Functional analysis of PrkA - a putative serine protein kinase from *Mesorhizobium alhagi* CCNWXJ12-2 - in stress resistance

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**Abstract**

**Background:** Serine/threonine protein kinases are highly conserved kinases with a wide distribution in microbes and with multiple functions. *Mesorhizobium alhagi* CCNWXJ12-2, a α-proteobacterium which could be able to form symbiosis with *Alhagi sparsifolia* in northwest of China, contains a putative PrkA-family serine protein kinase, PrkA. In our previous study, the expression of *prkA* was found to be downregulated in high-salt conditions. To elucidate the function of *M. alhagi* PrkA, a *prkA* deletion mutant was constructed and the phenotypes of the mutant were analyzed.

**Results:** The salt and alkaline tolerance and antioxidant capacity of the wild-type strain and the *prkA* deletion mutant was measured. Our results showed that the deletion mutant had higher salt and alkaline tolerance than the wild-type strain. The total cellular Na⁺ content was measured and showed no significant difference between the wild-type strain and the mutant. The *prkA* deletion mutant also showed a higher H₂O₂ tolerance than the wild-type strain. Therefore the activities of antioxidant enzymes were measured. Catalase activity was similar in the wild-type and the deletion mutant, while the superoxide dismutase activity in the mutant was higher than that in the wild-type.

**Conclusions:** We firstly analyze the function of a serine protein kinase, PrkA, in *M. alhagi*. Our data indicate that PrkA could reduce the survival of *M. alhagi* under environmental stress and deletion of *prkA* dramatically improved the salt and alkaline tolerance and antioxidant capacity of *M. alhagi*.

**Keywords:** Serine protein kinase, Salt resistance, Antioxygenation, Na⁺ concentration measurement

**Background**

Salinity and desiccation are major problems facing agriculture worldwide [1, 2]. *Mesorhizobium alhagi* CCNWXJ12-2 is a highly salt-tolerant and alkali-tolerant rhizobium which can form nodules with the desert plant *Alhagi sparsifolia* [3]. The nitrogen-fixing symbiosis formed between rhizobia and legumes can decrease the damage to plants caused by soil salinity; thus, there are an increasing number of studies on salt-tolerant rhizobia and their mechanism(s) of salt resistance [4, 5]. To determine the mechanism of salt resistance in *M. alhagi*, a global transcriptome comparison of *M. alhagi* was conducted in salt-free (no added salt) and high-salt conditions, and a downregulated putative serine kinase gene, *prkA*, was identified in high-salt [6].

PrkA is a highly conserved serine protein kinase with a wide distribution in bacteria and archaea [7]. The serine/threonine protein kinases play diverse roles in bacterial signal transduction and regulation by phosphorylating multiple substrates [8]. In the model bacterium *Escherichia coli*, the *prkA* homolog *yeaG* was regulated by leucine-responsive regulatory protein and may have a role in metabolic reprogramming to survive acidic and osmotic stress [9]. Recent reports showed that *yeaG* is also involved in adaptation to nutrient limitation [10, 11]. However, subsequent research showed that a *yeaG* deletion mutant showed no significant difference in salt tolerance and pH adaptation compared with the wild-type strain [12].
In *Mycobacterium tuberculosis*, the deletion mutant of *pknE* (a gene encoding a serine/threonine protein kinase) showed defective growth in conditions of neutral pH and on exposure to lysozyme, but a higher tolerance to acidic stress, sodium dodecyl sulfate (SDS), and kanamycin [13]. Proteomic and phosphoproteomic analysis of the *pknE* mutant of *M. tuberculosis* showed that PknE was involved in metabolism, dormancy, and suppression of some sigma factors and other kinases, and thus could play an important role in adaptive responses to hostile environments [14].

In *Bacillus subtilis*, PrkA was proved to be an inner spore coat protein and involved in spore formation, which is controlled by sigma factors [15, 16]. In *Streptococcus mutans*, the serine/threonine protein kinase annotated *pknB* was shown to play a significant role in biofilm formation, genetic competence, and acid resistance [17]. In *Rhizobium etli*, the expression of *prkA* was highly dependent on alarmones, guanosine, tetraphosphate, and guanosine pentaphosphate, but the Δ*pknA* mutant of *R. etli* showed no clear difference compared with the wild-type under osmotic, oxidative and heat stresses [7].

In this study, we constructed a Δ*pknA* (the *prkA* deletion mutant of *M. alhagi*) mutant and undertook a phenotypical analysis to understand the function of PrkA. Our results showed that the Δ*pknA* mutant grew better under high-salt (0.4 M NaCl) and alkaline (pH 9) conditions than the wild-type strain, and the survival rate of the mutant under oxidative stress was also higher than that of the wild-type. The total cellular Na⁺ content in the mutant was almost the same as that in the wild-type in high-salt conditions. Although the catalase (CAT) activity was similar in the wild-type and Δ*pknA* mutant, the superoxide dismutase (SOD) activity in Δ*pknA* mutant was higher than that in the wild-type. These results indicate that *prkA* could reduce the ability of *M. alhagi* in stress adaptation.

**Results and discussions**

**Increased Na⁺ and alkali tolerance of mutant Δ*pknA***

There is only one annotated PrkA family serine protein kinase in the genome of *M. alhagi*, denoted PrkA [18]. We have previously found that the expression of *prkA* was downregulated in high-salt conditions through RNA-Seq validated by RT-qPCR [6]. To determine the function of *M. alhagi* CCNWJX12-2 *prkA*, we constructed a deletion mutant and its tolerance to NaCl and alkali were tested.

The mutant Δ*pknA* had better salt and alkali resistance than the wild-type strain, while the complemented strain CΔ*pknA* had similar salt and alkali resistance to the wild-type strain (Fig. 1). The expression of *prkA* in the complementation strain was confirmed using reverse transcription PCR (data not shown).

In *Mycobacterium tuberculosis*, the *pknE* deletion mutant showed higher tolerance to acidic stress, SDS, and kanamycin than the wild-type, which means *pknE* is involved in stress adaptation [13]. Although the protein sequence similarity of *Mesorhizobium alhagi* PrkA and *Mycobacterium tuberculosis* PknE is only 12.9 %, the function of *pknE* in *M. tuberculosis* was to some degree similar to that of *prkA* in *M. alhagi* in stress adaption. However, our results showed that wild-type *M. alhagi* had the same resistance to antibiotics (kanamycin, gentamicin, ampicillin, tetracycline, streptomycin, and rifampicin; 50 ug/ml for all), SDS (0.01 %), and acid (pH 6) as did the mutant Δ*pknA* (data not shown).

In *E. coli*, the deletion mutant of *yeaG* showed no difference in salt tolerance compared with the wild-type [12], although the similarity in protein sequence between *E. coli* YeaG and *M. alhagi* PrkA is relatively high (65.5 %). Despite the similar genomic background of *prkA* in *R. etli* and *Mesorhizobium alhagi* (data not shown) and the high similarity in protein sequences (71.49 %), the *prkA* deletion mutant of *R. etli* showed no phenotype changes compared with the wild-type strain [7], in contrast to *M. alhagi* in the present work.

The functions of these genes in the different bacteria are obviously different. These results show that serine protein kinases can have very different functions in different bacteria.

**Measurement of total cellular Na⁺ content**

Bacteria can efflux the extra Na⁺ from the cells by Na⁺/H⁺ antiporters using energy of proton motive force [19]. We have previously found that the expression of a Na⁺/H⁺ antiporter gene, *nhaaA*, was upregulated in high-salt conditions [6]. Here we measured the total cellular Na⁺ content of the wild-type, Δ*pknA*, and CΔ*pknA* strains in salt-free and high-salt (0.4 M NaCl) conditions to find out whether PrkA influences the total cellular Na⁺ content. However, our results showed no significant difference among these three strains (p ≥ 0.05) when grown in the same conditions (Fig. 2). The Na⁺ content of the three strains grown on 0.4 M NaCl TY agar plates was almost 20-fold higher than that in the controls grown on salt-free medium. The similar Na⁺ content of the three strains implied that *prkA* does not depress the strain growth in high-salt conditions by adjusting the cellular Na⁺ content. Therefore, we speculate that the mechanism of PrkA depressing the growth of *Mesorhizobium alhagi* under salt stress is complex, which involves many components of metabolism.
Increased antioxidative capacity of mutant ΔprkA

Stressful conditions such as heat, acid and high-salt concentrations, can lead to secondary oxidative stress in bacteria [20]. Previous study has shown the intracellular level of reactive oxygen species (ROS) was increased significantly when cells were stressed by high salinity [21, 22]. Because high salinity can trigger a high level of intracellular ROS, we tested the oxidative resistance of the *M. alhagi* prkA deletion mutant to identify any antioxidant function of PrkA. The survival rates of the wild-type, ΔprkA and CΔprkA strains treated with 10 mM H₂O₂ for 30 min showed a significant difference (Fig. 3); survival of ΔprkA treated with H₂O₂ was significantly (*p* ≤ 0.05) higher than that of the wild-type and complemented strains. These results suggest that PrkA depresses the antioxidative capacity of *M. alhagi*.

Although H₂O₂ can be damaging for rhizobium, it appears that H₂O₂ is required for successful infection.

**Fig. 1** Sensitivity of the wild-type and mutant strains to NaCl and alkaline: Wild-type (WT), ΔprkA (prkA deletion mutant), and CΔprkA (complement of ΔprkA) bacterial cells were grown to OD₀₆₀₀ = 0.8 in TY broth medium, adjusted to OD₀₆₀₀ = 0.2, and then serially diluted and spotted onto TY agar plates containing no additional NaCl or 0.4 M NaCl at neutral pH, and TY agar plates at alkaline pH (pH 9). Ten-fold serial dilutions are shown. The plates with no additional NaCl were incubated at 28 °C for 3 days, while the plates with 0.4 M NaCl and the plates at pH 9 were incubated for 5 days at the same temperature.

**Fig. 2** Measurement of total cellular Na⁺ concentration. Wild-type (WT), ΔprkA (prkA deletion mutant), and CΔprkA (complement of ΔprkA) bacterial cells were grown to OD₀₆₀₀ = 0.8 in TY broth medium and then adjusted to OD₀₆₀₀ = 0.2. The diluted inocula were plated on TY agar plates with and without 0.4 M NaCl and inoculated at 28 °C for 5 days. Total cellular Na⁺ concentrations were measured using an atomic absorption spectrophotometer. The results are shown as the means of three biological replicates and the error bars indicate standard deviations. The significance of differences is shown at the *P* < 0.05 level (t-test). Different lowercase letters mean significant difference between two columns.
The overexpression of the housekeeping catalase in *Sinorhizobium meliloti* RsKatB++ results in a delay of symbiosis formation and has negative effects on the development of infection threads [23].

Unfortunately, the symbiosis formation of *M. alhagi* and *Alhagi sparsifolia* is very unstable. Great effort has been made to conduct the plant experiments, but the results are always unreliable and unauthentic (data not shown). Therefore, we can only hypothesize that PrkA has positive effects on symbiosis formation.

**Antioxidant enzyme activity determination**

Catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) are major antioxidases in bacteria, which can eliminate the intracellular ROS [24]. Therefore, we measured the CAT, SOD, and POD activities of *M. alhagi* in different conditions. Figure 4 shows the enzyme activity in the three strains (without treatment, H2O2 treated, or 0.4 M NaCl treated). The CAT and SOD activities could be detected in all strains in each condition, while the POD activity was not detectable.

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**Fig. 3** Survival rate of *M. alhagi* after H2O2 treatment. Wild-type (WT), ΔprkA (prkA deletion mutant), and CΔprkA (complement of ΔprkA) bacterial cells were grown to OD600 ≈ 0.8 in TY broth medium, adjusted to OD600 ≈ 0.1, and then treated with 10 mM H2O2 for 30 min or incubated without H2O2 in otherwise identical conditions as a control. The percentage survival rate of the three strains was calculated as follows: [(CFU per ml after treatment with H2O2)/(CFU per ml before treatment with H2O2)] × 100. Data shown are the means of three independent experiments and the error bars indicate standard deviations. The significance of differences is shown at the *P* < 0.05 level (*t*-test). Different lowercase letters mean significant difference between two columns.

**Fig. 4** Measurement of antioxidant enzyme activities. Measurement of catalase (CAT, (a)) and superoxide (SOD, (b)) activities in wild-type *M. alhagi* (WT), ΔprkA (prkA deletion mutant), and CΔprkA (complement of ΔprkA). Three independent biological experiments were conducted to measure the CAT and SOD activities. The error bars indicate standard deviations. The significance of differences is shown at the *P* < 0.05 level (*t*-test). Different lowercase letters mean significant difference between two columns.
We checked the RNA-Seq data and found that the genes coding CATs and SODs are highly expressed in *M. alhagi*, while PODs are only expressed at a low level [6]; thus POD activity in cells may have been below the detection limit of the kit used in our experiments.

In control cells (normal conditions), the CAT activities of the three strains were almost the same, while the SOD activity of ΔprkA was significantly higher \((p \leq 0.05)\) than that in the other two strains. Salt stress and \(H_2O_2\) treatment triggered increased CAT and SOD activities in all three strains (Fig. 4). The CAT and SOD activities under high-salt stress were markedly higher than those in the \(H_2O_2\) treatment group, which could also suggest that high-salt stresses trigger oxidative stress. The lower CAT and SOD activities in the \(H_2O_2\) treatment group were possibly caused by the low \(H_2O_2\) concentration or the short treatment time.

The CAT and SOD activities of ΔprkA under salt stress and \(H_2O_2\) treatment were extremely significantly higher \((p \leq 0.001)\) than those of the wild-type and CΔprkA (Fig. 4). The increase in CAT activity units of ΔprkA compared to the other two strains was much smaller than that in SOD activity units. Therefore, we hypothesize that PrkA influences the antioxidative capacity of *M. alhagi* mainly by affecting SOD activity. However, in favorable conditions (e.g., as in the control group), the high expression of SOD genes in ΔprkA could waste energy. PrkA may play a role in control of SOD gene expression. The adjustment of SOD gene expression may need the high expression of prkA in salt-free conditions.

**Conclusions**

A prkA deletion mutant has been constructed to study the function of PrkA in *M. alhagi*. The data suggest that the ability to survive some abiotic stresses was increased by knocking out prkA. Moreover, we showed similarities and differences in the functions of PrkA and its homologs in *R. etli* and other bacteria. Our results suggest that it is possible to increase the salt and alkali tolerance of a bacterium by constructing specific mutants in genes highlighted by RNA-Seq data. To our knowledge, this is the first report to identify the function of PrkA in stress adaption of *Mesorhizobium* and to construct an increased salt-tolerant rhizobial mutant. Most curious and interesting was that the high expression of prkA made *M. alhagi* more vulnerable to high salinity. Because of the unstable symbiosis formation in plant tests, the role prkA plays in symbiosis formation is unclear and needs more efforts to be illuminated.

**Methods**

**Bacterial strains and growth conditions**

Table 1 lists bacterial strains and plasmids used in this study. The purified bacteria were typically grown in tryptone-yeast extract (TY) broth (5 g tryptone, 3 g yeast extract, and 0.7 g \(CaCl_2\)·2\(H_2O\) per liter) at 28 °C for *M. alhagi* and its mutants, or Luria-Bertani broth (10 g tryptone, 5 g yeast extract, and 10 g \(NaCl\) per liter) at 37 °C for *Esherichia coli*. SM agar plates (10 g mannitol, 0.5 g \(K_2HPO_4\), 0.5 g \(KNO_3\), 0.2 g \(MgSO_4\)·7\(H_2O\), 0.1 g \(CaCl_2\), 0.1 g \(NaCl\), and 15 g agar per liter) was used to isolate the mutants. All bacteria were incubated in aerobic conditions. Where necessary, antibiotics were

| Strain or plasmid   | Description | Source or reference    |
|---------------------|-------------|-----------------------|
| Escherichia coli    |             |                       |
| DH5α                | endA hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deaR (880 diacΔ(lacZ)M15) | [27]                  |
| S17-1λpir           | Tn1’ Str’ recA thi pro hsdR hsdM’ RP4-2-Tc:MucKm Tn7 λpir lysogen | [28]                  |
| DH-PrkA             | DH5α carrying pK18prkA | This study            |
| DH-CPrkA            | DH5α carrying pBLprkA | This study            |
| Mesorhizobium alhagi|             |                       |
| XJ12-2              | Wild-type   | [18]                  |
| ΔprkA               | XJ12-2 ΔprkA | This study            |
| CΔprkA              | ΔprkA carrying pBLprkA | This study            |
| Plasmids            |             |                       |
| pK18mobsacB         | Suicide vector derived from plasmid pK18; Mob+ sacB Km’ | [25]                  |
| pBBR1MCS-5-S        | Broad-host-range cloning vector; Gm’ | [29]                  |
| pK18prkA            | pK18mobsacB:prkA | This study            |
| pBLprkA             | pBL carrying prkA | This study            |

\(^{a}\)Km’, kanamycin resistance; Gm’, gentamicin resistance
added as the following concentrations: kanamycin, 100 μg/ml; gentamicin 50 μg/ml.

**Plasmid construction**

The primers used to construct the prkA deletion mutant and the complementation strain are listed in Table 2. Primers PrkA-US and PrkA-UA were used to amplify a 208-bp upstream fragment of prkA with an EcoRI restriction enzyme site at the 5’ end and a BamHI site at the 3’ end. Primers PrkA-DS and PrkA-DA were used to amplify a 444-bp downstream fragment of prkA containing a BamHI restriction enzyme site at the 5’ end and an XbaI site at the 3’ end. The two fragments were then digested with the relevant restriction enzymes using standard protocols. The two digested fragments were then cloned into plasmid pK18mobsacB digested with the same enzymes to generate plasmid pK18prkA, which was verified by sequencing [25].

To construct the plasmid for prkA-complementation, a DNA fragment containing full-length prkA and a putative promoter of prkA (500 bp upstream of prkA) was amplified from genome DNA of M. alhagi using primers CPA and CPB. The PCR product was then purified using a Universal DNA Purification Kit (Tiangen, China). The purified PCR product was then cloned into plasmid pBRR1MCS-5 digested with Smal, using the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech, China), to generate the complementation plasmid pBLprkA, which was verified by sequencing.

**Mutant and complement construction**

To construct the prkA deletion mutant, we first transformed pK18prkA into E. coli strain S17-1λpir. Then a biparental mating procedure was used, as described previously [26], to transform pK18prkA from E. coli S17-1λpir into M. alhagi CCNWJ12-2. Briefly, cultures of E. coli S17-1λpir (OD₆₅₀ [optical density at 600 nm] = 0.6) and M. alhagi CCNWJ12-2 (OD₆₅₀ = 0.8) were mixed together in the ratio 1:2 (v/v) and cultured on a TY agar plate for 3 days. Single exchange (plasmid pK18prkA integrated into genome DNA of M. alhagi) cells of M. alhagi were selected using SM agar plates containing kanamycin. Double exchange mutants (prkA deletion mutant) were then isolated using TY agar plates containing sucrose (5 g/100 ml). Both of single-exchange and double-exchange mutants were verified by colony PCR and sequencing.

For genetic complementation, pBLprkA was transformed from E. coli S17-1λpir into M. alhagi CCNWJ12-2 by biparental mating. SM agar plates containing gentamicin were used to isolate the complementation mutant, and colony PCR and sequencing were used to verify the mutant strain.

**Salt and alkali resistance assays**

Wild-type M. alhagi, ΔprkA, and CΔprkA were first grown in 20 ml TY broth to OD₆₅₀ ≈ 0.8. Then suspensions (20 μl) of the three cultures were added to 20 ml TY broth and grown to OD₆₅₀ ≈ 0.8. The inocula were adjusted to OD₆₅₀ = 0.2 with sterile water and then serially diluted to 10-fold. Then 5 μl of each diluted inoculum were respectively spotted onto TY agar plates at pH 7 containing no NaCl (control) or 0.4 M NaCl (salt treatment), or TY agar plates at pH 9 containing no NaCl (alkaline treatment). The pH of TY agar medium was adjusted using NaOH solution (1 M) before sterilization.

**Measurement of total cellular Na⁺ content**

Inocula were prepared as described in the section on the salt and alkali resistance assay. Five hundred microliters of each inoculum (OD₆₅₀ = 0.2) were plated on TY agar plates with and without 0.4 M NaCl and inoculated at 28 °C for 7 days. The bacteria were collected in 1.5-ml sterile tubes and dried using an air dryer at 50 °C for 12 h. The total cellular Na⁺ content was measured using an atomic absorption spectrophotometer (Hitachi, Tokyo, Japan).

**Sensitivity assay of H₂O₂**

Inocula (OD₆₅₀ = 0.1) were treated with or without 10 mM H₂O₂ for 30 min, then serially diluted and plated on TY agar plates. The colonies were counted after 10 days of growth at 28 °C. The percentage survival rate of the three stains was calculated as follows: [(CFU per ml after treatment with H₂O₂)/(CFU per ml before treatment with H₂O₂)] × 100.

**Antioxidant enzyme activity assays**

Wild-type M. alhagi, ΔprkA and CΔprkA were grown in TY broth medium to OD₆₅₀ ≈ 0.8. Each strain (20 μl suspension) was added to two bottles of 20 ml TY broth medium without additional NaCl and one bottle of 20 ml TY broth medium containing 0.4 M NaCl. Then

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**Table 2** Primers used for mutant and complement strain construction

| Primer  | Sequence (5’-3’) |
|---------|-----------------|
| PrkA-US | CGGAATTCCTCTGTCGCCGGA |
| PrkA-UA | CGGATCCGGACCTCGTTCGCCGA |
| PrkA-DS | CCGGATCCGGCTTCCCATACAG |
| PrkA-DA | GCTCTAGACACTCTCCGGTGCAG |
| CPA     | ATGAAGTGGTTAATCCCAGGGGC |
| CPB     | TACGACACATGGGCCGACGCCGAGGT |

*Restriction enzyme sites are underlined*
the bacteria were grown to OD_{600} = 0.8. One bottle of TY broth medium without NaCl was set as the control group and the other was treated with 0.1 mM H_{2}O_{2} for 30 min (H_{2}O_{2} treatment). Then all groups for the three strains were collected in 50-ml tubes by centrifugation at 8000 g for 5 min. The cells were lysed by ultrasonication. The enzyme activities of CAT, SOD and POD were determined using a CAT Assay Kit, a SOD Assay Kit, and a POD Assay Kit (Suzhou Comin Biotechnology, China), respectively, according to the manufacturer's protocols. Total soluble protein concentration was measured using a BCA Protein Assay Kit (CWBIO, China).

Statistical analysis
Statistical differences between the control and treatment groups of different strains were assessed by t-test using SPSS software version 15 (SPSS Inc., Chicago, IL, USA). Differences were considered to be significant at a probability level of P <0.05.

Abbreviations
CAT: Catalase; CFU: Colony forming units; ΔprkA: The complemented strain of M. alhagi prkA; POD: Peroxidase; SDS: Sodium dodecyl sulfate; SOD: Superoxide dismutase. ΔprkA: The prkA deletion mutant of Mesorhizobium alhagi CCNWXJ12-2

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article. The DNA sequence of the M. alhagi CCNWXJ12-2 prkA sequence is available under the accession No AHAM010000144.1. The M. alhagi CCNWXJ12-2 and the mutant strains can be obtained from the lab of Gehong Wei upon request.

Authors’ contributions
XL, YL and GMH make conception and design of this work. XL and YL conduct the laboratory work. XL and ZL carry out the data analysis and manuscript writing. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

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