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Role of exopolysaccharide in salt stress resistance and cell motility of *Mesorhizobium alhagi* CCNWXJ12–2^T

Xiaodong Liu[1] · **Yantao Luo**[1] · **Zhefei Li**[1] · **Jiamei Wang**[1] · **Gehong Wei**[1]

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Abstract *Mesorhizobium alhagi*, a legume-symbiont soil bacterium that forms nodules with the desert plant *Alhagi sparsifolia*, can produce large amounts of exopolysaccharide (EPS) using mannitol as carbon source. However, the role of EPS in *M. alhagi* CCNWXJ12-2^T, an EPS-producing rhizobium with high salt resistance, remains uncharacterized. Here, we studied the role of EPS in *M. alhagi* CCNWXJ12-2^T using EPS-deficient mutants constructed by transposon mutagenesis. The insertion sites of six EPS-deficient mutants were analyzed using single primer PCR, and two putative gene clusters were found to be involved in EPS synthesis. EPS was extracted and quantified, and EPS production in the EPS-deficient mutants was decreased by approximately 25 times compared with the wild-type strain. Phenotypic analysis revealed reduced salt resistance, antioxidant capacity, and cell motility of the mutants compared with the wild-type strain. In conclusion, our results indicate that EPS can influence cellular Na^+ content and antioxidant enzyme activity, as well as play an important role in the stress adaption and cell motility of *M. alhagi* CCNWXJ12-2^T.

Keywords Exopolysaccharide · Mini-Tn5 · Cell motility · Antioxidant capacity · *Mesorhizobium alhagi*

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✉ Gehong Wei
weigehong@nwsuaf.edu.cn

Xiaodong Liu
lxd19861216@126.com

Yantao Luo
yantao.luo@163.com

Zhefei Li
lizhefei@hotmail.com

Jiamei Wang
18829841629@163.com

[1] College of Life Sciences, State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest Agriculture and Forestry University, Yangling, Shaanxi 712100, China

Introduction

Rhizobia are Gram-negative soil-dwelling bacteria that fix nitrogen in symbiosis with legumes. Rhizobia are α- or β-Proteobacteria, and most of them are able to synthesize and excrete exopolysaccharide (EPS) when using carbohydrates (e.g., mannitol, sucrose, or lactose) as a carbon source (Janczarek 2011; Kucuk and Kivanc 2009). Various functions of EPS are described in rhizobia such as *Rhizobium* sp. PRIM-18, *Rhizobium leguminosarum*, and *Sinorhizobium meliloti* (Priyanka et al. 2015; Rinaudi et al. 2010). Although the role of EPS in symbiosis was established very early (Battisti et al. 1992; Urzainqui and Walker 1992), other biological functions, such as stress resistance (Jaszek et al. 2014; Lehman and Long 2013; Rodriguez-Navarro et al. 2014), biofilm formation (Rinaudi and Gonzalez 2009), and bioremediation (Castellane et al. 2015; Ye et al. 2014), have been discovered in recent years.

In the absence of the host plant or when the host plant dies, rhizobia dwell within the rhizosphere and encounter numerous environmental stresses, such as desiccation, high salt concentration, pH changes, temperature fluctuations, and nutrient limitations (Deaker et al. 2004). Unfavorable environments will trigger the secondary oxidative stress responses inside living bacteria (Banjerdkij et al. 2005; Kim et al. 2008; Mols and Abee 2011). In *Bacillus cereus*, the acid environment can induce the formation of reactive oxygen species (ROS), such

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as hydroxyl radicals (OH·) and superoxide (O2−); these ROS are a common cause of cellular death for bacteria in severe stress conditions (Mols et al. 2010). Many reports suggest that EPS can help bacteria survive high salt stress, desiccation, and oxidative stress conditions (Jaszek et al. 2014; Lehman and Long 2013; Navarini et al. 1992; Ophir and Gutnick 1994; Vriezen et al. 2007). In *Sinorhizobium meliloti*, purified EPS decreased H_2O_2 levels in vitro (Lehman and Long 2013); thus, EPS could play an important role in the stress adaption of rhizobia. Additionally, EPS is a minor component of biofilms that can act as a physical barrier against harsh environmental stresses (Branda et al. 2005; Flemming 1993). This could explain why EPS helps bacteria survive environmental stresses (Rinaudi and Gonzalez 2009; Rinaudi et al. 2010).

EPS is also involved in the cell motility of bacteria. An *exoA* mutant of *Sinorhizobium fredii* HH103 that is incapable of EPS production was found to have decreased cell motility and increased osmosensitivity compared with the wild-type strain (Rodriguez-Navarro et al. 2014). EPS is essential for the formation of rhizobia-legume symbiosis, which may be caused by EPS, the effect of EPS on cell motility, or a combination of the two. In *R. leguminosarum* bv. *trifolii*, the cell motility of a *pssA* mutant, which showed a deficiency in EPS synthesis, was significantly decreased compared with the wild type; in this case, the mutant was unable to form a symbiotic relationship with the host plant (Janczarek and Rachwal 2013).

Mesorhizobium alhagi CCNWXJ12-2^T (=ACCC 14561^T=HAMBI 3019^T) is a salt-resistant rhizobium isolated from the root nodules of the desert plant *Alhagi sparsifolia* (Chen et al. 2010), with an available draft genome sequence (GenBank accession number AHAM00000000) (Zhou et al. 2012). Like most rhizobia, *M. alhagi* CCNWXJ12-2^T is able to synthesize and excrete EPS using mannitol as a carbon source (Kucuk and Kivanc 2009; Staudt et al. 2012). However, the role of EPS in this bacterium has not been described. In this study, EPS-deficient mutants were constructed to explore the role of EPS in *M. alhagi* CCNWXJ12-2^T. Six EPS-deficient mutants were isolated, and analysis of insertion sites showed that the disrupted genes belonged to two gene clusters. Here, we characterized the two gene clusters involved in EPS production and analyzed their functions in the cell motility and stress adaption of *M. alhagi* CCNWXJ12-2^T. An efficient method was proposed for identifying the insertion sites and characterizing the role of EPS in stress adaption. This work will contribute to understanding the stress resistance mechanism of EPS in rhizobia.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this work are listed in Table 1. The wild-type, EPS-deficient mutants and their complement strains of *M. alhagi* CCNWXJ12-2^T were grown in tryptone-yeast extract (TY) broth (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$ per liter) or modified minimal salt manntiol (SM) medium (0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2$, 0.5 g KNO_3, 0.5 g K_2HPO_4, 0.1 g NaCl, 10 g mannitol, 75 mg pantothenic acid, 75 mg biotin, and 75 mg thiamine per liter) at 28 °C. *Esherichia coli* was cultured in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) at 37 °C. All bacteria were incubated in aerobic conditions. Where necessary, antibiotics were added at the following: gentamicin 50 μg/mL, kanamycin 100 μg/mL, or ampicillin 100 μg/mL.

Transposon mutagenesis and isolation of EPS-deficient mutants

A suicide plasmid pRL27 which contains a mini-Tn5 was used to perform mutagenesis (Larsen et al. 2002). A slightly altered triparental mating procedure was conducted to transform the plasmid pRL27 from *E. coli* to *M. alhagi* CCNWXJ12-2^T (Dhooghe et al. 1995). Briefly, *E. coli* BW20767 containing the plasmid pRL27 [optical density at 600 nm (OD_{600}) ≈ 0.6], the MM249 strains containing the helper plasmid pRK2013 (OD_{600} ≈ 0.6), and the wild-type *M. alhagi* CCNWXJ12-2^T (OD_{600} ≈ 0.8) were mixed together (1:1:2 ratio by volume), spotted on TY agar plates, and cultured for 3 days. SM plates containing kanamycin were then used to isolate transconjugants. Transconjugants with an EPS-deficient colony morphology (i.e., rough surface) were selected.

Identification of Tn5 insertion site

A slightly modified single primer PCR method was used to identify the sequences flanking the insertion site (Karlyshev et al. 2000). Four primers (i.e., SPU1, SPD1, SPU2, and SPD2; Table S1) were used to amplify the flanking sequences at either end of the transposon. Genomic DNA was extracted following Wilson and Carson (2001). PCR amplifications of sequences flanking the insertion site were performed using 2×Taq MasterMix (Dye) (CoWin Biosciences, Beijing, China) according to the manufacture's protocol. A modified touch-up PCR reaction was performed as follows: (1) 95 °C, 5 min, (2) 95 °C 30 s, (3) 45 °C + 0.5 °C per cycle 30 s, (4) 72 °C, 5 min, (5) go to step 2 for 35 cycles, and (6) 72 °C, 10 min (Mansharamani et al. 2001). The PCR products were checked by 1% agarose gel electrophoresis. The dominant bands (>500 bp) were excised and purified using the Universal DNA Purification Kit (Tiangen, Beijing, China). The product was then cloned into the pMD 18-T vector (Takara, Otsu, Shiga, Japan) and transformed into a competent *E. coli* DH5α cell. Plasmids were extracted and sequenced (Sangon Biotech., Shanghai, China) using the M13R and M13F primers (Table S1). The obtained sequence was compared by BLAST analysis to the genome sequence of

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Esherichia coli strains		
DH5α	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169 deoR [Φ80 dlacΔ(lacZ)M15]*	Invitrogen
MM294	*supE44λ⁻ rfbD1 spoT thi-1 endA1 hsdR17 pro*	(Neidhardt 1987)
BW20767	RP4-2-Tc::Mu-1 kan::Tn7 integrant, leu-63::IS10 *recA1zbf-5 creB510 hsdR17endA1 thi uidA*(*MluI*)::*pir*+	(Larsen et al. 2002)
Mesorhizobium alhagi strains		
WT	Wild-type strain	(Zhou et al. 2012)
MAED1	XJ12-2 *galE* ::Tn5; Km^r	This study
MAED2	XJ12-2 *exoK*::Tn5; Km^r	This study
MAED3	XJ12-2 *exoP*::Tn5; Km^r	This study
MAED4	XJ12-2 *exoK*::Tn5; Km^r	This study
MAED5	XJ12-2 *exoK*::Tn5; Km^r	This study
MAED6	XJ12-2 *exoA*::Tn5; Km^r	This study
Plasmids		
pRL27	Km^r; mini-Tn5 plasposon (oriR6K) delivery vector	(Larsen et al. 2002)
pMD 18-T	Cloning vector; Amp^r	Takara, Japan
pBLprkA	pBBR1MCS-5 carrying a *prkA* promoter and the full-length *prkA*of XJ12–2	(Liu et al. 2016)
pBLED1	pBL::*galEoperon*	This study
pBLED3	pBL::*exoP*	This study
pBLED5	pBL::*exoKLAMONP*	This study
pBLED6	pBL::*exoAMONP*	This study

Km^r kanamycin resistance, *Amp^r* ampicillin resistance, *Gm^r* gentamicin resistance

M. alhagi CCNWXJ12-2^T to identify the mini-Tn5 genome junction (Altschul et al. 1990).

Mutant complement construction

Primers used to construct the plasmids for complementation of the EPS-deficient mutants are listed in Table S1. Plasmid pBLprkA was used to construct complementation plasmids (Liu et al. 2016). Primers CP1 and CP2 were used to generate the linearized vector (5268 bp) from the plasmid pBLprkA for complement plasmid construction. Primers CED1-U and CED1-D were used to amplify the DNA fragment of gal operon (fragment containing *galE* and the next five genes encoding alanine racemase, CapD, two hypothetical proteins, and sugar transferase, 7124 bp) for complementation of MAED1; CED3-U and CEDP-D were used to amplify the DNA fragment of *exoP* (2325 bp) for complementation of MAED3; CED5-U and CEDP-D were used to amplify the DNA fragment of *exoKLAMONP* (fragment containing genes *exoK* to *exoP*, 8400 bp) for complementation of MAED5; and CED6-U and CEDP-D were used to amplify the DNA fragment of *exoAMONP* (fragment containing genes *exoA* to *exoP*, 6380 bp) for complementation of MAED6. The PCR products were checked using 1% agarose gel electrophoresis, and the target bands were excised and purified using the Universal DNA Purification Kit (Tiangen). The purified PCR products were cloned into the plasmid pBLprkA DNA fragment using the ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech., Nanjing, China). The complementation plasmids (i.e., pBLED1, pBLED3, pBLED5, and pBLED6) were verified by sequencing. The complementation plasmids were transformed into the corresponding mutants using a triparental mating procedure. SM agar plates containing kanamycin and gentamicin were used to isolate the complemented strains, and colony PCR and sequencing were used to verify complementation.

Isolation and quantification of total soluble EPS

The isolation of EPS was performed following Sandal et al. with some modifications (Sandal et al. 2011). All strains were grown in TY broth to an OD_{600} of 0.8. Then, 100 μL of each inoculum was smeared on SM and TY agar plates containing the proper antibiotics and cultured at 28 °C for 5 days. The cells were collected, suspended in sterile distilled water, washed with sterile distilled water twice by vortexing vigorously at room temperature for 5 min, and centrifuged (12,000 rpm, 13,225×*g*) for 10 min at 4 °C. The supernatant was transferred into another tube for EPS isolation, while the cell pellet was dried and weighed. EPS was precipitated from

the supernatant (20 mL) with 96% cold ethanol at volumetric ratio of 1:4 (supernatant/ethanol). Then, EPS was collected by centrifugation (11,000 rpm, 11,384×*g*) at 4 °C for 30 min, and the pellet was washed three times with 100% ethanol. After the ethanol was evaporated, the EPS was dissolved in sterile deionized water, and total EPS content was measured using the phenol-sulfuric method (Dubois et al. 1956).

Analysis of salt resistance

Wild-type strain, EPS-deficient mutants, and the complementation strains of *M. alhagi* CCNWXJ12-2^T were grown in 20 mL of TY broth at 28 °C with shaking to an OD_{600} of 0.8. Suspensions (20 μL) of the three cultures were added to 20 mL of fresh TY broth and grown to an OD_{600} of 0.8. The inoculums were adjusted to an OD_{600} of 0.2 with sterile water and then serially diluted 10-fold. Then, 5 μL of each diluted inoculum were spotted onto TY agar plates without or with salt (0.4 M NaCl, 0.3 M LiCl, 0.3 M KCl, or 0.2 M Na_2SO_4).

Measurement of total cellular Na^+ content

Five hundred microliters of each inoculum ($OD_{600} \approx 0.2$ for wild-type strain and the complement strains and $OD_{600} \approx 0.5$ for the EPS-deficient mutants) was plated on TY agar plates containing the proper antibiotics with or without 0.4 M NaCl and grown at 28 °C for 5 days. The bacteria collection and pretreatment, as well as the Na^+ content measurements, were conducted as previously described (Liu et al. 2016).

Hydrogen peroxide sensitivity assay

Inoculums were treated with or without 10 mM H_2O_2 for 30 min at 28 °C, serially diluted, and plated on TY agar plates with the proper antibiotics. The colony-forming units (CFU) were counted after 10 days of growth at 28 °C. The percentage survival rate of all the stains was calculated as follows: [(CFU per mL after treatment with H_2O_2)/(CFU per mL before treatment with H_2O_2)] × 100.

Measurement of antioxidant enzyme activities

The wild-type strain and EPS-deficient mutants were grown in TY broth containing the proper antibiotics to an OD_{600} of 0.8. The bacteria growth, salt treatment, and H_2O_2 treatment were conducted according to previously described methods (Liu et al. 2016). Briefly, three bottles of 20-mL TY broth were used to culture the strains to an OD_{600} of 0.8. Two bottles did not contain additional NaCl and one bottle contained 0.4 M NaCl. One bottle without NaCl was used as the control, and the other was treated with 0.1 mM H_2O_2 for 30 min (H_2O_2 treatment). Then, all groups were collected and lysed by ultrasonication. Catalase (CAT) and superoxide dismutase (SOD) activities were measured using commercial kits (Comin Biotech., Suzhou, China) following the manufacturer's instructions. Total soluble protein concentration was measured using a BCA Protein Assay Kit (CoWin Biosciences, Beijing, China).

Cell motility assay

Cell motility was tested on semi-solid TY agar plates (0.3% agar). The inoculum (5 μL) was spotted onto soft TY agar plates and incubated for 3 days at 28 °C. The diameter of the colonies was measured in millimeters (mm). At least three plates were inoculated for each strain and cell motility assay was repeated three times.

Results

Isolation of *M. alhagi* CCNWXJ12-2^T EPS-deficient mutants

The wild-type *M. alhagi* CCNWXJ12-2^T can produce amount of EPS when grown on SM agar plates (Fig. 1a). The wild-type strain was subjected to random mutagenesis using plasmid pRL27. After being screened on SM agar plates containing kanamycin, six mutants with EPS-deficient phenotype were isolated; these isolates were termed MAED1–MAED6. The colony morphologies are shown in Fig. 1. The wild-type strain forms a mucous surface; meanwhile, the surfaces of the mutants are rough.

Insertion site identification and complementation strain construction

Mini-Tn5 insertion sites were identified in the EPS-deficient mutants by single primer PCR. In MAED1, a gene coding for an UDP-glucose 4-epimerase (GalE) was interrupted by mini-Tn5. MAED2, MAED4, and MAED5 showed disruption of the same gene, which codes for a 1,3-1,4-β-D-glucan-4 glucanohydrolase (ExoK), but with different insertion sites (Table 2). NaCl stress resistance of the three mutants was similar (data not shown); so, we randomly chose MAED5 for further studies. MAED3 carries a mini-Tn5 insertion of an EPS transporter-encoding gene (ExoP). Finally, in MAED6, mini-Tn5 was inserted in a glycosyltransferase protein gene (ExoA). Based on the analysis of the interrupted and surrounding genes, we propose two gene clusters that are involved in EPS production by *M. alhagi* CCNWXJ12-2^T (Fig. 2).

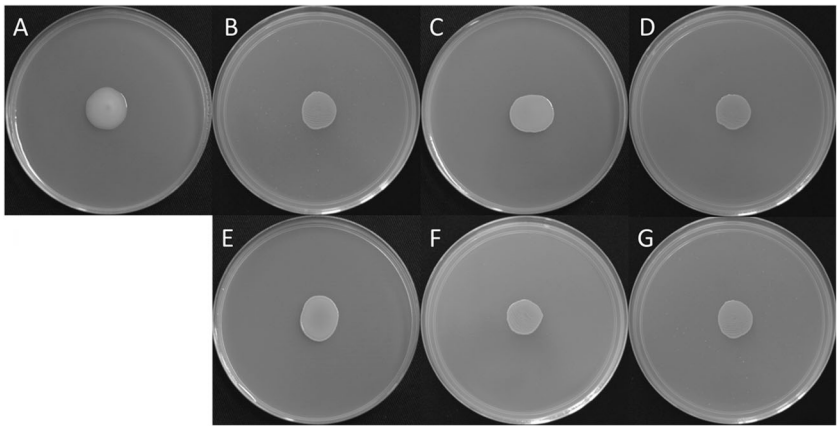

Fig. 1 Phenotypes of wild-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains on modified minimal (SM) medium agar plates. Wild-type and exopolysaccharide-deficient mutant strains were grown to $OD_{600} \approx 0.8$ in tryptone-yeast extract broth medium, and then, either 5 μL (wild-type) or 10 μL (mutants) inoculum was spotted onto SM agar plates containing 100 μg/mL kanamycin. Plates were incubated at 28 °C for 4 days. **a**–**g** The wild-type strain and MAED1 ~ MAED6 strains, respectively

EPS production

The total soluble EPS was isolated and quantified to determine the difference in EPS production between the wild-type and mutant strains (Fig. 3). The wild-type strain can produce up to 3500 μg EPS/mg dry bacteria on SM agar plates (Fig. 3a) and 90 μg EPS/mg dry bacteria on TY agar plates (Fig. 3b); meanwhile, the EPS-deficient mutant can only produce 100–200 μg EPS/mg dry bacteria on SM agar plates (Fig. 3a) and 50–65 μg EPS/mg dry bacteria on TY agar plates (Fig. 3b).

Effect of EPS on salt stress resistance

Salt stress resistance of the EPS-deficient mutants was queried to determine whether EPS produced by *M. alhagi* CCNWXJ12-2^T contributes to adaptations to environmental stresses. All EPS-deficient mutants showed decreased tolerance to salt stresses compared with the wild-type strain. KCl and Na_2SO_4 exhibited greater inhibition of the EPS-deficient mutants than LiCl or NaCl did. MAED3 had the highest sensitivity of all mutants to salt stress, and its growth was dramatically repressed under salt stress conditions. With 0.4 M NaCl, the suppression of MAED3 was higher than that of MAED1, MAED5, or MAED6 compared against the wild-type strain. A similar situation was found on TY agar plates containing 0.3 M LiCl. The growth of MAED1, MAED5, and MAED6 was suppressed on TY agar plates containing 0.3 M KCl; meanwhile, MAED3 growth was completely inhibited. The growth of the EPS-deficient mutants was similar under 0.2 M Na_2SO_4 and 0.3 M KCl. Meanwhile, similar to the wild-type strain, all the complementation strains had restored salt tolerance (Fig. 4).

Effect of EPS on cellular Na^+ content

Since the salt resistance of the EPS-deficient mutants was significantly decreased compared with the wild-type strain, we measured the total cellular Na^+ content of the strains to determine the influence of EPS on cellular Na^+ content. No significant differences were found in the total cellular Na^+ content between the wild-type strain and EPS-deficient mutants under the control condition (i.e., without additional NaCl). However, the cellular Na^+ contents of the EPS-deficient mutants were increased by one third compared with the wild-type strain under high salt conditions (0.4 M NaCl). For the wild-type strain and EPS-deficient mutants, the cellular Na^+ contents under high salt condition were increased by 15 and >20 times, respectively, compared with those under the control condition (Fig. 5).

Table 2 Identification of insertion sites in six exopolysaccharide (EPS)-deficient mutants of *Mesorhizobium alhagi* CCNWXJ12-2^T

Mutant name	Locus tag in genome	Gene annotation	Insertion location
MAED1	MAXJ12_10812	UDP-glucose 4-epimerase, GalE	94–95
MAED2	MAXJ12_01284	(1,3)-(1,4)-beta-D-glucan 4-glucanohydrolase, ExoK	361–362
MAED3	MAXJ12_01314	EPS transporter, ExoP	781–782
MAED4	MAXJ12_01284	(1,3)-(1,4)-beta-D-glucan 4-glucanohydrolase, ExoK	472–473
MAED5	MAXJ12_01244	(1,3)-(1,4)-beta-D-glucan 4-glucanohydrolase, ExoK	63–64
MAED6	MAXJ12_01294	glycosyltransferase protein, ExoA	237–238

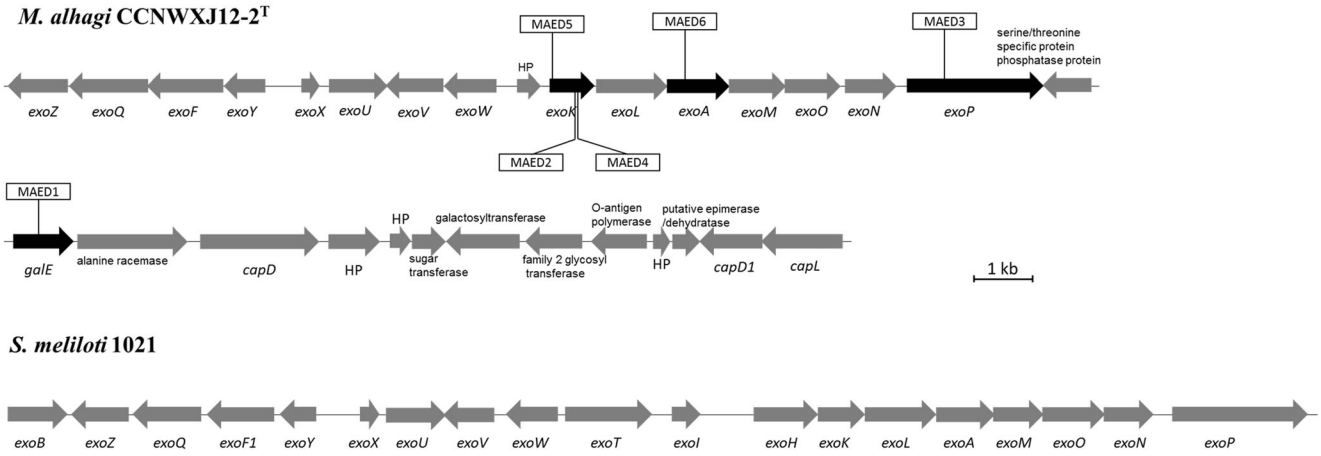

Fig. 2 Genetic organization of two gene clusters involved in exopolysaccharide production by *Mesorhizobium alhagi* CCNWXJ12-2^T. The disrupted genes are shown in *black*. The names and insertion positions of the exopolysaccharide-deficient mutants are indicated. The *exo* gene cluster of *Sinorhizobium melitoti* 1021 is shown at the *bottom*

Effect of EPS on oxidative stress tolerance

After treatment with H_2O_2, the survival rates of MAED3 and MAED5 were similar (one half that of the wild-type strain), while the survival rates of MAED1 and MAED6 were almost two thirds that of the wild-type strain. The survival rates of the complementation strains were slightly lower than the wild-type strain; however, the difference was not significant (Fig. 6). These results suggest that EPS can help bacteria survive under oxidative stress.

Effect of EPS on antioxidant enzyme activities

The antioxidant enzyme activities were measured to check the difference in antioxidant capacity between the wild-type strain and the EPS-deficient mutants. Under control conditions, the EPS-deficient mutants showed 30–42% lower CAT activities and almost the same SOD activities compared with the wild-type strain. Both the CAT and SOD activities were enhanced 50–180% by high salt and 0~100% by oxidative stress in all strains. The enzyme activities of EPS-deficient mutants remained 26–44% lower compared with the wild-type strain under stress conditions (Fig. 7).

Effect of EPS on cell motility

To investigate whether EPS produced by *M. alhagi* CCNWXJ12-2^T contributes to the cell motility, the wild-type strain and EPS-deficient mutants were assayed using motility tests. The colony diameter of the wild-type strain could reach

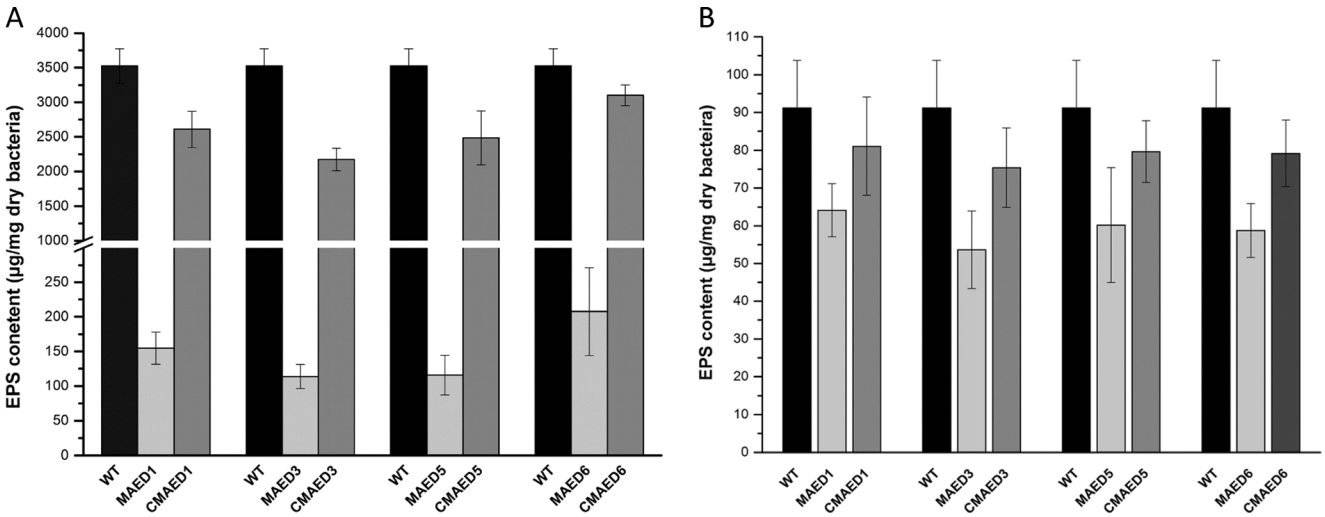

Fig. 3 Exopolysaccharide production of wide-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains on SM (**a**) and TY (**b**) agar plates. The wild-type (WT), exopolysaccharide-deficient (MAED), and complement (CMAED) strains are shown in *black*, *light gray*, and *dark gray*, respectively. All the strains were grown to $OD_{600} \approx 0.8$ in tryptone-yeast extract broth, and 100 μL of each strain was smeared on SM and TY agar plates containing proper antibiotics. The cells were collected after incubation at 28 °C for 5 days, and the EPS was extracted and quantified using the phenol-sulfuric method. Data shown are the means of three independent experiments, and the *error bars* indicate standard deviations

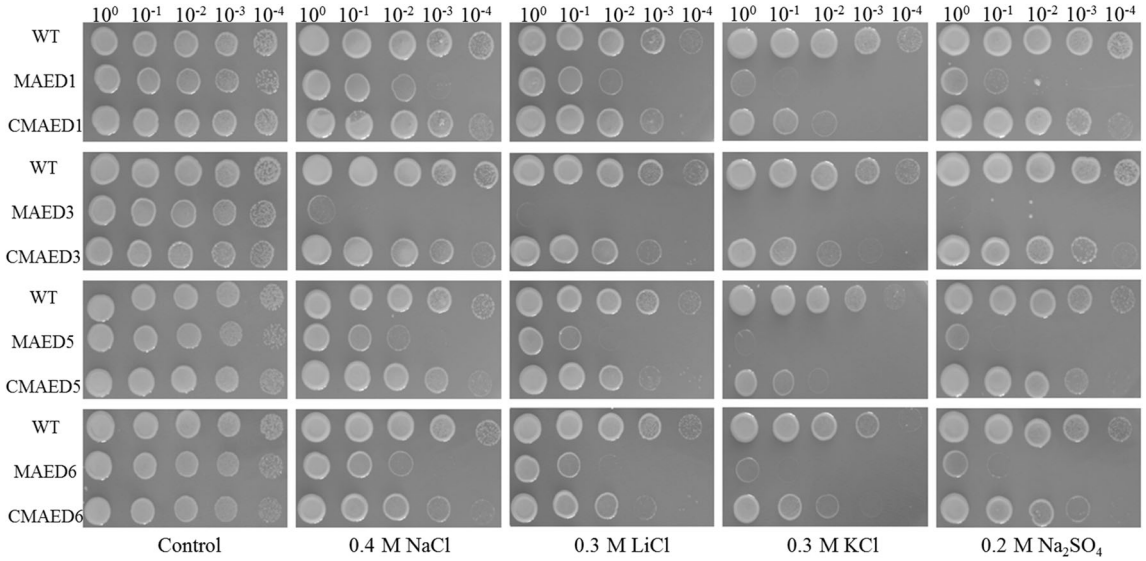

Fig. 4 Sensitivity of the wild-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains to different salts. The wild-type (WT), exopolysaccharide-deficient (MAED), and complement (CMAED) strains were grown to $OD_{600} \approx 0.8$ in tryptone-yeast extract broth and adjusted to $OD_{600} \approx 0.2$. The inoculums were serially diluted and spotted onto tryptone-yeast extract agar plates containing either no additional NaCl or different additional salts (i.e., 0.4 M NaCl, 0.3 M LiCl, 0.3 M KCl, or 0.2 M Na_2SO_4). Ten-fold serial dilutions are shown. The plates without additional NaCl were incubated at 28 °C for 3 days, while the plates with different salts were incubated for 5 days at the same temperature

to approximately 10 mm after incubating for 3 days. A dramatic decrease was found in the cell motility of the EPS-deficient mutants compared with the wild-type strain. In terms of the average colony diameter, MAED3 and MAED5 were almost the same and approximately one third of the wild-type strain; meanwhile, MADE1 and MAED6 were almost one half of the wild-type strain, respectively. The colony diameter of the complementation strains was similar to the wild-type strain (Fig. 8).

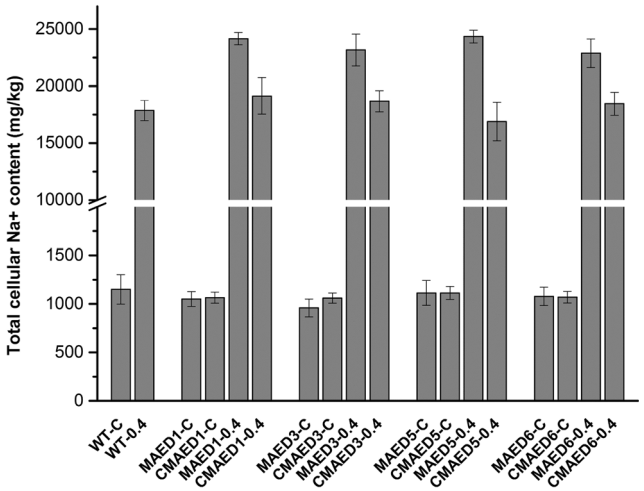

Fig. 5 Total cellular Na^+ content of the wild-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains. The wild-type (WT), exopolysaccharide-deficient mutants (MAED), and complement (CMAED) strains were grown to $OD_{600} \approx 0.8$ in tryptone-yeast extract broth. Five hundred microliters of each inoculum ($OD_{600} \approx 0.2$ for wild-type strain and the complement strains and $OD_{600} \approx 0.5$ for the EPS-deficient mutants) were plated on tryptone-yeast extract agar plates with or without 0.4 M NaCl containing proper antibiotics and inoculated at 28 °C for 5 days. The bacterial cells were collected, and cellular Na^+ content was measured using an atomic absorption spectrophotometer. The suffix "-C" stands for the control group and "-0.4" stands for the high salt treatment group (0.4 M NaCl). Three independent experiments were conducted, and the *error bars* indicate standard deviations

Discussion

M. alhagi CCNWXJ12-2^T is capable of high salt resistance and EPS production (Chen et al. 2010). It remains unclear whether EPS influences salt resistance and other traits in *M. alhagi* CCNWXJ12-2^T. We isolated six EPS-deficient mutants of *M. alhagi* CCNWXJ12-2^T (MAED1–MAED6) by transposon mutagenesis, in order to clarify the role of EPS in this rhizobium. The six mutants have disruptions in four genes (*exoK*, *exoA*, *exoP*, and *galE*). The four disrupted genes belong to two gene clusters that are involved in EPS production, indicating that there are at least two gene clusters responsible for EPS production in *M. alhagi* CCNWXJ12-2^T.

We successfully obtained the insertion sites of the EPS-deficient mutants using this method. The disrupted gene in MAED1 was *galE*, which encoded an UDP-glucose 4-epimerase involved in lipopolysaccharide or EPS production (Chang et al. 2008; Degeest and de Vuyst 2000; Li et al. 2014; Mozzi et al. 2003). In *M. alhagi* CCNWXJ12-2^T, our results indicate that *galE* plays a role in stress resistance and cell motility, and the EPS production of MAED1 was decreased dramatically (Fig. 3). The component and production

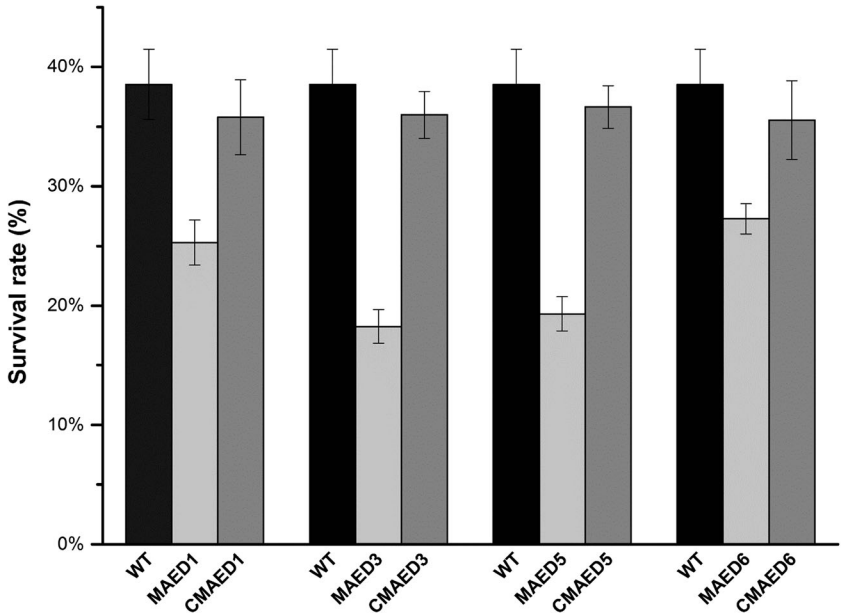

Fig. 6 Survival rate of the wild-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains after H_2O_2 treatment. The wild-type (WT), exopolysaccharide-deficient (MAED), and complement (CMAED) strains were grown to $OD_{600} \approx 0.8$ in tryptone-yeast extract broth and adjusted to $OD_{600} \approx 0.2$. The inoculums were treated with 10 mM H_2O_2 for 30 min or incubated without H_2O_2 treatment as a control. The percentage survival rate was calculated as follows: [(CFU per mL after treatment with H_2O_2)/(CFU per mL before treatment with H_2O_2)] × 100. The means of three independent experiments are shown, and the *error bars* indicate standard deviations

of lipopolysaccharide and EPS produced by MAED1 were both changed compared with the wild-type strain (data not shown). The other three mutants in the *exo* gene cluster (*exoK*, *exoA*, and *exoP*) had decreased EPS production, stress adaption, and cell motility. ExoK is a 1,3-1,4-β-D-glucan-4 glucanohydrolase (EC 3.2.1.73) belonging to glycosyl hydrolase family 16 and can hydrolyze linked β-D-glucans, such as lichenan and barley β-glucan (York and Walker 1998b). ExoA acts as the first glucosyltransferase that catalyzes the subunit assembly of carbohydrates (Leigh and Walker 1994). ExoP is a tyrosine kinase that contributes to polymerization and export of EPS succinoglycan (Jofré and Becker 2009). The *exo* gene clusters in *M. alhagi* CCNWXJ12-2^T and *S. meliloti 1021* are similar, although the latter contains four genes that are absent in the former: *exoB*, *exoT*, *exoI*, and *exoH*. The protein sequence similarities of ExoA, ExoK, and ExoP between

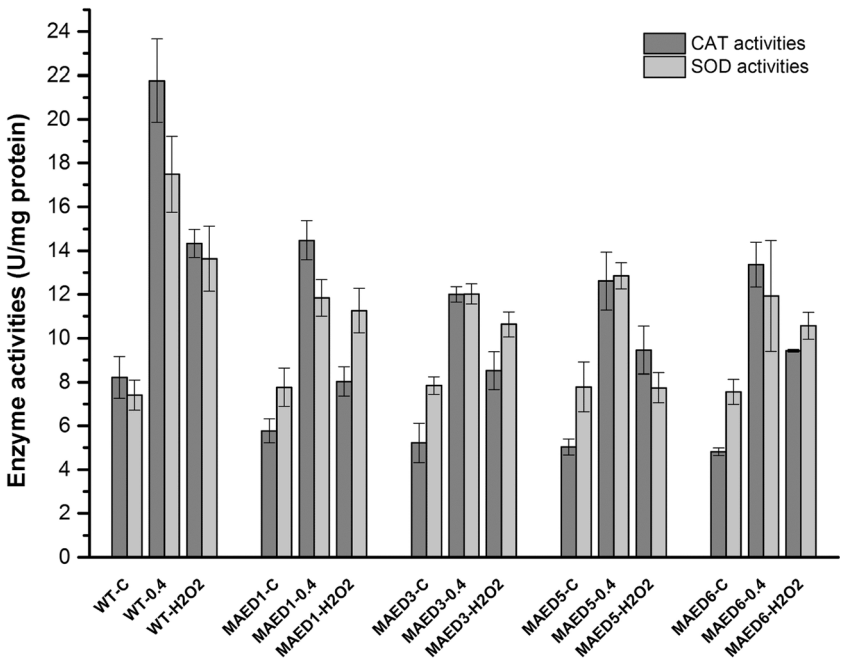

Fig. 7 Antioxidant enzyme activities of the wild-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains. Catalase (CAT) and superoxide dismutase (SOD) activity in the wild-type (WT), exopolysaccharide-deficient (MAED), and complement (CMAED) strains were measured in three independent biological experiments. The *error bars* indicate standard deviations. The suffix "-C," "-0.4," and "-H_2O_2" stands for control group, high salt treatment group (0.4 M NaCl), and H_2O_2 treatment group, respectively

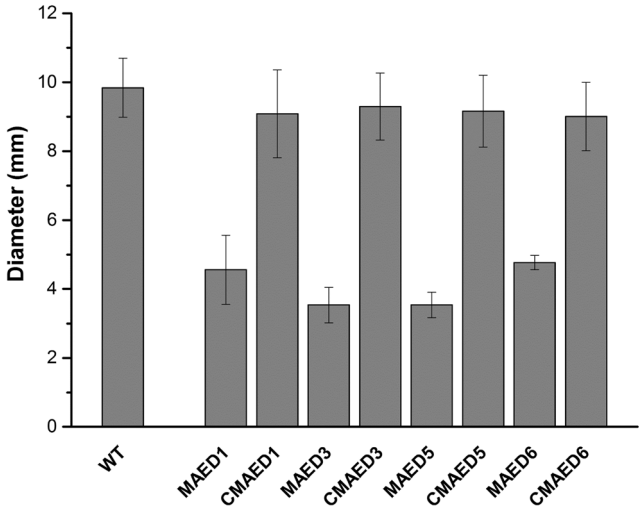

Fig. 8 Cell motility of the wild-type *Mesorhizobium alhagi* CCNWXJ12-2^T and mutant strains on semi-solid tryptone-yeast extract agar plates. The wild-type (WT), exopolysaccharide-deficient (MAED), and complement (CMAED) strains were grown to $OD_{600} \approx 0.8$ in tryptone-yeast extract broth and then adjusted to $OD_{600} \approx 0.2$. Inoculum (5 μL) was spotted onto soft tryptone-yeast extract agar plates (0.3% agar) and incubated for 5 days at 28 °C. Colony diameter was measured in millimeters. The means of three independent experiments are shown, and the *error bars* indicate standard deviations

M. alhagi CCNWXJ12-2^T and *S. meliloti* 1021 were 63.16, 59.35, and 51.97%, respectively. The high similarities of the protein sequences suggested the similar function of the genes. The EPS production of MAED3, MAED5, and MAED6 on SM agar plates was 4–6% of the wild-type strain (Fig. 3a). In *S. meliloti* 2011, the *exoA* and *exoP* mutants could not produce EPS and form effective nodules with *Medicago sativa* while the *exoK* mutant could still produce 50% EPS of the wild-type strain and form effective nodules with *M. sativa* (Becker et al. 1993). The lower EPS production of MAED5 compared with the *exoK* mutant of *S. meliloti* 2011 might be attributed to the polar effects of Tn5-insertion. Recent researches have shown that the *exo* gene cluster *S. meliloti* is involved in EPS production (especially low-molecular-weight succinoglycan) and symbiosis (Mendis et al. 2013; York and Walker 1997; York and Walker 1998a). There is a dependent relationship between the two gene clusters; when one gene cluster loses its function, the mutant will be EPS deficient. Thus, the two gene clusters likely cooperate to produce EPS in *M. alhagi* CCNWXJ12-2^T.

The EPS production markedly differed between *M. alhagi* CCNWXJ12-2^T strains grown on SM and TY agar plates. The EPS production of the wild-type and complement strains grown on TY agar plates were approximately one fourth of the same strains grown on SM agar plates; meanwhile, the EPS production of EPS-deficient strains grown on TY agar plates were almost one half of the same strains grown on SM agar plates (Fig. 3). These results indicate that carbon source significantly contributed to EPS production by *M. alhagi* CCNWXJ12-2^T. Numerous reports have also shown that carbon source plays an important role in EPS production by rhizobia (Janczarek et al. 2015; Janczarek and Urbanik-Sypniewska 2013). Notably, the EPS production by EPS-deficient mutants of *M. alhagi* CCNWXJ12-2^T grown on TY agar plates was approximately two thirds of the wild-type strain (Fig. 3).

Our results showed that the total cellular Na^+ content of the mutants under high salt conditions (0.4 M NaCl) was much higher than the wild-type strain; this could partially explain the decrease in salt resistance of the mutants. Stressful environmental conditions can trigger secondary oxidative stress in bacteria. Additionally, antioxidant enzymes can eliminate intracellular ROS and help the bacteria overcome environmental stresses (Harrison et al. 2012; Mols and Abee 2011). We were unable to detect POD activity in *M. alhagi* CCNWXJ12-2^T; therefore, we quantified CAT and SOD activity (Liu et al. 2016). This showed decreased antioxidant abilities of the EPS-deficient mutants compared with the wild-type strain. The antioxidant enzyme activities of the mutants were also lower than the wild-type strain, especially under stress conditions. EPS plays an important role in antioxidant regulation, and purified EPS has been shown to decrease H_2O_2 levels in vitro in *S. meliloti* (Lehman and Long 2013). The decrease in CAT and SOD activities could cause the decreased antioxidant capabilities and may also contribute to the salt sensitivity of the EPS-deficient mutants.

Cell motility has been described in many Gram-negative bacteria, including *Rhizobium*, *Pseudomonas*, *Agrobacterium*, and *Vibrio* (Daniels et al. 2006; Garrison-Schilling et al. 2014; Kohler et al. 2000; Sule et al. 2009). A few reports have shown that cell motility can be enhanced by EPS in some bacteria (Garrison-Schilling et al. 2014; Rodriguez-Navarro et al. 2014). The results presented here also showed that EPS production impacted the cell motility of *M. alhagi* CCNWXJ12-2^T on semi-solid TY agar medium and that the EPS-deficient mutants showed a dramatic decrease in cell motility compared with the wild-type strain. The growth rate of the EPS-deficient mutants was almost the same with the wild-type strain on TY agar plates (Fig. 4 control group) and in TY broth (data not shown). Under suitable conditions, the growth rate of the EPS-deficient mutants was also almost the same with the wild-type strain in other bacteria (Nogales et al. 2012). To have a better understanding of decreased cell motility in EPS-deficient mutants, four genes involved in flagella biosynthesis (flgA, flgB, fliI, and fliQ) were chosen to detect the expression pattern in the wild-type strain and EPS-deficient mutants. However, no obvious differences were found (data not shown). There are two types of cell motility in rhizobia: sliding, which mainly depends on the EPS production, and swarming, which mainly depends on the flagella (Nogales et al. 2012). We consider that the EPS-deficient mutants of *M. alhagi* CCNWXJ12-2^T might only

retain the swarming ability but lose the sliding ability, resulting in decreased cell motility.

EPSs are important for rhizobia-legume symbiosis. EPS-deficient mutants of *Mesorhizobium tianshanense* exhibit a severe reduction in nodulation capacity compared with the wild-type strain (Wang et al. 2008). However, the instability of symbiosis between *M. alhagi* CCNWXJ12-2^T and *A. sparsifolia* under laboratory conditions has hindered further investigation of the differences between EPS-deficient mutants and the wild-type strain. This phenomenon may be due to differing environmental conditions between the laboratory and the field. Since *A. sparsifolia* is a desert plant, its natural habitat is severe and dry, with high salinity levels and a large diurnal temperature difference (Zeng et al. 2002). Despite our best efforts to mimic these conditions in the laboratory, symbiosis was still unstable. However, our results suggest that the decrease in EPS production by the mutant strains will reduce the formation of a symbiotic relationship between the mutants and *A. sparsifolia*.

In *S. meliloti*, the whole *exo*HKLAMONP gene cluster was transcribed by a strong promoter at the upstream of *exoH* (Becker et al. 1993). A similar situation was also found in *M. alhagi* CCNWXJ12-2^T. Based on the RNA-Seq results, we found that the *exoK*, *exoL*, *exoA*, *exoM*, *exoO*, *exoN*, and *exoP* genes were in an operon, while the *galE* gene and its five downstream genes were in another operon (Fig. 2) (Liu et al. 2014). The expression of *exoP* could complement the phenotype of MAED3 for complementation construction because *exoP* was the last gene in the operon. Due to the polar effect of the mini-Tn5 insertion, the complement plasmids of pBLED1, pBLED5, and pBLED 6 were constructed using DNA fragments of the *galE* operon (a fragment containing *galE* and the five subsequent genes), *exoKLAMONP* (a fragment containing *exoK* to *exoP*), and *exoAMONP* (a fragment containing *exoA* to *exoP*); the complement strains restored the wild-type phenotype.

This is the first study to analyze the genes involved in EPS production by *M. alhagi* CCNWXJ12-2^T. Two gene clusters were found to be responsible for the EPS production. EPS helped the rhizobium to adapt to salt and oxidative stresses and increased the cell motility of *M. alhagi* CCNWXJ12-2^T.

Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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