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

By NORA IÑON DE IANNINO, ROBERTO O. COUSO AND 
MARCELO A. DANKERT*

Instituto de Investigaciones Bioquimicas ‘Fundacion Campomar’,
Facultad de Ciencias Exactas y Naturales and
Consejo Nacional de Investigaciones Cientificas y Tecnicas,
Antonio Machado 151, (1405) Buenos Aires, Argentina

(Received 22 December 1987)

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; 7–41 14 GBq mol⁻¹) and UDP-[14C]GlcA (200–309 Ci mol⁻¹; 7–41 14 G Bq 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 RCGr1 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 NICCh1 was isolated from RCGr1 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-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 6000g 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 (Rₑₑₚ = 0.5) (Couso et al., 1982). The glucan is not affected by this treatment and remains in the neutral area of the electrophoreogram. 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,
Lipid-linked sugars in acetan synthesis

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acetan [mg (mg wet cells)(^{-1})]</th>
<th>Cellulose [mg protein]</th>
<th>( ^{14} \text{C}) GlcA C.p.m.</th>
<th>Total c.p.m.</th>
<th>Hexasaccharide %</th>
<th>Neutral %</th>
</tr>
</thead>
<tbody>
<tr>
<td>B42</td>
<td>0.27</td>
<td>0.37</td>
<td>20870</td>
<td>85700</td>
<td>89.5</td>
<td>10.5</td>
</tr>
<tr>
<td>RCGrl</td>
<td>0.30</td>
<td>0.30</td>
<td>27710</td>
<td>125700</td>
<td>92.6</td>
<td>7.4</td>
</tr>
<tr>
<td>NICh1</td>
<td>1.22</td>
<td>0.32</td>
<td>925</td>
<td>2300</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>10821</td>
<td>1.22</td>
<td>0.32</td>
<td>288</td>
<td>2050</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>23769</td>
<td>0.41</td>
<td>0.41</td>
<td>5640†</td>
<td>2600</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>10245</td>
<td>0.16</td>
<td>0.16</td>
<td>100</td>
<td>1900</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>8747</td>
<td>–</td>
<td>–</td>
<td>3000†</td>
<td>800</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>

\( \text{Not detected.} \)

\(* \) 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\(^{14} \text{C}\) 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 RCGrl enzymes.
Table 2. Effect of ageing on synthesis of polysaccharide and lipid-linked sugar

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age of culture*</th>
<th>Size of colony selected</th>
<th>In vivo production of: [mg (mg wet cells)^{-1}]</th>
<th>In vitro incorporation of [^{14}C]GlcA into 1203[^{+}] extract [c.p.m. (mg protein)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCGrl</td>
<td>Fresh</td>
<td>Large</td>
<td>Acetan: 0.31</td>
<td>Cellulose: 0.30</td>
</tr>
<tr>
<td>RCGrl</td>
<td>Old</td>
<td>Large</td>
<td>Acetan: 0.29</td>
<td>Cellulose: 0.32</td>
</tr>
<tr>
<td>RCGrl</td>
<td>Old</td>
<td>Small</td>
<td>Acetan: 0</td>
<td>Cellulose: 0.78</td>
</tr>
<tr>
<td>NIChl</td>
<td>Fresh</td>
<td>Small</td>
<td>Acetan: 0</td>
<td>Cellulose: 0.80</td>
</tr>
<tr>
<td>NIChl</td>
<td>Old</td>
<td>Small</td>
<td>Acetan: 0</td>
<td>Cellulose: 0.85</td>
</tr>
</tbody>
</table>

- 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 \textit{in vitro} capacity to accumulate lipid-linked Xerox hexasaccharide, a probable precursor of acetan (Fig. 1, broken frame, and Fig. 2). These assays, done using either UDP-[^{14}C]GlcA or UDP-[^{14}C]Glc, showed that only the two acetan-producing strains were also able to synthesize lipid-linked hexasaccharide \textit{in vitro} (Table 1). The relatively high incorporations of [^{14}C]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 \textit{et al.}, 1986). With enzyme preparations from strain B42 this glucuronide is obtained in incubations done with UDP-[^{14}C]GlcA as the only sugar donor; if unlabelled UDP-Glc and GDP-Man are also present in the incubation mixture, [^{14}C]GlcA is only incorporated into the Xerox hexasaccharide-diphosphate-prenol and the formation of lipid-linked [^{14}C]glucuronide is negligible. This is not the case for strains 23769 and 8747, which cannot synthesize Xerox hexasaccharide-diphosphate-prenol. The lipid-linked [^{14}C]glucuronide does not participate in Xerox hexasaccharide biosynthesis and its function is unknown (Couso \textit{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 [^{14}C]GlcA (Couso \textit{et al.}, 1986).

Similarly the [^{14}C]glucose incorporation observed with strains other than B42 and RCGrl was due to the formation of an electrophoretically neutral compound: not even traces of the Xerox 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.

\textit{Isolation of strains that do not produce acetan}

During this study, in addition to the large, slightly mucoid colonies characteristic of strain RCGrl, 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 NIChl) never reverted to the large-colony type.

The ability to produce acetan and cellulose \textit{in vivo}, and to synthesize lipid-linked hexasaccharide \textit{in vitro}, was investigated in cultures from RCGrl and NIChl colonies of different size and age (Table 2). Only the RCGrl large colonies were able to produce acetan and lipid-linked intermediates. Small colonies, either from the NIChl strain or newly obtained from the old RCGrl 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.
Lipid-linked sugars in acetal synthesis

**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 acetal. Only the strains able to produce acetal *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 acetal 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 respective complex exopolysaccharides. However, the participation of prenyl-linked intermediates has been clearly established. The fact that most *A. xylinum* strains we tested do not produce acetal, 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).

The authors are indebted to Dr Sara Goldemberg for critically reading this manuscript and to Miss Susana Raffo and Marta Bravo for their excellent technical assistance in the preparation of labelled sugar nucleotides. M.A.D. is a Career Investigator of the Consejo Nacional de Investigaciones Científicas y Tecnicas (Argentina).

**REFERENCES**


