Generation of a novel polysaccharide by inactivation of the *aceP* gene from the acetan biosynthetic pathway in *Acetobacter xylinum*

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The acetan biosynthetic pathway in *Acetobacter xylinum* is an ideal model system for engineering novel bacterial polysaccharides. To genetically manipulate this pathway, an *Acetobacter* strain (CKE5), more susceptible to gene-transfer methodologies, was developed. A new gene, *aceP*, involved in acetan biosynthesis was identified, sequenced and shown to have homology at the amino acid level with β -D-glucosyl transferases from a number of different organisms. Disruption of *aceP* in strain CKE5 confirmed the function assigned above and was used to engineer a novel polysaccharide with a pentasaccharide repeat unit.

Keywords: *Acetobacter xylinum*, *aceP* gene, acetan, EPS biosynthesis, novel EPS

INTRODUCTION

Microbial exopolysaccharides (EPSs) are long-chain, high-molecular-mass polymers that are secreted into the environment by a large variety of different bacteria. These polymers are believed to play a protective role in the native state, and after isolation can be dissolved/dispersed in water to give interesting thickening, gelling and emulsifying effects: properties which are indispensable tools in the food and many other industries (Roller & Dea, 1992; Sutherland, 1998). Many EPSs secreted by bacteria are regular structures composed of complex chemical repeat units. These repeat units can contain a number of different sugars linked in a variety of ways; the structure can be branched or multi-branched, and may be decorated with non-carbohydrate substituents (Kenne & Lindberg, 1983; Lindberg, 1990). It is widely known that the functional properties of an EPS are determined by its primary structure, which dictates the subsequent folding and association under various environmental conditions. To assess the contribution of each sugar unit/linkage/substituent to the polysaccharide functionality it is desirable to engineer

novel polymers which are variants of the common structure (Griffin *et al.*, 1995). The acetan biosynthetic pathway in *Acetobacter xylinum* is an ideal model system for engineering novel bacterial polysaccharides for the following reasons. Firstly, the biochemical pathway for acetan biosynthesis has been elucidated and shown to be similar to that of xanthan biosynthesis in *Xanthomonas campestris* (de Lannino *et al.*, 1988). Secondly, acetan is structurally related to the commercially important polymer, xanthan (Fig. 1). It also has an advantage over xanthan for engineering studies because the repeat unit contains seven sugars (two more than xanthan), thereby increasing the scope for variation. Finally, many of the genes involved in acetan biosynthesis have been cloned and sequenced from the bacterial chromosome (Griffin *et al.*, 1996a, b, 1997a, b). It is envisaged that novel polymers could be produced either by inactivation of a selected sugar-transferase gene or by heterologous expression of transferase genes from other bacterial systems. In previous studies, random mutagenesis strategies were used to identify strains producing variants of the wild-type acetan and xanthan EPS repeat unit (MacCormick *et al.*, 1993; Betlach *et al.*, 1987; Hassler & Doherty, 1990). These data confirm that the bacterial biosynthetic machinery can correctly polymerize and export variant polysaccharide structures.

In this paper we report the identification and sequencing of *aceP*, a new gene encoding a glycosyl transferase involved in acetan biosynthesis. We report the de-

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Abbreviations: CTAB, cetyltrimethylammonium bromide; EPS, exopolysaccharide; PMA, partially methylated alditol acetate.

The EMBL accession number for the sequence reported in this paper is Y18467.

METHODS

Plasmids, bacterial strains and culture conditions. The plasmid vectors pAG18 (amp^R) (Griffin *et al.*, 1997a), pUCD2 (amp^R kan^R tet^R) (Close *et al.*, 1984) and pCR2.1 (amp^R) (Invitrogen) were used. *A. xylinum* strains C1 and CKE5 were grown in HS medium (Hestrin & Schramm, 1954) and incubated at 30 °C for 3 d. Broth cultures were shaken at 200 r.p.m. When required, antibiotics were added as follows: 4 µg tetracycline ml⁻¹, 20 µg ampicillin ml⁻¹. Incubation for up to 10 d was required in the presence of antibiotic selection.

(5 µg) was added to 40 µl aliquots of cells (in 0.1 cm cuvette). Using a Gene pulser apparatus (Bio-Rad laboratories), pulses of 1.8 kV, 200 Ω and 25 µF were applied, then 960 µl ice-cold HS broth was added immediately. Cells were incubated at 30 °C for 3 h to allow plasmid expression, then diluted and plated onto HS agar plates containing appropriate antibiotics. Plates were incubated at 30 °C for 3–10 d.

Construction of an *A. xylinum* strain with high electrotransformation frequencies. Strain C1 of *A. xylinum* was mutagenized by exposure to 0.04% methane sulfonic acid ethyl ester at 37 °C for 45 min. This procedure resulted in a 1% survival rate of cells. These were electrotransformed with the plasmid vector pUCD2, and one transformant, CK, was selected for a plasmid-curing procedure, which was carried out as follows: cultures in 10 ml HS broth containing 10 µg acriflavin ml⁻¹ were incubated at 30 °C for 24 h with agitation, then plated onto HS plates and incubated at 30 °C for 3 d. When subsequently tested, 90% of colonies obtained had lost plasmid-associated antibiotic resistance and were therefore 'cured' of the plasmid. The electroporation frequencies of six individual 'cured' isolates (CKE1 to CKE6) were tested.

Construction of pKE23. pCR2.1 (amp^R kan^R) does not replicate in *A. xylinum* CKE5 and was used to construct a suicide vector as follows: a 2.2 kb *Kpn*I fragment containing the tetracycline-resistance gene from pKT1a (J. Kok, pers. comm.) was cloned into the *Kpn*I site of pCR2.1 to generate pKE14 (tet^R amp^R kan^R). Primers a1 (5'-GATGACTGTTCCACCGAC-3') and a2 (5'-AGCCGCTGCAGTCCCTCG-3') were used to amplify a 700 bp fragment of the *aceP* gene from pAG18 using the PCR conditions described previously (Griffin *et al.*, 1996b) except that 1 unit Dynazyme (Flowgen) was used and cycling conditions were 25 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. The 700 bp PCR product obtained was purified, blunt ended (Sambrook *et al.*, 1989) and subsequently cloned into *Spe*I-digested blunt-ended pKE14 to generate pKE23.

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and purified by precipitation with cetyltrimethylammonium bromide (CTAB) as described previously for *A. xylinum* strains (MacCormick *et al.*, 1993). No attempt has been made to optimize growth conditions or to maximize polysaccharide production. Total carbohydrate content of crude and purified polymer from strain P2 was estimated colorimetrically by the phenol/sulphuric acid method (Dubois *et al.*, 1956), calibrated using glucose. The presence and level of uronic acid was determined using the 3-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991), calibrated using glucuronic acid. The presence of rhamnose was determined using the thiocarbamide method (Dische & Shettles, 1948; Baird & Smith, 1989). Neutral sugar composition was determined by subjecting the purified or crude P2 polysaccharide to acid hydrolysis in 2 M trifluoroacetic acid and derivatization to alditol acetates (MacCormick *et al.*, 1996). The linkage sites of all sugars were determined by methylation analysis as follows: the polymer was methylated (MacCormick *et al.*, 1993), extracted into $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1), split into three parts and vacuum dried over P_2O_5 overnight. The first part was reduced with lithium triethylborodeuteride in tetrahydrofuran ('Super Deuteride') at 64 °C for 4 h (York *et al.*, 1985). Excess reagent was treated with 2-propanol, water, then 1 M H_3PO_4 to pH 5, followed by filtration on glass-fibre paper and washing with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1). After drying, the first part (carboxy-reduced) and the second part of the methylated polymers were hydrolysed with trifluoroacetic acid and converted to partially methylated alditol acetates (PMAAs) as described previously (MacCormick *et al.*, 1996). The third portion of the methylated polymer was subjected to reductive cleavage using triethylsilane/trimethylsilyl trifluoromethanesulfonate/dichloromethane (Rolf *et al.*, 1985) and acetylated with acetic anhydride/acetic acid/*N*-methylimidazole (3:0.5:0.5 ml) at 30 °C for 30 min. This was extracted with water/dichloromethane in the usual way to yield partially methylated anhydroalditol acetates. The PMAAs and anhydroalditol derivatives were analysed by GC-MS as described previously (MacCormick *et al.*, 1996). A mixture of external PMAA standards for each sugar was prepared by deliberate undermethylation of the methyl glycosides (Doares *et al.*, 1991). 1,5-anhydroglucitol and 1,5-anhydromannitol were synthesized by the method of Ness *et al.* (1950). These were similarly undermethylated and acetylated to produce mixtures of standards. The identities of the PMAAs and partially methylated anhydroalditol acetates were confirmed from their electron-ionization mass spectra (Carpita & Shea, 1989; Gray, 1990). Polysaccharide harvested from strain CKE5 was prepared without CTAB precipitation. This was analysed as described above except that the reductive cleavage analysis was omitted.

RESULTS

Development of a strain of *A. xylinum* with high electrotransformation frequencies

Strain C1 of *A. xylinum* could not be transformed by either chemical-based protocols (Fukaya *et al.*, 1985) or by conjugation using broad-host-range mobilizable vectors (Valla *et al.*, 1986; Inoue *et al.*, 1985). Transformation frequencies obtained following electroporation using previously published protocols (Hall *et al.*, 1992; Wong *et al.*, 1990) and our own optimized protocol (see Methods) gave low and unreliable frequencies (max frequency 10^2 c.f.u. μg^{-1} DNA) (data not presented). Methane sulfonic acid ethyl ester treat-

	41	74
AceP	E IIIVDDC S TGTPDLLNRLARAHDSIRVMSTPY	
ExoO _{rm}	EVVVVDDCSADATPALVAIPDPR--VRLIALDR	
	*...*****.*.*.*. *	
Con	VVV D S R	
	75	108
AceP	N GGPSVARNVGLGAARGDWIAFLDADAFAPDRL	
ExoO _{rm}	N RGPGGARNAGIGAAARGRWIAVLSDDTVRPDRL	
	• • • • • * * * * * * * * * * * * * * *	
Con	N GP ARN A LD D PD L	

Fig. 2. An alignment of the regions of the amino acid sequences of AceP from *A. xylinum* and ExoO from *Sinorhizobium meliloti* (Becker *et al.*, 1993). The conserved sequence motif found in the N-terminal region of a number of β -glycosyl transferases (Becker *et al.*, 1998) is shown in bold. Asterisks (*) are used to identify positions of identity and dots are used to identify positions of conservative replacements.

ment of strain C1 and a plasmid-curing regime were used to isolate six new strains, CKE1 to CKE6. The electroporation frequencies of these strains were tested and reproducibly found to be as follows: CKE1, CKE2 and CKE4 had frequencies of 10^4 transformants μg^{-1} DNA, CKE3 and CKE6 had frequencies of 10^5 transformants μg^{-1} DNA, while CKE5 had a transformation frequency of 10^6 transformants μg^{-1} DNA.

Sequence analysis of the *aceP* gene

The identification and cloning of a plasmid clone, pAG18, containing genes involved in acetan biosynthesis has previously been reported (Griffin *et al.*, 1997a). The nucleotide sequence of a 1174 bp region of pAG18 was determined and is available from the EMBL database under accession number Y18467. Computer analysis of this region revealed the presence of an ORF that showed strong conformity to the *Acetobacter* codon usage table (not shown). This ORF, which was designated *aceP*, encodes a protein of 321 aa with a molecular mass of 35.37 kDa. There are two potential start codons for *aceP*; we assume the first ATG represents the actual start codon, since it is preceded at a reasonable distance by a strong potential ribosome-binding site (position 20–23) which has homology to the 3' end of *Acetobacter* 16S rRNA sequences (Sievers *et al.*, 1994). A stem-loop structure was detected downstream of *aceP*, at positions 1054 to 1080.

A comparison of the deduced amino acid sequence of *aceP* to the sequences in the EMBL/GenBank/DBJ databases revealed pronounced homology with glycosyl transferases from a variety of different organisms: for example, it had 56.54% similarity (33.98% identity) over the entire length of the 321 aa protein to ExoO from *Sinorhizobium meliloti* (previously *Rhizobium meliloti*) strain 2011 (Fig. 2) (Becker *et al.*, 1993), 56.62% similarity (33.44% identity) over 316 residues to ExoO from *Sinorhizobium fredii* strain USDA208 (accession number AF061245), 54.54% similarity (31.49% identity) over a length of 320 residues to ExoU from *Sinorhizobium meliloti* (Glucksmann *et al.*, 1993) and

Fig. 3. Strategy for the construction of an *aceP*⁻ strain of *A. xylinum*. At the top, the plasmid pKE23, described in Methods is drawn schematically. The truncated *aceP* gene fragment was used for integration into the *A. xylinum* genome by a single cross-over. Structure of the chromosomal acetan gene cluster (region of *aceP* gene) before integration is shown above the plasmid, while the structure after integration is shown below (the genes downstream of *aceA* are present, but not shown here). Location and transcription of genes from the plasmid and the chromosome are represented by large arrows and the small vertical arrowhead upstream of *aceA* indicates the position of a putative promoter. The chromosomal genes have been previously reported to encode the following activities: *aceM*, an UDP-glucose dehydrogenase (Griffin *et al.*, 1997a); *aceF*, a bifunctional enzyme with phosphomannose isomerase and GDP-mannose pyrophosphorylase activities (Griffin *et al.*, 1997b); *aceA*, *aceB* and *aceC*, transferase I, II and III of the acetan pathway, respectively, and *aceD* a function required for polysaccharide polymerization and/or export (Griffin *et al.*, 1996a, b).

52.54 % similarity (23.73 % identity) over 309 residues to Cps14J from *Streptococcus pneumoniae* (Klokman *et al.*, 1997). Interestingly, AceP displayed lower homology to AceA (48 % similarity) and AceB (50.44 % similarity), which are two glucosyl transferases involved in acetan biosynthesis in *A. xylinum* (Griffin *et al.*, 1996a, b). ExoO and ExoU have been demonstrated to act as β -D-glucosyl 1 $\rightarrow$ 6 transferases in the pathway for the biosynthesis of succinoglycan in *Sinorhizobium meliloti* (Reuber & Walker, 1993), while AceA and AceB are predicted to catalyse the transfer of β -D-glucosyl 1 $\rightarrow$ 4 linkages. Therefore it is likely that AceP catalyses the transfer of a β -D-glucose 1 $\rightarrow$ 6 linked glucose and, since only one β -D-glucosyl 1 $\rightarrow$ 6 linkage exists in the acetan repeat unit (Fig. 1), AceP is predicted to represent transferase 6 of the acetan pathway. A conserved sequence motif found in the N-terminal region of β -glucosyl transferases (Becker *et al.*, 1998) is located in the N-terminal region of AceP (Fig. 2).

Disruption of the *aceP* gene

To confirm the function predicted for AceP and to specifically engineer a novel acetan-based polymer, disruption of *aceP* was carried out using a truncated-gene strategy (see Methods and Fig. 3). *A. xylinum* CKE5 was transformed with pKE23 DNA and

tetracycline-resistant transformants were selected. As this plasmid is unable to replicate in *A. xylinum*, tetracycline-resistant colonies represent strains in which plasmid DNA has integrated into the chromosome by a homologous-recombination event. Twenty-one transformants were selected and shown to be tetracycline and ampicillin resistant. In two selected strains, P2 and P3, interruption of the *aceP* gene was confirmed by PCR using primers a3 and a4, which anneal to sites on the chromosome outside the fragment of *aceP* cloned in pKE23, in combination with the primers M13F and M13R (Fig. 3). Using chromosomal DNA as a template with primers a3 and a4, a 940 bp fragment was amplified from CKE5 DNA while no product was obtained from P2 and P3 DNA. A 900 bp fragment was amplified from chromosomal DNA from strains P2 and P3 using primer pairs a3+M13R and a4+M13F. No product was amplified from CKE5 DNA with these primer pairs. These data confirm that pKE23 had integrated into the chromosome resulting in a disruption of the *aceP* gene of *A. xylinum* CKE5.

Chemical analysis of EPS from strain P2

The yields of lyophilized, crude polysaccharide from strains P2, CKE5 and C1 of *A. xylinum* were 3.96 g l⁻¹, 4.62 g l⁻¹ and 4.38 g l⁻¹, respectively. It is noticeable that

the yield from the mutant P2 is not drastically reduced compared to that of the CKE5 parent strain. This is contrary to the common belief that disruption of biosynthetic genes drastically reduces the level of polysaccharide produced. This conclusion is largely based on the analysis of mutants produced by random mutagenesis strategies. Identification of mutants in such strategies usually relies on screening for individuals with a small colony morphology and/or a reduced mucoid character. Thus, the choice of screening conditions may force the selection of strains with multiple mutations, i.e. a reduced level of polysaccharide production in addition to disruption of the polysaccharide-assembly process. In the present case, the targeting of a particular gene appears to have only affected polysaccharide assembly and not the level of polysaccharide production.

The lyophilized, crude P2 polysaccharide preparation was found to contain 8.2% carbohydrate and only 0.86% uronic acid. The 6-deoxyhexose test was negative, indicating the absence of any detectable rhamnose. The neutral sugar analysis showed the presence of glucose and mannose in the ratios 1.5:1.0. The analysis of the crude polysaccharide from P2 was compared with that of the parent strain CKE5 which was used as a control. The crude preparation from CKE5 contained 19.7% carbohydrate and 2.6% uronic acid, and a positive 6-deoxyhexose test indicated the presence of rhamnose. Linkage analysis of the crude preparations from strains C1 (cellulose⁻, acetan⁺) and CKE5 confirmed that the CKE5 parental strain produced acetan: the linkage analysis showed the presence of t-rhamnose; 1,2-mannose; 1,6-glucose; 1,4-glucose; 1,3,4-glucose plus t-mannose and 1,2,6-mannose. The t-rhamnose; 1,2-mannose; 1,6-glucose; 1,4-glucose and 1,3,4-glucose are, together with the positive test for uronic acid, consistent with the acetan structure. Linkage analysis after carboxy reduction revealed the presence of additional deuterated 1,4,6-glucose indicating the presence of 1,4-glucuronic acid in the original C1 and CKE5 extracts. These studies confirmed the presence of acetan. The additional linkages (t-mannose, 1,2,6-mannose plus some 1,2-mannose) are due to the presence of a neutral branched mannan. These contributions are reduced after CTAB purification of the crude polysaccharide, a procedure which preferentially isolates charged polysaccharides from neutral polysaccharides.

Having established that the CKE5 parent strain produces acetan it was then possible to analyse the product of the new P2 strain to assess the effect of genetic modification on the structure of the polysaccharide produced. On purification by CTAB precipitation, the crude polysaccharide from P2 (1.13 g) yielded 75 mg of lyophilized material. This contained $82 \pm 2\%$ (w/w) carbohydrate (as glucose) and 27% uronic acid. The presence of the uronic acid leads to an underestimate of the total level of carbohydrate by about 20% because glucuronic acid has a lower response factor than glucose. Thus, the CTAB preparation was almost pure polysaccharide. Neutral sugar analysis showed an increase in the ratio of glucose:mannose to 3.9:1.0. The methylation analysis

Table 1. Linkage analysis of extracellular polysaccharides from *A. xylinum* P2.

CTAB, CTAB purified; C-red, carboxy reduced; red.cl, reductive cleavage analysis.

Linkages Molar ratio in ...	Crude	CTAB	CTAB C-red	CTAB red.cl*
t-Rha	—	—	—	—
t-Man	1.9	—	—	—
t-Glc	1.4	1.8	1.8	1.3
1,2-Man	1.7	0.83	2.5	1.6
1,4-Glc	1.0	1.0	1.0	1.0
1,3,4-Glc	0.69	0.50	0.68	1.1
1,4-GlcA	—	—	0.95	2.1
1,2,6-Man	0.87	—	—	—

* Not corrected using response factors.

for the P2 polysaccharide is shown in Table 1. The crude preparation shows the presence of the linkages t-mannose; t-glucose; 1,2-mannose; 1,4-glucose; 1,3,4-glucose and 1,2,6-mannose. CTAB purification eliminates t-mannose and 1,2,6-mannose, and reduces the content of 1,2-mannose. This can be explained by the removal of the neutral mannan material. The linkage analysis following carboxy reduction reveals the presence of 1,4-glucuronic acid due to the appearance of deuterated 1,4,6-glucose residues. No t-rhamnose residues were detected but there is a t-Glc residue instead. These conclusions are consistent with the proposed repeat unit shown in Fig. 1c. The basic carbohydrate repeat unit is a pentameric structure. The polysaccharide consists of a cellulosic backbone substituted on every second glucose residue with a charged trisaccharide side-chain terminating in a glucose residue. These data are consistent with the production of acetan by strain CKE5 and the production of a truncated acetan structure by strain P2. They demonstrate that disruption of *aceP* alters the structure of the extracellular polysaccharide, and the chemical analysis is consistent with formation of a truncated pentameric rather than heptameric repeat unit. The chromatograms for the CTAB-purified P2 and acetan are shown in Fig. 4. The experimental retention times on the columns are dependent on the detailed operating conditions and need to be corrected on the basis of known standards (not shown in Fig. 4). The relative retention times and mass-spectrometric data are used to assign the peaks. The linkage analysis of the P2 polysaccharide using the reductive cleavage method (Table 1) was particularly useful for several reasons. Firstly, it confirmed the linkage pattern revealed by conventional methylation analysis. Secondly, the retention times of the 1,5-anhydroalditol derivatives are distinct and identify the terminal sugar unambiguously as t-glucose. Thirdly, it also confirms the rings as pyranoses [except for glucuronic acid which rearranges to the furanose form during reductive cleavage (Vodonik & Gray, 1988)].

Fig. 4. Gas chromatograms of the PMAAs of CTAB-purified (a) acetan (Ojinaka *et al.*, 1996) and (b) P2 polysaccharide. The PMAAs correspond to the following sugar residues: 1, t-rhamnose; 2, t-glucose; 3, 1,2-mannose; 4, 1,6-glucose; 5, 1,4-glucose; 6, 1,3,4-glucose; 7, 1,4-glucuronic acid. The GC column was replaced between the two runs shown in (a) and (b), explaining the difference in retention time for equivalent residues. Identification of particular residues is based on the use of standards and mass spectrometric data.

DISCUSSION

Genetic manipulation of *Acetobacter* strains is widely known to be impaired by the low frequencies of transformation experienced with this species. In our case, the problems could not be overcome using procedures reported to be successful in other strains (Fukaya *et al.*, 1985; Valla *et al.*, 1986; Inoue *et al.*, 1985; Hall *et al.*, 1992; Wong *et al.*, 1990). In addition, the problem did not seem to be related to the presence of a restriction-modification system as described by Petroni *et al.* (1996) and Coucheron (1998), since plasmid DNA prepared from strain C1 did not result in higher electrotransformation frequencies of strain C1 when compared to frequencies obtained using DNA isolated from *Escherichia coli* (data not presented). The 10000-fold increase in electrotransformation frequency obtained with strain CKE5 compared to that of the wild-type and mutagenized strains was an essential prerequisite to the use of a suicide delivery vehicle for gene disruption

experiments. The basis of this increased frequency is unclear and may indicate that individual transformants (before curing) represented mutants with an enhanced ability to take up DNA. However, there are reports in the literature that many strains of *Acetobacter* contain cryptic plasmids, which may be incompatible with an incoming plasmid. Such a plasmid could also have been lost during the plasmid-curing regime. Although no cryptic plasmids were detected in our strain C1, we cannot rule out the possibility of one existing. Analysis of the polysaccharide produced by strain CKE5 confirmed that acetan biosynthesis was not altered by the mutagenesis or plasmid-curing procedure, making it ideally suitable for genetic manipulation studies.

AceP displayed a characteristic conserved motif in the N-terminal region, common to β -glycosyltransferases that use an α -linked nucleotide diphospho-sugar donor (in this case α ,D-UDP-glucose) in the transfer of the sugar to an acceptor (the growing repeat unit) to form a β -linked product (Saxena *et al.*, 1995; Becker *et al.*, 1998). These enzymes are members of the 'inverting' family of glycosyl transferases, as the reaction proceeds via inversion of the anomeric configuration at the reaction centre where a single nucleophilic substitution at the anomeric centre is sufficient to generate the β configuration (Sinnott, 1990; Saxena *et al.*, 1995). That AceP had a higher identity with ExoO and ExoU from *Sinorhizobium meliloti* than to AceA and AceB from the acetan pathway in *A. xylinum* led to the prediction that it catalyses the transfer of a β -D-glucose 1 $\rightarrow$ 6 linked glucose; i.e. step 6 of the acetan pathway involving linkage to a β -D-glucose linked 1 $\rightarrow$ 4 to β -D-glucuronic acid (Fig. 1a). Interestingly, ExoO, to which AceP had highest identity, also transfers a 1 $\rightarrow$ 6-linked β -D-glucose to β -D-glucose-(1 $\rightarrow$ 4)-glucuronic acid in the growing succinoglycan chain. It is tempting to speculate that the specificity of these transferase enzymes may be influenced not only by the sugar composition of the acceptor residue, but also by the micro-environment in which that residue is found. If this is found to be true, it could have important implications for genetic manipulation of EPS structure by heterologous gene expression.

Disruption of *aceP* confirmed the function predicted from the homology studies above. The disruption strategy did not result in polar effects on the expression of the acetan operon, since expression of the downstream genes *aceA*, *aceB* and *aceC* (Fig. 3) encoding transferases I, II and III, respectively (Griffin *et al.*, 1996a, b), was unaffected. Surprisingly, the yield of crude EPS obtained from the *aceP* mutant strain (P2) was close to that produced by wild-type strains. In our previous study, crude yields of a novel acetan-based polymer produced by strain CR1/4 were only 33% of those of the parent acetan-producing strain (MacCormick *et al.*, 1993); purified yields were approximately 10% of the parent yields (M. Ridout, pers. comm.). Others have also reported the yields as low as 1% those of wild-type for strains synthesizing novel polymers (Vanderslice *et al.*, 1990). Thus disruption of targeted genes may only influence polysaccharide as-

sembly and perhaps not affect the level of polysaccharide produced.

The results of the chemical analysis of the polysaccharide from the CKE5 strain are consistent with the production of acetan by this strain. The P2 polysaccharide, however, is clearly identified as being chemically distinct from that produced by the parent CKE5. The linkage analysis of CTAB-purified P2 polymer shows all the sugar residues expected to be present if the acetan has been truncated at the 1)- α -D-glucose-(6-1)- $\downarrow$ - β -D-glucose glycosidic linkage. This is consistent with blocking the biosynthetic pathway at transferase 6. Full structural characterization of the P2 polymer by NMR is under way to confirm the proposed structure by determining the sequence of sugars and the non-carbohydrate substitution of this novel polymer.

All possible variants of the acetan structure can be genetically engineered in this system for use in analysis of the polysaccharide structure-function relationship. In the longer term, heterologous gene expression could be used to engineer novel sugars or linkages in the acetan repeat unit.

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