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Identification of the *lrp* Gene in *Bradyrhizobium japonicum* and Its Role in Regulation of δ-Aminolevulinic Acid Uptake

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The heme precursor δ-aminolevulinic acid (ALA) is taken up by the dipeptide permease (Dpp) system in *Escherichia coli*. In this study, we identified a *Bradyrhizobium japonicum* genomic library clone that complemented both ALA and dipeptide uptake activities in *E. coli dpp* mutants. The complementing *B. japonicum* DNA encoded a product with 58% identity to the *E. coli* global transcriptional regulator Lrp (leucine-responsive regulatory protein), implying the presence of Dpp-independent ALA uptake activity in those cells. Data support the conclusion that the Lrp homolog induced the oligopeptide permease system in the complemented cells by interfering with the repressor activity of the endogenous Lrp, thus conferring oligopeptide and ALA uptake activities. ALA uptake by *B. japonicum* was effectively inhibited by a tripeptide and, to a lesser extent, by a dipeptide, and a mutant strain that expressed the *lrp* homolog from a constitutive promoter was deficient in ALA uptake activity. The data show that Lrp negatively affects ALA uptake in *E. coli* and *B. japonicum*. Furthermore, the product of the isolated *B. japonicum* gene is both a functional and structural homolog of *E. coli* Lrp, and thus the regulator is not restricted to enteric bacteria.

Bradyrhizobium japonicum is the bacterial endosymbiont of soybean that fixes atmospheric nitrogen to ammonia within a specialized plant organ called a root nodule. Nitrogen fixation is energy intensive, and symbiotic development involves the de novo synthesis of heme proteins that allow respiration in the hypoxic milieu of the nodule. ALA (δ-aminolevulinic acid) is the first universal heme precursor and is formed from glycine and succinyl coenzyme A via ALA synthase in the rhizobia (reviewed in reference 14). Interestingly, *B. japonicum hemA*, the gene encoding ALA synthase, is required for heme synthesis in culture but not in symbiosis with its homologous host, soybean, or with the heterologous hosts cowpea or mungbean (12, 18). Conversely, other rhizobia need *hemA* for symbiosis with their respective hosts (11, 16, 19). Evidence suggests that the *B. japonicum hemA* strain is rescued symbiotically by acquiring ALA synthesized by the plant host (7, 9, 18). Accordingly, a vigorous ALA uptake activity was found in *B. japonicum* that is deficient in rhizobial species that require the *hemA* gene for nodule formation (12, 18). Subsequently, ALA uptake activity in *Salmonella typhimurium* (8) and *Escherichia coli* (20) was described and found to be catalyzed by a dipeptide permease (Dpp) system. ALA is structurally similar to glycyl-glycine, and Dpp can transport any dipeptide containing L-amino acids (2).

Isolation of *B. japonicum* DNA that complements *E. coli dpp* strains for dipeptide and ALA uptake. *E. coli* E1769 is a proline auxotroph (*proC*) that can use prolyl-glycine (Pro-Gly) to fulfill its proline requirement because it can take up exogenous dipeptides by a Dpp system (15) (Table 1). A *dpp* derivative, strain E1772, cannot use Pro-Gly as a proline source; thus, we transformed the mutant with a *B. japonicum* expression library (7) and screened for transformants that grew on minimal medium supplemented with Pro-Gly. Plasmids p235 and p349 are overlapping genomic clones that conferred Pro-Gly-dependent growth on strain E1772, and a 0.8-kb *Dde*I-*Sal*I subclone of p349 (p349DS) was sufficient to complement the mutant as well (Fig. 1). We note that p349DS and the other complementing plasmids did not relieve the proline auxotrophy of strain E1772; therefore, it is unlikely that the cloned DNA encodes a *proC* homolog. The data show that p349DS confers dipeptide-dependent growth on strain E1772.

To test whether the *B. japonicum* clones can also complement ALA uptake, they were introduced into the *hemA* strain GE1387 and the *hemA dpp* strain EV149 (20). Both *E. coli* strains are ALA auxotrophs, but strain GE1387, which is dpp^+, requires 0.05 μg of ALA per ml in liquid media to support good growth, whereas strain EV149 needs 1 μg of ALA per ml for comparable growth, consistent with the conclusion that ALA is taken up by the Dpp system (8, 20). All plasmids that complemented strain E1772 for Pro-Gly-dependent growth also conferred on strain EV149 the ability to grow in low-ALA medium (Fig. 1). The complemented strains are ALA auxotrophs; thus, they did not acquire ALA synthesis activity. Complementation of strain EV149 for ALA uptake was measured directly by monitoring uptake of $[^{14}C]$ALA into cells. p349DS partially restored the ALA uptake defect of the *dpp* mutant to about 30% of the wild-type activity (Fig. 2), in agreement with the growth studies (Fig. 1). The experiments show that p349DS complements both dipeptide and ALA uptake activities in *E. coli*.

Identification of the cloned gene as an *lrp* homolog and evidence for a second ALA uptake activity in *E. coli*. The complementing *Dde*I-*Sal*I genomic fragment contained an open reading frame that encodes a peptide with 58% identity and 75% similarity to the *E. coli* leucine-responsive regulatory protein (Lrp) (Fig. 3). Lrp is a global transcriptional regulator found in numerous enteric bacteria that activates or represses many genes and operons (6, 13). Thus, the *dpp* mutants were complemented by a gene other than a *B. japonicum dpp* homolog, suggesting that *E. coli* has another system for dipeptide and ALA uptake that is induced in the presence of the plasmid-borne foreign gene. The oligopeptide permease (Opp) systems of *E. coli* and *S. typhimurium* take up peptides two to

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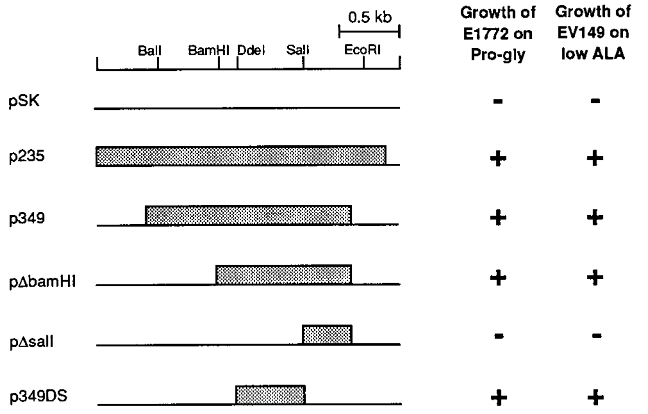

FIG. 1. Complementation of *E. coli* mutants with plasmids carrying *B. japonicum* genomic DNA fragments. Strain E1772 (*dppA proC*) harboring the plasmids shown was tested for the ability to use 50 μg of Pro-Gly per ml as a proline source. Strain EV149 (*dppA hemA*) harboring the plasmids shown was tested for the ability to grow with 0.05 μg of ALA per ml. Transformant strains of E1772 and EV149 retained their auxotrophies and required an exogenous source of proline and ALA, respectively.

TABLE 1. Bacterial strains used in this study[a]

Strain	Relevant genotype or trait	Source or reference
E. coli		
E1769	*proC*::Tn*10*	15
E1772	E1769, but *dppA*20::Kan	15
GE1387	*hemA*	20
EV149	GE1387, but *dppA30*::Kan	20
CV975	Δ(*lac-pro*) *ilvIH*::Mu dI1734	17
CV1008	CV975, but *lrp*::Tn*10*	17
MM584	*opp proC*::Tn*5*	1
B. japonicum		
I110	Small-colony derivative of USDA 3I1b110	10
ILRPc	*aph2-lrp* transcriptional fusion integrated in chromosome; also contains wild-type *lrp* gene	This study

[a] Some strains have auxotrophies not listed here. See original references for complete genotypes.

five amino acids in length, with a preference for tripeptides. The tripeptide prolyl-glycyl-glycine (Pro-Gly-Gly) is taken up by Opp (3, 4); thus, we assessed whether that tripeptide could satisfy the proline requirement in *proC* strains in various genetic backgrounds. Neither strain E1772 nor its isogenic dpp^+ parent strain E1769 could use 50 μg of Pro-Gly-Gly per ml as a proline source (Table 2), indicating that Opp activity was not sufficiently expressed under the defined growth conditions to support growth on the tripeptide and that the Dpp system could not support tripeptide-dependent growth. However, cells harboring p349DS acquired the ability to grow on Pro-Gly-Gly (Table 2), inferring the induction of Opp activity. This conclusion was supported by the observation that p349DS failed to confer tripeptide-dependent growth on the *opp* strain MM584 (Table 2). The latter observation argues against the possibility that the *B. japonicum lrp* homolog encodes a product that is in itself a peptide or ALA transporter. We suggest that expression of the *B. japonicum* gene results in the induction of *E. coli* Opp, permitting the uptake of dipeptides, tripeptides, and ALA.

ALA uptake activity is affected by Lrp in *E. coli*. The *E. coli oppABCDF* operon has been shown to be repressed by Lrp (3, 5); therefore, it was ironic that the plasmid-borne *lrp* homolog of *B. japonicum* resulted in the induction of Opp activity. We speculated that perhaps the *B. japonicum* Lrp homolog interferes with the repressor activity of the endogenous Lrp to derepress Opp. Thus, we examined peptide-dependent growth in the *lrp* mutant CV1008 (17) and its isogenic lrp^+ parent strain CV975. Like E1769, strain CV975 used Pro-Gly, but not Pro-Gly-Gly, as a proline source (Table 2). The *lrp* strain, however, used both Pro-Gly and Pro-Gly-Gly, a result consistent with the derepression of the Opp system (Table 2). Thus, the *lrp* mutant behaved similarly to an lrp^+ strain that overexpresses the *B. japonicum lrp* homolog with respect to peptide-dependent growth. Interestingly, overexpression of *E. coli lrp* from the high-copy-number plasmid pCV180 (17) repressed Dpp activity, as determined by the inability of dpp^+ strains E1769 and CV975 to use Pro-Gly as a proline source (Table 2). In addition, ALA uptake activity of strain E1769, which is catalyzed by Dpp (8, 20), was severely repressed in cells carrying pCV180 (data not shown). The data show that ALA uptake in *E. coli* is regulated by *lrp*; Lrp levels of wild-type *E. coli* cells grown in the defined media in this study were sufficient to repress Opp, but Dpp was discernibly repressed only when *lrp* was expressed from a high-copy-number plasmid.

ALA uptake in *B. japonicum* is competed by peptides and is affected by *lrp*. *B. japonicum* ALA uptake was measured in the presence of a dipeptide or tripeptide as a competitor of activity (Fig. 4). The tripeptide glycyl-histidyl-glycine (Gly-His-Gly) effectively competed with [^{14}C]ALA uptake, and Pro-Gly mod-

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B. japonicum       1          MELDRLDRRILSILQEDGRIANVELAERIGLSPTSIGERLK 41
                              :|||:|| ||  || |||| ||||  |:|||||   ||::
E. coli            1 MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVR 50

B. japonicum      42 RLQREGFVEGYGARLNPHRLGLGLLVFVEVLLDKTTPDNFERFARAVKlA 91
                     ||:|:||::|| | |||| |: :||||||: |::  || || |  ||
E. coli           51 RLERQGFIQGYTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNTAVQKL 100

B. japonicum      92 PEVLECHMVAGGFDYLVKARLADMTAYRRFLGETLLSMPGVRETRTYAVM 141
                      |: |||:| |:||||:| |::|| |||::|||||| :||| :|||| ||
E. coli          101 EEIQECHLVSGDFDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVM 150

B. japonicum     142 EEIKRDGPLPVG   153
                     ||:|    | :
E. coli          151 EEVKQSNRLVIKTR 164
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FIG. 2. Comparison of derived protein sequences between *E. coli* Lrp (bottom) and the *B. japonicum* Lrp homolog (top). Solid lines represent amino acid identities, and the dotted lines denote conserved amino acids.

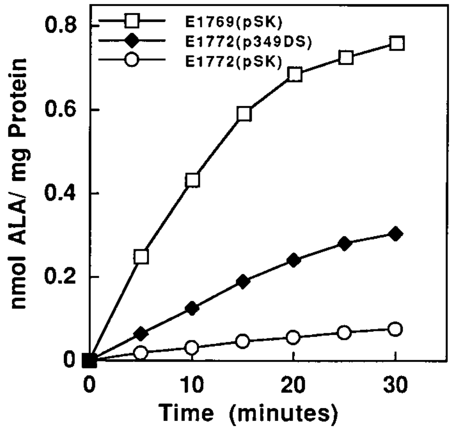

FIG. 3. Complementation of *E. coli* E1772 for ALA uptake. ALA uptake was measured in E1772 harboring either p349DS or pSK and in strain E1769(pSK). pSK is the control plasmid with no insert DNA. The data are expressed as nanomoles of ALA taken up per milligram of protein at the indicated time points.

estly inhibited activity, suggesting a mechanism that transports both ALA and peptides. The finding that the tripeptide was a better competitor may point to an Opp-dependent ALA uptake in *B. japonicum*. To determine whether *B. japonicum lrp* is involved in ALA uptake, we constructed a derivative of strain I110 that expresses *lrp* constitutively from a genomic gene fusion of the *aph2* promoter upstream of *lrp*, in addition to carrying a wild-type copy of the gene (ILRPc). Constitutive expression of *lrp* inhibited ALA uptake in cells grown in minimal medium (Fig. 5), implicating the Lrp homolog as a negative effector of transport activity. These data indicate that *B. japonicum* and *E. coli* are similar in that they take up ALA by systems that also take up peptides and that *lrp* negatively regulates the activities in each system.

TABLE 2. Complementation of *E. coli dpp*, *opp*, and *lrp* strains with plasmids encoding *lrp* genes from *B. japonicum* and *E. coli*

Strain and plasmid	Growth on medium with[a]:			
	No addition	Proline	Pro-Gly	Pro-Gly-Gly
E1769 (*dpp*$^+$ *opp*$^+$)				
pSK	−	+	+	−
p349DS	−	+	+	+
pCV180	−	+	−	−
E1772 (*dpp opp*$^+$)				
pSK	−	+	−	−
p349DS	−	+	+	+
pCV180	−	+	−	−
MM584 (*dpp*$^+$ *opp*)				
pSK	−	+	+	−
p349DS	−	+	+	−
CV975 (*lrp*$^+$)				
pSK	−	+	+	−
p349DS	−	+	+	+
pCV180	−	+	−	−
CV1008 (*lrp*)				
pSK	−	+	+	+
p349DS	−	+	+	+
pCV180	−	+	−	−

[a] Cells were grown on minimal medium plates containing 50 μg of proline or peptide per ml where indicated. +, growth on solid medium; −, no growth. All strains are proline auxotrophs.

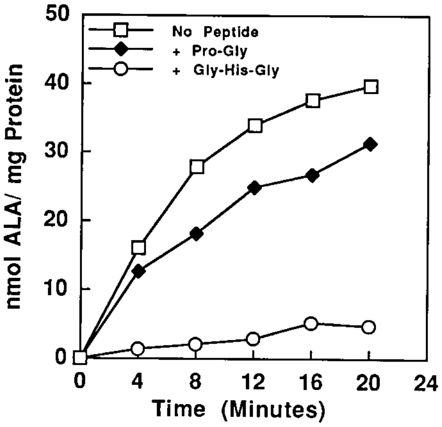

FIG. 4. Competition of ALA uptake in *B. japonicum* I110 with peptides. ALA uptake activity was measured as a function of time in the presence of no peptide, Pro-Gly, or Gly-His-Gly. The data are expressed as nanomoles of ALA taken up per milligram of protein at the indicated time points.

Conclusions. In this study, we found that overexpression of a *B. japonicum lrp* homolog complemented an *E. coli dpp* mutant for ALA and dipeptide uptake. Although unexpected, these findings were fruitful because they implicated an alternative route of ALA uptake in *E. coli* that may be the primary route in *B. japonicum*. We speculate that a *dpp* homolog was not obtained from our screen because the *E. coli dppA* strain E1772 carries a polar mutation and the entire *dpp* operon was unlikely to be represented in a single clone of the 1- to 3-kb *Sau*3A genomic library. Although the *B. japonicum lrp* homolog acted in an anomalous manner in a heterologous organism, its negative effect on ALA uptake in *B. japonicum* was consistent with the negative control of *E. coli* ALA uptake by its own *lrp* product. These observations suggest that the *B. japonicum* Lrp homolog is functionally similar to the *E. coli* protein as well as being structurally homologous. In support of this, we found that the *B. japonicum lrp* homolog positively affected *ilvIH* promoter activity, as does the *E. coli* gene. β-Galactosidase activity derived from a chromosomal *ilvI-lacZ* fusion (17) in the *lrp* strain CV1008 was 13-fold greater in a transformant carrying the plasmid-borne *B. japonicum lrp* homolog compared to the vector control (data not shown). We propose that the cloned *B. japonicum* gene product is a func-

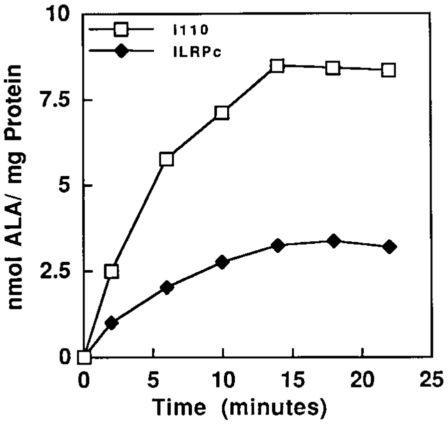

FIG. 5. ALA uptake in *B. japonicum* I110 and ILRPc. The data are expressed as nanomoles of ALA taken up per milligram of protein at the indicated time points.

tional and structural homolog of Lrp, and thus the gene should retain the name *lrp*. Furthermore, the findings show that Lrp is not confined to enteric bacteria.

Nucleotide sequence accession number. The nucleotide sequence of the *Dde*I-*Sal*I fragment bearing the *B. japonicum lrp* gene has been submitted to GenBank with accession number U85623.

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