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ORIGINAL PAPER

Bacterioferritin comigratory protein is important in hydrogen peroxide resistance, nodulation, and nitrogen fixation in *Azorhizobium caulinodans*

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Abstract

Reactive oxygen species are not only harmful for rhizobia but also required for the establishment of symbiotic interactions between rhizobia and their legume hosts. In this work, we first investigated the preliminary role of the bacterioferritin comigratory protein (BCP), a member of the peroxiredoxin family, in the nitrogen-fixing bacterium *Azorhizobium caulinodans*. Our data revealed that the *bcp*-deficient strain of *A. caulinodans* displayed an increased sensitivity to inorganic hydrogen peroxide (H_2O_2) but not to two organic peroxides in a growth-phase-dependent manner. Meanwhile, BCP was found to be involved in catalase activity under relatively low H_2O_2 conditions. Furthermore, nodulation and N_2 fixation were significantly impaired by mutation of the *bcp* gene in *A. caulinodans*. Our work initially documented the importance of BCP in the bacterial defence against H_2O_2 in the free-living stage of rhizobia and during their symbiotic interactions with legumes. Molecular signalling in vivo is required to decipher the holistic functions of BCP in *A. caulinodans* as well as in other rhizobia.

Keywords *Azorhizobium caulinodans* · ROS · *bcp* · Nodulation · N_2 fixing

Introduction

Rhizobium-legume symbiotic nitrogen (N_2) fixation within nodules is a major contributor to soil fertility and the global N_2 cycle (Kuypers et al. 2018). Nodules typically occur on roots, but a few legumes of the Papilionoideae and Mimosoideae families also form stem-located and aerial nodules (Boivin et al. 1997). Among these plants, *Sesbania rostrata* is an important legume green manure that can form both root and stem nodules induced by *Azorhizobium caulinodans* (Dreyfus and Dommergues 1981). In soil and during symbiosis, *A. caulinodans* must cope with varying stresses from the environment and from the plant defence against bacterial infections (Den Herder et al. 2006). This bacterium enters *S. rostrata* via root hairs or cracks (James et al. 2001). Upon first encountering the host plant, *A. caulinodans* is recognized as an invader, perhaps even as a pathogen, and triggers an oxidative burst in planta (D'Haeze et al. 2003). Notably, hydrogen peroxide (H_2O_2) is required for optimal symbiosis between *A. caulinodans* and *S. rostrata* (Capoen et al. 2010). From the crack entrance, the infection pocket formation induces local cell death due to the massive production of H_2O_2 (D'Haeze et al. 2003). Abundant H_2O_2 in

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infection threads of root hairs is also essential for the invasion of the deep tissue in *S. rostrata* (D'Haeze et al. 1998; Rubio et al. 2009).

Plant defences produce harmful reactive oxygen species (ROS), including H_2O_2, superoxide anion ($O_2^{\cdot-}$), and organic peroxides (OHPs), during symbiotic interactions, as reviewed in Apel and Hirt (2004) and Nanda et al. (2010). Beyond these interactions, legumes and rhizobia encounter ROS challenges from normal aerobic metabolic processes such as photosynthesis, respiration, and N_2 fixation (Apel and Hirt 2004). ROS are toxic forms of oxygen (O_2), often damage proteins, DNA, and cell membranes, and even interfere with symbiosis (Puppo et al. 2013). Indeed, numerous cellular defences against ROS stress are deployed by antioxidant systems, including catalase (Kat), peroxiredoxin (Prx), glutathione (Grx), thioredoxin (Trx) and superoxide dismutase (SOD), in bacteria (Imlay 2008). Among them, bacterioferritin comigratory protein (BCP) is one of the thiol-dependent peroxiredoxins (Mishra and Imlay 2012); BCP reduces a broad range of hydroperoxides with a high redox potential (Reeves et al. 2011) but with low catalytic efficiencies (Jeong et al. 2000).

BCP was first identified in *Escherichia coli* as a new member of the alkyl hydroperoxide reductase/thiol-specific antioxidant protein (AhpC/TSA) family that displays a general hydroperoxide peroxidase activity (Jeong et al. 2000). BCP reduces fatty acid hydroperoxide prior to H_2O_2 and *t*-butyl hydroperoxide (tBOOH) in *E. coli* (Jeong et al. 2000). BCP-deficient strains of *Helicobacter pylori* (Wang et al. 2005), *Campylobacter jejuni* (Atack et al. 2008), and *Porphyromonas gingivalis* (Johnson et al. 2011) grow slowly in aerobic culture and display an increased sensitivity to the cumene hydroperoxide (CuOOH) rather than to H_2O_2. *Thermococcus kodakaraensis* BCP has peroxidase activity towards all three substrates (Pham et al. 2015). Furthermore, the *bcp* mutant displays a growth-phase-dependent sensitivity to different oxidants in *E. coli* (Jeong et al. 2000) and *Burkholderia cenocepacia* (Clarke et al. 2010). However, there are limited reports about the role of BCP in rhizobia.

To investigate whether rhizobial BCP possesses similar functional properties to that of reported bacteria, we first knocked out the *bcp* gene (*AZC_1350*) in *A. caulinodans* ORS 571. Then, we tested the phenotype of the *bcp* mutant strain under H_2O_2, tBOOH and CuOOH stresses in vitro, as well as the symbiotic performance of the mutant strain in *S. rostrata*. We demonstrate that *A. caulinodans* BCP, which is involved in cellular protection against H_2O_2, is dependent on the growth phase and is also required for optimal symbiosis with the native host plant of *A. caulinodans*.

Materials and methods

Plant and bacterial culture condition

Escherichia coli strains were cultured in Luria–Bertani (LB) medium at 37 °C (Green and Sambrook 2012). *A. caulinodans* ORS571 (Lee et al. 2008) and its derivative strains were grown in tryptone-yeast extract (TY) medium at 28 °C (Beringer 1974). All solid media were supplemented with 1.2% agar. Indicated antibiotics were used for the selection at the final concentrations as following: kanamycin (Kan, 100 μg mL^{-1}), ampicillin (Amp, 100 μg mL^{-1}), spectinomycin (Spe, 20 μg mL^{-1}) and gentamicin (Gm, 20 μg mL^{-1}). Seeds of *S. rostrata* were surface sterilized as the description in (Fernández-López et al. 1998), and the seedlings were inoculated with *A. caulinodans* and grown in pots in the glasshouse (D'Haeze et al. 1998).

Plasmid and strain construction

Molecular techniques were performed following the standard protocols (Green and Sambrook 2012). Bacterial strains and the primer of the strain constructions from this work are listed in Tables 1 and 2. *E. coli* DH5α was used for general cloning, while *E. coli* SM10 λpir was used for cloning in conjugation. *A. caulinodans* was transformed by electroporation as described previously (Zhao et al. 2016).

The in-frame deletion was used to construct the *bcp*-defective mutant (Metcalf et al. 1996). First, two flanking fragments of *bcp*, a 739 bp of the upstream fragment (the primer pair of Ac-*bcp*-1/2) and a 724 bp of the downstream fragment (the primer pair of Ac-*bcp*-1/2), were cloned into pEX18Gm which is a *sacB* (gene encoding the levansucrase) suicide vector, and yielded the pEX18Gm-*bcp* plasmid. The mutant (Δ*bcp*) was selected for double-cross recombination and confirmed by PCR. For complementation plasmid for (Δ*bcp*), the P_{tac}-*bcp* plasmid was constructed through PCR amplification of the coding sequence (beginning with the ATG start codon) with the primer pair of pSRK-*bcp*-1/2 and cloned into pSRKGm (Khan et al. 2008). The open reading frame of *bcp* was amplified using indicated primers in Table 2. The amplicon was released by *Nde*I and *Hind*III and then cloned into pSRKGm plasmid. The correct orientation allowing the expression of *bcp* under the control of P_{tac} promoter was selected by PCR. The corresponding pSRK-*bcp* plasmid was introduced into the Δ*bcp* strain by the conjunction, yields the complementation strain bcp^c.

Table 1 Strains and plasmids in this work

Strains or plasmids	Characteristics	Source
Escherichia coli		
DH5α λpir	Host for cloning	Lab strain
SM10 λpir	Host for bacterial conjugation	Lab strain
Azorhizobium caulinodans		
AZC0	*Azorhizobium caulinodans* ORS571, wild-type, Amp^R	Lab strain
Δ*bcp*	In-frame deletion of *bcp* from AZC0	This study
Δ*bcp* (pSRKGm-*bcp*)	complementation of *bcp*, containing a recombinant plasmid pSRKGm-*bcp* Amp^R, Gm^R	This study
Plasmid		
pEX18Gm	Suicide cloning vector, Gm^R	Lab strain
pSRKGm	Expression vector	Lab strain
pEX18Gm-*bcp*	pEX18Gm carrying franking regions of *bcp*, Gm^R	This study
pSRKGm-*bcp*	Expressing *bcp* in pSRK, Gm^R	This study

Table 2 Primers in this work

Primer	Sequence	Description	Size (bp)
In-frame deletion of *bcp*			
Ac-*bcp*-1	CCCAAGCTTTTGACCTTCGCCCTGACGCT	Upstream fragment of *bcp*	739
Ac-*bcp*-2	CTCATGACACACGCGTCACCCGCATTGTCGAAGCGA		
Ac-*bcp*-3	AATGCGGGTGACGCGTGTGTCATGAGCCGGCCCATC	Downstream fragment of *bcp*	724
Ac-*bcp*-4	GCTCTAGAATCCTACAGCGGAGATGCGC		
Complementation of *bcp*			
pSRK-*bcp*-1	GGGAATTCCATATGCGGGTGTCAATGCGCCGCCTCGA	*bcp* complementation	593
pSRK-*bcp*-2	CCCAAGCTTTCATGACACCGCTCCTTCCGTGCGAACGCT		

Bacterial growth experiment in rich medium

Wild-type *A. caulinodans* and Δ*bcp* mutant strains used in this study were grown separately at 28 °C overnight (14–16 h) in 20 mL liquid TY medium. Bacterial cultures were kept in 100 mL conical flasks and shaken at 180 rpm in shaker incubator (SKY-211B, Sukun, China). The overnight cultures were harvested to prepare the bacterial suspension ($OD_{600}=0.01$) in TY liquid medium, and re-incubated for the growth curve experiment at above conditions. Growth was monitored by the determination of OD_{600} values in an UV-spectrophotometry (Philes Ltd, Nanjing, China) over time. Each experiment was repeated three times.

Peroxide disc diffusion assay

Three peroxides, H_2O_2, tBOOH and CuOOH were used to examine their inhibitions on the bacterial growth of *A. caulinodans*, Δ*bcp* and *bcp*^{c} via the disc diffusion assay (Wang et al. 2012). Overnight pre-cultures were prepared as described above and adjusted to the OD_{600} values of 1.0 (10^9 bacterial cells per mL). Then, those cells were mixed with TY top agar (0.6%) at 42 °C and poured onto the solid TY agar plates (55 mm in diameter). The sterilized Whatman AA disc (6 mm, Whatman International Ltd, Maidsotne, UK) was placed on the Petri plates for carrying 4 µL of 10 M H_2O_2, 5 M tBOOH, or 4.88 M CuOOH, then incubated at 28 °C for 24 h. The growth inhibition zones were measured and adjusted by subtracting the Whatman AA disc diameter. Each experiment was repeated three times.

Peroxide killing assay

This experiment was used to test the susceptibility of wild-type *A. caulinodans* and Δ*bcp* mutant strain to the mM levels of H_2O_2, tBOOH and CuOOH as reported previously in *Sinorhizobium meliloti* (Barloy-Hubler et al. 2004; Fontenelle et al. 2011). Overnight cultures of above strains were grown to the early-log phase ($OD_{600}\approx0.2$) or the stationary phase ($OD_{600}\approx1.0$) in TY liquid medium in described conditions. For peroxide treatments, H_2O_2, tBOOH and CuOOH were adjusted to the concentration of 8 mM, 1.5 mM, and 0.6 mM, respectively, for 2 h incubation in the shaker. Then, bacterial cultures were plated onto TY agar plate through a series of ten-time dilutions and incubated at 28 °C for 3 days

to determine the colony formation unit (CFU). Survival rate (%) was assessed based on the comparison with the control that was treated with the TY medium. Each trial contained triplicates per strain per condition.

Catalase assay

Overnight cultures of the wild-type strain and Δ*bcp* were inoculated at 1:100 into TY medium containing appropriate antibiotics and shaken at 28 °C until the early-log or the stationary phases. H_2O_2 was adjusted to 8 and 15 mM in bacterial cultures incubated at 28 °C for 1 h separately. Rinsed cells were collected and lysed by the sanitation. Equal amounts of lysates from the wild-type strain and Δ*bcp* were subjected to the catalase activity assays using Fluorometric Catalase Activity Assay Kit (Enco Scientific) according to the manufacturer's instructions.

Nodulation assay and nitrogenase activity

Overnight cultures of wild-type *A. caulinodans* and Δ*bcp* were diluted OD_{600} value of 0.1. Bacterial suspensions were stem spread for the stem nodulation or soil drenched onto *S. rostrata* for the root nodulation. Stem and root nodules were harvested to measure the nitrogen-fixing activity at 5 weeks post-inoculation. To measure the nitrogenase activity, 15 nodules per plant were placed into a 20 mL vial sealed with a butyl rubber septum. Each vial was injected with 2 mL acetylene by syringe and incubated at 28 °C for 2 h. Then, 1 mL gas from the vial was measured the acetylene and ethylene concentrations (Berrabah et al. 2014).

Transmission electron microscopy (TEM)

Five-week-old stem nodules were fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for 1 h, then in 2% osmium tetroxide with above buffer for 1 h at the room temperature and embedded with Epon812. Ultrathin sections (80 nm) of stem nodule were cut with a microtome (LKB 8800 Ultrotome III, Swiss) and stained with uranyl acetate for 20 min at 60 °C followed by lead citrate for 10 min at room temperature. The TEM of specimens was analysed in a Hitachi H-7650 transmission electron microscope (D'Haeze et al. 1998).

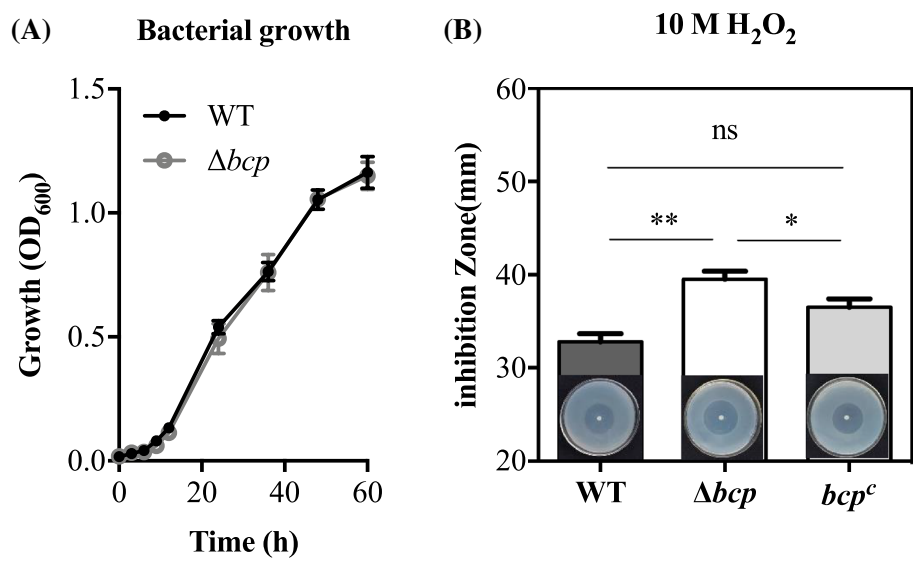
Results

The putative BCP is essential for resistance to H_2O_2 but not to tBOOH and CuOOH

In the *A. caulinodans* ORS571 genome (Lee et al. 2008), *AZC_1350* was identified as a putative gene encoding a Prx containing COG1225, i.e., the AhpC/TSA family protein BCP. To study the role of this putative *bcp* in the response to oxidative stress in *A. caulinodans*, we constructed an in-frame deletion mutant (Δ*bcp*) based on the genetic background of *A. caulinodans* ORS571 (WT). As shown in Fig. 1a, this *bcp*-deficient strain grew similarly to its parental strain ORS571 when it was incubated in liquid TY-rich medium. It is likely that BCP does not play a role in bacterial growth/fitness under normal conditions. Based on the results of disc diffusion assays, the growth of Δ*bcp* on agar plates was strongly inhibited by the addition of H_2O_2 but not CuOOH and tBOOH (Fig. 1b–d, *t* test). The inhibition diameter of the 10 M H_2O_2 treatment drastically increased from 32.8 ± 1.92 cm (WT) to 39.5 ± 1.73 cm (Δ*bcp*, *P* value = 0.0005). For bcp^c, the complemented strain of Δ*bcp*, the inhibition diameter of H_2O_2 was similar to that of the WT (*P* value = 0.0646) but significantly lower than that of Δ*bcp* (*P* value = 0.0125). However, Δ*bcp* grew similarly to the WT on the plate in the presence of 4.88 M CuOOH (Fig. 1c) and 5 M tBOOH (Fig. 2d). The inhibition diameter of CuOOH on Δ*bcp* (33.67 ± 0.73 mm) was not distinguishable from that 34.01 ± 0.01 cm inhibition diameter observed in the WT (*P* = 0.6611). Meanwhile, the inhibition diameter of Δ*bcp* (54.00 ± 0.58 mm) also showed no significant differences from that of WT (52.75 ± 0.78 mm) on the tBOOH plate (*P* value = 0.2827). The disc diffusion assays suggested the importance of BCP to the resistance of *A. caulinodans* to H_2O_2.

BCP confers resistance to H_2O_2 during the early growth phase

To further study the function of BCP in resistance to inorganic and organic hydroperoxides during the growth dynamics of *A. caulinodans*, we investigated the survival rates of the WT and Δ*bcp* strains in oxidative killing assays using 8 mM H_2O_2, 1.5 mM tBOOH and 0.6 mM CuOOH (Fig. 2, *t* test). Our data indicated that Δ*bcp* was more sensitive to H_2O_2 killing at the early-log stage of bacterial growth in the TY liquid culture (Fig. 2a). The survival rate of Δ*bcp* (0.5027% ± 0.17%) was significantly lower than that of the WT strain (15.17% ± 3.87%) in this stage (*P* value = 0.0021). In the stationary phase, there were no differences in the survival of the WT and Δ*bcp* strains following treatment with H_2O_2 (Fig. 2a, *P* value = 0.5264). As shown in Fig. 2b, c, the mutation of *bcp* did not alter the survival of *A. caulinodans* ORS571 relative to the organic peroxide killing at the log phase/stationary phase of growth. Indeed, both the WT and Δ*bcp* strains were equally sensitive to tBOOH and CuOOH. Following treatment with tBOOH there were no clear differences between the survival rates the WT and *bcp* strains at the log phase (*P* value = 0.7865) or the stationary stage (*P* value = 0.7492). For the CuOOH killing assay, the survival



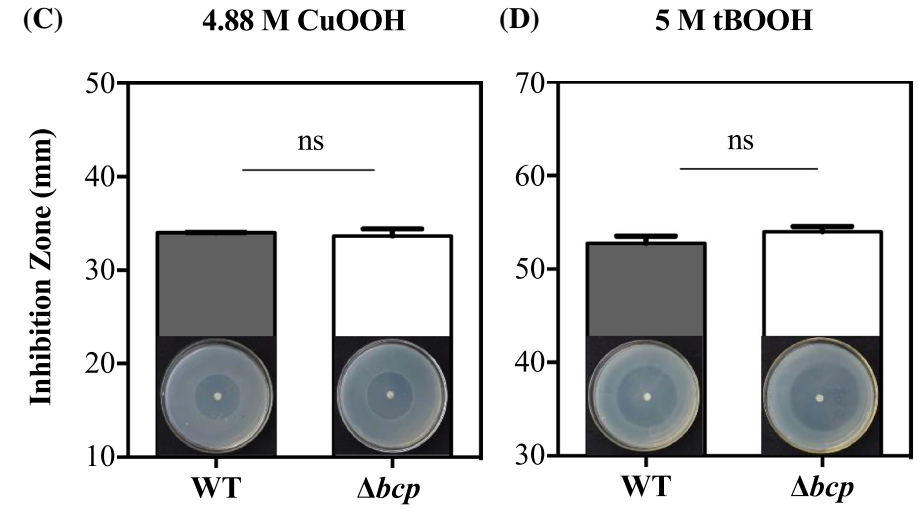


Fig. 1 Bacterial growth and disc diffusion assays. **a** Bacterial growth of *Azorhizobium caulinodans* ORS571 (WT) and the Δ*bcp* mutant strains. The cultures were grown in TY medium at 28 °C. OD_{600} was measured at the time points indicated. In the disc diffusion assay, approximately 10^7 bacterial cells of WT, Δ*bcp* and its complementary strain (*bcp*^{c}) were mixed and spread on the top of the TY agar plates. The disc was saturated with 4 µl of 10 M H_2O_2 (**a**), 4.88 M CuOOH (**b**), or 5 M tBOOH (**c**) and placed in the middle of the plate. Data are the means ± SDs of three independent experiments. $^{**}P < 0.01$; $^{*}P < 0.05$; *ns* no significant difference (*t* test)

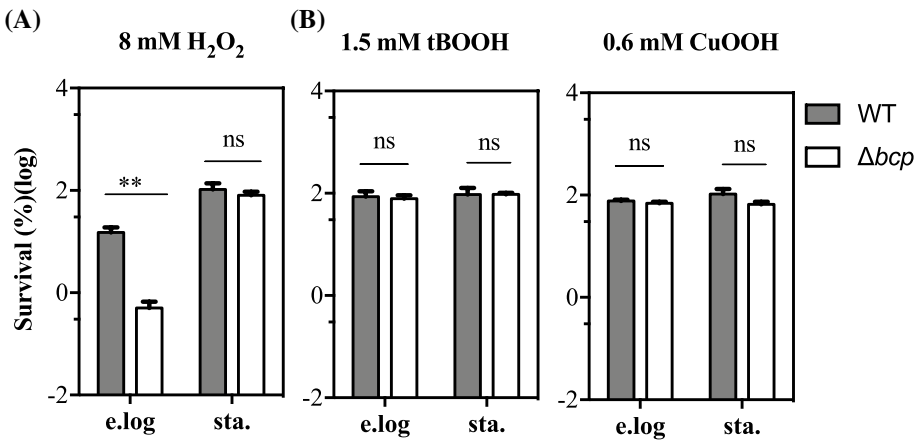


Fig. 2 Contributions of *bcp* to peroxide killing at early-log and stationary phases. Wild-type and Δ*bcp* strains were grown in TY liquid medium at 28 °C. The early-log (e.log) and stationary (sta.) phases of growth were defined as 5 h (OD_{600} of 0.2) and 24 h (OD_{600} of 1.0), respectively, after inoculation. After incubation, bacterial cultures were treated with 8 mM H_2O_2 (**a**), 0.6 mM CuOOH (**b**), and 1.5 mM tBOOH (**c**) for 2 h. The cells were then resuspended in sterile water and placed on TY agar plates to determine cell viability. Data are the means ± SDs of three independent experiments. $^{**}P < 0.01$; *ns* no significant difference (*t* test)

rate of Δ*bcp* was analogous to that of the WT strain at the log phase (*P* value = 0.3464) and the stationary phase (*P* value = 0.2325).

Δ*bcp* shows a reduction of catalase activity in the presence of H_2O_2

We found that BCP confers the resistance of *A. caulinodans* to H_2O_2 in both the disc diffusion and oxidative killing assays. To further study the role of BCP in H_2O_2 tolerance, we investigated the catalase activity of the WT and Δ*bcp* strains exposed to 8 mM H_2O_2 during the growth of *A. caulinodans* (Fig. 3a, b, *t* test). No differences were observed between the catalase activities of the WT and Δ*bcp* strains at the log phase/stationary phase of growth ($P_{WT\ vs\ \Delta bcp} > 0.05$). However, the catalase activity was significantly reduced for WT strain ($P_{WT\ (+)\ vs\ (-)} < 0.05$) or Δ*bcp* ($P_{\Delta bcp\ (+)\ vs\ (-)} < 0.05$) by the addition of H_2O_2 at both growth stages. Compared with the WT *A. caulinodans*, the Δ*bcp* strain displayed reduced catalase activity at both the early-log phase ($P_{WT\ vs\ \Delta bcp} = 0.0123$) and stationary phase ($P_{WT\ vs\ \Delta bcp} = 0.0064$). In *E. coli*, the peroxidase activity of BCP is increased with the concentration of H_2O_2 (Jeong et al. 2000). Thus, we further tested the catalase activity of Δ*bcp* at relatively high levels of H_2O_2 (15 mM) at the stationary phase because the growth of Δ*bcp* was strongly inhibited at the early-log phase under the stress of 8 mM H_2O_2 (Fig. 2a) and at both phases under the stress of 20 mM (data not shown). The *bcp*-defective strain displayed similar catalase activity as its parent strain at both the early-log phase ($P_{WT\ vs\ \Delta bcp} = 0.3119$) and the stationary phase ($P_{WT\ vs\ \Delta bcp} = 0.2786$). In this experiment, the catalase activity of Δ*bcp* was similar to that of the WT strain (*P* value > 0.05) when the bacteria entered the stationary phase. These results indicated that BCP plays an important role in catalase activity at low H_2O_2 concentrations.

BCP is required for nodulation and nitrogenase activity

The sensitivity of the *A. caulinodans* mutant to H_2O_2 is potentially relevant to symbiosis since *S. rostrata* cells respond to rhizobial infection with an enhanced production of ROS (D'Haeze et al. 2003; Zhao et al. 2016). To test the effect of *bcp* mutation on nodulation and nitrogen fixation, we inoculated ~ 10^8 WT and Δ*bcp* cells onto a *S. rostrata* stem. At 5 weeks post inoculation, fewer nodules were clearly observed in the plants infected with Δ*bcp* (Fig. 4a). The nodulation efficiency of Δ*bcp* was significantly reduced in comparison to that of plants inoculated with the parental strain ORS571 (*P* value = 0.0068, Fig. 4b). Moreover, stem nodules induced by the *bcp* mutant displayed substantially lower acetylene reduction activity than the WT-induced nodules (*P* value = 0.0294, Fig. 4c). However, there were differences between the nitrogenase activity of the 5-week-old root nodules formed by the Δ*bcp* and WT strains, even though the root nodule number was significantly reduced in the former (Fig. S1). To further investigate whether the lower nitrogenase activity of the nodules was impaired by the development of Δ*bcp* bacteroids, we performed a TEM test on the stem nodules induced by both the Δ*bcp* and WT strains. As shown in Fig. 4d, Δ*bcp* formed enlarged, rod-shaped, and round bacteroids within the cells of stem nodules. Our data offer that BCP is important for nodulation and

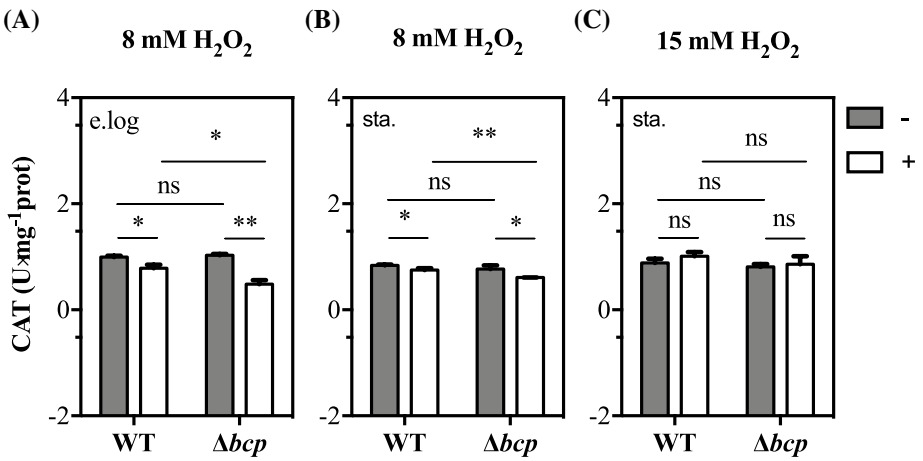


Fig. 3 Catalase activity of *Azorhizobium caulinodans* strains. Wild-type and Δ*bcp* were grown in TY medium at 28 °C. The early-log (e.log) and stationary (sta.) growth phases were defined as 5 h (OD_{600} of 0.2) and 24 h (OD_{600} of 1.0) after inoculation, respectively. When indicated, 8 mM H_2O_2 (e.log, **a**), 8 mM H_2O_2 (sta., **b**), and 15 mM H_2O_2 (sta., **c**) were added to the cell cultures and incubated for 1 h. Proteins were extracted from these two strains and determined as described in materials and methods. Data are the means ± SDs of three independent experiments. $^{**}P < 0.01$; $^{*}P < 0.05$; *ns* no significant difference (*t* test)

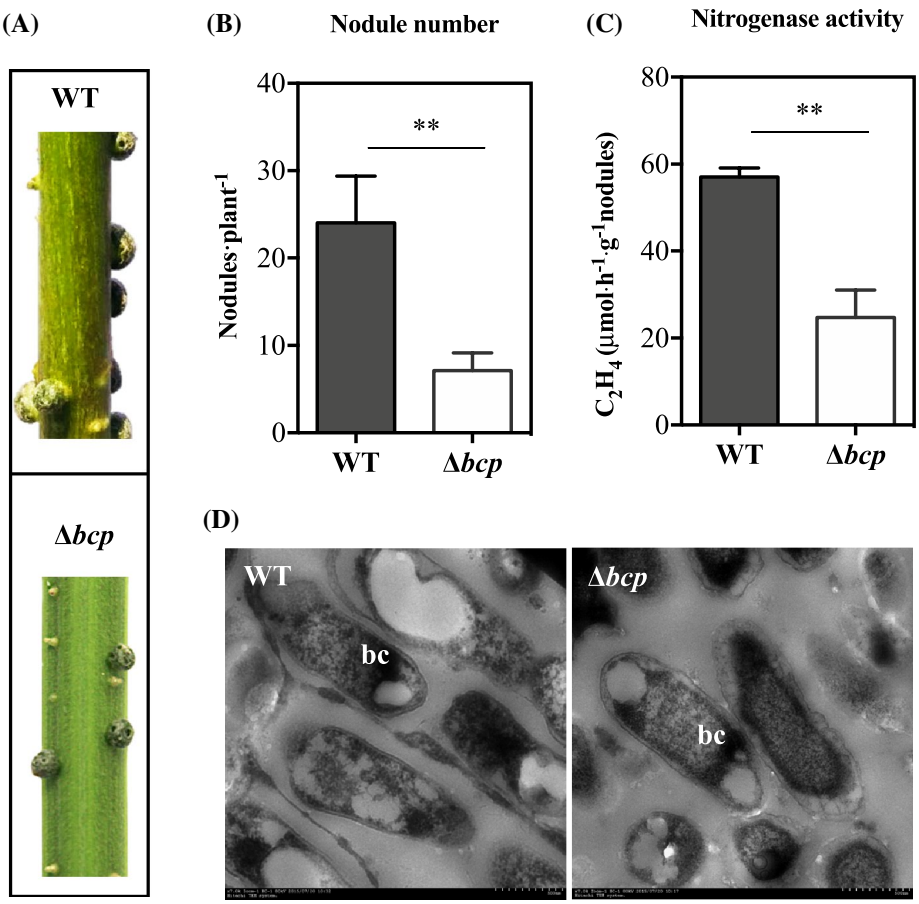


Fig. 4 Effect of BCP on stem nodule formation and nitrogenase activity. Stem nodule formation (**a**). Stationary cultures of wild-type (WT) and Δ*bcp* strains were inoculated on the stem surface of *S. rostrata*. Nodules were photographed 5 weeks post inoculation. The number of stem nodules on *S. rostrata* formed by WT and Δ*bcp* were assessed (**b**). Nitrogen fixation activities of stem nodules (**c**). The stem nodules formed by each strain were harvested at 5 weeks post inoculation for nitrogenase activity measurements. Data are the means ± SDs of three independent experiments. $^{**}P < 0.01$; *ns* no significant difference (*t* test). **d** TEM of the stem nodule structure. Transmission electron micrographs of stem nodules. The micrographs were taken from infected plant stem cells of 5-week-old nodules induced by WT and Δ*bcp* bacterial cells. Scale bars = 1 μm

fixation but has no impact on the development of bacteroids within stem nodules.

Discussion

In this work, we performed a functional analysis of the TSA/AhpC family protein BCP in *A. caulinodans*. Like other bacteria, *A. caulinodans* is often confronted with broad oxidative stresses generated in the environment or during host plant infection dynamics. Here, our data offer a preliminary report that BCP is essential for *A. caulinodans* defence against H_2O_2 rather than against two OHPs (i.e., CuOOH and tBOOH) under free-living conditions, and BCP is also required for nodulation and N_2 fixation during symbiotic associations with *S. rostrata*.

BCP contributed to the bacterial defence against high concentrations of H_2O_2 when *A. caulinodans* was grown on agar plates and against low H_2O_2 levels in cell culture (Fig. 3a). The roles of the *bcp*-associated phenotype seem to be heterogeneous among bacterial species. While *bcp*-deleted *A. caulinodans*, *E. coli* (Jeong et al. 2000) and *B. cenocepacia* (Clarke et al. 2010) are highly sensitive to H_2O_2, *bcp*-deleted *H. pylori* (Wang et al. 2005), *C. jejuni* (Atack et al. 2008), and *P. gingivalis* (Johnson et al. 2011) are not. This phenotypic heterogeneity of BCP is probably attributable to variations in the composition and the abundance of other antioxidant enzymes within the cells of different species. In *H. pylori*, BCP is considerably less abundant than another thiol-based Prx AhpCD. Compared to the wild-type strain of *H. pylori*, the AhpCD-defective mutant displays a strong oxidative stress resistance phenotype (Wang et al. 2005). Indeed, we also found functional overlap between BCP and AhpCD due to the antioxidant activities of AhpCD in *A. caulinodans* (submitted data). Furthermore, a high concentration of H_2O_2 can induce a more efficient Kat system in *E. coli* (Seaver and Imlay 2001), *S. meliloti* (Barloy-Hubler et al. 2004) and *A. caulinodans* (Zhao et al. 2016). This may explain why BCP-deficient *A. caulinodans* grows similar to its parent strain during bacterial growth under high H_2O_2 stress.

BCP appears to be unimportant for bacterial defence against organic ROS in *A. caulinodans*. The *bcp*-deleted mutant showed comparable growth to the wild-type *A. caulinodans* strain both on the agar plate and in liquid medium in the presence of CuOOH and tBOOH. This phenotype is inconsistent with previous reports that *E. coli* BCP can reduce a broad range of hydroperoxides, including

CuOOH and tBOOH (Jeong et al. 2000; Reeves et al. 2011). However, in addition to BCP, OHPs are reduced by other proteins, such as AhpCD in *E. coli* (Seaver and Imlay 2001), *Xanthomonas campestris* (Mongkolsuk et al. 2000) and *Bacillus subtilis* (Bsat et al. 1996) and Ohr (organic hydroperoxide resistance protein) in *B. subtilis* (Bsat et al. 1996), *X. campestris* (Mongkolsuk et al. 1998), *Agrobacterium tumefaciens* (Chuchue et al. 2006), and *Pseudomonas aeruginosa* (Ochsner UA 2001), as well as the N_2-fixing bacterium *S. meliloti* (Fontenelle et al. 2011). In our recent work, we also showed that Ohr, rather than AphCD (alkyl hydroperoxide reductase), is essential to the tolerance of stress from CuOOH and tBOOH in *A. caulinodans* (unpublished data). We argued that it would be ideal for *A. caulinodans* to produce different enzymes to cope with many types of ROS through an optimized metabolism and ROS signalling network, such as other bacteria or rhizobia (Mishra and Imlay 2012; Puppo et al. 2013).

In *A. caulinodans*, BCP is required for nodulation on stems and roots as well as N_2 fixation in stem nodules. In the early stage of infection, this bacterium induces the high production of H_2O_2 by defence reactions in *S. rostrate* cells (D'Haeze et al. 2003). It is possible that BCP reduces H_2O_2 and facilitates rhizobial infection *in planta*. The function of H_2O_2 in host plants during symbiosis is not only restricted to plant defence against bacteria but is also important for symbiotic processes (Nanda et al. 2010). In *S. rostrate*, the formation of infection pockets is induced by the *A. caulinodans* nod factors (D'Haeze et al. 1998) and involves the induction of local cell death associated with a massive production of H_2O_2 (D'Haeze et al. 2003). Thus, the balance between production and reduction of H_2O_2 is critical for deeper invasion of *S. rostrate* tissues. This characteristic explains why the H_2O_2-associated antioxidant proteins, such as BCP in this work, AphCD (submitted data) and KatG (Zhao et al. 2016) of *A. caulinodans* are required for the nodulation of *S. rostrate*. Furthermore, BCP significantly affects the nitrogenase activity of stem nodules (Fig. 4c) rather than that of root nodules (Fig. S1c) in *S. rostrate*. BCP is more effective at detoxifying ROS in stem nodules, which may have a lower oxygen microenvironment than root nodules (James et al. 1998).

Azorhizobium caulinodans encounters many ROS generated from environmental stresses under free-living conditions and during symbiosis. In this work, we preliminarily documented that BCP plays an important role in *A. caulinodans* in the defence reaction to oxidative stresses and in nodulation and N_2 fixation in *S. rostrate*. *A. caulinodans* has different antioxidant pathways to deal with ROS and eventually establishes normative symbiosis with the host plant. Our work shed light on deciphering the molecular bases of BCP to ROS resistance during symbiotic interactions between rhizobia and plants.

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Compliance with ethical standards

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

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