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# The Arginine Deiminase Pathway in *Rhizobium etli*: DNA Sequence Analysis and Functional Study of the *arcABC* Genes

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**Sequence analysis upstream of the *Rhizobium etli fixLJ* homologous genes revealed the presence of three open reading frames homologous to the *arcABC* genes of *Pseudomonas aeruginosa*. The *P. aeruginosa arcABC* genes code for the enzymes of the arginine deiminase pathway: arginine deiminase, catabolic ornithine carbamoyltransferase (cOTCase), and carbamate kinase. OTCase activities were measured in free-living *R. etli* cells and in bacteroids isolated from bean nodules. OTCase activity in free-living cells was observed at a different pH optimum than OTCase activity in bacteroids, suggesting the presence of two enzymes with different characteristics and different expression patterns of the corresponding genes. The characteristics of the OTCase isolated from the bacteroids were studied in further detail and were shown to be similar to the properties of the cOTCase of *P. aeruginosa*. The enzyme has a pH optimum of 6.8 and a molecular mass of approximately 450 kDa, is characterized by a sigmoidal carbamoyl phosphate saturation curve, and exhibits a cooperativity for carbamoyl phosphate. *R. etli arcA* mutants, with polar effects on *arcB* and *arcC*, were constructed by insertion mutagenesis. Bean nodules induced by *arcA* mutants were still able to fix nitrogen but showed a significantly lower acetylene reduction activity than nodules induced by the wild type. No significant differences in nodule dry weight, plant dry weight, and number of nodules were found between the wild type and the mutants. Determination of the OTCase activity in extracts from bacteroids revealed a strong decrease in activity of this enzyme in the *arcA* mutant compared to the wild-type strain. Finally, we observed that expression of an *R. etli arcA-gusA* fusion was strongly induced under anaerobic conditions.**

The degradation of arginine by the arginine deiminase pathway is widely distributed among prokaryotic organisms (6). The arginine deiminase pathway consists of the following three enzymes: arginine deiminase (EC 3.5.3.6), catabolic ornithine carbamoyltransferase (cOTCase; EC 2.1.3.3), and carbamate kinase (EC 2.7.2.2). Arginine is converted into ornithine and carbamoyl phosphate, which serves to generate ATP from ADP. The degradation of 1 mol of arginine results in the formation of 1 mol of ATP and 2 mol of ammonium.

cOTCase is closely related to anabolic OTCase. Anabolic OTCase is involved in arginine biosynthesis and catalyzes the reaction opposite to that catalyzed by the cOTCase, namely the formation of citrulline from ornithine and carbamoyl phosphate.

The genetics of the arginine deiminase pathway have been well studied in *Pseudomonas aeruginosa*. In this organism, the genes encoding the enzymes of the arginine deiminase pathway are organized in an operon, the *arcDABC* operon. The *arcA* gene encodes the arginine deiminase, the *arcB* gene encodes the cOTCase, and the *arcC* gene encodes the carbamate kinase (44, 55). The fourth gene, *arcD*, encodes a membrane-bound protein that is necessary for the uptake of arginine and the excretion of ornithine. This transport process does not consume energy but is driven by a concentration gradient (30, 57).

Expression of the *P. aeruginosa arcDABC* operon is induced under conditions of oxygen limitation (33). Anaerobic expression of the operon requires a functional Anr protein and the presence of the ANR box (TTGAC-$N_4$-ATCAG) in the promoter region (14, 15). The Anr protein is a transcriptional regulator and belongs to the FNR family (62). In *Escherichia coli*, the Fnr protein regulates the expression of genes involved in anaerobic respiration (50).

In *Rhizobium etli* CNPAF512 (41), the *arcABC* genes were identified upstream of the *fixLJ* homologous genes. The complete nucleotide sequence was determined. *R. etli arcA* mutants were constructed and evaluated in planta. The characteristics of the *R. etli* cOTCase were studied.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. Growth conditions and bacterial matings were performed as described by D'hooghe et al. (7). For the determination of the OTCase in the free-living bacteria, cells were grown in tryptone-yeast extract (TY) medium and TY medium supplemented with 20 mM arginine.

**DNA manipulations.** DNA manipulations were performed as described previously (48). Hybridizations were performed at 50°C by using a digoxygenin labeling and detection kit (Boehringer) according to the manufacturer's recommendations. DNA fragments were transferred to nylon membranes by vacuum blotting. After hybridization, a nonstringent washing procedure was used as described by D'hooghe et al. (7).

**DNA sequence analysis.** Overlapping restriction fragments covering the *arcABC* genes were subcloned in pUC18 and sequenced on both strands. Sequence analysis and the subsequent analysis of the data were performed as described by D'hooghe et al. (7).

**Construction of *R. etli arcA* mutants.** The 2.8-kb *Eco*RI fragment of pFAJ1309 containing part of the *R. etli fixL* gene and the 5′ end of the *arcA* gene (Fig. 1) was blunt-ended with Klenow polymerase and subsequently cloned into *Sma*I-digested pJQ200-UC1. The resulting plasmid was digested with *Bst*XI, and the 5′ overhanging ends were removed with $T_4$ polymerase. This fragment was ligated to a 2.5-kb *Bam*HI Ω-Km fragment of pHP45Ω-Km after filling in the overhanging ends of the Ω-Km fragment with Klenow polymerase. Restriction analysis allowed us to select two constructs differing in the orientation of the resistance gene to the *arcA* gene, namely pFAJ1315 (*nptII* reads in the same direction as *arcA*) and pFAJ1316 (*nptII* reads in the opposite direction to *arcA*). Plasmids pFAJ1315 and pFAJ1316 were introduced into *R. etli* CNPAF512. Double re-

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *R. etli* | | |
| CNPAF512 | Wild type | Embrapa, Brazil |
| RpFAJ1002 | Nm$^r$ CNPAF512 *fixL*::*nptII* | 7 |
| RpFAJ1003 | Nm$^r$ CNPAF512 *fixL*::*nptII* | 7 |
| RpFAJ1004 | Nm$^r$ CNPAF512 *fixJ*::*nptII* | 7 |
| RpFAJ1005 | Nm$^r$ CNPAF512 *fixJ*::*nptII* | 7 |
| FAJ1006 | Nm$^r$ CNPAF512 *arcA*::*nptII* | This work |
| FAJ1007 | Nm$^r$ CNPAF12 *arcA*::*nptII* | This work |
| CE3 | Str$^r$ derivative of wild-type strain CFN42 | 37 |
| *R. leguminosarum* bv. phaseoli LMG8820 | Wild type | LMG culture collection |
| *R. tropici* CIAT899 | Wild type | 18 |
| *R. leguminosarum* bv. trifolii LMG8819 | Wild type | LMG culture collection |
| *R. leguminosarum* bv. viciae RBL 1309 | | 59 |
| *S. meliloti* 1021 | Wild type | 32 |
| *Rhizobium* spp. | | |
| BR816 | Wild type | Embrapa, Brazil |
| NGR234 | Wild type | 54 |
| *A. caulinodans* ORS571 | Wild type | 9 |
| *E. coli* DH5α | F80*dlacZ*ΔM15 Δ(*lacZYA-argF*) *recA endA hsdr supE* | Gibco-BRL |
| Plasmids | | |
| pUC18 | Ap$^r$ | 38 |
| pLAFR1 | Tc$^r$ | 13 |
| pLAFR3 | Tc$^r$ | 51 |
| pRK2073 | Sm$^r$ *tra*$^+$ | 11 |
| pHP45Ω-Km | Ap$^r$ Km$^r$ | 10 |
| pJQ200-UC1 | Gm$^r$ *sacB lacZ* | 43 |
| pKW119 | Ap$^r$, *gusA* translational fusion vector | 60 |
| pFAJ1304 | pLAFR1 containing *fixLJ* and *arcABC* genes | 7 |
| pFAJ1305 | pLAFR1 containing *fixLJ* and *arcABC* genes | 7 |
| pFAJ1309 | pUC18 containing 2.8-kb *Eco*RI fragment of pFAJ1304 | 8 |
| pFAJ1315 | Gm$^r$ *sacB arcA*::*nptII* | This work |
| pFAJ1316 | Gm$^r$ *sacB arcA*::*nptII* | This work |
| pFAJ1318 | Ap$^r$, *arcA-gusA* fusion in pKW119 | This work |
| pFAJ1319 | Tc$^r$, *arcA-gusA* fusion in pLAFR3 | This work |

combinants were selected on medium containing neomycin and sucrose (50 g/liter) and were verified by Southern hybridization by using the 2.5-kb *Bam*HI fragment of pHP45Ω-Km and the 2.0-kb *Bam*HI fragment of pFAJ1304 (containing the *R. etli arcA* gene and part of the *arcB* gene) alternatively as probes. The resulting mutants were designated FAJ1006 and FAJ1007, and the mutated regions are shown in Fig. 1.

**Plant culture and acetylene reduction assay.** Plant experiments and acetylene reduction assays were performed as described by D'hooghe et al. (7). Bacteroids were isolated as outlined by Leyva et al. (28).

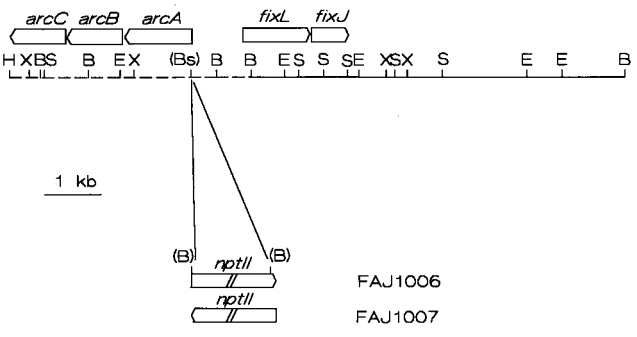


FIG. 1. Physical map of the region containing the *R. etli* CNPAF512 *arcABC* genes. The dotted line indicates the region that was sequenced. The structures of the *arcA* insertion mutants (FAJ1006 and FAJ1007) are shown below the map. The *nptII* cartridge is not drawn to scale. Abbreviations for the restriction sites are as follows: B, *Bam*HI; Bs, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. The restriction sites within parentheses were lost during cloning procedures.

**Construction of the *R. etli arcA-gusA* fusion.** An *R. etli arcA-gusA* translational fusion was constructed by cloning the 1.4-kb *Pst*I fragment of pFAJ1309 (containing 1,355 bp of the promoter region of the *arcA* gene and the first 18 codons of *arcA*) into the *Sma*I site of the translational fusion vector pKW119. The *Pst*I fragment was blunt-ended with $T_4$ polymerase. The resulting construct was referred to as pFAJ1316. The fusion junction was controlled by DNA sequence analysis using an oligonucleotide complementary to the *gusA* gene as described by Jefferson (24). The *arcA-gusA* fusion was recloned as a 3-kb *Hin*dIII fragment into the *Hin*dIII site of pLAFR3.

**β-Glucuronidase assay.** β-Glucuronidase activity was determined as described by Michiels et al. (34). The bacteria were incubated for 18 h in TY medium in increasing concentrations of oxygen (0 to 21% oxygen) as described previously (34).

**Assay of OTCase activity.** cOTCase activity was determined by measuring the formation of citrulline from ornithine and carbamoyl phosphate. Under the experimental conditions used, the enzyme is able to perform this reaction, which is the reverse of the in vivo reaction. The amount of citrulline formed in 15 min was quantified either by the colorimetric assay of Prescott and Jones (42) or by the Archibald methodology (1). Under the conditions tested, the enzyme assay is linear over a 30-min time period. The standard assay mixture (2.0 ml) contained 50 mM EDTA–NaOH buffer at pH 6.8 or pH 8.5, 10 mM ornithine, 10 mM carbamoyl phosphate (and 10 mM phosphate when appropriate), and cell extract. Cells were disrupted in 2 ml of extraction buffer (10 mM phosphate buffer [pH 7.5]). The carbamoyl phosphate solution was prepared just before use. The reaction was started by addition of the carbamoyl phosphate solution and was allowed to continue for 15 min at 37°C. Subsequently, the reaction was stopped by the addition of 1.0 ml of an antipyrine-diacetylmonoxime solution, the mixture was boiled for 20 min (42) and the absorbance at 466 nm was measured. When citrulline was detected by the method of Archibald (1), 2 ml of HCl (1 M) was added and absorbance was measured at 490 nm. Standard curves were constructed by appropriately diluting a stock solution of citrulline. Activity of the OTCase was defined as micromoles of citrulline formed per hour per milligram of protein.

**Molecular mass determination of the OTCases.** The molecular masses of the OTCases isolated from the bacteroids and the free-living cells were determined by gel filtration. The cellular extract was applied on an HR300 Sephacryl column (2.6 by 60 cm) equilibrated with phosphate buffer (20 mM, pH 7.0), as described by Nguyen et al. (36). The OTCases were detected by measuring the enzymatic activity at pH 6.8 for the bacteroid enzyme and at pH 8.5 for the free-living cell enzyme. The molecular masses were determined from a calibration curve established with cOTCase from *P. aeruginosa* (456 kDa), anabolic OTCase from *E. coli* (120 kDa) and the following markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa).

**Nucleotide sequence accession number.** The nucleotide sequence determined in this study has been deposited with the GenBank database under accession no. AF025543.

## RESULTS

**Identification and sequence analysis of the *R. etli arcABC* genes.** Sequence analysis of a 3.6-kb *Hin*dIII/*Bam*HI fragment upstream of the *R. etli fixLJ* homologous genes revealed the presence of three possible open reading frames (Fig. 1). The open reading frames were homologous with the *P. aeruginosa arcA*, *arcB*, and *arcC* genes and are therefore named the *R. etli arcA*, *arcB*, and *arcC* genes. So far no evidence has been found for the presence of the *R. etli* homolog of the *P. aeruginosa arcD* gene.

Translation initiation codons of the *R. etli arcA*, *arcB*, and *arcC* genes were assigned on the basis of the corresponding genes in *P. aeruginosa*. The three genes are each preceded by a putative Shine-Dalgarno sequence as follows: AGGAGGA, 11 bp upstream of the *arcA* translation initiation codon; GGAG, 11 bp upstream of *arcB*; and GGAGA, 10 bp upstream of *arcC*. The sequence TTGAT-$N_4$-CTCAA, similar to the consensus sequence for the FNR binding site in *E. coli* (TTGAT-$N_4$-ATCAA) (50), was identified 700 bp upstream of the *R. etli arcA* gene (50).

The *R. etli arcA* gene encodes a peptide of 409 amino acids with a calculated molecular mass of 46 kDa. The deduced amino acid sequence of the putative *R. etli* arginine deiminase (ArcA) showed 62 and 60% amino acid identities with the amino acid sequences of the arginine deiminases of *P. aeruginosa* (3) and *Pseudomonas putida* (GenBank accession no. U07185), respectively. Similarities with the *Mycoplasma arginini* (40), *Mycoplasma hominis* (19), and *Halobacterium salinarium* (47) arginine deiminases were considerably lower (approximately 20% identity).

The deduced amino acid sequence of the putative *R. etli* cOTCase (334 amino acids; 38 kDa) was aligned with the amino acid sequences of the cOTCases of *P. aeruginosa* (2) (72% identity), *Haemophilus influenzae* (12) (69% identity), and *H. salinarium* (46) (37% identity). From Fig. 2, it can be seen that, like all ornithine and aspartate carbamoyltransferases, the putative *R. etli* cOTCase contains two highly conserved regions: S-T-R-T-R (amino acids 57 to 61 in the *P. aeruginosa* protein) and H-P-T-Q (amino acids 135 to 138 in the *P. aeruginosa* protein) (2, 22).

Several conserved amino acids within and between these two regions have been shown to be involved in carbamoyl phosphate binding or catalysis, namely Ser-57, Arg-59, Thr-60, Lys-88, Arg-108, Gly-129, His-135, and Gln-138 (17, 21, 27). The numbering of the amino acids refers to the *P. aeruginosa* sequence.

The motif F-M/L-H-C-L-P (amino acids 271 to 276 in the *P. aeruginosa* protein) is present in all OTCases and the Cys-274 residue is the ornithine binding site (16, 31). All these motifs are highly conserved in both anabolic OTCases and cOTCases. The *Pseudomonas* cOTCase is unable to perform the anabolic reaction due to the high cooperativity and the poor affinity with respect to carbamoyl phosphate. However, when specific amino acid residues in the *P. aeruginosa* cOTCase, namely glutamate-106, methionine-322, and isoleucine-336, are changed, the protein can act as an anabolic OTCase in vivo, resulting from either reduced cooperativity or the absence of cooperativity with respect to carbamoyl phosphate (4, 35, 53). The glutamate-106 residue is also present in the *R. etli* protein but is absent in all but one anabolic OTCase. The methionine-322 and isoleucine-336 residues are not conserved in the putative *R. etli* cOTCase. On the contrary, the arginine residues R22, R29, R33, and R147 are conserved. They were shown to form the allosteric site for the nucleotide monophosphates in *P. aeruginosa* (58).

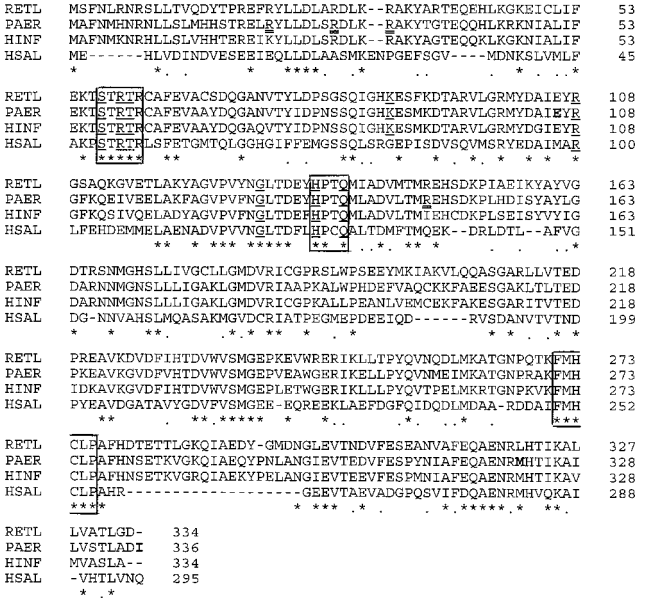


FIG. 2. Amino acid alignment of the cOTCases of *R. etli* (RETL), *P. aeruginosa* (PAER), *H. influenzae* (HINF), and *H. salinarium* (HSAL). Identical amino acids are marked with asterisks, and conservative substitutions (S-T-A/L-V-I-M/K-R/D-E/Q-N/F-Y-W) are indicated by dots. The conserved motifs S-T-R-T-R, H-P-T/C-Q, and F-M-H-C-L-P are boxed. The amino acids involved in carbamoyl phosphate binding are underlined. The residues involved in carbamoyl phosphate cooperativity in the *P. aeruginosa* OTCase are in bold. The arginine residues forming the allosteric site for the nucleotide monophosphates are double underlined.

The *R. etli arcC* gene encodes a peptide of 310 amino acids with a calculated molecular mass of 34 kDa. The putative amino acid sequence of the *R. etli* carbamate kinase was aligned with the sequences of the carbamate kinases of *P. aeruginosa* (3) (65% identity), *H. influenzae* (12) (62% identity), and *H. salinarium* (47) (31% identity). Since *arcABC* genes have not yet been described in *Rhizobium* species, we probed for the presence of *arc* genes in different *Rhizobium* species by hybridization. Genomic DNAs of *R. etli* CNPAF512 and CE3, *R. leguminosarum* bv. phaseoli LMG8820, *R. leguminosarum* bv. trifolii LMG8811, *R. leguminosarum* bv. viciae RBL 1309, *R. tropici* CIAT899, *Rhizobium* sp. strain BR816, *Rhizobium* sp. strain NGR234, *Sinorhizobium meliloti* 1021, and *Azorhizobium caulinodans* ORS571 were *Eco*RI restricted and hybridized with a 2-kb *Bam*HI fragment containing the *R. etli* CNPAF512 *arcA* gene and part of the *arcB* gene. Hybridization signals were only observed in CNPAF512 and in *R. leguminosarum* bv. phaseoli LMG8820. Surprisingly, no signals were detected in the other tested strains, not even in the *R. etli* strain CE3 or after long exposure of the hybridization blots (results not shown).

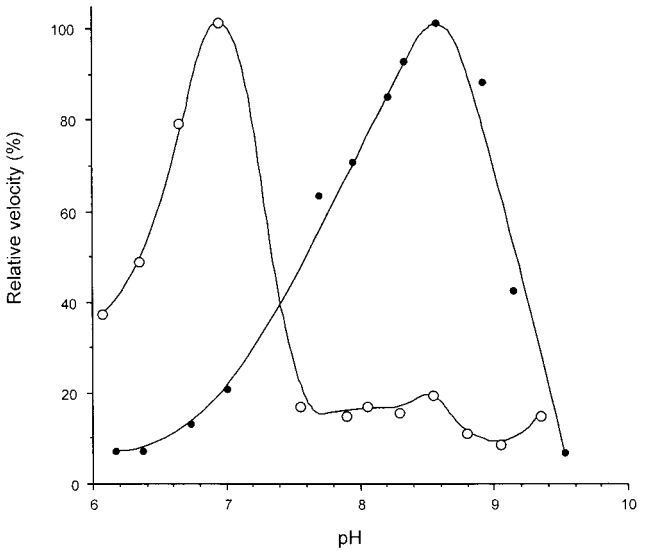


FIG. 3. pH dependence of the activity of the *R. etli* bacteroid carbamoyltransferase in the absence of an effector. Activities were determined as described in Materials and Methods and are expressed as a percentage of the maximal velocity. Protein extracts are from bacteroids (○) or free-living cells (●).

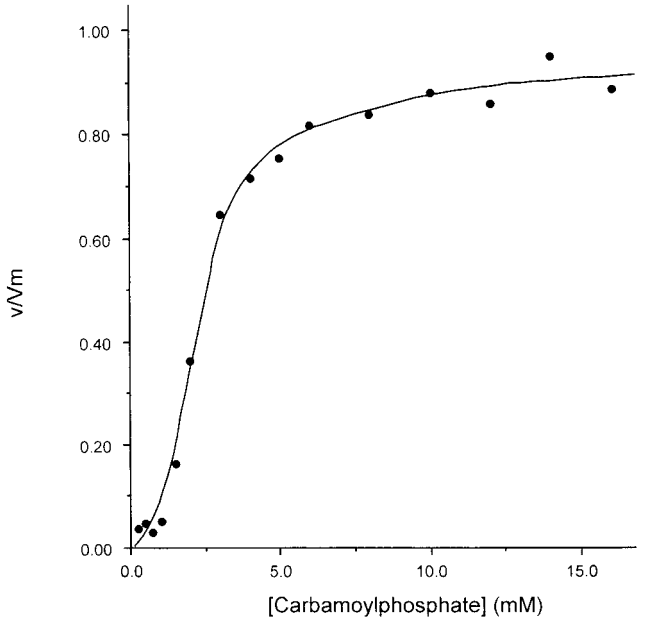


FIG. 4. Carbamoyl phosphate saturation curve of the *R. etli* bacteroid OTCase. Activities were determined in 150 mM imidazole–HCl, pH 6.8, in the presence of 10 mM ornithine. From the Hill plot $\{\log[V/(V_{max}-V)]$ versus log[CP]} (CP, carbamoyl phosphate), the Hill coefficient and the half-saturation coefficient were determined.

To check the possibility that multiple copies of the *arcABC* genes were present in *R. etli* CNPAF512, genomic DNA of CNPAF512 was digested with the restriction enzymes *Bam*HI, *Hin*dIII, *Pst*I, and *Sal*I. Hybridization on the restriction patterns revealed only those hybridizing fragments that could be expected from the restriction map for the *arcABC*-containing region. The presence of a second copy of the *arcABC* genes in *R. etli* CNPAF512 is therefore not very likely.

**Characterization of the *R. etli* OTCases.** We measured the OTCase activity in extracts of free-living cells as well as in bacteroids. Free-living cells were cultivated in TY medium supplemented with 20 mM arginine. The experimental data suggest that *R. etli* possesses two OTCase enzymes with different properties and different expression patterns of the corresponding genes. The enzyme in free-living cells had a maximum activity at pH 8.5, while the bacteroid OTCase had its maximum activity at pH 6.8 (Fig. 3).

Mass determination confirmed that the bacteroid OTCase was indeed different from the free-living cell enzyme. Gel filtration of a bacteroid cell extract revealed that the enzyme had a native molecular mass of approximately 450 kDa. Since the deduced *arcB* gene product has a calculated molecular mass of 38 kDa, this result suggests that the *R. etli* bacteroid OTCase has the same dodecameric structure as the cOTCase of *P. aeruginosa* (2). Gel filtration in the same conditions gave an estimation of 120 kDa for the enzyme present in the extract of free-living cells. Taking into account the good conservation of particular domains in the sequences of anabolic OTCases, this would indicate a trimeric structure for the free-living cell enzyme.

The bacteroid enzyme was characterized by a sigmoidal carbamoyl phosphate saturation curve (Fig. 4) with a carbamoyl phosphate concentration required for half-maximal velocity ($[S]^{CP}_{0.5}$) of 2.5 mM (CP, carbamoyl phosphate). A Hill coefficient ($n_H$) of 3.2 (20) confirmed that the bacteroid OTCase exhibits a cooperativity for the substrate carbamoyl phosphate. This cooperativity would account for the steep drop in activity at a pH higher than the optimum. It is known that an increase in pH strongly increases the cooperativity towards carbamoyl phosphate of the allosteric OTCase of *P. aeruginosa*, the threshold of concentration soon becoming too high to measure any activity (52). The activity of OTCase isolated from the bacteroids is stimulated by phosphate and nucleotide monophosphates and inhibited by polyamines such as spermidine (results not shown). With respect to ornithine, the bacteroid OTCase follows a Michaelis-Menten saturation kinetics, as deduced from the hyperbolic ornithine saturation (results not shown). Reciprocal velocities were plotted versus reciprocal ornithine concentrations (29), and a $K_m$ value of 2.5 mM was calculated. On the contrary, the free-living cell OTCase, which is active at pH 8.5, follows a Michaelis-Menten kinetics towards carbamoyl phosphate as well as ornithine, its physiological substrates. From reciprocal plots (29) a $K_m$ of 0.16 mM for carbamoyl phosphate and of 2.8 mM for ornithine could be estimated. Moreover, the enzyme in the free-living cells is inhibited by L-arginine ($K_i$ = 3 mM; data not shown). This inhibition is consistent with the hypothesis that the OTCase activity in free-living cells is part of the arginine biosynthetic pathway.

**Phenotype of the *R. etli arcA* mutants in planta.** The localization of the *arc* operon of *R. etli* upstream of the *fixLJ* homologous genes and the reported function of the corresponding proteins in other organisms could suggest a possible function in symbiotic nitrogen fixation. To verify this hypothesis, two *R. etli arcA* mutants, FAJ1006 (*nptII* gene in the opposite orientation to *arcA*) and FAJ1007 (*nptII* gene in the same orientation as the *arcA* gene), were constructed by insertion mutagenesis as outlined in Materials and Methods.

*Phaseolus vulgaris*, cultivar "Limburgse vroege," plants were inoculated with the *R. etli* wild-type strain CNPAF512 and the two *arcA* mutants, FAJ1006 and FAJ1007. No differences in time of nodulation could be observed. After 4 weeks of growth, plants were evaluated for acetylene reduction activity, plant dry weight, nodule dry weight, and number of nodules. From

TABLE 2. Acetylene reduction activity, plant dry weight, nodule dry weight, number of nodules, and OTCase activity in bacteriods of plants inoculated with the *R. etli* wild type (CNPAF512) and the *arcA* mutants FAJ1006 and FAJ1007

| Strain | Phenotypic characteristics[a] | | | | |
|---|---|---|---|---|---|
| | Acetylene reduction activity (nmol/h/plant) | Plant dry wt (g/plant) | Nodule dry wt (g/plant) | No. of nodules/plant | OTCase activity (U) |
| CNPAF512 | 4,330 (880) A | 0.45 (0.08) A | 0.071 (0.02) A | 263 (39) A | 7.8 (1.2) A |
| FAJ1006 | 2,830 (500) B | 0.51 (0.12) A | 0.066 (0.014) A | 300 (43) A | 1.95 (0.3) B |
| FAJ1007 | 3,590 (1,700) AB | 0.54 (0.15) A | 0.054 (0.017) A | 281 (93) A | 6.85 (1.2) A |
| Noninoculated plant | 0 (0) C | 0.45 (0.04) A | 0 (0) B | 0 (0) B | |

[a] Values are means for at least five plants. Standard deviations are given within parentheses. Results with the same letter are not significantly different from each other (Duncan $P \leq 0.05$).

Table 2, it can be seen that the *R. etli arcA* mutants are still able to fix nitrogen. However, both the FAJ1006 and FAJ1007 mutants show a lower acetylene reduction activity than the wild type. For FAJ1006, acetylene activity is significantly lower than the wild-type activity. No significant differences were found in the nodule dry weight, number of nodules, and plant dry weight.

We also determined OTCase activity in free-living cells and in extracts of bacteroids of the corresponding mutants. The bacteroids were isolated from nodules of plants inoculated with the wild-type strain and the two *arcA* mutants, FAJ1006 and FAJ1007. We measured OTCase activity at pH 6.8. For FAJ1007, OTCase activity at pH 6.8 is as high as in the wild-type, while FAJ1006 shows strongly reduced activity at this pH (Table 2). The mutation in the *arcA* gene has a polar effect on the *arcB* gene. The different behavior of the two mutants is probably due to read-through transcription from the *nptII* promoter in the FAJ1007 *arcA* mutant, since in this mutant the *nptII* gene was inserted in the same direction as the *arcA* gene. In the bacteroid extracts no OTCase activity was detected at pH 8.5. On the contrary, in the extracts of the free-living grown bacteria (the wild type and the *arcA* mutant FAJ1006) OTCase activity was observed at pH 8.5 but not at pH 6.8. At pH 8.5, OTCase activity was as high in the wild type as in the mutant. This indicates that the allosteric OTCase, active at pH 6.8 and present in the bacteroids, is the product of the *arcB* gene. To study the functional relationship between the *arcABC* genes and the *fixLJ* homologous genes, OTCase activity was also determined in bacteroids from plants inoculated with the *R. etli fixLJ* mutants RPFAJ1002 and RpFAJ1003 or with the *R. etli fixJ* mutants RpFAJ1004 and RpFAJ1005 (data not shown). The OTCase activity in the mutants was as high as in the wild-type strain. This means that the FixLJ two-component pair is not involved in the regulation of the *arcABC* operon.

**Expression of the *arcA-gusA* fusion in *R. etli*.** To study the expression of the *R. etli arcABC* genes, an *R. etli arcA-gusA* translational fusion was constructed in the broad-host-range plasmid pLAFR3. The expression of the fusion was examined in the wild-type strain CNPAF512, in the *arcA* mutants (FAJ1006 and FAJ1007), and in the *R. etli fixLJ* mutants (RPFAJ1002 and RpFAJ1003). Cultures of the wild-type strain carrying the *arcA-gusA* fusion were incubated at increasing oxygen concentrations (0 to 21% oxygen) (Fig. 5). Maximal *arcA* expression occurred at extremely low oxygen tensions. After anaerobic induction, the expression was approximately 10 times higher than in aerobic conditions. Mutations in the *fixLJ* homologous genes or in the *arcABC* genes did not affect the level of expression of the *arcA* genes, either in aerobiosis or in anaerobiosis. This result is in agreement with the fact that mutations in the *fixLJ* homologous genes do not affect the OTCase activity in bacteroids.

## DISCUSSION

The *R. etli* CNPAF512 *arcABC* genes were identified. The *arcA*, *arcB*, and *arcC* genes encode the enzymes of the arginine deiminase pathway, arginine deiminase, cOTCase, and carbamate kinase, respectively. The deduced amino acid sequences show similarity to the amino acid sequences of the corresponding proteins in other organisms, especially to the arginine deiminase, the cOTCase, and the carbamate kinase of *P. aeruginosa* (respectively 62, 72, and 65% identities). In *P. aeruginosa*, the operon also contains a fourth gene, the *arcD* gene, which encodes an arginine-ornithine antiporter, located upstream of the *arcA* gene (30, 57). In *R. etli*, we did not find an *arcD* gene upstream of the *arcA* gene. However, this does not mean that the *arcD* gene is absent in this bacterium. The organization of the *arc* genes in an *arcDABC* operon is not universal: in *H. salinarium* the *arcC* gene is located upstream of the gene encoding the OTCase (*arcB*) and not downstream (46). Another possibility is that the ornithine, formed during the arginine deiminase pathway, is further metabolized. In prokaryotes, L-ornithine can be directly converted into pyrroline-5-carboxylate by an ornithine transaminase, and in *S. meliloti*, ornithine is transformed into proline by means of the ornithine cyclodeaminase (49). Proline is then further converted by a proline dehydrogenase (25).

We identified a sequence motif (TTGAT-$N_4$-CTCAA) showing significant homology to the *E. coli* FNR box (50) 700 bp upstream of the *R. etli arcA*. In *P. aeruginosa* anaerobic induction of the *arcDABC* operon is dependent on the ANR regulatory protein, which is an Fnr-like transcriptional activator (14, 15). Expression analysis showed that the *R. etli arcA* gene is induced at extremely low oxygen tensions. Possibly, the expression of the *R. etli arc* genes is also controlled by an Fnr-like transcriptional activator. However, we do not have any experimental evidence for this hypothesis or for the importance of this FNR box motif, which is located at a rather

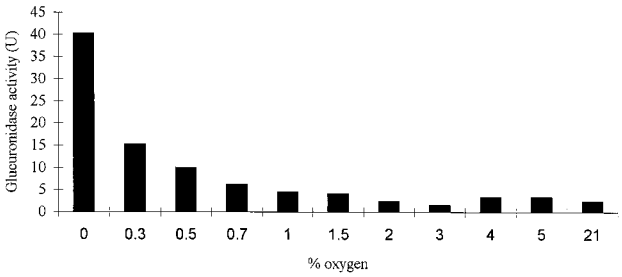


FIG. 5. Expression of *arcA-gusA* in the *R. etli* wild-type strain after incubation at different oxygen tensions. The β-glucuronidase activity is expressed in Miller units.

large distance from the *arcA* translation initiation codon. Analysis of the interjacent region did not reveal the presence of an additional open reading frame. An Fnr homolog has been identified in *R. leguminosarum* bv. viciae but not yet in *R. etli* (5). Moreover, we cannot exclude the possibility that the identified FNR motif is involved in the Fnr-dependent regulation of the *fixLJ* homologous genes, which are located upstream of the *arcABC* operon, oriented in the opposite direction.

Hybridization experiments on genomic DNA of different *Rhizobium* strains revealed the presence of the *arc* operon in *R. leguminosarum* bv. phaseoli LMG8820. None of the other tested strains had a sequence which was homologous with this operon. We can assume that the *arcABC* genes are not present in these strains, taking into account the high degree of homology between the *R. etli arcABC* genes and the *arcABC* genes of unrelated species.

*R. etli arcA* mutants are still able to fix nitrogen, albeit at a reduced level. No significant differences were found in speed of nodulation, nodule dry weight, number of nodules, and plant dry weight between the mutants and the wild-type strain. We were also able to demonstrate OTCase activity in the bacteroids of plants inoculated with the wild-type strain. This enzyme, which has a pH optimum of 6.8, is probably encoded by the *arcB* gene, since in an *arcA* insertion mutant almost no OTCase activity (reduced to background levels) was detected under these conditions. On the contrary the enzyme which is active at pH 8.5 is probably the anabolic OTCase encoded by the *argF* gene (23, 56). Activity of this enzyme was only detected in free-living cells.

The bacteroid OTCase was partially purified to study the characteristics of the enzyme. The enzyme has a native molecular mass of approximately 450 kDa. This indicates that the *R. etli* OTCase has a dodecameric structure as is the case for the cOTCase of *P. aeruginosa* (2). In addition, the conserved arginine residues R22, R29, R33, and which were shown to form the allosteric site for the nucleotide monophosphates in *P. aeruginosa* OTCase, are also conserved in R147, the *R. etli* protein (36, 58) (Fig. 2).

The dodecameric quaternary structure of the native enzyme and the strong cooperative saturation by carbamoyl phosphate indicate that the bacteroid OTCase is a catabolic enzyme that is not able to perform the anabolic reaction in *Rhizobium.*

All these features of the bacteroid OTCase are comparable to the properties of the *P. aeruginosa* cOTCase (52, 53). The *H. salinarium* cOTCase, on the other hand, does not bind carbamoyl phosphate cooperatively but exhibits a Michaelis-Menten kinetic for both carbamoyl phosphate and ornithine, and the native enzyme functions as a hexamer.

From this work, we have indications that the arginine deiminase pathway is active in nitrogen-fixing *R. etli* and could be required for efficient nitrogen fixation. In *P. aeruginosa* the arginine deiminase pathway is induced by a shift to extremely low oxygen concentrations, to allow the bacteria to generate energy, by the degradation of arginine at extreme conditions of nutrient depletion (33). In *R. etli* the pathway could have an identical function. The arginine deiminase pathway possibly becomes active at a certain stage of the symbiosis. We have shown that the *R. etli arcABC* genes are expressed at extremely low oxygen concentrations. It has been suggested before that amino acids serve as additional carbon and energy sources for the bacteria during nodule formation and invasion (26, 61). $C_4$-dicarboxylic acids, which are the major carbon source in symbiosis, are not needed for nodule invasion (39, 45). *S. meliloti* mutants unable to catabolize ornithine or proline are affected in nodulation efficiency and in competitiveness (25, 49).

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