing cloned chromosomal DNA isolated from complemented 2444 mutant. Dashed lines denote additional DNA fragments that are not contiguous with the rest of the region shown. (B) mMu-*tac* transpositions. Vertical lines indicate the location of each insertion, and their positions show the orientation of the *tac* promoter (above the line from right to left and below the line from left to right). mMu-*tac* transpositions which result in insertions plus deletions are also shown. The unfilled triangle indicates insertion pGB108.240. Solid boxes represent the cosmid vector. (C) Localization of the *aceA* gene. The restriction fragments were subcloned into pRK404 and introduced into strain 2444 to test for complementation (mucoidy). The directions of transcription from the P_{lac} are indicated by arrows. Unfilled boxes represent a mMu-*tac* fragment. (D) The enlarged 2.3-kb fragment of pAP8, which was sequenced. Box arrows indicate the positions of the ORFs and the directions in which they are transcribed. Restriction sites relevant to the construction of plasmids are indicated by the following abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; P, *Pst*I; Sm, *Sma*I. EPS production by *X. campestris* 2444 containing each construction is indicated as follows: ○ (panel B) and + (panel C), production of EPS; ● (panel B) and − (panel C), no production of EPS.

carbohydrate chain. The alignment shows (Fig. 6) that there are common amino acid residues close to the carboxy termini of the O antigen α-mannosyltransferases of *Salmonella enterica* RfbU (serogroup B), RfbW, and RfbZ (serogroup C2) (35). The α-mannosyltransferase consensus motif, which has the sequence $EXFGX_4E$, was also found to be present in the α-mannosyltransferases MtfA (twice), MtfB, and MtfC of the O9 polysaccharide of *E. coli* (28).

DISCUSSION

To better understand the assembly and translocation of bacterial biopolymers, we are studying this process in *A. xylinum*. In this work, we have identified a chromosomal DNA region (*tax* region) which restored both the mucoid phenotype and EPS production to an α-mannosyltransferase-deficient mutant strain of *X. campestris* by employing a genomic library of *A. xylinum*. The EPS produced in vivo by the genetically transformed mutant had a totally indistinguishable hexose composition and about the same degree of pyruvylation as that of xanthan. In addition, we found that the polyprenol-bound trisaccharide synthesized by the complemented mutant is functionally equivalent to the mannosylα1-3-cellobiosyl-P-P-polyprenol synthesized by the wild-type strain, since it could be used as an acceptor of two more sugars, and that the resulting polyprenol-bound pentasaccharide could be polymerized to produce xanthan. At present, we do not have an explanation for the reduced amount of polysaccharide produced in vitro by

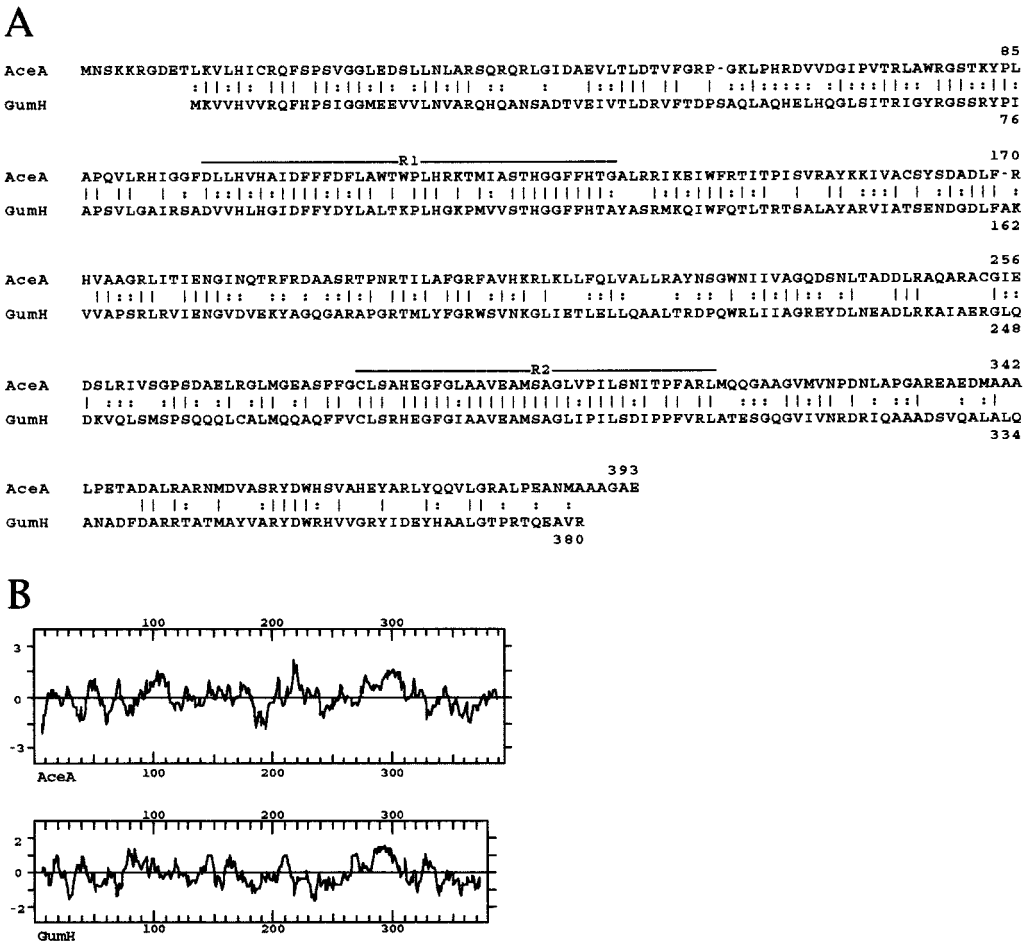

FIG. 5. (A) Alignment of amino acid sequences of AceA and GumH (GenBank accession number U22511, 1995) from *A. xylinum* and *X. campestris*, respectively. The alignment was generated by use of the Lipman and Pearson algorithm (34). Vertical bars and colons indicate positions with identical and conserved residues, respectively. R1 and R2 represent highly conserved regions. (B) Hydropathy plots of AceA and GumH. Hydrophobic domains are above each center line, and hydrophilic domains are below. Plots were obtained by the method of Kyte and Doolittle (30) by use of a window of 11 amino acids.

strain 2444(pGB108.2). A similar effect was observed when mutants of *X. campestris* defective in sugar nucleotide synthesis were studied (20). The absence of rhamnose detected in the analysis of EPS produced in vivo by 2444(pGB108.2) and the absence of in vitro incorporation of labeled rhamnose to lipid-linked intermediates support the conclusion that the *tax* region would be able to synthesize only xanthan, not acetan or a new distinct EPS mixture.

Four lines of evidence support the conclusion that *aceA* constitutes the GDP-Man:cellobiosyl-diphosphopolyprenol α-mannosyltransferase gene, which transfers a mannose residue from GDP-Man to Glcβ1-4Glc-P-P-polyprenol to form Manα1-3Glcβ1-4Glc-P-P-polyprenol. First, insertions of mMu-*tac* within the coding sequence for *aceA* abolish complementation of the 2444 mutant. Second, plasmid pAP240.1, carrying the *aceA* ORF in the same orientation as that of the expression vector promoter, complements the 2444 mutant, but pAP240.2, which harbors the same fragment in the opposite orientation, does not. Third, clone pAP9, which has an insert of 2.1 kb, also complements strain 2444 deficiency, while complementation is abolished in deletion clone pAP10, which harbors only 261 codons of *aceA* (Fig. 4C). Fourth, there is significant homology between AceA (42% identical amino acid residues) and *X. campestris* GumH, which possesses similar catalytic activity (1, 51).

Several reports have shown that EPS biosynthetic genes are clustered in a short region of the bacterial genome (2, 25, 32). Plasmids pGB108.2 and pGB108.3 did not complement xanthan production of *X. campestris* 2445 (UDP-Glc glucosylphosphotransferase defective) and 2452 (UDP-GlcUA β-glucuronyltransferase defective) (18, 22) (Fig. 1, reactions 1 and 4, respectively). Introduction of the *A. xylinum* genomic library into each of these mutants resulted in restoration of EPS production for strain 2452 alone, suggesting that *A. xylinum* DNA encodes a protein functionally equivalent to *X. campestris* UDP-GlcUA β-glucuronyltransferase. At present, we have no evidence indicating that this new region is linked to the *tax* region. These observations raise the possibility that glycosyltransferase activity in a heterologous background may be dependent on several host factors. The inability of the *A. xylinum* genomic library to complement *X. campestris* 2445 may reflect a requirement for efficient expression in *X. campestris* cells. Alternatively, some glycosyltransferases may require additional functional regions for a correct insertion into the cytoplasmic membrane or may require contact with other components of their respective systems.

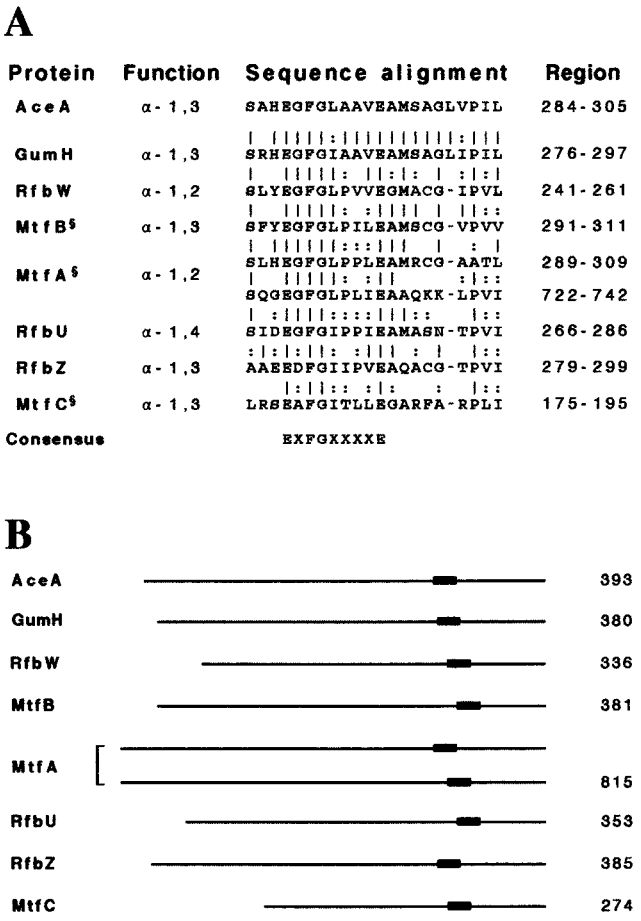

FIG. 6. Sequence similarities of the AceA polypeptide to other members of α-mannosyltransferase proteins. (A) Fragment sequences around the conserved domain are presented in the single-letter amino acid code. The alignment was generated by use of the Lipman and Pearson algorithm (34). Vertical bars and colons indicate positions with identical and conserved residues, respectively. The consensus shows the amino acid residues that are identical in the compared sequences. (B) Solid bars represent the locations of the amino acid sequence shown in panel A on the polypeptide chain. Numbers to the right of each line indicate the length in amino acid residues of each protein. §, the complete amino acid sequences of MtfA, MtfB, and MtfC were obtained from DNA sequencing analysis (28).

δ*aceB*, *aceA*, and ORF3 are located at the right end of pGB108.2 and are transcribed from right to left. In addition, there is an overlap of the start and stop codons of the genes flanking *aceA*. These observations suggest that δ*aceB*, *aceA*, and ORF3 are transcribed as a single unit and may represent a portion of a cluster involved in acetan biosynthesis.

The *aceA* gene has G+C content (65%) similar to the average G+C content of *Acetobacter* genus chromosomal DNA (51 to 65%) (9). The deduced amino acid sequence of AceA was not found to have significant homology within glycosyltransferase families, and no significant homology with any protein in the database, other than GumH, has been found. Lack of homology among glycosyltransferases which have similar acceptor or donor substrates has been observed (28, 38). However, alignments between AceA and α- and β-mannosyltransferases revealed a shared sequence, $EXFGX_4E$. This motif, which was first found to be present in MtfA, MtfB, and RfbW by Kido et al. (28), was also present in GumH, RfbU, RfbZ, and MtfC and was not found in β-mannosyltransferases. The conserved peptide was also found in the gene product ORF6.17, which is suspected to participate in O-antigen synthesis of *Salmonella enterica* serogroup C1, although its specific function is unknown (31). Possible functions for this common sequence include formation of the mannose α-linkage to the lipid-oligosaccharide acceptor. Alternatively, it may be required for proper insertion of the GDP-Man α-mannosyltransferase into the inner membrane cytoplasmic domain, since the conserved sequence occurs in potential membrane-spanning regions. A detailed analysis of the sequences of bacterial α-mannosyltransferases revealed the possible existence of a family of glycosyltransferases (15).

Although we have not determined the complete sequence of the δ*aceB* gene, we found that the putative δAceB polypeptide shows sequence similarity to proteins that have been implicated in the translocation of several polysaccharides across the cytoplasmic membrane, such as ExoP and NdvA in *R. meliloti* (4, 16, 46), ChvA in *Agrobacterium tumefaciens* (5), CpsC in *Streptococcus agalactiae* (42), and KpsT in *E. coli* K-1 (39). This analysis suggests that δAceB could be involved in energy-dependent translocation of acetan across the inner membrane.

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REFERENCES

1. **Betlach, M. R., M. A. Capage, D. H. Doherty, R. A. Hassler, N. M. Henderson, R. W. Vanderslice, J. D. Marrelli, and M. B. Ward.** 1987. Genetically engineered polymers: manipulation of xanthan biosynthesis, p. 35–50. *In* M. Yalpani (ed.), Progress in biotechnology, vol. 3. Industrial polysaccharides. Elsevier Science B.V., Amsterdam.
2. **Boulnois, G. J., and I. S. Roberts.** 1990. Genetics of capsular polysaccharide production in bacteria. Curr. Top. Microbiol. Immunol. **150:**1–18.
3. **Brede, G., E. Fjaervik, and S. Valla.** 1991. Nucleotide sequence and expression analysis of the *Acetobacter xylinum* uridinediphosphoglucose pyrophosphorylase gene. J. Bacteriol. **173:**7042–7045.
4. **Buendía, A. M., B. Enenkel, R. Köplin, K. Niehaus, W. Arnold, and A. Pühler.** 1991. The *Rhizobium meliloti* exoZl exoB fragment of megaplasmid 2: ExoB functions as a UDP-glucose 4-epimerase and ExoZ shows homology to NodX of *Rhizobium leguminosarum* biovar *viciae* strain TOM. Mol. Microbiol. **5:**1519–1530.
5. **Cangelosi, G. A., G. Martinetti, J. A. Leigh, C. C. Lee, C. Theines, and E. W. Nester.** 1989. Role for *Agrobacterium tumefaciens* ChvA protein in export of β-1,2-glucan. J. Bacteriol. **171:**1609–1615.
6. **Coucheron, D. H.** 1993. A family of IS1031 elements in the genome of *Acetobacter xylinum*: nucleotide sequences and strain distribution. Mol. Microbiol. **9:**211–218.
7. **Couso, R. O., L. Ielpi, and M. A. Dankert.** 1987. A xanthan-gum-like polysaccharide from *Acetobacter xylinum*. J. Gen. Microbiol. **133:**2123–2135.
8. **Couso, R. O., L. Ielpi, R. C. García, and M. A. Dankert.** 1982. Biosynthesis of polysaccharides in *Acetobacter xylinum*. Sequential synthesis of a heptasaccharide diphosphate prenol. Eur. J. Biochem. **123:**617–627.
9. **De Ley, J., J. Swings, and F. Gosselé.** 1984. Family VI. Acetobacteraceae, p. 268–274. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
10. **de Mendoza, D., and A. L. Rosa.** 1985. Cloning of mini-Mu bacteriophage in cosmids: in vivo packaging into phage lambda heads. Gene **39:**55–59.
11. **Ditta, G.** 1986. Tn*5* Mapping of *Rhizobium* nitrogen fixation genes. Methods Enzymol. **118:**519–578.
12. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, L. Xiao-Wu, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmid related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13:**149–153.
13. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
14. **Fukaya, M., K. Tayama, H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Improved transformation method for *Acetobacter* with plasmid DNA. Agric. Biol. Chem. **49:**2091–2097.

15. **Geremia, R. A., E. A. Petroni, L. Ielpi, and B. Henrissat.** Toward a classification of glycosyl transferases based on amino acid sequences similarities. Prokaryotic α-mannosyl-transferases. Biochem. J., in press.
16. **Glucksmann, M. A., T. L. Reuber, and G. C. Walker.** 1993. Genes needed for the modification, polymerization, export, and processing of succinoglycan by *Rhizobium meliloti*: a model for succinoglycan biosynthesis. J. Bacteriol. **175:**7045–7055.
17. **Gramajo, H. C., A. M. Viale, and D. de Mendoza.** 1988. Expression of cloned genes by in vivo insertion of *tac* promoter using a mini-Mu bacteriophage. Gene **65:**305–314.
18. **Harding, N. E., J. M. Cleary, D. K. Cabañas, I. G. Rosen, and K. S. Kang.** 1987. Genetic and physical analysis of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. J. Bacteriol. **169:**2854–2861.
19. **Harding, N. E., J. M. Cleary, and L. Ielpi.** 1995. Genetics and biochemistry of xanthan gum production by *Xanthomonas campestris*, p. 495–514. *In* Y. H. Hui and G. G. Khachatourians (ed.), Food biotechnology: microorganisms. VCH Publishers, New York.
20. **Harding, N. E., S. Raffo, A. Raimondi, J. M. Cleary, and L. Ielpi.** 1993. Identification, genetic and biochemical analysis of genes involved in synthesis of sugar nucleotide precursors of xanthan gum. J. Gen. Microbiol. **139:**447–457.
21. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33:**933–938.
22. **Ielpi, L.** Unpublished data.
23. **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.
24. **Iversen, T., R. Standal, T. Pedersen, and D. H. Coucheron.** 1994. IS1032 from *Acetobacter xylinum*, a new mobile insertion sequence. Plasmid **32:**46–54.
25. **Jann, K., and B. Jann.** 1991. Biochemistry and expression of bacterial capsules. Biochem. Soc. Trans. (London) **19:**623–628.
26. **Jansson, P. E., L. Keene, and B. Lindberg.** 1975. Structure of the extracellular polysaccharide from *Xanthomonas campestris*. Carbohydr. Res. **45:**275–282.
27. **Jansson, P. E., J. Lindberg, S. Wimalasiri, and M. A. Dankert.** 1993. Structural studies of acetan, an exopolysaccharide by *Acetobacter xylinum*. Carbohydr. Res. **245:**303–310.
28. **Kido, N., V. I. Torgov, T. Sugiyama, K. Uchiya, H. Sugihara, T. Komatsu, N. Kato, and K. Jann.** 1995. Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. coli* O9 rfb gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. J. Bacteriol. **177:**2178–2187.
29. **Knauff, V., and E. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8:**45–54.
30. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
31. **Lee, S. J., L. K. Romana, and P. R. Reeves.** 1992. Sequence and structural analysis of the *rfb* (O antigen) gene cluster from a group C1 *Salmonella enterica* strain. J. Gen. Microbiol. **138:**1843–1855.
32. **Leigh, J. A., and D. L. Coplin.** 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. **46:**307–346.
33. **Leigh, J. A., and G. C. Walker.** 1994. Exopolysaccharides of *Rhizobium*: synthesis, regulation and symbiotic function. Trends Genet. **10:**63–67.
34. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227:**1435–1440.
35. **Liu, D., A. M. Haase, L. Lindqvist, A. A. Lindberg, and P. R. Reeves.** 1993. Glycosyl transferases of O-antigen biosynthesis in *Salmonella enterica*: identification and characterization of transferase genes of groups B, C2, and E1. J. Bacteriol. **175:**3408–3413.
36. **Miller, G., and M. Feiss.** 1981. Cohesive end annealing and the helper-mediated transformation system of phage λ. Virology **109:**379–390.
37. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Paulson, J. C., and K. J. Colley.** 1989. Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. J. Biol. Chem. **264:** 17615–17618.
39. **Pavelka, M. S., S. F. Hayes, and R. P. Silver.** 1994. Characterization of KpsT, the ATP-binding component of the ABC-transporter involved with the export of capsular polysialic acid in *Escherichia coli* K1. J. Biol. Chem. **269:** 20149–20158.
40. **Petroni, E. A., S. N. Bocca, and L. Ielpi.** Sequence-specific DNA modification in *Acetobacter xylinum*. Cell. Mol. Biol., in press.
41. **Petroni, E. A., and L. Ielpi.** 1993. Isolation and characterization of a cellulose-negative, acetan-producer mutant of *Acetobacter xylinum*. An. Asoc. Quím. Argent. **81:**225–234.
42. **Rubens, C. E., L. M. Heggen, R. F. Haft, and M. R. Wessels.** 1993. Identification of *cpsD*, a gene essential for type III capsule expression in group B streptococci. Mol. Microbiol. **8:**843–855.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Saxena, I. M., K. Kudlicka, K. Okuda, and R. M. Brown.** 1994. Characterization of genes in the cellulose-synthesizing operon (*acs* operon) of *Acetobacter xylinum*: implications for cellulose crystallization. J. Bacteriol. **176:** 5735–5752.
45. **Semino, C. E., and M. A. Dankert.** 1993. *In vitro* biosynthesis of acetan using electroporated *Acetobacter xylinum* cells as enzyme preparations. J. Gen. Microbiol. **139:**2745–2756.
46. **Standfield, S. W., L. Ielpi, D. O'Brochta, D. R. Helinski, and G. S. Ditta.** 1988. The *ndvA* gene product of *Rhizobium meliloti* is required for β-(1-2)glucan production and has homology to the ATP-binding export protein HlyB. J. Bacteriol. **170:**3523–3530.
47. **Stankowski, J. D., B. E. Mueller, and S. G. Zeller.** 1993. Location of a second *O*-acetyl group in xanthan gum by the reductive-cleavage method. Carbohydr. Res. **241:**321–326.
48. **Sutherland, I. W., and M. I. Tait.** 1992. Biopolymers, p. 339–349. *In* J. Lederberg (ed.), Encyclopedia of microbiology, Academic Press, Inc., San Diego, Calif.
49. **Tait, M. I., I. W. Sutherland, and A. J. Clarke-Sturman.** 1990. Acid hydrolysis and high-performance liquid chromatography of xanthan. Carbohydr. Polym. **13:**133–148.
50. **Tolmasky, M. E., and J. H. Crosa.** 1984. Molecular cloning and expression of genetic determinants for the iron uptake system mediated by the *Vibrio anguillarum* plasmid pJM1. J. Bacteriol. **160:**860–866.
51. **Vanderslice, R. W., D. H. Doherty, M. A. Capage, M. R. Betlach, R. A. Hassler, N. M. Henderson, J. Ryan-Graniero, and M. Tecklenburg.** 1989. Genetic engineering of polysaccharide structure in *Xanthomonas campestris*, p. 145–156. *In* V. Crescenzi, I. C. M. Dea, S. Paoletti, S. S. Stivala, and I. W. Sutherland (ed.), Biomedical and biotechnological advances in industrial polysaccharides. Gordon & Breach, New York.
52. **Wong, H. C., A. L. Fear, R. D. Calhoon, G. H. Eichinger, R. Mayer, D. Amikam, M. Benziman, D. H. Gelfand, J. H. Meade, A. W. Emerick, R. Bruner, A. Ben-Bassat, and R. Tal.** 1990. Genetic organization of the cellulose synthase operon in *Acetobacter xylinum*. Proc. Natl. Acad. Sci. USA **87:**8130–8134.