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Genetic and Physical Analyses of a Cluster of Genes Essential for Xanthan Gum Biosynthesis in *Xanthomonas campestris*

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Xanthomonas campestris **produces copious amounts of a complex exopolysaccharide, xanthan gum. Nonmucoid mutants, defective in synthesis of xanthan polysaccharide, were isolated after nitrosoguanidine mutagenesis. To isolate genes essential for xanthan polysaccharide synthesis (*xps*), a genomic library of *X. campestris* DNA, partially digested with *SalI* and ligated into the broad-host-range cloning vector pRK293, was constructed in *Escherichia coli*. The pooled clone bank was conjugated en masse from *E. coli* into three nonmucoid mutants by using pRK2013, which provides plasmid transfer functions. Kanamycin-resistant exconjugants were then screened for the ability to form mucoid colonies. Analysis of plasmids from several mucoid exconjugants indicated that overlapping segments of DNA had been cloned. These plasmids were tested for complementation of eight additional nonmucoid mutants. A 22-kilobase (kb) region of DNA was defined physically by restriction enzyme analysis and genetically by ability to restore mucoid phenotype to 10 of the 11 nonmucoid mutants tested. This region was further defined by subcloning and by transposon mutagenesis with mini-Mu(Tet^r), with subsequent analysis of genetic complementation of nonmucoid mutants. A region of 13.5 kb of DNA was determined to contain at least five complementation groups. The effect of plasmids containing cloned *xps* genes on xanthan gum synthesis was evaluated. One plasmid, pCHC3, containing a 12.4-kb insert and at least four linked xanthan biosynthetic genes, increased the production of xanthan gum by 10% and increased the extent of pyruvylation of the xanthan side chains by about 45%. This indicates that a gene affecting pyruvylation of xanthan gum is linked to this cluster of *xps* genes.**

Xanthan gum is an extracellular polysaccharide produced by the gram-negative bacterium *Xanthomonas campestris*. This biopolymer has unique rheological properties resulting from its high viscosity, pseudoplastic behavior (reversible decrease in viscosity with shear rate increases), and tolerance to a wide range of temperatures, pHs, and salt concentrations (19, 21). It is therefore used in a variety of food and industrial applications as a viscosifying, thickening, stabilizing, or suspending agent (30).

The chemical structure of xanthan gum has been extensively studied and shown to consist of a cellulosic (1→4)-β-D-glucose backbone with trisaccharide side chains composed of two mannose residues and one glucuronic acid residue attached to alternate glucose residues in the backbone (18, 22, 24) (Fig. 1).

The internal mannose residues are acetylated at the O-6 position. Approximately one-third to one-half of the terminal mannose residues may be substituted with a pyruvate ketal at positions 4 and 6. However, the extent of acetylation and particularly pyruvylation can vary considerably, depending on fermentation conditions or strain variants (3, 18, 31, 37). The actual distribution pattern of the pyruvate groups is unknown.

Detailed in vitro studies of the biosynthesis of xanthan gum by Ielpi and co-workers (15–17) have shown that the pentasaccharide subunit is first synthesized, attached to a lipid carrier (prenyl diphosphate), in a sequential manner from the precursors UDP-glucose, GDP-mannose, UDP-glucuronic acid, acetyl coenzyme A, and phosphoenolpyruvate. The pentasaccharide units are subsequently polymerized into a high-molecular-weight polysaccharide and released from the cell.

Thus, the biosynthesis of xanthan gum is a complex and precise process which should require a multitude of enzymes and regulatory controls (35, 36). Essentially nothing is known about the structure, organization, functions, or regulation of the genes involved in xanthan gum synthesis. Several mutants altered in xanthan gum production have been isolated. Whitfield et al. (40) described nonmucoid mutants of NRRL B-1459 which form smaller colonies than do the wild type, lack the characteristic glossy mucoid appearance, and produce trace amounts of normal xanthan polysaccharide, and crenated mutants with unusual colonial morphology which produce a polysaccharide containing sugars normally found in lipopolysaccharide. Sutton and Williams (38) investigated a mutant of an *X. campestris* strain isolated from cabbage plants, which produces smooth, compact colonies on agar, is weakly virulent on cabbage, and does not produce exopolysaccharide in culture. With *X. campestris* NRRL B-1459, under certain culture conditions, variants develop which produce smaller-than-normal mucoid colonies. These have been found to produce about 30% less xanthan gum than do the normal large mucoid colonies, with the same basic structure but with reduced amounts of acetate and pyruvate and lower intrinsic viscosity (3, 31). To better understand the biosynthesis and control of xanthan

→4)-β-D-mannose-(1→4)-β-D-glucose-(1→

β-D-mannose-(1→4)-β-D-glucuronic acid-(1→2)-α-D-mannose-(1→3)

FIG. 1. Trisaccharide side chain of xantham gum attached to the cellulosic (1→4)-β-D-glucose backbone.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
JZ279	*recA56 lacY galK2 galT22 metB1 trpR55 supE44 supF58 hsdR514*	14; J. Zyskind
3051	MC4100::mini-Mu(Tet^r) (P1 *clr100*Cm)	2; B. Belas
3054	M8820 with Mu	B. Belas
X. campestris		
0543	Wild type	NRRL B-1459
0100	Stable Xps$^+$ mutant from 0543	This study
2444	0100 Rif^r *xps-108*	This study
2445	0100 Rif^r *xps-109*	This study
2446	0100 Rif^r *xps-110*	This study
2447	0100 Rif^r *xps-111*	This study
2448	0100 Rif^r *xps-112*	This study
2449	0100 Rif^r *xps-113*	This study
2450	0100 Rif^r *xps-114*	This study
2451	0100 Rif^r *xps-115*	This study
2452	0100 Rif^r *xps-116*	This study
2453	0100 Rif^r *xps-117*	This study
2895	0100 Rif^r *xps-261*	This study
2896	N1 Rif^r *xps-262*	I. Sutherland
ATCC 31313	Pyruvate-free xanthan gum	32; American Type Culture Collection
Plasmids		
pRK293	Tc^r Km^r Mob$^+$ Tra$^-$	10; D. Helinski
pRK2013	ColE1 Mob$^+$ Tra$^+$ (RK2) Km^r	11; D. Helinski

[a] Abbreviations: Km, kanamycin; Tc, tetracycline; Rif, rifampin; Tra$^+$, self-transmissible; Mob$^+$, mobilizable for transfer.

gum production, we have initiated a study of the genes essential for this process.

In this paper, we describe the cloning of several of the genes essential for xanthan gum synthesis, as defined by their ability to rescue mutants deficient in xanthan gum production, and show that some of these genes are clustered in the *X. campestris* genome. We also show that a plasmid containing a portion of this gene cluster elicits enhanced xanthan gum production and increased pyruvylation.

(A preliminary report of this work has been presented [N. E. Harding, J. M. Cleary, D. K. Cabañas, I. G. Rosen, and K. S. Kang, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, O-64, p. 272].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. Strain 0100 is a stable xanthan gum-producing strain derived from the naturally occurring *X. campestris* NRRL B-1459 (19). The xanthan gum-deficient mutants were derived from strain 0100 after mutagenesis with nitrosoguanidine (100 to 250 μg/ml for 2 to 5 h). After overnight growth, cultures were diluted and plated as single colonies on YM solid medium with 1% glucose to screen for the appearance of nonmucoid colonies. Spontaneous rifampin-resistant isolates of these mutants were selected.

Media. *Escherichia coli* cells were grown in YT medium of Miller (26). *X. campestris* cells were grown either in YT medium, in YM medium (3) containing yeast extract (3 g/liter; Difco Laboratories), Bacto-Peptone (5 g/liter; Difco), malt extract (3 g/liter; Difco), and glucose (10 g/liter), or in minimal M9 medium (26) with 2 g of glucose per liter. The media were supplemented as needed with kanamycin (50 μg/ml; Calbiochem-Behring), tetracycline (6 μg/ml; Calbiochem-Behring), or rifampin (100 μg/ml; Sigma Chemical Co.). *X. campestris* strains were incubated at 30°C; *E. coli* strains were incubated at either 30 or 37°C.

DNA isolation and biochemistry. *X. campestris* chromosomal DNA was prepared by the procedure of Okita et al. (28), except that 100 mM NaCl–10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA was substituted for 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) buffer. Preparative amounts of plasmid DNA were made by cleared lysis, phenol extraction, and banding in CsCl-ethidium bromide gradients (20). Small amounts of plasmid DNA were isolated by the Triton X-100 lysis method (20) or by a modification (1) of the alkaline denaturation procedure of Currier and Nester (5), but with 1% Triton X-100 instead of sodium dodecyl sulfate.

Restriction endonucleases and ligase were purchased from commercial suppliers (Bethesda Research Laboratories, Inc., and New England BioLabs, Inc.) and used as recommended. Electrophoresis was performed on 0.4 to 0.8% agarose horizontal gels in Tris-borate-EDTA buffer (23).

To prepare partial *Sal*I fragments of *X. campestris* DNA, the chromosomal DNA was prepared as described by Maniatis et al. (23). The DNA digest was fractionated after centrifugation through a 10 to 40% linear sucrose gradient in 100 mM Tris hydrochloride (pH 8.0)–100 mM NaCl–1 mM EDTA, for 16 h at 35,000 rpm in an SW41 rotor. Fractions containing DNA fragments of 10 to 20 kilobases (kb) were combined. A fivefold excess of chromosomal DNA fragments was ligated to pRK293 DNA linearized at the *Sal*I site.

Genetic procedures. *E. coli* JZ279 was transformed with plasmid DNA according to the method of Dagert and Ehrlich (6) except that the cells were used within 2 h instead of after a 24 h incubation at 0°C. For conjugal transfer of pRK293 (10) and its derivatives into *X. campestris*, a helper plasmid pRK2013 in *E. coli* was used to provide transfer functions (11). Matings were performed on solid medium (YT) for 6 to 18 h, with approximately equal volumes of logarithmic-phase *E. coli* cells and overnight cultures of *X. campestris*. The mating mixtures were suspended and plated for single colonies on selective agar. For large numbers of complementation assays, the cultures were mixed in wells of microtiter dishes, spotted onto nonselective (YT) agar for expression, and then transferred to selective agar.

Assays for xanthan gum, carbon source concentration, and pyruvate. *X. campestris* strains were grown for 72 h in 100 ml of complex medium containing hydrolyzed corn syrup as the carbon source and soy protein concentrate (Promosoy 100; Central Soya Co.) and NH_4NO_3 as nitrogen sources, as described by W. H. McNeely in U.S. patent 3,433,708, March, 1969. The yield of xanthan gum was determined gravimetrically after precipitation with 2 volumes of isopropanol and drying. The initial and final concentrations of corn syrup in the fermentation medium, measured as reducing sugar equivalents after hydrolysis with glucoamylase, were determined by the dinitrosalicylic acid assay (25). The amount of pyruvate covalently attached to the polysaccharide was determined by a lactate dehydrogenase enzymatic assay (13) after hydrolysis of xanthan in 0.1 M HCl at 100°C for 4 h.

TABLE 2. Complementation of *xps* mutations by the *X. campestris* gene library

Strain	Mutation	No. of Km^r transconjugants[a]/ no. of recipient (Rif^r) cells[b] (%)	No. of mucoid colonies/no. of Km^r transconjugants (%)
2453	*xps-117*	14.0	0.56
2445	*xps-109*	2.6	2.67
2450	*xps-114*	21.0	0.14

[a] Kanamycin-resistant transconjugants were isolated on YM-rifampin-kanamycin agar.
[b] The number of recipient *X. campestris* cells were determined on YM-rifampin plates.

RESULTS

Isolation of nonmucoid mutants. Mutants nonmucoid by appearance on YM agar plates were isolated after mutagenesis with nitrosoguanidine at a frequency of about 1 in 10^3. The mutants described in this study were morphologically distinct from the mucoid wild type, forming dark-yellow, flat, slightly shiny colonies, smaller than those of the wild type.

Cloning of genes essential for xanthan gum synthesis. A genomic library of *X. campestris* 0100 was constructed by ligating partial *Sal*I fragments, of approximately 10 to 20 kb, into the broad-host-range cloning vector pRK293. This vector, derived from RK2 by G. Ditta and co-workers (10), is 21.4 kb in size and is not self-transmissible, but can be complemented in *trans* for transfer; pRK293 confers tetracycline and kanamycin resistance and contains a number of unique restriction enzyme sites, in particular, a single *Sal*I site in the gene for tetracycline resistance. The ligation mixture was used to transform *E. coli* JZ279 to kanamycin resistance, and the transformants were screened for tetracycline sensitivity. Approximatley 2,500 Km^r Tc^s colonies in *E. coli* were pooled to form the *X. campestris* gene library. Of 28 Km^r Tc^s transconjugants selected at random, 27 contained plasmids larger than pRK293, indicating the presence of insert DNA. Seven of these plasmids were digested with *Sal*I. The insert size ranged from 6.9 to 18.6 kb, with a mean of 14.1 kb.

As a first step in the isolation of genes required for xanthan polysaccharide synthesis (*xps*), the *X. campestris* gene library was mated en masse from *E. coli* into several rifampin-resistant nonmucoid *X. campestris* mutants by conjugation on solid medium. Initially, three nonmucoid mutants (2445 [*xps-109*], 2450 [*xps-114*], and 2453 [*xps-117*] were selected at random as recipients of the gene library. The mating mixtures were suspended, diluted, and plated for single colonies on YM agar containing rifampin and kanamycin to select *X. campestris* exconjugants. Mucoid colonies, in a background of nonmucoid colonies, were isolated (Table 2).

Plasmid DNA was isolated from several mucoid exconjugants from each of the three matings, and the sizes of the *Sal*I inserts were determined (Table 3). For each of the three nonmucoid mutants, the complementing plasmids isolated contained at least one *Sal*I fragment in common. Fragments of the same size were identified in plasmids from matings with different mutants.

These plasmids were transferred into *E. coli* by transformation to kanamycin resistance and then reintroduced into the three nonmucoid mutants above and an additional nine independently isolated nonmucoid mutants (Table 1) by solid-surface matings. In each case, when a plasmid obtained by complementation of a particular Xps mutant was reintroduced from *E. coli* into that mutant, all Km^r transconjugants were mucoid (Xps$^+$). Of the total 12 mutants, 10 were found to be rescued (mucoid phenotype restored) by one or more of these plasmids. Each plasmid complemented several of the *xps* mutations (Fig. 2). Mutant 2451 (*xps-115*) was leaky, giving rise to mucoid colonies at a high frequency, and was therefore not mapped. These results suggested that several of the loci involved in xanthan gum synthesis might be clustered.

Mutant 2447 (*xps-111*) was not complemented by any of these plasmids, but was subsequently determined to be rescued by a plasmid containing a different DNA fragment not adjacent to those shown in Fig. 2, by restriction enzyme mapping (data not shown). Therefore, this mutation is presumably located in a separate region of the *X. campestris* chromosome.

Restriction analysis of plasmids containing cloned *xps* genes. The plasmids obtained from the *X. campestris* gene library by complementation of nonmucoid mutants were digested with *Sal*I, and other restriction enzymes (*Bgl*II, *Bam*HI, *Eco*RI, *Hin*dIII, *Sma*I, *Sst*I, and *Xho*I) as needed, to derive the composite map shown in Fig. 2, and to determine the size and orientation of each of the inserts. Since overlapping segments of DNA were obtained in unique plasmids, we conclude that this DNA is colinear on the *X. campestris* chromosome. *Sal*I fragments present in only one of the plasmids shown here (e.g., the 0.8-kb *Sal*I fragment in pCHC3 and the 5.8- and 1.4-kb *Sal*I fragments in pCHC22) were confirmed to be colinear by their presence in unique plasmids isolated from two other independently constructed *X. campestris* gene banks (data not shown), indicating that these fragments were not joined to the *xps* region by random ligation of noncontiguous fragments.

Subcloning of the *xps* genes. To further define the number and order of the *xps* genes and to correlate specific DNA regions with genetic complementation groups, the cloned DNA was subcloned. A plasmid which complemented the greatest number of Xps mutants (pCHC3) was used as a source of DNA for subcloning with the restriction endonucleases *Sal*I and *Bam*HI. *Sal*I fragments from a complete digest of pCHC3 were subcloned into the *Sal*I site of pRK293, selecting in *E. coli* for Km^r Tc^s transconjugants.

To obtain a variety of insert sizes, pCHC3 was digested

TABLE 3. Characterization of recombinant plasmids containing *xps* DNA

Strain	Mutation	Plasmids	Size of cloned *Sal*I fragments (kb)[a]
2453	*xps-117*	pCHC1, pCHC9, pCHC14, pCHC16	5.4,* 1.65*
		pCHC3	5.4,* 4.5, 1.65,* 0.8
2445	*xps-109*	pCHC23, pCHC30, pCHC35	4.5,* 1.65
		pCHC29	5.4, 4.5,* 1.65, 0.8
		pCHC27	10, 4.5,* 2.0, 0.7
		pCHC22	5.8, 4.5,* 2.0, 1.65, 1.4, 0.7
2450	*xps-114*	pCHC45, pCHC46, pCHC48, pCHC50, pCHC51	5.4,* 4.5,* 1.65,* 0.8*

[a] *Sal*I fragments marked by asterisks are those in common in all the plasmids obtained by complementation of a particular *xps* mutation.

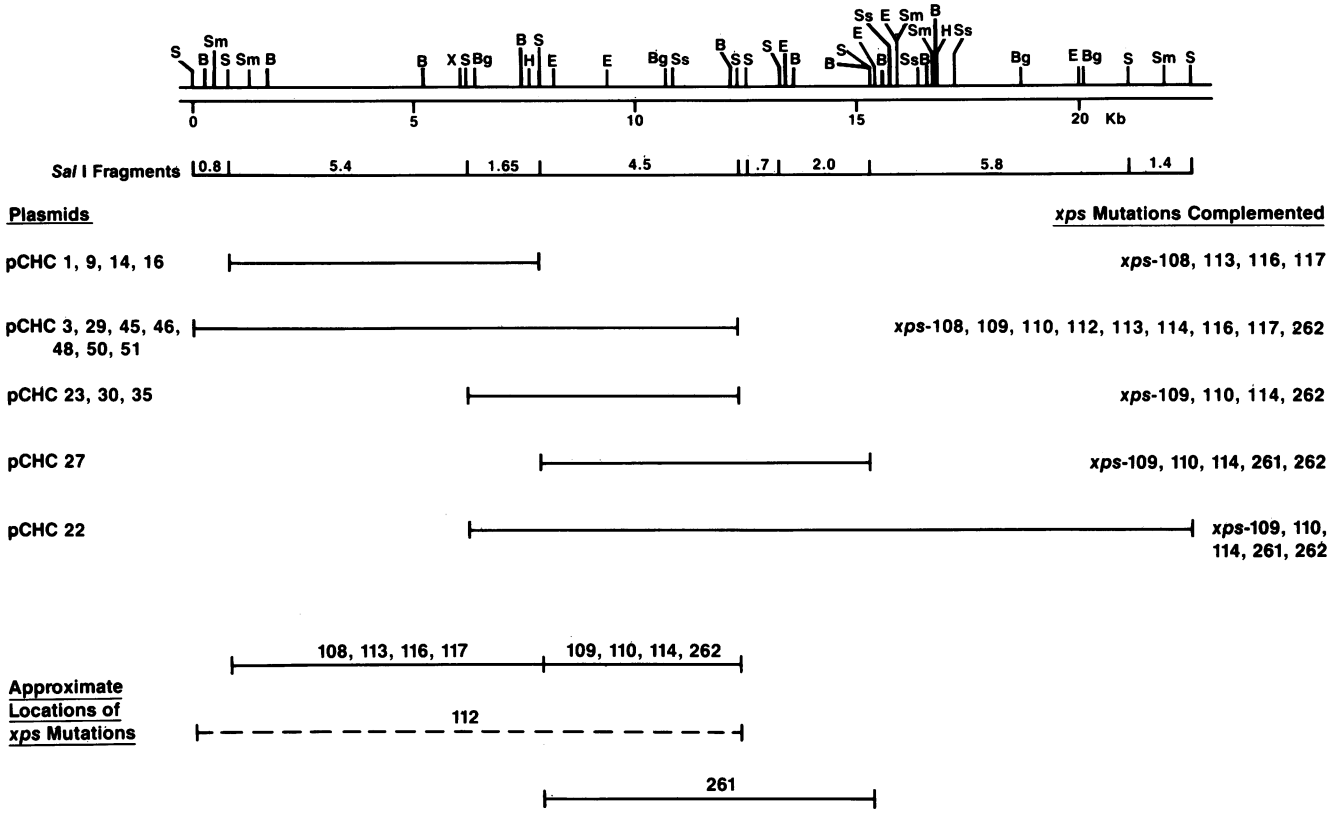

FIG. 2. Physical map of the inserts in plasmids isolated from an *X. campestris* gene library by complementation of nonmucoid (Xps) mutants, assembled as a composite map. Restriction sites are shown for *Sal*I (S), *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sma*I (Sm), *Sst*I (Ss), and *Xho*I (X). The *Sal*I fragments cloned in each plasmid are represented by the lines below the physical map. The *xps* mutations complemented by each plasmid are listed. pCHC27 contains an additional 10-kb *Sal*I fragment obtained during cloning which does not map to this region. It is probably the result of a trimeric ligation reaction involving two unlinked chromosomal *Sal*I fragments and the vector.

partially with *Bam*HI, and the fragments thus generated were ligated into the *Bgl*II site of pRK293. Insertion into the *Bgl*II site of pRK293 did not lead to inactivation of a selectable marker. Thus, it was not possible to select directly for plasmids with inserts. In this case, recombinant plasmids were detected based on complementation of Xps mutants. The ligation mixture was used to transform *E. coli* to Km^r Tc^r; transformants were pooled and mated into selected Xps mutants (2445 [*xps-109*], 2448 [*xps-112*], and 2452 [*xps-116*]). Mucoid exconjugants were isolated, and the plasmids were analyzed for insert size and orientation. Unique plasmids were transferred back into *E. coli* by transformation to use as donors for conjugal matings into the other Xps mutants.

Results of complementation of the 10 linked *xps* mutations with these subclones and with representative isolates of the original plasmids isolated from the gene library are summarized in Fig. 3. Mutant 2452 (*xps-116*) was complemented by plasmids containing the 3.5-kb *Bam*HI fragment or the 5.4-kb *Sal*I fragment. Mutant 2449 (*xps-113*) was complemented by the 5.4-kb *Sal*I fragment, but required both the 3.5- and 2.3-kb *Bam*HI fragments for restoration of the mucoid phenotype; thus, this gene probably spans this *Bam*HI site. Mutants 2444 (*xps-108*) and 2453 (*xps-117*) were rescued by plasmids containing the 2.3-kb *Bam*HI fragment and DNA on either side of this region. (The 2.3-kb *Bam*HI fragment alone was not present in our collection because of the technique used to isolate the subclones, i.e., rescue of selected mutants.) Both *Sal*I fragments 5.4 and 1.65 were required to rescue mutations *xps-108* and *xps-117*, suggesting that this gene spans the *Sal*I site joining these fragments.

The minimum region which complemented mutant 2448 (*xps-112*) consisted of the 2.3- and 4.6-kb *Bam*HI fragments. However, three *Sal*I fragments were required to restore the mucoid phenotype: 5.4-, 1.65-, and 4.5-kb fragments. Since large segments were required for complementation, mutant 2448 (*xps-112*) may contain more than one mutation in the *xps* region. The 4.6-kb *Bam*HI and the 4.5-kb *Sal*I fragments complemented mutations *xps-109*, *-110*, *-114*, and *-262*. The size of this region could contain more than one gene, but this has not yet been shown by subcloning. Mutant 2895 (*xps-261*) was rescued by plasmids (pCHC22 and pCHC27) containing DNA extending to the right of the region analyzed by subcloning, including the 2.0-, 0.7-, and 0.2-kb *Sal*I fragments.

Thus, the genetic analysis suggests that this cluster of *xps* genes consists of a minimum of five distinct complementation groups represented by the following mutations: (i) *xps-116*; (ii) *xps-113*; (iii) *xps-108* and *-117*; (iv) *xps-109*, *-110*, *-114*, and *-262*; and (v) *xps-261*.

Genetic mapping of *xps* genes by insertional inactivation. The locations of several of the *xps* mutations were confirmed by inactivation of the gene on the plasmid by insertion of a transposon. Plasmid pCHC3, which rescued all the linked mutations except *xps-261*, was mutagenized in *E. coli* with the transposon mini-Mu(Tet^r) (2), and the positions of the insertions were mapped by restriction analysis. pCHC3::mini-Mu(Tet^r) isolates were then mated into the

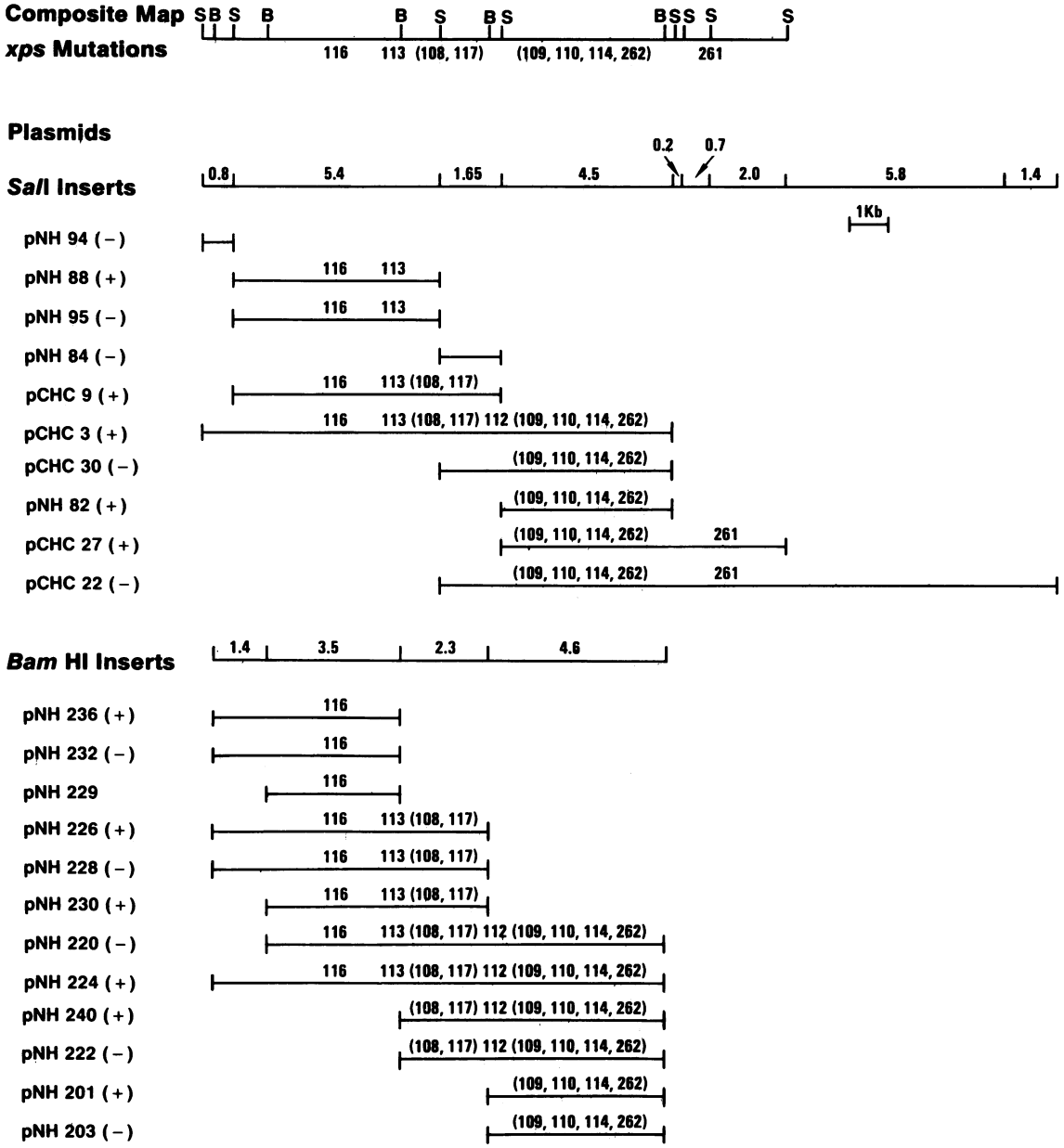

FIG. 3. Correlation of *Sal*I and *Bam*HI fragments with the *xps* mutations complemented. Each plasmid was introduced into each Xps mutant by conjugal matings. Km^r transconjugants were selected and screened for mucoid phenotype. The segments of DNA cloned in each plasmid are designated by brackets; the relative orientation of the insert in the vector is indicated (+ and −). Inserts in the (+) orientation are defined as those with the *Eco*RI site of the vector to the right (shortest distance) of the insert mapped as shown. The orientation of pNH229 could not be determined since none of the restriction enzymes used cut in the 3.5-kb *Bam*HI fragment. Mutations complemented by each plasmid are indicated. Mutant 2446 (*xps-110*) was rescued by plasmids (pNH201, pNH224, and pNH240) with inserts in the (+) orientation; however, similar plasmids with inserts in the (−) orientation (pNH203, pNH220, and pNH222) did not fully restore the wild-type mucoid phenotype, but resulted in a mixture of mucoid and nonmucoid colonies. The cause of this orientation effect is not known at present. B, *Bam*HI; S, *Sal*I.

Xps mutants complemented by pCHC3. Km^r Tc^r transconjugants were examined for the presence or absence of complementation by the mutagenized plasmids. Failure to complement a mutation which was previously complemented by pCHC3 indicates insertion of the transposon into the corresponding gene on the plasmid. Although these insertions were very stable in *E. coli*, some instability, resulting in deletions, was observed in *X. campestris* after growth in the absence of tetracycline. This was prevented by maintaining selective pressure by inclusion of tetracycline (1 to 3 μg/ml) in the media.

A single transposon insertion in the 5.4-kb *Sal*I fragment resulted in failure to restore the normal mucoid phenotype to mutant 2452 (*xps-116*) (Fig. 4). One transposon insertion in the 1.65-kb *Sal*I fragment prevented complementation of both mutants 2444 (*xps-108*) and 2453 (*xps-117*). Two transposons, approximately 1.5 kb apart within the 4.5-kb *Sal*I fragment, each failed to restore the normal mucoid

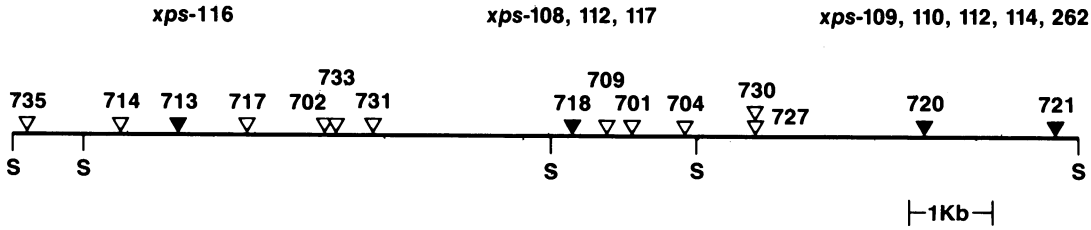

FIG. 4. A physical map of the *X. campestris* insert in the plasmid pCHC3 with triangles representing mini-Mu(Tet^r) insertions. Each plasmid, containing a single transposon insertion, was tested for ability to complement each *xps* mutation. Symbols: ▲, insertions which failed to restore the wild-type mucoid phenotype for the mutations indicated directly above; △, insertions which restored the wild-type mucoid phenotype S, *Sal*I.

phenotype to mutants 2445 (*xps-109*), 2446 (*xps-110*), 2450 (*xps-114*), and 2896 (*xps-262*). These results confirmed the location of these mutations determined by complementation analysis of the subclones.

Complementation of mutant 2448 (*xps-112*) was prevented by transposon insertions in either of two distinct locations, corresponding to previously identified complementation groups (*xps-108* and *-117*) and (*xps-109*, *-110*, *-114*, and *-262*). Thus, this mutant apparently harbors two mutations in the *xps* region. Mutant 2449 (*xps-113*) was complemented by all the plasmids containing transposon insertions shown in Fig. 4. Thus, none of these transposon insertions occurred in this gene. There is a gap of about 2.2 kb in which no transposon insertions were obtained, in the approximate location expected for the gene complementing mutant 2449 (*xps-113*), determined by genetic analysis of the subclones. Thus, the results of insertional inactivation correlate with the genetic locations based on subclone analysis for all the *xps* mutations complemented by plasmid pCHC3.

Effects of cloned *xps* genes on xanthan gum synthesis. Genetic and physical analysis of the cloned *xps* DNA indicated that a cluster of genes involved in xanthan gum biosynthesis had been cloned. Increasing the copy number of genes involved in xanthan gum biosynthesis by cloning them into the multicopy vector pRK293 (5 to 10 copies per cell in *E. coli* and in *X. campestris* [unpublished data]) could potentially increase the production of xanthan gum, if one or more of the genes cloned encodes an enzyme which is rate limiting for xanthan gum synthesis.

All the plasmids shown in Fig. 3 were transferred from *E. coli* to a xanthan gum-producing strain of *X. campestris* (NRRL B-1459 or 0100) by triparental conjugal matings as described above, except that the *E. coli* strains were counterselected by isolation of the transconjugants on minimal-kanamycin plates rather than by using rifampin. The *X. campestris* transconjugants were evaluated for their xanthan gum production compared with that of the parental strain.

In quadruplicate fermentations containing 3.5% corn syrup, one plasmid, pCHC3, resulted in a 10% increase in yield of xanthan gum compared with that of controls with no plasmid or with the vector alone (Table 4). The yield increase was reproducible in subsequent experiments both in shake flasks and small-scale (10-liter) fermentors (unpublished data). This strain also consumed slightly more of the available carbon source (Table 4). Since xanthan gum production by *X. campestris* is already a very efficient process with a 70 to 80% conversion efficiency (27, 34), relatively small increases would be expected and are important to the commercial production of xanthan gum. Plasmid pCHC3 complements the large majority of the *xps* mutations which map to this cluster (except *xps-261*) and thus contains at least four of the five linked *xps* complementation groups mapped to this region of the *X. campestris* chromosome. No other plasmids tested significantly enhanced xanthan gum synthesis.

TABLE 4. Effect of plasmid pCHC3 on xanthan gum

Strain	Yield (g/100 ml)[a]	Residual reducing sugar (g/100 ml)[b]	% Pyruvate (g/100 g)[c]
NRRL B-1459	2.53 ± 0.04	0.32	3.8 ± 0.1
NRRL B-1459(pRK293)	2.58 ± 0.02	0.32	3.7 ± 0.2
NRRL B-1459(pCHC3)	2.85 ± 0.01	0.26	5.5 ± 0.1

[a] Xanthan gum yield was determined gravimetrically after precipitation with isopropyl alcohol.

[b] Residual reducing sugar was determined by dinitrosalicylic acid assay after hydrolysis with glucoamylase.

[c] Pyruvate concentration was determined by lactate dehydrogenase enzymatic assay.

Polysaccharide composition analysis of the xanthan gum produced by *X. campestris* containing pCHC3 showed that the molar ratio of mannose to glucose, as well as the glucuronic acid content of the polymer was the same as that produced by a plasmidless control (data not shown), indicating that the basic structure of xanthan gum was the same for both strains.

In xanthan gum produced by NRRL B-1459, about one-third to one-half the terminal mannose residues are pyruvylated (3, 18). Since plasmid pCHC3 contains a number of genes affecting xanthan gum synthesis, we considered that a gene controlling the addition of pyruvate might be in this gene cluster. A significant increase (45%) in the pyruvate content of the xanthan gum was observed when pCHC3 was present (Table 4), suggesting that a gene involved in pyruvylation of xanthan gum is located within this gene cluster. Fully pyruvylated xanthan gum would contain about 9% pyruvate by weight; thus, the xanthan gum produced by NRRL B-1459(pCHC3) has approximately 60% of its terminal mannose residues pyruvylated. In support of this hypothesis, a mutant *X. campestris* strain, ATCC 31313, which produces essentially pyruvate-free xanthan gum (32; W. C. Wernau, U.S. patent 4,296,203, October 1981) was complemented by plasmid pCHC3. Pyruvate contents of xanthan gum from this strain, with and without pCHC3, were 3.2% and less than 0.1%, respectively.

DISCUSSION

The production of xanthan gum by *X. campestris* is a very efficient process (approximately 70 to 80% conversion of carbon source to polysaccharide), which occurs during growth and in the stationary phase under conditions of high carbohydrate-to-nitrogen ratio (27, 34). The biosynthesis of xanthan gum involves first the uptake of glucose (or other

hexoses) and then synthesis of nucleotide sugar precursors, phosphoenolpyruvate, and acetyl coenzyme A; formation of lipid-linked sugar intermediates; polymerization to polysaccharide; and release of the high-molecular-weight polysaccharide from the cell.

Ielpi and co-workers have shown that in vitro the assembly of the pentasaccharide precursor follows an ordered process starting with transfer of a glucose residue from UDP-glucose to a lipid prenyl carrier in the membrane, followed by sequential transfer of a second glucose residue from UDP-glucose, a mannose residue from GDP-mannose, glucuronic acid from UDP-glucuronic acid, and a second mannose residue from GDP-mannose (15). Acetylation (from acetyl coenzyme A) of the internal mannose residue (17) and pyruvylation (from phosphoenolpyruvate) of the terminal mannose of the pentasaccharide (16) occur before assembly of the polysaccharide.

Thus, the biosynthesis of xanthan gum is a complex pathway which should involve a multitude of enzymes. From the proposed pathway for xanthan gum synthesis (36), eight enzymes will be involved in polymer synthesis (five specific transferases, acetylase, ketal transferase, and polymerase). A specific enzyme may be involved in liberating the completed polysaccharide from the lipid carrier. Acetylase and ketal transferase are not essential for polymerization of the pentasaccharides to occur, since xanthan which lacks pyruvate or acetate can be produced (21). Enzymes specific for xanthan gum production include those required for synthesis of specific precursors: UDP-glucuronic acid and GDP-mannose (36). Availability of the prenyl lipid carrier may be one of the most critical factors affecting synthesis of the exopolysaccharide (35).

In this study, evidence has been obtained for a cluster of genes essential for xanthan polysaccharide synthesis. Plasmids containing cloned *xps* genes were isolated from an *X. campestris* genomic library by their ability to restore mucoid phenotype to nonmucoid *X. campestris* mutants. By subcloning and genetic complementation, a region of 13.5 kb of DNA has been shown to contain a minimum of five complementation groups. A plasmid containing four of these complementation groups increased the production of xanthan gum. Smaller segments of this region did not significantly increase xanthan gum production. Thus, this region contains several genes encoding enzymes which may be involved in a rate-limiting step in xanthan synthesis. This cluster was also shown to include a gene involved in pyruvylation of xanthan gum, which restored pyruvate addition to a strain which produces pyruvate-free xanthan gum and increased the degree of pyruvylation of xanthan gum in a wild-type strain. The pyruvate content of xanthan gum has been implicated as an important parameter affecting the solution viscosity, thermostability, and salt compatibility of the polymer (29, 31, 33).

The biochemical defects in these Xps mutants have not yet been determined; thus, the nature of the genes cloned is not known. These nonmucoid mutants grew on minimal media with glucose, fructose, mannose, mannitol, or succinate, except mutant 2446, which did not grow on any of the minimal media tested (data not shown); thus, they are not defective in enzymes required for metabolism of these carbohydrates. One of the cloned genes specifically affects modification (i.e., pyruvylation) of the polymer. The simplest assumption is that this region encodes enzymes for assembly of the pentasaccharide or, alternatively, for polymerization of the pentamers, release of the polysaccharide from the cell surface, or precursor synthesis.

One of the mutants in this study was complemented by a segment of DNA which was not adjacent to this gene cluster. Work in progress indicates that there are at least six other unlinked genes involved in xanthan gum synthesis (unpublished results).

The genetics of polysaccharide biosynthesis in a variety of gram-negative bacteria has been of increasing interest in recent years. Several genetic loci involved in biosynthesis of alginic acid by *Pseudomonas aeruginosa* have been identified by gene cloning (8, 12), and one region was shown to consist of a cluster of six genes (9). Genes involved in exopolysaccharide synthesis in *Rhizobium trifolii* have been cloned and used to study the involvement of the polysaccharide in nodule formation (4). A cluster of genes encoding three glycogen biosynthetic enzymes of *E. coli* has been cloned in a high-copy vector, resulting in four- to sevenfold overproduction of these enzymes and enhanced glycogen synthesis (28).

X. campestris has only recently been studied by molecular genetic technology, and little is known about the structure of operons in this organism. Cloned genes have been identified which restored yellow pigmentation to white mutants, prototrophy to certain amino acid auxotrophs, and pathogenicity toward turnip plants to nonpathogenic mutants (7). Some of the genes involved in pathogenicity of *X. campestris* have been shown to be clustered (39).

This study has demonstrated the utility of recombinant DNA technology for cloning of xanthan biosynthetic genes and the potential use of the cloned genes to improve the production and alter the physical structure (pyruvate content) of xanthan gum. Additional studies of the cloned *xps* genes and their gene products will contribute to understanding the biosynthetic pathway and the regulatory controls of xanthan gum synthesis. Detailed knowledge of the biosynthetic pathway and its regulation will be essential for successful genetic manipulation of xanthan polysaccharide synthesis and structure.

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