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

ANTONIO D. UTTARO, LUIS IELPI and RODOLFO A. UGALDE*

Instituto de Investigaciones Bioquímicas 'Fundación Campomar', Facultad de Ciencias Exactas y Naturales and CONICET, Antonio Machado 151, 1405 Buenos Aires, Argentina

(Received 3 August 1992; accepted 14 January 1993)

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 $\beta(1-2)$ 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 $\beta(1-2)$ 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 $\beta(1-2)$ -glucan (Staneloni *et al.*, 1984; Tolmasky *et al.*, 1982; Zorreguieta *et al.*, 1985). *exoB* mutants are also pleiotropic on the synthesis of different polysaccharides. They produce normal

* Author for correspondence. Tel. 1 88 4015; fax 1 865 2246.

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 phenotype (Finan *et al.*, 1985; Matthysse, 1983).

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 285.1 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 (1:1:0.3, 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), NaCl (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 Ultrasphere 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 1. Bacterial strains

Strain	Relevant marker(s)	Source or reference
<i>A. tumefaciens</i>		
A348	Rp ^r Cm ^r pTiA6	Parent strain; Garfinkel <i>et al.</i> (1981)
A5094	ExoB ⁻ Km ^r	Tn5 mutagenesis of A348; Cangelosi <i>et al.</i> (1987)
A5126	ExoB ⁻ Km ^r	
A5129	ExoC ⁻ Km ^r	
<i>R. meliloti</i>		
Rm 1021	Sm ^r derivative of SU47	Parent strain; Leigh <i>et al.</i> (1985)
Rm 7025	ExoC ⁻ Km ^r	Tn5 mutagenesis of Rm 1021; Leigh & Lee (1988)
Rm 7094	ExoB ⁻ Km ^r	
<i>E. coli</i>		
Y 1090	Tc ^r Ap ^r Sm ^r Trp ⁻	Young & Davis (1983)

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 DNAaseI (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-glucose to UDP-galactose 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 285.1 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 uridylyltransferase 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 [1-¹⁴C]galactose [55 Ci mol⁻¹ (2.04 TBq ml⁻¹)], 1 mM-ATP, or 8 × 10⁴ c.p.m. [U-¹⁴C]galactose 1-phosphate [285.1 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 dehydratase were assayed according to Arias & Cerveñansky (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/1 M-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 P₂ 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

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.

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

Strain	Substrate	Products
		[c.p.m. (mg protein) ⁻¹ in 1103 extracts]
A348	UDP-[¹⁴ C]Glc	19205
	UDP-[¹⁴ C]Gal	10170
	UDP-[¹⁴ C]Glc + UDP-Gal	10561
A5094	UDP-[¹⁴ C]Glc	1851
	UDP-[¹⁴ C]Gal	22893
	UDP-[¹⁴ C]Glc + UDP-Gal	46562
A5126	UDP-[¹⁴ C]Glc	2322
	UDP-[¹⁴ C]Gal	27510
	UDP-[¹⁴ C]Glc + UDP-Gal	44676

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 P₂ 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-[^{14}C]glucose or UDP-[^{14}C]galactose (Fig. 2, lanes 1 and 2) and *exoB* mutant cells incubated with UDP-[^{14}C]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,

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_x 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-[14 C]glucose (lanes 1, 3, 6), UDP-[14 C]galactose (lanes 2, 4, 7) or UDP-[14 C]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_2 chromatography. The products with mobility similar to octasaccharide (●) or acylated octasaccharide (○) produced by strain A5126 were eluted from TLC plates (Fig. 2) and subjected to chromatography on a Bio-Gel P_2 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_2 column. Fig. 3 shows the elution profile of octasaccharides from mutant A5126 incubated with UDP-

[14 C]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

Fig. 4. HPLC profile of endogenous content of sugar nucleotides in formic acid extracts from the wild-type strain A348 (a), *exoB* mutant strain A5126 (b) and *exoC* mutant strain A5129 (c).

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

Strain	Substrate	Products (c.p.m.)		UDP-Glc/ UDP-Gal
		UDP-Glc	UDP-Gal	
A348	UDP-Glc	109 680	35 360	3.1
	UDP-Gal	98 500	33 560	2.9
A5094	UDP-Glc	151 800	< 100	—
	UDP-Gal	< 100	102 450	—
A5126	UDP-Glc	165 230	< 100	—
	UDP-Gal	< 100	100 050	—
Rm 1021	UDP-Glc	95 777	34 306	2.8
	UDP-Gal	89 229	32 503	2.7
Rm 7094	UDP-Glc	119 090	< 100	—
	UDP-Gal	< 100	98 731	—

Table 4. Enzyme activities of *A. tumefaciens* wild-type strain A348

Carbon source	Enzyme activity [nmol product formed min ⁻¹ (mg protein) ⁻¹]		
	UDP-Glc 4-epimerase	Galactose dehydrogenase	Galactonate dehydratase
D-Glucose	2.1	0	2
D-Galactose	2.0	8.1	35
L-Arabinose	2.6	9.6	16

* 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 Cellufluor 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.*

Fig. 5. Metabolic pathway of sugars. Pgm, phosphoglucomutase; Epi, UDP-glucose 4-epimerase; * indicate intermediates in the Leloir pathway; † (dagger) indicates intermediates in the De Ley–Doudoroff pathway; Glc, glucose; KDGal, 2-keto-3-deoxygalactonate; KDPGal, 2-keto-3-deoxy-6-phosphogalactonate; P, phosphate.

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.*, 1962).

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

detected in reactions without ATP or with cytosolic extract from cells grown on D-glucose.

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 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

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-glucuronic acid and a subsequent epimerization by an UDP-glucuronate 4-epimerase activity (EC 5.1.3.6). This epimerase has been demonstrated in *R. meliloti* 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 Tolmasky *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. meliloti* 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 & Cerveñansky (1986) in *R. meliloti* (see Fig. 5). In *A. tumefaciens*, as in *P. saccharophila* and *R. meliloti*, this pathway was induced by D-galactose and L-arabinose (Table 4).

The significant homology between *R. meliloti 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. meliloti* (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 & Cerveñansky, 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 & Cerveñansky, 1986; Canter-Cremers *et al.*, 1990; De Ley & Doudoroff, 1957; and this work). Preliminary results (unpublished) indicate that *Azospirillum brasilense* uses a similar route.

We thank Susana Raffo and Maria de los Angeles Curto for preparing ¹⁴C-labelled compounds and Marta Bravo and Alejandra Raimondi for technical assistance. We also thank Eugene W. Nester and Graham C. Walker for providing *A. tumefaciens* and *R. meliloti* mutant strains respectively.

This study was supported by a PID 294 grant from CONICET (Argentina). A.D.U. is fellow of CONICET; L.I. and R.A.U. are members of Carrera del Investigador (CONICET, Argentina).

References

- ADHYA, S. (1987). The galactose operon. In *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*, pp. 1503–1512. Edited by F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- AMAN, P., MCNEIL, M., FRANZEN, L., DARVILL, A. G. & ALBERSHEIM, P. (1981). Structural elucidation, using HPLC–MS and GLC–MS, of the acidic polysaccharide secreted by *Rhizobium meliloti* strain 1021. *Carbohydrate Research* **95**, 263–282.
- ARIAS, A. & CERVEÑANSKY, C. (1986). Galactose metabolism in *Rhizobium meliloti* L5-30. *Journal of Bacteriology* **167**, 1092–1094.
- BUENDIA, A. M., ENENKEL, B., KOPLIN, R., NIEHAUS, K., ARNOLD, W. & PUHLER, A. (1991). The *Rhizobium meliloti exoZ/exoB* fragment of megaplasmid 2: ExoB functions as a UDP-glucose 4-epimerase and ExoZ shows homology to NodX of *Rhizobium leguminosarum* biovar *viciae* strain TOM. *Molecular Microbiology* **5**, 1519–1530.
- BURROWS, S., GRYLLS, F. S. M. & HARRISON, J. S. (1952). Paper chromatography of phosphoric esters. *Nature, London* **170**, 800–801.
- CANGELOSI, G. A., HUNG, L., PUVANESARAJAH, V., STACEY, G., OZGA, A. D., LEIGH, J. A. & NESTER, E. W. (1987). Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. *Journal of Bacteriology* **169**, 2086–2091.
- CANGELOSI, G. A., MARTINETTI, G., LEIGH, J. A., LEE, C. C., THEINES, C. & NESTER, E. W. (1989). Role of *Agrobacterium tumefaciens* ChvA protein in export of β -1,2-glucan. *Journal of Bacteriology* **171**, 1609–1615.
- CANTER-CREMERS, H. C. J., BATLEY, M., REDMOND, J. W., EYDEMS, L., BREEDVELD, M. W., ZEVENHUIZEN, L. P. T. M., PEES, E., WIJFELLMAN, C. A. & LUGTENBERG, B. J. J. (1990). *R. leguminosarum exoB* mutants are deficient in the synthesis of UDP-glucose 4-epimerase. *Journal of Biological Chemistry* **265**, 21122–21127.
- DE LEY, J. & DOUDOROFF, M. (1957). The metabolism of D-galactose in *Pseudomonas saccharophila*. *Journal of Biological Chemistry* **227**, 745–757.
- DELL, A., YORK, W. S., MCNEIL, M., DARVILL, A. G. & ALBERSHEIM, P. (1983). The cyclic structure of β -D-(1,2)-linked D-glucans secreted by *Rhizobia* and *Agrobacteria*. *Carbohydrate Research* **117**, 185–200.
- DYLAN, T., IELPI, L., STANFIELD, S., KASHYAP, L., DOUGLAS, C. J., YANOFSKY, M., NESTER, E. W., HELINSKI, D. R. & DITTA, G. (1986). *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 4403–4407.
- FINAN, T. M., HIRSCH, A. M., LEIGH, J. A., JOHANSEN, E., KULDAU, G. A., DEEGAN, S., WALKER, G. C. & SIGNER, E. R. (1985). Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. *Cell* **40**, 869–877.
- GARFINKEL, D. J., SIMPSON, R. B., REAM, L. W., WHITE, F. F., GORDON, M. P. & NESTER, E. W. (1981). Genetic analysis of crown gall: fine structure map of T-DNA by site-directed mutagenesis. *Cell* **27**, 143–153.

- GEREMIA, R. A., CAVAGNAC, S., ZORREGUIETA, A., TORO, N., OLIVARES, J. & UGALDE, R. A. (1987). A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β -(1-2) glucan. *Journal of Bacteriology* **169**, 880–884.
- GLAZEBROOK, J. & WALKER, G. C. (1989). A novel exopolysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. *Cell* **56**, 661–672.
- HISAMATSU, M., ABE, J., AMEMURA, A. & HARADA, T. (1980). Structural elucidation of succinoglycan and related polysaccharides from *Agrobacterium* and *Rhizobium* by fragmentation with two special β -D-glycanases and methylation analysis. *Agricultural and Biological Chemistry* **44**, 1049–1055.
- LEIGH, J. A. & LEE, C. C. (1988). Characterization of polysaccharides of *Rhizobium meliloti* *exo* mutants that form ineffective nodules. *Journal of Bacteriology* **170**, 3327–3332.
- LEIGH, J. A., SIGNER, E. R. & WALKER, G. C. (1985). Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 6231–6235.
- LONG, S., REED, J. W., HIMAWAN, J. & WALKER, G. C. (1988). Genetic analysis of a cluster of genes required for synthesis of the Calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. *Journal of Bacteriology* **170**, 4239–4248.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MATTHYSSE, A. G. (1983). Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. *Journal of Bacteriology* **154**, 906–915.
- MAXWELL, E. S., KURAHASHI, K. & KALCKAR, H. M. (1962). Enzymes of the Leloir pathway. *Methods in Enzymology* **5**, 174–189.
- PAYNE, S. M. & AMES, B. N. (1982). A procedure for rapid extraction and high-pressure liquid chromatographic separation of the nucleotides and other small molecules from bacterial cells. *Analytical Biochemistry* **123**, 151–161.
- POSTMA, P. W. (1977). Galactose transport in *Salmonella typhimurium*. *Journal of Bacteriology* **129**, 630–639.
- PUTNOKY, P., PETROVICS, G., KERESZT, A., GROSSKOPF, E., THI CAM HA, D., BANFALVI, Z. & KONDOROSI, A. (1990). *Rhizobium meliloti* lipopolysaccharide and exopolysaccharide can have the same function in the plant–bacterium interaction. *Journal of Bacteriology* **172**, 5450–5458.
- PUVANESARAJAH, V., SCHELL, F. M., STACEY, G., DOUGLAS, C. J. & NESTER, E. W. (1985). Role for 2-linked- β -D-glucan in the virulence of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **164**, 102–106.
- REUBER, T. L., LONG, S. & WALKER, G. C. (1991). Regulation of *Rhizobium meliloti* *exo* genes in free-living cells and in planta examined by using *TnphoA* fusions. *Journal of Bacteriology* **173**, 426–434.
- SALKINOJA-SALONEN, M. & BOECK, R. (1978). Characterization of lipopolysaccharides isolated from *Agrobacterium tumefaciens*. *Journal of General Microbiology* **105**, 119–125.
- STANELONI, R. J., TOLMASKY, M. E. & LELOIR, L. F. (1984). Lipid-bound saccharides containing glucose and galactose in *Agrobacterium tumefaciens*. *Journal of General Microbiology* **130**, 869–879.
- TOLMASKY, M. E., STANELONI, R. J., UGALDE, R. A. & LELOIR, L. F. (1980). Lipid-bound sugars in *Rhizobium meliloti*. *Archives of Biochemistry and Biophysics* **203**, 358–364.
- TOLMASKY, M. E., STANELONI, R. J. & LELOIR, L. F. (1982). Lipid-bound saccharides in *Rhizobium meliloti*. *Journal of Biological Chemistry* **257**, 6751–6757.
- TREVELYAN, W. F., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugar on paper chromatograms. *Nature, London* **166**, 444–445.
- UGALDE, R. A., COIRA, J. A. & BRILL, W. J. (1986). Biosynthesis of a galactose and galacturonic acid-containing polysaccharide in *Rhizobium meliloti*. *Journal of Bacteriology* **168**, 270–275.
- UTTARO, A. D., CANGELOSI, G. A., GEREMIA, R. A., NESTER, E. W. & UGALDE, R. A. (1990). Biochemical characterization of avirulent *exoC* mutants of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **172**, 1640–1646.
- WHATLEY, M., BODWIN, J., LIPPINCOTT, B. & LIPPINCOTT, J. A. (1976). Role for *Agrobacterium* cell envelope lipopolysaccharide in infection site attachment. *Infection and Immunity* **13**, 1080–1083.
- WILSON, D. B. & HOGNESS, D. S. (1964). The enzymes of the galactose operon in *Escherichia coli*. I. Purification and characterization of uridine diphosphogalactose 4-epimerase. *Journal of Biological Chemistry* **239**, 2469–2481.
- YOUNG, R. A. & DAVIS, R. W. (1983). Yeast RNA polymerase II genes: isolation with antibody probes. *Science* **222**, 778–782.
- ZEVENHUIZEN, L. P. T. M., SCHOLTEN-KOERSELMAN, J. & POSTHUMUS, M. A. (1980). Lipopolysaccharides of *Rhizobium*. *Archives of Microbiology* **125**, 1–8.
- ZHAN, H., LEVERY, S. B., LEE, C. C. & LEIGH, J. A. (1989). A second exopolysaccharide of *Rhizobium meliloti* strain SU47 that can function in root nodule invasion. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3055–3059.
- ZORREGUIETA, A., UGALDE, R. A. & LELOIR, L. F. (1985). An intermediate in cyclic β 1-2glucan biosynthesis. *Biochemical and Biophysical Research Communications* **126**, 352–357.