A Chemotaxis Receptor Modulates Nodulation during the *Azorhizobium caulinodans*-*Sesbania rostrata* Symbiosis

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ABSTRACT

Azorhizobium caulinodans ORS571 is a free-living nitrogen-fixing bacterium which can induce nitrogen-fixing nodules both on the root and the stem of its legume host *Sesbania rostrata*. This bacterium, which is an obligate aerobe that moves by means of a polar flagellum, possesses a single chemotaxis signal transduction pathway. The objective of this work was to examine the role that chemotaxis and aerotaxis play in the lifestyle of the bacterium in free-living and symbiotic conditions. In bacterial chemotaxis, chemoreceptors sense environmental changes and transmit this information to the chemotactic machinery to guide motile bacteria to preferred niches. Here, we characterized a chemoreceptor of *A. caulinodans* containing an N-terminal PAS domain, named IcpB. IcpB is a soluble heme-binding protein that localized at the cell poles. An *icpB* mutant strain was impaired in sensing oxygen gradients and in chemotaxis response to organic acids. Compared to the wild-type strain, the *icpB* mutant strain was also affected in the production of extracellular polysaccharides and impaired in flocculation. When inoculated alone, the *icpB* mutant induced nodules on *S. rostrata*, but the nodules formed were smaller and had reduced N₂-fixing activity. The *icpB* mutant failed to nodulate its host when inoculated competitively with the wild-type strain. Together, the results identify chemotaxis and sensing of oxygen by IcpB as key regulators of the *A. caulinodans*-*S. rostrata* symbiosis.

IMPORTANCE

Bacterial chemotaxis has been implicated in the establishment of various plant-microbe associations, including that of rhizobial symbionts with their legume host. The exact signal(s) detected by the motile bacteria that guide them to their plant hosts remain poorly characterized. *Azorhizobium caulinodans* ORS571 is a diazotroph that is a motile and chemotactic rhizobial symbiont of *Sesbania rostrata*, where it forms nitrogen-fixing nodules on both the roots and the stems of the legume host. We identify here a chemotaxis receptor sensing oxygen in *A. caulinodans* that is critical for nodulation and nitrogen fixation on the stems and roots of *S. rostrata*. These results identify oxygen sensing and chemotaxis as key regulators of the *A. caulinodans*-*S. rostrata* symbiosis.

Chemotaxis is a stimulated process enabling motile bacterial species to detect chemical gradients and to move in a beneficial direction. The bacterial chemotactic system of *Escherichia coli* is so far the best understood. This bacterium possesses four attractant-specific transmembrane chemoreceptors, named methyl-accepting chemotaxis proteins (MCPs) (1), as well as a fifth chemoreceptor, Aer, which contains an N-terminal Per-Arnt-Sim (PAS) domain that binds a flavin adenine dinucleotide (FAD) cofactor to sense redox changes (2, 3). The chemoreceptors convey sensory information to the flagellar motors across a complex signal transduction pathway encompassing six soluble chemotaxis proteins (named CheA, CheB, CheR, CheY, CheW, and CheZ) (4, 5). All chemotaxis receptors have highly similar cytoplasmic domains that are essential for the formation of ternary signaling complexes with the histidine kinase CheA and the adaptor protein CheW. These signaling complexes are large molecular ultrastructures that can be seen at the cell poles by fluorescent labeling of chemotaxis proteins and of chemoreceptors (6).

Chemoreceptors are functional signaling proteins located at the input end of the signaling pathway. They detect specific effectors with high specificity and transduce chemotactic signals to the downstream proteins (7, 8). Although membrane-bound chemoreceptors represent the largest class of chemotaxis receptors found in bacterial genomes (9, 10), soluble cytoplasmic chemoreceptors

are also broadly distributed (11). Soluble chemotaxis receptors either appear to localize with other receptors at the cell poles (12), or they can localize as separate cytoplasmic clusters (13).

Azorhizobium caulinodans ORS571 is a symbiont of the aquatic tropical legume *Sesbania rostrata*. *A. caulinodans* is capable of inducing nodule formation on the roots, as well as at stem-located root primordia of the host plant (14, 15). In addition to nitrogen fixation in roots and stem nodules, *A. caulinodans* ORS571 is capable of fixing nitrogen in the free-living state, providing it can locate conditions where oxygen concentrations are very low (14). Chemotaxis plays a key role in the establishment of symbiotic relationships of diverse bacteria with plants (16, 17), but its role in the *A. caulinodans*-*S. rostrata* symbiosis has not been investigated.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5α	F [−] <i>supE44 ΔlacU169</i> (φ80 <i>lacZΔM15</i>) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Transgen
BL21	<i>E. coli</i> B F [−] <i>ompT hsdS</i> (r _B [−] m _B [−]) <i>dcm</i> ⁺ Tet ^r gal λ(DE3) <i>endA</i> Hte [<i>argU proL</i> Cm ^r]	Transgen
<i>A. caulinodans</i>		
ORS571	Type strain	15
AC001	ORS571 derivative; Δ <i>cheA</i>	This study
AC301	ORS571 derivative; Δ <i>icpB</i>	This study
AC302	AC301 derivative, complemented <i>icpB</i> ; Tc ^r	This study
AC303	AC301 derivative, contained mutant plasmid pLAI <i>icpB</i> ^{H154A} ; Tc ^r	This study
Plasmids		
pK18 <i>mobsacB</i>	Suicide vector for gene disruption; <i>lacZ mob sacB</i> Km ^r	21
pLAFR3	IncP broad-host-range cosmid; Tc ^r	23
pRK2013	Helper plasmid, ColE1 replicon; Tra ⁺ Km ^r	22
pET30a	General expression vector, His tag; <i>lacI</i> Km ^r	Novagen
pUC19-GFP	Amp ^r ; source of the GFP gene	This laboratory
pPR9TT	9,388-bp broad-host-range plasmid carrying promoterless <i>lacZ</i> without ATG; Amp ^r Cm ^r	25
pIG3718	pPR9TT expressing IcpB-GFP with 736-bp upstream promoter of <i>icpB</i> ; Amp ^r Cm ^r	This study
pLAI <i>icpB</i>	pLAFR3 with <i>icpB</i> open reading frame and 736-bp upstream promoter region; Tc ^r	This study
pLAI <i>icpB</i> ^{H154A}	pLAI <i>icpB</i> carried H154A substitution	This study
pIN1	pET-30a derivative expressing PAS domain of IcpB attached to an N-terminal His ₆ -SUMO tag	This study
pIN2	pIN1 derivative with the histidine at position 154 mutated to alanine	This study

^a Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance.

In the present study, we characterize a PAS-containing chemoreceptor in *A. caulinodans* that we named IcpB (internal chemotaxis protein B) and show that IcpB senses oxygen via a heme-bound cofactor and that it modulates aerotaxis and chemotaxis. We also provide evidence that supports a critical role for IcpB in the establishment of a functional symbiosis between *A. caulinodans* and its host plant.

MATERIALS AND METHODS

Media, bacterial strains, and growth conditions. The bacterial strains and plasmids are listed in Table 1. *A. caulinodans* ORS571 and its derivatives were grown at 37°C in TY medium (10 g/liter tryptone, 5 g/liter yeast extract, and 4 g/liter CaCl₂·2H₂O) (18) or in L3 minimal medium (10 mM KH₂PO₄, 10 mg/ml D,L-sodium lactate, 100 μg/ml MgSO₄·7H₂O, 50 μg/ml NaCl, 40 μg/ml CaCl₂·2H₂O, 5.4 μg/ml FeCl₃·6H₂O, 5 μg/ml Na₂MoO₄·2H₂O, 2 μg/ml biotin, 4 μg/ml nicotinic acid, and 4 μg/ml pantothenic acid) (19), which was either supplemented with 10 mM NH₄Cl (L3+N medium) or lacked any nitrogen source (L3−N medium). When indicated in the text, sodium lactate was replaced with other carbon sources as the sole carbon source in L3 medium. The growth medium of *A. caulinodans* was supplemented with ampicillin (final concentration, 100 μg/ml) and nalidixic acid (final concentration, 25 μg/ml).

Behavioral assays. The soft agar plate and temporal gradient assays for chemotaxis in *A. caulinodans* were performed essentially as previously described (24), with some modifications. For the soft agar assay, cells were grown to mid-log phase in TY medium, washed, and resuspended in chemotaxis buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, 0.1 mM EDTA [pH 7.0]) to an optical density at 600 nm (OD₆₀₀) of ~0.6. Aliquots of 5 μl of this bacterial suspension were inoculated at the center of L3 minimal soft agar plates solidified with 0.3% agar and containing different carbon sources added at a final concentration of 10 mM. The inoculated soft agar plates were incubated for 3 to 5 days at 37°C before being photographed.

The temporal assay for aerotaxis was essentially carried out according to the method described by Greer-Phillips and coworkers (17). A 10-μl drop of bacterial suspension adjusted to an OD₆₀₀ of 0.2 was placed on a microscope slide, inside a microchamber that was ventilated with humid-

ified N₂ or air gas (flow rate, 800 ml min^{−1}). The cell suspension was equilibrated with air for 2 min. After that, the ventilating gas was switched to N₂ for 1 to 3 min and then changed to air again by means of a controlling gas valve. The motion of bacteria was digitally recorded using Cellsens Dimension 1.7 (Olympus Corp.). The time it took for swimming bacteria to return to a prestimulus swimming pattern after stimulation was determined by measuring the average reversal frequency (RF) of free-swimming cells, using CellTrak 1.1 (Motion Analysis Corp., Santa Rosa, CA). The removal of air caused a transient increase in the RF, and the addition of air caused a transient decrease in the RF. Experiments were performed three times, with a minimum of six replicates per sample.

Flocculation assay. Flocculation was estimated using the method described by Burdman et al. (20) with the following modifications. Overnight cultures in liquid TY medium were normalized to an OD₆₀₀ of 1.0, and 200 μl was inoculated into 10 ml of L3 medium added to a 40-ml conical sterile tube. These conical tubes were incubated vertically in a rotary shaker (180 rpm) at 37°C. After incubation for 24 and 48 h, the tubes were removed from the shaker and left standing for 30 min. After this period, flocculated cells had settled to the bottom of the tube, while the nonflocculated cells remained in suspension. The turbidity of the supernatant (OD_s) and the total turbidity (OD_t) of the culture obtained after mechanical dispersion of the flocs by treatment in a tissue homogenizer were measured by spectrophotometry as OD₆₀₀. The percentage of flocculation was calculated as follows: % flocculation = [(OD_t − OD_s) × 100]/OD_t. The experiment was carried out three times, with three replicates per sample.

Construction of the mutants and complemented strains. To construct the *icpB* mutant, a 736-bp upstream fragment (UF) and an 807-bp downstream fragment (DF) of the *icpB* gene were amplified by PCR by using two primer pairs, *icpB*UF-*icpB*UR and *icpB*DF-*icpB*DR (Table 2). The amplicons were digested with appropriate restriction enzymes (UF, BamHI and EcoRI; DF, EcoRI and XbaI) before linking them together to generate a BamHI-XbaI fragment. The DNA fragment obtained was inserted into the suicide vector pK18*mobsacB* digested with BamHI and XbaI (21). This construct was introduced into the wild-type strain by triparental conjugation for allelic exchange, as described previously (22).

TABLE 2 PCR primers used in this study

Primer	Sequence (5'–3') ^a
icpB _{UF} BamHI	CGCGGATCCCTTGAACCAACCGTAGCT
icpB _{URE} EcoRI	CGGAATTCTCGGCAGGCACCGCCGA
icpB _{DF} EcoRI	CGGAATTCATCGCGCAGGCGGATTGG
icpB _{DR} XbaI	GCTCTAGAAATCTCGTCTCGGTCTCAAG
icpB _{comF} EcoRI	CCGGAATTCGGTCTGTTGGAAGGCGAAG
icpB _{comR} HindIII	CCCAAGCTTTCAGGACTGCGTGCAGG
cheA _{UF} EcoRI	GCGGAATTCATCTCGGCTCAGGTTTCC
cheA _{UR} BamHI	CAAGGATCCCGGAACCTGTCCATCAGCG
cheA _{DF} BamHI	CGAGGATCCTAAGGAATTCGCCGCCA
cheA _{DR} XbaI	CGCTCTAGACCTCTCTTGAATTCGAG
pasF _{Bgl} III	GGAAGATCTGAATCCTCGGATCTCGCG
pasR XhoI	CCGCTCGAGTCAGGCGACGCTGTTTCATGTC
SDM _{pasF}	TTCGAGGTGATCGCCGCCGTGCGACCCCTTACC
SDM _{pasR}	GGTGAAGGGTCGCACGGCGCGATCACCTCGAA
GicpF _{HindIII}	CCGAAGCTTCGGTCTGGAAGGCGAAG
GicpR EcoRI	CGGAATTCGGACTGCGTGCAGGTTG
GfpF EcoRI	CGGAATTCATGAGTAAAGGAGGAGGA
GfpR XbaI	CGTCTAGATAATTTGTATAGTTCATCC

^a Engineered restriction sites are underlined.

Homologous recombinants lacking the *icpB* gene were recovered on TY plates containing 10% sucrose, and correct recombination was verified by PCR. One resulting mutant strain was named AC301 (Table 1) and used in subsequent experiments.

To construct a mutant lacking a functional *cheA* gene, a 766-bp UF and a 545-bp DF of *cheA* were amplified by PCR using two primer pairs, cheA_{UF}-cheA_{UR} and cheA_{DF}-cheA_{DR}, respectively (Table 2). The UF was digested with EcoRI and BamHI and the DF was digested with BamHI and XbaI, followed by ligating the two fragments at their BamHI sites. The integrated fragment was then cloned into the suicide vector pK18*mobsacB*. Allelic exchange and positive recombinant selection were carried out as described for the *icpB* mutant construction above. Such a *cheA* mutant strain was named AC001 (Table 1).

In order to complement the *icpB* mutant strain AC301, a fragment encompassing the 738-bp region upstream of the *icpB* gene and the intact open reading frame for IcpB were amplified by PCR using the primer pair icpB_{comF}-icpB_{comR} (Table 2). The amplified fragment was cloned into the EcoRI and HindIII sites of the broad-host-range vector pLAFR3 (23), and the DNA sequence was verified by sequencing. The resulting plasmid was introduced into AC301 via triparental mating, selecting for tetracycline resistance. One such resulting strain was named AC302 (Table 1).

Site-directed mutagenesis. We replaced the conserved histidine residue at position 154 of the PAS domain of IcpB with alanine using site-directed mutagenesis. A 738-bp region immediately upstream of the *icpB* gene and including 472 bp from the predicted ATG start codon was amplified with the primer pair icpB_{comF}EcoRI and SDM_{pasR}. A 951-bp region beginning from the end of *icpB* was amplified with the primer pair SDM_{pasF} and icpB_{comR}HindIII. The *icpB* fragment containing the desired site-directed replacement was generated by a two-step, overlap PCR procedure (24). After verification by sequencing, the fragment was cloned into the pLAFR3 vector at appropriate restriction sites, yielding pLAIcpB^{H154A}. Using the same method, the primer pairs pasF_{Bgl}III-SDM_{pasR} and SDM_{pasF}-pasRXhoI (Table 2) were used to amplify the PAS fragment containing the H154A mutagenesis cloning into the expression vector pET-30a and create pIN2. Both candidate plasmids were verified by sequencing before being transferred into AC301 or *E. coli* BL21 by triparental mating and chemical transformation, respectively.

Generation of IcpB-GFP fusions and fluorescence microscopy. The broad-host-range plasmid pPR9TT (25) was used as the expression vector for fusing the gene coding for IcpB with the gene encoding green fluorescent protein (GFP) in frame, to generate an IcpB-GFP chimeric protein. A 2,130-bp DNA fragment, including the *icpB* open reading frame but lack-

ing the stop codon and 736 bp of the 5' sequence upstream of the *icpB* translational start, was amplified by PCR using the primers GicpF_{HindIII} and GicpR_{EcoRI} (Table 2). The GFP gene was amplified from pUC19-GFP using the primers set GfpF_{EcoRI} and GfpR_{XbaI} (Table 2). These two amplicons were then cloned into pPR9TT to yield pIG3718, which was verified by sequencing. *E. coli* DH5 α competent cells were transformed with pIG3718 and used as donors for triparental mating experiments with *A. caulinodans* derivatives. Fluorescent images were acquired with a Leica DM5000B fluorescence microscope (Wetzlar, Germany) and Leica Application Suite version 4.3 (Leica Microsystems, Switzerland) at $\times 100$ magnification. Fluorescence signals from GFP (excitation at 488 nm) were detected using a 525- to 550-nm band-pass filter.

Quantification of biofilm formation. Biofilm formation was assayed using crystal violet (CV) staining essentially as described previously (26). Microtiter plates were filled with bacterial suspensions, in L3+N or L3-N medium, adjusted to an OD₆₀₀ of 1.0. After inoculation, plates were incubated at 37°C for 3 days. After staining of the biofilms with CV, 1 ml of 95% ethanol was added to each well of the microplate to dissolve the CV-stained biofilms. The absorbance at OD₅₉₅ was measured to determine the amount of CV-stained biofilm recovered by using a microplate reader (Tecan Infinite M200). The experiment was repeated three times, with six replicates per sample.

Quantification of EPSs. For qualitative evaluation of changes in exopolysaccharide (EPS) production, L3-grown cells were inoculated as 5- μ l drops onto solid L3 plates containing Congo red (40 μ g/ml) and supplemented with a nitrogen source (citric acid) or without any combined nitrogen (nitrogen fixation conditions). The plates were allowed to grow at 37°C for 3 days before being photographed. Quantification of EPS production was performed as described by Nakajima et al. (19). Supernatants containing the EPS soluble fraction were first treated with 1 ml of concentrated sulfuric acid containing 0.2% anthrone, mixed, and incubated for 7 min at 100°C before being quickly chilled on ice. The OD₆₂₀ of the chilled mixture was measured. D-Glucose was used to prepare a standard curve. The EPS concentration of the samples was evaluated by normalizing to the OD₆₀₀ of the collected cell suspension.

Protein expression and purification. The DNA corresponding to the PAS domain fragment (residues 50 to 177 of IcpB; see Fig. 2A) was amplified from the ORS571 genomic DNA using the primers pasF_{Bgl}III and pasRXhoI and then cloned into the BglIII and XhoI sites of pET-30a (Novagen) with an engineered N-terminal His₆-SUMO tag to create pIN1. The protein was overexpressed from the pET-30a-derived plasmid in *E. coli* BL21 cells by induction with 100 μ M IPTG and incubation on a rotary shaker at 37°C for 5 h. After sonication, the cells were centrifuged for 1 h (13,000 rpm) at low temperature to isolate the soluble proteins in supernatant. The His₆-SUMO-tagged fusion proteins (the wild-type protein expressed from pIN1 and the mutant protein expressed from pIN2) were purified using Ni-nitrilotriacetic acid (Novagen) and eluted with a buffer containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 250 mM imidazole. The concentration of the eluted proteins was determined using NanoDrop 2000c (Thermo). Spectrophotometric assays were conducted for heme detection in NanoDrop 2000c at room temperature. Absorbance spectra between 350 and 650 nm were recorded by scanning 100 μ g of the purified proteins dissolved in 1 ml of cleavage reaction buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 250 mM imidazole). Deoxygenation was achieved by the addition of a few grains of sodium dithionite (Na₂S₂O₄) to 1 ml of each protein solution before recording a new absorption spectrum.

Plant growth and bacterial inoculation. *S. rostrata* seeds were surface sterilized by treatment with concentrated sulfuric acid for 20 min, followed by three washes with sterile water. All seeds were germinated in sterile trays in the dark at 37°C for 48 to 72 h. Germinated seeds were planted in vermiculite moisturized with a low-N nutrient solution in Leonard jars (27). *A. caulinodans* cells were grown overnight in TY liquid medium to an OD₆₀₀ of 0.8 to 1.0, and 1 ml of bacterial culture was inoculated per plant. For stem nodules, a bacterial culture adjusted at an

OD₆₀₀ of 0.8 was used for inoculating onto the stems of plants 2 weeks after transplantation in vermiculite. All plants were grown at 26°C in a greenhouse, with a daylight illumination period of 12 h. Nodules were harvested at 28 days postinoculation.

Nodulation competition assays were carried out according to Yost et al. (28). Briefly, surface-sterilized seedlings were coinoculated with parental strain ORS571 plus the *icpB* mutant strain or plus the complemented strain in 1:1 and 1:10 ratios. The correct proportion of wild-type to mutant strain was confirmed by viable plate counts on the inocula. Bacteria were reisolated from surface-sterilized nodules after 5 to 6 weeks of plant growth and identified by PCR amplification of the *icpB* gene. For each competition experiment, at least 100 nodules were crushed and plated.

ARA assays. Free-living acetylene reduction activity (ARA) was determined by cultivating bacterial cells in 3 ml of L3–N medium containing 0.3% agar in sealed test tubes (5 ml). Then, 200 µl of 10% (vol/vol) acetylene was added 8 h after bacterial inoculation. After 4 h of incubation at 37°C, 100 µl of the gas phase was analyzed by gas chromatography (Agilent Technologies, 7890A). Nitrogenase activity was expressed as nmol of C₂H₄ produced h⁻¹ mg of protein⁻¹. Protein concentrations were determined using the BSA protein assay (Bio-Rad) according to the manufacturer's instructions.

To measure the symbiotic ARA, 10 root nodules per plant were harvested and placed into a 20-ml tube sealed with a butyl rubber septum. Then, 2 ml of 10% (vol/vol) acetylene was added to each tube, and the harvested root nodules were incubated in the tubes at 37°C for 3 h. After incubation, 100 µl of the gas phase was sampled from the tubes and the concentration of ethylene was determined by gas chromatography. The nitrogenase activity is expressed as µmol of C₂H₄ produced h⁻¹ g of fresh nodules⁻¹.

Bioinformatic analysis. Chemotaxis genes and proteins present in the *A. caulinodans* ORS571 genome were identified in the MIST2 database by using key words such as “MCP” to identify chemotaxis receptors, “CheA” for chemotaxis proteins, etc. (http://www.mistdb.com/bacterial_genomes/summary/951) (29). Protein domains were predicted by using Pfam (<http://pfam.janelia.org/>) (30). Amino acid sequences of selected proteins were aligned by using MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>) (31).

Statistical analysis. Statistical analyses for behavioral assays, expression assays and nodulation competition experiments were performed using GraphPad (Prism 5.0). A Student *t* test assuming equal variances (*P* < 0.05) was used to determine significant differences between conditions. A chi-square test was used to determine whether there were significant differences between inoculation and recovery ratios (*P* < 0.001 and *P* < 0.05 were tested).

RESULTS

Chemoreceptor genes in the *A. caulinodans* ORS571 genome.

The versatile lifestyle of *A. caulinodans* combined with its chemotactic abilities prompted us to analyze its complete genome sequence to search for chemotaxis receptors that could contribute to such a lifestyle. We used the MiST2 database as described in Materials and Methods to identify all chemotaxis receptors encoded in the genome of *A. caulinodans* ORS571 (GenBank accession no. AP009384.1). Nitrogenase is extremely sensitive to oxygen, which can rapidly inactivate its activity (32). Free-living bacteria with an aerobic metabolism must thus be able to locate oxygen tensions compatible with the functioning of the enzyme. Given previous work on the role of an aerotaxis soluble receptor in a diazotrophic bacterium (33) and the role of PAS domain-containing proteins in regulation of nitrogen fixation in soil bacteria (11), we hypothesized that soluble chemoreceptors with PAS domains could mediate a similar lifestyle in *A. caulinodans*. Of the 43 chemoreceptors we detected in the genome, six were predicted to be soluble, and among them, five possessed one or two PAS domains at their N

FIG 1 DNA region encompassing the *icpB* gene (top) and the protein domains found in IcpB (bottom). The *icpB* gene (AZC_3718, 1,395 bp) is flanked by *sppA* (AZC_3717) and *rps1* (AZC_3719), which are predicted to encode a signal peptide peptidase and a small ribosomal protein, respectively. The arrows indicate the direction of transcription. Domains of the IcpB protein were predicted by the Pfam database. The predicted protein has no transmembrane domains and contains a heme pocket in the PAS domain (MA, methyl-accepting chemotaxis-like domain).

terminus (AZC_0573, AZC_1026, AZC_1546, AZC_3153, and AZC_3718). Of the five PAS-domain containing soluble chemotaxis receptors, IcpB (AZC_3718) was the only one that possessed a single N-terminal PAS domain. Furthermore, the IcpB PAS domain was predicted to contain a putative heme-binding pocket, suggesting that it could sense oxygen, which prompted us to select IcpB for further characterization in the present study (Fig. 1).

The IcpB PAS domain binds heme. The IcpB PAS domain is predicted to bind heme (Fig. 1). To further test this hypothesis, we constructed a plasmid (pIN1) to recombinantly express the N-terminal complete PAS domain of IcpB in frame with an N-terminal poly-His–SUMO tag to facilitate recombinant protein purification. After overexpression and purification of the protein to homogeneity (Fig. 2B), we analyzed the UV-Vis spectrum of the recombinant protein. This spectrophotometric analysis confirmed that the IcpB N-terminal PAS domain possessed an absorption spectrum typical of oxygen-bound heme proteins, which are characterized by the presence of a Soret band at 401 nm and weak bands at 485 and 615 nm (Fig. 2C). We further confirmed the presence of heme by repeating the UV-Vis spectral analysis after the addition of an excess of sodium dithionite, which is expected to completely reduce a ferric (and thus heme-containing) protein. As expected for a heme-bound protein, this treatment resulted in shifts of Soret, α , and β bands at 414, 556, and 530 nm, respectively, confirming that the N-terminal PAS domain of IcpB binds a heme cofactor (Fig. 2C).

In PAS domains, hemes are typically coordinated by conserved histidine residues (34). The PAS domain of IcpB contains only two histidine residues at positions 154 and 165. To identify which of these two histidine residues may be involved in heme binding, we aligned the protein sequence of the PAS domain of IcpB with those of a few well-characterized and related heme-bound protein domains previously shown to be implicated in O₂ sensing (35) (Fig. 2A). As shown in Fig. 2A, the histidine residue at position 154 in the IcpB PAS domain is the only histidine residue that is strictly conserved among the selected aligned sequences. To confirm the role of His154 in heme binding, we substituted alanine for histidine at the 154 position of the protein and recombinantly expressed the corresponding variant protein. As expected, the characteristic absorption peak (Soret band at 401 nm) was absent from the UV-Vis spectrum, implicating His154 as the residue respon-

FIG 2 IcpB PAS sequence alignment, purification and electronic absorption spectra. (A) Sequence alignment of the IcpB PAS domain with PAS domains of related proteins. Conserved residues are shown in boldface, and the proximal histidine residue required for heme binding is indicated by an asterisk. Abbreviations: Bs-HemAT, *Bacillus subtilis* HemAT (GI 505065322); Gs-GCS, *Geobacter sulfurreducens* GCS (GI 499246383); Ec-YddV, *Escherichia coli* YddV (GI 902634910); Av-Greg, *A. vinelandii* (GI 502027541). (B) Coomassie blue-stained SDS-PAGE gel of purified IcpB PAS protein (residues 50 to 177, 14 kDa). (C) Optical absorption spectra of purified IcpB PAS protein in the reduced (Fe²⁺) or oxidized (Fe³⁺) state. The inset shows an enlarged view of peaks between 450 and 650 nm. (D) Optical absorption spectrum of the purified PAS domain of IcpB with the H154A substitution.

sible for heme binding (Fig. 2D). This finding further suggests that the heme-binding PAS domain of IcpB confers oxygen binding/sensing ability to this chemoreceptor.

The *icpB* mutant is impaired in chemotaxis and aerotaxis. We constructed an *icpB* deletion mutant (AC301) and characterized its role in taxis responses using qualitative and quantitative behavioral assays. Chemotaxis to various carbon sources known to be attractants for rhizobacteria was tested on soft agar plates supplemented with or without ammonium as the nitrogen source to compare chemotaxis under nitrogen-replete and nitrogen fixation conditions (Fig. 3). Compared to the wild type, the AC301 strain lacking a functional IcpB chemoreceptor was significantly impaired in chemotaxis to all carbon sources tested, regardless of the presence of a source of combined nitrogen in the medium (Fig. 3A and B), with the exception of chemotaxis to galactose that did not seem to be affected when tested under conditions of nitrogen fixation (Fig. 3B). We also noted that the *icpB* mutant strain chemotaxis defect was greater in the presence of malate, glucose, and glycerol when cells were grown under nitrogen fixation conditions than under nitrogen-replete conditions (Fig. 3B). This could suggest that the contribution of IcpB to chemotaxis toward these rapidly oxidizable substrates varies with growth conditions, notably, with nitrogen availability. The chemotactic ability of the *icpB* mutant complemented with a plasmid carrying the parental IcpB or its IcpB^{H154A} variant (AC302 and AC303) was also assayed under similar conditions. The chemotaxis defects could be rescued

by expressing the parental *icpB* from a broad-host-range plasmid under both conditions (Fig. 3A and B), but expression of the IcpB^{H154A} failed to restore chemotaxis abilities to the AC301 strain (Fig. 3C), indicating that heme binding to the PAS domain of IcpB is essential for chemotaxis under these conditions.

Next, we directly tested the role of IcpB as an oxygen sensor using a temporal assay for aerotaxis. In this assay and using strains grown under nitrogen fixation conditions, the response time of the *icpB* mutant (AC301) cells to the removal or addition of air was much shorter than that of the wild-type strain (Table 3). This defective behavior could be complemented by the IcpB (AC302) but not the mutated IcpB carrying an H154A (AC303) substitution. No obvious difference between the mutant and the wild type was observed when cells were grown in the presence of ammonium. Taken together, these results indicate that IcpB functions in aerotaxis and has a major role under nitrogen fixation conditions.

Subcellular localization of IcpB using a fusion to GFP (IcpB-GFP). To visualize the subcellular localization of IcpB *in vivo*, we expressed GFP fused to the C-terminal region of IcpB (IcpB-GFP) under the control of the upstream promoter of the *icpB* gene (pIG3718). The subcellular localization of IcpB in *A. caulinodans* and its derivative cells was assessed by fluorescence microscopy. IcpB-GFP localized to the cell poles in *A. caulinodans*, but it failed to localize in a mutant lacking the sole *cheA* gene in the genome (strain AC001) (Fig. 4A), indicating that the localization of IcpB depended on the presence of CheA. The IcpB-GFP chimeric pro-

FIG 3 Comparison of chemotactic behavior between the wild-type strain, the *icpB* mutant (AC301), and the *icpB* complemented mutant (AC302). (A) Swimming plates containing ammonium. (B) Swimming plates without nitrogen source. The percentages of the chemotactic ring diameters of the mutants relative to that of the wild-type strain were measured after 72 h of incubation at 37°C. Representative soft agar plates for each strain and condition are shown on the right. (C) Chemotactic ring of the wild-type *A. caulinodans* and the *icpB* mutant (AC301) carrying an empty pLARF3 vector (controls) or complemented with wild-type *IcpB* (AC302) or *IcpB* containing the H154A point mutation expressed from its own promoter on pLARF3 (AC303). The soft agar plates contained malate as the carbon source and ammonium chloride as the source of combined nitrogen. In all panels, error bars indicate standard errors calculated from at least six repetitions. Asterisks indicate values significantly different from the wild type ($P < 0.05$) using the Student *t* test.

tein was functional in chemotaxis since it could complement the chemotaxis defect of the mutant strain (data not shown). This result suggests that the localization of *IcpB*-GFP detected here as foci at the cell poles corresponds to chemotaxis signaling complexes and further implies that *IcpB*-GFP localizes with other chemoreceptors. In addition, the fluorescence of the polar foci was qualitatively (Fig. 4A) and quantitatively (Fig. 4B) reduced when cells were grown in the presence of ammonium compared to growth under conditions of nitrogen fixation. These observations

are consistent with the greater chemotaxis defects of the *icpB* mutant when tested under conditions of nitrogen fixation and indicate that the contribution of *IcpB* to chemotaxis is greater under these conditions.

The *icpB* mutation impairs flocculation and biofilm formation. *A. caulinodans* ORS571 is capable of flocculation under conditions of growth at high aeration in minimal medium (19). Chemotaxis receptors and proteins have been previously implicated in the regulation of cell-cell aggregation (36) and biofilm formation

TABLE 3 Role of IcpB in aerotaxis in *A. caulinodans*

Growth condition and strain	Response time (s) to adaptation $\pm$ SD in a temporal assay for aerotaxis ^a	
	Without air	With air
Ammonium		
ORS571	62.6 $\pm$ 4.1	57.2 $\pm$ 2.0
AC301	69.9 $\pm$ 1.2	61.8 $\pm$ 4.0
AC302	62.6 $\pm$ 1.3	58.8 $\pm$ 1.7
AC303	69.2 $\pm$ 3.3	62.5 $\pm$ 2.1
Nitrogen fixation		
ORS571	72.6 $\pm$ 2.2	62.6 $\pm$ 2.1
AC301	48.9 $\pm$ 1.4*	40.6 $\pm$ 1.5*
AC302	70.0 $\pm$ 3.2	62.4 $\pm$ 1.0
AC303	47.2 $\pm$ 3.6*	41.7 $\pm$ 2.3*

^a Cells were grown in minimal medium containing 10 mM chemical to be tested as the sole carbon and energy source. *, statistically significant difference ($P < 0.05$).

in diverse bacteria (37). Given the obligately aerobic metabolism of *A. caulinodans*, we hypothesized that IcpB may affect cell-cell aggregation and cell-surface interactions. To test this hypothesis, we compared the ability to flocculate between the wild-type strain

FIG 4 IcpB-GFP localization in *A. caulinodans*. (A) Fluorescence micrographs of strain ORS571 derivatives in different culture conditions. Left panel, bacteria grown with ammonium; right panel, nitrogen fixation condition. In each panel, representative differential interference contrast (DIC) and fluorescence images, respectively, are shown. (B) The fluorescence intensity of IcpB-GFP in wild-type *A. caulinodans* and the *icpB* mutant at the polar foci was analyzed using ImageJ (a.u., arbitrary units). The error bars represent the standard deviations from the means. WT, wild type.

FIG 5 Surface properties of the *A. caulinodans* wild type and its *icpB* mutant strain. (A) Percent flocculation. The detailed measuring method is described in Materials and Methods. The error bars represent the standard deviations from the means. (B) Quantification of ethanol-solubilized CV from polyvinyl chloride (PVC) plate biofilms. OD₅₉₅ was recorded after 3 days of incubation. Biofilm was quantified using CV staining as described in Materials and Methods. Asterisks indicate significant differences between the wild-type strain and the *icpB* mutant. WT, wild type.

and the *icpB* mutant. We found that the *icpB* mutant initiated flocculation earlier than the wild type but yielded quantitatively similar amounts of flocculated cells after 48 h (Fig. 5A).

To determine whether IcpB was affected in biofilm formation, the wild type, the *icpB* mutant, and the complemented strains were compared for biofilm formation using an *in vitro* assay (Fig. 5B). The results showed that the *icpB* mutant strain produced more biofilm than the wild type and the complemented strains ($P < 0.05$). Together, the results suggest that lack of IcpB caused the cell to aggregate to other cells or abiotic surfaces at greater rates.

The *icpB* mutant has an increased production of EPS. The aggregation and biofilm formation phenotypes of the *icpB* mutant strain (AC301) prompted us to test if EPS production was affected by lack of IcpB function. EPS production of wild-type *A. caulinodans* was compared to that of the *icpB* mutant strain (AC301) by first using a qualitative assay based on the ability of colonies to bind Congo red (Fig. 6A). Dramatic differences in the appearance of colonies formed by the *icpB* mutant strain in comparison to the wild type were visible when cells were grown in medium lacking nitrogen and thus under conditions of nitrogen fixation (Fig. 6A). Such differences were not observed when cells were grown in the presence of ammonium (data not shown). The morphology of the colonies formed by the *icpB* mutant strain was drastically different from that of the wild type and complemented strains when grown under nitrogen fixation conditions, with the colonies formed by the *icpB* mutant having a “wet” appearance.

The quantitative assay for EPS production confirmed these qualitative observations: the *icpB* mutant (AC301) produced significantly more total EPS than did the other strains under nitrogen fixation conditions (Fig. 6B). The amounts of EPS produced by

FIG 6 The *icpB* mutant has an increased production of EPS. (A) Colony morphologies of *A. caulinodans* derivatives spotted on the L3–N Congo red plates. Photographs were taken after 3 days of incubation. There were distinct differences in the Congo red binding pattern produced by bacteria between the wild-type strain and the *icpB* mutant. (B) Quantitative analysis of the EPS. The extraction and quantification of EPS is described in Materials and Methods. The error bars indicate the standard deviations from the means for each sample. Asterisks represent statistically significant differences compared to the wild-type strain ($P < 0.05$). WT, wild type.

the wild type and the complemented strain (AC302) were similar and were almost half of that produced by the *icpB* mutant. Therefore, lack of IcpB correlates with changes in EPS production, which may explain the greater propensity for flocculation and biofilm formation of the mutant.

The *icpB* mutant is disadvantaged in symbiotic properties.

Rhizobial surface polysaccharides are necessary for plant-microbe symbiotic interactions and root invasion (38). Since the EPS content and biofilm formation of the *icpB* mutant (AC301) differed from those of the wild-type strain, we expected that it would cause defects in the ability of *A. caulinodans* to nodulate its host. To test this hypothesis, we compared the wild-type and *icpB* mutant (AC301) strains for nodulation of *S. rostrata* when inoculated alone or in competition with one another. The growth rate of the *icpB* mutant in the free-living state did not differ from that of the wild-type strain (data not shown), excluding that any effect on nodulation would directly result from defects in growth rates. As shown in Fig. 7A, the *icpB* mutant induced nodule formation on the roots and stems of its host plant with similar numbers of nodules formed. However, the morphology of nodules formed by the *icpB* mutant was different from that of nodules formed by the wild-type strain (Fig. 7A). The stem nodules formed by the *icpB* mutant (AC301) were smaller than those formed by the wild-type strain. Furthermore, the nodules induced by the *icpB* mutant had a pale inner region compared to the bright red color of the wild-type nodules (Fig. 7A). The pale color of the nodules induced by the *icpB* mutant suggested that they lacked sufficient leghemoglobin, which should also cause a defect in the rate of nitrogen fixation.

Consistent with the hypothesis, the measured ARA of nodules formed by the *icpB* mutant strain was significantly lower than that

FIG 7 Properties of bacteria in a symbiotic interaction with host. (A) Typical appearances of stem nodules induced by *A. caulinodans* ORS571 (left), *icpB* mutant AC301 (center), and complemented strain AC302 (right). Natural leghemoglobin (Lb) shows a characteristic orange-brown color. (B) Acetylene reduction activities (ARAs) of *A. caulinodans* ORS571, the *icpB* mutant AC301, and the complemented strain AC302 at the free-living state (left) and ARAs of root nodules induced by them (right). Data are the means of six replicates. Asterisks indicate significant difference from the wild type ($P < 0.05$). (C) Nodulation competition between *icpB* mutants and the parent strain. The *icpB* mutant was rarely recovered from the harvested nodules. Inoculation with suspensions containing a 10-fold excess of the *icpB* mutant compared to the wild type could not retrieve its capability of nodulation competition. The complemented strain AC302 ($\Delta icpB + icpB$) restored the ability to compete with the parent strain. Statistically significant ($P < 0.05$) differences between the inoculation ratio and recovery ratio in a chi-square test are indicated by asterisks. WT, wild type.

of nodules formed by the wild-type strain ORS571 (Fig. 7B). As expected from this impaired ability to form functional nodules, the *icpB* mutant was severely impaired in competitive nodulation with the parent strain ORS571 (Fig. 7C): the mutant strain was outcompeted by the wild-type strain even when the inoculum ratio between the *icpB* mutant and wild-type strain was increased to 10:1. The wild-type phenotype for nodulation was restored when the complementing plasmid (pLAIcpB) was introduced into the *icpB* mutant strain (Fig. 7C). Altogether, these data suggest that IcpB is required for effective nodulation of *S. rostrata* and critical for competitive nodulation.

DISCUSSION

In this study, we characterized the role of a soluble chemoreceptor of *A. caulinodans* in oxygen sensing during chemotaxis and nodulation of its host legume *S. rostrata*. We showed that IcpB binds heme in its PAS domain and functions to sense oxygen, with this ability being required for efficient chemotaxis and aerotaxis. PAS domains are the most prevalent sensing domains occurring in

cytoplasmic chemoreceptors, where they perform various functions (11). For example, *Geobacter sulfurreducens* utilizes heme-containing sensors to propagate signals under anaerobic conditions (39). The Aer-2 chemoreceptor of *Pseudomonas aeruginosa* also possesses a PAS domain that is sandwiched between three N-terminal and two C-terminal HAMP domains (40). Although the exact role of Aer-2 in *P. aeruginosa* remains unclear, it was able to mediate aerotaxis when expressed in *E. coli*, suggesting that it has a similar function in *P. aeruginosa*. Similar to *A. caulinodans* IcpB, *B. subtilis* senses oxygen directly using a heme-based aerotactic transducer, HemAT (41), but the HemAT heme is coordinated within a globin-coupled domain rather than a PAS domain. Similar to *A. caulinodans*, *Azospirillum brasilense* can fix N₂ under low-oxygen conditions, and it monitors conditions of low oxygen concentrations using AerC; however, the *A. brasilense* AerC chemoreceptor does not sense oxygen by binding this molecule but rather senses changes in intracellular redox via FAD cofactors present in each of two N-terminal PAS domains (33). In both diazotrophs, PAS-containing soluble chemoreceptors mediate the ability to locate low-oxygen-concentration conditions to support nitrogen fixation, but these species detect these conditions using different strategies, as reflected in the presence of different cofactors within the PAS domains of the receptors that guide these cells under diazotrophic conditions.

A. caulinodans, which belongs to the family *Xanthobacteraceae*, is taxonomically distant from the other rhizobia of the *Alphaproteobacteria* subgroup. Moreover, it differs by its ability to fix nitrogen in the free-living state, in addition to within nodules (42). The *icpB* mutant showed greater behavioral defects under nitrogen-limiting conditions than in the presence of ammonia (Fig. 3A and B and Table 3), and the IcpB chemoreceptor appeared to contribute most to aerotaxis and chemotaxis to oxidizable substrates under nitrogen fixation conditions. These results suggest that IcpB plays a major role under conditions of a low oxygen concentration. For chemotaxis to be observed in the soft agar plate assay, the inoculated cells must first grow to establish a concentration gradient of the chemical present as the sole carbon source, linking chemotaxis to growth in this assay. The defects in chemotaxis to oxidizable substrates thus strongly suggest that IcpB, which possesses an oxygen-sensing PAS domain, confers on *A. caulinodans* the ability to locate the best oxygen conditions in the soft agar to metabolize the carbon sources available. However, a direct role for IcpB in the chemotaxis response to carbon sources cannot be ruled out, although the sensory mechanism implicated in this case would remain to be established.

The production and composition of extracellular polysaccharide (EPS) is closely related to bacterial motility and chemotaxis (43). However, the relationship between chemotaxis and changes in EPS production is likely indirect. First, chemotaxis receptors signal to modulate flagellar motor activity and thus changes in the motility pattern (6). Second, changes in the motility pattern of several bacteria as a result of chemotaxis signaling modulate transient cell-cell interactions and cell-surface interactions, which indirectly and ultimately cause changes in EPS production (36). The results obtained here suggest that IcpB affected flocculation, biofilm formation, and EPS production by a similar mechanism. The following results support this hypothesis. First, IcpB functioned to regulate temporal responses to changes in oxygen concentrations in the cells' atmosphere and directly modulated the swimming pattern and chemotaxis responses (Table 3 and Fig. 3A and B).

Second, *A. caulinodans* is an obligate aerobe and fixes nitrogen under free-living conditions only under microaerobic conditions. Given the defect of IcpB in aerotaxis under nitrogen fixation conditions, it is likely that cells failed to locate optimum positions in oxygen gradients under nitrogen fixation conditions. Therefore, the increased production of EPS could be a compensatory response to this defect. Consistent with this hypothesis, the *icpB* mutant strain was impaired in nitrogen fixation under free-living conditions, despite the observation that it produced more EPS under these conditions. This hypothesis is also consistent with the precocious flocculation of the *icpB* mutant under conditions of high aeration since flocculation is induced by elevated aeration and limitation in combined nitrogen availability, which are conditions likely to represent a stress for the bacterium which will need to fix nitrogen (44).

The results obtained here not only show that a chemoreceptor is essential for competitive nodulation, as demonstrated for other rhizobial species (16, 45, 46), but also establish the role of aerotaxis mediated by IcpB in the formation of efficient nitrogen-fixing nodules induced by *A. caulinodans*. The *icpB* mutant formed nodules with a reduced leghemoglobin content and nitrogenase activity despite being able to produce more EPS and to form denser biofilms. There are several possibilities for the defective nitrogen-fixing phenotype of nodules formed by the *icpB* mutant: (i) the bacteria may be unable to reach the plant cortex cells and thus fail to form bacteroids in sufficient numbers (47, 48) or (ii) the production of EPS and/or lack of oxygen sensing in the *icpB* mutant may alter metabolism and bacteroid function during the developing stages of the nodules (49). A combination of these two possibilities cannot be excluded.

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