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Identification of a putative LPS-associated cation exporter from *Rhizobium leguminosarum* bv. *viciae*

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Abstract

A gene, *cpaA*, with similarity to calcium proton antiporters has been identified adjacent to *lpcAB* in *Rhizobium leguminosarum*. LpcA is a galactosyl transferase while LpcB is a 2-keto-3-deoxyoctonate transferase, both of which are required to form the lipopolysaccharide (LPS) core in *R. leguminosarum*. Mutations in *lpcAB* result in a rough LPS phenotype with a requirement for elevated calcium concentrations to allow growth, suggesting that truncation of the LPS core exposes a highly negatively charged molecule. This is consistent with the LPS core being one of the main sites for binding calcium in the Gram-negative outer membrane. Strain RU1109 (*cpaA*::Tn*5-lacZ*) has a normal LPS layer, as measured by silver staining and Western blotting. This indicates that *cpaA* mutants are not grossly affected in their LPS layer. LacZ fusion analysis indicates that *cpaA* is constitutively expressed and is not directly regulated by the calcium concentration. Over-expression of *cpaA* increased the concentration of calcium required for growth, consistent with CpaA mediating calcium export from the cytosol. The location of *lpcA, lpcB* and *cpaA* as well as the phenotype of *lpcB* mutants suggests that CpaA might provide a specific export pathway for calcium to the LPS core.

Keywords: Calcium; Antiporter; Lipopolysaccharide

1. Introduction

The lipopolysaccharide (LPS) layer is one of the principal barriers between Gram-negative bacteria and the outside environment. Interactions between anionic groups in the LPS and calcium have long been implicated as having a major role in stabilising the structural integrity of LPS [1]. The LPS core of *Rhizobium leguminosarum* is a good model because it is well characterised both genetically and biochemically (Fig. 1) [2,3]. In these studies, we identified a cluster of LPS core biosynthetic genes that map close to the *dct* region of *R. leguminosarum*. Two of these genes, *lpcA*, which encodes a galactosyl transferase, and *lpcB*, which encodes a 2-keto-3-deoxyoctonate transferase, are separated by approximately 10 kb from *lpcC*, which encodes a mannosyl transferase (Fig. 1B). Mutations in these genes result in truncated core structures that lack the O-antigen. Mutants of *lpcAB* require elevated levels of calcium to grow [4]. The requirement for higher concentrations of calcium might be a result of the truncated LPS core structures in these mutants exposing negatively charged groups from the core region. Consistent with this, mutations affecting the O-antigen, but not the core, do not alter the calcium concentration required for growth [4]. It is not currently known how calcium is delivered to the LPS. Alternative explanations include: calcium simply diffuses into the LPS from outside the cell; or that calcium is actively exported from the cytoplasm. Calcium is normally present at very low concentrations inside the cell, consistent with its active export. In this study, we have identified a putative calcium export system transcribed towards *lpcB*. To our knowledge, this is the first example of a specific export system that might provide calcium for the LPS core.

2. Materials and methods

2.1. Strains and growth conditions

The strains and plasmids used in this work are described in Table 1. *R. leguminosarum* was routinely incubated at

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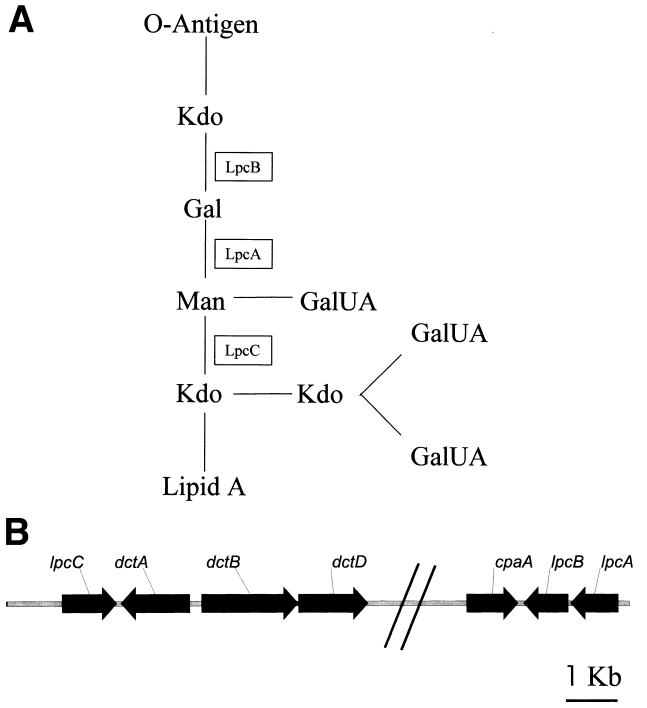

Fig. 1. A: The structure of the LPS core of *R. leguminosarum*. Steps catalysed by LpcA, B, C are shown boxed. Abbreviations are: Kdo, 2-keto-3-deoxyoctonate; Gal, galactose; Man, mannose; GalUA, galacturonic acid. B: Structure of the *lps-cpaA* region of *R. leguminosarum*. The double line indicates an unsequenced region of approximately 10 kb. Arrows refer to sequenced genes with the direction of transcription indicated.

26°C on tryptone yeast extract media (TY) [5]. Acid minimal salts medium (AMS) was used with succinate and ammonium chloride as the sole carbon and nitrogen sources, respectively (both at 10 mM) [4]. Calcium chloride (0.17 mM) and magnesium chloride (0.5 mM) were added routinely. However, these concentrations were varied as described for some experiments.

The growth of strains 3841 and RU1109 was measured over a pH range of 5.5–8.5 on agar (1.5%) plates containing AMS buffered with MOPS/MES (20 mM). Single colonies of each strain were picked from a TY plate and streaked onto minimal plates in duplicate. Growth was recorded after incubation at 26°C for 3 days.

Various strains were tested for their growth at a range of calcium concentrations. Cultures from TY slopes were suspended in AMS (5 ml) lacking calcium, and washed twice to remove contaminating calcium. After final resuspension in calcium-free AMS (5 ml), inoculum (0.01%) was added to AMS (10 ml) containing $MgSO_4$ (0.1 mM) and $CaCl_2$ (0.17–5 mM). Antibiotics were omitted as they slow growth and might affect cation utilisation. Cultures were grown at 26°C for 2–3 days.

Antibiotics were added at the following concentrations (μg ml^{-1}): streptomycin 500, kanamycin 40, gentamicin 20 for *R. leguminosarum* and 10 for *Escherichia coli*, tetracycline 5. *E. coli* was incubated at 37°C on Luria Bertani medium.

2.2. Genetic analysis

All routine DNA analysis was performed essentially according to Sambrook et al. [6]. Conjugations were performed as previously described [4]. Cosmid pIJ1848 was mutagenised in *cpaA* with Tn*5-lacZ* by using λ containing the transposon Tn*5* derivative B20 [7]. Transposon-mutated cosmids were screened using a pair of oligonucleotide primers designed to *lpcB* (GCAGATGGCGGCTTCAACGTCCTG) and IS*50* (GTTCCGTTCAGGACGCTACTTGTG), encoded by Tn*5-lacZ*. PCR fragments between 500 and 1200 bp indicated the transposon had inserted into *cpaA*. Several mutated cosmids were isolated, digested with restriction enzymes and Southern-blotted to confirm that an insertion was present in *cpaA*. Southern blotting with the 1.7-kb *Sph*I fragment from pRU72, which contains all of *lpcB* and part of *cpaA*, confirmed the PCR bands were homologous to the *cpaA* region. The exact position and orientation of Tn*5-lacZ* in pRU3079 was determined by cloning the transposon as a *Sal*I fragment into pBluescript SK−. The chromosomal junctions were sequenced using primers that bind to IS*50*, p113 (AGGTCACATGGAAGTCAGATC), and *lacZ*, p15 (GGATCCATAATTTTTTCCTCC). To create a chromosomal mutation, pRU3079 was conjugated into *R. leguminosarum* strain 3841. After purification, the incompatible plasmid pPHJI1 was conjugated into each strain and the homogenotes isolated [8]. The homogenote was confirmed by Southern blotting of chromosomal DNA with the 1.7-kb *Sph*I fragment from pRU72.

DNA sequencing was performed using an ALF automated sequencer as described previously [2]. Sequence alignment was performed with ClustalW [9]. PCR amplification of *cpaA* was performed using Bio-X-Act Taq polymerase (Bioline) and with the primers GCGCGCGGATGGCTGC and GCTGAGGCTTGAGGATCGCGTC. The PCR product was isolated from agarose using a QIAEX DNA isolation kit (Qiagen), cloned into pGEM-T and transferred, as a *Spe*I–*Apa*I fragment, into the broad host range vector pBBR1-MCS-5, producing pRU480.

2.3. General techniques

LPS was analysed as previously described [4]. β-Galactosidase fusions were assayed as described previously [10], using cultures grown overnight to mid-exponential growth phase (5×10^8 cfu ml^{-1}). Strains were tested for symbiotic performance by inoculation onto vetch (*Viciae sativa*) seeds, essentially as previously described [4]. After 6 weeks, plants were harvested and the dry weights determined.

3. Results and discussion

3.1. Sequencing and genetic analysis of cpaA

Whilst sequencing immediately downstream of *lpcB*, an open reading frame (ORF) transcribed in the opposite direction was identified (Fig. 1B). The 999-bp ORF was sequenced on both strands (EMBL AJ006753). It encodes a putative protein of 333 amino acids predicted to have a relative molecular mass of 35 396 and a p*I* of 5.3. Hydropathy analyses using the TMPRED, TOPPRED and THMM programs all predict there are 10 membrane-spanning regions characteristic of membrane transport proteins. A notable feature of all predictions is a large central loop, between transmembrane helices 5 and 6, from residue 151 to 186. This loop is highly hydrophilic and is predicted to be cytoplasmic. BLAST analysis reveals that the putative translation product of this ORF has 38.8% identity over 325 residues to ChaA of *E. coli* [11] and 29.9% identity over 334 residues to a ChaA homologue (y4hA) from the NGR234a sym plasmid sequence [12]. ChaA is a calcium proton antiporter, initially identified by its ability to suppress a sodium proton antiporter mutant (*nhaA*) at alkaline pH [11,13]. ChaA is a 39.2-kDa protein predicted to have 11 membrane-spanning domains. This topology is not common and is similar to that predicted for the Na^+/Ca^{2+} antiporter [14] and NhaA [15]. ChaA was demonstrated to cause efflux of calcium and was inhibited by magnesium. Due to its similarity to a wide range of calcium proton antiporters, we have designated the ORF *cpaA*, for calcium proton antiporter. Phylogenetic analysis shows that *cpaA* is similar to a broad range of calcium proton antiporters from bacteria, yeast and plants (data not shown). There are four very broad groups of putative calcium transporters: a general bacterial group, yeast, *Synechocystis* and *Bacillus* and finally plant systems. As expected, CpaA from *R. leguminosarum* belongs to the general bacterial group. Unfortunately, the gene designation *chaA* has been used for a number of systems for which function has not been established, presumably because they are homologous to *chaA* from *E. coli*. When the bacterial sequences alone are aligned, it can be seen that there is broad similarity between CpaA and other calcium export systems over much of the sequence (Fig. 2). There are 11 highly conserved residues common to prokaryotic and eukaryotic transporters: A64 (T64 in eukaryotes), G72, E88, G124, Y189, F192 (sometimes a Y in eukaryotes), H200, P282 (A282 in eukaryotes), E283, G305 and G355 (Fig. 2). Notably, CpaA lacks the N-terminal transmembrane domain predicted in ChaA of *E. coli* and several other members of the bacterial group. The central hydrophilic loop of CpaA (residues 151–186) corresponds to one of the least conserved regions of the molecule. This might partly reflect that it lacks the constraints of a transmembrane location but it is also possible that this region has a specific role in regulation.

The presence of a gene encoding a protein with similarity to a class of calcium efflux transporters, adjacent to a region of DNA that encodes proteins involved in the synthesis of a calcium-dependent LPS core structure, is intriguing. While calcium is known to be important for stabilisation of the LPS layer, there is no evidence concerning the mechanism of calcium insertion. Furthermore, there are no known transport systems involved in calcium export to the LPS core.

To determine whether *cpaA* encodes a calcium export system, a transposon mutation was introduced into *cpaA*.

Table 1
Bacterial strains and plasmids used

Strain or plasmid	Description	Reference
R. leguminosarum		
3841	Str^R derivative of *R. leguminosarum* biovar *viciae* strain 300	[17,18]
RU1109	*cpaA*::Tn*5*-*lacZ*	This work
E. coli		
DH5α	*sup*E44 D*lac*U169 (ϕ80 *lac*ZDM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Bethesda Research Laboratories (BRL)
Plasmids and phage		
pRK2013	ColEI replicon with RK2 *tra* genes, helper plasmid used for mobilising IncP and IncQ group plasmids. Km^R	[19]
Bluescript II SK−	Phagemid, fl(−) origin of replication, ColEI replicon, SK polylinker, 2.96 kb. Standard cloning vector. Amp^R	Stratagene
Bluescript II pBC−	Phagemid, fl(−) origin of replication, ColEI replicon, KS polylinker, 3.4 kb. Cm^R	Stratagene
pIJ1848	Cosmid containing *dct*-*lps* region from *R. leguminosarum*. Tc^R	[20]
pGEM-T	T-overhang cloning vector. Amp^R	Promega
pBBRMCS-5	Broad host range cloning vector. Gm^R	[21]
pPHJI1	IncP broad host range plasmid. Gm^R	[22]
pRU72	2.4-kb *Pst*I fragment from pIJ1848 containing *cpaA* cloned in pBC. Cm^R	This work
pRU480	1.1-kb fragment containing *cpaA* cloned in pBBRMCS-5	This work
pRU3097	pIJ1848 *cpaA*::Tn*5*-*lacZ*	This work
λ::Tn*5*-*lacZ*	λ carrying the Tn*5*-B20 transposon	[7]

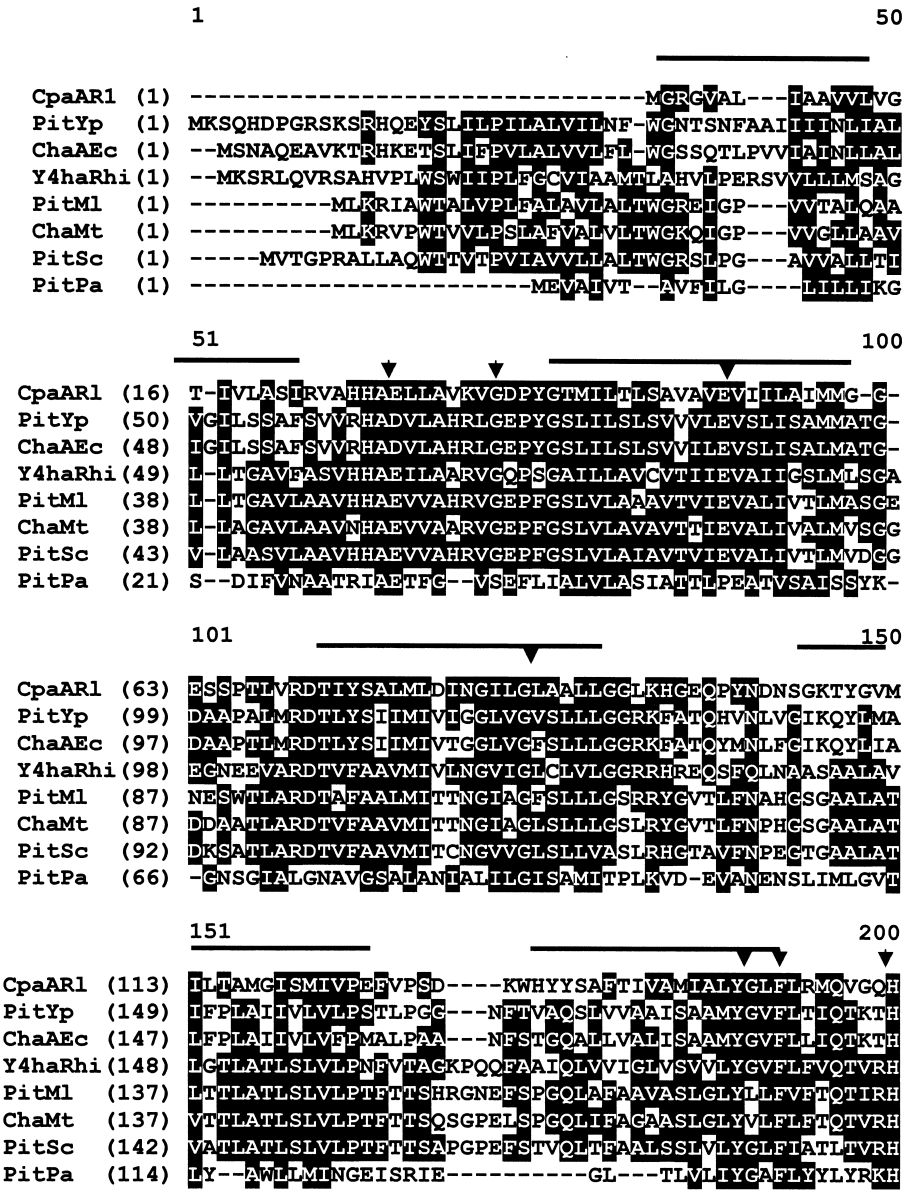

Fig. 2. A ClustalW alignment of the predicted peptide sequences from the bacterial cluster of ionic transporters showing homology to CpaA. Abbreviations with accession numbers shown in parentheses, which refer to the EMBL database unless otherwise stated, are as follows: CpaARl, calcium proton antiporter from *R. leguminosarum* (AJ006753); PitYp, probable calcium proton antiporter from *Yersinia pestis* (AL031866); ChaAEc, calcium proton antiporter from *E. coli* (L28709); Y4haRhi, putative ionic transporter from *Rhizobium* spp. NGR234 (SwissProt P55471); PitMl, putative ionic transporter from *Mycobacterium leprae* (AL049913); ChaAMt, putative calcium proton antiporter from *Mycobacterium tuberculosis* (AL022001); PitSc, putative ionic transporter from *Streptomyces coelicolor* (AL035591); PitPa, putative ionic transporter from *Pyrococcus abyssi* (AJ248288). Transmembrane domains of CpaA, predicted by the TOPPRED program, are overlined. Conserved residues and conservative substitutions are blocked in black. The absolute numbering of each sequence is shown in parentheses at the beginning of each line. The bacterial sequences shown here were also aligned with the following proteins from eukaryotic organisms: vacuolar calcium proton exchanger from *Saccharomyces cerevisiae* (SwissProt S61933), putative ionic transporter from *Schizosaccharomyces pombe* (AL035247), calcium proton exchanger from *Neurospora crassa* (AF053229), proton/calcium exchanger from *Synechocystis* spp. (D90912), putative ionic transporter from *Bacillus subtilis* (D83967), proton/Ca exchanger from *Ipomoea nil* (AB018526), low affinity calcium antiporter from *Arabidopsis thaliana* (U57412), calcium proton exchanger from *Vigna radiata* (AB012932), high affinity calcium antiporter from *Arabidopsis thaliana* (AC003028). Highly conserved residues from both bacterial and eukaryotic proteins are indicated with an arrow and discussed in the text.

To do this, cosmid pIJ1848 was subjected to saturation mutagenesis with Tn*5*-*lacZ*. Cosmid pRU3097 has an active fusion of Tn*5*-*lacZ* located in *cpaA*, with a 9-bp repeat of ATGCCGAAC (80–89 bp of EMBL AJ006753). A *cpaA* mutant strain, RU1109, was constructed by homogenotisation of *cpaA*::Tn*5*-*lacZ*. Strain RU1109, like the wild-type, has smooth colony morphology. This contrasts to the rough colony morphology of *lpcA* and *lpcB* mutants. Periodate silver stains and Western blots, using the O-antigen specific monoclonal antibody JIM39, of out-

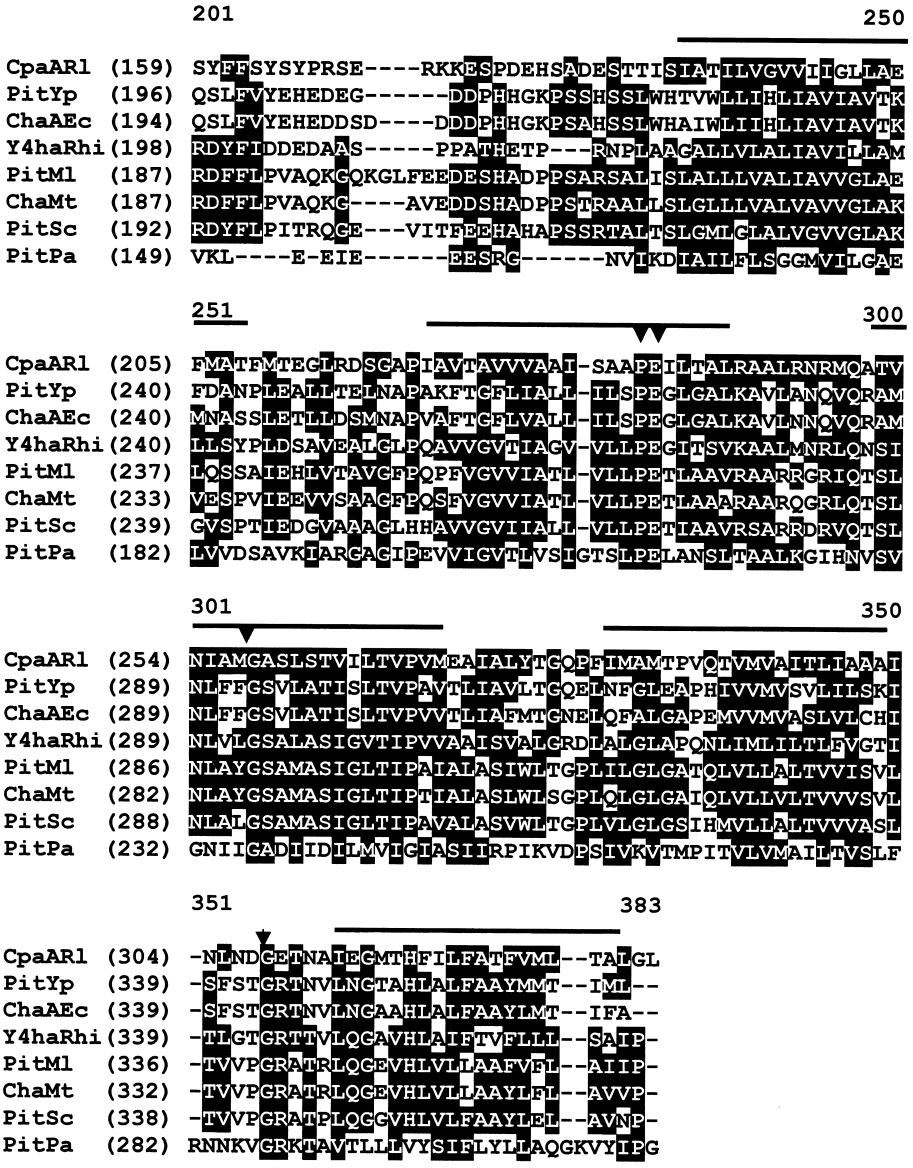

Fig. 2 (continued).

er membrane preparations showed no difference between the wild-type and RU1109 (data not shown). This indicates there is no gross change in LPS structure.

The growth medium was precipitated with two volumes of ethanol and screened by Western blotting with JIM39 for the presence of O-antigen. Traces of O-antigen were present but there was no significant difference between strains 3841 and RU1109. This indicates that LPS is not sloughed off in the *cpaA* mutant, which might occur if the LPS attachment was weakened.

3.2. CpaA is not required for pH regulation and growth

Since CpaA has similarity to ChaA, which can affect growth of *E. coli* under alkaline conditions in an *nhaA* background [11,13], strain RU1109 was grown over a pH range of 5.5–8.5. No difference was observed in growth between RU1109 and 3841 over the entire range, indicating that *cpaA* is not required for pH regulation. However, other systems might also regulate pH and while such systems are active, a role for *cpaA* in pH regulation cannot be dismissed.

3.3. Over-expression of CpaA prevents growth at low calcium and magnesium concentrations

In a previous study, it has been shown that there is no active uptake system for calcium in *R. leguminosarum* strain 3841 [4]. This means that simple measurements of $^{45}Ca^{2+}$ uptake in the wild-type and *cpaA* mutant are not possible. However, an active efflux system should reduce the cytosolic calcium concentration, which might impair growth when calcium is at a low concentration in the medium. A mutant with a defective calcium efflux system

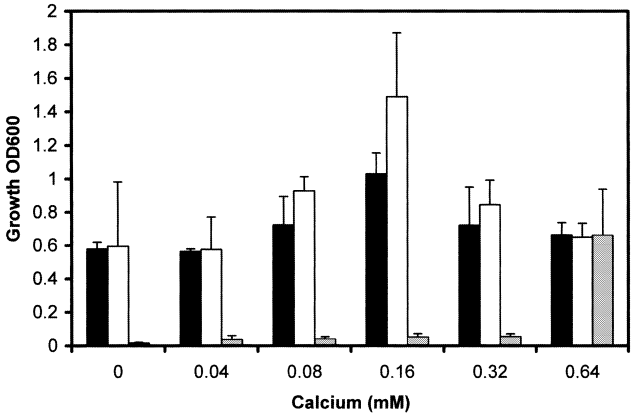

Fig. 3. Effect of over-expression of CpaA on growth of *R. leguminosarum*. ■, 3841; □, RU1109; ▨, RU1109 pRU480. Results are the average of three separate cultures (±S.E.M.) and were determined after 72 h growth. It should be noted that the magnesium chloride concentration was 0.1 mM in this experiment.

might maintain a higher cytosolic calcium concentration, enabling growth. To test whether RU1109 was less sensitive to low cytosolic calcium concentrations, a growth experiment was carried out over a calcium range (0–0.32 mM). The magnesium concentration was decreased to 0.1 mM in this experiment as it can substitute for calcium in many cytosolic functions. There were no growth differences at 0.04 mM calcium and growth at no added calcium was erratic so that no phenotype of the *cpaA* mutant was apparent. The difficulty of measuring a clear phenotype with efflux systems, unless they extrude a toxin, is well documented. For example, the *chaA* locus was only identified because it suppressed an *nhaA* mutant, enabling growth at alkaline pH. No clear calcium phenotype for the *chaA* mutant has been reported [11,13]. One of the main reasons for this is that there are multiple calcium efflux systems in *E. coli*, making the detection of clear phenotypes difficult [16]. It is possible that *R. leguminosarum* also has more than one calcium efflux system, explaining why a *cpaA* mutant does not have a gross phenotypic effect on the cellular calcium requirement.

Strain 3841 containing the cosmid pIJ1848 consistently required the calcium concentration of the growth medium to be above 0.17 mM, in which the wild-type grows normally. We considered that this might be due to over-expression of CpaA but this is complicated by the presence of other genes in the 30 kb of insert DNA. To see if this effect is due to CpaA, its gene was PCR-amplified and cloned to produce pRU480. The vector (pBBRMCS-5) contains a *lac* promoter ensuring constitutive expression of CpaA. Expression of CpaA in strain RU1109 via the multicopy plasmid pRU480 consistently increased the concentration of calcium required for growth (Fig. 3). Since the insert in plasmid pRU480 only encodes the PCR product of *cpaA*, this suggests *cpaA* encodes a functional calcium efflux system. Apart from ChaA, this is the only bacterial system for which there is experimental evidence that it exports calcium. All other systems have been identified by sequence similarity only (see Fig. 2).

The requirement for elevated concentrations of calcium for growth by the *lpcB* mutant RU301 has previously been described [4]. This mutation prevents attachment of the final 2-keto-3-deoxyoctonate residue in the core and therefore prevents attachment of the O-antigen (Fig. 1). The truncated core in an *lpcB* mutant, which is rich in potential metal chelating sites such as galacturonic acid, might increase the calcium required for growth. The presence of this cluster of LPS biosynthetic genes next to *cpaA* is consistent with a role for it in calcium export.

The Tn*5-lacZ* fusion in strain RU1109 enabled transcriptional analysis of the *cpaA* promoter. Strain RU1109 grown with no added, 0.17 and 5.0 mM calcium had β-galactosidase activity of 115 ± 25, 107 ± 23 and 109 ± 3 nmol min^{-1} mg^{-1} protein ($n = 6$), respectively. Since strain RU1109 has a chromosomal mutation in *cpaA*, which might affect the regulation of *cpaA* by calcium, strain RU1109 was complemented with pIJ1848 and β-galactosidase activity measured in cultures grown in the presence of 5 mM calcium. The activity was 100 ± 43 nmol min^{-1} mg^{-1} protein ($n = 6$). As stated above, this strain will not grow unless the added calcium is above 0.17 mM. There is no significant difference in these values, indicating that under the growth conditions we have tested ChaA is expressed at a constitutively low level. This would be consistent with *cpaA* having a housekeeping role in insertion of calcium into the LPS core.

3.4. Plant analysis

To determine whether there are any effects on the symbiosis with its plant host, strain RU1109 was inoculated onto vetch. Strain RU1109 nodulated vetch normally forming healthy pink nodules. Plants were harvested after 6 weeks and the dry weights of vetch infected with 3841, RU1109 and uninoculated controls were 0.031 S.E.M. = 0.003, 0.025 S.E.M. = 0.004 and 0.012 S.E.M. = 0.002 g dry weight per plant, respectively. From this, it can be seen that there is no significant effect on symbiosis.

In summary, we have identified a putative new calcium export system, CpaA. The evidence for this is based on its similarity to other calcium export systems, such as ChaA and eukaryotic calcium exporters and its ability to alter the calcium required for growth when over-expressed. The location of *cpaA* next to a cluster of LPS core biosynthetic genes whose product is one of the principal sites for calcium binding is possibly significant. However, mutation of *cpaA* does not cause a clear loss of or damage to the LPS layer. This can be contrasted to mutation of any of the sugar transferases (*lpcA, B* or *C*) that results in the formation of a rough colony morphology. This is not unexpected, as rough mutants are unable to form specific glycosidic bonds in the core or O-antigen structure. Calcium is thought to be important for stabilisation of the LPS

layer, rather than having a direct role in its assembly. Prevention of proper calcium insertion into the LPS core might only have the effect of altering the stability of the LPS. Such changes are subtler than loss of the O-antigen and very difficult to detect. It cannot be excluded that other systems might export calcium to the core region or that under laboratory growth conditions sufficient calcium can enter the core by diffusion. This study has emphasised our almost total lack of knowledge of how calcium is inserted into the LPS layer of Gram-negative bacteria.

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