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Expression and biochemical characterisation of recombinant AceA, a bacterial α-mannosyltransferase

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Abstract Biosynthesis of repeat-unit polysaccharides and N-linked glycans proceeds by sequential transfer of sugars from the appropriate sugar donor to an activated lipid carrier. The transfer of each sugar is catalysed by a specific glycosyltransferase. The molecular basis of the specificity of sugar addition is not yet well understood, mainly because of the difficulty of isolating these proteins. In this study, the *aceA* gene product expressed by *Acetobacter xylinum*, which is involved in the biosynthesis of the exopolysaccharide acetan, was overproduced in *Escherichia coli* and its function was characterised. The *aceA* ORF was subcloned into the expression vector pET29 in frame with the S·tag epitope. The recombinant protein was identified, and culture conditions were optimised for production of the soluble protein. The results of test reactions showed that AceA is able to transfer one α-mannose residue from GDP-mannose to cellobiose-P-P-lipid to produce α-mannose-cellobiose-P-P-lipid. AceA was not able to use free cellobiose as a substrate, indicating that the pyrophosphate-lipid moiety is needed for enzymatic activity.

Key words Bacterial polysaccharide · Glycosyltransferase · Acetan · *Acetobacter xylinum* · Biosynthesis

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Introduction

Many bacterial cell surface polysaccharides [O-antigens, capsular polysaccharides (CPS), and exopolysaccharides (EPS)] consist of repeats of an oligosaccharide unit (for a review see Whitfield and Valvano 1993). Some of these polysaccharides have been shown to possess biological activity, e.g. *Rhizobium meliloti* succinoglycan is involved in symbiosis (Leigh and Walker 1994; van Rhijn and Vanderleyden 1995), xanthan plays a role in plant pathogenesis by *Xanthomonas campestris* (Katzen et al. 1998), and CPS serves as a virulence factor in *Streptococcus pneumoniae* (Watson and Musher 1990). Some EPSs display particular rheological properties in solution, making them attractive for industrial use (Becker et al. 1998; Sutherland 1998).

Assembly of the repeating oligosaccharide subunit occurs by sequential addition of the sugars to a lipid-carrier intermediate (polyprenol-phosphate, hereafter referred to as P-lipid) by specific enzymes. The first step consists of the transfer of the glycosyl-1-phosphate from the corresponding nucleotide sugar to the P-lipid by a specific glycosyl-1-phosphate transferase, forming the glycosyl-P-P-lipid. The following sugars are sequentially transferred by specific glycosyltransferases, using the corresponding nucleotide sugars. The repeating unit is presumably translocated to the periplasmic face of the membrane and polymerised there. The polymer is ligated to a membrane-bound acceptor or secreted in a process that is not well understood. In recent years, the genetic loci that encode the enzymes of many polysaccharide biosynthetic pathways have been cloned and sequenced (for reviews on enterobacterial LPS see Schnaitman and Klena 1993; Heinrichs et al. 1998). In most cases putative functions were indirectly assigned to the gene products involved in the assembly of the lipid-bound oligosaccharide, by characterisation of radio-labelled repeat units formed either by mutant strains of the native organism or in heterologous strains harbouring different combinations of genes (Reuber and Walker

1993; Katzen et al. 1998; van Kranenburg et al. 1999). These studies were performed using permeabilised cells. Few of the glycosyltransferases have been studied using cell-free systems (Liu et al. 1993; Lellouch and Germia 1999).

To date very little is known about the structure of glycosyltransferases and their mechanism of action, as only one X-ray structure has been solved (Vrielink et al. 1994). Techniques of sequence comparison have been used to search for conserved motifs in glycosyltransferases and have revealed a DXD motif that is conserved in several families and is essential for activity (Breton et al. 1998; Busch et al. 1998; Wiggins and Munro 1998). This motif has been proposed to bind the pyrophosphate moiety of the nucleotide sugar donor, using a bridging divalent cation such as Mg^{2+} or Mn^{2+}.

Acetan is an EPS produced by *Acetobacter xylinum* (Couso et al. 1987), composed of glucose (Glc), mannose (Man), glucuronic acid and rhamnose in the ratio 4:1:1:1. The heptasaccharide-repeating unit is depicted in Fig. 1A. The polymer consists of a cellulose backbone with alternating pentasaccharide branches (Jansson et al. 1993). Structurally acetan is closely related to xanthan. Xanthan is made up of pentasaccharide repeating units (Fig. 1B) and consists of a cellulose backbone with alternating branches of trisaccharides (Jansson et al. 1975). In both cases the biosynthetic steps leading to the assembly of the lipid-linked oligosaccharides and polymerisation have been studied using permeabilised or electroporated cells (Couso et al. 1982; Ielpi et al. 1993; Semino and Dankert 1993). While the genetic organisation of the xanthan biosynthetic cluster is well known (for a review see Becker et al. 1998), little is known about acetan biosynthesis. A putative polysaccharide-related gene cluster has been cloned from *A. xylinum* and partially sequenced, but no biochemical evidence has been presented to show that it encodes enzymes involved in the biosynthesis of acetan (Griffin et al. 1996a, 1996b, 1997).

Recently Petroni and Ielpi (1996) isolated and characterised the gene *aceA*, by complementation of a *X. campestris* mutant that is deficient in a homologous gene, *gumH*. The *gumH* gene product (Katzen et al. 1998) is thought to catalyse the addition of an α-(1–3) mannose residue to the cellobiose-pyrophosphate-polyprenol (hereafter referred to Glc_2-P-P-lipid) (Fig. 1C). The *aceA* ORF codes for a 393-amino acid protein (molecular weight 43,219 , pI 9.7), which is 42% identical to GumH (Petroni and Ielpi 1996). AceA displays sequence similarity with other bacterial α-mannosyltransferases involved in the assembly of the *Salmonella* O antigen subunit, as well as with other prokaryotic and eukaryotic glycosyltransferases, such as LpcC – a mannosyltransferase from *R. leguminosarum* that is involved in LPS core biosynthesis – or the human PigA, which is involved in the biosynthesis of the phosphatidylinositol anchor of lipid-bound proteins and is mutated in individuals who suffer from paroxysmal nocturnal haemoglobinuria (Geremia et al. 1996; Petroni and Ielpi 1996; Breton et al. 1998). The sequence similarity among these proteins is strongest in two regions located in their C-terminal halves; one region contains a conserved EX_7E motif, while in the other a lysine residue is conserved. However a detailed study by hydrophobic cluster analysis revealed

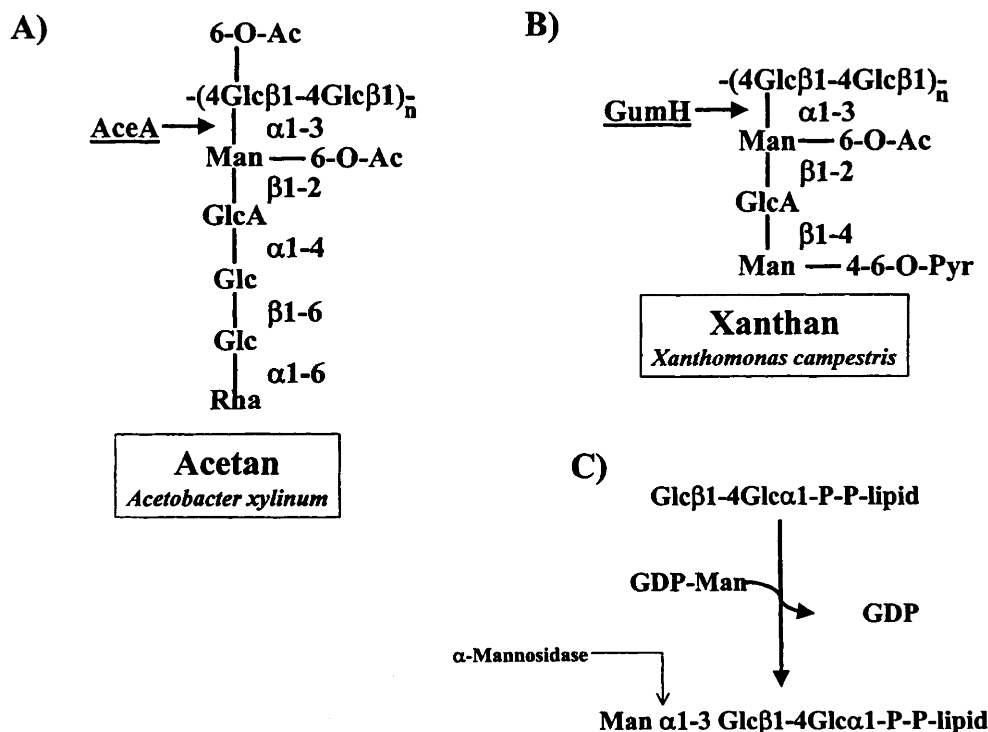

Fig. 1 **A** Structure of acetan. The step presumably catalysed by AceA is indicated. **B** Structure of xanthan. The step thought to be catalysed by GumH is indicated. **C** Reaction presumably catalysed by AceA. The *arrow* shows the site of action of jackbean α-mannosidase

that the similarity extends also to the N-terminal half (Geremia et al. 1996) of the proteins. Sequence similarity in a region including the EX_7E motif is also found in sucrose (phosphate) synthases, and glycogen synthases (Geremia et al. 1996; R. Geremia, unpublished results), which are also α-glycosyltransferases. Although the significance and role of this motif remains to be established, it has been suggested that the conserved residues are involved in catalysis (Geremia et al. 1996). The DXD motif is absent in the family that includes AceA (Breton et al. 1998), which led us to speculate that the pyrophosphate moiety may bind to these glycosyltransferases via a different sequence motif(s).

In spite of recent progress in gene isolation and identification, the molecular basis for the diversity of bacterial polysaccharides is not well understood, due mainly to the lack of structural data for glycosyltransferases. The low expression level of these enzymes precludes direct purification from the natural producers, for subsequent biochemical studies. An alternative strategy for the production of sufficient amounts of protein involves overexpression in *Escherichia coli*, as has been done for glycosyltransferases involved in biosynthesis of the core oligosaccharide of lipopolysaccharide (Gilbert et al. 1996; Wakarchuk et al. 1996, 1998; Kadrmas and Raetz 1998; Kadrmas et al. 1998). In this paper we report the production of an active, soluble form of recombinant AceA, and the characterisation of the in vitro reaction product.

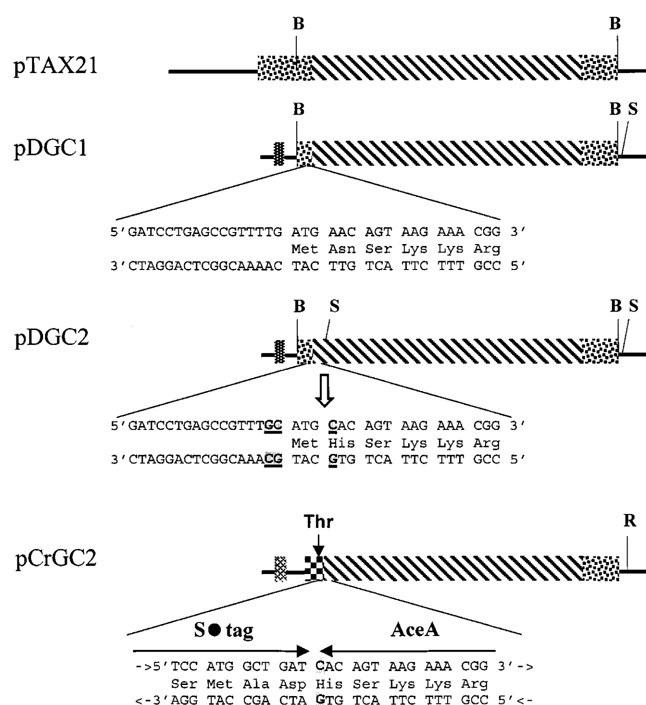

Fig. 2 Construction of pCrGC2. The construction of the plasmids is described in Materials and methods. The predicted N-terminal sequence of AceA and the site of the fusion between AceA and the S·tag are shown. Bases that were mutated to create the *Sph*I site (*stippled*) are *underlined*. B, *Bam*HI; S, *Sph*I; R, *Eco*RI. Thr is the thrombin cleavage site contributed by the S·tag. The *aceA* ORF is indicated by *hatching*, the flanking *A. xylinum* DNA by the *dispersed black squares*, P_{Lac} in pDGC1 and pDGC2, and P_{T7} in pCrGC2 are indicated by the different *chequered patterns*, the S·tag in pCrGC2 is depicted by *the chessboard pattern*, and the 5′ end of the blunted *Sph*I fragment is indicated by the *vertical arrow*

Materials and methods

General procedures

DNA subcloning was performed in *E. coli* XL1-Blue cells (Stratagene, La Jolla, Calif.). *E. coli* XL1-Blue and BL21(DE3)/pLysS (Novagen, Madison, Wis.) were grown in LB (Sambrook et al. 1989) on a rotary shaker at 37° C and 210 rpm unless otherwise indicated. *X. campestris* was grown in YM medium (3 g of malt extract, 3 g of yeast extract, 5 g of bactopeptone and 10 g of glucose/l). Antibiotics were used at the following concentrations: kanamycin (Km), 30 μg/ml; ampicillin (Amp), 100 μg/ml; chloramphenicol (Cm) 34 μg/ml. GDP-[U-^{14}C]mannose, UDP-[U-^{14}C]glucose, the USE mutagenesis kit, and Ligation and Blunting kits were from Amersham Pharmacia Biotech (Les Ulis, France). The pET29a expression vector and the S·tag Western Blot kit were from Novagen. Restriction enzymes were from New England Biolabs (Beverly, Mass.). SDS/PAGE and other molecular biological procedures were performed according to established protocols (Sambrook et al. 1989). All other chemicals were obtained from Sigma (L'isle d'Abeau Chesnes, France). Prediction of putative transmembrane domains in AceA was done using the Dense Alignment Surface method (Cserzo et al. 1997) available at http://www.biokemi.su.se/~server/DAS/.

Construction of pCrGC2

Construction of pCrGC2 is shown in Fig. 2. The ORF coding for AceA (Genbank Accession No. U37258) was subcloned from pTAX21 (Petroni and Ielpi 1996) into pUC18, to obtain pDGC1, as follows: a *Bam*HI fragment of 1254 bp encompassing the *aceA* ORF, 18 bp of upstream and 30 bp of downstream sequence, and a 20-bp segment from the polylinker of pBCSK+, was subcloned into the *Bam*HI site of the pUC18 multiple cloning site (under the control of P_{Lac}). A *Sph*I site overlapping the start ATG codon of *aceA* was created by site-directed mutagenesis using 5′-GTTTCTTACTGT**G**CAT**GC**AAACGGCTCAGGATC-3′ (Oligo-Express, Paris, France) as a mutagenic primer (the mutated bases are indicated in bold), thus generating pDGC2. To construct pCrGC2 a 1277-bp *Sph*I fragment encompassing *aceA* was blunted and cloned into the *Eco*RV site of pET29a, generating a protein fusion with the S·tag (AceA-S·tag). A unique thrombin cleavage site is present in the fusion peptide, facilitating removal of the S·tag peptide from AceA, if required.

Expression of AceA-S·tag

E. coli BL21(DE3) or BL21(DE3)/pLysS cells were transformed with pCrGC2, or with pET29 as a control. To optimise the expression, cells were grown at 37° C in 20 ml of LB until an OD_{600} value of 0.5, 0.8, 1.5, 2.0 or 2.5 was reached, at which point protein expression was induced by adding 0.5 mM IPTG; the cultures were subsequently incubated at 37° C, 30° C or 17° C. Aliquots (2 ml) of the culture were withdrawn at various times after induction. Cells were collected by centrifugation, resuspended in denaturing buffer, and subjected to SDS-PAGE on 10% gels. Gels were stained with Coomassie Brilliant Blue R250. For detection of the S·tag epitope, the proteins were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and treated with the Novagen S·tag Western Blot kit, using alkaline phosphatase S-protein conjugate for detection of the tagged protein according to the manufacturer's instructions.

Preparation of cell extracts and subcellular fractionation

For determination of enzymatic activity, *E. coli* BL21(DE3)/pLysS cells harbouring either pCrGC2 or pET29 were grown in 500 ml of LB at 37° C. When the OD_{600} value reached 0.8, IPTG was added, and cells were grown for 2 h at 37° C. The cells were collected by centrifugation, washed once with 70 mM TRIS-HCl pH 8, resuspended in 70 mM TRIS-HCl, 10 mM EDTA, pH 8.2 at 40 OD equivalents/ml and disrupted by three passages through a French press (at 18,000 psi). The lysate (approximately 5 ml) was submitted to sequential centrifugation at $2000 \times g$ (5 min), $15,000 \times g$ (15 min), and $100,000 \times g$ (2 h). The pellets from each centrifugation step were resuspended in 5 ml of buffer (70 mM TRIS-HCl, 10 mM EDTA, pH 8.2).

Protein sequence determination

Cell extracts of *E. coli* BL21(DE3)/pCrGC2 were digested with thrombin (1.2 U; restriction grade, Novagen) in the buffer provided with the enzyme, for 16 h at 20° C. Proteins separated by SDS-PAGE were electrophoretically transferred onto PVDF (Matsudaira 1987) according to the protocol for ProBlott membranes (Applied Biosystems, Foster City, Calif.). Amino acid sequences were determined using a gas-phase Sequencer Model 477A (Applied Biosystems). Phenylthiohydantoin amino acid derivatives were identified and quantitated on-line with a Model 120A HPLC system (Applied Biosystems) as recommended by the manufacturer.

Preparation of glycolipid acceptors

Permeabilised *X. campestris* NRRL B-1459 cells were used for in vitro synthesis of cellobiose-P-P-lipid. These cells were prepared as described previously (Ielpi et al. 1993). Briefly, 100 ml of YM was inoculated with 10 ml of a stationary-phase culture of *X. campestris*, and incubated for 20–26 h. Cells were collected by centrifugation, washed with 70 mM TRIS-HCl pH 8.2, 10 mM EDTA, resuspended in 5 ml of the same buffer and submitted to three cycles of freezing and thawing. The permeabilised cells were recovered by centrifugation, and resuspended in 1.5 ml of 70 mM TRIS-HCl, 10 mM EDTA, pH 8.2. Aliquots of the suspension were stored at −20° C. To prepare the polyprenol-linked intermediates, 30 μl of permeabilised cells was incubated in the presence of 8.5 mM $MgCl_2$, 25 mM TRIS-HCl pH 8.2, UDP-Glc, and GDP-Man, either labelled or unlabelled [4 mM UDP-Glc, 0.135 μCi of UDP-[$U^{14}C$]Glc, (specific activity 231 Ci/mol); 0.14 mM GDP-Man, 0.25 μCi GDP-[$U^{14}C$]Man, (specific activity 301 Ci/mol)]. Incubation was carried out at 20° C for 30 min. The reaction was stopped by adding 0.2 ml of 70 mM TRIS-HCl pH 8.2, 10 mM EDTA. Cells were recovered by centrifugation, and washed twice with the same buffer. The acceptor was recovered by extracting the cells three times with 150 μl of chloroform:methanol:water (1:2:0.3), as previously described (Couso et al. 1982). The extract containing the acceptor was dried to a volume of 10 μl, and stored at −20° C until use.

Mannosyltransferase assay

The reaction mixture (100 μl) contained: the *E. coli* $100,000 \times g$ supernatant (200 μg of total protein), 1% Triton X-100, 6 mM $MgCl_2$, 10 μl of Glc_2-P-P-lipid acceptor, and GDP-Man, either labelled or unlabelled as indicated. Incubation was carried out at 37° C for 45 min. The reaction was stopped by the addition of 100 μl of water and 200 μl of a 1:1 mixture of chloroform and methanol. The lipid-bound oligosaccharides were isolated from the organic phase and protein pellet as previously described (Lellouch and Geremia 1999). The oligosaccharide was released by mild acid hydrolysis (in 200 μl of 0.01 N aqueous trifluoroacetic acid for 10 min at 100° C). The hydrolysate was neutralised with 2 μl of 1 M NH_4OH, and digested with 5 U of bovine alkaline phosphatase (at 37° C overnight) in the presence of 10 mM glycine-NaOH pH 9 and 10 mM $MgCl_2$ to remove any remaining sugar-linked phosphates. The released lipid moiety was recovered by extraction with 0.5 ml of chloroform:methanol (2:1), and salts were removed by incubation with the mixed-bed resin TM8. The aqueous phase containing the released oligosaccharides was concentrated, resuspended in a volume of 10 μl, and subjected to thin-layer chromatography (TLC). For competition assays, 1 mM cellobiose (Glc_2) was added to the reaction mixture. For the assays using Glc_2 (1 mM) as the sole acceptor, Glc_2-P-P-lipid was omitted from the mixture, and the reaction was stopped by adding 200 μl of water. Unreacted GDP-[^{14}C]Man was removed using DEAE-Sephadex as previously described (Geremia et al. 1994).

α-Mannosidase treatment

For α-mannosidase treatment, the glycolipid product was hydrolysed and de-phosphorylated as described above. The digestion was performed in a buffer containing 0.1 M citrate buffer pH 4.5, 0.2 U of jackbean α-mannosidase (M7257, Sigma), at 20° C for 12 h. The sample was desalted and delipidated as indicated above, and analysed by TLC.

Thin-layer chromatography and fluorography

Samples (5000–10,000 cpm in 5–10 μl) were supplemented with 50 μg of malto-oligosaccharides (dimer to pentamer), and spotted on a TLC plate (silica gel 60, 0.25 mm; Sigma). Glc, Man, Glc_2, maltose, and the malto-oligosaccharides were used as standards. The TLC plate was developed twice in 1-propanol:nitromethane:water (50:20:20 v/v). Standards were revealed by charring after treatment with 5% H_2SO_4 in ethanol. Radioactivity was detected by fluorography, using 0.4% 2, 5 diphenyloxazole (PPO) in 2-methylnaphtalene as scintillator. Photographic films (Hyperfilm *β*max, Amersham Pharmacia Biotechnology), were exposed for 24–96 h before development.

Results and discussion

Production of recombinant S·tag-AceA

Previous studies of AceA were based on assays carried out using a whole-cell system, in which the Glc_2-P-P-Lip was produced in vivo by the reporter strain, e.g. a *X. campestris gumH* mutant. Xanthan production, as well as Man-Glc_2-P-P-Lip synthesis was restored in the *gumH* strain harbouring *aceA*. However, the activity of AceA was not assessed in a system lacking endogenous acceptor. To test the enzymatic activity of AceA in a cell-free assay, the *aceA* gene product was overexpressed in *E coli*. The AceA ORF was subcloned into pET29a, to yield pCrGC2 (see Materials and methods, Fig. 2). This plasmid encodes the fusion protein S·tag-AceA (423 amino acids, 46.5 kDa), under the control of the T7 promoter. The SDS/PAGE protein profile of the *E. coli* BL21(DE3)/pCrGC2 strain was studied before and after induction with IPTG (Fig. 3). A prominent band of about 46 kDa was present in the IPTG-induced cell extract, but absent in the *E. coli* BL21(DE3)/pET29 extracts. Western analysis showed that this protein contains the S·tag (data not shown). In order to firmly establish that the 46-kDa protein corresponded to S·tag-AceA, the S·tag was cleaved by digestion with thrombin. SDS/PAGE revealed the presence of a novel peptide of

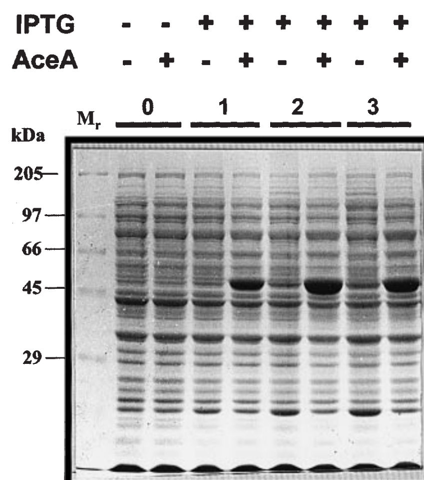

Fig. 3 Production of AceA-S·tag. *E. coli* BL21(DE3) cells harbouring either pET29 (AceA−) or pCrGC2 (AceA+) were grown as described in Materials and methods. When the OD_{600} value reached 0.8, 0.1 mM IPTG was added, and the culture was incubated further at 37° C. Samples (2 ml) were withdrawn before (0) and at various times (1, 2, or 3 h) after addition of IPTG. Proteins were stained with Coomassie Brilliant Blue. The leftmost lane contains molecular weight markers

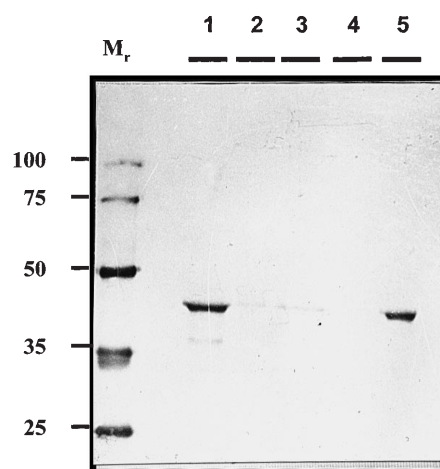

Fig. 4 Subcellular location of AceA-S·tag in *E. coli*. *E. coli* BL21(DE3)/pLysS cells harbouring pCrGC2 were grown, disrupted, and fractionated, as described in Materials and methods. Samples from each fractionation step were separated by SDS-PAGE and transferred to PVDF membranes. The AceA-S·tag was detected using alkaline phosphatase-conjugated S-protein as probe. Lane 1, total cell extract; lane 2, $2000 \times g$ pellet; lane 3, $5000 \times g$ pellet; lane 4, $100{,}000 \times g$ pellet; lane 5, $100{,}000 \times g$ supernatant. The leftmost lane contains molecular weight markers

43.5 kDa. The N-terminal sequence of this peptide was found to agree with that of the expected fusion protein (data not shown). The best cell density for induction of the recombinant protein was equivalent to 0.8 OD_{600} units, and optimal production was attained 2 h after addition of IPTG (data not shown).

Production of soluble AceA-S·tag

AceA-S·tag was first produced by using *E. coli* BL21(DE3)/pCrGC2 cells. However, differential centrifugation to investigate the subcellular localisation of the recombinant protein revealed that AceA-S·tag was found exclusively in the $2000 \times g$ and $15000 \times g$ pellets, strongly suggestive of the formation of inclusion bodies. As native protein is preferable for biochemical studies, we sought conditions that would yield the native form of the protein. First, pCrGC2 was introduced into the *E. coli* BL21(DE3)/pLysS strain. pLysS encodes the T7 lysozyme, which allows tighter control of the expression of proteins driven by the T7 promoter (Studier et al. 1990). Secondly, since previous results showed that the best optical density for induction was 0.8, and that production was optimal 2 h after IPTG addition, these conditions were used to obtain the native protein. Cell extracts of the *E. coli* BL21(DE3)/pLysS/pCrGC2 strain were analysed on Western blots (Fig. 4). The AceA-S·tag was not detected in the pellets obtained by low-speed centrifugation ($2000 \times g$ and $15{,}000 \times g$) or in the membrane fraction ($100{,}000 \times g$ pellet), but remained in the $100{,}000 \times g$ supernatant, strongly suggesting a cytosolic location. It has previously been reported that AceA contains a putative transmembrane region (Petroni and Ielpi 1996). The DAS prediction method predicts a transmembrane alpha-helix in AceA spanning amino acids 298–306, with a score of 1.7. The presence of the S·tag does not change the prediction. It was therefore surprising to find AceA-S·tag in the soluble fraction.

Many glycosyltransferases involved in the biosynthesis of polysaccharides have potential transmembrane domains (Whitfield and Valvano 1993). The involvement of membrane-bound multi-protein complexes in the biosynthesis of polysaccharides has previously been suggested (Whitfield and Valvano 1993; Leigh and Walker 1994). An interesting case is that of the mannosyltransferases WbaU, WabW and WabZ from *Salmonella enterica*. These proteins do not have predicted transmembrane regions; however, their enzymatic activity is located in a membrane fraction (Liu et al. 1993). These proteins may bind to membranes either by electrostatic interaction with phospholipids, via non-classical transmembrane domains or through interaction with other membrane-bound biosynthetic proteins, forming a multiprotein complex. In the case of AceA, the protein expressed in *E. coli* was found in the soluble fraction despite the putative presence of a transmembrane helix. Since the participation of cytoplasmic proteins in the putative biosynthetic complex has already been proposed (Whitfield and Valvano 1993), it is tempting to speculate that AceA forms part of such a membrane-associated complex. Establishing the location of AceA in *A. xylinum* will shed more light on this question.

In vitro activity of AceA-S·tag

Analysis of the biosynthetic intermediates produced by the *X. campestris gumH* mutant harbouring *aceA*

(Petroni and Ielpi 1996), suggested that AceA is an α-mannosyltransferase that uses GDP-Man as the sugar donor, and cellobiose-P-P-lipid as acceptor. In order to confirm this prediction, the enzymatic activity of recombinant AceA-S·tag was investigated in vitro by incubation of extracts containing S·tag-AceA together with the predicted substrates. Since the pure putative acceptor is not commercially available, and chemical synthesis is difficult, we used a glycolipid extract from *X. campestris* that is enriched in the acceptor (see Materials and methods). Permeabilised *X. campestris* cells incubated with UDP-[^{14}C]Glc as the only sugar donor accumulate mainly Glc_2-P-P-lipid in the organic extract. A lower amount of Glc-P-P-lipid is also produced. A detailed analysis of Glc_2-P-P-lipid has previously been reported (Ielpi et al. 1981).

Cell extracts *of E. coli* BL21(DE3)/pLysS harbouring either pCrGC2 or pET29 were incubated with GDP-[^{14}C]Man in the presence or absence of a *X. campestris*-lipid acceptor. The incorporation of [^{14}C]Man into the organic phase was determined (Table 1). Radioactivity was systematically excluded from the organic phase when extracts of *E. coli* BL21(DE3)/pLysS/pET29 cells were used, indicating that no homologous mannosyltransferase activity is present in *E. coli*. Radioactivity was also absent from the organic phase when *E. coli* BL21(DE3)/pLysS/pCrGC2 extracts were incubated in the absence of added *X. campestris* lipid acceptor, indicating that there is no endogenous acceptor for AceA in *E. coli*. In contrast, radioactivity was incorporated into the organic phase in reaction mixtures containing both AceA-S·tag and glycolipid acceptor, indicating that AceA is a mannosyltransferase. The oligosaccharides were released from the lipid anchor into the aqueous phase by mild acid hydrolysis using 0.01 N TFA, indicating that the sugars are bound to the lipid moiety by a pyrophosphate bridge (Couso et al. 1982). This behaviour is similar to that of the authentic Man-Glc_2-P-P-lipid produced by *X. campestris* (Ielpi et al. 1993). The released [^{14}C] oligosaccharide was analysed by TLC. A major radioactive product that comigrates with authentic Man-Glc_2 was detected (Fig. 5A), indicating that the lipid-linked carbohydrate is a trisaccharide containing Man.

[^{14}C]Glc_2-P-P-lipid was prepared in the *X. campestris* system in order to confirm that it is indeed the acceptor

Table 1 Incorporation of [^{14}C]Man into organic extracts

E. coli strain	Glycolipid acceptor	Man (pmol/min per mg protein)[a]
BL21(DE3)/pLysS/pET29	−	≤0.001
BL21(DE3)/pLysS/pCrGC2	−	≤0.001
BL21(DE3)/pLysS/pET29	+	≤0.001
BL21(DE3)/pLysS/pCrGC2	+	1.1

[a] Cell extracts from the indicated strains were incubated in the presence or absence of glycolipid acceptor. Lipid-linked products were extracted as described Materials and methods, and the level of radioactivity was determined

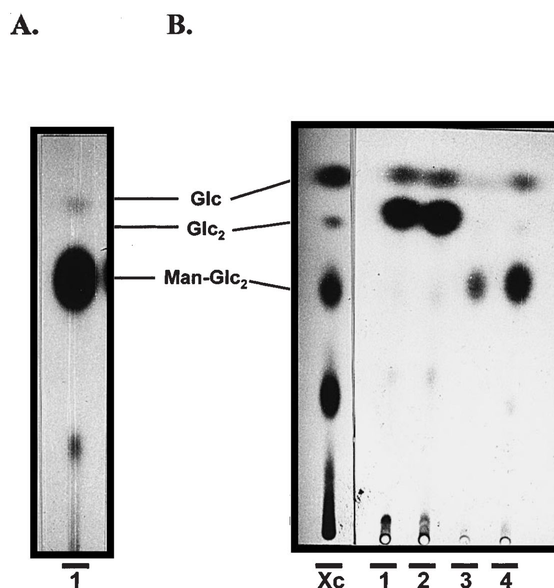

Fig. 5A, B Thin-layer chromatography of oligosaccharides formed in vitro by the action of AceA. **A** The 100,000 × *g* supernatant obtained by fractionation of BL21(DE3)/pLysS/pCrGC2 cells (lane 1) was incubated in the presence of unlabeled Glc_2-P-P-Lip and GDP-[^{14}C]Man. **B** The 100,000 × *g* supernatants from BL21(DE3)/pLysS/pET29 (lanes 1 and 2) and BL21(DE3)/pLysS/pCrGC2 (lanes 3 and 4) cells were incubated in the presence of [^{14}C]Glc_2-P-P-Lip and unlabeled GDP-Man. Oligosaccharides were extracted and released from the lipid as described in Materials and methods. Lipid-linked oligosaccharides from *X. campestris* were used as standards (lane Xc)

of the Man residue. The labelled acceptor was incubated in the presence of GDP-Man and cell extracts from *E. coli* BL21(DE3)/pLysS harbouring either pCrGC2 or pET29. The radioactive oligosaccharides were released by acid hydrolysis and analysed by TLC (Fig. 5). In the presence of *E. coli* BL21(DE3)/pLysS/pET29 extracts, the unmodified Glc_2 was found (Fig. 5B, lanes 1 and 2). In contrast, a newly formed trisaccharide that comigrated with Man-Glc_2 was found on incubation with *E. coli* BL21(DE3)/pLysS/pCrGC2 cell extracts (Fig. 5B, lanes 3 and 4). These results strongly suggest that AceA catalyses the transfer of a mannose residue from GDP-Man to Glc_2-P-P-lipid to generate Man-Glc_2-P-P-lipid.

Characterisation of the mannosyl glycosidic linkage

The Glc_2-P-P-Lip acceptor was not purified because it is present in small amounts, and is probably highly unstable due to the pyrophosphate linked to the unsaturated α-isoprene unit. Since both the use of a crude acceptor fraction and the small amounts of product formed precluded the chemical analysis of the trisaccharide, we used jackbean α-mannosidase to determine the stereochemistry of the Man linkage formed by AceA. The trisaccharide obtained using either a radioactive donor or a radioactive acceptor, was digested with jackbean α-mannosidase. As shown in Fig. 6A, the [^{14}C]Man-labelled trisaccharide released [^{14}C]Man,

A. B.

AceA Xc AceA 1 2 3

+ + Man-[14C]Glc2 - -
- - [14C]Man-Glc2 + +
- + α-mannosidase - +

Fig. 6 **A** Sensitivity of Man-Glc_2 oligosaccharides to jackbean α-mannosidase. Oligosaccharides synthesised either in vivo by *X. campestris* or in vitro in the presence of recombinant AceA were incubated with jackbean α-mannosidase as described in Materials and methods. The site of α-mannosidase cleavage is shown in Fig. 1. **B** Acceptor specificity of AceA. Recombinant AceA was incubated in the presence of unlabeled Glc_2-P-P-Lip and GDP-[^{14}C]Man (lane 1); unlabeled Glc_2-P-P-Lip GDP-[^{14}C]Man and 1 mM cellobiose (lane 2); or GDP-[^{14}C]Man and 1 mM cellobiose (lane 3). The monosaccharide in B Lane 3 is Man

while the [^{14}C]Glc-labelled trisaccharide released a radioactive disaccharide that co-migrated with Glc_2. The authentic Man-Glc_2-P-P-Lipid is also sensitive to jackbean α-mannosidase (Fig. 6A) (Ielpi et al. 1993). These results are consistent with an α-mannosyl linkage. Taken together these results indicate that AceA is an α-mannosyltransferase.

Acceptor specificity of AceA

The ability of AceA to use free Glc_2 (cellobiose) as an acceptor was assessed in two different ways. First, the ability to transfer Man to lipid-free Glc_2 was studied by incubating *E. coli* cell extracts containing AceA in the presence of GDP-[^{14}C]Man and Glc_2. As shown in Fig. 6B, no trisaccharide was found on incubation with Glc_2. Secondly, the capacity of Glc_2 to block the formation of Man-Glc_2-P-P-Lip was tested by incubating AceA in the presence of GDP-[^{14}C]Man, glycolipid acceptor (Glc_2-P-P-Lip) and Glc_2. As shown in Fig. 6B (lane 2), free Glc_2 did not inhibit the formation of Man-Glc_2-P-P-Lip. These results strongly suggest that the aglycon moiety of Glc_2-P-P-Lip is essential for the reaction. The requirement for the -P-P-Lip moiety has previously been observed for the rhizobial α-(1,4) glucosyltransferase ExoM (Lellouch and Geremia 1999). The need for complete oligosaccharide-P-P-Lip acceptor seems not to be universal, since the α-(1,4) galactosyltransferase Cps14J uses as acceptor soluble FCHASE-α-Lac-GlcNAc (Kolkman et al. 1997; for a detailed discussion see Lellouch and Geremia 1999).

In conclusion, we have produced a recombinant soluble form of *A. xylinum* AceA protein in *E. coli*, and confirmed the enzymatic activity of AceA using a recently developed (Lellouch and Geremia 1999) in vitro assay. The availability of recombinant active AceA opens the way for the elucidation of its mechanism of action, and determination of its structure and the role of the conserved amino acids in enzymatic activity. Such knowledge will further our understanding of glycosyltransferases of this class.

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