Lipid-linked Intermediates and the Synthesis of Acetan in *Acetobacter xylinum*

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Several strains of *Acetobacter xylinum* were screened for *in vivo* cellulose and acetan production, and for *in vitro* synthesis of a prenyl-diphosphate-hexasaccharide, using UDP-Glc, UDP-GlcA and GDP-Man as sugar donors. The lipid-bound saccharide was synthesized only by acetan-producing strains. Previous work has shown that the *in vitro*-synthesized lipid-linked saccharides have the same structure as the acetan repeating unit. The present results strongly suggest a precursor-product relationship. The strains that produced acetan lost their ability to do so by ageing of the culture.

INTRODUCTION

Acetobacter xylinum is a Gram-negative organism, which characteristically synthesizes cellulose. Couso *et al.* (1987) reported that strain B42 also produces acetan, a complex exopolysaccharide containing glucose, mannose, glucuronic acid and rhamnose in a molar ratio of 4:1:1:1. The proposed structure is represented in Fig. 1. Furthermore, previous work from this laboratory has shown that EDTA-treated cells of *A. xylinum* B42 are able to synthesize *in vitro* a lipid-linked heptasaccharide having the same structure as the repeating unit of acetan (Fig. 1, solid frame) suggesting a precursor-product relationship. The step-wise assembly of the heptasaccharide on a diphosphate prenol was obtained using UDP-Glc, GDP-Man, UDP-GlcA and TDP-Rha as sugar donors, and all the compounds formed at the different intermediate steps were isolated and characterized (Garcia *et al.*, 1974; Couso *et al.*, 1980, 1982). However, all attempts to obtain the *in vitro* polymerization of the lipid-linked heptasaccharide in order to synthesize acetan (Fig. 2) were unsuccessful.

In the present work several strains of *A. xylinum* have been screened for *in vivo* production of cellulose and acetan, and for *in vitro* accumulation of prenyl-diphosphate-hexasaccharide, the lipid intermediate, which is easier to characterize (Couso *et al.*, 1982). In addition, it is shown that both the *in vivo* synthesis of acetan and the *in vitro* formation of a lipid-linked hexasaccharide may be irreversibly lost by ageing of the culture.

METHODS

Chemicals. UDP-[¹⁴C]Glc (200–309 Ci mol⁻¹; 7.4–11.4 GBq mol⁻¹) and UDP-[¹⁴C]GlcA (200–309 Ci mol⁻¹; 7.4–11.4 GBq mol⁻¹) were prepared as described by Garcia *et al.* (1974) and Couso *et al.* (1980). All other chemicals were commercial products.

Bacterial strains and culture media. Cellulose-forming *Acetobacter xylinum* strains were NRRL B42 and ATCC 10821, 23769 and 10245. Strain NCIB 8747 does not produce cellulose. All these strains were purchased from the American Type Culture Collection. Strain RCGrl was isolated from plates of B42 as a mucoid and relatively large colony (2.0–2.5 mm in diameter after one week at 28 °C). Strain NICH1 was isolated from RCGrl cultures; it produces small dry colonies (0.5–1.0 mm diameter after one week at 28 °C).

Fig. 1. Proposed structure for acetan (Couso *et al.*, 1987). The solid frame indicates the repeating unit. The broken frame shows the X₆ hexasaccharide.

The liquid and solid media developed by Hestrin & Schramm (1954) were used.

Enzyme system. Cellulose-producing cells were grown in unshaken cultures and freed from cellulose by filtering through cheesecloth as described by Hestrin & Schramm (1954), with slight modifications. The cell suspension filtrates were centrifuged and acetan was isolated from the supernatants (see below). Strain 8747 was grown with rotary shaking (200 r.p.m.) at 28 °C and the filtration step was omitted. The cell pellets, washed and treated with EDTA as previously reported (Garcia *et al.*, 1974), were used as enzyme source.

Protein was determined by the Lowry method, with bovine serum albumin as standard.

Cellulose. The cellulose fibres retained in the cheesecloth as described above were removed, treated with alkali and washed as reported by Hestrin & Schramm (1954). They were dried *in vacuo* after washing first with ethanol and then with diethyl ether. Cellulose production was expressed as mg dried cellulose (mg wet cells)⁻¹.

Acetan. KCl was added to the culture supernatants to give a final concentration of 1% (w/v). The polysaccharide was precipitated by adding 2 vols 96% (v/v) ethanol. The precipitate was dissolved in distilled water, reprecipitated three times, dialysed and lyophilized as in previous work (Couso *et al.*, 1987). This preparation was protein-free, as judged by the Lowry method. Yields were expressed as mg dried material (mg wet cells)⁻¹.

Incubations. Standard assays were done in 70 mM-Tris/HCl buffer pH 8.2, with EDTA-treated cells (200–500 µg protein), 8 mM-MgCl₂, 0.035 mM-GDP-Man, 0.035 mM-UDP-GlcA and 0.010 mM-UDP-[¹⁴C]Glc. When UDP-[¹⁴C]GlcA (0.010 mM) was used, the UDP-Glc concentration was 0.350 mM. TDP-Rha, the donor of the last sugar of the repeating unit, was not added in this study since its absence did not alter the results (Couso *et al.*, 1982). The reactions were performed in a final volume of 0.070 ml at 30 °C for 30 min, stopped by addition of a fourfold molar excess of EDTA over the Mg²⁺ concentration and centrifuged at 6000 *g* for 5 min. The cell pellets were washed three times with 1 ml 70 mM-Tris/HCl buffer pH 8.2 and extracted three times with 0.1 ml chloroform/methanol/water (1:2:0.3, by vol.) (1203 solvent). Radioactivity incorporated into the 1203 extract was counted in Bray's solution (Bray, 1960), with a Packard Tri-Carb scintillation counter.

In incubations done with UDP-[¹⁴C]Glc, unlabelled UDP-GlcA and GDP-Man as sugar donors and enzyme from B42 cells, under the standard conditions used, the main product extracted with 1203 solvent is X₆ hexasaccharide-diphosphate-prenol (Couso *et al.*, 1982), but small amounts of a neutral glucan which is also formed may be present (Garcia *et al.*, 1974). To separate these two components, the 1203 extracts were hydrolysed in 0.01 M-HCl at 100 °C for 10 min and submitted to paper electrophoresis with 1.2 M-pyridinium acetate buffer pH 6.5 as previously described (Couso *et al.*, 1982). Under these mild hydrolysis conditions only the phosphate linkages are split (Fig. 2), releasing the labelled X₆ hexasaccharide (Fig. 1) which is readily characterized by its electrophoretic mobility (*R*_{UMP} = 0.5) (Couso *et al.*, 1982). The glucan is not affected by this treatment and remains in the neutral area of the electrophoretogram. The relative amounts of neutral and charged compounds were calculated from the respective peak areas.

RESULTS

Correlation between in vivo acetan production and in vitro accumulation of lipid-linked sugars

The *in vivo* synthesis of acetan and cellulose was investigated in the different strains of *A. xylinum* (Table 1). The only strains that produced acetan were B42 and its derivative RCGr1,

Fig. 2. Proposed scheme for the stepwise assembly of the X_7 heptasaccharide-diphosphate-prenol (Couso *et al.*, 1982). The empty arrows in the lipid-linked X_6 hexasaccharide indicate the linkages labile to mild acid hydrolysis (0.01 M-HCl, 100 °C, for 10 min). The dashed arrow indicates the proposed polymerization step leading to the formation of acetan.

Table 1. Comparison between *in vivo* polysaccharide production and *in vitro* synthesis of lipid-linked hexasaccharide

Strain	<i>In vivo</i> production [mg (mg wet cells) ⁻¹] of:		Radioactivity incorporated <i>in vitro</i> into 1203* extract			
			From [¹⁴ C]GlcA C.p.m. (mg protein) ⁻¹	From [¹⁴ C]glucose:		
	Acetan	Cellulose		Total c.p.m. (mg protein) ⁻¹	Hexasaccharide (%)	Neutral (%)
B42	0.27	0.37	20870	85700	89.5	10.5
RCGr1	0.30	0.30	27710	125700	92.6	7.4
NICr1	—	1.22	925	2300	—	100
10821	—	0.32	288	2050	—	100
23769	—	0.41	5640†	2600	—	100
10245	—	0.16	100	1900	—	100
8747	—	—	3000†	800	—	100

—, Not detected.

* 1203, chloroform/methanol/water (1:2:0.3, by vol.).

† This material was characterized as a lipid-linked glucuronide of unknown function, identical to that obtained with enzyme from strain B42 and UDP-[¹⁴C]GlcA as the only sugar donor (Couso *et al.*, 1982, 1986). This glucuronide was not detected when the incubations were performed in the presence of the three donors either using B42 or RCGr1 enzymes.

Table 2. *Effect of ageing on synthesis of polysaccharide and lipid-linked sugar*

Strain	Age of culture*	Size of colony selected	<i>In vivo</i> production [mg (mg wet cells) ⁻¹] of:		<i>In vitro</i> incorporation of [¹⁴ C]GlcA into 1203† extract [c.p.m. (mg protein) ⁻¹]
			Acetan	Cellulose	
RCGr1	Fresh	Large	0.31	0.30	21 100
RCGr1	Old	Large	0.29	0.32	21 800
RCGr1	Old	Small	—	0.78	1 000
NICH1	Fresh	Small	—	0.80	800
NICH1	Old	Small	—	0.85	640

—, Not detected.

* 'Fresh' and 'old' cultures were agar plates grown for 7 d and for 45 d, respectively. In each case a colony with the morphology indicated was selected and cultivated in unshaken cultures for 7 d to obtain the polysaccharides and the enzyme preparations.

† 1203, chloroform/methanol/water (1:2:0.3, by vol.).

which formed cellulose also. The other strains tested produced cellulose but not acetan, except for strain 8747, which did not synthesize either polysaccharide.

These results were compared with the *in vitro* capacity to accumulate lipid-linked X₆ hexasaccharide, a probable precursor of acetan (Fig. 1, broken frame, and Fig. 2). These assays, done using either UDP-[¹⁴C]GlcA or UDP-[¹⁴C]Glc, showed that only the two acetan-producing strains were also able to synthesize lipid-linked hexasaccharide *in vitro* (Table 1).

The relatively high incorporations of [¹⁴C]GlcA observed with strains 23769 and 8747 are due to the formation of a lipid-linked glucuronide identical to that already described for strain B42 (Couso *et al.*, 1986). With enzyme preparations from strain B42 this glucuronide is obtained in incubations done with UDP-[¹⁴C]GlcA as the only sugar donor; if unlabelled UDP-Glc and GDP-Man are also present in the incubation mixture, [¹⁴C]GlcA is only incorporated into the X₆ hexasaccharide-diphosphate-prenol and the formation of lipid-linked [¹⁴C]glucuronide is negligible. This is not the case for strains 23769 and 8747, which cannot synthesize X₆ hexasaccharide-diphosphate-prenol. The lipid-linked [¹⁴C]glucuronide does not participate in X₆ hexasaccharide biosynthesis and its function is unknown (Couso *et al.*, 1982). It is insoluble in water but soluble in butanol and other organic solvents. Mild acid (0.01 M-HCl, 100 °C, for 10 min) or mild alkaline (0.06 M-NaOH, at room temperature for 5 min) hydrolysis releases free [¹⁴C]GlcA (Couso *et al.*, 1986).

Similarly the [¹⁴C]glucose incorporation observed with strains other than B42 and RCGr1 was due to the formation of an electrophoretically neutral compound: not even traces of the X₆ hexasaccharide were detected. This neutral compound was probably a glucan similar to that observed with strain B42, (see Methods). The small amounts of radioactivity present in this fraction did not permit a more detailed study.

Isolation of strains that do not produce acetan

During this study, in addition to the large, slightly mucoid colonies characteristic of strain RCGr1, small opaque colonies were often detected in cultures from old plates of this strain. On plates inoculated with material from old large colonies (more than 40 d at 28 °C), small colonies were always detectable after 7 d incubation at 28 °C. This procedure could be repeated several times, starting always with an inoculum from an old large colony: after one week at 28 °C small colonies were observed. No such small colonies were observed when starting with fresh large colonies (5–7 d old). On the other hand, small-colony strains (such as NICH1) never reverted to the large-colony type.

The ability to produce acetan and cellulose *in vivo*, and to synthesize lipid-linked hexasaccharide *in vitro*, was investigated in cultures from RCGr1 and NICH1 colonies of different size and age (Table 2). Only the RCGr1 large colonies were able to produce acetan and lipid-linked intermediates. Small colonies, either from the NICH1 strain or newly obtained from the old RCGr1 colonies, were unable to produce acetan and lipid-linked intermediates. Small colonies, on the other hand, systematically produced more cellulose than did the respective large mother colony.

DISCUSSION

The *in vitro* accumulation of lipid-linked saccharides with the structure of the repeating unit of the respective complex exopolysaccharides produced has been described for systems from *Acetobacter xylinum* (Couso *et al.*, 1982), *Rhizobium meliloti* (Tolmasky *et al.*, 1982), *Agrobacterium tumefaciens* (Staneloni *et al.*, 1984), *Rhizobium trifolii* (Bossio *et al.*, 1986), *Aerobacter aerogenes* (Troy *et al.*, 1971) and *Xanthomonas campestris* (Ielpi *et al.*, 1981). However, only in the two latter cases has the polymerization step, leading to the formation of the exopolysaccharide, been described *in vitro*.

For the *Acetobacter xylinum* system the present results provide evidence for the assumption that the prenylphosphosugars described in previous papers (Garcia *et al.*, 1974; Couso *et al.*, 1980, 1982) are intermediates in the biosynthesis of acetan. Only the strains able to produce acetan *in vivo* (B42 and RCGr1) are able to synthesize lipid-linked X₆ hexasaccharide *in vitro* (Table 1). The biosynthetic mechanism proposed (Fig. 2) is similar to that reported for *Aerobacter* and *Xanthomonas* but different from the one recently described in *Acetobacter xylinum* for the production of cellulose, in which lipid-linked intermediates do not participate (Ross *et al.*, 1986). Since acetan can be considered as a substituted cellulose it is remarkable that the same organism has different machinery for producing two structurally related exopolysaccharides. It could be argued that homopolysaccharides like cellulose do not need to preassemble repeating units on prenyl derivatives, and can be produced by the classical stepwise addition of the corresponding monosaccharides. But this is not the case for the well-studied systems that make another exo-homopolysaccharide, namely the polysialic capsular polysaccharide from *Escherichia coli* (Troy *et al.*, 1975) and *Neisseria meningitidis* (Masson & Holbein, 1985), in which the participation of prenyl-linked intermediates has been clearly established.

The fact that most *A. xylinum* strains we tested do not produce acetan, and that in those which do the ability to produce it may be lost in old cultures (Table 2), may explain the failure of other groups to detect *in vitro* synthesis of lipid-linked sugars in other strains (Delmer, 1983).

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