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Identification of Structural Genes Involved in Bacterial Exopolysaccharide Production

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Introduction

Bacterial exopolysaccharides (EPS) are the extracellular capsules and slimes excreted by many bacteria. They form an interfacial barrier between the cell and its environment and have been assigned a number of important biological functions. Nowdays there is increasing interest in the isolation and exploitation of these polymers for industrial purposes. In recent years considerable data have been accumulated on the chemical structures of many of these polysaccharides. Despite this knowledge and the importance of the materials, there is still little understanding of the relationship between the chemical structure and the conformation and of the inter- or intramolecular associations of these polymers, and of how these are responsible for their functional properties. This understanding is complicated by the complex chemical structure of EPS. Fortunately many of the polysaccharides consist of complex but defined chemical repeat units. Recently it has been found that some of these polymers can be grouped into families of structures, within which individual members of the family show small naturally arising modifications of the chemical structure. Such systems provide a basis for identifying critical structural features which determine polymer functionality. The similarity of such structures suggests a similar biosynthetic pathway. Manipulation of the pathways in individual bacteria offers an oppportunity for selectively manipulating the polysaccharide structure by deletion or inactivation of genes. The production of a complete range of structures obtained by the deletion of individual sugars or non-carbohydrate substituents would permit a thorough study of the structure-function relationship. Manipulation of related pathways in different bacteria offers a route to assessing the feasibility of expressing heterologous genes in these organisms and hence creating new pathways for the genetic engineering of polysaccharide structure.

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Functions of bacterial exopolysaccharides

A variety of natural roles have been suggested for bacterial polysaccharides, many of which involve protection of the host in one way or another. EPS may act as receptor sites for bacteriophage (Lin, Cunneen and Lee, 1994; Lindberg, 1977) and conversely, differentation of the polysaccharide structure may block or mask bacteriophage attachment (Lin, Cunneen and Lee, 1994; Lindberg, 1977; Radke and Siegel, 1971). Similarly, EPS together with the lipopolysaccharide O-antigens constitute the principal immunogens and antigens of bacteria (Bishop and Jennings, 1982). The diversity of the polysaccharide structures formed probably reflects the evolution of bacteria in order to evade the immune system. Capsular polysaccahrides have, and will continue to provide cost effective vaccines (Bishop and Jennings, 1982). The secreted slimes and capsules are also important virulence factors whose role is to protect the organism from phagocytosis (Dudman, 1977; Roantree, 1967; Rottini *et al.*, 1975; Smith, 1977b). Extracellular polysaccharides have been implicated in the selective and non-selective adhesion of bacteria to surfaces, including inert surfaces, cultured human mucoid epithelial layers and plant cell walls (Costerton *et al.*, 1978; Costerton, Ingram and Cheng, 1974; Fletcher and Floodgate, 1973; Orskov *et al.*, 1977; Schiffer *et al.*, 1976; Smith, 1977a; Swanson, 1977; Morris *et al.*, 1977; Costerton *et al.*, 1987; Zottola, 1994). They are considered to play important roles in the penetration, invasion and colonization of both plant and mammalian tissue (Orskov *et al.*, 1977; Schiffer *et al.*, 1976; Rees, 1976) and as receptor sites for surface located biosynthetic enzymes (Mukasa and Slade, 1974). Roles in specific recognition steps in plant host-pathogen interactions have also been suggested involving binding to plant lectins (Albersheim and Anderson-Prouty, 1975) or plant cell wall polysaccharides (Morris *et al.*, 1977; Rees, 1976). Extracellular polysaccharides may accumulate moisture, preventing cell dehydration (Rees, 1976; Wilkinson, 1958). In the case of soil microorganisms, the organism may help retain water in soils, promote soil adhesion and inhibit soil erosion (Lynch and Bragg, 1985; Morris, 1993). The mucoid excretions may be responsible for pathogenicity in certain organisms. Thus polysaccharide production by plant pathogens may block vascular systems and similar behaviour is associated with the appearance of mucoid strains of *Pseudomonas aeruginosa* found in accompanying respiratory tract infections associated with cystic fibrosis (Evans and Linker, 1973; Dogget and Harrison, 1969). Production of polysaccharide by fermentation provides a natural texturisation of food fermentation products (Alaban, 1962; Cerning, 1990). On a wider industrial scale bacterial polysaccharides provide a potential source of useful polysaccharides with controlled chemical and physical properties, cost and supply. Present industrial usage includes microencapsulation (Sandford, Cottrell and Pettit, 1984; Chilvers and Morris, 1987), blood plasma substituents (Sandford and Baird, 1983), emulsifiers (Sandford and Baird, 1983) and the replacment of traditional plant and animal gelling, thickening and suspending agents (Sandford and Baird, 1983). Because of the importance of their various roles, the majority of research on structure-function relationships as well as studies on biosynthesis and genetics, have been concentrated on pathogenic bacterial polysaccharides, polysaccharides produced by bacteria involved in important agricultural symbiotic processes and those bacterial polysaccharides of industrial importance.

Structure-function studies

Studies on the structure functional relationships of bacterial polysaccharides are difficult. Certain bacteria secrete polysaccharides resembling those produced by plants. Examples include β(1→3) glucans (Kiniura *et al.*, 1973), cellulose (Brown, 1979) and alginates (Evans and Linker., 1973; Linker and Jones, 1964; Carlson and Matthews, 1966; Lynch and Bragg, 1985; Larsen and Hang, 1971; Page and Sadoff, 1975). For these polymers it is possible to deduce structure function relationships from data accumulated on the related plant polysaccharides. In the majority of cases the chemical structures of bacterial polysaccharides are complicated but are organised into well defined repeat units. For these polysaccharides it has been found that related polysaccharides produced by different bacteria can be grouped into families of structures showing small variations about a common structure (Morris and Miles, 1986; Chandrasekaran *et al.*, 1992; Millane, 1992; Morris, 1992). These families can be extended by chemical, enzymic or genetic manipulation (Morris, 1994). Most methods of inducing mutants, such as chemical or transposon mutagenesis, are not highly selective. It would be better to use a more direct approach to creating families of structures. Such an approach would require the isolation, identification and selective deletion or inactivation of biosynthetic genes in order to produce all the variants of the family of structures possible i.e., by deletion of individual sugars or non-carbohydrate substituents. An understanding of the structure-function relationship based on a study of such families, coupled with detailed genetic understanding of the biosynthetic pathway, provides a route to assessing the ability to design rationally new useful polysaccharide structures.

Strategies for identifying structural genes involved in EPS biosynthesis

The structural genes involved in polysaccharide biosynthesis are clustered in a large number of gram negative organisms, for example *Escherichia coli* (Trisler *et al.*, 1984), *Xanthomonas campestris* (Barrere, Barber and Daniels, 1986; Harding *et al.*, 1987; Hotte *et al.*, 1990; Thorne, Tansey and Pollock, 1987; Vanderslice *et al.*, 1990), *Rhizobium meliloti* (Chen *et al.*, 1988), *Zooglea ramigera* (Easson, Sinskey and Peoples, 1987b), *Erwinia stewartii* (Coplin *et al.*, 1990b), *Pseudomonas solanacearum* (Coplin *et al.*, 1990a) and *Acetobacter xylinum* (Wong *et al.*, 1990; Standal *et al.*, 1994). Thus strategies that allow the identification and isolation of one gene ensure identification of others. This is particularly useful for the identification and isolation of genes for which no screening method can be devised. In order to find genes involved in polysaccharide biosynthesis, a number of different strategies have been employed. These can be divided broadly into two types; the indirect and the direct approaches.

Indirect or mutation based approaches rely on the prior isolation of mutants. Such mutants may arise spontaneously or be induced by the use of a variety of agents, such as chemicals, ultraviolet light, or transposons. Spontaneous mutations arise from different sources including errors in DNA replication, spontaneous lesions and indigenous transposable genetic elements. Such mutations are found to occur naturally at low frequencies in most hosts. Induced mutations occur at higher frequencies, and unless a spontaneous mutant is already available, most researchers will induce mutations in the host. However they are derived, mutants will have to be screened to

STRUCTURES OF ACETAN AND XANTHAN

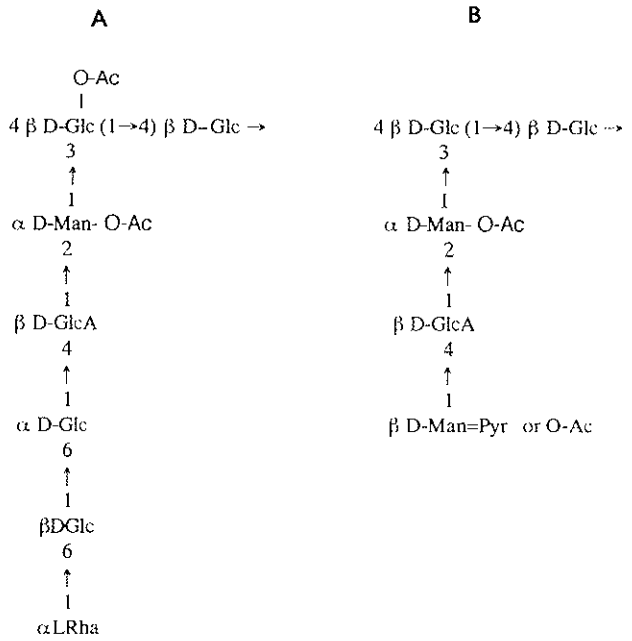

Figure 1. (A) The structure of acetan produced by *X. xylinum*. (B) Xanthan produced by *X. campestris*. The terminal sugar in xanthan may be linked either to a pyruvate group (Pyv) or an acetyl group (-O-Ac). Sugar abbreviations; Glc, glucose; GlcA, glucuronic acid; Man, mannose, Rha, rhamnose.

A. xylinum NRRL B42 produces both cellulose and another exopolysaccharide called acetan. Acetan is a heteropolysaccharide that is structurally related to the commercially important polymer xanthan which is produced by *X. campestris* (*Figure 1*). The NRRL B42 strain of *A. xylinum* was mutagenised with NTG and the survivors were screened for the appearance of large mucoid colonies (i.e., cellulose producing strains have a small, dry colony morphology). One cel$^-$ mutant, the C1 strain, was further mutagenised with NTG and screened for colonies which were either less mucoid or non-mucoid compared to the parent strain (MacCormick *et al.*, 1993). Such mutants were predicted to have either lost acetan production completely or to produce polymers with truncated side-chains (MacCormick *et al.*, 1993). Polysaccharide was harvested from the culture medium of three mutants and analysed for the presence of rhamnose (the terminal sugar in the repeat unit). Absence of rhamnose in all three mutants, CR1/4, CR1/29 and CR1/74 indicated that these strains produced acetan polymers with truncated side-chains.

Genes involved in acetan biosynthesis were later identified using a direct PCR based approach (Griffin, Morris and Gasson, 1994). Degenerate PCR primers, when used in a PCR reaction with genomic DNA from *A. xylinum* NRRL B42, generated the predicted 180bp product. This was sequenced and the translated sequence confirmed

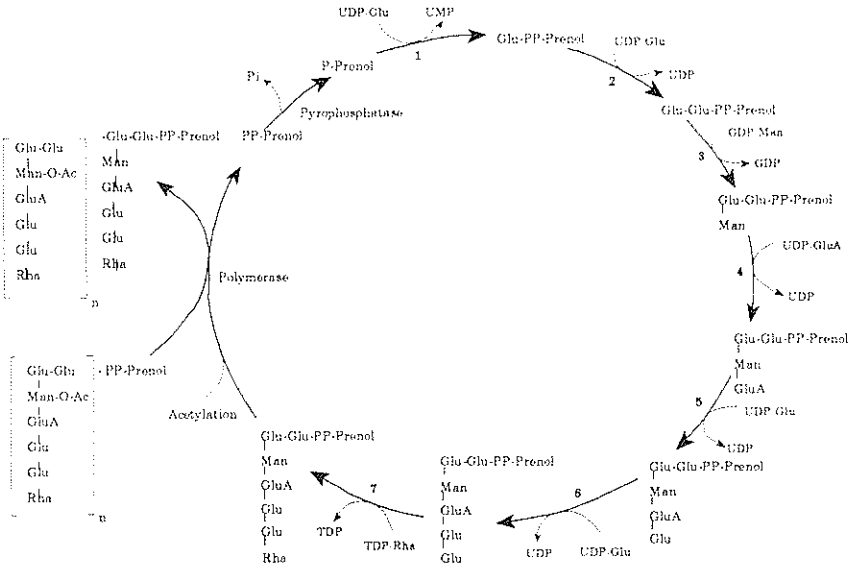

Figure 2. The biochemical pathway for the synthesis of acetan in the gram-negative organism, *A. xylinum* involves the stepwise assembly of the heptasaccharide repeat unit on a diphosphate lipid carrier. Steps 1 to 7 are catalysed by sugar transferases. Abbreviations as given in Figure 1.

that the fragment originated from a gene involved in polysaccharide biosynthesis. This fragment was labelled with fluorescein and used to probe a genomic lambda library. A positive lambda clone containing an 8.4 kb fragment containing genes involved in acetan biosynthesis was identified. The fragment was subcloned into a plasmid vector and nucleotide sequenced. Six open reading frames were identified and comparison of the translated nucleotide sequences of open reading frames to the amino acid sequences of the published sequences of exopolysaccharide biosynthetic genes from other gram negative organisms was used to assign functions to three of these (Muller *et al.*, 1993; Capage, 1987; Jiang *et al.*, 1991; Koplin *et al.*, 1992). The translated sequence of *aceA* was homologous to that of the *gumD* gene from *X. campestris* (Capage, 1987) and is therefore predicted to encode the phosphate-prenyl glucose-1-phosphate transferase catalyzing the first step in the pathway (*Figure 2*). The deduced amino acid sequence of the *aceC* gene was homologous to that of *gumH* from *X. campestris* (Capage, 1987), therefore it is likely that *aceC* encodes a GDP-mannosyl transferase (step 3 of the pathway; *Figure 2*). The *manB* gene was predicted to encode the bifunctional enzyme, phosphomannose isomerase and GDP-mannose pyrophos-phorylase, since the predicted amino acid sequence of this gene was homologous to the translated *xanB* gene (Koplin *et al.*, 1992). The availability of a method for screening for variants with truncated side-chains should simplify the process of generating a family of acetan polymers using a genetic approach such as gene inactivation.

IDENTIFICATION OF GENES INVOLVED IN BIOSYNTHESIS OF EPS IN *X. CAMPESTRIS*

Xanthan gum is an exopolysaccharide produced by the gram negative microorganism *X. campestris*. This polymer enjoys wide commercial application as a thickening and

suspending agent in aqueous solutions (Hassler and Doherty, 1990). The structure proposed for this polysaccharide indicates that it may be considered as a substituted cellulose; with a pentasaccharide repeat unit (*Figure 1*). Because of its commercial importance there has been great interest in finding the genes involved in xanthan biosynthesis and in manipulating these to generate polymers with novel structures.

Several groups have reported the isolation of mutants of *X. campestris* that are defective in xanthan biosynthesis (Harding *et al.*, 1993; Thorne, Tansey and Pollock, 1987; Harding *et al.*, 1987; Hotte *et al.*, 1990; Marzocca *et al.*, 1991; Barrere *et al.*, 1986; Vanderslice *et al.*, 1995; Koplin *et al.*, 1992; Capage, 1987). Mutagenesis with EMS (Barrere, Barber and Daniels, 1986; Thorne, Tansey and Pollock., 1987) and NTG (Harding *et al.*, 1987) was used to generate EPS^- mutants of *X. campestris*. Restoration of the mucoid phenotype by complementation of EPS^- mutants with cloned genomic DNA, was used to identify fragments containing polysaccharide biosynthetic genes (Thorne, Tansey and Pollock, 1987; Harding *et al.*, 1987; Barrere *et al.*, 1986). Transposon mutagenesis of the insert DNA with Tn5 (Barrere, Barber and Daniels, 1986) and mini-Mu (Harding *et al.*, 1987) was used to isolate the complementation regions of the insert DNA. Marker exchange of Tn5 insertions from cloned DNA into the *X. campestris* genome (Barrere *et al.*, 1986) or the complementing plasmid (Harding *et al.*, 1987) provided evidence that the genes involved in xanthan biosynthesis were clustered (Barrere, Barber and Daniels, 1986; Harding *et al.*, 1987). Harding *et al* predicted that this cluster is in the region of 13.5 kb. Thorne, Tansey and Pollock (1987) found three unlinked loci involved in xanthan biosynthesis; some such genes may be involved in xanthan biosynthesis directly, others may encode auxiliary structural proteins or positive regulatory factors. Expression of a plasmid (pCHC3) with a 12.4kb insert, containing the xanthan biosynthetic cluster in *X. campestris* resulted in increased yields of polymer with increased pyruvate content (Harding *et al.*, 1987). Thorne, Tansey and Pollock (1987) postulated that pCHC3 must contain a ketal pyruvate transferase; this was later confirmed and the gene was cloned and sequenced (Marzocca *et al.*, 1991).

Transposon mutagenesis (Tn5) was used to generate 'different colony morphology' mutants of *X. campestris* (Hotte *et al.*, 1990). Two colony types were isolated; mutants B100–152 had a smooth colony surface and mutants B100–13 and B100–22 were characterized as rough colony types. Complementation with cloned wild-type DNA led to the identification of a 35.5kb genomic fragment containing a new DNA region involved in xanthan biosynthesis (Hotte *et al.*, 1990). By fragment-specific Tn5-*lac* mutagenesis, 12 complementation groups were identified. Since many of the mutants showed, in addition to altered EPS phenotype, agglutination and reduced motility, Hotte *et al.* predicted that many of the affected genes might encode enzymes or regulatory factors involved in the synthesis of both EPS and lipopolysaccharide. In *X. campestris* the genes encoding nucleotide sugar biosynthesis were shown to be physically separated from those involved in the assembly and polymerization of the pentasaccharide repeat unit (Coplin and Cook, 1990a; Harding *et al.*, 1993). Nucleotide sugars are key intermediates for both EPS and lipopolysaccharide biosynthesis, therefore a regulatory mechanism that enables the microorganism to channel intermediates from one polymer to another may exist at this level (Koplin *et al.*, 1992). A 3.4 kb fragment from the 35.5 kb insert, which could restore the EPS phenotype, was subcloned and analysed in more detail (Koplin *et al.*, 1992). Nucleotide sequencing

revealed the presence of two genes, *xanA* and *xanB*. The deduced amino acid sequence of *xanB* was homologous to the translated sequence of the *algA* from *P. aeruginosa*, therefore *xanB* is likely to encode the bifunctional enzyme phosphomannose isomerase and GDP-mannose pyrophosphorylase (PMI-GMP). This putative function of *xanB* was confirmed by enzymic assays on cell extracts i.e., PMI-GMP activity was present in mutants complemented by *xanB*. Biochemical analysis was used to assign a function to *xanA*. It is thought to encode an enzyme, phosphoglucomutase-phosphomannomutase (PGM-PMM) responsible for the biosynthesis of glucose-1-phosphate and mannose-1-phosphate (Koplin *et al.*, 1992). The levels of PGM and PMM were drastically reduced, though not abolished in the *xanA* mutants, indicating that one enzyme or its reaction product may be responsible for the induction of the other or *xanA* may encode a regulatory factor which modulates the activities of both PGM and PMM.

The relationship between these enzymic activities was also detected in a later study (Harding *et al.*, 1993). In this study EMS mutagenesis was used to generate non-mucoid mutants of *X. campestris*. These were divided into seven different complementation groups following conjugation of a genomic library of *X. campestris* DNA into non-mucoid muants. Complementation and biochemical analysis and DNA mapping were used to identify and characterize the *xpsIII, IV* and *VI* regions. These mutants were able to synthesize both the lipid intermediates and xanthan gum *in vitro* when nucleotide sugars were provided as substrates (Harding *et al.*, 1993). *XpsVI* was defective in the synthesis of UDP-glucuronic acid (due to loss of UDP-glucose dehydrogenase activity) and produced a small amount of polymer (5% of the wild-type level). Structural analysis of the polymer revealed a structure identical to that of polytrimer (Betlach *et al.*, 1987) i.e., the absence of glucuronic acid, and a glucose/mannose ratio of approximately two. A gene encoding an UDP-glucose dehydrogenase has recently been cloned and sequenced from the Xc11 strain of *X. campestris* (Lin *et al.*, 1995). The *xpsIII*-A group of mutants, showing a defect in UPD-glucose, UDP-glucuronic acid and GDP mannose biosynthesis, displayed a loss of PGM–PMM enzymic activity. The *xpsIII*-B group were defective in GDP-mannose biosynthesis and were found to have lowered activities of mannose isomerase (MI) and phosphomannoseisomerase (PMI) enzymes involved in the biosynthesis of this sugar. Mutations affecting the activity of a UDP-glucose pyrophosphorylase enzyme were defective in the UDP-glucose and UDP-glucuronic acid. All three complementation groups characterized in this study involved defects in genes involved in the biosynthesis of the nucleotide sugars required for xanthan biosynthesis.

Screening for a reduction in polysaccharide yield following transposon mutagenesis with Tn10 and Tn903 was used to identify and isolate mutants defective in xanthan gum biosynthesis in *X. campestris* (Betlach *et al.*, 1987). Biochemical analysis of freeze-thaw lysates of each mutant *in vitro* was used to separate the mutants into two groups (Betlach *et al.*, 1987). One group produced normal xanthan *in vitro* when sugar nucleotides were provided; thus these have defects in genes other than those involved in the assembly and polymerization of the pentasaccharide repeat unit. The other group were blocked at various stages of xanthan production, hence different lipid-linked intermediates accumulated *in vitro*. Complementation of mutants in this latter group was used to identify a 16 kb (approx) chromosomal fragment containing a cluster of 12 genes involved in xanthan biosynthesis (Hassler and Doherty, 1990).

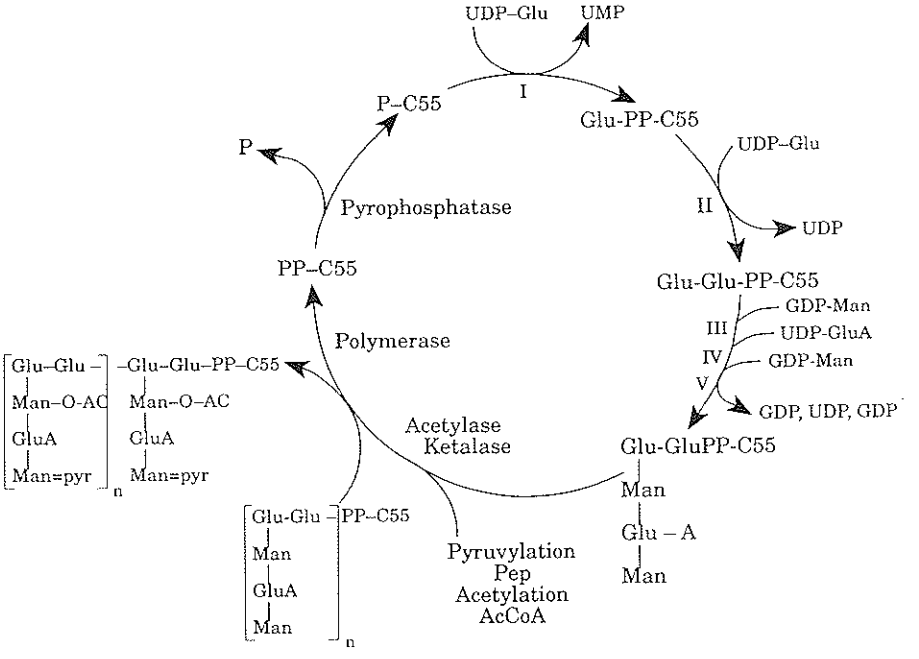

Figure 3. The biochemical pathway for the synthesis of xanthan in the gram-negative organism, *X. campestris* involves the stepwise assembly of the pentasaccharide repeat unit on a diphosphate lipid carrier. Steps 1 to 5 are catalysed by transferases I–I. Abbreviations as given in Figure 1.

The nucleotide sequence of this region was determined (Capage, 1987) and the cloned genes were extensively mutagenised *in vivo* with Tn10 (Hassler and Doherty, 1990; Doherty, 1987). Plasmid-borne mutated genes were transferred back into *X. campestris* and subsequently mutations were identified in which the mutated gene had been inserted into the chromosome by homologous recombination. *In vitro* analysis of xanthan deficient mutants led to the identification of strains that accumulate glucose, cellobiose or polytrimer (Vanderslice *et al.*, 1990). Thus these mutants are each blocked at the respective steps for transferase II, III or IV (*Figure 3*) and accumulate the appropriate lipid-linked intermediate *in vitro*. Transferase II, III and IV are encoded by *gumM, gumH* and *gumK* respectively. The strain in which the lipid carrier is not charged with lipid is defective in the gene encoding transferase I (*gumD*). Identification of mutants defective in transferase V (producing polytetramer) using the *in vitro* system to detect lipid linked intermediates was not efficient i.e., tetrameric repeat units were efficiently polymerized. However, it was possible to detect defects in genes responsible for polymerization (*gumB*, *gumC* and *gumE*) and polymer export (*gumJ*) (Vanderslice *et al.*, 1990). To identify strains producing polytetramer, non-pyruvylated and non-acetylated polymers, it was necessary to isolate polysaccharide from the culture medium of mutants for use in structural studies. Genes encoding the enzymes involved in these processes are *gumI, gumL* and *gumF* respectively.

IDENTIFICATION OF EPS BIOSYNTHETIC GENES IN *RHIZOBIUM*

Bacteria within the family Rhizobiaceae can infect plants and are involved in the symbiotic process of nitrogen fixation. The cell surface polysaccharides of these bacteria have been shown to be involved in this process (Gray and Rolfe, 1990; Breedveld and Miller, 1994; Halverson and Stacey, 1986; Hirsch, 1992; Leigh and Coplin, 1992). The major type of polysaccharide molecules found loosely attached to the bacterial outer membrane surface are the acidic EPS, while the neutral β(1→2) linked glucans are released into the environment (usually upon cell-lysis/cell-damage). Genetics of EPS biosynthesis has been studied in three different strains of *Rhizobium*, namely *R. leguminosarum* bv. *phaseoli*, *R. meliloti* strain Rm1021 and *R. sp* strain NGR234. Since the genetic systems are similar, we will concentrate on studies based on *R. meliloti*. Tn5 mutagenesis of *R. meliloti* was used to isolate 102 transposon insertions in a 48 kb region previously shown to contain genes involved in polysaccharide biosynthesis (Long *et al.*, 1988). Mutations were screened for either a loss of calcofluor binding or reduced/altered calcofluor binding. Mutations affecting biosynthesis of the exopolysaccharide, succinoglycan, were clustered in a 22kb region and fell into 12 complementation groups. Strains carrying mutations in seven complementation groups (*exoA*, *exoB*, *exoF*, *exoL*, *exoM*, *exoP* and *exoQ*) produced no calcofluor binding EPS. Mutants in an eighth complementation group (*exoH*), fluoresced on calcofluor plates. However the colonies were not surrounded by a halo. As previously reported, these mutants produced altered polysaccharide that is not succinylated (Leigh *et al.*, 1987). Mutants in the four remaining complementation groups (*exoN*, *exoG* and *exoJ*) produced less calcofluor binding polysaccharide than the wild type. Biochemical analysis of lipid-linked intermediates that accumulated in *R. meliloti exo* mutants was subsequently used to identify the genes that function in each stage of assembly (Reuber and Walker, 1993). Radiolabelled precursors were added to permeabilized cells and the identity of the intermediates that accumulated defined the function of each gene: for example *exoF* or *exoY* mutants did not accumulate any lipid-linked intermediates, therefore these genes are postulated to function in the initial glycosyl transfer step (Leigh *et al.*, 1994; Reuber and Walker, 1993). Lipid-linked galactose was accumulated by *exoA* mutants, therefore *exoA* is thought to encode an enzyme that adds the second sugar (glucose) to the growing subunit (Reuber and Walker, 1993). *ExoP*, *exoQ* and *exoT* mutants accumulated the entire lipid-linked subunit, therefore these genes are required for polymerization and export. Acetylation failed to occur in *exoZ* mutants implying that this gene encodes an acetyl transferase. The *exoB*, *exoC* and *exoN* were predicted to be involved in the biosynthesis of the nucleotide sugar precursors required for EPS biosynthesis (Leigh *et al.*, 1994; Reuber and Walker, 1993; Glucksmann *et al.*, 1993a). Putative genes (*exoO*, *exoU* and *exoW*) that add respectively the fifth, sixth, and seventh sugars of the subunit were later identified (Glucksmann *et al.*, 1993b). Other studies also used transposon mutagenesis to identify, clone and subsequently sequence genes involved in succinoglycan biosynthesis in *R. meliloti* (Becker *et al.*, 1993a,b; Muller *et al.*, 1993; Buendia *et al.*, 1991; Reed, Capage and Walker, 1991). Combined with the 16kb of sequence data presented by Glucksmann *et al.* (1993b), sequence information for the entire 25kb *exo* gene cluster, found on the second symbiotic plasmid, was now available. The genetic map of the *exo* genes correlated with DNA sequence. Transposon

mutagenesis was used by two different groups to isolate mutants of *R. meliloti* that produced mucoid colonies that did not fluoresce in the presence of calcofluor (Galzebrook and Walker, 1989; Zhan *et al.*, 1989). These mutants were found to produce an alternative polysaccharide termed EPS II or EPSb. Genetic analysis indicated that EPS II synthesis required the products of at least seven loci on the second symbiotic plasmid (Galzebrook and Walker, 1989), one of which was the previously characterized *exoB* gene.

IDENTIFICATION OF GENES INVOLVED BIOSYNTHESIS OF EPS IN *Z. RAMIGERA*

Z. ramigera produces significant quantities of EPS that have been studied for their role in bioflocculation (Dugan, 1975) and their unique ability to absorb heavy metal ions (Dugan, 1975; Norberg and Persson, 1984). The genetics of EPS biosynthesis has been studied in two different strains of *Z. ramigera* (Easson, Peoples and Sinskey, 1987; Easson, Sinskey and Peoples, 1987). Strain I-16-M produces a cellulose-like fibrillar polysaccharide (Dugan, 1975; Easson *et al.*, 1986) whilst strain 115 produces an exopolysaccharide composed of glucose, galactose and pyruvate (Franzen and Norberg, 1984; Ikeda *et al.*, 1982; Sinskey *et al.*, 1986). Screening for loss of fluorescence in the presence of calcofluor was used to detect an EPS$^-$ variant of *Z. ramigera* I-16-M (Easson, Sinskey and Peoples, 1987). Calcofluor binds specifically to $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ glycosidic linkages (Easson *et al.*, 1986; Haigler, Brown and Benziman, 1980) and thus is concentrated around cells and colonies producing β-linked polysaccharides. Spontaneous non-fluorescing mutants were obtained at a frequency of approximately 1 in 10 000 cells. The mutation was shown to be due to a spontaneous deletion in the gene region encoding polysaccharide genes by Easson, Sinskey and Peoples (1987), who then extended this screening method to the isolation of exo-mutants, generated by the use of transposon mutagenesis. By complementing different Tn5 mutants with host chromosomal DNA, a 14kb region of chromosomal DNA containing the biosynthetic genes was identified.

The genetics of polysaccharide biosynthesis in strain 115 is not so advanced. In this strain EPS- mutants were isolated following transposon Tn5 mutagenesis and mutants were complemented following conjugation with cloned wild-type DNA. However genetic analysis of insert DNA complementing EPS-phenotype was not reported (Easson, Peoples and Sinskey, 1987).

Conclusion

With the availability of sequence data for an increasing number of genes involved in exopolysaccharide biosynthesis, the goal of analyzing structure–function relationships using families of related polymers becomes feasible. A new family of polymers based on the structures of acetan and xanthan would make an excellent model system for this type of study. With the availability of the nucleotide sequences of genes involved in the biosynthesis of these polymers it should be possible to design systematically a family of polymers which differ about the central structure by selectively inactivating and/or deleting genes. Such polymers would differ from the parent in the number of sugar units present in the repeat unit and/or in the presence of non-carbohydrate substituents. This has already been achieved in a limited, random

fashion in both *A. xylinum* and *X. campestris* using mutagenesis, proving that these organisms can correctly assemble and polymerize such structures. The availability of sequences for genes involved in EPS production by bacteria producing polysaccharides with related chemical structures provides an opportunity for assessing the practicality of designing or engineering new polysaccharide structures. Such changes would require heterologous expression of genes from different bacteria. This approach could be tested initially by transferring genes encoding enzymes catalyzing equivalent functions from one bacterium to another. An example would be to transfer the gene encoding transfer of 1,2 mannose to either acetan or xanthan (*Figure 1*). If this led to correct assembly and polymerization of the polysaccharide molecule then it should be possible to introduce new genes leading to the assembly of new polysaccharide structures. Knowledge of structure–function relationships derived from studies of families of related polymers could lead to the ability to predict physical properties for polymers with specific structural alterations.

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