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Full Length Research Paper

Mutants of the hybrid sensor kinase CstA affect cyst cell development in *Azospirillum brasilense* Sp7

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We previously characterized a gene (originally named *org35*, renamed *cstA*) encoding a PAS/PAC sensor hybrid histidine kinase (HK) which interacts with *Azospirilum brasilense* NifA in a yeast two-hybrid system. The predicted CstA is a hybrid sensor kinase, comprising two N-terminal PAS domains, a histidine kinase core domain and a response regulator receiver (RR) domain. To determine the function of the *cstA*, two mutants were constructed including two in-frame deletion mutants *ΔHK* and *ΔRR*. A microscopic analysis revealed that both *cstA* mutants developed mature cyst cells more quickly than the wild type. Moreover, *cstA* mutants are affected in colony morphology which are essential for *A. brasilense* cells to differentiate into cyst-like forms. These observations suggested that *cstA* is involved in cyst development in *A. brasilense.*

Key words: Two-component system protein, cyst development, nitrogen fixation, *Azospirillum brasilense.*

INTRODUCTION

Azospirillum brasilense is a nitrogen-fixing and plant growth-promoting α-proteobacterium which mainly colonizes the rhizosphere of cereals and grasses in tropical and subtropical regions (Steenhoudt and Vanderleyden, 2000). To resist to desiccation and other environmental stresses, the bacterial cells are known to change their metabolic activities swiftly and to form resistant resting cysts (Sadasivan and Neyra, 1987; Berleman et al., 2004). In *Azospirillum* spp., cysts are cells which lost motility, assumed an enlarged spherical form, and accumulated abundant poly-hydroxybutyric acid (PHB) granules and developed an outer undifferentiated layer (coat) of polysaccharides. Previous studies on *Rhodospirillum centenum* and *Azotobacter vinelandii* revealed that cyst formation is a process in which there is a large hierarchy of signal transduction components that regulate encystment (Sadoff, 1975; Stadtwald-Demchick et al., 1990; Berleman et al., 2004; Berleman and Bauer, 2005).

We had previously discovered a gene of *A. brasilense* Sp7, originally named *org35*, renamed *cstA* (Cui et al., 2010; Wu et al., 2011), which was originally identified to be able to interact with NifA in yeast-two-hybrid system (Chen et al., 2005). The *cstA* encodes a hybrid histidine kinase (HK), including N-terminal PAS domains, a HK core domain and a response regulator receiver (RR) domain in C-terminal (Cui et al., 2010). The HK domain of CstA possesses autokinase activity and the phosphorylated HK is able to transfer phosphate groups to the RR domain (Cui et al., 2010). Our previous experiments demonstrated that *cstA* mutant containing deletion of the N-terminal PAS domains and insertion of Ω fragment showed reduced chemotaxis ability compared to

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Abbreviations: HK, Histidine kinase; **RR,** regulator receiver; **PHB,** poly-hydroxybutyric acid; **TDFs,** transcript-derived fragments; **Ap,** antibiotics ampicilline; **Km,** kanamycin; **Tc,** tetracycline; **Nx,** nalidixic acid; **Sp,** spectinomycin; **DNA,** deoxyribonucleic acid; **PCR,** polymerase chain reaction; **MSM,** minimal salts medium; **EPS,** exopolysaccharides.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics [a]	Source/ Reference
E. coli JM109	Strain that supports blue-white screening of colonies for cloning experiments	Novagen
E. coli S17-1	*mob*; stain capable of mobilizing the suicide vector into host cells; Sm^r Sp^r	Simon et al. (1983)
A. brasilense Sp7	Wild type of *A. brasilense*; Ap^r Nx^r	ATCC 29145
A. brasilense Δ*HK*	In-frame deletion mutant lacking the HK domain of *cstA*; Ap^r Nx^r	This work
A. brasilense Δ*RR*	In-frame deletion mutant lacking the RR domain of *cstA*; Ap^r Nx^r	This work
A. brasilense Δ*(HK-RR)::Km*	Mutant lacking both the HK domain and RR domains of *cstA* and inverse insertion with Km cassette; Ap^r Nx^r	This work
A. brasilense Δ*PAS::Ω* (Sp7353)	Mutant lacking PAS domain of *cstA* and insertion with the Ω cassette; Ap^r Nx^r Sp^r	Cui et al. (2010)
pUC4K	Source plasmid for Kanamycin cassette; Ap^r Km^r	Amersham Biosciences
pK18*mobsacB*	Allelic exchange suicide vector; *lacZ mob sacB*; Km^r Suc^s	Schafer et al. (1994)
pKS-HK	pK18*mobsacB* derivative carrying in-frame deletion of HK domain of *cstA*; Km^r Suc^s	This work
pKS-RR	pK18*mobsacB* derivative carrying in-frame deletion of RR domain of *cstA*; Km^r Suc^s	This work
pPHU281	Suicide vector; *lacZ mob*; Tc^r	Hübner et al. (1993)
pBS354	pPHU281 derivative carrying Δ*(HK-RR)::Km* partial of *cstA*; the Km cassette insert inversely; Tc^r	This work

[a] Ap^r, ampicilline resistance; Nx^r, nalidixic acid resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance; Sm^r , streptomycin resistance; Suc^s, sucrose-sensitive.

that of wild-type (Cui et al., 2010). *cstA* is located downstream and cotranscribed with *cstB* whose predicted product displays sequence similarity with CheB/CheR fusion protein (Wu et al., 2011). A *cstB* mutant has reduced swarming motility and develops mature cyst cells more quickly than the wild-type (Wu et al., 2011).

Our previous study indicated differentially expressed genes in *cstA* mutants comparing with wild type Sp7 by means of cDNA-AFLP (Li et al., 2011). Three down-regulated transcript-derived fragments (TDFs) AB46, AB58 and AB63 were similar to PHB de-polymerase C-terminus, cell shape-determining protein and flagellin domain protein in the mutants of *cstA* relative to the wild type Sp7.

These results suggested that *cstA* may be involved in cyst development. Thus the function of the *cstA* in cyst formation was here determined. In the present study, to reveal the role of *cstA* in cyst formation and development, two mutants of *cstA* including two in-frame deletion mutants Δ*HK* and Δ*RR* were here constructed. The effect of mutating *cstA* on cyst formation in *A. brasilense* Sp7 was estimated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. *A. brasilense* strains were routinely grown at 30°C in LD medium (per liter: tryptone, 10 g; yeast extract, 5 g; NaCl, 2.5 g). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Antibiotics ampicilline (Ap), kanamycin (Km) and tetracycline (Tc) were used for *E. coli* at 100 µg/ml, 50 µg/ml and 12.5 µg/ml, respectively. Ampicillin, nalidixic acid (Nx) and spectinomycin (Sp) were used for *A. brasilense* at 25, 5 and 100 µg/ml, respectively.

Construction of *cstA* mutants

Two mutants including two in-frame deletion mutants Δ*HK* and Δ*RR* were here constructed. To construct Δ*HK* mutant containing in-frame deletion of the HK domain, a deoxyribonucleic acid (DNA) segment containing the HK domain (from the 469th to 723th codon) was deleted by polymerase chain reaction (PCR) amplification of chromosomal sequences flanking the HK domain from wild-type *A. brasilense* DNA. To do this, a 1058-bp fragment which contains the upstream region flanking of the HK domain was PCR amplified using primers DH_A-F [5'-TAAGGATCCCTGGACCATGGGCGCCTG-3' (a *Bam*HI site underlined)] and DH_A-R [5'-GCAGAATTCGCGCTCCATCGACTTGAT-3' (an *Eco*RI site underlined)], and a 1382-bp fragment which contains the downstream region flanking of HK domain was PCR amplified using primers DH_D-F [5'-TATGAATTCCTGCTGGTCGAGGACGAT-3' (an *Eco*RI site underlined)] and DH_D-R [5'-TATCTCGAGCTGGTCATGCTGCTGTT-3' (a *Xho* I site underlined)]. The two PCR products were successively ligated to suicide vector pK18*mobsacB*, resulting in the recombinant plasmid pKS-HK. Plasmid pKS-HK carrying the upstream and downstream regions around the HK domain in the correct orientation is confirmed by enzyme digestions and PCR amplification.

To construct Δ*RR* mutant with in-frame deletion of the RR domain

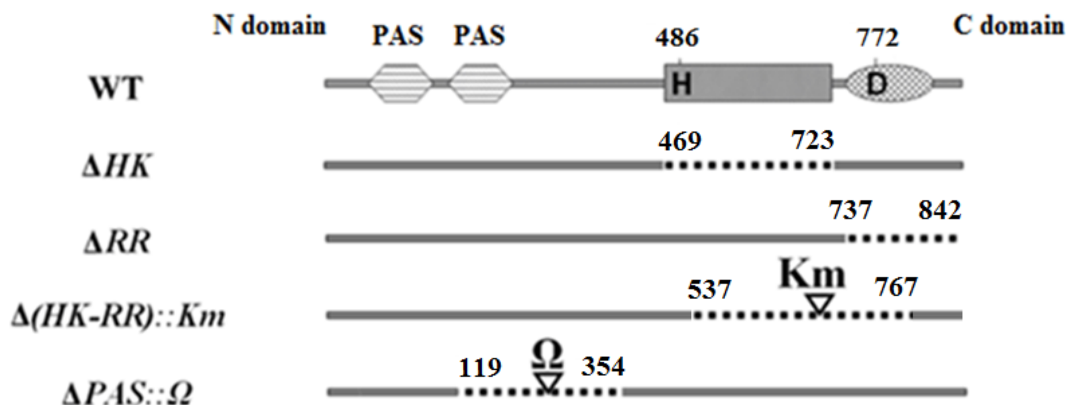

Figure 1. A schematic diagram of the *A. brasilense cstA* mutants.The dots represent the regions deleted in each mutant. The triangles represent the insertion of antibiotic resistance as described in detail in the Material and Methods. H, the phosphate group acceptor His-486; D, a site of phosphorylation Asp-772; Km, kanamycin resistance; Ω; spectinomycin resistance.

(from the 737th to 842th codon) of CstA, a 860-bp fragment upstream of RR domain was PCR amplified with primers DR_A-F [5'-CTAGAATTCAAGTCGATGGAGCGCGA-3' (an *Eco*RI site underlined)] and DR_A-R [5'-ACTAAGCTTTCAGAAGGCCATGCGGATCAC-3' (a *Hin*dШ site underlined)], and a 1021-bp fragment immediately downstream of the RR domain was PCR with primers DR_D-F [5'-TTTAAGCTTTGGAGACGGAGGCGACGT-3' (a *Hin*dШ site underlined)] and DR_D-R [5'-TATCTCGAGCTGGTCATGCTGCTGTT-3' (a *Xho*I site underlined)]. The two PCR products were successively ligated to pK18mobsacB with appropriately orientation to produce the recombinant plasmid pKS-RR.

The two recombinant plasmids pKS-HK and pKS-RR, as described above were transferred by conjugation from *E. coli* S17-1 to *A. brasilense* Sp7, respectively. Colonies were streaked onto 10% (w/v) sucrose plates and sucrose resistant colonies were selected and patched onto agar plates containing kanamycin (50 μg/mL). Sucrose resistant and kanamycin sensitive colonies were screened for the double crossing-over mutants *ΔHK* and*ΔRR* and verified by PCR amplification.

The Δ*(HK-RR)::Km* mutant containing deletion of HK-RR domains (from the 537th to 767th codon) and insertion of Km gene, and the insertion-deletion mutant Δ*PAS::Ω* containing deletion of the N-terminal PAS domains and insertion of Ω fragment, were constructed in our previous study (Li et al., 2011; Cui et al., 2010).

Cyst induction and desiccation resistance assays

To induce cyst production, the mid-exponential cultures of *A. brasilense* strains were harvested by centrifugation at 4,000 × *g* for 5 min, washed three times in 100 mM potassium phosphate buffer (pH 6.8) to give an OD_{600} of 1.0, and then 5 μl aliquots were pipetted onto 0.3% semisolid minimal salts medium (MSM) (Sadasivan and Neyra, 1985) with 8 mM malate as a carbon source and inductor. Cyst cells from colonies on 0.3% semisolid cyst-inducing medium MSM were examined microscopically by preparing wet mounts at 3, 6 and 9 days after growth and checking for the presence of cysts with phase-contrast microscopy. Cells were viewed with a Nikon E800 light microscope equipped with a 100 × Plan Apo oil objective.

To quantify the amount of mature cyst cell formation, *A. brasilense* wild type and mutants were subjected to desiccation resistance analysis according to the method described previously (Sadasivan and Neyra, 1987; Berleman et al., 2004). The cells at 6 days after growth on semisolid MSM were processed and the cyst cells number was determined by calculating the amount of surviving cells on LD medium. Before desiccation, the total number of vegetative cells plus cyst cells should be determined. The resuspended cells at the stated time were serially diluted onto LD plates and incubated at 37°C for 3 days. Total colonies before and after desiccation were counted with assays repeated in triplicate. The conversion rate of vegetative cells into cyst cells was calculated.

The detection of production of exopolysaccharides and colony morphology

Colony morphology at 3 days after growth on agar-solidified cyst-inducing medium MSM and nutrient-rich medium LD lacking NaCl, containing Congo red (which was used to show the distinct ridge colonies), was photographed using a Nikon PL5100 digital camera.

Nitrogenase activity

Nitrogen fixation was measured by the acetylene reduction assay according to the derepression protocol described by Galimand et al. (1989). The data presented below were the results obtained from at least three independent experiments.

RESULTS

CstA mutants were constructed

To determine the functions of *CstA* and its different domains, mutants were constructed and were illustrated in Figure 1. The Δ*HK* or Δ*RR* mutants contain a deletion of the HK domain or RR domain in frame, respectively. The Δ*(HK-RR)::Km* mutant contained deletions of the HK-RR domains and an inverse insertion of Km cassette (Li et al.,

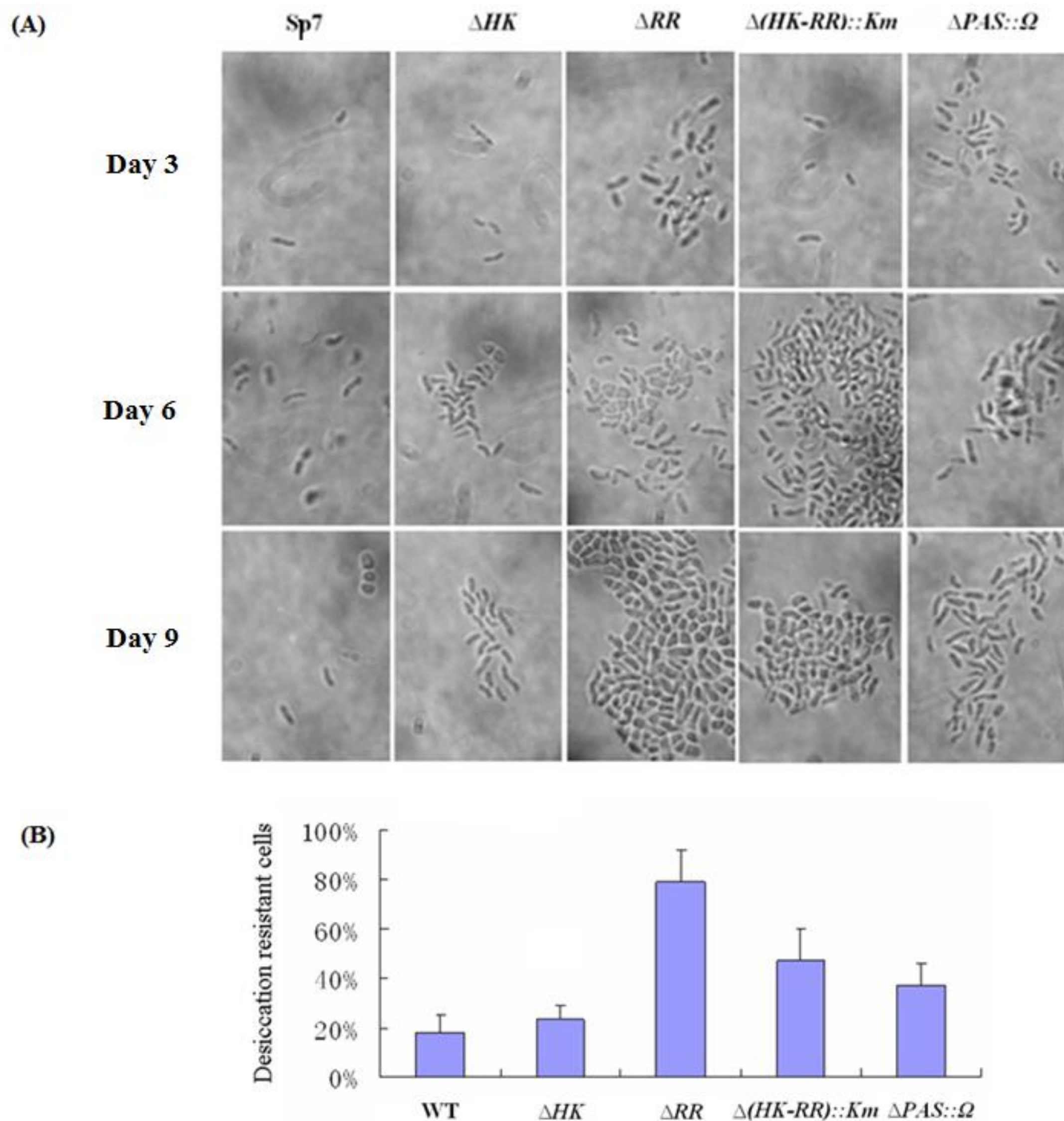

Figure 2. Cellular morphology and desiccation resistance of the *A. brasilense cstA* mutants during cyst induction.(A) Phase-contrast microscopy of wet mounts of the *A. brasilense* Sp7 (wild-type) and *cstA* mutants after 3, 6 and 9 days growth on semi-solidified MSM. (B) Desiccation resistance analysis of *cstA* mutants after 6 days growth on MSM medium.

2011). A insertion-deletion mutant Δ*PAS::*Ω containing a deletion of the N-terminal PAS domains and an insertion of Ω fragment was constructed in our previous study (Cui et al., 2010).

Mutations in *cstA* are affected in cyst cell development

Since the predicted *cstA* gene product of *A. brasilense* showed sequence similarity with hybrid TCSs which were found to be involved in cyst formation, wild-type and *cstA* mutants were analyzed for cyst formation in wet mounts with cells grown on cyst inducing malate medium (MSM). Microscopic analysis showed that *cstA* mutants except Δ*HK* mutant, developed mature cyst cells more quickly than the wild-type Sp7 (Figure 2A).

At day 3, the Δ*RR* mutant showed a mixture of vegetative and cyst types, while the other mutants and the wild-type Sp7 exhibited only vegetative cells. At day 6, mutants Δ*RR* and Δ*(HK-RR)::Km* exhibited large quantities of multicelled cyst cluster. At day 9, *A. brasilense* wild-type Sp7 had a mixture of vegetative and cyst types, while Δ*RR* and Δ*(HK-RR)::Km* exhibited large quantities of cyst clusters indicative of mature cysts, while Δ*HK* and Δ*PAS::*Ω have a moderate cyst phenotype with

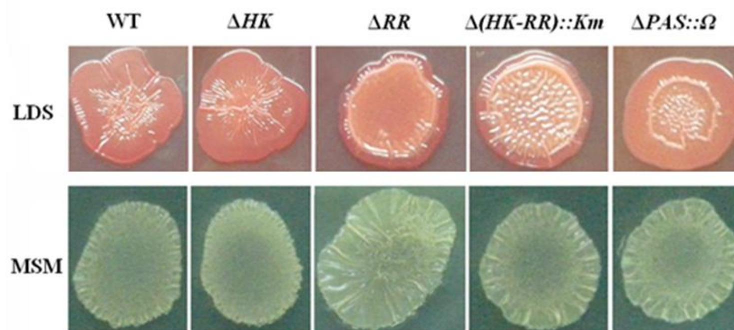

Figure 3. The *cstA* mutants are affected in colony morphologies. Colony morphologies of the wild-type and the *cstA* mutants observed on LDS solid agar plates containing Congo Red (top) or MSM medium (bottom), after 3 days of incubation.

the majority of oblong-shaped cells containing intracellular PHB granules and a small percentage of nature cyst cells.

To quantify the amount of mature cyst cell formation, *A. brasilense* wild type and mutants were subjected to desiccation resistance analysis after 6 days of growth on cyst-inducing MSM medium, according to the previous method (Sadasivan and Neyra, 1987; Berleman et al., 2004). Under these conditions, only 18% of the cells from wild type survived 3 days of desiccation. Survivability was increased in the *cstA* mutants, ranging from 23% of the total viable cell count in Δ*HK*, 37% in Δ*PAS::Ω*, 47% in Δ*(HK-RR)::Km*, to 79% in Δ*RR*. These observations indicated a 1.3-, 2.1-, 2.6-, to 4.4-fold elevation in cyst formation in these mutants (Figure 2B). The Δ*RR* can produced cyst cells in shorter time compared with wild type and other mutants. These results were consistent with changes in cellular morphology of the *A. brasilense cstA* mutants during cyst induction.

cstA mutants are affected in colony morphology

The exopolysaccharides (EPS) on cell surface are essential for *A. brasilense* cells to aggregate, to form large clumps, to flocculate and to differentiate into cyst-like forms (Valverde et al., 2006). Congo red-binding EPS has been reported to be correlated with the ability of cells to flocculate under certain conditions (Sadasivan and Neyra, 1985). Thus, we compared *cstA* mutants with the wild type for the ability of colonies to bind the Congo red dye. We noticed differences in the appearance of colonies formed by the *cstA* mutant strains in comparison with the wild type (Figure 3). Wild type formed wrinkled colonies after 3 days of growth on the nutrient-rich medium (LDS) containing Congo red, while mutants formed wrinkled and ridged colonies. In detail, the Δ*HK* has a tiny ruffle around the colony similar to the wild-type; the Δ*RR* and Δ*(HK-RR)::Km* have the most marked wrinkles on the surface; while Δ*PAS::Ω* has a more obvious ruffle around the colony than the Δ*HK* and wild-type colonies. But the colonies of mutants have not clear differences with the wild type in colony color. So the ability of mutants and wild type to bind the Congo red dye is similar. These observations suggest that the mutants are not affected in the amount of production of at least some Congo red-binding EPS.

Even on the agar-solidified cyst-inducing MSM medium, *cstA* mutants exhibited difference from wild type in colony morphology (Figure 3). After 3 days of growth on the MSM medium, colonies of *cstA* mutants Δ*RR* and Δ*(HK-RR)::Km* , and Δ*PAS::Ω* are more wrinkled than those of wild type, which are features typical of mature cyst cells (Berleman et al., 2004; Berleman and Bauer, 2005). These results indicated that *cstA* was probably involved in cyst cell development.

cstA mutants are affected in nitrogenase activity

Previous studies indicated that *A. brasilense* mutants that were impaired in cyst formation affected the efficiency of root colonization and nitrogenase activity (Katupitiya et al., 1995; Pereg et al., 2000). In present study, nitrogenase activity of *cstA* mutants and wild-type were comparably studied (Figure 4). The mutant Δ*RR* showed a decrease in nitrogenase activity compared to wild type, while nitrogenase activity of Δ*(HK-RR)::Km* and Δ*HK* were similar to those of wild type, Δ*PAS::Ω* has a higher nitrogenase activity than those of wild type. These observations were consistent with the differences of these mutants in cyst formation. Our results indicated that cyst development may impaired nitrogenase activity as expected from non-vegetative, cyst cells and are consistent with the previous studies (Eskew et al., 1977).

DISCUSSION

Protein sequences analysis showed that the C-terminal

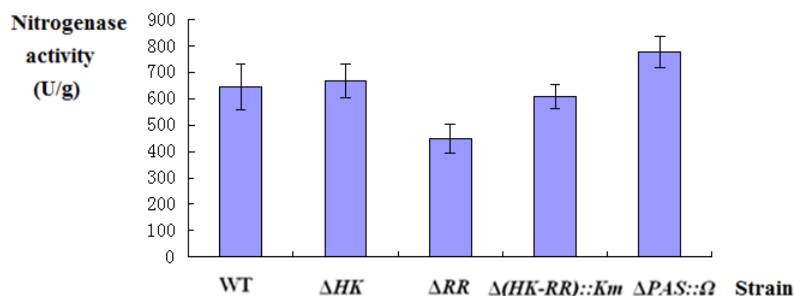

Figure 4. Nitrogenase activities of the *cstA* mutants and Sp7.

region of CstA predicted product has structural and sequence similarity to the CstS1 of *R. centenum* (Cui et al., 2010). The CstS1 was a hybrid TCS protein containing a C-terminal sensor HK-RR hybrid domain and a N-termainl GAF region (Berleman et al., 2004). Our results showed that the Δ*HK* or Δ*(HK-RR)::Km* mutant which break down the HK domains or totally destroyed the CstA can elevate cyst formation in varying degrees. Moreover, the in-frame deletion mutant Δ*RR*, which only broke down the RR domain, has the most prominent effect on regulating the timing of cyst cells formation. All these experimental results indicated that the *A. brasilense* CstA may be a CstS1-like protein, involving the regulation of cyst development and the RR domain played an inhibitory effect on the CstA activity in cyst cells development. This phenomenon had been observed in *M. xanthus* FrzE, a CheA-CheY fusion chemotaxis protein, in which removal of the receiver domain can induce the FrzE kinase domain autophosphorylation. So the RR domain of FrzE negatively regulated the CheA histidine kinase activity and the downstream signaling to the A- and S-motility systems (Inclan et al., 2008). Other hybrid kinase/response, such as ArcB and VirA, had been shown to function with an inhibitory receiver domain, so removal of the RR domain from them appeared to increase their activities (Chang et al., 1996; Iuchi and Lin, 1992). Thus the information confirmed that the receiver domain in these proteins had similar receiver modules, and genetic ablation of these modules caused acceleration effect on the kinase activity and thus may function to a more efficient response to environmental stimuli.

CstA lacks any DNA-binding domain, thus we suggested that other proteins are involved in a multi-step phosphorelay signal transduction pathway (more-complex types of two-component-based systems), and a protein can be interacted with a regulated promoter after phosphorylation. Usually the sensor and response regulator are adjacent, and as expected, a region flanking *cstA* may be a likely partner response regulator gene. Recently, the upstream sequences of *cstA* was confirmed and encoded a sensor protein CstB harboring a CheB-CheR fusion domain and two PAS domains (Wu et al., 2011). Our assay showed that *cstB* affected cyst cell development. And studies confirmed that *cstB* and *cstA* were cotranscribed by means of RT-PCR analysis (Wu et al., 2011). These observations thus suggested that *cstB* and *cstA* were involved together in cyst development.

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