Symbiosis-specific expression of *Rhizobium etli casA* encoding a secreted calmodulin-related protein

Chuanwu Xi*, Eric Schoeters†, Jos Vanderleyden*, and Jan Michiels*‡

*F. A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001, Heverlee, Belgium; and †Zoological Institute, Katholieke Universiteit Leuven, Naamsestraat 59, B-3000, Leuven, Belgium

Edited by Sharon R. Long, Stanford University, Stanford, CA, and approved August 7, 2000 (received for review April 20, 2000)

Symbiosis between *Rhizobium* and its leguminous host requires elaborate communication between the partners throughout the interaction process. A calmodulin-like protein, termed calsymin, was identified in *Rhizobium etli*; a calmodulin-related protein in a Gram-negative bacterium had not been described previously. Calsymin possesses three repeated homologous domains. Each domain contains two predicted EF-hand Ca^{2+}-binding motifs. Ca^{2+}-binding activity of calsymin was demonstrated on purified protein. *R. etli* efficiently secretes calsymin without N-terminal cleavage of the protein. The gene encoding calsymin, *casA*, is exclusively expressed during colonization and infection of *R. etli* with the host. Expression of *casA* is controlled by a repressor protein, termed CasR, belonging to the TetR family of regulatory proteins. Mutation of the *casA* gene affects the development of bacteroids during symbiosis and symbiotic nitrogen fixation.

The symbiotic interaction between leguminous plants and rhizobia leads to the formation of a new plant organ, the nodule. Within this structure, the bacteria differentiate into bacteroids that fix molecular nitrogen for the benefit of the plant. A sustained molecular dialogue between both partners during the infection process is a prerequisite for a successful symbiosis. During the initial step of the interaction, the presence of plant root-secreted flavonoids induces the expression of rhizobial nodulation (*nod*) genes. The *nod* genes encode enzymes involved in the synthesis of lipochitooligosaccharide molecules, also termed Nod factors (1–3). These rhizobial Nod factors are secreted and act as signal molecules that provoke several physiological and morphological alterations in the roots of the host plant (4, 5). During infection of the root, the bacteria are released from the unwalled tip of the infection thread in the plant cell cytoplasm by endocytosis of the membrane surrounding the infection thread. As a result, the bacteria, now termed bacteroids, are enclosed by a plant membrane. This new subcellular compartment is named a symbiosome. The generation and maintenance of this subcellular compartment is an essential facet of the coexistence between rhizobia and living plant cells.

Ca^{2+} is a common intracellular second-messenger molecule in eukaryotic cells and modulates a myriad of cellular processes (6). The earliest responses of root hairs to Nod factors include influx of Ca^{2+} and efflux of chloride and potassium ions (7) and intracellular calcium spiking (8). Changes in intracellular Ca^{2+} concentrations in eukaryotes are transduced through high-affinity Ca^{2+}-binding proteins. Calmodulin, a primary Ca^{2+} receptor, is the most ubiquitous member of the largest family of Ca^{2+}-binding proteins. It is a small acidic protein present in all eukaryotes, regulating the activity of many vital enzymes (9). Calmodulin has a dumbbell-shaped structure with two globular domains connected by a flexible central tether. Each of these lobes contains two Ca^{2+}-binding motifs consisting of two nearly perpendicular α-helices separated by a 12-residue loop. This structure is known as the EF-hand helix–loop–helix motif (10). Ca^{2+} binding induces a large conformational change in calmodulin and causes the exposure of two hydrophobic patches involved in target recognition.

Although the presence of bacterial proteins with calmodulin-like properties has been reported repeatedly, calmodulin-like genes with authentic EF-hands appear to be very uncommon in prokaryotes (11). The only example so far described is a calmodulin-like protein from the Gram-positive bacterium *Saccharopolyspora erythraea* that contains four EF-hands (12). The biological role for this protein is presently unknown. Here we describe the isolation of a calmodulin-like gene from *Rhizobium etli*, *casA*, that is implicated in symbiosis and regulated by a TetR-type of repressor.

Materials and Methods

Growth Conditions. *R. etli* strains were routinely grown in liquid tryptone/yeast extract (TY) or acid minimal salts (AMS) medium at 30°C and maintained on yeast-mannitol agar plates (13). *Escherichia coli* was grown in Luria–Bertani medium at 37°C.

Screening of a Mutant Expression Library. The *R. etli* mutant library was constructed as described by Xi *et al.* (14). Approximately 4,000 mutants were screened for differential induction of the *gusA* gene when the cultures were grown in a microoxic environment with 0.3% oxygen and/or in the presence of nodule extracts. Details on the screening procedure are described by Xi *et al.* (15).

Plant Culture and Bacteroid Isolation. *Phaseolus vulgaris* cv. Limburgse vroege plants were grown in the plant growth room essentially as described by Michiels *et al.* (16) and analyzed 3 weeks after inoculation. For symbiotic expressions, bacteroids were purified from plant material by differential centrifugation (13).

Cloning of the *casA–casR* Gene Region. Standard methods were used for *in vitro* DNA manipulations (17). Total DNA from the selected mutant strain FAJ1806 was digested with *Xho*I and ligated into the *Sal*I site of pUC18. Inserts containing part of the mTn*5gusA-pgfp21* transposon were selected. From the cloned fragment, the partial sequence flanking the mTn*5gusA-pgfp21* insertion was determined, using the *gusA* primer 5′-GATTTCACGGGTTGGTTCT-3′. To isolate the corresponding wild-type *R. etli* CNPAF512 gene, the sequenced fragment was amplified by PCR using the primers OJM142 (5′-GGCTGTCGTCAATGCTCTCCGATCTCGATACCG-3′) and OJM143 (5′-CATGAACTCTTCCAACGACACCAGC-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: spc^R, spectinomycin resistance; km^R, kanamycin resistance; PVDF, poly(vinylidene difluoride).

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF288533).

‡To whom reprint requests should be addressed. E-mail: jan.michiels@agr.kuleuven.ac.be.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.210181097. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.210181097

CCATCCC-3′). The resulting 360-bp PCR fragment was used as a probe to hybridize a λ phage EMBL3 gene library of CNPAF512. DNA from a positive plaque was hybridized with the 360-bp digoxigenin-labeled probe. Two positive *Sal*I bands (1.8 kb and 0.7 kb) were cloned in pUC18 and sequenced.

Construction of Mutants. To construct *R. etli casR* mutants, the 1.8-kb *Sal*I fragment containing the complete *casR* gene was first cloned in the *Sal*I site of pUC19, yielding plasmid pFAJ1822. The 2.1-kb spectinomycin resistance (spc^R) cartridge from pHP45Ω (18) was removed with *Hin*dIII and ligated in the *Fse*I and *Psh*AI sites of pFAJ1822 to obtain plasmids pFAJ1824 (the orientation of the spc^R gene is opposite to *casR*) and pFAJ1825 (the orientation of the spc^R gene is the same as *casR*) after blunting of the fragments. The resulting *Sal*I fragments from pFAJ1824 and pFAJ1825 were cloned in the *Sal*I site of the suicide plasmid pJQ200SK (19), generating plasmids pFAJ1826 and pFAJ1827, respectively. These plasmids were introduced into strain CNPAF512 and double recombinants were selected as described (13). The *casR* mutants were FAJ1802 (spc^R gene in *Fse*I site in the same orientation as *casR*) and FAJ1803 (spc^R gene in *Psh*AI site, opposite orientation to *casR*).

For the construction of an *R. etli casA* mutant strain, a 2.5-kb fragment containing the *casA* and *casR* genes was amplified by PCR using primers OJM161 (5′-GATCCTCGAGCGCCGGTCAGGCGAGGCAACAGCC-3′) and OJM162 (5′-GATCCTCGAGGAAGTCGCCGGCCCGCGTCAGTACCG-3′) and, after digestion with *Xho*I, ligated into the *Sal*I site of pUC19-ΔE (a pUC19 derivative from which the *Eco*RI site was removed), yielding plasmid pFAJ1828. Plasmid pFAJ1828 was digested with *Eco*RI, thereby removing the *casA* internal *Eco*RI fragment, and ligated to the 2.2-kb *Hin*dIII fragment from pHP45Ω-km, containing a kanamycin resistance (km^R) gene, by blunt-end ligation, generating plasmids pFAJ1829 (the orientation of the km^R gene is the same as *casA*) and plasmid pFAJ1830 (the km^R gene and *casA* have opposite orientations). The *Bam*HI fragments containing the inserts of pFAJ1829 and pFAJ1830 were cloned into the *Bam*HI site of pJQ200SK to obtain plasmids pFAJ1831 and pFAJ1832, respectively. Plasmids pFAJ1831 and pFAJ1832 were used to mutate the wild-type strain CNPAF512, yielding the *casA* mutant strains FAJ1804 and FAJ1805, respectively. *R. etli casAR* double mutants are described in the section on the construction of *casA–gusA* and *casR–gusA* fusions.

Construction of *gusA* Fusions. The *R. etli casA*::*gusA* strain FAJ1806 carries the insertion of mTn*5gusA-pgfp21* between the nucleotides 838 and 839 from the ATG start codon of the *casA* gene.

To construct the *R. etli casA*::mTn*5gusA-pgfp21 casR*.::Ω-Spc strains FAJ1807 and FAJ1808, plasmids pFAJ1826 and pFAJ1827 were introduced into the *R. etli* mutant strain FAJ1806 and double recombinants were selected, generating the *casAR* double mutant strains FAJ1807 and FAJ1808, respectively.

To construct a genomic *casR*::*gusA* fusion, the 4.5-kb *Bam*HI fragment from pWM6 containing a promoterless *gusA* gene and a km^R cassette were ligated into the *Psh*AI site of pFAJ1822, yielding plasmid pFAJ1836. The resulting 6.3-kb *Kpn*I fragment from pFAJ1836 was cloned in the *Sma*I site of pJQ200-UC1 by blunt-end ligation, yielding plasmid pFAJ1837. This plasmid was introduced into the *R. etli* wild-type strain CNPAF512 and double recombinants were selected to obtain the *casR*::*gusA* mutant strain FAJ1809.

Plasmid-borne P_{casA}–*gusA* (pFAJ1842) and P_{casR}–*gusA* (pFAJ1843) fusions were constructed. For this, the 450-bp *casAR* intergenic region, amplified by PCR using the primers OJM163 (5′-GATCTCTAGAGGATCCGGTGGAGACTTTCGGCCGGCCTCG-3′) and OJM164 (5′-GATCTCTAGAGACATCGGACGGCCGGGCCGCGACG-3′), was digested with *Xba*I and ligated into the *Xba*I site of pUC18NotI (20), yielding plasmid pFAJ1839. The 4.5-kb *gusA*-km^R *Sma*I fragment from pWM6 was inserted into the *Sma*I and *Sal*I sites of pFAJ1839 to obtain plasmids pFAJ1840 and pFAJ1841, respectively. The *Not*I fragments containing the P_{casA}–*gusA* and P_{casR}–*gusA* fusions from the respective plasmids were inserted into the *Bam*HI site of pLAFR3 (21) by blunt-end ligation, yielding plasmids pFAJ1842 and pFAJ1843.

Constructs for Complementation Analysis. A 1.6-kb fragment containing the complete *casA* gene sequence but not the *casR* gene was obtained by PCR using primers OJM162 (5′-GATCCTCGAGGAAGTCGCCGGCCCGCGTCAGTACCG-3′) and OJM163 (5′-GATCTCTAGAGGATCCGGTGGAGACTTTCGGCGGCCTCG-3′). This fragment was digested with *Xba*I and *Xho*I and ligated with *Xba*I/*Sal*I-digested pUC18NotI, yielding plasmid pFAJ1833. To facilitate subsequent cloning in pLAFR3, the 2.1-kb *Sma*I fragment containing the spc^R gene from pHP45Ω was inserted into the *Sma*I site of pFAJ1833. From the resulting plasmid pFAJ1834, the 3.8-kb *Not*I fragment containing the *casA* gene was cloned into the *Bam*HI site of pLAFR3 by using blunt-end ligation, yielding pFAJ1835. Plasmid pFAJ1838 was obtained by inserting the 4.2-kb *Bam*HI fragment from pFAJ1829 containing the complete *casR* gene into the *Bam*HI site of pLAFR3.

Light and Electron Microscopy. For light microscopic analysis, 3-μm sections of 3-week-old nodules were prepared as described (16).

To obtain transmission electron micrographs, 3-week-old nodules were fixed overnight at 4°C in 2% cold glutaraldehyde solution in 10 mM sodium cacodylate buffer (pH 7.3) and postfixed in osmium tetroxide in the same buffer. Then the sample was block-stained in uranyl acetate (in 10% aqueous acetone) and dehydrated in a graded acetone series, followed by embedding in Araldite. Serial semithin sections were stained with methylene blue and thionin. Thin sections, made with a Reichert Ultracut E microtome, were stained with uranyl acetate and lead citrate in an LKB 2168 Ultrostainer and examined in a Zeiss EM 900 electron microscope.

Purification of Proteins. Bacterial cells and culture supernatant were collected from the wild type or the CasA-overproducing *casR* strain FAJ1802 grown overnight at 30°C in TY medium. Proteins were precipitated from the growth medium supernatant by incubation with trichloroacetic acid (10%, wt/vol) for 2 h on ice and pelleted by centrifugation at 15,000 × *g* for 20 min. The cell pellets were washed twice with PBS at pH 6.8 and resuspended in the same buffer. The mixture was passed three times through a French press (SLM Instruments, Rochester, NY) at 10,000 psi (69 MPa). Cell debris was removed by centrifugation for 20 min at 10,000 rpm. The proteins were precipitated as described above. Proteins were separated by electrophoresis on SDS/polyacrylamide gels (5% stacking gel, 15% separating gel, 0.1% SDS). After electrophoresis, proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes by electroblotting.

To overexpress calsymin in *E. coli*, a 900-bp fragment was amplified by PCR using primers OJM186 (5′-GTCACTCGAGATGACGACCATTTTCTGCTGCAACATC-3′) and OJM187 (5′-GTCACTGCAGCTCGCGGCGGACTGGACGG-3′) and cloned as a *Xho*I/*Pst*I fragment in the corresponding sites of pBAD/HisA, yielding pFAJ1845. To express the recombinant gene, *E. coli* Top10 cells carrying pFAJ1845 were grown exponentially and subsequently induced for 6 h in the presence of 0.2% arabinose. The recombinant calsymin fusion protein carrying the N-terminal polyhistidine tag was purified under denaturing conditions on ProBond as recommended by the manufacturer (Invitrogen).

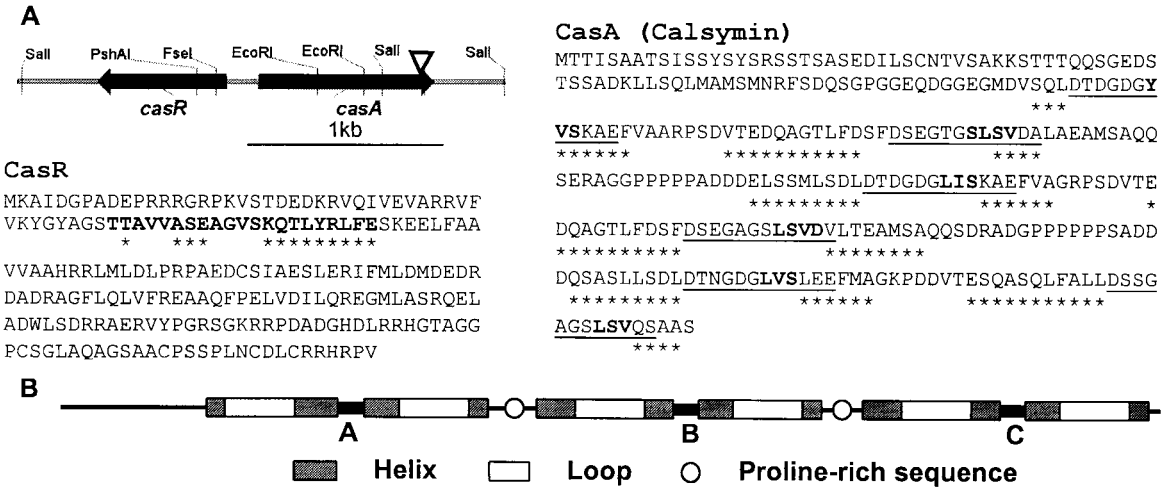

Fig. 1. Sequence and structure of the *R. etli* CasA (calsymin) and CasR proteins. (*A*) Schematic representation and physical map of the divergently transcribed *casA* and *casR* genes. The arrowhead indicates the position of the mTn*5* insertion in the *casA* mutant FAJ1806. Predicted products of the *casA* and *casR* genes showing the six putative EF-hand calcium-binding sites of CasA and the helix–turn–helix structure of CasR. The secondary structure predictions are based on the algorithm of Geourjon and Deleage (28). Amino acids in boldface in the CasR sequence represent the conserved helix–turn–helix structure as found in TetR-related proteins (27). The amino acids in the CasR and CasA proteins marked with asterisks are part of predicted α-helices. Underlined amino acids in CasA are similar to EF-hand calcium-binding motifs (10). Amino acids forming putative short β-strands within the loops are in boldface. (*B*) Schematic representation of the three homologous calcium-binding domains of calsymin and localization of the EF-hand helix–loop–helix calcium-binding motifs and proline-rich domains.

Calcium-Binding Assay. After electroblotting of the proteins on PVDF membranes, the same membrane was first used in a $^{45}Ca^{2+}$-binding assay, then stained with ruthenium red, and finally stained with Coomassie brilliant blue. The $^{45}Ca^{2+}$-binding assay was performed essentially as described by Maruyama *et al.* (22). Ruthenium red staining was carried out as described by Charuk *et al.* (23). To confirm the identity of the ≈45-kDa $^{45}Ca^{2+}$-binding band, it was excised (50 μg) from several lanes from PVDF blots and N-terminally sequenced by Edman degradation on a pulsed liquid-phase 477A/120A protein sequencer (Perkin–Elmer) using *N*-methylpiperidine as a coupling base.

The NAD kinase activity assay was performed according to Harmon *et al.* (24), using purified chicken NAD kinase (Sigma). Calsymin was prepared from the culture supernatant of FAJ1803 as described above. Aliquots were tested for induction of NAD kinase activity.

Results

Sequence of *casA* and *casR*. *R. etli* is the nodulating symbiont of *P. vulgaris*, the common bean plant. To identify new symbiotic genes in this species, *R. etli* was mutagenized with the miniTn*5* transposon derivative mTn*5gusA-pgfp21* (14) carrying a promoterless *gusA* gene suitable for promoter trapping. Expression of the *gusA* gene in the mutants was assessed under conditions of aerobiosis or microaerobiosis in the presence or absence of nodule extracts. One of the strains displaying a differential induction pattern was selected for further characterization.

Sequence analysis of a 2442-bp segment containing the region flanking the mTn*5gusA-pgfp21* insertion in mutant FAJ1806 indicated the presence of two divergently transcribed genes (Fig. 1*A*). The *casA* gene (*ca*lmodulin-like *s*ymbiosis gene *A*), which was inactivated in FAJ1806, codes for a protein of 293 amino acids (M_r 29,891) that is highly acidic (pI 3.6) (Fig. 1*A*). This protein, named calsymin, is composed of three domains (A, B, and C), containing 57 or 58 amino acids, structurally similar to the two domains of calmodulin, and a fourth nonhomologous N-terminal domain (Fig. 1*B*). Each of the three domains of calsymin contains two EF-hand helix–loop–helix structural motifs (Fig. 1). The 12-residue Ca^{2+}-binding loops I, III, and V of calsymin contain the calmodulin hallmark residues at positions 1, 3, 5, 7, 9, and 12. These residues provide one (positions 1, 3, 5, 7, and 9) or two (position 12) oxygen ligands for the coordination of Ca^{2+} in calmodulin. Loops II, IV, and VI are atypical because of the absence of the conserved glutamic acid bidendate ligand at position 12. The conserved glycine residue at position 6, enabling side-chain and main-chain ligations at positions 5 and 7, respectively, is found in the six Ca^{2+}-binding loops of calsymin. Structure predictions indicate the presence of short β-strands of 3 or 4 residues in calsymin at positions 7–11 in all loops (Fig. 1*A*). In calmodulin, two β-strands (residues 7–9 in both loops) from each domain interact and form an antiparallel sheet structure allowing cooperativity of Ca^{2+} binding. The amino acid sequence is more conserved between alternate loops (I, III, V and II, IV, VI) than between adjacent loops, similar to what is observed in calmodulin. In addition, highly similar linkers connect domains A to B (20 amino acids between loops II and III) and B to C (22 amino acids between IV and V). Calsymin may therefore have arisen by successive duplications and fusions of an ancestral gene coding for one Ca^{2+}-binding domain giving rise to a protein composed of three homologous domains.

Calsymin also possesses two proline-rich stretches, between amino acids 148–152 and 226–231, with five and six consecutive proline residues, respectively. These sequences contain the SH3 domain core recognition consensus sequence Pro-Xaa-Xaa-Pro (Xaa is any amino acid) (25). In the case of class I peptides, an arginine residue is found three amino acids N-terminal from the core recognition sequence. Also in calsymin, arginines are located N-terminal from the core sequences; however, they are separated by three amino acids instead of two. Differences in the number of arginine residues are known to affect the affinity for the SH3 domain (26).

A divergently transcribed gene, *casR* (*R*, repressor), is located in the region upstream from *casA* (Fig. 1*A*). The *casR* gene product contains 214 amino acids (M_r 24,240) and is homologous to transcriptional repressors belonging to the TetR family of bacterial regulatory proteins [21% identical and 28% conserved residues with TetR(E), accession no. X14035.1] (Fig. 1*A*). Sequence conservation is particularly strong in the helix–turn–

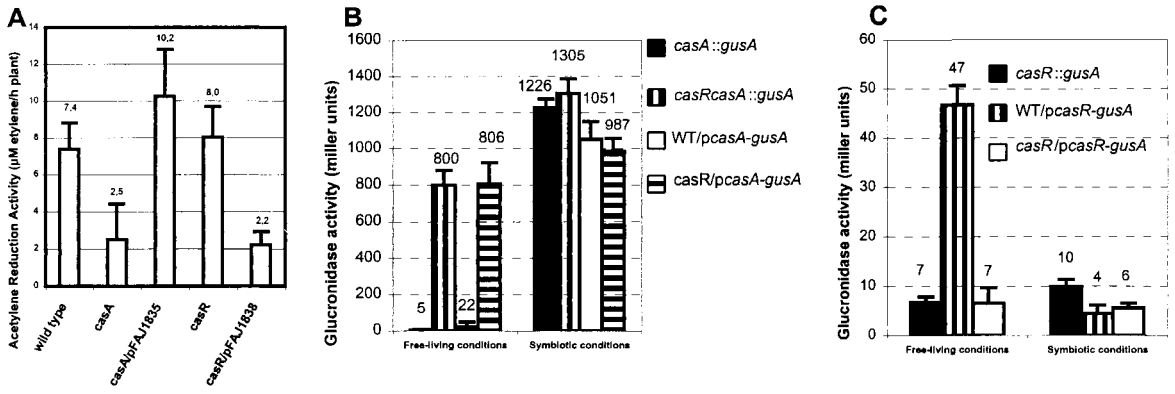

Fig. 2. Symbiotic phenotype of mutants and expression of *R. etli casA* and *casR* genes. (*A*) Nitrogen fixation of *P. vulgaris* plants inoculated with the *R. etli* wild-type strain, *casA* (FAJ1804) and *casR* (FAJ1802) mutant strains, and their complemented derivatives. Values are the mean ± SD ($n = 10$). The means are indicated above the bars. Plasmids pFAJ1835 and pFAJ1838 carry *casA* and *casR* genes, respectively. Similar results were obtained between strains FAJ1802 and FAJ1803 (*casR* mutants) and between strains FAJ1804 and FAJ1805 (*casA* mutants). (*B* and *C*) Expression of fusions between *gusA* and the *casA* (*B*) and *casR* (*C*) genes in free-living aerobic cultures (AMS) medium (13) supplemented with 10 mM sodium succinate, $OD_{595} = 0.1–0.2$, read with 100 μl culture in a $Versa_{max}$ microplate reader (Molecular Devices) and during symbiosis. Direct comparison of GusA activities under the two conditions may be difficult. Expressions were determined by using plasmid-borne P_{casA}–*gusA* (pFAJ1842) and P_{casR}–*gusA* (pFAJ1843) fusions and chromosomally located *casA*::*gusA* (FAJ1806 and FAJ1807) and *casR*::*gusA* (FAJ1809) fusions. β-Glucuronidase activities are expressed in Miller units. Values are the mean ± SD ($n = 3$).

helix structure localized in the N-terminal region of the protein and responsible for DNA binding in the TetR protein (27).

Symbiotic Phenotype. To study the role of calsymin during symbiosis with *P. vulgaris*, *R. etli casA* and *casR* insertional mutants were constructed and tested on plant. Symbiotic acetylene reduction activity (ARA), a measure for nitrogen fixation, was reduced by approximately 70% in nodules infected with the *casA* mutant strain versus wild type (Fig. 2*A*). A similar phenotype was observed when multiple copies of the *casR* gene, supplied on a multicopy plasmid, were present (Fig. 2*A*), which is in agreement with the postulated role of CasR as a repressor of CasA (see below). The ARA phenotype of a *casA* mutant could be restored to the wild-type level by complementation. No effect of a *casR* mutation on ARA was observed (Fig. 2*A*). Nodule numbers on plants inoculated with *R. etli* wild type or *casA* or *casR* mutants were not statistically different.

Light microscopic examination of stained nodule sections suggested a clearly reduced number of bacteroids in nodules infected with *casA* mutants compared with those infected with wild-type *R. etli* (Fig. 3 *A* and *B*). This was confirmed by a transmission electron microscopic analysis showing a reduction of the number of bacteroids by approximately 40% in plant cells colonized by the *casA* mutant (3.3 bacteroids per 10 μm^2, sample standard deviation 1.1, 3 samples of 750 μm^2 each analyzed) compared with the wild type (5.7 bacteroids per 10 μm^2,

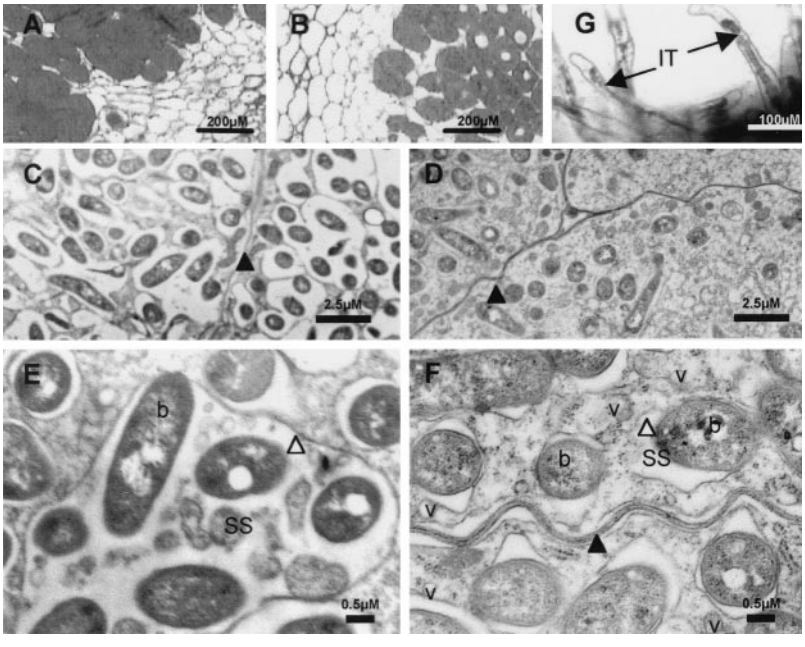

Fig. 3. Microscopic analysis of nodules and infection threads formed by *R. etli* wild-type (*A, C,* and *E*) and *casA* mutant FAJ1805 (*B, D, F,* and *G*) strains. (*A* and *B*) Toluidine blue stainings of 3-μm-thick sections of *P. vulgaris* nodules. (*C–F*) Transmission electron micrographs of 3-week-old nodules. (*G*) GusA staining of FAJ1806 bacteria expressing a *casA–gusA* fusion inside the infection threads. Black arrowhead, plant plasma membrane; white arrowhead, symbiosome membrane; black arrow, infection thread (IT); b, bacteroid; SS, symbiosome space; v, vesicle.

standard deviation 0.7), 12 days after inoculation. Furthermore, these images indicated that *casA* mutant bacteroids are packed individually in the symbiosome membrane (SM), whereas wild-type symbiosomes often contain multiple (2–10) bacteroids (Fig. 3 *C–F*). The SM was always found closely associated with the bacteroid membrane of *casA* mutants. As a result, the *casA* mutant symbiosomes were virtually lacking any symbiosome space (Fig. 3*F*). We can, however, not exclude that the observed differences are reinforced by the experimental protocol used.

Expression Patterns. The expression patterns of *casA* are consistent with a role during symbiosis. In the absence of the plant, expression of chromosomally integrated as well as plasmid-borne fusions between the *casA* promoter and *gusA* (P_{casA}–*gusA*) in a wild-type or a *casA* mutant background was repressed under aerobic or microaerobic (0.3% oxygen) conditions in the absence or presence of nodule extracts (Fig. 2*B*). Inhibition of *casA* expression under free-living conditions is relieved by the inactivation of *casR*, indicating that the expression of *casA* is negatively controlled by CasR (Fig. 2*B*). At the moment we cannot exclude that the *casA* gene could be induced under yet-unknown specific free-living growth conditions.

In contrast, plasmid-borne or chromosomal *casA-gusA* fusions in a wild-type or a *casA* mutant background are strongly expressed on the root surface of *P. vulgaris* plants approximately 1 day after inoculation (not shown). The expression is specific for *P. vulgaris* plants as no induction of the fusions was observed under similar conditions on *Medicago sativa* or wheat rootlets. GusA activity of P_{casA}–*gusA* was maintained in the infection threads (Fig. 3*G*) and inside the nodules of *P. vulgaris* (Fig. 2*B*).

Under free-living conditions, a plasmid-borne *casR-gusA* fusion is expressed in the wild type but GusA activity is strongly reduced in the *casR* mutants. Therefore, expression of *casR* is positively autoregulated permitting efficient repression of *casA* in the absence of the plant (Fig. 2*C*). The expression of *casR-gusA* was also reduced during symbiosis both in the wild type and in the *casR* mutant. Likely, under these conditions, the activator function of CasR on *casR* expression is inhibited. Expression of the *casR* gene occurred independently of the oxygen tension (results not shown).

Plant root expression of *casA* occurs independently of *nod* gene regulation as the activity of a P_{casA}–*gusA* (pFAJ1842) fusion is not reduced in a pSym-cured *R. etli* background whereas, under the same conditions, expression of a P_{nodA}–*gusA* fusion is reduced to background levels (results not shown). Also, no effect of a mutation in *fixL, nifA,* and *fnrN*, regulators of symbiotic gene expression in *R. etli*, on *casA* expression was observed. In addition, expression of the *casA–gusA* fusion was not induced by a low oxygen tension as it is the case with *nif/fix* genes (results not shown).

Calsymin Is a Calcium-Binding and Secreted Protein. The Ca^{2+}-binding activity of calsymin was demonstrated on overexpressed and purified protein from *E. coli* after polyacrylamide gel electrophoresis (PAGE) and transfer to a nylon membrane, using a $^{45}Ca^{2+}$ overlay technique (22) (Fig. 4). Ca^{2+}-binding activity of calsymin was not abolished/or could be restored after treatment by several protein denaturing treatments, including boiling, trichloroacetic acid precipitation, and denaturing PAGE. Unexpectedly, when overproduced in an *R. etli casR* mutant, $^{45}Ca^{2+}$-binding activity was detected only in the growth-medium supernatant, indicating that the protein was secreted (Fig. 5*B*). The same result was obtained when a ruthenium red binding assay was used (Fig. 5*C*). The identity of the secreted $^{45}Ca^{2+}$-binding protein as calsymin was confirmed by N-terminal sequence analysis. The amino acid sequence obtained, TTI-SAATSISSYSY, corresponds to the predicted N terminus of the protein based on the DNA sequence. Calsymin is therefore

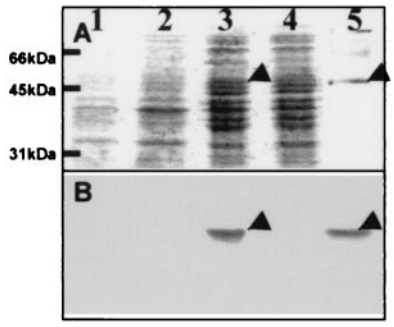

Fig. 4. Overexpression and calcium binding of recombinant calsymin in *E. coli*. Coomassie brilliant blue staining (*A*) and autoradiograph (*B*) after incubation with $^{45}Ca^{2+}$, of protein profiles immobilized on a PVDF membrane. Crude protein extracts were obtained from the culture supernatant (lanes 1 and 2) or from the cell pellet (lanes 3 and 4). Exponential cell cultures were either uninduced (lanes 2 and 4) or induced with 0.2% arabinose for 6 h (lanes 1 and 3) before harvesting. The recombinant protein was extracted under denaturing conditions from a crude protein extract of *E. coli* Top10 cells (Invitrogen) induced with arabinose and purified by Ni-chelation chromatography (lane 5). The calsymin band is marked with an arrowhead.

secreted from *R. etli* without cleavage of the *casA* gene product. Calsymin migrates on PAGE with a molecular mass of approximately 45 kDa, slightly higher than the calculated molecular mass. Aberrant migration may be caused by the acidity of the protein as noticed previously for other calmodulin-like proteins. Secretion of calsymin is efficient, because the protein could never be detected inside of cultured cells carrying a *casR* mutation. In agreement with the expression analysis of *casA*, no calsymin was secreted from *R. etli* wild-type cultures. In the mutant strain FAJ1808 (*casR* mutant derived from FAJ1806),

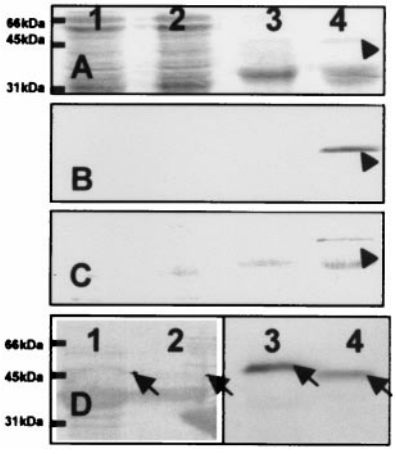

Fig. 5. Calcium binding and secretion of calsymin. (*A–C*) Staining of whole-cell proteins (lanes 1 and 2) and proteins secreted into the culture supernatant (lanes 3 and 4) from the *R. etli* wild-type CNPAF512 (lanes 1 and 3) and the *casR* mutant strain FAJ1803 (lanes 2 and 4). Proteins were subjected to electrophoresis on an SDS/polyacrylamide gel and transferred to PVDF membranes by electroblotting. The membranes were stained with Coomassie brilliant blue (*A*) or incubated with $^{45}Ca^{2+}$ and autoradiographed (*B*), or the calcium-binding proteins were stained with ruthenium red (*C*). The protein band indicated with an arrow was identified as calsymin by N-terminal sequence analysis of the isolated protein. (*D*) Calcium binding and secretion of a truncated calsymin protein from *R. etli* cultures. Proteins were isolated from the culture supernatant of *R. etli* FAJ1803 (lanes 1 and 3) and strain *casA*::mTn*5gusA-pgfp21 casR*::Ω-Spc FAJ1808 (lanes 2 and 4). The membrane was stained with Coomassie brilliant blue (lanes 1 and 2) or incubated with $^{45}Ca^{2+}$ and autoradiographed (lanes 3 and 4). The wild-type (lanes 1 and 3) and truncated (lanes 2 and 4) calsymin proteins are marked with an arrow.

the miniTn*5* insertion is located in codon 280 in the loop of the sixth calcium-binding site of the *casA* gene and therefore lacks the 13 C-terminal amino acids found in wild-type calsymin. As a result, the truncated form has a slightly reduced molecular mass (Fig. 5*D*). However, this truncated protein is still able to bind calcium and is secreted in the growth medium (Fig. 5*D*). Overproduction of CasA in free-living *R. etli* cells does not produce any obvious phenotype. Calsymin protein, purified from the *casR* mutant FAJ1803 culture supernatant, was not able to activate chicken NAD kinase.

Discussion

Two divergently transcribed genes, *casA* and *casR*, were identified in *R. etli*. Transcription of the *casA* gene is negatively controlled by the gene product of *casR*. The CasR repressor belongs to the TetR family of regulators. In the case of TetR, a helix–turn–helix motif on the N terminus is responsible for protein binding to an operator sequence in the *tetA* promoter, which inhibits transcription of the latter gene (27). Recognition of the antibiotic metabolite tetracycline by TetR causes dissociation of the repressor–DNA complex and induces transcription of the *tetA* gene, conferring tetracycline resistance. Other members of this family, including BarA from *Streptomyces virginiae* and ArpA from *Streptomyces griseus,* also bind to small effector molecules such as the butyrolactone autoregulators IM-2 and the A-factor. The *casA* gene is expressed on the roots of *P. vulgaris* plants, in the infection threads, and inside the bacteroids but not under the free-living conditions tested, suggesting that the inducing compound is plant derived. The expression pattern of *casA* is not altered in a pSym-cured *R. etli* strain and was not affected by mutations in *nif* and *fix* regulatory genes. Therefore, *casA* regulation occurs independently of known regulatory mechanisms of nodulation and nitrogen fixation genes and constitutes a type of symbiotic regulation in *R. etli* that has not been described previously.

The *casA* mutant was affected in the production of a calmodulin-like protein named calsymin. Reports on this type of protein in prokaryotes are very uncommon, and, to our knowledge, calsymin constitutes the first example of a calmodulin-like protein with canonical EF-hand motifs in a Gram-negative bacterium. In other prokaryotes, proteins with calmodulin-like properties have been reported previously (11, 29). However, the amino acid sequences of the corresponding proteins are still unknown. In the Gram-positive bacterium *Saccharopolyspora erythraea*, a 20-kDa calcium-binding protein, named calerythrin, was previously shown to possess a structural organization similar to calmodulin with four potential helix–loop–helix EF-hand motifs (12). More specifically, the protein belongs to the subfamily of eukaryotic sarcoplasmic Ca^{2+}-binding proteins and might therefore function as an intracellular calcium buffer (30).

Calsymin belongs to the calmodulin superfamily. Calsymin possesses a three-domain structure similar to the organization of calbindin D_{28k} and calretinin. Pairs of EF hands in each of the domains of calsymin may interact through short β-strands present in the loops and form a compact structure. Proteins belonging to the calmodulin superfamily are often divided into sensor and buffer proteins. The latter class may act as calcium buffers in the cell and control the concentration of free cytosolic Ca^{2+}. The sensor proteins change their conformation upon binding Ca^{2+} and interact directly with target proteins to modulate their activity. It is presently unclear to which class calsymin belongs.

Other calcium-binding rhizobial proteins have been implicated in symbiosis previously (31, 32). Secretion of the calcium-binding NodO protein requires a specialized secretion system homologous with ABC transporters involved in the secretion RTX proteins (32). N-terminal amino acid sequence analysis of calsymin isolated from the culture supernatant indicated that, similarly to NodO, calsymin is secreted without cleavage of an N-terminal transit peptide. The mechanism by which calsymin is secreted is presently unknown. However, the observation that calsymin possesses an N-terminal region of approximately 100 amino acids that is different from the three other homologous Ca^{2+}-binding domains suggests that determinants for secretion might be located within the N-terminal part of the protein. Also, in mutant FAJ1808, a truncated calsymin protein lacking part of the C terminus is still efficiently secreted.

Calcium has been implicated in a number of symbiosome functions, arguing for an important role of this ion in this compartment (33, 34). Complexation of Ca^{2+} by calsymin could have a direct or indirect effect on Ca^{2+}-dependent processes in the plant. The identified gene may therefore constitute a valuable tool to study the communication between plant and bacteria at the late stages of symbiosis.

We thank Dr. W. Broekaert, Dr. J. Cox, and Dr. F. Wuytack for the critical reading of the manuscript and their valuable comments, and Dr. P. Proost for the sequence analysis of the N terminus of calsymin. C.X. is a recipient of a doctoral scholarship from the Research Council of the Katholieke Universiteit Leuven. J.M. and E.S. are postdoctoral fellows of the Fund for Scientific Research–Flanders.

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