dpp Genes of *Rhizobium leguminosarum* Specify Uptake of δ -Aminolevulinic Acid

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An operon with homology to the *dppABCDF* genes required to transport dipeptides in bacteria was identified in the N₂-fixing symbiont, *Rhizobium leguminosarum*. As in other bacteria, *dpp* mutants were severely affected in the import of δ -aminolevulinic acid (ALA), a heme precursor. ALA uptake was antagonized by adding dipeptides, indicating that these two classes of molecule share the same transporter. Mutations in *dppABCDF* did not affect symbiotic N₂ fixation on peas, suggesting that the ALA needed for heme synthesis is not supplied by the plant or that another uptake system functions in the bacteroids. The *dppABCDF* operon of *R. leguminosarum* resembles that in other bacteria, with a gap between *dppA* and *dppB* containing inverted repeats that may stabilize mRNA and may explain why transcription of *dppA* alone was higher than that of *dppBCDF*. The *dppABCDF* promoter was mapped and is most likely recognized by σ^{70} .

Additional keywords: ABC transporter, gluconate kinase.

In *Escherichia coli*, three sets of closely related ATP-binding cassette (ABC) systems (Linton and Higgins 1998), encoded by *dpp*, *tpg*, and *opp*, import dipeptides, tripeptides, and oligopeptides, which may be exogenous or breakdown products of bacterial cell wall proteins (Goodell and Higgins 1987). In several bacteria, genes encoding all the components of ABC transporters are in single operons (Abouhamad and Manson 1994; Abouhamad et al. 1991; Mathiopoulos et al. 1991). Thus, in the *E. coli* *dpp* system, DppA is the periplasmic dipeptide-binding protein and DppB and DppC are located in the inner membrane. The two ABC domains of Dpp permeases are usually encoded by two separate but similar genes, *dppD* and *dppF*. In some bacteria, expression of *dppA* alone is greater than the full-length *dppABCDF* transcript, due to sequences in the *dppA-dppB* intergenic region that contain potential secondary structures affecting transcription, mRNA stability, or both (Abouhamad and Manson 1994). In addition to its role in transport, the DppA protein of *Rhodobacter sphaeroides* may be a molecular chaperone protecting the periplasmic dimethyl-sulfoxide reductase (Matsuzaki et al. 2000), and in *Haemophilus influenzae*, the heme-binding protein HbpA resembles DppA (Hanson et al. 1992).

In enteric bacteria, Dpp transporters also import δ -aminolevulinic acid (ALA), a heme precursor with structural

similarity to dipeptides (Elliot 1993; Verkamp et al. 1993). In *Sinorhizobium meliloti*, the symbiont of alfalfa, *hemA* mutants, which fail to make ALA, are Fix⁻ (Leong et al. 1982), leading to the initial, though erroneous, suggestion that rhizobia make the heme for the leghemoglobin, the most abundant protein in the plant cytosol. In contrast, in *Bradyrhizobium japonicum*, which nodulates soybeans, *hemA* mutants are Fix⁺ on soybeans (Guerinot and Chelm 1986). This apparent paradox was resolved by McGinnis and O'Brian (1995), who showed that *hemA* mutants of *B. japonicum* import "surplus" ALA from the plant while those of *S. meliloti* do not (O'Brian 1996).

In this text, we describe a *dpp* operon of *Rhizobium leguminosarum* bv. *viciae*, the symbiont of peas and vetches. The effects of *dpp* mutations on the uptake of ALA and its antagonism by exogenous peptides are described, as is the symbiotic phenotype of *dpp* mutants.

RESULTS

Identification of the *dpp* genes of *R. leguminosarum*.

In the course of other work, we identified and sequenced part of a cosmid, pBIO1095, that contained cloned *R. leguminosarum* DNA (deLuca et al. 1998). This region contained a cluster of five genes whose products resembled the Dpp transport proteins that had been identified in other bacteria. These genes, *dppA*, *dppB*, *dppC*, *dppD*, and *dppF*, are in the same orientation (Fig. 1) and, with the exception of a gap between *dppA* and *dppB*, are very close to each other (discussed below). The 6,916-bp sequence is deposited at the EMBL database (accession number AJ310184).

The 3' end of the gene (*orfI*) was separated by 338 bp from *dppA*. The product of *orfI* is 84% identical to the product of an open reading frame (ORF) of unknown function, SMc00785, in the chromosome of *S. meliloti*. The 5' start of *idnK* is separated by 21 bp from *dppF*, and its product resembles those of two *E. coli* genes, *idnK* and *gntK*, that encode different gluconate kinases (Fig. 1).

The arrangement of the *R. leguminosarum* *dppABCDF* operon resembles that of other bacteria, such as *E. coli* and *Bacillus subtilis* (Abouhamad and Manson 1994; Mathiopoulos et al. 1991). In *E. coli*, *dppA* and *dppB* are separated by 310 bp containing inverted repeats. The smaller, 87-bp *dppA-dppB* intergenic region of *R. leguminosarum* also has inverted repeats (Fig. 2), as do corresponding regions in *dppABCDF* operons of *S. meliloti* and *Mesorhizobium loti* (which nodulates *Lotus* spp.). However, the actual sequences do not resemble each other in any of these genera (Fig. 2). The calculated-binding energies of the two *R. leguminosarum* stem-loop structures are -11.23 and -13.23 kcal per mol, suggesting that both form loops within cells (Fig. 2). As in other bacteria,

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dppC and *dppD* of *R. leguminosarum* may be translationally coupled.

Isolation of *dpp* mutants.

Plasmid pBIO1095 was mutagenized with the transposon *Tn**lac*, and insertions in the *dpp* genes were mapped by restriction digests and then by DNA sequencing, using a primer at the 5' end of *lacZ* in *Tn**lac*. Mutant plasmids with insertions in *dppA* (pBIO1149), *dppB* (pBIO1108), *dppC* (pBIO1168), and *dppD* (pBIO1150) were obtained. By marker exchange, these plasmid-borne mutations were transferred to the chromosome of wild-type *R. leguminosarum* bv. *viciae* to form mutants J327 (*dppA*::*Tn**lac*), J328 (*dppB*::*Tn**lac*), J329 (*dppC*::*Tn**lac*), and J330 (*dppD*::*Tn**lac*).

All the *dpp* mutants were reduced by approximately 80% in their import of ¹⁴C-labeled ALA (Table 1). Dipeptides, and to a lesser extent the tripeptide Gly-His-Gly, greatly reduced ALA uptake (Table 2). Thus, in *R. leguminosarum* as in other bacteria, ALA and dipeptides share a Dpp transporter. The *dpp* mutants were used to inoculate peas. Compared with the wild type, nodules induced by the mutants developed at the same time, were similar in sizes and numbers, and fixed N₂ to equivalent levels. Thus, *dpp* mutants appear to be unaffected in the symbiosis.

Expression of the *dpp* genes.

In the *dpp*::*Tn**lac* mutant plasmids pBIO1149 (*dppA*::*lac*), pBIO1108 (*dppB*::*lac*), and pBIO1168 (*dppC*::*lac*), the promoterless *lacZ* was in the same orientation as the mutated

dpp gene. To measure expression of these fusions, derivatives of wild-type *R. leguminosarum* with these three plasmids were grown in minimal medium to midlog phase and assayed for β-galactosidase activity. This was greatest for *dppA*::*lac* compared with *dppB*::*lac* and *dppC*::*lac*, just as in *dppABCDF* of *E. coli* (Abouhamad and Manson 1994). Similar results were found when each fusion was marker-exchanged into the genome, with expression being greater for *dppA*::*lac* (324 ± 10 Miller units) than for *dppB*::*lac* (90 ± 1) or *dppC*::*lac* (54 ± 2).

The promoter for the *dppABCDF* operon was located between *dppA* and *orf1* by cloning a fragment that spanned these two genes into the wide-host-range, *lacZ*-fusion plasmid pMP220 (Spaink et al. 1987) to form pBIO1144 (Fig. 1). When transferred to wild-type *R. leguminosarum*, pBIO1144 expressed β-galactosidase at high levels (data not shown). The strain with pBIO1144 was assayed at different times during the growth phase. As with *E. coli* (Abouhamad and Manson 1994), expression of *dppA*::*lac* was approximately twofold higher in cells entering the stationary phase than when in the early log phase. In contrast, expression of the *lac* fusions in *dppB* and *dppC* was unaffected by the growth stage (data not shown). The *dppA*-*dppB* intergenic region has no promoter since, when cloned into pMP220 to form pBIO1142, a *Kpn*I-*Pst*I fragment spanning it (Fig. 1) yielded no β-galactosidase activity above background in *R. leguminosarum*.

To map the *dppA* promoter precisely, mRNA was harvested from wild-type *R. leguminosarum* harboring pBIO1095 (to enhance levels of the *dppABCDF* message) and was used as the template in a primer-extension experiment (Fig. 3) using a

Fig. 1. Representation of the *dpp* region of *Rhizobium leguminosarum*. Directions of transcription and dimensions (in base pairs) of genes in the *dppABCDF* operon and the flanking genes *orf1* and *idnK* are shown. Dimensions of DNA cloned into pMP220 in fusion plasmids pBIO1142 and pBIO1144 are indicated. Sites of *Tn**lac* insertions in pBIO1095 are shown, as are the directions of *lacZ* transcription and the names of the resultant mutant plasmids.

Fig. 2. Sequences of the *dppA*-*dppB* intergenic region in *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Mesorhizobium loti*. The TAA stop codons of the *dppA* genes and the ATG start codons of the *dppB* genes are underlined. The dimensions of the inverted repeats are shown as arrows. *R. leg* = *R. leguminosarum* bv. *viciae*; *M. lot* = *M. loti*; and *S. mel* = *S. meliloti*. The calculated-binding energies of potential stem-loop structures are shown.

primer within *dppA*. There is a single transcript-start site, at a G residue 63 bp upstream of the *dppA* AUG codon (Fig. 3). The DNA upstream of the *dppA* transcription-start site is relatively AT-rich, with potential -10 (TTCCAT) and -35 (TTGCTT) RNA polymerase recognition sequences that could serve as a promoter, perhaps for a “housekeeping” σ^{70} -containing holoenzyme.

DISCUSSION

The *dppABCDF* operon identified here in *R. leguminosarum* is similar in function and organization to that of *dpp* genes of other bacteria, such as *E. coli* and *Bacillus subtilis*, that contain a single version of this ABC transporter.

The genomes of two rhizobia, *M. loti* and *S. meliloti*, have been sequenced (Galibert et al. 2001; Kaneko et al. 2000). *M. loti* and *S. meliloti* possess no fewer than 26 and 27 members, respectively, of the peptide/opine/nickel uptake transporter (PepT) subfamily of ABC transporters. The individual DppA, DppB, DppC, DppD, and DppF proteins of *R. leguminosarum* were most similar to products of five corresponding genes in

single operons of both *S. meliloti* (ORFs SMc00786, SMc00787, SMc00788, SMc00789, and SMc00790) and *M. loti* (ORFs Mlr5416, Mlr5417, Mlr5418, Mlr5419, and Mlr5420). The genomes of *R. leguminosarum* and *S. meliloti*, but not of *M. loti*, show significant synteny in the regions that about these three *dpp* operons.

The sequences of the *R. leguminosarum* DppD and DppF proteins (the ABC subunit ATPases) were included in a phylogenetic-tree analysis with other members of the PepT family of transporters of *M. loti*, *S. meliloti*, and selected members of other bacteria. The *R. leguminosarum* DppD and DppF proteins were each in a cluster that contained, as expected, the deduced gene products of *S. meliloti* and *M. loti* mentioned above (Fig. 4). These two clusters also contained DppD or DppF of *E. coli* and *H. influenzae*. Taken together, the sequence comparisons and the data obtained on ALA uptake indicate that, of all the rhizobial PepT-like transporters, the one identified here is an orthologue, as well as a homologue, of *dppABCDF* of e.g., *E. coli*.

It remains to be seen if the residual ALA uptake in *R. leguminosarum dpp* mutants is due to minor contributions by

Table 1. Uptake of labeled δ -amino-levulinic acid (ALA) by *Rhizobium leguminosarum* wild type and *dpp* mutants

Strain	Genotype	Rate of ALA uptake (nmol per mg of protein per min)
8401pRL1JI	Wild type	4.91 $\pm$ 0.16
J327	<i>dppA</i> mutant	0.96 $\pm$ 0.02
J328	<i>dppB</i> mutant	1.01 $\pm$ 0.07
J329	<i>dppC</i> mutant	1.01 $\pm$ 0.10
J330	<i>dppD</i> mutant	0.98 $\pm$ 0.09

Table 2. Effects on δ -amino-levulinic acid (ALA) uptake of exogenous di- and tripeptides in the wild-type strain

Competing solute (1 mM)	Rate of ALA uptake (nmol per mg of protein per min)	Percentage of control
None	4.97 $\pm$ 0.26	100
Pro-gly	0.28 $\pm$ 0.02	6
Ala-ser	0.35 $\pm$ 0.03	7
Ala-val	0.43 $\pm$ 0.04	9
Gly-his-gly	1.89 $\pm$ 0.56	38

Fig. 3. Determination of the transcription initiation site of the *dppA* mRNA. **A**, Primer extension analysis of total RNA isolated from *Rhizobium leguminosarum* harboring pBIO1095 was performed. Transcription initiates from a guanosine residue, which is part of the sequence shown on the left. **B**, A portion of the DNA sequence of the *dppA* regulatory region. The angled arrow designates the location of the transcription initiation site. Putative -10 and -35 promoter-recognition sequences are underlined, and the translation start site of the *dppA* mRNA is shown in bold.

one or more of the many other *dpp*-like operons. Given that some proteins whose sequences resemble peptide transporters actually transport sugars or other nonpeptide molecules (Elferink et al. 2001; Gage and Long 1998), it will be necessary to perform transport experiments with different substrates on mutants defective in individual “*dpp*” genes to determine their precise specificities.

King and O’Brian (1997) obtained indirect evidence that the global regulator Lrp represses expression of the *dpp* operon of *Bradyrhizobium*. In *R. leguminosarum*, *M. loti*, and *S. meliloti*, there are several homologues of the *E. coli lrp* gene (Allaway et al. 2000; (P. S. Poole and A. H. F. Hosie, unpublished data); it will be of interest to see if one or more of these affect expression of *dppABCDF*.

The *dpp* mutants of *R. leguminosarum* are apparently unaffected in symbiotic N₂ fixation on peas, showing that bacteroids make sufficient ALA and do not depend on the plant

for a supply of this molecule. However, the possibility that there is a bacteroid-specific ALA-uptake system or that the residual ALA-uptake ability of the *dpp* mutants may satisfy the bacteroids, or both, has not been excluded. Nonetheless, the lack of a symbiotic phenotype is consistent with the Fix⁻ phenotype of *hemA* mutants of the closely related *S. meliloti*. These fail to synthesize ALA, and alfalfa plants cannot supply sufficient ALA to the bacteroids. In contrast, soybeans appear to supply ample ALA to *B. japonicum* bacteroids (O’Brian 1996). It would be of interest to investigate the symbiotic properties of *dpp* mutants of *B. japonicum*; such mutants might be Fix⁻.

MATERIALS AND METHODS

Microbiological growth and in vivo genetic manipulations.

R. leguminosarum and *E. coli* were grown as described in Beringer (1974). Cosmid pBIO1095 was obtained from a gene

Fig. 4. Phylogenetic tree of selected members of the PepT family of ATP-binding cassette (ABC) transporters. Amino acid sequences of the highly conserved region of the ATP-binding proteins (Saurin et al. 1999) of selected members of the PepT family were aligned using Vector NTI Suite version 6. The resulting phylogenetic tree was produced with Treeview version 1.6.1. The *Rhizobium leguminosarum* sequences described here are boxed. Sequences are identified by designated protein names or protein numbers in sequencing projects. at = *Agrobacterium tumefaciens*; bs = *Bacillus subtilis*; ec = *Escherichia coli*; hi = *Haemophilus influenzae*; mlr = *Mesorhizobium loti*; PA = *Pseudomonas aeruginosa*; rl = *R. leguminosarum*; SMA, SMb, and SMc = *Sinorhizobium meliloti*; st = *Salmonella typhimurium*. The two ABC domains of fused dimers are split and the N-terminal and C-terminal domains indicated by “-C” and “-N”. The transporter classifications are in parentheses next to representative members of transporter subfamilies (Saier, 2000).

bank of *R. leguminosarum* DNA cloned in the wide-host-range vector pLAFR1 (deLuca et al. 1998). pBIO1095 was mutagenized with Tnlac (Simon et al. 1989), and marker exchange of Tnlac mutations was done essentially as described by Downie et al. (1983). β -Galactosidase was assayed as described in Rossen et al. (1985), and peas were grown and assayed for N₂ fixation as described in Beynon et al. (1980).

DNA manipulations.

Routine DNA manipulations were done as described in Sambrook et al. (1989). DNA was sequenced by the dideoxy-chain-termination method, either by cloning fragments into pUC18 and using a semi-automated ALF Pharmacia sequencer (Pharmacia, Peapack, NJ, U.S.A.) or by cycle-sequencing from cosmid templates (MWG Biotech, Ebersberg, Germany).

Analysis of RNA transcripts.

Total RNA was isolated from *R. leguminosarum* grown in "high" and "low" iron, using RNA Wiz kits (Ambion, Austin, TX, U.S.A.). Primer extension analysis (Sawers and Böck, 1989) used 10 μ g of total RNA and 0.2 pmol of ³²P-labeled oligonucleotide primer. The primer had the sequence (5'-CCAGCCGACGTCGCCATGAC-3'), and hybridized within the coding sequence, of *dppA* at position +47 to +67 relative to the AUG codon. The labeled primer was also used to generate the sequence ladder using pBIO1095 as template DNA.

Uptake assays.

ALA uptake assays were performed by rapid filtration (Poole et al. 1985). The final concentration of ALA was 25 μ M (0.125 μ Ci ¹⁴C-); competing di- and tripeptides were added to 1 mM final concentrations 5 s before adding radiolabeled ALA. Samples (100 μ l) were taken at 0, 1, 2, and 3 min, filtered through Whatman glass-fiber filters (Whatman, Maidstone, England), washed in minimal salts medium, and then added to scintillation fluid and counted. Peptides were from Sigma (Sigma, Poole, England) and δ -[4-¹⁴C]-ALA HCl (1.76 GBq mmol⁻¹) was from NEN (Life Science Products, Boston, MA, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Department of Plant Gene Research Kazusa DNA Research Institute:

<http://www.kazusa.or.jp/rhizobase>

Sinorhizobium meliloti strain 1021 Genome Project:

<http://sequence.toulouse.inra.fr/meliloti.html>