Azorhizobium caulino dons c-di-GMP phosphodiesterase Chp1 involved in motility, EPS production, and nodulation of the host plant

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Abstract
Establishment of the rhizobia-legume symbiosis is usually accompanied by hydrogen peroxide (H₂O₂) production by the legume host at the site of infection, a process detrimental to rhizobia. In Azorhizobium caulino dons ORS571, deletion of chp1, a gene encoding c-di-GMP phosphodiesterase, led to increased resistance against H₂O₂ and to elevated nodulation efficiency on its legume host Sesbania rostrata. Three domains were identified in the Chp1: a PAS domain, a degenerate GGDEF domain, and an EAL domain. An in vitro enzymatic activity assay showed that the degenerate GGDEF domain of Chp1 did not have diguanylate cyclase activity. The phosphodiesterase activity of Chp1 was attributed to its EAL domain which could hydrolyse c-di-GMP into pGpG. The PAS domain functioned as a regulatory domain by sensing oxygen. Deletion of Chp1 resulted in increased intracellular c-di-GMP level, decreased motility, increased aggregation, and increased EPS (extracellular polysaccharide) production. H₂O₂-sensitivity assay showed that increased EPS production could provide ORS571 with resistance against H₂O₂. Thus, the elevated nodulation efficiency of the Δchp1 mutant could be correlated with a protective role of EPS in the nodulation process. These data suggest that c-di-GMP may modulate the A. caulino dons-S. rostrata nodulation process by regulating the production of EPS which could protect rhizobia against H₂O₂.

Keywords C-di-GMP · Azorhizobium caulino dons ORS571 · Chp1 · Sesbania rostrata

Introduction
Legumes have the ability to establish a symbiotic interaction with N₂-fixing rhizobia resulting in the formation of root nodules. Within nodules, internalized rhizobia can find suitable environmental conditions (with respect to oxygen concentrations) to fix atmospheric nitrogen which can be subsequently used for plant nutrition. Legume-rhizobia interaction is initiated by signal exchange between bacteria and plant (Dénarié et al. 1996; Oldroyd 2013). After perceiving flavonoid compounds exuded by the plant root, rhizobia can produce specific molecules named “Nod factors” which are recognized by the host and turn on the plant nodulation program. The next rhizobial invasion can proceed using different routes; the best known being the intracellular invasion via root hairs, with the formation of an infection thread (Goormachtig et al. 2004a). However, another type of invasion (intercellular invasion) which occurs at bases of lateral or adventitious roots is far less characterized. This particular mode of infection is found in the case of the Azorhizobium caulino dons-Sesbania rostrata interaction, and this interaction has been a classic model for the study of intercellular invasion (Capoen et al. 2010; Goormachtig et al. 2004b; Lievens et al. 2001). A. caulino dons ORS571 is a versatile nitrogen fixer which could fix nitrogen not only in free-living state under microaerobic conditions but also in symbiotic interaction with...
S. rostrata (Lee et al. 2008). Nitrogen-fixing nodules formation by A. caulino dons ORS571 through intercellular invasion requires the formation of the outer cortical infection pocket which is characterized by release of a massive amount of hydrogen peroxide (H₂O₂) (D’Haeze et al. 2003). In addition, oxidative burst and accumulation of H₂O₂ also exists in the process of root hair invasion (Santos et al. 2001). It was shown that H₂O₂ played a positive role in the infection processes and was required for the successful establishment of final symbiosis (D’Haeze et al. 2003; Jamet et al. 2007). However, since H₂O₂ is detrimental to bacteria, it is of great importance for rhizobia to develop survival mechanisms during the process of nodulation.

Depending on physiological/environmental conditions, bacteria can be embedded in an extracellular matrix composed of complex extracellular polymeric substances including extracellular polysaccharides (EPSs) (Flemming and Wingender 2010). EPSs could confer survival advantages by protecting bacteria from various biotic and abiotic stresses including oxidative stress (Perez-Mendoza and Sanjuan 2016). As a major part of the extracellular matrix, EPSs are also involved in cell-cell interactions and surface adhesion (Koo et al. 2013; Matthysse 2014). Two gene clusters involved in EPS synthesis, exp cluster I (AZC_1831-1834) and exp cluster II (AZC_3319-3332), are present in A. caulino dons ORS571. These two clusters are both corresponding to the exp gene clusters of Sinor hizobium meliloti, which have been analysed extensively (Tsukada et al. 2009). The AZC_1831 (oac3) and AZC_1832 (oac2) genes of the exp cluster I are encoding dTDP-d-glucose synthase and dTDP-L-rhamnose synthase enzymes, respectively, and their involvement was demonstrated in the synthesis of EPSs essential for successful nodulation on its legume host S. rostrata (Gao et al. 2001; Goethals et al. 1994).

The secondary messenger c-di-GMP (cyclic bis-(3’-5’) dim 4-ric guanosine monophosphate) has been extensively studied for its multiple regulatory functions including involvement in cell motility, cell aggregation, biofilm formation, and virulence (Galperin 2004; Hengge 2009) as well as in regulation of EPSs synthesis (Liang 2015; Perez-Mendoza and Sanjuan 2016). Generally, high levels of c-di-GMP stimulate the production of various exopolysaccharides. c-di-GMP is synthesized by diguan ylate cyclases (DGCs) from two molecules of GTP and hydrolysed by phosphodiesterases (PDEs) into 5’-phosphoguananylyl-(3’-5’)guanosine (pGpG) (Chan et al. 2004; Rao et al. 2008). DGC activity is associated with a domain containing a conserved motif of amino acids “GGDEF”, and PDE activity is associated with a domain containing the motif “EAL” (Ryjenkov et al. 2005; Schmidt et al. 2005; Tal et al. 1998). Both motifs (GGDEF and EAL) are essential for the enzymatic activity being parts of the enzyme active sites. In addition, the GGDEF and EAL domains are often accompanied by regulatory domains such as PAS, REC, and GAF (Kazmierczak et al. 2006; Lee et al. 2007). These regulatory domains could respond to specific intracellular or external signals to regulate the activity of DGCs and PDEs. As a second messenger, c-di-GMP must be recognized by different receptors to perform its regulatory role. The receptors of c-di-GMP are also various including the well-known PilZ domain, degenerate GGDEF and EAL domains, and some c-di-GMP-specific riboswitches (Krsteva et al. 2012).

In this study, we identified a c-di-GMP PDE named Chp1 (c-di-GMP hydrolysis protein 1) in A. caulino dons ORS571. The Δchp1 mutant had decreased motility, increased EPS production, increased resistance against H₂O₂, and increased nodulation efficiency on the legume host S. rostrata. The elevated nodulation efficiency suggests a protective role of EPS against hydrogen peroxide in the nodulation process which favours our conclusion that c-di-GMP may modulate the nodulation process by regulating the EPS production.

Materials and methods

Bacterial strains, plasmids and culture medium

The strains and plasmids used in this study are listed in Supplemental Table S1. The wildtype (WT) A. caulino dons ORS571 (ATCC 43989) and mutants were grown at 37 °C in TY medium (tryptone 10 g/L, yeast extract 5 g/L, CaCl₂ · 2H₂O 4 g/ L, and agar 1.5% for plate) with corresponding antibiotics (ampicillin 100 μg/mL, nalidixic acid 25 μg/mL, gentamycin 50 μg/mL). L3 minimal medium (KH₂PO₄ 1.36 mg/mL, MgSO₄ · 7H₂O 100 μg/mL, NaCl 50 μg/mL, CaCl₂ · 2H₂O 40 μg/mL, FeCl₃ · 6H₂O 5.4 μg/mL, Na₂MoO₄ · 2H₂O 5 μg/ mL, biotin 2 μg/mL, nicotinic acid 4 μg/mL, pantothentic acid 4 μg/mL, succinate 1.18 mg/mL as carbon source, and supplemented with 10 mM NH₄Cl or not for L3+N/-N) was also used when mentioned. The Escherichia coli strains used for cloning and expression were routinely grown at 37 °C in LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and agar 1.5% for plate). In addition, different kinds of antibiotics (ampicillin 100 μg/mL, kanamycin 25 μg/mL, gentamycin 50 μg/mL, tetracycline 10 μg/mL) were added to the LB medium to select the bacterial strains with correct plasmids.

Protein expression and purification

The expression plasmid used in this study is pET30a with an engineered N-terminal His6-SUMO tag (named pET30a-SUMO). The DNA fragments corresponding to the PAS, GGDEF, EAL domain, and the full-length Chp1 (BAF86306.1) were amplified from the ORS571 genomic DNA with primer pairs PAS-BamHI-F/XhoI-R, GGDEF-BamHI-F/XhoI-R, EAL-BamHI-F/XhoI-R, and Chp1-BamHI-F/XhoI-R, respectively. The fragments were cloned into the BamHI/XhoI site of pET30a-SUMO to create recombinant expression plasmids and then be transformed into the expression strain E. coli BL21 (DE3). The PASH45A and Chp1H45A mutants (H45 was labelled by an
asterisk in Fig. 1c) were generated by using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) with primers pairs PASH45A-F/R and Chp1H45A-F/R, respectively. Proteins were overexpressed by induction with 50 μmol/L IPTG at 16 °C for 20 h. The harvested cells were resuspended in binding buffer (50 mmol/L Tris, 100 mmol/L NaCl, 10 mmol/L imidazole, pH 8.0) and lysed by sonication. The debris was removed by centrifugation and the supernatant was collected and loaded on a His-trap FF crude column (GE Healthcare, Wauwatosa, USA). After removing impurities by washing buffer (50 mmol/L Tris, 100 mmol/L NaCl, 100 mmol/L imidazole, pH 8.0), the target protein was harvested by elution buffer (50 mmol/L Tris, 100 mmol/L NaCl, 500 mmol/L imidazole, pH 8.0). The imidazole present in protein solution was then removed by using the Amicon® Ultra-15 10 K centrifugal filter device (Merck KGaA, Darmstadt, Germany), and the protein was finally dissolved in storage buffer (20 mmol/L Tris, 5 mmol/L EDTA, 5% glycerol, pH 8.0). For the final purified target protein, the purity was analysed by using the SDS-PAGE, the concentration was quantified by using the Nanodrop 2000c (Thermo Fisher Scientific, Waltham, USA) combined with the standard BCA protein assay (Pierce, Rockford, USA).

**Absorption spectroscopy** The absorption spectra were all performed in reaction buffer (50 mmol/L Tris, 100 mmol/L NaCl, pH 8.0) and measured by using the UV-Vis spectrophotometer of the Nanodrop 2000c (Thermo Fisher Scientific, Waltham, USA). The apo-PAS and apo-PASH45A were measured first

**Fig. 1** Chp1 schematic representation, purification, and sequence alignment. 
(a) Transcription directions are indicated by the arrows, and the chp1 gene (AZC_0308) is flanked by two genes with different transcription directions (AZC_0307 and AZC_0309). Domain structures of Chp1 were predicted by using the SMART program. Abbreviations: PAS, Per-Arnt-Sim domain; GGDEF, diguanylate cyclase domain; EAL, phosphodiesterase domain. 
(b) Proteins with SUMO tag: Chp1 (residue 1–566, 74.3 kDa), EAL domain (residue 322–564, 41.6 kDa), GGDEF domain (residue 132–320, 35.2 kDa), PAS domain (residue 20–120, 25.8 kDa). 
(c) Sequence alignment of PAS domain of the Chp1 with two other heme-binding PAS domains from different proteins. A. cau, Azorhizobium caulinodans; C. cre, Caulobacter crescentus; Dos (GI 1036410138); A. xyl, Acetobacter xylinus; P. aeru, Pseudomonas aeruginosa; RoaR (GI 1176904427). The asterisk indicates the key residue histidine responsible for heme binding. Sequence alignment of GGDEF domains. A. cau, Azorhizobium caulinodans; C. cre, Caulobacter crescentus, DgcA (GI 221236330); A. xyl, Acetobacter xylinus, Dgc1 (GI 3659615); C. cre, Caulobacter crescentus, PdeA (GI 221236443); P. fluo, Pseudomonas fluorescens, WspR (GI 18535672). The asterisks indicate the conserved GGDEF motif. Sequence alignment of EAL domains. A. cau, Azorhizobium caulinodans; E. coli, Escherichia coli, Dos (GI 1036410138); C. cre, Caulobacter crescentus, PdeA (GI 221236443); K. pneu, Klebsiella pneumoniae, BlpR1 (GI 241913377); P. aeru, Pseudomonas aeruginosa, RoaR (GI 1176904427). The asterisks indicate the conserved EAL motif. The backgrounds of conserved residues are labelled black and grey according to their conservation degree.
in reaction buffer. Hemin (Sigma-Aldrich, Munich, Germany) was dissolved in 100 mmol/L NaOH, and aliquots were added into the sample of blank buffer, the sample containing 2 μmol/L purified PAS protein, 2 μmol/L purified PASH45A protein and 50 μmol/L haemoglobin (Sigma-Aldrich, Munich, Germany), respectively. Purchased haemoglobin was used here as positive control. The mixed samples were then incubated at room temperature for 30 min before being measured. Absorption spectra between 250 and 800 nm were recorded, and the absorption spectra were analysed.

**Enzymatic activity assays by HPLC-MS** The in vitro purified GGDEF domain and EAL domain proteins were assayed for the diguanylate cyclase (DGC) activity and the phosphodiesterase (PDE) activity as described previously with some modification (Russell et al. 2013). HPLC-MS was used to detect the level of c-di-GMP present in the reaction buffer. The DGC and PDE activity were all investigated in the same reaction buffer (25 mmol/L Tris, 250 mmol/L NaCl, 10 mmol/L MgCl₂, pH 7.9). For DGC activity assay, 2 μmol/L purified protein and 10 μmol/L GTP were added and the reaction mixture was incubated at 37 °C for 2 h. For PDE activity assay, 2 μmol/L purified protein and 10 μmol/L c-di-GMP were added and incubated at 37 °C for 2 h.

Purified apo-Chp1 and apo-Chp1H45A protein were first titrated with hemin. Hemin was dissolved in 100 mmol/L NaOH, and aliquots of solution were added to protein solutions. The absorptions of protein solutions were read at 411 nm by Nanodrop 2000c after each addition, and the titrations were completed when the absorptions remained constant. The deoxy proteins were prepared by adding 10 μmol/L dithiothreitol to reconstituted proteins solutions which were flushed with nitrogen gas in a glovebox. The oxy proteins were then prepared by treating the deoxy proteins with O₂. For anaerobic and aerobic conditions, the reactions were performed in glovebox and air, respectively. For investigation of NO regulation, the deoxy proteins were treated with NO, and all reactions were performed in the glovebox. To investigate the GGDEF domain regulation of neighbouring EAL domain by binding GTP, 10 μmol/L GTP was added into reaction buffer. After 30 min incubation at 37 °C, all reaction mixtures were stopped by boiling for 5 min and then centrifuged at 15,000g for 5 min to remove any debris. The supernatant was then measured by HPLC-MS with a reverse phase Hypersil GOLD™ C18 column (3 μm particle size, Thermo Fisher Scientific, Waltham, USA) according to procedures reported previously. The mobile phase consisted of solvent A: 15 mmol/L acetic acid in 97% water and 3% methanol and solvent B: 100% methanol. Then, a 50-min gradient was created by these solvent as follows: t = 0 min, 100% solvent A; t = 5 min, 100% solvent A; t = 10 min, 80% solvent A; t = 15 min, 80% solvent A; t = 30 min, 35% solvent A; t = 33 min, 5% solvent A; t = 38 min, 100% solvent A; t = 50 min, 100% solvent A. In addition, the column temperature was 25 °C, the inject volume was 10 μL, the flow rate was 200 μL/min and the UV absorbance of c-di-GMP was measured at 254 nm. Under these chromatography conditions, c-di-GMP had a retention time of 18.70 min, pGpG (the product of PDE activity) had a retention time of 18.37 min and GTP had a retention time of 9.56 min. Accurate mass measurement of the c-di-GMP, pGpG, and GTP in negative ion mode was made on tandem quadrupole mass spectrometer LCQ Fleet (Thermo Fisher Scientific, Waltham, USA).

**Construction of the mutants** The gentamycin substitution mutants were constructed by using the allelic exchange vector pCM351, a vector carrying cre/lox system and a gentamycin cassette (Marx and Lidstrom 2002). DNA fragments flanking upstream and downstream of each gene were amplified from the genomic DNA of ORS571 and cloned into pCM351. The recombinant vectors were introduced into A. caulinodans ORS571 by tri-parental conjugation with the helper plasmid pRK2013. Subsequent allelic exchange between ORS571 chromosome and the recombinant plasmid led to the substitution of target genes with gentamycin. The resulting gentamycin substitution mutants were further confirmed by PCR with corresponding primer pairs. The chp1 gene and oac cluster deletion mutants were generated by using the suicide vector pK18mobSACB, a vector carrying a sacB gene conferring sensitivity to sucrose which allows selection for vector loss (Schäfer et al. 1994). To construct the deletion mutants, upstream fragments and downstream fragments of the target genes were linked in tandem before inserted into the suicide vector pK18mobSACB. The following procedures to construct deletion mutants were the same with substitution mutants. The primers used in the construction are all listed in Supplemental Table S2.

**Extraction and quantification of intracellular c-di-GMP** The extraction of intracellular c-di-GMP was performed as described by Russell et al. (2013). The concentration of extracted c-di-GMP was analysed by HPLC-MS and normalized with the amount of whole cell protein.

**Motility assay** The motility assay was performed as previously described with some modification (Vicario et al. 2015). The A. caulinodans ORS571 and mutants grown in TY medium to mid-log phase were washed with L3-N minimal liquid medium and normalized to an OD₆₀₀ of 1.0. Aliquots of 5 μL of bacterial suspensions were inoculated at the centre of TY and L3+L3-N minimal soft agar plates (agar 0.3%). The inoculated soft agar plates were then incubated for 48 h at 37 °C before the swimming ring of strains being measured. For microscopic observation, an aliquot of 5 μL of bacterial suspension was added to a microscope slide, and the swimming...
behavior was recorded using cellSens Dimension 1.7 imaging software on an Olympus BX53 system microscope (Olympus Optical Ltd., Tokyo, Japan) as previously described (Liu et al. 2017b). The swimming velocity of each strain was then calculated by tracking at least 30 cells on video recordings.

**Aggregation assay** The aggregation assay was performed as previously described (Jiang et al. 2016).

**Quantification of EPS production** The EPS produced by ORS571 and mutants were measured based on the method described by Nakajima et al. (2012). The Congo red plates were used as qualitative assay of EPS production. The colonies grown on plates without Congo red were processed to quantitatively determine the EPS production.

**H₂O₂ sensitivity assay** The sensitivity assay was performed based on the previous reported method (D’Haese et al. 2004). Wild type and mutants were grown in L3+N/L3-N medium overnight to mid-log phase and then normalized to an OD₆₀₀ of 1.0. To remove the EPS, strains were washed by λ buffer (10 mmol/L Tris, 10 mmol/L MgSO₄, pH 7.0) and then washed with minimal liquid medium twice. The unwashed and washed cell suspensions were untreated or incubated with 10 mmol/L H₂O₂ at room temperature for 20 min. After incubation, cells were diluted serially and 100 μL aliquot of each dilution was plated onto TY plates to count the CFU (colony forming unit). The viability of cells treated with H₂O₂ was then calculated.

**Plant growth and bacterial inoculation** The seeds of *S. rostrata* were treated with concentrated sulphuric acid for 30 min and washed three times with sterile water. The seeds were then soaked in sterile water on petri dish and incubated in the dark at 37 °C for 2 days. After germination, the seedlings were planted in Leonard jars full of water. For single bacterial nodulation, the germinated seeds were inoculated with cell mixtures containing wild type and mutants. For competitive nodulation, the germinated seeds were inoculated with cell mixtures of wild type and mutants at a 1:1 ratio. Then, the cell mixtures were inoculated onto the germinated seedlings. All plants were grown in greenhouse, and the root nodules were harvested after 3 weeks.

**Acetylene reduction activities (ARAs) assay** The assay was performed by using the protocol developed by Akiba et al. (2010) with modifications. For measurement of ARAs under symbiotic state, the root nodules of plants inoculated with a single strain were used. For measurement of ARAs of strains under free-living state, bacteria were cultivated under microaerobic conditions. The acetylene reduction activities were measured by using gas chromatography 7890A (Agilent Technologies, Santa Clara, USA) and defined as C₂H₄ production/h/g of fresh nodules and C₂H₄ production/h/g of protein, respectively.

**RNA isolation and qRT-PCR analysis** Overnight grown cells of ORS571 and the mutant Δchp₁ were inoculated into fresh TY liquid medium with or without 2 mmol/L H₂O₂ and grown for another 5 h. The cells were harvested by centrifuging at low temperature and quickly frozen with liquid nitrogen before RNA isolation. RNA was isolated by using the SV Total RNA Isolation System (Promega, Madison, USA) according to the manufacturer’s instructions. The isolated RNA was then used as template for the synthesis of cDNA with the GoScript™ Reverse Transcription System (Promega, Madison, USA). For RT-PCR analysis, the GoTaq® qPCR Master Mix (Promega, Madison, USA) was used and the cDNA was diluted differently to determine the expression level of 16S rRNA and other genes of interest. Primer pairs used in RT-PCR analysis are all listed in Supplemental Table S2.

**Results**

**GGDEF and EAL domains of A. caulinodans ORS571**

The whole genome sequence of *A. caulinodans* ORS571 had been determined earlier (Lee et al. 2008). According to the prediction of SMART database (http://smart.embl.de/), there were a total of 37 proteins carrying GGDEF and/or EAL domains in ORS571, including 23 and 4 proteins containing GGDEF and EAL domains alone, respectively, and 10 composite proteins containing both domains (Supplemental Fig. S1). In addition, there were different regulatory domains, such as PAS, GAF, REC, and PAC, present in 14 proteins. Previous studies have shown that these domains are responsible for binding of signal and regulating function of proteins (Bouret RB 2010; Heikaus et al. 2009; Henry and Crosson 2011). Therefore, six proteins containing characteristic combination of these regulatory domains were selected. The encoding genes were AZC_0308, AZC_3085, AZC_3226, AZC_4658 (encoding four composite proteins) and AZC_2412 and AZC_2765 (encoding two GGDEF single domain proteins), respectively. The gentamycin resistance substitution mutants of selected genes were constructed to investigate their functions. As shown in Supplemental Fig. S2, the swimming motility of the mutant 0308::gent was impaired most seriously compared with other mutants. In particular, high level of c-di-GMP was reported to suppress cell motility (Simm et al. 2004). The impaired motility of the mutant 0308::gent may suggest the function of AZC_0308, and this gene was subjected to further investigation.

**The chp1 gene is encoding a multidomain protein related to c-di-GMP metabolism**

Since the protein encoded by the gene AZC_0308 was named as Chp1 (c-di-GMP hydrolysis protein 1) in the introduction...
part, the gene AZC_0308 was renamed as chp1 herein for convenience. As shown in Fig. 1a, the chp1 gene is flanked by two genes AZC_0307 and AZC_0309 encoding a polyhydroxyalkanoate synthesis repressor and a protein of unknown function, respectively. According to prediction of the SMART program, the chp1 gene encodes a multidomain protein containing putative PAS, GGDEF, and EAL domains (Fig. 1a). To further characterize Chp1, gene portions encoding the three domains of Chp1 as well as the full-length Chp1 were subcloned to overexpress the respective polypeptides products for purification (Fig. 1b) and in vitro assay of their individual catalytic activity.

Chp1 is a c-di-GMP PDE and it is devoid of DCG activity

GGDEF and EAL domains have long been identified as enzyme domains catalysing the synthesis and hydrolysis of c-di-GMP, respectively. Emerging evidences support the hypothesis that proteins containing both GGDEF and EAL domains have variable functionality. Several composite proteins have been reported to have only one function, either cyclase or esterase (Garcia et al. 2004; Kuchma et al. 2007; Weber et al. 2006). But, other composite proteins were shown to be bifunctional enzymes (Ferreira et al. 2008; Tari et al. 2006).

The protein sequence of the putative Chp1 GGDEF domain was aligned with other GGDEF domains (Fig. 1c). The highly conserved GGDEF (often GGEEF) motif is known to constitute the active site of the enzyme responsible for binding of substrates (Jenal and Malone 2006). Alignment data revealed a degenerate motif containing SGNKF residues showing a poor conservation with the consensus sequence, suggesting that Chp1 may not have DGC activity. This was indeed the case. The DGC activity of the purified GGDEF domain and full-length Chp1 protein were assayed with GTP as substrate, but there was no production of c-di-GMP and only the substrate GTP were detected by HPLC-MS (Supplemental Fig. S3).

Multiple sequence alignment of the putative EAL domain shown that the key residues in the motif of Chp1 EAL domain were fully conserved (Fig. 1c). The PDE activity of purified EAL domain was assayed with c-di-GMP as substrate, which led to production of significant amounts of pGpG in the reaction buffer after incubation, suggesting that c-di-GMP was hydrolysed into pGpG (Fig. 2). The results indicate that Chp1 is a phosphodiesterase with a functional EAL domain. Previous studies have shown that the catalytically inactive neighbouring GGDEF domain could allosterically regulate the activity of EAL domain by binding to its substrate GTP (An et al. 2010; Christen et al. 2005). To explore the possible regulatory role of Chp1 protein “GGDEF” domain, the activity of the purified full-length Chp1 protein was assayed. However, the PDE activity of Chp1 was not affected by the addition of GTP (Supplemental Fig. S4), suggesting that the GGDEF domain did not have a regulatory role under the conditions of our assay.

The activity of Chp1 is regulated by the oxygen-sensing PAS domain

PAS domains, which are widely distributed in proteins from different species, sense changes in oxygen concentrations, light, redox potential (Taylor and Zhulin 1999). A variety of ligands are known for PAS domains, such as heme, FAD, metal ion, and even specific dicarboxylic acids (Cho et al. 2006; Gong et al. 1998; Rebbapragada et al. 1997; Zhou et al. 2008). PAS domains are commonly located at the N-terminus of signalling proteins such as histidine kinases, methyl-accepting chemotaxis proteins, and c-di-GMP synthases/hydrolases (Galperin 2004). The E. coli Dos protein is a c-di-GMP PDE containing a PAS domain binding heme (Nixon et al. 2000). The sensor histidine kinase FixL of rhizobia is also known to sense oxygen via a heme ligand of its PAS domain (Key and Moffat 2005). The protein sequence of Chp1 PAS domain was then aligned with PAS domains of Dos and FixL. Similarity between the aligned sequences was limited (<12% identity), but the histidine residue required for heme binding was fully conserved (labelled with an asterisk in Fig. 1c). To confirm the role of histidine at position 45 in heme binding, the mutant PAS45A and Chp1H45A were constructed by replacing histidine with alanine (Supplemental Fig. S5).

Heme-binding activity was investigated using purified Chp1 PAS domain protein and mutant PASH45A, and the UV-Vis spectra were analysed. The fresh purified PAS and PASH45A showed no trace of heme, which suggested that the PAS domain was purified primarily as an apoprotein (Fig. 3a). After heme addition to the preparation, the spectrum of PAS revealed a specific band corresponding to heme binding with an absorption at 411 nm, similar to what appeared with the positive control haemoglobin (Fig. 3a) (Sekine et al. 2016). However, the absorption peak of PASH45A with heme was similar to that of the free heme. The spectrometric analysis confirmed that the PAS domain could bind heme by His45, indicating that the heme-binding PAS domain may regulate the function of Chp1.

To further document the regulatory role of the PAS domain, purified apo-proteins were first reconstituted with hemin, deoxy, and oxy proteins were then prepared. As shown in Fig. 3b, the amount of c-di-GMP hydrolysed by oxy-Chp1 was about 30% of deoxy-Chp1 within the same time, which meant that the PDE activity of Chp1 was greatly reduced when oxygen bound to heme. As negative controls, the PDE activities of EAL single domain protein and Chp1H45A showed no difference in anaerobic and aerobic conditions. In addition to oxygen, heme could also bind nitric oxide and carbonic oxide (Gilles-Gonzalez et al. 2005). Furthermore, there is nitric oxide production in the process of legume-
rhizobia symbiosis (Nagata et al. 2008). Thus, the nitric oxide was tested for its potential regulatory role. However, the PDE activities of all assayed proteins with nitric oxide were about the same with the activities in anaerobic conditions (Supplemental Fig. S6). These results suggested that the heme-binding PAS domain could sense oxygen and regulate the activity of Chp1.

The Δchp1 mutant shows increased intracellular c-di-GMP level, decreased motility, increased aggregation, and EPS production

Construction of a full-length chp1 gene deletion mutant was achieved to identify phenotypic changes between the wild type and the mutant strain. The intracellular c-di-GMP level was first determined. As shown in Fig. 4a, the intracellular c-di-GMP concentration of Δchp1 mutant was about 1.6-fold higher than WT, and the result confirmed that Chp1 was truly a functional PDE in ORS571.

In addition, several phenotypes usually associated with second messenger c-di-GMP were assayed. Swimming motilities of the wild type and the Δchp1 mutant were examined on 0.3% soft agar plates of both rich medium (TY) and minimal medium (L3) containing or not a nitrogen source (+N, -N). As shown in Fig. 4b, the swimming motility of Δchp1 was apparently impaired on all three kinds of medium tested. Also, the impaired motility of Δchp1 was in accordance with mutant 0308::gent. On TY plates, the swimming diameter of the Δchp1 mutant was about 60% of the wild type. The swimming diameters of the Δchp1 mutant on L3+N and L3-N plates were 75% and 68% of the wild type, respectively. In addition, the impaired swimming motility of Δchp1 mutant was also confirmed by microscopic observations (Supplemental Fig. S7).

c-di-GMP was also reported to positively regulate cell aggregation of several bacterial species (such as Clostridium difficile, Pseudomonas aeruginosa) (Purcell et al. 2012; Ueda and Wood 2009). Since A. caulinodans ORS571 is capable of auto-aggregating and forming flocs (Nakajima et al. 2012), the cell aggregation of the wild type and the Δchp1 mutant was measured. Cell aggregation was enhanced over time with both strains and the flocs formed at 24 h were larger than flocs formed at 12 h (Fig. 4c). However, the Δchp1 mutant strain showed superior aggregation ability than the wild type, both after 12 h (aggregation ratio 34% vs 11%) and 24 h (54% vs 16%) incubation.
Increased EPS production confers ORS571 with resistance against H$_2$O$_2$

The process of nodulation is accompanied by a massive production of H$_2$O$_2$ toxic to bacteria (D’Haeze et al. 2003; Santos et al. 2001). Previous data showed that EPSs were associated with protection against reactive oxygen species including H$_2$O$_2$ (Jang et al. 2016; Lehman and Long 2013). Viability of the wild type and the _Δchp1_ mutant strains were compared after 20-min exposure to H$_2$O$_2$. The mutant strain, which is producing more EPS than the wild type, was found more resistant to H$_2$O$_2$ than the wild type, in all the conditions assayed (Fig. 5). In addition, decreased viability was observed when EPS were extensively washed prior the H$_2$O$_2$ treatment confirming the protective role of EPS (Fig. 5). It is assumed that the slightly higher viability of _Δchp1_ mutant (in L3-N washing condition) may result from the residual EPS. EPS-related genes are highly expressed in _Δchp1_ mutant.

EPS production was reported to be regulated by c-di-GMP both at transcriptional and post-translational levels (Hickman and Harwood 2008; Perez-Mendoza et al. 2017). Thus, quantitative RT-PCR was used to determine if c-di-GMP played a role in EPS gene expression in the case of ORS571. For _exp_ gene cluster I (_AZC_1831-1834), all of four genes were selected for analysis (Supplemental Fig. S8a). According to Fig. 6a, the expression levels of _exp_ cluster I genes were at least 10-fold higher in the _Δchp1_ mutant than in the wild type. Among them, the _AZC_1832 was especially highly expressed with 20-fold higher than the wild type. The expression levels of these genes were also analysed in H$_2$O$_2$-treated condition. When both strains were subjected to H$_2$O$_2$ treatment, all four genes of the wild type were expressed to higher extent (2- to 3-fold), indicating that bacteria could respond to external oxidative stress by increasing production of self-protect EPS. The expression levels of genes in the _Δchp1_ mutant did not increase as much as the wild type, and this may result from the increased EPS function as physical barrier against H$_2$O$_2$. Among _exp_ gene cluster II (_AZC_3319-3332), four genes (_AZC_3321, 3322, 3326, 3330) were selected for analysis (Supplemental Fig. S8b). Different from genes of _exp_ cluster _I_, the expression levels of four genes of _exp_ cluster _II_ in the _Δchp1_ mutant only displayed 3-fold increment above the wild type (Fig. 6b).

The EPS produced by _A. caulinodans_ ORS571 has been characterized as a linear homopolysaccharide of α-1, 3-linked 4, 6-O-(1-carboxyethylidene)-d-galactosyl residue (D’Haeze et al. 2004). As mentioned in the introduction section, _AZC_1831 (_oac3_) and _AZC_1832 (_oac2_) of the _exp_ cluster _I_ (named _oac_ cluster) were reported to be involved in the synthesis of EPSs essential for successful nodulation. Moreover, the positive correlation between increased intracellular c-di-GMP level and a high expression level of _oac_ cluster genes in the _Δchp1_ mutant suggested that the _oac_ cluster may be highly

EPS production is reported to be positively regulated by c-di-GMP (Perez-Mendoza and Sanjuan 2016), and Congo red is known to bind to polysaccharides. Congo red staining of the colonies of the _Δchp1_ mutant was stronger than that of the wild type suggesting an increased EPS production by the _Δchp1_ mutant (Fig. 4d). It was previously shown that the genes involved in EPS synthesis in _A. caulinodans_ ORS571 were expressed to higher extent in minimal medium than in rich medium (Tsukada et al. 2009). Consistent with this, the Congo red staining of the colonies of both strains on minimal agar plates were stronger than that on rich agar plates. In addition, the quantitative anthrone-sulphuric acid colorimetric assay used to quantify EPS production of both stains confirmed that the _Δchp1_ mutant produced more EPS than the wild type. The EPS production levels of the _Δchp1_ mutant were about 40%, 25%, and 50% higher than that of the wild type on rich (TY) and minimal media (L3+N and L3-N) plates, respectively (Fig. 4d).
induced by c-di-GMP. In order to further investigate the vital role of oac cluster, an oac cluster deletion mutant Δoac was constructed. As expected, the EPS production of Δoac was less than of the wild type and the corresponding viabilities after H2O2 exposure were also decreased (Supplemental Fig. S9). However, the EPS production of Δoac was still increased when grown on minimal plates (L3+N/-N plates). According to the above results, we could say that the oac cluster I was mainly regulated by c-di-GMP, while the exp cluster II may be mainly regulated by the limitation of nutrients.

The Δchp1 mutant has advantage in nodulation compared to the wild type

The nodulation efficiencies of WT and mutants were then measured to further examine the protective role of EPS during the intercellular invasion. First, Δchp1 and Δoac mutants were inoculated together with the wild type with ratio 1:1 on the plant host S. rostrata, respectively. The quantitative analysis showed that the nodulation efficiency of Δchp1 mutant outcompeted the wild type with about 1.7-fold in numbers of root nodules, while the nodulation efficiency of Δoac was significantly reduced down to 10% of the wild type (Fig. 7a). The positive correlation between EPS production and competitive nodulation efficiency suggested that EPS may play a role in the nodulation process. Next, both mutants and the wild type were inoculated separately on the plant host. The number of nodules formed by the wild type and mutants was not comparable since nodule formation varied from plant to plant, while the size of root nodules could be comparable. As shown in Fig. 7b, the nodule fresh weight per plant of the Δchp1 mutant was heavier than the wild type. But the nodules formed

Fig. 4 The phenotypes of A. caulinodans ORS571 and its mutant Δchp1. a Intracellular c-di-GMP concentration of WT and Δchp1. c-di-GMP was extracted from WT and Δchp1, and the concentration was then analysed by HPLC-MS. b Swimming motility assay. The swimming motility of WT and Δchp1 was detected on 0.3% soft agar plates (TY, L3+N, and L3-N with succinate as carbon resource), and the swimming diameters were measured after 2 days of incubation at 37 °C. c Flocculation assay. Top panel, flocculation conditions of WT and Δchp1 after shaking for 12 h and 24 h in L3+N (with succinate as carbon resource) liquid medium. Bottom panel, quantification of the flocculation percentage. d EPS production assay. Top panel, colonies of WT and Δchp1 grown for 72 h on TY, L3+N, and L3-N (with succinate as carbon resource) Congo red plates, respectively. Bottom panel, quantification of EPS production of different plates. Error bars indicate standard deviations, and asterisks represent significant differences (*P < 0.05; **P < 0.01)
by Δoac were much smaller than the wild type, indicating the reduced fitness caused by the decreased EPS production.

A. caulinodans ORS571 could not only fix nitrogen in nodules formed on S. rostrata, but was also able to fix nitrogen in free-living state under microaerobic conditions (Dreyfus et al. 1988). Thus, the nitrogen fixation efficiencies of strains in symbiotic state and free-living state were assayed using the acetylene reduction test (ARA; see the “Materials and methods” section). Although the ARAs of Δoac had no difference with wild type in free-living state, the ARAs of Δoac in symbiotic state was severely impaired (Fig. 7c, d). Combined with the impaired nodule formation of Δoac, the results further suggested that the EPS played vital roles in both the nodulation process and the symbiosis. The ARAs of the Δchp1 and the wild type were about the same both in symbiotic and free-living state, indicating that the deletion of chp1 genes had no effect on nitrogen fixation efficiency of ORS571. While with higher weight of nodules, the Δchp1 mutant was still superior to the wild type in nitrogen fixation efficiency.

Discussion

Genome-wide screening of A. caulinodans ORS571 revealed 37 proteins containing either a GGDEF or EAL domain or both. Such vast number of c-di-GMP metabolic genes is common in rhizobia, ranging from 51 in Bradyrhizobium japonicum and 21 in S. meliloti (Gao et al. 2014). In S. meliloti Rm2011, only six putative c-di-GMP metabolic genes were proved to have an impact on the intracellular c-di-GMP level (Schüper et al. 2016). Further, the results may suggest a redundancy of GGDEF and EAL domains. For A. caulinodans ORS571, the systematic genome-wide investigation of all c-di-GMP-related genes needs to be further conducted.

As a GGDEF-EAL composite protein, the Chp1 protein is actually a phosphodiesterase with a single EAL domain possessing PDE activity. Previously, the heme-binding PAS domain-containing protein AxPDEA1 provides an example of oxygen regulation of PDE activity (Chang et al. 2001). When the heme iron is coordinated with oxygen, the PDE activity of AxPDEA1 is reduced. In some bacteria, extracellular H2O2 can enter cells and be hydrolysed into H2O and O2 by catalase activity (Seaver and Imlay 2001). In this study, there exists a possibility that the O2 generated from hydrolysis of H2O2 functions as a negative regulatory factor of the Chp1 and leads
to the elevated c-di-GMP levels. The highly expressed EPS-related genes of the wild type when treated with H$_2$O$_2$ may result from the H$_2$O$_2$-caused elevated c-di-GMP levels. Besides H$_2$O$_2$, the NO production in the nodulation process has also been reported, and the NO production was induced transiently in the first 4 h and decreased to its basal level at 10 h (Nagata et al. 2008). Our study showed that NO had no effect on the PDE activity of Chp1. This indicates that the potential regulatory role of NO in the nodulation process may be performed through different mechanisms.

As an important signalling molecule, H$_2$O$_2$ is also involved in many other physiological processes in plants. For example, root hair growth and stomatal closure are all regulated by H$_2$O$_2$ (Cardenas et al. 2008; Pei et al. 2000). The accumulation of H$_2$O$_2$ during the infection process suggests that rhizobia should have a mechanism to deal with H$_2$O$_2$. Previously, the H$_2$O$_2$ scavenging enzyme catalase of rhizobia has been studied for its crucial role in the infection process (Jamet et al. 2003). However, the catalase is accommodated inside of the cells and only functions when H$_2$O$_2$ diffuses into cells (Seaver and Imlay 2001). EPSs are located on the bacterial surface. A previous study has shown that EPSs have H$_2$O$_2$ scavenging ability and could provide the first line of defence against exogenous H$_2$O$_2$ (Gao et al. 2015). Thus, EPSs and catalase may work together to protect rhizobia against H$_2$O$_2$ during the nodulation process. For root hair invasion, the protective function of S. meliloti EPSs against H$_2$O$_2$ has been reported, and EPS may function during the early stage of symbiosis with Medicago species (Lehman and Long 2013). The initial rhizobia invasion is reminiscent of pathogen infection especially in terms of the host-generated oxidative stress (Flint et al. 2016). Previous studies showed that increased c-di-GMP level could confer resistance of the opportunistic pathogen P. aeruginosa against H$_2$O$_2$ which is part of the host immunity system. The resistance is attributed to the c-di-GMP regulated EPSs which function as physical barrier to reduce H$_2$O$_2$ penetration into cell membranes (Chua et al. 2016). In the present work, we come to the conclusion that deletion of the phosphodiesterase Chp1 leads to the elevated intracellular c-di-GMP level. Then, the resulting increased EPS production confers A. caulinodans ORS571 with survival advantage against H$_2$O$_2$ during the infection process (Fig. 8). Finally, the nodulation efficiency of mutant Δchp1 outcompetes the wild type.

Many studies have shown that the c-di-GMP regulation of EPS synthesis functions at different levels. For example, the repression of the EPS synthesis operon by transcriptional regulator FleQ could be relieved by the direct binding of c-di-GMP on the repressor (Hickman and Harwood 2008). The post-translational regulation of c-di-GMP on EPS synthesis...
has also been demonstrated in *S. meliloti*. The EPS synthase BgsA is allosterically activated by c-di-GMP binding to its C-terminal domain (Perez-Mendoza et al. 2017). In this study, the results of qRT-PCR analysis suggest that the c-di-GMP regulation of EPS synthesis in *A. caulinodans* ORS571 may occur partially at the transcriptional level.

Deletion of the *chp1* gene impairs swimming motility while promoting cell aggregation of *A. caulinodans* ORS571. Similarly, the inverse regulation of cell motility and aggregation by c-di-GMP has been reported in many other species (Lee et al. 2010; Purcell et al. 2012; Ueda and Wood 2009). The swimming motility is dependent on bacterial flagellae, and the c-di-GMP regulation functions at different levels including transcriptional level and allosteric level. Transcriptional factors such as FleQ from *P. aeruginosa* and VpsT from *Vibrio cholera* can directly bind c-di-GMP to regulate the expression level of flagellar biosynthesis genes (Hickman and Harwood 2008; Krasteva et al. 2010). The PilZ domain-containing YcgR from *E. coli* and *Salmonella* functions as a molecular brake to reduce the flagellar motor output (Boehm et al. 2010; Paul et al. 2010; Ryjenkov et al. 2006). According to previous reports, cell aggregation is also dependent on other factors under the control of c-di-GMP, for example, type IV pili in *C. difficile*, cellulose production in the cyanobacterium *Thermosynechococcus* or Psl polysaccharide of *P. aeruginosa* (Colley et al. 2016; Enomoto et al. 2015). In addition, the reduced swimming motility also contributes to the cell aggregation (Caldara et al. 2012; Qi et al. 2013). In the case of the ORS571 Δchp1 mutant, increased EPS production and impaired swimming motility may function together to finally lead to the increased cell aggregation.

Previous work of our lab has identified several chemotaxis proteins including CheZ and chemotaxis receptor IcpB and TlpA1 (Jiang et al. 2016; Liu et al. 2017a; Liu et al. 2017b). Deletion of these chemotaxis proteins also affected motility, EPS production, and nodulation of *A. caulinodans* ORS571. In *P. aeruginosa* PAO1, the interaction between chemotaxis protein CheR and c-di-GMP receptor MapZ was reported (Xu et al. 2016). This favours the hypothesis that the complex network of c-di-GMP may also cross talk with bacterial chemotaxis in ORS571, a hypothesis which needs to be further documented.

In conclusion, our study revealed the regulatory role of c-di-GMP in the nodulation process, which provided a new perspective to further investigate the rhizobia-legume symbiosis.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with ethical standards** This article does not contain any studies with animals performed by any of the authors.

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