Galactose metabolism in *Rhizobiaceae*: characterization of *Agrobacterium tumefaciens exoB* mutants

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*Agrobacterium tumefaciens exoB* mutants (deficient in succinoglycan or EPS I synthesis) were characterized. The defect was determined to be the lack of UDP-glucose 4-epimerase (EC 5.1.3.2) activity. This defect results in absence of UDP-galactose, which is the obligatory intermediate as galactose donor for initiation of the succinoglycan repeating unit on a lipid intermediate. It was determined that *A. tumefaciens* does not have the Leloir pathway enzyme activities galactokinase (EC 2.7.1.6) and galactose-1-phosphate uridylyltransferase (EC 2.7.7.10). A De Ley–Doudoroff oxidative pathway for galactose metabolism was observed in *A. tumefaciens*. This pathway is not constitutive and its expression required D-galactose or L-arabinose. However, UDP-glucose 4-epimerase is constitutive and did not require growth on galactose for full expression.

Introduction

The family *Rhizobiaceae* includes the genera *Agrobacterium*, which induces crown gall tumour formation on dicotyledonous plants and *Rhizobium*, which forms nitrogen-fixing root nodules in association with legumes. Bacterial surface polysaccharides are involved in these processes. *A. tumefaciens* and *R. meliloti* synthesize the periplasmic oligosaccharide cyclic β(1-2)glucan, and the exopolysaccharide succinoglycan (EPS I) (Dell et al., 1983; Hisamatsu et al., 1980). EPS I is a polymer formed by repetitive units of an octasaccharide that contains glucose, galactose, pyruvic acid and acyl substituents (acetyl and succinyl) in the proportion of 7:1:1:1 (Aman et al., 1981; Hisamatsu et al., 1980). Mutants have been obtained that do not produce these polysaccharides, or produce defective exopolysaccharide, lipopolysaccharide or glucan (Cangelosi et al., 1987, 1989; Geremia et al., 1987; Leigh & Lee, 1988; Leigh et al., 1985; Putnoky et al., 1990; Whatley et al., 1976). Genetic information required for the synthesis of these polysaccharides is interchangeable between *Agrobacterium* and *Rhizobium* (Cangelosi et al., 1987; Dylan et al., 1986). Mutants that do not form or excrete β(1-2)glucan are avirulent in *A. tumefaciens* and form ineffective empty nodules in *R. meliloti*. The same phenotype was described for *R. meliloti* exo mutants that do not synthesize succinoglycan. In *A. tumefaciens*, however, exo mutants are not affected in virulence (Cangelosi et al., 1987, 1989; Leigh & Lee, 1988; Leigh et al., 1985; Puvanesarajah et al., 1985).

A recent report described the isolation and characterization of *R. meliloti* mutants that do not produce EPS I but form nitrogen-fixing nodules. These mutants synthesize a different exopolysaccharide (EPS II) containing galactose, glucose and pyruvic acid in a ratio of 1:1:1. EPS II can replace EPS I in the nodule invasion process (Glazebrook & Walker, 1989; Zhan et al., 1989).

exo mutants have been assigned to several genetic complementation groups which are functionally equivalent in *A. tumefaciens* and *R. meliloti* (Cangelosi et al., 1987; Leigh et al., 1985). So far, the number of mutant complementation groups described in *R. meliloti* is 16 (Leigh et al., 1985; Long et al., 1988; Reuber et al., 1991). Two of these complementation groups (exoB and exoC) showed a pleiotropic defect on the synthesis of other polysaccharides (Cangelosi et al., 1987; Leigh & Lee, 1988). We have characterized exoC mutants of *A. tumefaciens* (Uttaro et al., 1990). These mutants lack phosphoglucomutase activity (EC 2.7.5.1), which catalyses the conversion of glucose 6-phosphate to glucose 1-phosphate, an obligatory intermediate in the synthesis of UDP-glucose, a precursor for the synthesis of succinoglycan and β(1-2)-glucan (Staneloni et al., 1984; Tolmasky et al., 1982; Zorregueta et al., 1985). exoB mutants are also pleiotropic on the synthesis of different polysaccharides. They produce normal
β(1-2)glycan but do not produce either EPS I or EPS II, and *R. meliloti* exoB mutants produce an abnormal lipopolysaccharide (Glazebrook & Walker, 1989; Leigh & Lee, 1988; Zhan et al., 1989).

EPS I was found to be synthesized on a lipid-pyrophosphate intermediate in which galactose is the first residue incorporated from UDP-galactose as donor (Staneloni et al., 1984; Tolmasky et al., 1980, 1982). Here, we present evidence regarding the nature of the *exoB* lesion. (A preliminary summary of this work was presented at the VI congress of the Pan-American Association of Biochemical Societies, February 1990, S.P., Brazil.)

Methods

Bacterial strains and culture conditions. Strains used in this study are listed in Table 1. Bacterial strains were grown at 28 °C in Luria broth or M9 minimal medium with 0.2% of the indicated hexoses as carbon sources (Maniatis et al., 1982). When required, media were supplemented with chloramphenicol (20 μg ml⁻¹), streptomycin (100 μg ml⁻¹) and neomycin (100 μg ml⁻¹). Luria agar plates containing 0.02% Cellufluor white (Polysciences Inc.) were used for detection of the Exo

Preparation of permeabilized cells. Cells grown in Luria broth (200 ml) until late exponential phase were harvested by centrifugation, and pellets washed four times with 5 ml 10 mM-EDTA/Tris buffer (pH 8.0) at 0 °C. Pellets were resuspended in the same buffer and frozen and thawed three times before using.

In vitro assays for lipid-bound saccharides synthesis. Permeabilized cells (1 mg protein) were incubated with 70 mM-Tris/HCl buffer (pH 8.2), 12 mM-MgCl₂ and 3 × 10⁵ c.p.m. UDP-[U-¹⁴C]glucose or UDP-[U-¹⁴C]galactose [specific activity 285 Ci mol⁻¹ (10.55 TBq mol⁻¹) in both cases] in a final volume of 50 μl. When required, 1 mM-UDP-galactose was added. Incubations were carried out at 10 °C for 30 min and stopped with 1 ml 10 mM-EDTA/Tris buffer (pH 8.0), centrifuged and washed twice with the same buffer. The pellet was extracted twice with chloroform/methanol/water (1:1:0.9, by vol.). This fraction, referred to as 1103 extract and containing lipid-bound oligosaccharides, was evaporated and hydrolysed with 10 mM-HCl at 100 °C for 10 min. Under these conditions, radioactive water-soluble oligosaccharides were obtained and submitted to thin-layer chromatography with solvent C (Staneloni et al., 1984).

Preparation of formic acid extracts. Strains of *A. tumefaciens* were grown until late exponential phase in 200 ml of medium containing mannitol (1%, w/v), sodium glutamate (2.33 g l⁻¹), KH₂PO₄ (0.5 g l⁻¹), KCl (0.2 g l⁻¹), MgSO₄·7H₂O (0.2 g l⁻¹) and biotin (2 mg l⁻¹), supplemented with 0.3% glucose. Cells were harvested by centrifugation (6000 g, 15 min), washed twice and resuspended in the same medium with 20 mM-glucose, and incubated for 10 min with strong agitation. Formic acid was added to a final concentration of 10% (v/v), immediately vortexed and stored at −20 °C overnight. These suspensions were thawed, centrifuged for 10 min at 5000 g and supernatants freeze-dried to eliminate formic acid. The lyophilized powder was dissolved in 300 μl of water and submitted to HPLC (Pharmacia-LKB system) using a UtraSphere 5 μm ODS (4.6 × 250 mm) column (Beckman) (Payne & Ames, 1982), eluted with 40 mM-triethylamine/phosphoric acid buffer, pH 6.5. Sugar nucleotides were detected with a UV detector at 260 nm.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant marker(s)</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>A. tumefaciens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A348</td>
<td>Rp' Cm' pTiA6</td>
<td>Parent strain; Garfinkel et al. (1981)</td>
</tr>
<tr>
<td>A5094</td>
<td>ExoB⁻ Km'</td>
<td>Tn5 mutagenesis of A348; Cangelosi et al. (1987)</td>
</tr>
<tr>
<td>A5126</td>
<td>ExoB⁻ Km'</td>
<td></td>
</tr>
<tr>
<td>A5129</td>
<td>ExoC⁻ Km'</td>
<td></td>
</tr>
<tr>
<td>R. meliloti</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rm 1021</td>
<td>Sm' derivative of SU47</td>
<td>Parent strain: Leigh et al. (1985)</td>
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<td>Tn5 mutagenesis of Rm 1021;</td>
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<td>ExoB⁻ Km'</td>
<td>Leigh &amp; Lee (1988)</td>
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<td>E. coli</td>
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<tr>
<td>Y 1090</td>
<td>Te' Ap' Sm' Trp⁻</td>
<td>Young &amp; Davis (1983)</td>
</tr>
</tbody>
</table>

Preparation of cytosolic fractions. Cells from an exponential phase culture (1 litre) were harvested by centrifugation (6000 g, 15 min) and suspended in 10 ml 30 mM-Tris/HCl buffer (pH 8.0), 20% (w/w) sucrose, 10 mM-EDTA and 10 mg chicken egg-white lysozyme (Sigma). Cells were incubated for 45 min at 0 °C and centrifuged for 10 min at 10000 g. Pellets were suspended in 10 ml 30 mM-Tris/HCl buffer (pH 8.0), 10 mM-MgCl₂ with 10 mg DNAase I (bovine pancreas type IV; Sigma), disrupted with a French press and centrifuged at 100000 g for 5 h. Supernatants recovered were termed the cytosolic fraction.

Enzyme assays. The reversible epimerization of UDP-galactose to UDP-glucose was assayed in reaction mixtures containing 50 mM-Tris/HCl (pH 7.5), 8 mM-MgCl₂, cytosolic fractions (0.15 mg protein) and 3 × 10⁵ c.p.m. UDP-[U-¹⁴C]glucose or UDP-[U-¹⁴C]galactose [specific activity 285 Ci mol⁻¹ (10.55 TBq mol⁻¹) in both cases] in a final volume of 50 μl. Reactions were carried out at 30 °C for 30 min and stopped by adding 100 μl ethanol. Supernatants obtained after centrifugation at 3000 g for 5 min were submitted to paper chromatography with solvent A. Compounds with the mobility of UDP-hexoses were eluted from the paper strip with water, submitted to acid hydrolysis with 100 mM-HCl for 10 min at 100 °C, and chromatographed with solvent B to identify hexoses. Radioactivity recovered was counted and the percentage of glucose and galactose calculated. Experiments were repeated three times. UDP-Glucose 4-epimerase was also assayed spectrophotometrically according to Postma (1977).

Galactokinase and galactose-1-phosphate uridylytransferase were assayed in reaction mixtures containing 50 mM-Tris/HCl (pH 7.5), 8 mM-MgCl₂, cytosolic fraction (0.15 mg protein), 1 mM-UTP, 1 mM-UDP-glucose and 3 × 10⁵ c.p.m. of [¹⁴C]galactose [55 Ci mol⁻¹ (2.04 TBq mol⁻¹)], 1 mM-ATP, or 8 × 10⁴ c.p.m. [¹⁴C]galactose 1-phosphate [285 Ci mol⁻¹ (10.55 TBq mol⁻¹)] respectively, in a final volume of 50 μl. The reactions were carried out at 30 °C for 30 min and products analysed as described above for the epimerase assay.

Galactose dehydrogenase and galactonate dehydrogenase were assayed according to Arias & Cerveñasky (1986).

6-Phospho-2-keto-3-deoxygalactonate aldolase was assayed by an indirect procedure. Because the substrate (6-phospho-2-keto-3-deoxygalactonic acid) was not available, cytosolic fractions were incubated in a coupled reaction with lactic dehydrogenase. The reaction mixture in a final volume of 0.5 ml, containing 50 mM-Tris/HCl (pH 7.5), 8 mM-MgCl₂, cytosolic fraction (0.15 mg protein), 2 mM-ATP, 10 mM-galactonic acid, 0.2 mM-NADH and lactic dehydrogenase (9 U), was incubated in a UV cuvette at room temperature.
The reduction of pyruvate was determined by the diminution in absorbance at 340 nm in a Gilford Response II spectrophotometer.

**Chromatography.** Descending paper chromatography on Whatman no. 1 paper was carried out with the following solvents: solvent A, ethanol/1M-ammonium acetate, pH 7.4 5:2, v/v; solvent B, 1-butanol/pyridine/water (6:4:3, by vol.). Radioactivity was detected with a model 7201 radiochromatogram scanner (Packard). Thin-layer chromatography was carried out on silica gel 60 (0.25 mm, Merck) with solvent C: 1-propanol/nitromethane/water (5:2:2, by vol.). Radioactive compounds were localized by radioautography.

UDP-Glucose standard was observed under UV light and sugar phosphate and hexose standards were developed by the methods of Burrows et al. (1952) and Trevelyan et al. (1950), respectively.

Gel-filtration chromatography was carried out on a Bio-Gel P, column (9 × 1.2 cm) and eluted with 0.1 M-pyridine acetate (pH 5.5).

Glucose, sucrose, raffinose, stachyose and radioactive octasaccharide of A. tumefaciens (Staneloni et al., 1984) were used as standards.

### Results

**In vitro synthesis of lipid-bound saccharides**

Strains containing the exoB mutation are defective in the formation of different polysaccharides containing galactose (Cangelosi et al., 1987; Glazebrook & Walker, 1989; Leigh & Lee, 1988; Zhan et al., 1989). For example, EPS I is synthesized through the polymerization of an intermediate repetitive unit, formed by glucose and galactose in a ratio of 7:1, which is synthesized on an undecaprenol-phosphate lipid to which galactose-1-phosphate is transferred from UDP-galactose. Glucose moieties are then successively added and the repetitive unit polymerized through an unknown process (Fig. 1) (Staneloni et al., 1984; Tolmasky et al., 1982). The inability of exoB mutants to synthesize galactose-containing polysaccharides may be the consequence of a blockage in the transfer of galactose from UDP-galactose to the polysaccharides or in an earlier step. To distinguish between these possibilities, we tested the ability of permeabilized cells from exoB mutants to synthesize lipid-bound intermediates of EPS I, in the presence of exogenously added UDP-galactose and UDP-glucose under the conditions described in Methods.

When permeabilized cells of the wild-type strain were incubated with radioactive sugar nucleotides, radioactive lipid-bound saccharides that could be extracted with organic solvents were produced in vitro (Staneloni et al., 1984; Tolmasky et al., 1980, 1982). Results obtained with permeabilized cells of the wild-type and exoB mutant strains of A. tumefaciens incubated with UDP-[14C]glucose or UDP-[14C]galactose are shown in Table 2. A. tumefaciens wild-type strain A348 and exoB mutants A5094 or A5126 strains synthesized extractable lipid-bound sugars when UDP-[14C]galactose was used as substrate. As shown in Table 2, only A348 synthesized radioactive lipid when UDP-[14C]glucose was the sole substrate. However, when the reaction mixture was supplemented with non-radioactive UDP-galactose a small decrease in the accumulation of lipid-bound saccharides was observed with the wild-type strain; on the other hand, with mutant cells an increase in the formation of extractable lipids was observed.

To confirm that the substances extractable with organic solvent were EPS I lipid-octasaccharide intermediates, 10000 c.p.m. of organic extract was hydrolysed with 10 mM-HCl at 100 °C for 10 min and the resulting water-soluble radioactivity was submitted to TLC with solvent C and autoradiography (Fig. 2). Traces of radioactivity were incorporated with exoB mutant permeabilized cells incubated with UDP-[14C]glucose. Although the identities of these compounds (Fig. 2, lanes 3 and 6) were not established, they eluted as a monosaccharide in Bio-Gel P, chromatography. When UDP-[14C]galactose was used as substrate, [14C]galactose and traces of other oligosaccharides were detected (Fig. 2, lanes 4 and 7).

Permeabilized cells of the wild-type strain incubated with UDP-[14C]glucose or UDP-[14C]galactose (Fig. 2, lanes 1 and 2) and exoB mutant cells incubated with UDP-[14C]glucose supplemented with 1 mM non-radioactive UDP-galactose (Fig. 2, lanes 5 and 8) yielded similar lipid-oligosaccharides. These products were similar to those described previously as intermediates in the synthesis of succinoglycan of A. tumefaciens and R. meliloti (Staneloni et al., 1984; Tolmasky et al., 1980, 1982).}

![Fig. 1. Structure of the octasaccharide repeating unit of A. tumefaciens and R. meliloti EPS I (taken from Hisamatsu et al., 1980). Acyl substituents are not shown.](image-url)

### Table 2. In vitro production of extractable lipid-bound sugars

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Products [c.p.m. (mg protein)⁻¹ in 1103 extracts]</th>
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<tr>
<td>A348</td>
<td>UDP-[14C]Glc</td>
<td>19205</td>
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<td></td>
<td>UDP-[14C]Gal</td>
<td>10170</td>
</tr>
<tr>
<td></td>
<td>UDP-[14C]Glc+UDP-Gal</td>
<td>10561</td>
</tr>
<tr>
<td>A5094</td>
<td>UDP-[14C]Glc</td>
<td>1851</td>
</tr>
<tr>
<td></td>
<td>UDP-[14C]Gal</td>
<td>22893</td>
</tr>
<tr>
<td></td>
<td>UDP-[14C]Glc+UDP-Gal</td>
<td>46562</td>
</tr>
<tr>
<td>A5126</td>
<td>UDP-[14C]Glc</td>
<td>2322</td>
</tr>
<tr>
<td></td>
<td>UDP-[14C]Gal</td>
<td>27510</td>
</tr>
<tr>
<td></td>
<td>UDP-[14C]Glc+UDP-Gal</td>
<td>44676</td>
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Fig. 2. Thin-layer chromatography of the saccharide portion of lipid-bound sugar EPS I intermediates. The organic solvent extract (10000 c.p.m.) obtained as described in Methods (1103 extract) was treated with 10 mM-HCl for 10 min at 100 °C. Water-soluble radioactive products were chromatographed on a TLC plate with solvent C. Compounds were detected by autoradiography. M, indicates the degree of polymerization of the maltooligosaccharides; Glc, glucose; Oc and OcA indicate the mobility expected for octasaccharide and acylated octasaccharide. Permeabilized cells were incubated with UDP-[14C]glucose (lanes 1, 3, 6), UDP-[14C]galactose (lanes 2, 4, 7) or UDP-[14C]glucose plus unlabelled UDP-galactose (lanes 5, 8). A348 wild-type strain (lanes 1, 2); A5094 exoB mutant (lanes 3, 4, 5); A5126 exoB mutant (lanes 6, 7, 8).

Fig. 3. Bio-Gel P, chromatography. The products with mobility similar to octasaccharide (●) or acylated octasaccharide (O) produced by strain A5126 were eluted from TLC plates (Fig. 2) and subjected to chromatography on a Bio-Gel P, column (1.2 × 100 cm). The column was eluted with 0.1 M pyridine/acetic acid buffer (pH 5.5). Standards: Glc, glucose; Suc, sucrose; Raf, raffinose; Sta, stachyose; Oc, octasaccharide from A. tumefaciens.

1982). Compounds with the mobility expected for acylated and non-acylated octasaccharides were eluted from TLC plates and chromatographed in a Bio-Gel P, column. Fig. 3 shows the elution profile of octasaccharides from mutant A5126 incubated with UDP-[14C]glucose supplemented with non-radioactive UDP-galactose. Similar profiles were obtained with mutant A5094 and wild-type A348 octasaccharides.

These results indicated that the wild-type and exoB mutant strains synthesized EPS I lipid-octasaccharide intermediates when sugar-nucleotides were added exogenously. A strict requirement for UDP-galactose was observed with exoB mutants, which is the expected result if exoB mutants are defective in UDP-galactose synthesis. These results also confirm that lipid-bound octasaccharide initiation occurs through incorporation of galactose from UDP-galactose (Staneloni et al., 1984; Tolmasky et al., 1982).

**In vivo content of sugar-nucleotides**

Since exoB mutants synthesized EPS I intermediates in vitro when UDP-galactose and UDP-glucose were added exogenously, their inability to synthesize EPS I in vivo may be due to an inability to synthesize these sugar nucleotides. To test this possibility, we analysed the endogenous sugar nucleotide content in exoB and exoC mutant cells compared to the wild-type strain.

Cells of the wild-type and of exoB and exoC mutants of A. tumefaciens were extracted with formic acid and subjected to HPLC as described in Methods. As shown in Fig. 4, exoB mutants contain UDP-glucose but no UDP-galactose. On the other hand, exoC mutants contain neither UDP-glucose nor UDP-galactose as expected, since these mutants were previously
characterized as deficient in UDP-glucose synthesis (pgm mutant) (Uttaro et al., 1990).

In vitro synthesis of UDP-galactose

UDP-galactose is synthesized from UDP-glucose by the enzyme UDP-glucose 4-epimerase (EC 5.1.3.2). The absence of endogenous UDP-galactose may have been due to inactivity of this enzyme. To test this possibility, we incubated cytosolic extracts from mutants and wild-type cells of *A. tumefaciens* and *R. meliloti* grown in Luria media with UDP-[¹⁴C]glucose or UDP-[¹⁴C]galactose as substrates. The products of these incubations were analysed by paper chromatography as described in Methods. Wild-type extracts yielded a ratio of UDP-glucose to UDP-galactose of about 3:1, which is similar to the value described for *E. coli*, yeast and calf liver enzymes (Maxwell et al., 1962; Wilson & Hogness, 1964). However, when exoB mutant extracts were used, no epimers were detected with either substrate (Table 3).

![Table 3. In vitro determination of sugar nucleotide epimerization](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>UDP-Glc</th>
<th>UDP-Gal</th>
<th>UDP-Glc/UDP-Gal</th>
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<td>151800</td>
<td>&lt;100</td>
<td>–</td>
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<tr>
<td></td>
<td>UDP-Gal</td>
<td>&lt;100</td>
<td>102450</td>
<td>–</td>
</tr>
<tr>
<td>A5126</td>
<td>UDP-Glc</td>
<td>165230</td>
<td>&lt;100</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>UDP-Gal</td>
<td>&lt;100</td>
<td>100050</td>
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<td>UDP-Gal</td>
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</table>

* Determined spectrophotometrically.

The wild-type strain of *A. tumefaciens* was grown in minimal medium with different carbon sources (D-galactose, D-glucose or L-arabinose) and cytosolic extract assayed for UDP-glucose 4-epimerase activity by a spectrophotometric method (Postma, 1977). No significant difference was obtained with extracts obtained from cells grown on these carbon sources (Table 4), thus indicating that in *A. tumefaciens* the synthesis of this enzyme is constitutive and not inducible by these carbon sources.

Galactose metabolism

As described by us previously (Uttaro et al., 1990), *A. tumefaciens* exoC mutants grown on Celluflo White/Luria agar plates supplemented with galactose remained dark under UV light. Similar results were obtained with exoB mutants of *A. tumefaciens* and *R. meliloti*.

exoB and exoC *A. tumefaciens* or *R. meliloti* mutants grew on minimal media with galactose as sole carbon source with no production of exopolysaccharide. These results indicate that the Leloir pathway is absent in *A.
was added to the reaction mixture. No pyruvate was pyruvate min⁻¹ (mg protein)⁻¹, respectively, only if ATP

confirm these results, cytosolic extracts of wild-type

incubated with [14C]galactose or [14C]galactose

strains of Pseudomonas saccharophila

in Table 4, these enzymes were inducible in

of the Leloir pathway, galactokinase (EC 2.7.1.6) and

et al., 1982), EPS I (see Fig. 1) (Staneloni et al., 1984; Tolmasky et al., 1982), EPS II (Glazebrook & Walker, 1989; Zhan et al., 1989) and lipopolysaccharides (Salkinoja-Salonen & Boeck, 1978; Zevenhuizen et al., 1980) of A. tumefaciens and R. meliloti. The absence of UDP-galactose justified the lack of synthesis of EPS I observed with A. tumefaciens and R. meliloti exoB mutants and the formation of a defective lipopolysaccharide in R. meliloti mutants (Leigh & Lee, 1988). Presumably, exoB mutants of A. tumefaciens also have a defective lipopolysaccharide.

The endogenous pools of UDP-galactose, UDP-glucose and other sugar nucleotides were measured. While in wild-type extracts UDP-glucose, UDP-galactose, GDP-mannose, UDP-glucuronic acid and UDP-galacturonic acid were observed, in exoB mutant extracts UDP-galactose only was lacking, which is consistent with a defective UDP-glucose 4-epimerase (Fig. 5). On the other hand, exoC mutants extracts lacked UDP-glucose, UDP-galactose, UDP-glucuronic acid and

Discussion

Our results indicate that in A. tumefaciens exoB mutants the enzyme UDP-glucose 4-epimerase is inactive or missing. This confirms the results of Canter Cremers et al. (1990) and Buendia et al. (1991), who characterized exoB mutants of R. leguminosarum and R. meliloti respectively.

UDP-Glucose 4-epimerase catalyses the reversible epimerization of UDP-glucose to UDP-galactose. Both sugar nucleotides are obligatory intermediates in the synthesis of EPS I (see Fig. 1) (Staneloni et al., 1984; Tolmasky et al., 1982), EPS II (Glazebrook & Walker, 1989; Zhan et al., 1989) and lipopolysaccharides (Salkinoja-Salonen & Boeck, 1978; Zevenhuizen et al., 1980) of A. tumefaciens and R. meliloti. The absence of UDP-galactose justified the lack of synthesis of EPS I observed with A. tumefaciens and R. meliloti exoB mutants and the formation of a defective lipopolysaccharide in R. meliloti mutants (Leigh & Lee, 1988). Presumably, exoB mutants of A. tumefaciens also have a defective lipopolysaccharide.

The production of pyruvate from galactose was also assayed. Cytosolic extracts of strain A348 grown on D-galactose or L-arabinose produce 14 and 13 nmol pyruvate min⁻¹ (mg protein)⁻¹, respectively, only if ATP was added to the reaction mixture. No pyruvate was detected in reactions without ATP or with cytosolic extract from cells grown on d-glucose.

tumefaciens and R. meliloti, and an alternative pathway for galactose dissimilation must be present (see Fig. 5) (Arias & Cerveñansky, 1986; Uttaro et al., 1990). To confirm these results, cytosolic extracts of wild-type strains of A. tumefaciens, R. meliloti and E. coli grown on minimal medium with galactose as carbon source were incubated with [14C]galactose or [14C]galactose 1-phosphate as substrates and the products analysed as described in Methods. Both substrates were totally converted to UDP-[14C]glucose and UDP-[14C]galactose by E. coli extracts. However, extracts of A. tumefaciens and R. meliloti did not produce detectable amounts of sugar nucleotides, indicating that the first two enzymes of the Leloir pathway, galactokinase (EC 2.7.1.6) and galactose 1-phosphate uridylyltransferase (EC 2.7.7.10), are not present (Adhya, 1987; Maxwell et al., 1982).

An alternative pathway for dissimilation of galactose has been described by De Ley & Doudoroff (1957) in Pseudomonas saccharophila and detected in R. meliloti by Arias & Cerveñansky (1986). To determine the presence of this pathway in A. tumefaciens, we assayed cytosolic extract for galactose dehydrogenase (EC 1.1.1.48) and galactonate dehydratase (EC 4.2.1.6) activities. Extracts were obtained from wild-type cells grown on M9 minimal medium supplemented with D-glucose, D-galactose or L-arabinose as sole carbon source. As shown in Table 4, these enzymes were inducible in A. tumefaciens by D-galactose and L-arabinose.

The production of pyruvate from galactonate was also assayed. Cytosolic extracts of strain A348 grown on D-galactose or L-arabinose produce 14 and 13 nmol pyruvate min⁻¹ (mg protein)⁻¹, respectively, only if ATP was added to the reaction mixture. No pyruvate was
UDP-galacturonic acid, which confirms our previous characterization of exoC mutants as lacking phosphoglucomutase activity (Uttaro et al., 1990), an enzyme that converts glucose 6-phosphate to glucose 1-phosphate, a direct intermediate in the synthesis of UDP-glucose and UDP-galactose (see Fig. 5). These results are consistent with the synthesis of UDP-glucuronic acid and UDP-galacturonic acid through the oxidation of UDP-glucose to UDP-glucofronic acid and a subsequent epimerization by an UDP-glucuronic acid 4-epimerase activity (EC 5.1.3.6). This epimerase has been demonstrated in R. mellioti extracts (Ugalde et al., 1986).

exoB mutants synthesized EPS I lipid-octasaccharide intermediates in vitro when UDP-glucose and UDP-galactose were added exogenously, showing that probably the only defect of these mutants is the absence of endogenous UDP-galactose. exoB mutants were shown to have a strict requirement for UDP-galactose in order to synthesize lipid-octasaccharide in vitro. These results are in agreement with that described by Tolmasy et al. (1982), who reported that the first step in the synthesis of the EPS I repetitive unit (Fig. 1) is the transfer of galactose 1-phosphate from UDP-galactose to undecaprenol-phosphate. The endogenous pool of UDP-glucose justified the traces of intermediates observed in incubations with exoB extracts and UDP-[14C]galactose (Fig. 2, lanes 4 and 7).

exoB and exoC mutants of A. tumefaciens and R. melliloti grow on galactose as sole carbon source without reversion of their pleiotropic phenotype (Uttaro et al., 1990), indicating that an alternative route for dissimilation of galactose must be present. We have detected in A. tumefaciens an oxidative pathway for galactose metabolism, similar to that described by De Ley & Doudoroff (1957) in Pseudomonas saccharophila and Arias & Cerveñas (1986) in R. melliloti (see Fig. 5). In A. tumefaciens, as in P. saccharophila and R. melliloti, this pathway was induced by D-galactose and L-arabinose (Table 4).

The significant homology between R. melliloti exoB and E. coli galE loci (Buendia et al., 1991) confirms that exoB is the structural gene of UDP-glucose 4-epimerase. However, in A. tumefaciens (Table 4) and R. melliloti (Buendia et al., 1991) the expression of this enzyme is not induced by galactose as carbon source. On the other hand, the enzymes galactokinase and galactose-1-phosphate uridylyltransferase are absent (see above), indicating that the Leloir pathway is absent (Adhya, 1987; Arias & Cerveñas, 1986; Uttaro et al., 1990), justifying the absence of a toxic effect when galactose was used as carbon source, because galactose 1-phosphate could not be accumulated (Adhya, 1987). The absence of this route and the presence of an oxidative pathway for galactose metabolism seems to be frequent in Rhizobiaceae and other soil bacteria (Arias & Cerveñas, 1986; Canter-Cremers et al., 1990; De Ley & Doudoroff, 1957; and this work). Preliminary results (unpublished) indicate that Azospirillum brasiliense uses a similar route.

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References


Agrobacterium tumefaciens exoB mutants