Stringent Expression Control of Pathogenic R-body Production in Legume Symbiont Azorhizobium caulinitodans

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ABSTRACT

R bodies are insoluble large polymers consisting of small proteins encoded by reb genes and are coiled into cylindrical structures in bacterial cells. They were first discovered in Caedibacter species, which are obligate endosymbionts of paramecia. Caedibacter confers a killer trait on the host paramecia. R-body-producing symbionts are released from their host paramecia and kill symbiont-free paramecia after ingestion. The roles of R bodies have not been explained in bacteria other than Caedibacter. Azorhizobium caulinitodans ORS571, a microsymbiont of the legume Sesbania rostrata, carries a reb operon containing four reb genes that are regulated by the repressor PraR. Herein, deletion of the praR gene resulted in R-body formation and death of host plant cells. The rebR gene in the reb operon encodes an activator. Three PraR binding sites and a RebR binding site are present in the promoter region of the reb operon. Expression analyses using strains with mutations within the PraR binding site and/or the RebR binding site revealed that PraR and RebR directly control the expression of the reb operon and that PraR dominantly represses reb expression. Furthermore, we found that the reb operon is highly expressed at low temperatures and that oxoglutarate induces the expression of the reb operon by inhibiting PraR binding to the reb promoter. We conclude that R bodies are toxic not only in paramecium symbiosis but also in relationships between other bacteria and eukaryotic cells and that R-body formation is controlled by environmental factors.

IMPORTANCE

Caedibacter species, which are obligate endosymbiotic bacteria of paramecia, produce R bodies, and R-body-producing endosymbionts that are released from their hosts are pathogenic to symbiont-free paramecia. Besides Caedibacter species, R bodies have also been observed in a few free-living bacteria, but the significance of R-body production in these bacteria is still unknown. Recent advances in genome sequencing technologies revealed that many Gram-negative bacteria possess reb genes encoding R-body components, and interestingly, many of them are animal and plant pathogens. Azorhizobium caulinitodans, a microsymbiont of the tropical legume Sesbania rostrata, also possesses reb genes. In this study, we demonstrate that A. caulinitodans has ability to kill the host plant cells by producing R bodies, suggesting that pathogenicity conferred by an R body might be universal in bacteria possessing reb genes. Furthermore, we provide the first insight into the molecular mechanism underlying the expression of R-body production in response to environmental factors, such as temperature and 2-oxoglutarate.

KEYWORDS

R body, legume, pathogenesis, reb gene, rhizobia, symbiosis
**R** bodies are bacterial inclusion bodies and are large proteinaceous ribbons that are coiled into cylindrical structures. R bodies were first observed in *Caedibacter* species, which are obligate endosymbiotic bacteria that inhabit paramecia (1). Paramecia that harbor R-body-producing *Caedibacter* cells are referred to as killer paramecia and release bacterial cells via the cytopyge. Subsequently, sensitive nonendosymbiotic paramecia are killed following ingestion of the released bacteria (2), conferring the “killer trait” of paramecia (3). R bodies play a major role in this trait, because paramecia harboring mutant *Caedibacter* strains that are defective in R body production do not express the killer trait (4).

The genes involved in R-body production were originally identified in *Caedibacter taeni spiralis* (5) and include rebA, rebB, rebC, and rebD (5, 6). Moreover, genes that are homologous to rebA, rebB, and rebD of *C. taeni spiralis* have been found in species of the phylum Proteobacteria and in *Kordia algicida* OT-1 (7), which belongs to the phylum Bacteroidetes, whereas no rebC-homologous genes have been identified in bacteria other than *C. taeni spiralis* (8, 9). It is hypothesized that reb-homologous genes were passed on by horizontal gene transfer—i.e., by phages or plasmids (2, 8, 10, 11). Although many bacterial species that carry reb-homologous genes are pathogenic to plants and animals (e.g., *Xanthomonas axonopodis* pv. *citri*, *Stenotrophomonas mal to phila*, *Burkholderia pseudomallei*, and so on) (8), the rhizobium *Azorhizobium caul inodans* ORS571, a mutualistic microsymbiont of the tropical legume *Sesbania rostrata*, possesses reb-homologous genes (8, 12). To date, reb-homologous genes have not been found in rhizobia other than *A. caul inodans* ORS571.

*A. caul inodans* ORS571 fixes atmospheric nitrogen in free-living and symbiotic states (13–15) and forms nitrogen-fixing nodules at the sites of adventitious root primordia on roots and stems. Bacteria enter stems via fissures at root primordia and colonize cortical infection pockets (16). From these infection pockets, infection threads guide the bacteria toward the nodule meristematic zone, where they are released into host cells and surrounded by plant-derived peribacteroid membranes (16). Subsequently, infected host cells are filled with differentiated bacteroids and infected areas enlarge with nodule maturation (15, 16).

The *A. caul inodans* ORS571 strain has a gene cluster containing four reb-homologous genes (8, 12) that are strongly suppressed by PraR, which is a conserved transcription factor among *Alphaproteobacteria* (8). In a previous study, stem nodules harboring *A. caul inodans* praR mutants that expressed high levels of reb genes were defective in nitrogen fixation (8). Furthermore, praR knockout in these nodules altered the interactions between these bacteria and their host cells according to two distinctly abnormal patterns. Specifically, host cells maintained normal shapes, and the bacteria disappeared with increasing host vacuolar sizes, or bacteria predominantly occupied host cells that had shrunken gradually (8). These observations suggest that derepression of reb genes at least partially reverts pathogenic traits for the symbiont. The regulatory mechanism underlying the expression of reb genes, however, is yet to be elucidated.

In *Caedibacter* spp., reb genes are responsible for the production of R bodies, which likely mediate eukaryotic cell death (4, 5). Although reb genes have been identified in many pathogenic bacteria, pathogenic roles of R bodies have not been directly demonstrated. On the other hand, observations of disordered intracellular symbiosis in plant hosts following derepression of reb genes in *A. caul inodans* suggest that *A. caul inodans* kills host cells by producing R bodies. Herein, we investigated the regulatory mechanism of reb gene expression in *A. caul inodans* and defined the roles of R bodies in reb-associated pathogenic traits, with particular emphasis on transcription repressor-activator interactions.

**RESULTS**

**Genetic organization of the reb operon.** The reb operon includes genes from AZC_3781 to AZC_3788 on the genome of *A. caul inodans* ORS571 (Fig. 1), and transcription units and start sites were determined using reverse transcription-PCR (RT-PCR) and primer extension analyses (see Fig. S1 in the supplemental material). A compre-
The comprehensive phylogenetic analysis revealed that the proteins encoded by reb genes in A. caulinodans did not belong to clusters, including RebA, RebB, and RebD of C. tae-niospiralis (see Fig. S2 in the supplemental material). Accordingly, the reb-homologous genes (AZC_3781, AZC_3782, AZC_3783, and AZC_3786) were renamed rebAZC1, rebAZC2, rebAZC3, and rebAZC4, respectively. An InterProScan analysis revealed that the AZC_3788 gene, which was designated rebR, encoded a putative transcription factor of the cyclic AMP receptor protein-fumarate and nitrate reduction regulator (Crp-Fnr) superfamily (17). The AZC_3781-7 genes had no similarities to known reb genes, although BLASTp searches identified AZC_3784 homologues in Rhizobium sp. strain AAP43, Oceanicaulis sp. strain HTCC2633, and Oceanicaulis alexandrii DSM 11625, as well as an AZC_3787 homologue in Inquilinus limosus. However, no AZC_3785 homologues were found in the database.

**Contributions of the reb operon to R-body formation.** To identify roles of the reb operon in R-body formation, we generated a praR deletion (ΔpraR) mutant, a deletion mutant with deletion of a region from AZC_3781 to AZC_3787 (ΔAZC_3781-7), and a ΔpraR ΔAZC_3781-7 double mutant and observed phenotypes of stem nodules at 14 days postinoculation (dpi) with these mutant and wild-type (WT) bacteria. The nitrogen-fixation-defective (Fix/H11002) phenotype of the reb operon expression levels (Fig. 2B). Transmission electron microscopy (TEM) observations showed that R bodies were produced in many ΔpraR bacterial cells in shrunken host cells, and many R-body-containing bacterial cells had collapsed appearances (Fig. 2C). R bodies were not observed in stem nodules harboring the double mutant (Fig. 2C), suggesting that genes that are essential for R-body formation are located in the region from AZC_3781 to AZC_3787.
cells (Fig. 3A and D), in which nuclei were collapsed (Fig. 3E), indicating that R-body production is associated with host cell death in the nodules.

Requirements of the non-reb-homologous genes AZC_3784, AZC_3785, and AZC_3787 and the reb-homologous genes rebAZC1, rebAZC2, rebAZC3, and rebAZC4 for R-body production were determined in ΔpraR mutant derivatives with the deletions of these genes. In these experiments, AZC_3784 was essential for R-body formation and AZC_3785 and AZC_3787 were not (see Fig. S3 in the supplemental material). In addition, both rebAZC3 and rebAZC4 and either rebAZC1 or rebAZC2 were essential for R-body production (see Fig. S4 in the supplemental material).

In investigations of the contributions of RebR to nodule formation, deletion of rebR from the WT strain did not alter the phenotypic expression of stem nodules, but abolished the Fix− phenotype of nodules harboring the ΔpraR mutant so that the ΔpraR ΔrebR mutant produced the Fix+ phenotype (Fig. 4A). R bodies were not
observed in the ΔpraR ΔrebR cells in the stem nodules (Fig. 4B). However, in symbiotic stem nodules, reb operon expression in the ΔpraR mutant was more than 10-fold higher than that in the ΔpraR ΔrebR mutant and was about 2 orders of magnitude higher than that in the WT strain, whereas levels of reb operon expression were similar in the WT

![Image](image1.png)

**Fig 3** TEM observations of stem nodules harboring the ΔpraR mutant at 7 dpi. (A) Normally shaped host cells (NC) with expanding vacuoles (v) and shrunken host cells (SC). (B and C) Bacterial cells (B) and host nuclei (C) in normally shaped cells. (D and E) Bacterial cells (D) and nuclei (E) in shrunken host cells. Arrows in panel B indicate bacterial cells, and arrowheads in panel D indicate R bodies. v, vacuole; N, nuclei.

![Image](image2.png)

**Fig 4** Phenotypes of praR and/or rebR mutants. *S. rostrata* plants were inoculated with the WT or ΔpraR, ΔrebR, or ΔpraR ΔrebR mutant strains and grown at 30°C, and stem nodules were analyzed at 14 dpi. (A) Hand-cut images (upper) and ARAs (lower) of stem nodules. Values are presented as means ± standard deviations from five separate plants. Different letters indicate significant differences (P < 0.05, Tukey-Kramer). (B) OM observations of infected host cells in stem nodules and TEM observations of harbored bacterial cells. (C) Relative expression levels of the reb operon in stem nodules and free-living cultures. Total RNAs were isolated from bacteria residing in stem nodules and from bacterial cultures after growth to an OD600 of approximately 1.0 at 38°C. Expression levels of the reb operon were estimated using quantitative RT-PCR and were normalized to 16S rRNA. Data are presented as means ± standard deviations of three replicate cultures and plants and are expressed relative to mRNA levels in free-living WT cultures. Statistical analyses were carried out for stem nodules and free-living cultures, respectively. Different letters indicate significant differences (P < 0.05; Tukey-Kramer).
and ΔrebR mutant strains (Fig. 4C). In contrast, in the free-living state, reb operon expression in the ΔpraR mutant was similar to that in the ΔpraR ΔrebR mutant. These results indicate that although PraR predominantly represses the expression of the reb operon, RebR acts as an activator of the operon under conditions of symbiosis.

PraR and RebR directly control the reb operon. Following a systematic evolution of ligands by exponential enrichment (SELEX) analysis, Frederix et al. (18) proposed that PraR of R. leguminosarum binds the consensus palindrome CAAC-NGTTG. In the present SELEX analysis using N-terminally His6-tagged PraR (His6-PraR) from A. caulinodans, the consensus PraR sequence of A. caulinodans was also CAAC-NGTTG (see Fig. S5A in the supplemental material). However, no sequence on the promoter region of the reb operon (reb promoter) matched this consensus sequence perfectly or with a base substitution, whereas four sequences matched with two or three substitutions, and these were examined as candidates for PraR binding sites. Subsequent electrophoretic mobility shift assays (EMSAs) revealed that His6-PraR binds strongly to a sequence designated PraR-bs-A and weakly to the sequences of PraR-bs-B and -C (Fig. S5B and C). Meanwhile, SELEX analysis using N-terminally His6-tagged RebR (His6-RebR) revealed that RebR potentially binds to a consensus palindrome, GT(A/G)(A/C)-N4-G(T/G)(T/C)AC (Fig. S5D). A sequence (designated RebR-bs) matching this consensus palindrome was present on the reb promoter, and the EMSA revealed that His6-RebR binds to this sequence (Fig. S5E). The positions of PraR-bs-A, -B, and -C and RebR-bs on the reb promoter are shown in Fig. 1.

To investigate whether PraR and RebR actually bind to the reb promoter, we carried out EMSAs using a double-stranded DNA (dsDNA) probe that covers the intergenic noncoding region upstream of the reb operon (reb promoter dsDNA probe) as shown in Fig. 5A. The molecular weight (MW) of the reb promoter dsDNA probe increased with the increasing quantity of His6-PraR, whereas addition of His6-RebR to the promoter probe increased not only the MW of the probe but also the amount of stacked probe in the wells of the gel in a concentration-dependent manner (Fig. 5B). Even in the presence of His6-PraR, the addition of His6-RebR to the promoter probe increased the MW of the probe (Fig. 5C), indicating that PraR does not interfere the binding of RebR to the RebR-bs.

To confirm the involvement of PraR and RebR in reb operon expression, the significance of the promoter sequences was assessed in stem nodules that were formed...
after inoculation with mutants carrying base substitutions of $P_{reb}(P_{reb}(PraR-bs-A^-) - RebR-bs^-)$ double mutants and free-living cells grown in the defined medium at 38°C to an OD$_{600}$ of approximately 1.0. Expression levels of the $reb$ operon were estimated using quantitative RT-PCR, normalized to 16S rRNA levels, and expressed relative to corresponding mRNA levels in free-living WT cultures and are presented as means ± standard deviations from three replicate cultures and plants. Statistical analyses were carried out for stem nodules and free-living cultures, respectively. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer).
those in the WT strain (Fig. 6D). The consistency of phenotypic expression between deletion mutants of praR and rebR (ΔpraR, ΔrebR, and ΔpraR ΔrebR mutants in Fig. 4) and corresponding promoter sequence mutants strongly indicates that PraR and RebR directly control the expression of the reb operon.

**Identification of environmental factors that abolish reb repression by PraR.** In the experiments described above, symbiotic R-body production was observed in ΔpraR and PrebR(Prab-ΔA−) mutants but was not present in the WT strain under symbiotic or free-living conditions, suggesting that R-body production is subjected to environmental conditions. Thus, we investigated environmental factors that induce RebR-dependent activation of the reb operon in the ΔpraR mutant and then identified factors that attenuate PraR-dependent repression of the reb operon in WT cells grown under the identified favorable environmental conditions.

Initially, we constructed a reb operon-lacZ transcriptional fusion (reb-lacZ) on chromosomes of the WT and ΔpraR strains, namely reb-lacZ and reb-lacZ ΔpraR strains, respectively. During these manipulations, the reb-lacZ ΔpraR strain expressed β-galactosidase in the free-living state at room temperature (around 26°C) but not at 38°C, suggesting that activation by RebR is temperature dependent. Accordingly, when reb-lacZ and reb-lacZ ΔpraR strains were grown at various temperatures between 26 and 41°C, β-galactosidase activity was highly induced below 35°C in the reb-lacZ ΔpraR strain, but not in the reb-lacZ strain (Fig. 7A). Moreover, expression levels of the reb operon were about 30-fold higher at 26°C than at 38°C in the ΔpraR strain, whereas activation at 26°C was not observed in either the ΔrebR or ΔpraR ΔrebR mutant (Fig. 7B). Under free-living conditions, R bodies were observed in up to 10% of ΔpraR cells grown at 26°C, but not in those grown at 38°C (Fig. 7C). Similarly, ΔpraR cells in stem nodules (symbiotic state) failed to produce R bodies when plants were grown at 38°C, and reb operon expression was lower than that in ΔpraR cells under symbiotic conditions at 30°C (see Fig. S6 in the supplemental material). Taken together, these data indicate that activation of the reb operon by RebR is temperature dependent under both free-living and symbiotic conditions. However, binding of RebR to the reb promoter was not affected by temperature (see Fig. S7 in the supplemental material).

In subsequent experiments, the effects of host-derived tricarboxylic acid (TCA) cycle, nitrogen, and oxygen metabolites on reb expression were investigated in reb-lacZ and reb-lacZ ΔpraR strains. In the absence of praR (the reb-lacZ ΔpraR strain), all organic acids except for citrate promoted reb expression under nitrogen-sufficient conditions at either 21 or 3% oxygen (Fig. 8). However, even in the presence of praR (reb-lacZ strain), reb expression was induced in medium containing 2-oxoglutarate (2OG) with sufficient nitrogen sources (Fig. 8). Moreover, induction was increased with 2OG concentrations even in the presence of succinate as a carbon source (Fig. 9A), suggesting that 2OG induces reb irrespective of its metabolism as a carbon source, although these effects of 2OG were observed at 26°C but not at 38°C (Fig. 9B). In agreement, TEM observations showed that R bodies were produced in WT cells grown in the presence of 2OG at 26°C but not at 38°C (Fig. 9C). Taken with the absence of a response to 2OG in ΔrebR mutant cells (Fig. 9D), these observations indicate that rebR is essential for 2OG-mediated induction of the reb operon. Moreover, the ΔpraR mutants did not respond to 2OG, further suggesting that 2OG derepresses the reb operon by attenuating PraR-dependent repression. Western blotting of an rgs-his6-praR transformant (19) showed that expression levels of RGS-His6-PraR protein were not affected by 2OG (Fig. 9E). However, binding of PraR to the reb promoter dsDNA probe decreased with increasing 2OG concentration, whereas RebR binding was impervious to 2OG (Fig. 9F; Fig. S7). These observations strongly suggest that 2OG derepresses the reb operon directly by concentration-dependently inhibiting PraR binding to the reb promoter.

**DISCUSSION**

In this study, we demonstrated that reb-driven pathogenicity is associated with R-body production by *A. caulinodans* and suggest that R-body production is a widespread trait among bacterial pathogens that carry reb operons. Although the reb...
operon is predominantly repressed by PraR, we identified biological and environmental factors that derepress reb gene expression and thus R-body production under free-living conditions.

R bodies are rolled up at neutral pH, but reportedly unroll to form needle-shaped structures at low pH (10). Recombinant R bodies from *Escherichia coli* act as pistons that puncture spheroplasts of *E. coli* at low pH (20). In paramecia, the role of R bodies in the killer trait follows release of R-body-containing bacteria from killer paramecia and ingestion by sensitive paramecia. Subsequently, internalized bacteria enter acidified food vacuoles, and R bodies are unrolled and penetrate the phagosomal membrane to deliver lethal toxins to the cytoplasm (9, 21). This scenario may also be applicable to interactions of *A. caulinodans* and *S. rostrata*, in which the peribacteroid space (mi-
fumarate, or malate as carbon sources and 10 mM NH4 remain poorly understood, they are likely to be components of R bodies (25) and warrant further compositional analyses of R bodies and investigations of the molecular mechanisms of R-body formation. These observations strongly support the hypothesis that differences in the expression of the virulence genes according to temperature (July/August 2017 Volume 8 Issue 4 e00715-17) mediates the formation of crown galls at temperatures below 32°C, and the VirA/VirG two-component system regulates the expression of virulence genes according to temperature (26, 27). In agreement, the reb operon was mainly regulated (suppressed) in the free-living state—probably at the translation level or at the R-body assembly level—and that A. caulinodans R bodies play more important roles in the symbiotic state.

Although R-body formation was not observed in WT bacterial cells in the symbiotic state, the present environmental factors that derepress the praR regulatory system have significant implications for the understanding of reb operon evolution during microsymbiosis of A. caulinodans with S. rostrata. It is widely accepted that virulence genes are regulated by temperature in many pathogenic bacteria and are upregulated in mammalian bacterial pathogens at around host body temperature (37°C) (23, 24). In contrast, most plant-pathogenic bacteria express virulence genes at ambient temperatures that are generally lower than their optimal growth temperatures (25). For example, Agrobacterium tumefaciens mediates the formation of crown galls at temperatures below 32°C, and the VirA/VirG two-component system regulates the expression of virulence genes according to temperature (26, 27). In agreement, the reb operon was frequently produced by ΔpraR mutants in the symbiotic state, but were observed in fewer than 10% of free-living cells, even at the optimum temperature (26°C) for reb operon expression. These results suggest that R-body formation is more strongly regulated (suppressed) in the free-living state—probably at the translation level or at the R-body assembly level—and that A. caulinodans R bodies play more important roles in the symbiotic state.

The present series of mutant analyses showed that essential genes for R-body formation in A. caulinodans include both reb-homologous and non-reb-homologous genes. Although the roles of the proteins encoded by the reb-homologous genes remain poorly understood, they are likely to be components of R bodies (5). Among non-reb-homologous genes, AZC_3784 was found in the reb operon and was also essential to R-body formation. In C. taeniospiralis, RebC is encoded by a non-reb-homologous gene that may be involved in the assembly of R bodies (5). Similarly, the AZC_3784 protein is not a component of R bodies but is likely involved in their assembly, although it may lack homology to rebC from C. taeniospiralis. Taken together, these observations warrant further compositional analyses of R bodies and investigations of the molecular mechanisms of R-body formation.

croenvironment surrounding bacteroids) is progressively acidified during nodule morphogenesis (22), likely triggering the conformational change of R bodies into the needle-shaped structure that penetrates membranes.

FIG 8 Effects of carbon and nitrogen sources and oxygen concentrations on the expression of the reb operon under free-living conditions. Mutant reb-lacZ and reb-lacZ ΔpraR strains were grown in medium containing 50 mM pyruvate, citrate, 2-oxoglutarate (2OG), succinate, fumarate, or malate as carbon sources and 10 mM NH4 or NO3 as nitrogen sources or without a nitrogen source (−N) under aerobic (21% O2) or microaerobic (3% O2) conditions. Initial OD600 values of cultures were adjusted to 0.02 for pyruvate, succinate, fumarate, and malate media with NH4 or NO3 supplementation, 0.1 for supplementation with 2OG and NH4 or NO3, and 0.2 for supplementation with citrate medium and NH4 or NO3 or nitrogen-deficient media. After incubation at 26°C for 24 h, β-galactosidase activities were measured. Data are presented as means ± standard deviations from three replicate cultures. Different letters indicate significant differences (P < 0.05, Tukey-Kramer).
FIG 9 Effects of 2OG on the expression of the reb operon and R-body formation in bacteria under free-living conditions. (A) β-Galactosidase activities of the reb-lacZ strain in the presence of various 2OG concentrations. The reb-lacZ strain was grown in medium with the indicated concentrations of 2OG (0 to 50 mM) at 26°C for 24 h to an OD600 of approximately 1.0. Data are presented as means ± standard deviations from three replicate cultures. Different letters indicate significant differences (P < 0.05, Tukey-Kramer). (B) Quantitative RT-PCR analysis to investigate the effects of 2OG and temperature on the reb operon expression in the WT strain. Cultures of the WT strain were supplemented with 10 mM 2OG (+2OG) or no 2OG (−2OG) and were grown at 26 or 38°C. The data are expressed relative to those from the cultures under −2OG conditions at 26°C and are presented means ± standard deviations from three replicate cultures. Asterisks indicate significant difference (**, P < 0.01, Student’s t test). (C) TEM observations of the WT cells cultured with 2OG at 26 and 38°C. Bacterial cells were collected from the cultures that were used for the quantitative RT-PCR conducted in panel B. Arrowheads indicate R bodies. (D) Quantitative RT-PCR analysis of the reb operon in the WT, ΔpraR, ΔrebR, and ΔpraR ΔrebR strains. These strains were grown under −2OG or +2OG conditions at 26°C. The data are expressed relative to corresponding values in the WT strain under −2OG conditions at 26°C and are presented as means ± standard deviations from three replicate cultures. Asterisks indicate significant difference (**, P < 0.01, Student’s t test). (E) Western blotting with an anti-His antibody to investigate the effects of 2OG on RGS-His6-PraR expression. Whole-cell lysates from the rgs-his6-praR strain grown under +2OG or −2OG conditions and from the WT strain grown under −2OG conditions were electrophoresed. (F) EMSA analysis to investigate the effects of 2OG on binding activities of His6-PraR to the reb promoter. FITC-labeled dsDNA probe corresponding to the reb promoter was incubated with the purified His6-PraR in the presence of 2OG (0.01 to 10 mM) or succinate (10 mM).

expression in the ΔpraR mutant of A. caulinodans at temperatures below 35°C and within the optimal range for the growth of the host plant (around 30°C). Moreover, because regulation by PraR was derepressed by 2OG in the present free-living WT cells at 26°C, the reb operon may also be induced during symbiosis in host nodules, wherein the 2OG is accumulated in the host plant cells, although we have not found the conditions under which 2OG actually accumulates in the host plant cells. Plant 2OG levels reflect cellular C/N status and may play a signaling role in the coordination of C and N metabolism (28). Alterations in the activities of nitrogen fixation by bacteria and ammonia assimilation by plant cells may lead to 2OG accumulation in host cells. To elucidate the roles of reb operon in the symbiotic state, we need to conduct more investigations to estimate the conditions wherein 2OG is accumulated in the host plant cells.

Although PraR homologues are widespread among Alphaproteobacteria (8), the roles of PraR have not been wel characterized. In particular, the praR homologue phfR was originally identified in the acid-tolerant rhizobiun Sinorhizobium medicae WSM419 as a gene that is induced at low pH (29). However, praR expression is not pH sensitive in A. caulinodans and Rhizobium leguminosarum (8, 18). Moreover, R. leguminosarum PraR directly represses the expression of the quorum-sensing genes rhiR and raiR and the biofilm formation genes rpaA2, rpaB, and rpaC (18, 30), whereas the homologous genes in A. caulinodans are not controlled by PraR (8). Hence, although PraR homologues are widely distributed, the roles of PraR have diversified during the evolution of Alphaproteobacteria. The ubiquity of chemical and environmental factors that regulate

July/August 2017 Volume 8 Issue 4 e00715-17 mbio.asm.org 11
praR expression in the Alphaproteobacteria, such as the effects of A. caulinodans factors, also requires investigation in the context of the evolution of prar regulatory systems.

RebR belongs to a novel subfamily of the Crp-Fnr superfamily, and all Crp-Fnr members carry putative DNA-binding helix-turn-helix domains on their C terminus and ligand-binding domains on their N terminus (17). Various intracellular and exogenous signals activate Crp-Fnr members via their ligand-binding domains, including 2OG and temperature (17). In A. caulinodans, however, binding activity of RebR to the reb operon was not affected by 2OG and temperature, indicating that in addition to 2OG and temperature, as yet unidentified factors are involved in the activation of reb operon expression via RebR.

Herein, we demonstrated the roles of R bodies in the pathogenicity of bacteria that harbor the reb operon, although the ensuing roles in nodule symbiosis and the related evolutionary implications remain uncharacterized. Because bacterial genomes are plastic, endosymbionts may become pathogenic after acquiring the reb operon if they do not suppress its expression. Although we did not determine whether R bodies threaten biodiversity or ecosystems, this possibility may require solutions in the future. Unlike obligate endosymbionts of paramecia, A. caulinodans can be cultured in vitro and genetic manipulation techniques have been established in this bacterium, warranting further use of A. caulinodans as a model for studies of R-body/prar genes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table S1 in the supplemental material. A. caulinodans strains were grown in tryptone-yeast extract (TY) medium (31) or in basal defined (BD) medium containing 30 mM disodium succinate, 10 mM NH₄Cl, 10 mM potassium phosphate (pH 7.0), 100 mg liter⁻¹ MgSO₄·7H₂O, 50 mg liter⁻¹ NaCl, 40 mg liter⁻¹ CaCl₂·2H₂O, 5.4 mg liter⁻¹ FeCl₂·6H₂O, 5 mg liter⁻¹ Na₂MoO₄·2H₂O, 2 mg liter⁻¹ biotin, 4 mg liter⁻¹ nicotinic acid, and 4 mg liter⁻¹ pantothenic acid. To vary the carbon and nitrogen contents of BD medium, disodium succinate was replaced with sodium pyruvate, trisodium citrate, disodium 2-oxoglutarate, disodium fumarate, or disodium ε-malate, and NH₄Cl was replaced with KNO₃ or omitted. In some experiments, BD medium was further supplemented with disodium 2OG. To grow A. caulinodans strains under aerobic conditions, test tubes containing medium were sealed with butyl rubber septums, and the contained air was replaced with N₂ gas with 3% O₂. Before inoculation into BD medium, bacterial cells were cultured overnight in TY medium and were washed twice in 10 mM potassium phosphate buffer (pH 7.0). Unless otherwise noted, initial optical density at 600 nm (OD₆₀₀) values of cultures were adjusted to 0.1 or 0.02 for growth at 26 or 38°C, respectively, and OD₆₀₀ values were approximately 1.0 after 24 h of incubation.

Construction of deletion and substitution mutants. The plasmids and primers used for strain construction are listed in Tables S1 and S2 in the supplemental material, respectively.

To construct A. caulinodans deletion mutants of AZC_3784, AZC_3785, AZC_3787, and rebR genes, two DNA fragments containing upstream and downstream regions of each gene were amplified from the WT genomic DNA by PCR using appropriate primer pairs and were then directionally cloned into a suicide vector, pK18mob sacB (32), using the In-Fusion cloning kit (Clontech, Mountain View, CA). The linearization of pK18mob sacB was performed by inverse PCR using the PrimeSTAR Max (TaKaRa-Bio, Shiga, Japan) with primer pair Tp73-Tp74. The resulting plasmids were conjugated into the WT or ΔpraR (B) strains via E. coli S17-1(λpir) (33), and gene deletions were introduced by allelic exchange.

To construct deletion mutants of the rebAZC1, rebAZC2, rebAZC3, and rebAZC4 genes, a series of plasmids were constructed as follows. DNA fragments containing the WT AZC_3781-7 region with its upstream and downstream regions were amplified by PCR and cloned into the linearized pK18mob sacB. Genes on plasmids containing the WT region were deleted by inverse PCR using the PrimeSTAR Mutagenesis Basal kit (TaKaRa-Bio). To introduce double deletions, second inverse PCRs were conducted using plasmids harboring single mutations. Constructed plasmids were conjugated into the ΔpraR ΔAZC_3781-7 mutant, and deletion mutants were obtained after allelic exchange.

To construct mutants with base substitutions in PraR-bs-A and/or RebR-bs, a series of plasmids were constructed as follows. A DNA fragment containing the WT reb promoter region was amplified by PCR and cloned into the linearized pK18mob sacB. An XbaI site was generated within the PraR-bs-A on the plasmid containing the reb operon by inverse PCR using the PrimeSTAR Mutagenesis Basal kit. Similarly, an EcoRI site was generated within the RebR-bs on the plasmid containing the reb promoter. The resulting plasmids were conjugated into the WT strain, and mutants were obtained after allelic exchange.

To construct strains that express the reb-lacZ fusion gene, two fragments containing rebR and AZC_3789 and a lacZ fragment were amplified by PCR from the WT genomic DNA and the plasmid pTA-MTL (34), respectively. Fragments were then cloned into the linearized pK18mob sacB in the direction of the rebR, lacZ, and AZC_3789 fragments using the In-Fusion cloning kit. The plasmid containing reb-lacZ was conjugated into the WT and ΔpraR strains, and strains with lacZ at the position immediately downstream of the rebR open reading frame (ORF) were obtained after allelic exchange.
Plant growth and bacterial inoculation for nodule formation. S. rostrata stems were inoculated with A. caulinodans strains as described previously (19) and were then grown at 30 or 38°C under a 24-h light regime. Acetylene reduction activities (ARAs) of stem nodules were assayed as described previously (19).

Optical microscopy observation. Stem nodules were longitudinally cut into three pieces. The middle pieces of each sample were chemically fixed with 4% paraformaldehyde and 2% glutaraldehyde, dehydrated through a graded ethanol series, and then embedded in Technovit 7100 (Heraeus Kulzer). The embedded samples were sliced into 5-μm sections, stained with 0.05% toluidine blue O, and then observed using a bright-field microscope (DMLB; Leica).

TEM observation. Bacterial cells were collected from culture media by centrifugation. Stem nodules were cut into small pieces. These samples were chemically fixed as described above, postfixed with 2% OsO4, dehydrated through a graded ethanol series, and finally embedded in Spurr low-viscosity embedder. Small pieces of each sample were chemically fixed with 4% paraformaldehyde and 2% glutaraldehyde, dehydrated through a graded ethanol series, and then embedded in Technovit 7100 (Heraeus Kulzer). Optimal sections were cut into small pieces. These samples were chemically fixed as described above, postfixed with 2% OsO4, dehydrated through a graded ethanol series, and finally embedded in Spurr low-viscosity embedder. These samples were cut into small pieces. These samples were chemically fixed as described above, postfixed with 2% OsO4, dehydrated through a graded ethanol series, and finally embedded in Spurr low-viscosity embedder.

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Total RNA extraction and quantitative RT-PCR. Total RNA was isolated from bacterial cultures and stem nodules, and cDNA was synthesized according to previously described methods (19). Quantitative PCR was performed with a LightCycler system (Roche, Basel, Switzerland) using the Quantitect SYBR green PCR kit (Qiagen, Hilden, Germany) with primer pairs Acp326-Acp714 for the reb operon and Tp35-Tp36 for 16S rNRA. Standard curves were generated using genomic DNA that was isolated from the WT strain using the Nucleospin tissue kits (Macherey-Nagel, Düren, Germany). To determine expression levels of the reb operon, copy numbers of reb operon transcripts were normalized to those of 16S rRNA.

Purification of His-Tagged PraR and RebR. The proR ORF was amplified by PCR from the WT genomic DNA using primer pair Acp375-Acp161 and was then cloned into BamHI and XbaI restriction sites of the pCold I vector (TaKaRa-Bio, Shiga, Japan). The resulting plasmid was designated pTAC9. The rebR ORF was amplified by PCR from WT genomic DNA using primer pair Acp669-Acp670 and cloned into pCold I using the In-Fusion cloning kit (Clontech, Mountain View, CA). The linearization of pCold I was performed by inverse PCR using the PrimeSTAR Max (TaKaRa-Bio) with primer pair Tp78-Tp79. The resulting plasmid was designated pTAC133. pTAC9 and pTAC133 were transferred into E. coli BLR(DE3) (Novagen, Darmstadt, Germany). Subsequently, His6-PraR and His6- RebR were extracted and purified using Ni2+-charged magnetic beads (His Mag Sepharose Ni; GE Healthcare, Little Chalfont, United Kingdom). Eluted proteins were then concentrated using Vivasin 500 kits (molecular weight cutoff [MWCO] of 5,000; Sartorius, Göttingen, Germany) and were mixed with 5 volumes of 1.2× storage buffer (24 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1.2 mM dithiothreitol [DTT], 60% glycerol). EMSA of the reb promoter. Fluorescein isothiocyanate (FITC)-labeled dsDNA probes corresponding to the reb promoter region were prepared as follows. Initially, the reb promoter region was amplified by PCR using primer pair Acp646-Acp702 and genomic DNA from WT or derivative strains. Acp702 has an end. PCR products from each strain were purified using the MinElute PCR purification kit (Qiagen) and were used as the templates for the second round of PCR using primer pair Acp646-M13R_FITC. Finally, FITC-labeled PCR products were purified. EMSAs were then performed by incubating 1-pmol aliquots of FITC-labeled dsDNA probes with various amounts of His6-PraR and/or His6- RebR in 20 μl of EMSA buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 60 mM NaCl, 5 mM MgCl2, 1 mM DTT, 10 ng μl−1 poly(dI-dC), 4% glycerol, 0.05% Tween 20) for 30 min at 26°C. Binding reaction mixtures were then electrophoresed in 4% native polyacrylamide gels containing 2.5% glycerol in 0.5× TBE buffer, and FITC-labeled DNAs were detected using the LAS3000 system (Fuji Film, Tokyo, Japan).

Western blotting analyses of RGS-His6-PraR. Bacterial cells were collected from cultures by centrifugation, sonicated in phosphate-buffered saline (150 mM NaCl, 10 mM NaH2PO4, 20 mM NaH2PO4 [pH 7.4]), and then fractionated by SDS-PAGE using 14% polyacrylamide gels. Fractionated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes, incubated with mouse anti-His6 antibody (Qiagen), and detected using horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibodies (GE Healthcare) and the ECLWestLumi Plus reagents (Atto, Tokyo, Japan).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00715-17.

FIG S1, PDF file, 0.2 MB.

FIG S2, PDF file, 0.2 MB.
FIG S3, PDF file, 1.2 MB.
FIG S4, PDF file, 1.1 MB.
FIG S5, PDF file, 0.3 MB.
FIG S6, PDF file, 0.4 MB.
FIG S7, PDF file, 0.2 MB.
TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.

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