Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*

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In *Rhizobium leguminosarum* both the C₄-dicarboxylate transport system and wild-type lipopolysaccharide layer (LPS) are essential for nitrogen fixation. A Tn5 mutant (RU301) of *R. leguminosarum* bv. *viciae* 3841, was isolated that is only able to synthesize LPS II, which lacks the O-antigen. Strain RU301 exhibits a rough colony morphology, flocculates in culture and is unable to swarm in TY agar. It also fails to grow on organic acids, sugars or TY unless the concentration of calcium or magnesium is elevated above that normally required for growth. The defects in the LPS and growth in strain RU301 were complemented by a series of cosmids from a strain 3841 cosmid library (pRU3020–pRU3022) and a cosmid from *R. leguminosarum* bv. *phaseoli* 8002 (pIJ1848). The transposon insertion in strain RU301 was shown to be located in a 3 kb *EcoRI* fragment by Southern blotting and cloning from the chromosome. Sub-cloning of pIJ1848 demonstrated that the gene disrupted by the transposon in strain RU301 is located on a 2.4 kb *EcoRI*–*PstI* fragment (pRU74). *R. leguminosarum* bv. *viciae* VF39-C86, which is one of four LPS mutants previously isolated by U. B. Priefer (1989, *J Bacteriol* 171, 6161–6168), was also complemented by sub-clones of pIJ1848 but not by pRU74, suggesting the mutation is in a gene adjacent to that disrupted in strain RU301. Complementation and Southern analysis indicate that the region contained in pIJ1848 does not correspond to any other cloned *lps* genes. Two *dctA* mutants, RU436 and RU437, were also complemented by pIJ1848 and pRU3020. Mapping of pIJ1848 and Southern blotting of plasmid-deleted strains of *R. leguminosarum* revealed that *dctD* and the region mutated in strain RU301 are located approximately 10 kb apart on the chromosome. Analysis of homogenotes demonstrated that there is not a large region important in calcium utilization, organic acid metabolism or LPS biosynthesis located between the gene disrupted in strain RU301 and *dctD*. Strain VF39C-86 also required an elevated concentration of calcium for growth on succinate, while strains mutated in the α -chromosomal or β -plasmid group of *lps* genes grew at the same calcium concentrations as the wild type, demonstrating that the additional calcium requirement is not a property of all LPS rough mutants. Strain RU301 nodulates peas, but does not reduce acetylene, demonstrating that the gene mutated in this strain is essential for nitrogen fixation.

Keywords: *Rhizobium leguminosarum*, lipopolysaccharide, *dct*, organic acids, dicarboxylates, calcium

INTRODUCTION

The lipopolysaccharide (LPS) layer of *Rhizobium leguminosarum* is essential for the formation of effective nodules, with *lps* mutants being severely impaired in nodule development and nitrogen fixation (Noel *et al.*, 1986; Prierer, 1989; de Maagd *et al.*, 1989b; Diebold & Noel, 1989; Cava *et al.*, 1989, 1990; Brink *et al.*, 1990; Kannenberg *et al.*, 1992). Several roles have been suggested for the LPS layer during infection, which include a signal molecule, protection from toxic hydrophobic molecules or from being recognized as foreign by plant defences (reviewed in Noel, 1992). In the determinate nodules of bean plants, LPS rough mutants of *R. leguminosarum* bv. *phaseoli* do not fix nitrogen and bacteria accumulate in the infection thread without forming bacteroids (Noel *et al.*, 1986; Diebold & Noel, 1989; Cava *et al.*, 1989). On indeterminate hosts such as peas and vetch, infection proceeds much further and while nitrogen fixation is severely affected, low rates can often be detected (Diebold & Noel, 1989; Prierer, 1989; de Maagd *et al.*, 1989b). Some bacteroids can be seen in sections of indeterminate nodules and it has been suggested that there may be two distinct functions for the LPS layer, with only one being required in indeterminate nodules, possibly for bacteroid proliferation (Noel, 1992).

The LPS of *R. leguminosarum* can be classified into LPS I and LPS II. LPS I contains lipid A, core tetra- and trisaccharide as well as O-antigen which produces several bands on an SDS gel (Zhang *et al.*, 1992; Noel, 1992). LPS II lacks the O-antigen and only produces a single rapidly migrating band on an SDS gel. It is apparent that the composition and amount of the O-antigen are highly variable, with low pH, low O₂ tension and low phosphate changing the levels of expression of some O-antigens (Kannenberg & Brewin, 1989; de Maagd *et al.*, 1989a; Sindhu *et al.*, 1990; Tao *et al.*, 1992). In addition, growth substrates such as succinate can at least indirectly change the O-antigen by increasing the pH of the medium during growth (Kannenberg & Brewin, 1989). Calcium deficiency has also been shown to cause a loss of LPS from the outer membrane of *R. leguminosarum* bv. *viciae*, with a possible accumulation in the growth medium (de Maagd *et al.*, 1989a). Curing one of the native plasmids of all three biovars of *R. leguminosarum* also causes a loss of the O-antigen (Hynes & McGregor, 1990; Baldani *et al.*, 1992; Brom *et al.*, 1992; Chen *et al.*, 1993). In *R. leguminosarum* bv. *trifolii*, curing this plasmid also causes the loss of the ability to grow on several different carbon sources including malate (Baldani *et al.*, 1992). However, with such a large deletion it is difficult to tell whether the nutritional effects are attributable to the lack of LPS itself or to other genes on the plasmid.

Nutrient exchange in the nodule is a complex process, where the bacteroid reduces N₂ to ammonia in return for a carbon source from the plant. The most likely carbon source provided by the plant for use by the bacteroid is a C₄-dicarboxylic acid, either L-malate, succinate or fumarate. This conclusion is based on the observation that C₄-

dicarboxylates support high rates of respiration in isolated bacteroids (Glenn & Dilworth, 1981) and mutations in either malic enzyme or the structural gene for the dicarboxylate transport system (*dctA*) abolish nitrogen fixation, while mutations preventing sugar catabolism have no effect (Ronson & Primrose, 1979; Ronson *et al.*, 1981; Finan *et al.*, 1983; Arwas *et al.*, 1985; Engelke *et al.*, 1987; Glenn *et al.*, 1984; Bolton *et al.*, 1986). Furthermore, gluconeogenic enzymes are expressed in bacteroids of *R. leguminosarum*, *R. meliloti* and *Rhizobium* strain NGR234, indicating significant quantities of sugars are not present in the cytoplasm (McKay *et al.*, 1985; Finan *et al.*, 1991; Osteras *et al.*, 1991). The dependence of nitrogen fixation on the transport and catabolism of dicarboxylates has led to intensive study of the *dct* system (Arwas *et al.*, 1985; Finan *et al.*, 1983; Glenn *et al.*, 1980; Ronson *et al.*, 1981). In *R. leguminosarum* and *R. meliloti*, *dctA* encodes a highly hydrophobic protein with several membrane-spanning helices that is almost certainly the dicarboxylate transport protein (Ronson *et al.*, 1984, 1987; Watson, 1990; Jiang *et al.*, 1989; Engelke *et al.*, 1989). *dctB* and *dctD* encode a two-component sensor and regulator, respectively, transcribed divergently from *dctA*, which activate transcription of *dctA* in response to the presence of dicarboxylates in the environment (Ronson & Astwood, 1985; Ledebur & Nixon, 1992; Wang *et al.*, 1989; Batista *et al.*, 1992; Ledebur *et al.*, 1990; Ronson, 1988; Jording *et al.*, 1992; Yarosh *et al.*, 1989). Dicarboxylates are presumably detected by DctB, either by directly binding the substrate itself or via detection of the substrate binding state of DctA. Most models suggest that DctB undergoes autophosphorylation and in turn phosphorylates DctD, which binds to the tandem upstream activator sites of *dctA* enabling it to activate transcription (Ronson, 1988; Yarosh *et al.*, 1989; Jording *et al.*, 1992; Ledebur *et al.*, 1990; Ledebur & Nixon, 1992). In *R. meliloti* the *dct* system is located on the *exo* megaplasmid, but its location in *R. leguminosarum* is unknown (Watson *et al.*, 1988).

While examining the regulation of dicarboxylate utilization, we isolated an unusual mutant (RU301) which does not grow on organic acids. Growth of strain RU301 is critically dependent on the concentration of divalent metal ions in the medium, with its growth on organic acids being rescued by increased levels of magnesium and calcium. In this work we demonstrate that strain RU301 is an LPS rough mutant. Furthermore, the gene mutated in strain RU301 is clustered with the *dct* genes on the chromosome of *R. leguminosarum*. The significance of the physiological and genetic linkage of these two regions, both of which are essential for nitrogen fixation, is discussed.

METHODS

Bacterial strains and culture conditions. The strains used are described in Table 1. Bacteria were grown on either TY with CaCl₂ (6 mM) (Beringer, 1974) or on acid minimal salts (AMS) medium, which is derived from that of Brown & Dilworth (1975) with the changes being; potassium phosphate (0.5 mM), MgSO₄ (2 mM), CaCl₂ (0.17 mM) and buffering provided by

MOPS (20 mM), pH 7.0. It is indicated in the text when calcium and magnesium levels were altered. All carbon and nitrogen sources were at 10 mM. Antibiotics were used at the following concentrations (in $\mu\text{g ml}^{-1}$): kanamycin, 40; streptomycin, 500; tetracycline, 2 (in AMS), 5 (in TY); gentamycin, 20; ampicillin, 50; unless otherwise stated. Motility was measured in TY with agar at 0.3% and is referred to as motility agar.

DNA and genetic manipulations. All routine DNA analysis was done according to Sambrook *et al.* (1989). Southern transfer of DNA to positively charged nylon membrane and hybridization were done using an Amersham ECL kit according to the manufacturer's instructions. Conjugations were performed using either *Escherichia coli* strain S17-1 as the donor strain according to Simon *et al.* (1983) or as triparental matings according to Figurski *et al.* (1979) with either *E. coli* strains 803 or DH5 α as the donor, and strain 803 containing pRK2013 providing the transfer functions. Transductions were performed according to Buchanan-Wollaston (1979) using the phage RL38. Transductants were selected for on TY agar containing kanamycin (80 $\mu\text{g ml}^{-1}$). DNA sequencing was performed by the cycle sequencing method using a Promega fmol kit according to the manufacturer's instructions.

Transport assays. Cells were prepared and succinate transport assayed as previously described (Poole *et al.*, 1985), using 18.5 kBq [2,3- ^{14}C] succinate (4.0 GBq mmol $^{-1}$) at a total substrate concentration of 25 μM . Calcium transport was measured as previously described (Poole *et al.*, 1985), except that cells were washed and resuspended in HEPES (5 mM) instead of minimal salts. Calcium transport was measured using 18.5 kBq [^{45}Ca] CaCl_2 (126.1 MBq mmol $^{-1}$) at total substrate concentrations of 50 μM , 1 mM and 5 mM.

Mutagenesis. Transposon mutagenesis was carried out on *R. leguminosarum* bv. *viciae* 3841 with Tn5 using the suicide vector pSUP202-1 as described by Simon *et al.* (1983). Mutations in pIJ1848 were produced by first transforming the cosmid into *E. coli* strain MC1061. Transformants were mutagenized with Tn5-*lacZ* by using λ containing the transposon derivative B20 as described by Simon *et al.* (1989). Kanamycin-resistant colonies were pooled, and the cosmids isolated by the alkaline lysis technique. The mutated cosmids were transformed into *E. coli* strain DH5 α and kanamycin-resistant colonies purified. Cosmids were isolated from each colony and the location and orientation of transposons determined by restriction mapping and Southern blotting using pRU47 and pRU48 as probes (Fig. 3). Mutated cosmids were conjugated into *R. leguminosarum* 3841. After purification the incompatible plasmid pPHJ11 was conjugated into each strain and the homogenotes isolated by the technique of Ruvkun & Ausubel (1981).

Plant assays. Seeds of *Pisum sativum* cv. meteor and *Vicia sativa* were surface-sterilized and germinated in sterile water before adding them to cotton-wool-plugged 250 ml conical flasks containing 100 ml sterile vermiculite and wetted with sterile nitrogen-free rooting solution. This contained 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 100 μM KCl, 800 μM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 μM Fe EDTA, 35 μM H_3BO_3 , 9 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.8 μM ZnCl_2 , 0.5 μM $\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$, 0.3 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 7.2 mM KH_2PO_4 and 7.2 mM Na_2HPO_4 . The phosphate solutions were autoclaved separately and added to the main stock just prior to use. After 3 d the seeds were inoculated with a bacterial culture and seedlings kept in the dark until the shoot reached the top of the flask, after which the shoot was pulled through, and the flask wrapped with foil to exclude light from the roots. Plants were incubated at 25 °C in a growth room with illumination provided

by a Philips Sont-Agro grow light. Four weeks after inoculation, plants were harvested and acetylene reduction carried out on whole plants as described by Trinick *et al.* (1976). Sample nodules were removed and surface-sterilized by immersion in calcium hypochlorite (0.7%) for 10 min. Nodules were then washed three times in sterile distilled water, crushed and bacteria streaked on TY agar. Isolated bacteria were then replica plated and screened for appropriate antibiotic and nutritional markers.

Preparation and PAGE of LPS samples. *R. leguminosarum* strains were grown in liquid culture overnight or taken directly from 3-d-old slope cultures and re-suspended in sterile water. Cells were centrifuged and washed twice in MOPS (10 mM), pH 7.5, buffered saline. The pellets were then re-suspended in MOPS (25 mM), pH 7.5, containing EDTA (5 mM). Cells were broken by two passages through a French press at 69000 kPa and unlysed cells were removed by centrifuging twice at 1100 g for 5 min. A sample of the supernatant was kept as the total cell fraction and the rest was centrifuged at 17400 g for 30 min at 5 °C to precipitate the outer membrane fraction, which was resuspended in MOPS (10 mM), pH 7.5. All samples were stored at -20 °C and the protein concentration determined by the Bradford assay (Bradford, 1976). Samples were digested by proteinase K (50 $\mu\text{g ml}^{-1}$) by incubation at 37 °C for 90 min. These samples were then heated to 100 °C for 10 min, cooled and incubated again with proteinase K (100 $\mu\text{g ml}^{-1}$) at 37 °C for 60 min. To detect LPS, samples were mixed with an equal volume of Laemmli solubilization buffer where the SDS was replaced by taurodeoxycholate (TDOC) (4%) and treated as described by Kannenberg *et al.* (1992). The protein loading for total cell extract was 5–10 μg of protein and for partially purified outer membrane, 1–2 μg . Samples were run on 15% polyacrylamide gels according to the technique of Laemmli (1970) except TDOC (0.1%) replaced the SDS. LPS was then detected by the periodate-silver staining technique of Dzandu *et al.* (1984).

RESULTS

Isolation of *dct* and LPS mutants of *R. leguminosarum* bv. *viciae* 3841

Strain 3841 was mutagenized with Tn5 and 10000 colonies screened for lack of growth on either malate, fumarate or succinate as the sole carbon source (Table 2). Strains RU436 and RU437 were unable to transport succinate at wild-type rates, suggesting that they are *dct* mutants. The ability of strain RU301 to grow on sugars, but not on organic acids, and actively transport succinate suggested it may be a gluconeogenic mutant. However, strain RU301 was rescued for growth on organic acids by elevated levels of calcium or magnesium (Table 3). Colonies of strain RU301 grown on TY agar lacked the normal mucoid appearance of *Rhizobium*; the cells clumped together when cultured and they were unable to swarm in motility agar (Fig. 1a and b). Strain RU301 is motile when observed under the microscope, suggesting that the inability to swarm may be due to cells adhering to one another in the agar, rather than any defect in either flagella assembly or the flagellar motor. These characteristics are all indicative of an LPS rough mutant. To confirm this, crude preparations of outer membranes were isolated from strains 3841 and RU301 and run on TDOC-PAGE gels. After periodate oxidation the gels were silver-stained to allow detection of LPS (Fig. 2, lanes 1

Table 1. Bacterial strains, phages and plasmids used

Strain, bacteriophage or plasmid	Description	Source or reference
<i>R. leguminosarum</i>		
1.4d	Strain W14-2 bv. <i>trifolii</i> cured of plasmid a	Baldani <i>et al.</i> (1992)
3841	Str-resistant derivative of strain 300 bv. <i>viciae</i>	Johnston & Beringer (1975)
RU4000	Spontaneous derivative of strain 3841 that grows at low calcium	This work
CR534	Strain 3855 bv. <i>viciae</i> <i>dctA</i> ::Tn5	Ronson <i>et al.</i> (1987)
CR535	Strain 3855 bv. <i>viciae</i> <i>dctB</i> ::Tn5	Ronson <i>et al.</i> (1987)
CR538	Strain 3855 bv. <i>viciae</i> <i>dctD</i> ::Tn5	Ronson <i>et al.</i> (1987)
LRS39301	Strain VF39 bv. <i>viciae</i> cured of plasmid pRleVF39c	Hynes & McGregor (1990)
RU301	Strain 3841 <i>lps</i> ::Tn5	This work
RU337	pIJ1848::Tn5- <i>lacZ</i> homogenote in strain 3841	This work
RU344	pIJ1848::Tn5- <i>lacZ</i> homogenote in strain 3841	This work
RU353	pIJ1848::Tn5- <i>lacZ</i> homogenote in strain 3841	This work
RU363	pIJ1848::Tn5- <i>lacZ</i> homogenote in strain 3841	This work
RU427	pIJ1848::Tn5- <i>lacZ</i> homogenote in strain 3841	This work
RU436	Strain 3841 <i>dctA</i> ::Tn5	This work
RU437	Strain 3841 <i>dctA</i> ::Tn5	This work
VF39	Wild type bv. <i>viciae</i>	Priefer (1989)
VF39-23	Strain VF39 <i>lps</i> ::Tn5	Priefer (1989)
VF39-32	Strain VF39 <i>lps</i> ::Tn5	Priefer (1989)
VF39-51	Strain VF39 <i>lps</i> ::Tn5	Priefer (1989)
VF39-C86	Strain VF39 <i>lps</i> ::Tn5	Priefer (1989)
W14-2	Wild type bv. <i>trifolii</i>	Baldani <i>et al.</i> (1992)
<i>E. coli</i>		
803	<i>met⁻ gal⁻</i>	Wood (1966)
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	Hanahan (1983); BRL (1986)
MC1061	<i>hsdR mcrB araD139 Δ(<i>araABC-leu</i>)7679 ΔlacX74 <i>galU galK rpsL thi</i></i>	Meissner <i>et al.</i> (1987)
S17-1	<i>pro hsdR recA</i> [RP4-2(Tc::Mu) (Km::Tn7)]; RP4 integrated into its chromosome	Simon <i>et al.</i> (1983)
Plasmids and phage		
Bluescript SK -	Phagemid, fl(-) origin of replication, ColE1 replicon; Amp ^R	Stratagene
pCOS109.11	Cosmid containing α - <i>lps</i> region from <i>R. leguminosarum</i> bv. <i>phaseoli</i> CFN42	Cava <i>et al.</i> (1989)
pCOS126	Cosmid containing β - <i>lps</i> region from <i>R. leguminosarum</i> bv. <i>phaseoli</i> CFN42	Cava <i>et al.</i> (1989)
pCOS309.1	Cosmid containing γ - <i>lps</i> region from <i>R. leguminosarum</i> bv. <i>phaseoli</i> CFN42	Cava <i>et al.</i> (1989)
pIJ1848	Cosmid from strain 8002 containing <i>dctA-B-D</i> and <i>lps</i>	Mavridou (1992)
pIJ1969	pIJ1848 <i>dctB</i> ::Tn5- <i>lacZ</i>	Mavridou (1992)
pIJ1970	pIJ1848 <i>dctA</i> ::Tn5- <i>lacZ</i>	Mavridou (1992)
pLAFR1	Broad host range P-group cloning vector, mobilizable RK2 cosmids; Tc ^R	Freidman <i>et al.</i> (1982)

Table 1. (cont.)

Strain, bacteriophage or plasmid	Description	Source or reference
pMP2602	pTJS133 containing <i>exoB</i> gene from <i>R. leguminosarum</i> bv. <i>viciae</i>	Canter-Cremers <i>et al.</i> (1990)
pPHJ11	P-group chaser plasmid	Hirsch & Beringer (1984)
pRK2013	ColE1 replicon with RK2 <i>tra</i> genes, helper plasmid used for mobilizing P- and Q-group plasmids; Nm ^R Km ^R	Figurski & Helinski (1979)
pRK415	Broad host range P-group cloning vector; Tc ^R	Keen <i>et al.</i> (1988)
pRU8	pBluescript SK — carrying 0.9 kb <i>EcoRI</i> fragment from strain 3841 containing <i>dctA-B</i> intergenic region	This work
pRU29	pBluescript SK — carrying <i>EcoRI</i> fragment containing Tn5 from RU301	This work
pRU47	pBluescript SK — carrying 10 kb <i>HindIII</i> fragment from pIJ1848	This work
pRU48	pBluescript SK — carrying 9.4 kb <i>HindIII</i> fragment from pIJ1848	This work
pRU67	pRK415 carrying 4.4 kb <i>BamHI-HindIII</i> fragment from pIJ1848	This work
pRU68	pRK415 carrying 3.8 kb <i>EcoRI-HindIII</i> fragment from pIJ1848	This work
pRU74	pRK415 carrying 2.4 kb <i>EcoRI-PstI</i> fragment from pIJ1848	This work
pRU75	pBluescript SK — carrying 1 kb <i>HpaI-HindIII</i> fragment from IS50	This work
pRU86	pBC KS — carrying 3 kb <i>HindIII-EcoRI</i> fragment from pIJ1848	This work
pRU3000	Cosmid from strain 3841 containing <i>dctA</i>	This work
pRU3001	Cosmid from strain 3841 containing <i>dctA-B-D</i>	This work
pRU3020	Cosmid from strain 3841 containing <i>dctA-B-D</i> and <i>lps</i>	This work
pRU3022	Cosmid from strain 3841 containing <i>lps</i>	This work
pSUP202-1::Tn5	<i>mob</i> Km ^R	Simon <i>et al.</i> (1983)
λ::Tn5- <i>lacZ</i>	λ carrying the Tn5-B20 transposon	Simon <i>et al.</i> (1989)
RL38	Generalized transducing phage of <i>R. leguminosarum</i>	Buchanan-Wollaston (1979)

Table 2. Properties of organic acid utilization mutants of *R. leguminosarum*

Growth was measured at 0.17 mM calcium and 2 mM magnesium. Succinate transport was measured in cells grown on minimal medium with glucose and aspartate which induces the DCT system. +, growth; —, no growth.

Strain	Growth on:						Transport of succinate
	TY	Glucose	Succinate	Malate	Fumarate	Pyruvate	
3841	+	+	+	+	+	+	+
RU301	+	+	—	—	—	—	+
RU436	+	+	—	—	—	+	—
RU437	+	+	—	—	—	+	—

Table 3. Effect of magnesium and calcium concentration on growth of *R. leguminosarum* on succinate

Growth medium was AMS with carbon and nitrogen sources at 10 mM. +, good growth; ±, poor growth; —, no growth.

Strain	Magnesium (mM)	Calcium (mM)	Growth	Swarm
3841	0.5	0.17	—	—
RU301			—	—
3841	1	0.17	±	—
RU301			—	—
3841	2	0.17	+	+
RU301			—	—
3841	5	0.17	+	+
RU301			±	—
3841	10	0.17	+	±
RU301			±	—
3841	2	0.08	+	—
RU301			—	—
3841	2	0.16	+	—
RU301			±	—
3841	2	0.32	+	—
RU301			+	—
3841	2	0.64	+	±
RU301			+	±
3841	2	2	+	+
RU301			+	+

and 2). It can be seen that strain RU301 only has an LPS II fraction, which consists of part or all of the core oligosaccharides and the lipid A component, but lacks LPS I which includes the O-antigen. That the stained bands were unlikely to be due to protein was confirmed by proteinase K digestion (Fig. 2, lanes 3 and 4).

When grown on glucose minimal medium, strain RU301 was not as mucoid as strain 3841, although this was less apparent than when grown on TY which suppresses exopolysaccharide synthesis. Analysis of the LPS of strains 3841 and RU301 grown on glucose or succinate minimal medium confirmed the absence of LPS I in strain RU301 (data not shown). Thus the loss of LPS I in strain RU301 is not dependent on whether cells are grown on a rich or minimal medium. The medium used for growth in these studies was highly buffered, so pH-dependent changes in LPS associated with growth on organic acids were not observed.

Effect of calcium and magnesium levels on growth and LPS

Increasing the magnesium level in the medium to 10 mM, while maintaining the calcium level at 0.17 mM, partially rescued the growth of strain RU301 on succinate as the sole carbon source (Table 3). Increasing the calcium level to 0.32 mM, with the magnesium level kept at 2 mM, was even more effective at rescuing the growth of strain RU301 (Table 3). Increasing the pH of the growth

medium, which decreases the solubility of calcium and magnesium, also increased the total amount of these ions required for growth. Clearly the precise concentration of both calcium and magnesium required for growth are interdependent. Furthermore the effect is altered by any factor, such as phosphate concentration or pH which alters the availability of either ion. However, growing strain RU301 with either magnesium at 5 mM or calcium at 2 mM did not restore LPS I (data not shown). This clearly indicates that elevating the concentration of metal ions does not rescue growth on organic acids by restoring normal synthesis of LPS.

R. leguminosarum strains 3841 and RU301 were grown on minimal medium with glucose as the carbon source and either 0.08 or 0.16 mM CaCl_2 to determine calcium accumulation by the cells. Under these growth conditions there was no active accumulation of calcium in either strain at final calcium concentrations of 50 μM , 1 mM or 5 mM. While this implies that the mutation in RU301 is unlikely to be in an active transport system, it cannot be excluded that the mutation alters binding or passive accumulation of calcium.

Strain RU301 also grows very poorly compared to strain 3841 on glucose minimal medium with magnesium at 0.5 mM and in TY with the calcium level decreased to 3 mM. Thus, while the added requirement for divalent metal ions is most noticeable when growth is measured on organic acids, it is not specific to this class of compounds. Throughout this study, succinate was used as a carbon source to measure the calcium dependence of growth but this is not meant to indicate that it is in any way specific to this compound. It is possible that the higher requirement for divalent metal ions for growth on dicarboxylic acids compared to a sugar for either strain RU301 or 3841 may be due to chelation of the calcium by the carboxyl groups.

Spontaneous mutants of *R. leguminosarum* that grow at lower levels of magnesium and calcium than the wild type were readily obtained by plating cells on succinate minimal medium containing 0.5 mM magnesium and 0.17 mM calcium. Unless scrupulous precautions are taken, any routine sub-culture of *R. leguminosarum* on minimal media containing dicarboxylates appears likely to produce such mutants. One such mutant of strain 3841, designated strain RU4000 was retained for further study. Compared to strain 3841, strain RU4000 grows at lower levels of magnesium or calcium with either succinate, glucose or glutamate as the carbon source. This suggests that the spontaneous mutation in strain RU4000 affects calcium acquisition or utilization rather than utilization of any specific carbon source.

The addition of extra calcium to minimal medium containing succinate as the carbon source, enabled both strains 3841 and RU301 to swarm (Table 3). On TY agar, swarming could not be restored to strain RU301 by levels of calcium from 2 to 10 mM. These results confirm that the loss of LPS does not directly prevent motility or swarming. The ability to swarm is actually dependent on

Fig. 1. Swarm phenotype of strain RU301 and complemented derivatives measured in TY motility agar (0.3%). (a) Strain 3841. (b) Strain RU301. (c) Strain RU301(pIJ1848).

the media composition as well as the ion content. While the ability to swarm on TY can be restored to some rough mutants of *R. leguminosarum* by lowering the calcium level to 1 mM (Priefer, 1989), this could not be tested in strain RU301 as this level of calcium did not support growth.

Plant properties

Strain RU301 nodulated peas and of 30 nodule isolates, only one did not retain both kanamycin resistance and a rough colony morphology. In four separate experiments, *R. leguminosarum* strain 3841 reduced acetylene at a rate of $0.58 \pm 0.1 \mu\text{mol h}^{-1}$ per plant, while there was no detectable acetylene reduction by strain RU301. These results are consistent with previous studies that have shown that LPS mutants do not reduce acetylene at

significant rates and thus are presumably impaired in nitrogen fixation (Diebold & Noel, 1989; Priefer, 1989; de Maagd *et al.*, 1989b).

Complementation and mapping of strain RU301

The Tn5 insertions in strains RU301, RU436 and RU437 were transduced into the wild-type strain 3841, using the generalized transducing phage RL38. All the kanamycin-resistant transductants of strain RU301 (> 100) had a rough colony morphology. Of these, six colonies were purified twice and all failed to grow on succinate at the same divalent metal ion concentration as strain 3841. Likewise, all 37 and 17 transductants of strains RU436 and RU437, respectively, were unable to grow on dicarboxylic acids. This confirms that in all three mutants a single

Fig. 2. Silver-stained TDOC-PAGE gels of crude outer membrane preparations. Lanes: 1 and 3, strain 3841; 2 and 4, strain RU301; 1 and 2, crude membranes without further treatment; 3 and 4, membranes digested with proteinase K.

transposon insertion is tightly linked to the respective phenotype.

The putative *dct* mutant strains RU436 and RU437 were complemented by pIJ1848, which is known to contain the *dct* genes (Mavridou, 1992), but not by pIJ1969 which contains a transposon insertion in *dctA*. This demonstrates that they are both *dctA* mutants.

While strain RU301 is not impaired in its ability to transport succinate, the interaction between growth on succinate and divalent metal ion utilization suggested that the effect of cosmids which complement *dct* mutants should be tested for their ability to complement the mutation in strain RU301. Strain RU301 was restored to wild-type colony morphology, the ability to swarm on TY and growth on succinate at the same concentrations of divalent metal ions as strain 3841 by pIJ1848 (Fig. 1). However, pRU3001, which contains all three *dct* genes, did not complement strain RU301. This suggested that there may be a second group of genes that map close to the *dct* system that is involved in or regulates LPS biosynthesis. The insert DNA in pIJ1848 is derived from a cosmid library of *R. leguminosarum* bv. *phaseoli* 8002. To ensure that the clustering of genes for the *dct* system and for LPS biosynthesis is not specific to a strain of *R. leguminosarum*, strain RU301 was complemented with a cosmid library derived from *R. leguminosarum* bv. *viciae* 3841. A series of cosmids (pRU3020–3022) were isolated that restored smooth colony morphology and swarming in TY motility agar. These appear to be overlapping on the basis of common bands in restriction digests. Of these,

pRU3020 complemented strains CR534 (*dctA*), CR535 (*dctB*), CR538 (*dctD*) and RU301, while pRU3022 only complemented strain RU301.

Southern blotting of *Eco*RI-digested chromosomal DNA of strain RU301 with pRU75, which contains an internal fragment of IS50, revealed a single hybridizing band of approximately 9 kb. This is consistent with a single copy of Tn5 being located in a 3 kb *Eco*RI fragment. Chromosomal *Eco*RI digests of strains 3841 and RU301 were Southern blotted with pIJ1848 and it was found that a hybridizing band of approximately 3 kb in the DNA from strain 3841 was absent in strain RU301, but was replaced by a new band of approximately 9 kb. This demonstrates that the insert DNA in pIJ1848 is homologous to the DNA in the region of the transposon insertion in strain RU301 and indicates that the cosmid complements, rather than suppresses, the mutation. To confirm this conclusion, the Tn5 insertion in strain RU301 was cloned from the chromosome as a 9 kb *Eco*RI fragment (pRU29), by selection for kanamycin resistance. Plasmid pRU29 was then used as a probe in Southern blots against a series of sub-clones of pIJ1848 and was found to hybridize specifically to a 3.8 kb *Hind*III/*Eco*RI fragment of pRU48 (Fig. 3). It should be noted that the cosmid pIJ1848 is derived from *R. leguminosarum* bv. *phaseoli* 8002, not *R. leguminosarum* bv. *viciae* 3841, which explains the different restriction sites in the DNA of this region from the two strains. To confirm the exact position and orientation of the Tn5 insertion in pRU29 relative to pIJ1848, sequencing was carried out across the *Eco*RI sites of pRU29, and two subclones of pIJ1848 (pRU67 and pRU68) (Fig. 3).

Fig. 3. Map of the *dct*-*lps* region of *R. leguminosarum*. Below the map of pIJ1848 (DNA from strain 8002) are all the sub-clones made from this cosmid, while above are clones from the chromosome and cosmids derived from strain 3841. The locations of chromosomal Tn5 (>) and Tn5-*lacZ* (>) insertions are shown with the RU strain number in which they are inserted. The arrowhead points in the direction of transcription of the neomycin phosphotransferase gene. Restriction sites are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I. *, Restriction sites present in DNA from strain 3841 but not in pIJ1848.

A detailed restriction map of the *dct-lps* region of pIJ1848 was determined from overlapping *Hind*III and *Bam*HI subclones of pIJ1848 (Fig. 3). The position of the *dct* genes and the region that affects the LPS in pIJ1848 were confirmed to be 10 kb apart by Southern blotting with pRU29 and a clone of the *dctA-B* intergenic region (pRU8) (Fig. 3). The presence of the *dct* region in pRU3020 was confirmed by hybridization with pRU86, which consists of a *Hind*III-*Eco*RI fragment containing *dctA* and part of *dctB* (Fig. 3). A sub-clone of pIJ1848 (pRU67) (Fig. 3), which contains the region that affects the LPS layer, hybridized to a 3 kb *Eco*RI fragment of pRU3020. This confirms that the clustering of these genes is not dependent on the strain of *R. leguminosarum*. To further define the region mutated in strain RU301, a series of sub-clones of pRU48 were made (pRU67, pRU68 and pRU74) and tested for complementation of growth on succinate at low calcium, the ability to swarm and smooth colony morphology of strain RU301 (Fig. 3). All of these properties were complemented by the three sub-clones and in addition for pRU68 it was confirmed by TDOC-PAGE that LPS I synthesis had been restored (data not shown). The 2.4 kb *Eco*RI-*Pst*I fragment contained in pRU74 was the smallest able to complement RU301 (Fig. 3).

In an attempt to see whether the mutation in strain RU301 corresponds to an already defined mutation, a group of known *lps* mutations from *R. leguminosarum* bv. *viciae* VF39 (strains VF39-23, VF39-32, VF39-51 and VF39-C86) were tested to see if pIJ1848 complemented any of the mutations for growth on succinate at low calcium, the ability to swarm and smooth colony morphology (Priefer, 1989). Only the mutation in strain VF39-C86 was complemented. As reported by Priefer (1989) the three mutant strains VF39-23, VF39-32 and VF39-51 were complemented by pCOS4 and are clustered together on the chromosome, in what is probably the α -group defined by Noel (1992). No complementing cosmid was isolated for strain VF39-C86, so the location of the mutation was not determined (Priefer, 1989). The plasmids pRU67 and pRU68 complemented the mutations in both strain VF39-C86 and strain RU301 for growth on succinate at low calcium, the ability to swarm and smooth colony morphology. However, pRU74 did not complement strain VF39-C86 for any of the three measured parameters. This indicates that RU301 and VF39-C86 are mutated in separate genetic units.

Noel (1992) has defined five clusters of genes known to be required for normal LPS biosynthesis in *R. leguminosarum*. These are the α -chromosomal group which contains a large number of genes required for core and O-antigen synthesis, the plasmid-borne β -group, the chromosomal γ -group, a poorly defined region which contains the *lps-166* mutation of Noel (1992) and finally the *exoBC* region (Cava *et al.*, 1989; Diebold & Noel, 1989; Priefer, 1989; Cava *et al.*, 1990; Canter-Cremers *et al.*, 1990). Cosmids pCOS109.11, pCOS126, pCOS309.1 and pMP2602 (Table 1) which correspond to the α -*lps*, β -*lps*, γ -*lps* and *exoB* regions, respectively, all failed to complement RU301.

Furthermore Southern blotting showed that pRU68 does not hybridize with pCOS109.11, pCOS126 or pCOS309.1, confirming that the genes disrupted by RU301 and VF39-C86 do not correspond to any of the previously defined *lps* regions.

It is possible that higher concentrations of divalent metal ions are required for growth in any LPS rough mutant, perhaps due to an enhanced loss of ions from the cell surface. To test this possibility, LPS rough mutants from three different genetic regions were tested for their ability to grow on succinate, relative to the wild type *R. leguminosarum* bv. *viciae* VF39, at various calcium levels. Mutant strain VF39-C86, whose transposon insertion maps to the same region as that in strain RU301, was rescued for growth on AMS agar with succinate as the sole carbon source by elevating the calcium concentration to 0.25 mM. By comparison the wild type grew at 0.04 mM calcium. Strain VF39-32, which appears to be mutated in the main α -chromosomal group of *lps* genes, and strain LRS39301, which lacks the β -plasmid-borne *lps* genes, both grew at the same calcium concentration as the wild type. This demonstrates that the higher metal ion requirement for growth on succinate is specific to the region mutated in strains RU301 and VF39-C86 and is not a general property of LPS rough mutants.

Localization of the *dct* and *lps* genes of *R. leguminosarum*

It has been demonstrated that the *dct* genes of *R. meliloti* are clustered on the *exo* megaplasmid, while in *R. leguminosarum* their location is unknown (Watson *et al.*, 1988). They are not located on the *nod* plasmid since the *dct* system is still present in *R. leguminosarum* strain 8401 which is cured of its *nod* plasmid (Mavridou, 1992).

Strains of all three biovars of *R. leguminosarum* have now been cured of their endogenous plasmids (Hynes & McGregor, 1990; Brom *et al.*, 1992; Baldani *et al.*, 1992). One particular plasmid in each biovar, pRleVF39c in bv. *viciae* VF39, pb in bv. *phaseoli* strain CFN42 and plasmid a in bv. *trifolii* strain W14-2, cause a rough colony morphology due to loss of LPS I (Hynes & McGregor, 1990; Brom *et al.*, 1992; Baldani *et al.*, 1992). Strain W14-2 cured of plasmid a (strain 1.4d) was also reported to be unable to grow on malate (Baldani *et al.*, 1992). To test the possibility that this may be due to the loss of the region reported here, that affects both LPS biosynthesis and contains the *dct* genes, pIJ1848 was conjugated into strain 1.4d and found to be unable to complement the loss of LPS. Using pRU68 as a probe for the *lps* region, genomic *Eco*RI digests of strains W14-2, 1.4d and 3841 were Southern blotted and all three had a strongly hybridizing band at approximately 3 kb (data not shown). Since strain 1.4d lacks plasmid a, which carries the only plasmid-borne *lps* genes, these results are consistent with the clustering on the chromosome of the genes for the *dct* system and those mutated in strains RU301 and VF39-C86.

Characterization of directed mutations of the *dct-lps* region

The clustering of genes for *dct* and those that affect LPS, both of which are required for normal growth on dicarboxylates, implied there may be other genes in this region that alter either the LPS layer, divalent metal ion utilization or the ability to grow on organic acids. To test this, Tn5-*lacZ* insertions were made in pIJ1848, which were then mapped by restriction analysis and Southern blotting (Fig. 3). These were conjugated into *R. leguminosarum* strain 3841 and five homogenotes isolated. Loss of pIJ1848-dependent tetracycline resistance and Southern blotting confirmed the location of the transposons. All the homogenotes, strains RU344, RU337, RU363, RU353 and RU427, had a smooth colony morphology and swarmed normally on TY swarm agar, indicating that none of them were grossly affected in their LPS layer (Fig. 3). They all grew on succinate at the same levels of magnesium and calcium as strain 3841. While it cannot be concluded from this that there are not more subtle effects on the LPS layer and organic acid metabolism in these mutants, it suggests that there is a discrete region that affects calcium utilization and LPS biosynthesis between the boundaries defined by the position of transposon insertions in strains RU363 and RU353 (Fig. 3). The three homogenotes, strains RU344, RU337 and RU363, also demonstrate that there is not a large region lying between *dctD* and the transposon insertion in strain RU301 that affects either the LPS layer, calcium utilization or organic acid metabolism (Fig. 3).

DISCUSSION

On the basis of rough colony morphology, the inability to swarm in TY agar and the absence of bands corresponding to the O-antigen on silver-stained TDOC-PAGE gels, strain RU301 lacks the ability to make a complete LPS I (Figs 1 and 2). It is also unable to grow at the same concentration of calcium and magnesium as the wild type (Table 3). While increasing the concentration of magnesium or calcium restored growth, it did not restore LPS I biosynthesis. This indicates that the mutation in strain RU301 does not prevent growth because of the absence of a complete LPS alone. Instead the loss of the O-antigen might change either the availability of, or requirement for, metal ions at the cell surface. Alternatively the mutation in strain RU301 may regulate another factor, which affects both the utilization of divalent metal ions and LPS biosynthesis. The transduction and complementation data imply that the pleiotropic effects are due to a single Tn5 insertion. If there are other point or insertion sequence mutations they would have to map to the 2.4 kb region (pRU74) that already contains Tn5 in strain RU301.

The mutation in strain RU301 does not map to any of the already defined regions that are responsible for LPS biosynthesis in *R. leguminosarum*. However, the previously identified mutation in strain VF39-C86 appears to lie in a gene which is probably contiguous with the gene

disrupted in strain RU301 (Priefer, 1989). The mutation in VF39-C86 results in a truncation of the core tetrasaccharide to a disaccharide (Zhang *et al.*, 1992). Since strain RU301 lacks the O-antigen, it may also be defective in core synthesis; however, to be sure of this would require chemical analysis of the core oligosaccharides.

The requirement for a higher concentration of divalent metal ions for growth as measured with succinate, is specific to *R. leguminosarum* bv. *viciae* strains RU301 and VF39-C86, which are mutated in the same region. LPS rough mutants of *R. leguminosarum* bv. *viciae* strains VF39-32 and LRS39301 which are mutated in the α and β regions, respectively, were unaffected in growth on succinate relative to the wild type. It is particularly relevant that the LPS defects in strains VF39-C86 and VF39-32 have been chemically defined (Zhang *et al.*, 1992). In strain VF39-32 there is a complete loss of the core tetrasaccharide, while strain VF39-C86 forms a truncated core disaccharide. This indicates that the higher divalent metal ion requirement in strain VF39-C86 is not simply caused by it having more severe damage to the LPS layer than in strain VF39-32. It is still possible though that the mutations in strains RU301 and VF39-C86 change the charge on the cell surface more than other LPS rough mutants. This might increase the requirement for divalent metal ions for stabilization of the cell surface. It is possible that the genes mutated in strains RU301 and VF39-C86 may encode or regulate a factor which is pleiotropic in its effects on both the outer membrane and calcium utilization. It does not appear to encode an active uptake system for calcium but this does not preclude a role in passive accumulation or binding of calcium and other metal ions or possibly a structural role in the outer membrane.

Calcium and magnesium are required at high levels at the cell surface by rhizobia (Humphrey & Vincent, 1962; Vincent & Humphrey, 1963), with insufficient calcium and magnesium causing *R. leguminosarum* to swell and elongate (Humphrey & Vincent, 1962; Vincent, 1962; Vincent & Humphrey, 1968). Free-living cells of *R. meliloti* will form bacteroid-like shapes when grown on high concentrations of succinate (Gardioli *et al.*, 1987). To prevent the effect being due to a deficiency of cations, high levels of magnesium and calcium are required in the growth medium for these experiments. Calcium-deficient cells have also been shown to adsorb antibodies more effectively (Vincent & Humphrey, 1968) and interestingly appear to lose membrane-associated LPS to the medium (de Maagd *et al.*, 1989a). One of these antibodies, the protein-binding monoclonal antibody mAb 38, only binds under calcium-deficient conditions (de Maagd *et al.*, 1989a). The gene encoding the outer membrane protein to which mAb 38 binds has been cloned and the protein product shown to have a relative molecular mass of 36000. It forms high molecular mass oligomers that are stabilized most effectively by calcium but also by magnesium, manganese and strontium (de Maagd *et al.*, 1989c, 1992). The ability of the 36 kDa protein and another 40 kDa protein to form calcium-stabilized oligomers is

similar to that of porins in other Gram-negative bacteria. It is evident that the mutation in strain RU301 may affect these porin-like proteins either directly or possibly via changes in the availability of divalent metal ions for their stabilization. Calcium therefore plays an essential role in maintaining the correct structure of the cell surface in rhizobia. Such a role may be essential for proper synthesis and attachment of LPS as well as affecting growth.

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