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Biosynthesis of a substituted cellulose from a mutant strain of *Xanthomonas campestris*☆

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Abstract

In *Xanthomonas campestris* the genes involved in polysaccharide (xanthan) biosynthesis are located in a gene cluster (*gum*) of 16 kb. A Tn5 insertion mutant with a reduced slimy phenotype has been characterized. This mutant failed to produce the pentasaccharide repeating-unit of xanthan. Only three sugars were transferred to the prenyl phosphate intermediate. Several lines of evidence suggested that the lipid-associated saccharide was the trisaccharide reducing end of the pentasaccharide from the wild-type strain. This trisaccharide was built up from UDP-Glc and GDP-Man, and a glucose residue was at the reducing end, linked to an allylic prenol through a diphosphate bridge. Results from one- or two-stage reactions showed that the trisaccharide-P–P-polyprenol was the precursor of the polymer. This new polymer, a polytrisaccharide, was detected also in vivo. The transposon responsible for the mutation was located within *gumK* gene. Therefore, this gene encodes for the glycosyltransferase IV, which catalyses the transfer of glucuronic acid to the lipid-linked β-D-Man*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p* trisaccharide. A recombinant plasmid with the whole *gum* cluster restored the wild type phenotype. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Polysaccharide; Xanthan; Biosynthesis; *gum* genes

1. Introduction

Xanthomonas campestris is a plant pathogen that produces an exopolysaccharide (EPS), xanthan, liberated into the culture medium. It has a wide variety of applications as a thickener and emulsifier in activities as diverse as the food, pharmaceutical, and oil industries.[1]

The chemical structure of xanthan has been studied by several laboratories and shown to consist of a cellulosic backbone with (1→4)-β-D-Glc*p* linkages and a trisaccharide side-chain, β-D-Man*p*-(1→4)-β-D-GlcA-(1→2)-α-D-Man*p*, (1→3)-linked to every two glucose residues (Fig. 1). About half of the terminal mannose residues are substituted with ketal-pyruvate residues, and all of the internal ones with acetyl residues.[2,3] However other proportions of substituents have been described.[4] A recent publication has shown that a second acetyl residue is present in the non-pyruvated terminal mannose.[5]

Most of the biochemical details of the biosynthesis of this polysaccharide have been described, lipid-linked oligosaccharides being involved.[6] In the first step, a linear pentasaccharide repeating-unit is assembled on a polyprenol lipid acceptor by sequential addition of the

Abbreviations: UDP-Glc, uridine 5′-(α-D-glucopyranosyl diphosphate); UDP-GlcA, uridine 5′-(α-D-glucopyranosyluronic diphosphate); GDP-Man, guanosine 5′-(α-D-mannopyranosyl diphosphate); UDP-GalA, uridine 5′-(α-D-galactopyranosyluronic diphosphate); AMP, adenosine 5′-monophosphate; UMP, uridine 5′-monophosphate; GalA, D-galactopyranosyluronic acid; GlcA, D-glucopyranosyluronic acid; ManA, D-mannopyranosyluronic acid; EPS, exopolysaccharide; Glc, D-glucopyranose; Man, D-mannopyranose.

☆ A substituted cellulose from *Xanthomonas campestris*.

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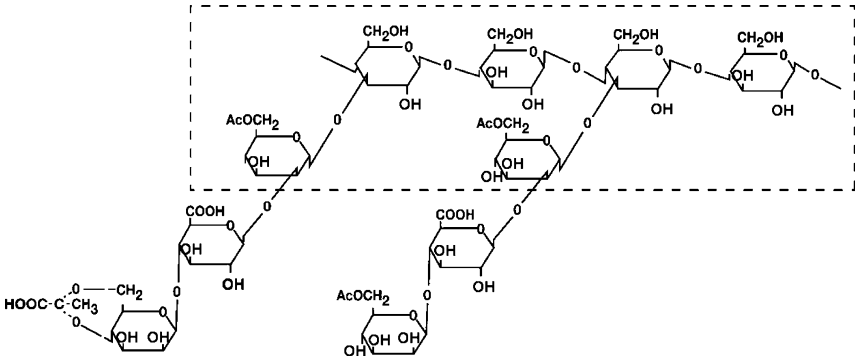

Fig. 1. Xanthan structure. Two repeating units are shown with the respective nonglycosidic substituents.[2,5] The dotted lines encircle the truncated polymer here described.

individual sugars from the corresponding sugar nucleotide precursors UDP-Glc, UDP-GlcA, and GDP-Man. Recent studies have established that these sugar nucleotides are synthesized by *X. campestris* consistent with a role in the biosynthesis of xanthan.[7] At the lipid intermediate stage, the ketal-pyruvate and acetyl residues are also incorporated from enolpyruvate phosphate and acetyl CoA, respectively.[8,9] The second step consists of the subsequent polymerization of the repeating units to produce the polysaccharide. A striking result observed was that the new repeating units are incorporated at the reducing end of the growing chain,[10] a mechanism observed for the first time in the biosynthesis of the O-antigen polysaccharide in Salmonella.[11] All of these studies were performed with a wild-type strain NRRL B-1459.

Several genetic loci are involved in the biosynthesis of xanthan. These include at least four DNA regions located in the chromosome of *X. campestris*.[12–19] The genes that encode for the enzymes involved in the transfer of the sugars and of the nonglycosidic substituents are located in a cluster[12,13,17] which comprises of 12 predicted open-reading frames, *gumB–gumM*.[18] Transcriptional analysis has shown that the *gum* genes are mainly expressed as an operon from a promoter upstream of the first gene, *gumB*,[19] Secondary (weak) promoters may exist upstream of *gumK*[19] and *gumD*.[18,20] The function of some of the gene products has been established. GumD, GumH, GumI, GumK, and GumM are known to be involved in the assembly of the pentasaccharide lipid intermediate. GumL is the pyruvate-ketal transferase,[21] GumF and GumG are involved in acetyl transfer from acetyl CoA,[22] *gumB* and *gumC* genes have been demonstrated to operate together in the production of the final polymer.[23] The function of the *gumE* and *gumJ* genes, originally proposed to have a role in the polymerisation and translocation of xanthan,[18] is still unclear.

Xanthan has also been implicated as a pathogenicity factor in black rot.[24,25] An EPS-defective mutant was shown to have diminished virulence in broccoli[26] and recently, the expression of the *gum* promoter have been checked in turnip, using the β-glucuronidase reporter gene.[27]

Xanthan-deficient mutants of *X. campestris* pv. *campestris* have been derived from another wild-type strain, 8004, by Tn5 mutagenesis.[12] In a previous report, the structural analysis of the polysaccharide produced in vivo by one such Tn5 mutant, the 8396, indicated the presence of a new polymer containing glucose and mannose in a molar ratio of 2.0:1.0 instead of glucose, mannose, and glucuronic acid (2.0:2.0:1.0) in xanthan produced by *X. campestris*.[28] A mutant with similar properties has been described in the pioneering work by Betlach et al.,[29] but unfortunately a detailed study was not performed.

In this report, it is shown that this mutant is unable to transfer glucuronic acid residues to the lipid-linked repeating unit, producing truncated side chains. Nevertheless, the trisaccharide repeating unit is polymerized into a high molecular-weight polysaccharide, with a substituted cellulose-like structure, that is released into the growth medium, although the amount of polymer is much less than in the wild type (1–2% glucose equivalents). It is also demonstrated that the mutation lies in the *gumK* gene.

2. Results

Incorporation of the different polymer components using permeabilized cells from the wild-type and mutant strains.—The results presented with the wild-type 8004 strain are similar to those obtained in previous studies with the B-1459 strain.[10] Incorporation of radioactivity into the polymer was observed from all the precursors tested and the expected molar ratios of the different components of the EPS (2:2:1:1 D-glucose–D-mannose–D-glucuronic acid–acetate), were approximately observed (Table 1).

The results obtained with the mutant 8396 differ from those of the wild type in several aspects. On the

Table 1
Incorporation of radioactivity into organic extracts and polysaccharides (EPS) using wild type (8004) and mutant (8396) *X. campestris* pv *campestris* EDTA-treated cells, in the presence of the three sugar donors, one of them labeled as indicated

Radioactive precursor	Strain							
	Wild type				Mutant			
	Organic extracts		EPS		Organic extracts		EPS	
	cpm mg^{-1} protein		(pmol mg^{-1} protein)					
UDP-[^{14}C]GlcA	54,000	(87.40)	28,200	(45.60)	2000	(3.20)	<5	
UDP-[^{14}C]Glc	52,000	(84.00)	43,400	(70.00)	97,013	(156.00)	2500	(4.04)
GDP-[^{14}C]Man	62,000	(125.00)	48,000	(97.00)	63,020	(128.00)	1540	(3.12)
[^{14}C]acetyl-CoA	5400	(47.00)	4378	(38.60)	7735	(68.00)	265	(2.00)
UDP-[^{14}C]Glc UDP-GlcA omitted	82,000	(132.00)	2825	(4.60)				
GDP-[^{14}C]Man UDP-GlcA omitted	54,000	(109.0)	1600	(3.24)				

The amounts of the synthesized polysaccharide were calculated from BioGel A 5m gel-filtration data (as for the 8396 mutant in Fig. 6). Numbers in brackets indicate pmol mg^{-1} protein assuming that the specific radioactivity of the different incorporated sugars is that of the respective sugar nucleotide precursor.

one hand, the incorporation of D-[^{14}C]-glucose and D-[^{14}C]-mannose into the organic extract are high but in the polymer fraction are 20–30 times lower than the wild-type strain. On the other hand, the incorporation of D-[^{14}C]-glucuronic acid into the organic extract is low (about 25 times less than for the wild type) and practically absent in the polymer fraction (Table 1).

It should be taken into account that the enzyme preparation used, EDTA treated cells, contains undetermined amounts of water-soluble precursors, as well as partially assembled lipid-linked intermediates. For this reason, the figures shown in brackets may underestimate the true activity, since the specific radioactivity of the precursor could be smaller than that used for the calculation (Table 1).

Analysis of the organic extract obtained from the 8396 mutant.—The labeled products, from the 8396 mutant strain, were analyzed in several ways. Upon paper electrophoresis with the untreated organic extracts, all of the radioactivity, independently of the label, remained at the origin of the run. This suggested that the labeled materials in these fractions were lipid-associated and not water-soluble (Fig. 2(I-A, II-A, III-A and IV-A)).

Most of the radioactivity released from the lipid, after mild-acid hydrolysis, migrated as neutral material. A small amount of a compound $R_{UMP} = 0.7$ was also observed (Fig. 2(I-B, II-B and III-B)). Paper chromatography of the neutral material, D-[^{14}C]-glucose and D-[^{14}C]-mannose labeled, showed mainly a compound with the mobility of maltotriose (Fig. 2(I-C and II-C)). In addition, with the D-[^{14}C]-glucose-labeled material two more compounds were observed: one running like D-glucose and the other with a chromatographic mobility near that of the maltose standard (Fig. 2(IC)). Mild alkaline treatment of this latter compound produced the trisaccharide-like material (data not shown), suggesting it was an acetyl substituted trisaccharide.

In incubations with unlabeled sugar nucleotides and [^{14}C]-acetyl CoA neutral material and traces of the compound with $R_{UMP} = 0.7$ were observed in the mild acid-treated organic extracts (Fig. 2(III-B)). Upon paper chromatography of the neutral material, only the compound with the mobility near that of maltose could be detected, a result that confirmed the observation already made that it was an acetylated derivative (Fig. 2(III-C)). The patterns of the oligosaccharides released from the mutant were clearly different from the wild-type strain. Mainly pentasaccharide and pyruvated pentasaccharide were observed with similar treatment on 8004 or NRRL B-1459 wild-type strains (see arrows in Fig. 2 and Ref. 10).

In incubations performed with UDP-[^{14}C]GlcA and unlabeled UDP-Glc and GDP-Man, the only material obtained by mild-acid hydrolysis ran almost at the position of D-glucuronic acid ($R_{UMP} = 1.1$) upon paper electrophoresis (Fig. 2(IV-B)) and turned out to be D-galacturonic acid when it was submitted to a similar analysis with sodium molybdate buffer (Fig. 2(IV-C)). A lipid-bound galacturonate, was already characterized in the NRRL B-1459 wild-type strain (also detected in the 8004 strain, not shown) of *X. campestris*, and it was unrelated to EPS biosynthesis.[30]

Analysis of the organic extract by DEAE-cellulose column chromatography.—Submitted to DEAE-cellulose column chromatography, both D-[^{14}C]-glucose- and D-[^{14}C]-mannose-labeled organic extracts of wild-type strains (either 8004 or NRRL B-1459) produced a pattern with a main radioactive compound eluting at 0.95–1.0 M and a smaller one in the 1.1–1.15 M ammonium acetate area, which were characterized as the lipid diphosphate derivatives of the pentasaccharide

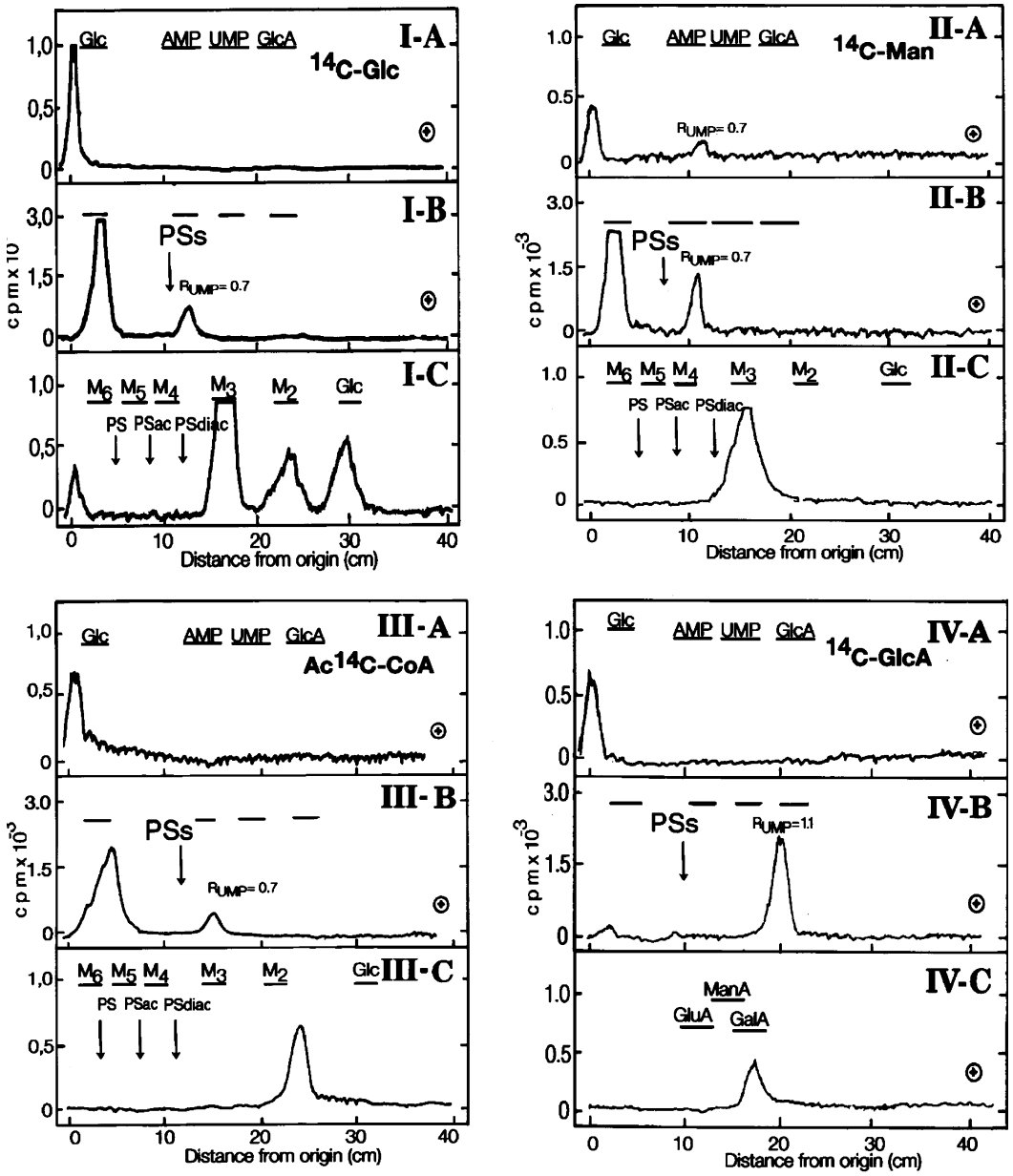

Fig. 2. Analyses of the organic extracts. The 8396 mutant organic extracts, obtained as in Table 1, were analyzed by paper electrophoresis with pyridine acetate buffer either directly (A) or after mild acid treatment (B) I, II, III and IV: extracts [^{14}C]Glc, [^{14}C]Man, [^{14}C]acetyl and [^{14}C]GlcA labeled, respectively. The neutral compounds from I-B, II-B and III-B were submitted to paper chromatography with the 2-propanol–AcOH–water solvent: I-C, II-C and III-C, respectively. The compound with $R_{UMP} = 1.1$ from IV-B was submitted to paper electrophoresis with sodium molybdate buffer: IV-C ManA. Where indicated AMP and UMP were added as internal standards; glucose (Glc), glucuronic acid (GlcA), and malto-oligosaccharides (M_2–M_6) were run as external standards. The arrows indicate the position of the wild type products obtained after mild-acid hydrolysis of the organic extracts: PSs, mixture of pentasaccharides (non, mono, and, diacetylated); PS, pentasaccharide; PSac, monoacetylated pentasaccharide; PSdiac, diacetylated pentasaccharide.

and of the pyruvated pentasaccharide repeating-units, respectively [not shown[10]].

When the untreated organic extracts from the 8396 mutant, labeled with either D-[^{14}C]-glucose, D-[^{14}C]-mannose, or [^{14}C]acetyl, were analyzed by column chromatography on DEAE-cellulose, the profiles observed were different. For all the labels, a sharp peak was obtained at 0.7 M ammonium acetate (Fig. 3). Paper electrophoresis of pH 2-treated material labeled with D-[^{14}C]-glucose showed that the main compound liberated was neutral. The compound behaved, by paper chromatography, mostly as a trisaccharide, but a small amount of its acetylated derivative was also detected. Similar results were obtained when the D-[^{14}C]-man-

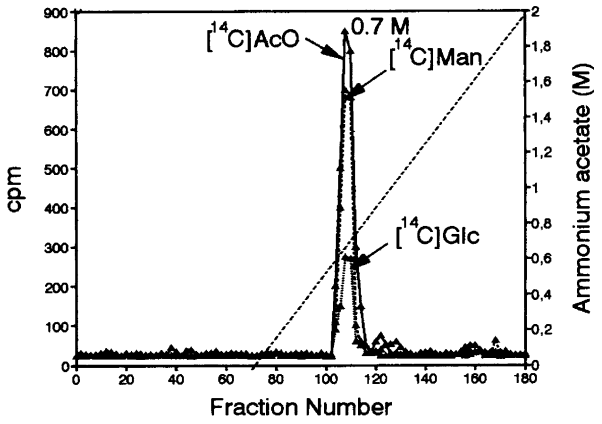

Fig. 3. Column chromatography on DEAE cellulose. The organic extracts labeled with either D-[^{14}C]-Glc (broken line), [^{14}C]acetyl (full line) and D-[^{14}C]-Man (dotted line) obtained from incubations with the 8396 mutant strain as in Table 1 were analyzed. The ammonium acetate gradient was initiated at fraction 70.

nose-labeled material purified by DEAE-celullose (Fig. 3, dotted line) was analyzed (data not shown). Finally, and as expected, mild-acid degradation of the [^{14}C]acetate labeled material from a DEAE column (Fig. 3, full line) produced only the [^{14}C]acetylated derivative, electrophoretically neutral and running near maltose upon paper chromatography (results not shown). In short, with any of the labels, the mutant product behaved as described above for the directly mild acid-treated organic extracts.

In addition, in all of the cases, the above-mentioned compound with $R_{UMP} = 0.7$ was again observed (data not shown). It turned out to be an oligosaccharide cyclic phosphate ester (see below), produced from mishandling of the 0.7 M ammonium acetate-eluted material.[31]

All of these results led to the conclusion that the 8396 mutant EDTA-treated cells failed to synthesize the lipid-linked pentasaccharide intermediate. Instead, a truncated lipid-linked 'trisaccharide' and its acetylated derivative were accumulated.

Structural analysis of the lipid-linked 'trisaccharide'.—As just mentioned, the lipid-linked 'trisaccharide' was eluted at 0.7 M ammonium acetate on DEAE-celullose column chromatography. This high-ionic strength was consistent with the presence of a diphosphate bridge expected for a polyprenol intermediate.[10] To confirm the diphosphate bond, the D-[^{14}C]-mannose-labeled material was submitted to paper chromatography under mild alkaline conditions (EtOH–NH_4OH). In this alkaline solvent, prenyl-diphospho sugars are degraded to the respective oligosaccharide cyclic phosphate esters, provided the hydroxyl group at C-2 and the phosphate bridge are in the cis configuration. Prenyl monophosphate sugars are insensitive to this treatment.[31] Under these chromatographic conditions, a compound with R_f 0.56 was obtained (Fig. 4(A)). This compound migrated faster ($R_{UMP} = 0.7$) than a neutral oligosaccharide upon paper electrophoresis with pyridine–AcOH buffer. It was insensitive to degradation by alkaline phosphatase. However, if it was previously treated with mild acid to open the phosphate ring and then with alkaline phosphatase, the compound became neutral (data not shown). These

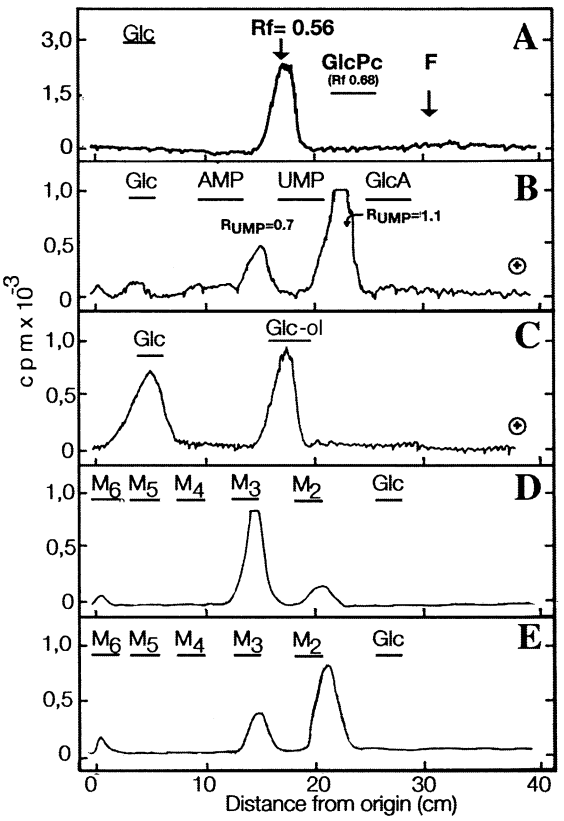

Fig. 4. Characterization of the acetylated trisaccharide-P-P-prenol. Paper chromatography with EtOH–NH_4OH solvent of an organic extract from a standard incubation with 8396 enzyme and GDP-[^{14}C]Man as labeled precursor (A). An aliquot of the same organic extract was submitted to catalytic reduction and the resulting water layer was analyzed by paper electrophoresis with pyridine–AcOH buffer (B). [^{14}C]Glucose-labeled trisaccharide was reduced with sodium borohydride, totally acid-hydrolyzed and the products fractionated by paper electrophoresis with sodium molybdate solvent (C). Two series of incubations were performed with enzymes from the wild type 8004 strain (not shown) and from the 8396 mutant in the presence of UDP-Glc and GDP-[^{14}C]Man (UDP-GlcA was omitted, see Table 1), either in the absence (D) or in the presence (E) of unlabeled acetyl-CoA. The organic extracts were fractionated by paper electrophoresis and the neutral components analyzed by paper chromatography with 2-propanol–AcOH–water solvent. Where indicated, AMP and UMP were added as internal standards and glucose 1,2-cyclic phosphate (GlcPc), glucose (Glc), glucuronic acid (GlcA), glucitol (Glc-ol), and malto-oligosaccharides (M_2–M_6) were run as external standards. The F (arrow) indicates the solvent front.

results indicated that a cyclic phosphoric ester was present in the product of mild-alkaline degradation, which was removed by the combined acid and enzymatic treatments and that a diphosphate bridge was present in the lipid-linked 'trisaccharide' eluted at 0.7 M ammonium acetate.

Catalytic reduction with hydrogen, in the presence of platinum, of the lipid-diphosphate 'trisaccharide' released all the radioactivity as water-soluble material. Two compounds were observed upon paper electrophoresis (Fig. 4(B)). One migrated with $R_{UMP} = 0.7$ (a degradation product, see foregoing), and the main one migrated with $R_{UMP} = 1.1$. This latter compound, treated with alkaline phosphatase, produced a neutral saccharide. These results confirmed that a diphosphate bridge links the 'trisaccharide' to an allylic prenol; this technique breaks the original compound between the lipid and the phosphate residue provided the lipid is an allylic prenol, liberating the diphosphate saccharide.[31,32] Dolichol derivatives are insensitive to this treatment.[31,32]

The saccharide was confirmed to be a trisaccharide by paper chromatography (data not shown) and by gel filtration of the material labeled either with D-[^{14}C]-glucose or with D-[^{14}C]-mannose, through a BioGel P-2 column (Fig. 5(A)). In both cases, the oligosaccharide behaved as the trisaccharide used as standard.

Upon borohydride reduction of the D-[^{14}C]-glucose-labeled trisaccharide and total acid-hydrolysis, a 1:1 relation between glucitol and glucose was obtained (Fig. 4(C)), indicating that a glucose residue was at the reducing end of the trisaccharide and that the other one was internal. Thus, the trisaccharide was assumed to be mannosyl-cellobiose linked to the lipid diphosphate through a glucose residue. This result was confirmed by permethylation of the D-[^{14}C]-glucose-labeled trisaccharide: 2,3,6-tri-*O*-methylglucose and 2,4,6-tri-*O*-methylglucose were detected (not shown).

In summary, incubations with EDTA-treated cells of the 8396 mutant accumulated a mannosyl-cellobiose-diphosphate-α-insaturated-polyprenol, in which the oligosaccharide moiety was linked to phosphate through a glucose residue. This corresponds to the reducing trisaccharide end of the wild-type pentasaccharide repeating-unit (Fig. 1).

Complementary enzymatic studies.—To investigate whether the trisaccharide could enzymatically accept more than one acetyl residue, incubations were performed with each label, in the presence of excess acetyl-CoA donor. A significant increase was observed in the acetylated derivative (Fig. 4(E)) as compared with those products formed upon incubation without acetyl-CoA (Fig. 4(D)).

In order to mimic the failure of the mutant to incorporate glucuronic acid on the repeating unit, permeated cells of wild-type strains were incubated omitting UDP-GlcA as donor. As shown in Table 1 for the 8004 strain, incubations performed either with UDP-[^{14}C]Glc or with GDP-[^{14}C]Man in the absence of unlabeled UDP-GlcA, incorporated radioactivity into the organic extracts and in similar amounts to those observed with the 8396 mutant in the presence of unlabeled UDP-GlcA. Identical results were observed with the wild-type B-1459 strain (not shown). The elec-

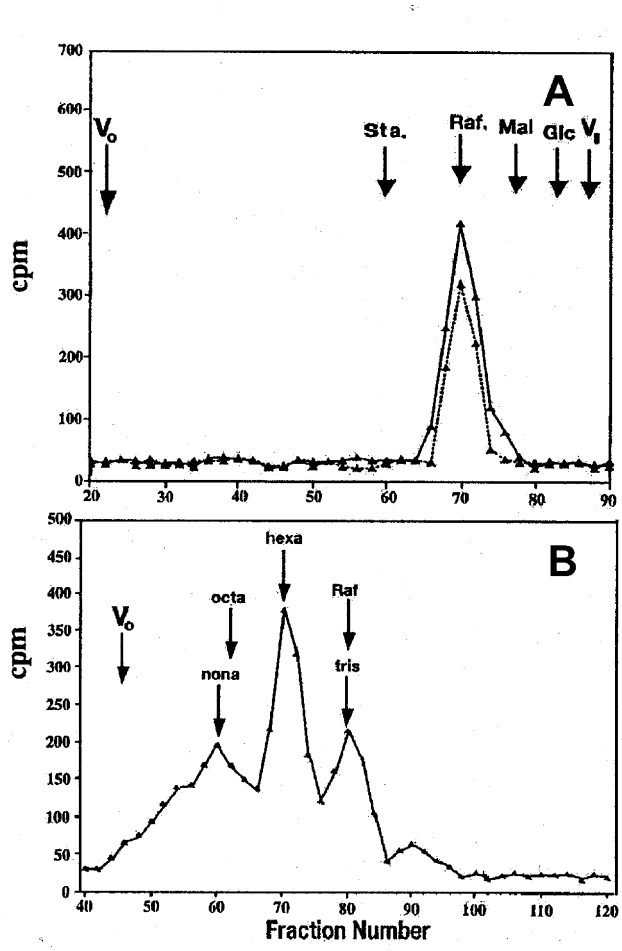

Fig. 5. BioGel P-2 gel filtration. (A) Gel filtration of the 'trisaccharide': The compounds running as maltotriose (M_3) upon paper chromatography with 2-propanol–AcOH–water solvent labeled with either [^{14}C]glucose (full line) (13,000 cpm) or [^{14}C]mannose (dotted line) (10,000 cpm) (Fig. 2(I-C and II-C, respectively)) were filtered through a BioGel P-2 column. Glucose (Glc), maltose (Mal), raffinose (Raf) and stachyose (Sta) were added as internal standards of mono-, di-, tri- and tetrasaccharides and located by the phenol–sulfuric acid method.[33] Blue dextran (Vo) and $CoCl_2$ (Vi) were added as total-exclusion and total-inclusion indicators, respectively. (B) Oligosaccharide gel filtration: The radioactive material obtained in the included volume of a BioGel A-5m from the second-step incubation experiment (Table 2) was submitted to gel filtration through a BioGel P-2 column as above. Raffinose (Raf) was used as a trisaccharide and the repeating unit of succinoglycan (octa) as octasaccharide standards, respectively. Blue dextran (Vo) and Cl_2Co (Vi) were used as above.

trophoretic patterns of the mild acid-treated organic extracts obtained with the wild-type strains in the absence of UDP-GlcA were identical to those observed with 8396 mutant. A small amount of pentasaccharide and of its pyruvated derivative were the only differences detected (not shown). Pentasaccharide formation and pyruvic substitution in these strains was probably due to the presence of endogenous UDP-GlcA and enolpyruvate phosphate, the pyruvate-ketal donor.[8] Furthermore, the chromatographic profiles obtained with mild acid-treated organic extracts were identical to those observed with the mutant. Under these conditions, excess acetyl-CoA in the incubation mixture only increased the amount of the acetylated trisaccharide both for the mutant (Fig. 4(D and E), already mentioned) and for the wild type (not shown). These results indicated that in the absence of the second (and terminal) mannose, only one acetyl residue is transferred.

Polymer formation.—Polymer formation both in vivo and in vitro was investigated. The culture supernatants of 8004 and 8396 strains contained large amounts of polymer, being much higher than the amounts obtained from the wild type. Paper electrophoresis and chromatographic analysis of these materials after total acid-hydrolysis showed D-glucose, D-mannose, and D-glucuronic acid for the wild type but only D-glucose and D-mannose for the mutant 8396 polymer (data not shown). A detailed analysis of the polymer produced in vivo by the mutant strain has been performed and showed that D-glucose and D-mannose are in a 2:1 molar ratio and that acetyl groups are present.[28]

With permeated cells from both strains, the formation of polysaccharide in vitro was also investigated. The incubation supernatant was fractionated on a column of BioGel A-5m to separate the different components. For all the labeled precursors, a high molecular-weight compound was seen when the 8004 enzyme preparation was utilized (not shown).

Supernatants from standard incubations with the mutant strain also produced clearly detectable polymer, eluting at the same position as xanthan when D-[^{14}C]-glucose-, D-[^{14}C]-mannose- or [^{14}C]-acetate-labeled material was analyzed (Fig. 6(A, B and D)), but this polymer was not observed when UDP-[^{14}C]GlcA was used as labeled precursor (Fig. 6(C)).

The amount of radioactivity present in the polysaccharide fraction was much smaller than with the wild-type strain, but again equivalent for the three markers incorporated (Table 1). As expected, the mutant polymer was neutral: it was not retained by a mixed bed Amberlite MB3 column, as was the wild-type xanthan (data not shown).

To mimic the mutant lesion, the supernatant obtained from incubations with the wild-type enzyme,

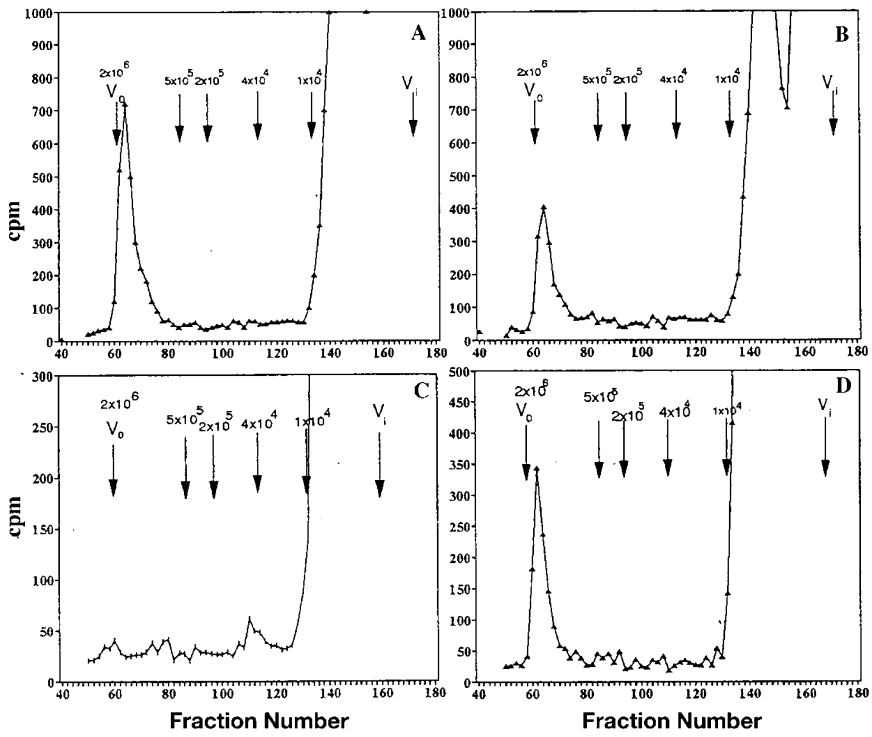

Fig. 6. Gel filtration of in vitro synthesized polymer. The incubations were performed and processed as in Table 1 using either UDP-[^{14}C]Glc (A), GDP-[^{14}C]Man (B), UDP-[^{14}C]GlcA (C), and [^{14}C]-acetyl-CoA (D) as the labeled donor, and enzymes from the 8396 mutant. The respective aqueous incubation supernatants were filtered through a BioGel A-5m column. Autoclaved *Rhizobium meliloti* cells (Vo) and $CoCl_2$ (Vi) were used as exclusion and inclusion volume indicators, respectively. The numbers on top of the arrows indicate the approximate M_W of dextrans standards running in that position, respectively.

performed in the absence of UDP-GlcA was analyzed by gel filtration as before (not shown). With either D-[^{14}C]Man or D-[^{14}C]Glc label, a smaller amount of polymer was formed, similar to that obtained with the mutant (Table 1). These results indicated that a neutral polymer, lacking glucuronic acid, could be synthesized in vitro both by enzymes from the mutant under the standard conditions, and from the wild type when the sugar donor UDP-GlcA was omitted. It was concluded that in both cases, a 'polytrisaccharide', instead of a polypentasaccharide, was formed, although less efficiently.

Two step incubations.—To check that the trisaccharide diphosphate polyprenol was the precursor of the neutral polysaccharide, two-step incubation experiments were performed. In the first incubation D-[^{14}C]-mannose-labeled trisaccharide diphosphate polyprenol was accumulated. After washing off the excess of sugar nucleotide donors and the polymer formed (2.75 pmol of [^{14}C]mannose mg^{-1} protein, Table 2), the incubation was continued without additions for 30 min at 20 °C: a similar amount of polymer was formed again (2.88 pmol of D-[^{14}C]-mannose mg^{-1} protein, Table 2). Of the initial radioactivity associated to the trisaccharide-P–P-polyprenol (118.6 pmol mg^{-1} protein) formed in the first step 41% was detected in the supernatant of the second step, but most of the label (38%) was included in a BioGel A-5m column (Table 2). This material turned out to be a mixture of one-, two-, and three-linked trisaccharide repeating-units (tri-, hexa-, and nonasaccharides Fig. 5(B)), released during the second incubation, probably due to an incomplete polymerization process.

This assay showed that, in spite of the fact that the polyprenol diphosphate trisaccharide was not a good substrate for the polymerizing enzyme(s), a certain amount was used as precursor for the synthesis of a polymer, most likely a 'polytrisaccharide'.

Location of the transposon.—It was hypothesized by Capage et al.[34] that *gumK*, in the 3.4 kb *Bam*HI fragment (Fig. 7(A)), coded for the glucosyluronic transferase or transferase IV. Therefore the Tn5 insertion might be expected to be in *gumK*. This possibility was investigated through Southern blot experiments. Two probes were employed, gumK (Fig. 7(C, panel I)) and Tn5p. The gumK (gumKp) probe consisted of the whole *gumK* gene (about 900 bp) (Fig. 7 (legend)) and the other probe (Tn5p) was a fragment of the Tn5 transposon (500 bp) (Fig. 7(B)). Both wild-type (Fig. 7(C, line 1, legend)) and mutant genomic DNA (Fig. 7(C, line 2)) digested with BamHI were hybridized with the gumK probe (Fig. 7(C, panel I)) and with the Tn5 probe (Fig. 7(C, panel II)). With the wild-type DNA, using the gumK probe, only a 3400 bp fragment was seen, as expected (Fig. 7(C, panel I, lane I)). The same probe, with the mutant DNA (the Tn5 has one BamHI restriction site, Fig. 7(B)), detected two bands (Fig. 7(C, panel I, lane 2)). One of 5750 bp and another of 3400 bp indicating that the BamHI site at the transposon (Fig. 7(B)) had generated two fragments with *gumK* material. It was therefore concluded that Tn5 had inserted in the *gumK* gene. With the Tn5 probe (Fig. 7(C, panel II)) no bands were detected with the wild-type run, as expected (Fig. 7(C, panel II, lane 1)) and only the 5750 bp band with the mutant DNA (Fig. 7(C, panel II, lane 2)). It should be taken into account that the Tn5 probe (Tn5p) was a fragment that did not contain the BamHI site (Fig. 7(B)).

To locate the Tn5 insertion point, three fragments of different size were obtained by PCR using a combination of several Tn5 and *gumK* primers. Since both sequences, *gumK* and Tn5, were known [34 and accession number U00004, respectively], the precise location of the Tn5 insertion was determined by DNA sequencing of one of these PCR products (T IVa + Tn5a) (Fig. 7(A and B)). The exact insertion position of the Tn5 transposon, was at 240 pb of *gumK* gene (Fig. 7(D)).

Strain 8396 was complemented by recombinant plasmid pIZD15-261 which contains the whole *gum* cluster, *gumK* gene included. The wild-type slime phenotype was recovered by the 8396/pIZD15-261 strain and from its culture supernatants, large amounts of polymer could be isolated. Total acid-hydrolysis of this material produced D-glucose, D-mannose, and D-glucuronic acid, as with wild-type xanthan (not shown).

Table 2
Two-step incubation of EDTA-treated 8396 cells with radioactive precursors

Incorporated [^{14}C]Man (pmol mg^{-1} protein)				
First step		Second step		
Organic extract	EPS	Organic extract	Included volume	Excluded volume (EPS)
118.60	2.75	69.70	45.80	2.88

For details see text.

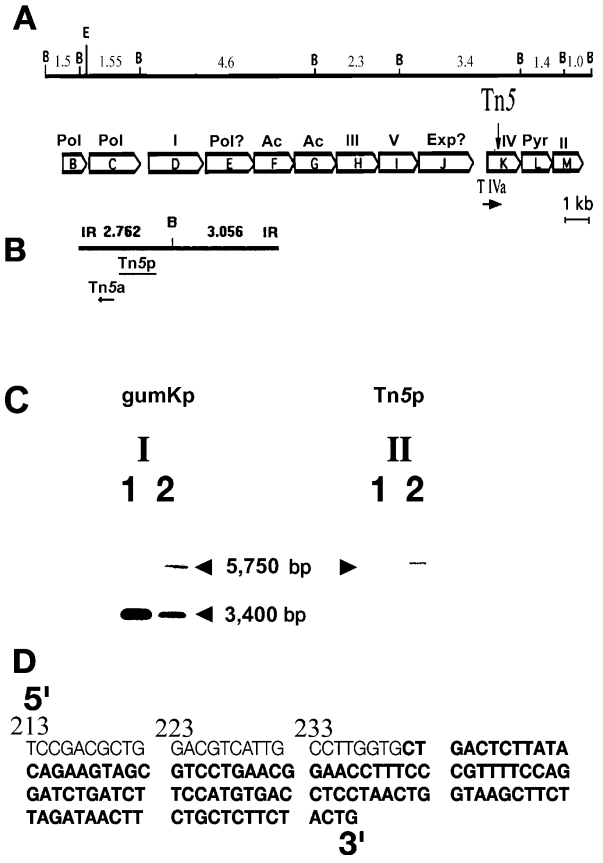

Fig. 7. Location of the Tn5 transposon. (A) Organization of the gene cluster responsible for xanthan *gum* biosynthesis: B indicates BamHI restriction sites and numbers the fragment sizes, in kb. TheTn5 transposon insertion in *gumK* and the position of TIVa primer are indicated. GumKp = *gumK*. The letters show the different open reading frames and the proposed gene functions: Pol, polymerization; Exp, export; Pyr, ketal pyruvation; roman numbers (I–V) indicate the different glycosyltransferases. (B) Tn5 transposon: B indicates the BamHI restriction site and the numbers the fragment sizes, in bp; Tn5p shows the position of the [^{32}P]labeled Tn5 probe and Tn5a the primer used to obtain the PCR fragment sequenced. (C) Southern blot experiment: Both wt 8004 (panel I lane 1 and panel II lane 1) and mutant 8396 (panel I lane 2 and panel II lane 2) DNA were digested with BamHI and the fragments separated through 0.7% agarose gel electrophoresis, transferred to a nitrocellulose filter and hybridized using the whole *gumK* gene as probe (gumKp) (panel I) and a fragment of the Tn5 (panel II Tn5p) as ^{32}P labeled probes. Both probes were obtained by PCR amplification using primers and conditions described in Section 4. (D) The Tn5 insertion site: The sequence corresponding to *gumK* gene is indicated with light letters and that of the Tn5 with bold letters.

3. Discussion

Biosynthesis of xanthan gum involves the formation of a prenol diphosphate pentasaccharide and the polymerization of its saccharide moiety. Nonglycosidic substituents are added at the lipid-linked intermediate stage. This process initially studied with EDTA-treated cells from *X. campestris* pv *campestris*, strain NRRL B-1459[6,8–10] has now been extended to another wild-type strain, 8004, and to its derivative 8396. In all the aspects analyzed, strain 8004 behaved as the B-1459 wild-type strain. In contrast, the mutant strain failed to produce the pentasaccharide and only three sugars were transferred to the prenyl phosphate acceptor. Acetyl residues, produced by both endogenous and exogenous acetyl CoA, were also incorporated to some extent (Figs. 2 and 3).

Several lines of evidence suggest that in the 8396 mutant the lipid-associated saccharide was the trisaccharide reducing end of the pentasaccharide (Figs. 1 and 4). It was built from UDP-Glc and GDP-Man (Table 1), glucose was at the reducing end, that was linked to an allylic prenol through a diphosphate bridge, as shown by borohydride reduction (Fig. 4(C)), catalytic hydrogenation (Fig. 4(B)), and sensitivity to mild alkali (Fig. 4(A)).

The trisaccharide could also be obtained partially acetylated (Fig. 2(I-C and II-C)), but even in the presence of excess acetyl-CoA only one acetylated species was observed (Fig. 4(D and E)). With the wild-type strains, two acetyl groups were incorporated (Fig. 2(I-C, II-C and IV-C arrows)).[35]

The polymerization process was clearly more efficient with the wild-type strain than with the mutant (Table 1). The possibility that this impairment was due to partial damage of the polymerizing enzyme(s) (Table 1) was ruled out by the experiments performed with the wild-type preparation in the absence of UDP-GlcA: the amounts and nature of the products obtained were identical to those obtained with the mutant (Table 1 and results not shown). It was concluded that the low yield obtained were a consequence of the high specificity of the polymerisation system, which did not recognize the trisaccharide as good a substrate as is the wild-type pentasaccharide. In the polymer obtained from the mutant, both in vivo (not shown) and in vitro (Fig. 6), only D-glucose and D-mannose were detected and in the latter, a 2:1 ratio of glucose–mannose was found, indicating that a 'polytrisaccharide' polymer was produced. No [^{14}C]glucuronic acid was incorporated into the polymers (Fig. 6(C)). Taking into consideration the well-established structure of xanthan,[2,10] it is proposed that the structure of this 'polytrisaccharide' is that of a mannosylated cellulose (water soluble) (Fig. 1). Some results indicated that about 25% of the repeating units are acetylated (not shown). Recently, the structure of the in vivo produced polytrisaccharide was determined by methylation and nuclear magnetic resonance analyses, confirming a cellulosic backbone with (1→4) β-glucose linkages and an α-mannose residue (1→3)-linked to every two glucoses.[28]

It has been reported that the genes that code for the enzymes involved in the transfer of the sugars and of

the nonglycosidic substituents are located in a cluster.[12,13,17] This cluster was sequenced[34] and a preliminary study of xanthan-deficient mutants predicted the presence of 12 open reading frames (*gumB–gumM*) associated to xanthan *gum* biosynthesis.[18] Unfortunately this excellent work was only presented as brief communications to meetings, and detailed data has not been available.

Recently some other gene functions and promoters of this gene cluster have been characterized,[19,23] as well as genes involved in the synthesis of the sugar nucleotide precursors of xanthan.[7]

In this report it is established clearly that the insertion point of the Tn5 transposon is within the *gumK* gene (240 bp from the ATG site, Fig. 7(D)). It should be mentioned that this insertion in the *gumK* gene, that completely blocks the entrance of GlcA does not affect the incorporation of the second glucose by an enzyme supposedly coded by *gumM*, to the right of the *gumK* gene (Fig. 7(A)), implying that a different promoter controls it.

Finally, the fact that truncated repeating units are able to polymerize to form an 'acetylated polytrisaccharide' opens the way to obtain new polymers with interesting rheological properties.

4. Experimental

Strains and microbiological techniques.—*X. campestris* NRRL B-1459 and 8004 (wild types) and a 8396 (a 8004::Tn5 mutant)[12] were grown as described by Cadmus et al.[36] The *Escherichia coli* strain used in transformation was DH5α[37] and was grown in Luria–Bertani (LB) medium.[38] When necessary, appropriate antibiotics were added: kanamycin (50 μg mL^{-1}), rifampicin (50 μg mL^{-1}) and ampicillin (200 μg mL^{-1}).

DNA manipulations.—Preparations of plasmid DNA from *E. coli*, agarose gel-electrophoresis, cloning procedures and Southern hybridization were carried out following established protocols.[38] Total DNA from *X. campestris* was isolated according to Hull et al.[39] and *X. campestris* plasmid DNA was isolated as reported previously.[13]

Preparation of ^{32}P labeled probes.—The sequence of the primers used to amplificate the *gumK* gene was taken from the 16 kb xanthan *gum* cluster sequence of *X. campestris* determined by Capage et al.[34]

Sequences of the Tn5 primers were obtained from data published (accession number U00004) The sequences of the primers used were the following (kindly made by F. Volpatti, ICGEB, Trieste, Italy).

To amplify *gumK* genes:

5′-CTGCAGCGCCATGTTCCGCTGGTATG-3′, and 5′-GAACAATGCGCGACGTTC-3′

To amplify a fragment of Tn5:

5′-CCGTGTTCCGGCTGTCA-3′, and 5′-GGCCACAGTCGATGAAT-3′

The probes were obtained by PCR and ^{32}P labeled with an oligolabeling kit (Pharmacia) following the instructions of the manufacturers.

DNA sequencing.—A fragment of about 1.5 kb resulting from PCR with T IVa (5′-GGGCTGAGCA-3′) and Tn5a (5′-GGAAGTCAGATCCTGGTT-3′) primers, was cloned in pUC18 and sequenced with a Sequencing kit (Pharmacia).

Chemicals.—UDP-[^{14}C]Glc (300 Ci mol^{-1}), UDP-[^{14}C]GlcA (300 Ci mol^{-1}), and GDP-[^{14}C]Man (238.9 Ci mol^{-1}) were prepared as described previously.[31,40] UDP-D-Glc, GDP-D-Man, UDP-D-GlcA, and AcCoA were purchased from Sigma Chemical. The octasaccharide repeating-unit of succinoglycan was obtained from in vitro incubations with EDTA-treated *Agrobacterium radiobacter* cells.[41] Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs. Enzymes were used under the conditions specified by the suppliers.

Enzyme preparations.—Cells from *X. campestris* NRRL B-1459, 8004, and 8396 were grown as reported[35] and harvested by centrifugation in late exponential phase. The enzyme preparation (EDTA-treated cells) consisted of the cell pellet resuspended in 0.01 M EDTA–Tris buffer (pH 8.2), frozen and thawed several times.[6]

Assay procedure.—*(i) One-step incubations.* The standard incubation mixture contained 70 mM Tris–HCl buffer (pH 8.2), 5 mM $MgCl_2$, EDTA treated cells (about 0.6–0.8 mg of protein), and UDP-[^{14}C]Glc (17 μM) or UDP-[^{14}C]GlcA (17 μM) or GDP-[^{14}C]Man (17μM), as indicated in each case. The unlabeled sugar nucleotides, UDP-Glc, UDP-GlcA, and GDP-Man (357 μM each) were added where indicated. The reactions were performed in a total volume of 70 μL for 30 min at 20 °C and were stopped by adding 0.5 mL of 70 mM Tris–HCl buffer (pH 8.2) containing 30 mM EDTA. The mixtures were centrifuged in a microcentrifuge at 14,000 rpm for 2 min, and the pellets were resuspended and washed twice with Tris–HCl buffer without EDTA. The combined supernatants were hydrolyzed at pH 2 to decompose the excess of sugar nucleotide donors, and dialyzed twice against 70 mM Tris–HCl (2 L) for 2 h and then overnight, against water. The dialyzed samples were concentrated under diminished pressure using a Rotavapor Buchi RE 120 to determine polysaccharide formation by gel filtration. Material at the exclusion area was considered exopolysaccharide.

The washed cell pellets were then extracted three times (0.2 mL each) with 1:2:0.3 (v/v) $CHCl_3$–MeOH–water (organic solvent).[6] This extract, which contains

the polyprenol-linked [^{14}C]oligosaccharides, is referred to as organic extract. Aliquots were counted for radioactivity.

(ii) Two-step incubations. The first step was a standard incubation scaled up two to three times. The incubation mixtures were processed as described above, and the supernatants, containing the excess sugar nucleotides and the possible polysaccharides formed, were analyzed as before. For the second incubation, the washed cell pellets were resuspended in the original volume of 70 mM Tris–HCl buffer (pH 8.2) containing 8 mM $MgCl_2$ (but not sugar nucleotides donors), aliquots were taken for analysis, and the reminder were reincubated for 30 min at 20 °C. The reactions were stopped with EDTA and processed as described for the standard assay. In this case, for the combined supernatants the dialysis and hydrolysis steps were omitted.

Chemical treatments.—Mild-acid hydrolysis, to break the sugar–phosphate linkage of prenyl-phosphosugars, was carried out at pH 2 (0.01 M HCl) and 100 °C for 10 min.[42] Cyclic phosphoric esters were opened and diphosphoric esters were cleaved by treatment at pH 1 (0.1 M HCl) and 100 °C for 10 min. Total hydrolysis was performed with 1 M HCl at 100 °C for 16 h in sealed tubes.

Trisaccharide permethylation was performed by the Hakomori method and the products detected by TLC as in previous work.[33]

Chromatography and electrophoresis.—Paper chromatography and electrophoresis techniques, as well as the location procedures for the different compounds, were as described previously.[31,33] The following solvents were used: 7:3 EtOH (96%)–concd NH_4OH; 27:4:9 2-propanol–AcOH–water; 1:0.04:9 pyridine–AcOH–water); pH 6.5; sodium molybdate (0.1 M), pH 5.0.

Gel filtration through columns of BioGel P-2 (200–400 mesh) (107×0.8 or 1.0 cm), and BioGel A5m (110×1.2 cm) utilized 0.1 M pyridinium–AcOH buffer, pH 5.0, collecting fractions of 0.5 mL at a rate of 0.25 mL min^{-1}. The radioactivity was determined with 0.1-mL aliquots. The DEAE-cellulose column chromatography (1×60 cm) was performed in 99% MeOH eluted with a linear gradient of NH_4OAc in 99% MeOH (0–2 M) in a total volume of 400 mL, as reported previously.[31] Aliquots (0.25 mL) of each fraction (3 mL) were counted for radioactivity.

Enzymatic treatments.—Treatments with alkaline phosphatase (from *E. coli*, Sigma Chemical) were performed as previously described.[31] Reactions were ended by adding 1 volume of EtOH.

Radioactivity measurement.—Aliquots of the different fractions of incubations or columns were measured in Bray's solution with a 1214 Rackbeta–Wallac liquid scintillation counter. The radioactivity from paper electrophoresis or paper chromatography was detected with a Packard radiochromatogram scanner, model 7201.

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