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ORIGINAL ARTICLE

CRISPR/Cas9 genome editing shows the important role of *AZC_2928* gene in nitrogen-fixing bacteria of plants

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Abstract

*AZC_*2928 gene (GenBank accession no. BAF88926.1) of *Azorhizobium caulinodans* ORS571 has sequence homology to 2,3-aminomutases. However, its function is unknown. In this study, we are for the first time to knock out the gene completely in *A. caulinodans* ORS571 using the current advanced genome editing tool, CRISPR/Cas9. Our results show that the editing efficiency is 34% and *AZC_2928* plays an extremely important role in regulating the formation of chemotaxis and biofilm. CRISPR/Cas9 knockout of *AZC_2928* (*ΔAZC_2928)* significantly enhanced chemotaxis and biofilm formation. Both chemotaxis and biofilm formation play an important role in nitrogen-fixing bacteria and their interaction with their host plants. Interestingly, AZC_2928 did not affect the motility of *A. caulinodans* ORS571 and the nodulation formation in their natural host plant, *Sesbania rostrata*. Due to rhizobia needing to form bacteroids for symbiotic nitrogen fixation in mature nodules, *AZC_2928* might have a direct influence on nitrogen fixation efficiency rather than the number of nodulations.

Keywords Genome editing · *Azorhizobium caulinodans* ORS571 · *AZC_*2928 · Symbiotic nitrogen fixation · CRISPR/Cas9 · Plant

Introduction

Plants need nitrogen for growth and development. Rhizobia are widely accepted endosymbionts that induce nitrogen-fixing nodules on the roots or stems of legume hosts (Masson-Boivin et al. 2009; Boivin et al. 1997). Legumes and rhizobia can establish a symbiotic relationship, in which legumes provide a carbon source for rhizobia to survive through its own nitrogen-fixing function; at the same time, rhizobia convert N_2 in the air into NH_4^+, which can directly be used by plants (Oldroyd and Downie 2008, Oldroyd et al. 2011; Margaret et al. 2011). *Azorhizobium caulinodans* ORS571 not only fixes nitrogen by itself but could also fix nitrogen with its natural host, *Sesbania cannabina*, symbiotically. Chemotaxis is the way bacteria move, helping bacteria to adapt quickly to the living environment. The uneven distribution of nutrients, heterogeneous pollutants, and water conditions leads to the prevalence of bacterial chemotaxis in the soil and affects the community composition and spatial and temporal distribution of soil microbes at all times (Bianciotto et al. 2001). In the 1980s, Caetano and Favelukes (1986) found that bacterial motility and characterization play an important role in root colonization, suggesting that the movement and chemotaxis of bacteria were important factors in the initial "chemical-physical contact" between bacteria and plant root surface.

A biofilm is a large amount of bacterial aggregate film formed by bacteria adhering to a contact surface, secreting a polysaccharide matrix, fibrin, a lipid protein, and wrapping itself (Kolter and Greenberg 2006). In a recent report by Kakkanat and colleagues (Kakkanat et al. 2017), YjeA acts as a Lys-2,3-aminomutase in *E. coli*. After mutation of YjeA,

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it actually enhances the exercise capacity of EC958. In the study of *Salmonella*, a serotype opposite phenotype against the YjeA mutant has been reported, and mutations in these genes result in impaired exercise capacity (Hayrapetyan et al. 2015). The above studies confirmed the effect of Lys-2,3-aminomutase on bacterial motility, but the specific mechanism is still unclear. Zhang et al. (2014a) found that the transcriptional regulation of the lysine 2,3-aminomutase gene was regulated by σ^{54}. σ^{54} is a transcriptional regulator that may play a role in physical interaction of bacteria with their environment, including virulence and biofilm formation. σ^{54} is also a gene encoding a mutant that is also involved in the degradation of branched chain amino acids (Hasmik et al. 2015). Therefore, we suspect that biofilms provide a stable living environment in addition to bacteria and are closely related to the chemotaxis and motility of bacteria.

A. caulinodans ORS571 has about 87.6 Kb symbiosis island (Tsukada et al. 2009) containing almost all genes related to nodulation. It is interesting that according to the biosignal analysis, the protein sequence homology of AZC_2928 of *Azorhizobium caulinodans* ORS571 and 2,3-aminomutase of *Escherichia coli* was 42.5%. Therefore, we hypothesize that lysine 2,3-aminomutase may affect bacterial biofilm formation and chemotaxis. Among those genes, *AZC_2928* is a putative lysine 2,3-aminomutase; however, its function has not been verified yet. In this study, we employed the modern advanced clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas) (Jinek et al. 2012) technology to knockout the *AZC_2928* gene completely and studied its biological functions.

CRISPR/Cas9 system is a newly developed genome editing tool and it is widely used since 2013 in many fields as it shows higher editing efficiency than other genome editing tools (Zhang and Zhang 2020); these tools include zinc finger nucleases (ZFNs) (Urnov et al. 2010), transcription activator-like effector nucleases (TALENs) (Bogdanove and Voytas 2011), and meganucleases (Stoddard 2005). CRISPR/Cas9 is also affordable and easier to use, which can be used to edit both coding and non-coding genes, including microRNAs (Budak and Zhang 2017). Scientists have employed this technology to conduct many scientific and clinic research, including successfully inactivating HIV-1 in cells infected by HIV-1 (Hu et al. 2014), and repairing *Fah* gene mutated in mice (Yin et al. 2014). In animals, CRISPR/Cas9 technology has been successfully employed to edit genes in many species, including *Mus musculus* (Qiu et al. 2013), *Rattus norvegicus* (Qiu et al. 2013; Hu et al. 2013), *Caenorhabditis elegans* (Friedland et al. 2013), *Macaca fascicularis* (Niu et al. 2014), and *Plasmodium yoelii* (Zhang et al. 2014b). In plants, CRISPR/Cas9 has also been successfully applied to edit genes in *Arabidopsis thaliana*, tobacco, rice, banana, wheat, and cotton (Kim et al. 2018; Kaur et al. 2018; Li et al. 2013; Shan et al. 2013; Li et al. 2017). CRISPR/Cas9 is also employed to edit genes in microorganisms instead of traditional methods (Schäfer et al. 1994; York et al. 1998; Pienkowska et al. 1993), such as *Saccharomyces cerevisiae* (DiCarlo et al. 2013), *Streptomyces* (Cobb et al. 2015), and *Cyanobacteria* (Yao et al. 2016). However, there is no report on employing CRISPR/Cas9 to knockout gene in *A. caulinodans*.

A. caulinodans ORS571 is a newly identified rhizobium, which is more tolerant to oxygen than other rhizobia and could form unique stem nodules on natural host *Sesbania* (Boivin et al. 1997). In this study, we employed CRISPR/Cas9 genome editing technology to successfully knockout *AZC_2928* gene completely for the first time and study its function in *A. caulinodans* ORS571 via the homologous recombination (HR) mechanism (Cobb et al. 2015). Our results show that *AZC_2928* gene plays an important role in the nitrogen-fixing bacteria *A. caulinodans* ORS571, particularly on its chemotaxis and biofilm formation. This shines light on potential prospects for nitrogen fixation in non-legume crops with *A. caulinodans* ORS571 mutants.

Materials and methods

Strain and plasmid

All strains and plasmids used in this study were listed in Table 1.

Plant seed sterilization and culture

Mature *S. rostrata* seeds were obtained from the laboratory of Professor Zhihong Xie. Firstly, seeds were treated with 95% sulfuric acid to remove the seed coat. Then, the seeds were sterilized with 75% alcohol for 60 s, followed by 1% sodium hypochlorite for 10 min. Finally, the seeds were rinsed 3 times with sterilized water to completely remove the residents of sulfuric acid, alcohol, and sodium hypochlorite.

The sterilized seeds were grown on a plate with half strength of Hoagland's nutrient medium (Hoagland and Arnon 1950; Spomer et al. 1997). The seeds were then grown in the dark at 37 °C. Five biological replicates were performed, each containing six seeds. The germinated seeds were transplanted into a 9-cm-diameter mushroom bag containing two-thirds of the sterilized vermiculite. When the plants had two fully opened leaves, the wild-type strains and the genome editing strains were used to infect the natural host plant *S. rostrata*, respectively. Then, the plants were cultured at 26 °C for 16 h in the light and 8 h in the dark per day. After 2 weeks of infection, the number of nodules was counted.

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Sources
E. coli DH5α	Standard cloning host	From TIANGEN
A. caulinodans ORS571	Wild-type Amp^+, Tet^+	This study
▵*AZC_2928*/*A. caulinodans* ORS571	*AZC_2928* deletion mutation of *A. caulinodans* ORS571 Amp^+, Tet^+, Apr^+	This study
AZC_2928/*A. caulinodans* ORS571	*AZC_2928* complement of ▵*AZC_2928*Amp^+, Tet^+, Gen^+	This study
pBBR1-MCS5-*AZC_2928*	pBBR1-MCS5 ligates genome of *AZC_2928* gene Gen^+	This study
pCRISPomyces-2	Knockout vector Apr^+	From BIO SCI

Bacteria culture

E. coli strains DH5α was grown in LB liquid medium (10 g·L^{-1} tryptone, 5 g·L^{-1} yeast extract, and 10 g·L^{-1} NaCl). *A. caulinodans* ORS571 was then grown on TY liquid medium (5 g·L^{-1} tryptone, 3 g·L^{-1} yeast powder, and 0.88 g·L^{-1} $CaCl_2 \cdot 2H_2O$ with pH 7.4). Both LB and TY mediums were solidified by adding 15 g·L^{-1} agar. As the mediums cooled, about 70 °C, 100 μg/mL ampicillin, 100 μg/mL tetracycline, 100 μg/mL apramycin, and 100 μg/mL gentamicin were added into each medium followed by rhizobia culture condition: 28 °C, 220 rpm for liquid medium, or 28 °C constant temperature incubation on plate to an exponential growth period (about 1.0×10^9 per milliliter) for reservation. *E. coli* strains DH5α culture condition: 37 °C, 220 rpm for liquid medium, or 37 °C for solid culture.

A. caulinodans ORS571 AZC_2928 sequence analysis

Both genome and protein sequences of *A. caulinodans* ORS571 *AZC_2928* gene were extracted from the NCBI GenBank database. BLASTn was employed to search the homologues of *A. caulinodans* ORS571 *AZC_2928* gene. ClustalW was employed to construct the phylogenetic tree of the identified sequences related to *A. caulinodans* ORS571 *AZC_2928*.

SWISS-MODEL (http://swissmodel.expasy.org/) was used to predict the three-dimensional structures of lysine 2,3-aminomutases, collected for *A. caulinodans* ORS571 lysine 2,3-aminomutase *AZC*_2928 and other 12 lysine 2,3-aminomutases from NCBI database GenBank, with homologous modeling principle. PROSITE (http://prosite.expasy.org/) was used for domain analysis. ProtParam (http://cn.expasy.org/tools/protparam.html) was used to analyze the relative molecular mass and isoelectric point. TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) was used to analyze the transmembrane motif. PROTSCALE (http://www.expasy.org/cgi-bin/protscale.pl) was used to analyze hydrophobicity. SignaIP (http://www.cbs.dtu.dk/services/SignalP/) was used to analyze signal peptide.

sgRNA design and plasmid construction

Online software E-CRISP (http://www.e-crisp.org/E-CRISP/designcrispr.html) was employed to design sgRNAs for the *AZC_2928* gene (Heigwer et al. 2014; Jiang et al. 2015). The sgRNAs were chemically synthesized by TSINGKE Biotechnology Limited Company. The *AZC_2928*-gRNA was designed for targeting − 162 position at the upstream of *AZC_2928* CDS, meanwhile, adding *Bbs*I sticky end on *AZC_2928*-gRNA 5′-end and 3′-end, respectively. The homologous arm primers (*AZC_2928*-LF, *AZC_2928*-LR, *AZC_2928*-RF, and *AZC_2928*-RR) were designed online for the left and right arms of *AZC_2928* (http://nebuilder.neb.com/). Other primers were also designed as *AZC_2928*-HB-F and *AZC_2928*-HB-R for amplification of *AZC_2928* to construct complementary plasmid. All primers and sgRNAs were synthesized by TSINGKE Biotechnology Limited Company. All primers and sgRNAs were listed in Table 2.

The annealing reaction buffer was added to the mixtures of sgRNA upstream and downstream primers (final concentration 10 μM/L for each primer). The anneal program was set to first denature the mixture at 95 °C for 10 min, then it was slowly cooled to room temperature, and finally the annealed sgRNA insert and pCRISPomyces-2 (knockout plasmid, https://www.addgene.org/61737) were ligated via Golden Gate assembly (Cobb et al. 2015). The Golden Gate reaction mixtures are pCRISPomyces-2 0.5 μL (100 ng), annealed sgRNA 0.5 μL, T4 ligase buffer (NEB) 2 μL, T4 ligase (NEB) 1 μL, *Bbs*I-HF (NEB) 1 μL, ddH_2O 15 μL, and total 20 μL system. The Golden Gate Program was set as follows: 37 °C 10 min, 16 °C 10 min; 50 °C 5 min, repeating the 3 steps for 9 times, then 65 °C 20 min; the final step was to keep at 4 °C. Heat shocking was used to transfer the Golden Gate assembly production (5 μL) into DH5α competent cell. Selective plates which contain 0.25 μM/mL IPTG, 40 μg/mL X-gal (in DMSO), and 100 μg/mL apramycin sulfate were cultured at 37 °C. The white colonies and recombinant plasmids were picked up for sequencing. Figure 1 shows the sequencing result of sgRNA. The recombinant plasmid was named as pCRISPomyces-2-sgRNA.

Table 2 Primers used in this study

Primer names	Primer sequences* (5′➡3′)
AZC_2928-LF	tcggttgccgccgggcgtttttatctagaGCCCCAGAAGGGCGCGCG
AZC_2928-LR	gcccggctcgcacCGTCCGTCGAGGCTCTGGCTTG
AZC_2928-RF	gcctcgacggacgGTGCGAGCCGGGCTGCAAAG
AZC_2928-RR	gcggccttttacggttcctggcctctagaATCTCGGGCGGGTTGGCG
AZC_2928-HB-F-*Hind* III	CCCAAGCTTGTCCAGCTCAAGCCAGAGC
AZC_2928-HB-R-*Bam*H I	CGC GGATCC GCCAGATATAGGGCTGGGC
SgRNA-F	ACGC GGGCGCGCGCTATGCGGTGG
SgRNA-R	AAAC CCACCGCATAGCGCGCGCCC

*The restriction sites are underlined

The underlined lowercase letters are overlapping segments

AZC_2928 gene homologous arm (donor) plasmid construction

Using *A. caulinodans* ORS571 genome as template and *AZC_2928*-LF, *AZC_2928*-LR, *AZC_2928*-RF, and *AZC_2928*-RR as primers, *AZC_2928* gene homologous arms were obtained respectively via PCR (Fig. 2a). Fifty microliters of PCR reaction included 20 μL ddH_2O, 25 μL 2 × HiProof 2G PCR Master Mix (JIEYI BIOTECH), 2 μL forward and reverse primers (10 μM/L), respectively, 1 μL DNA template, and ddH_2O was the negative control. The PCR program was setup as follows: the first run was set at 98 °C 5 min, then 98 °C 30 s, 55 °C 30 s, 72 °C 1 min, repeating it 30 times, and then 72 °C for 10 min. The nucleic acid marker was DL2000 (TIANGEN Biotech (Beijing) Co., Ltd.).

The PCR products of *AZC_2928*-L, *AZC_2928*-R and pCRISPomyces-2-sgRNA were digested by *Xba*I at 37 °C for 4 h followed by column purification kit (TIANGEN Biotech (Beijing) Co., Ltd.). The purified homologous arms and pCRISPomyces-2 were ligated by Lightning Cloning Kit (Beijing Biodragon Immunotechnologies Co., Ltd.). Ligation reaction system was as follows: linear vector 1 μL, 2 μL left and right homology arms, and 5 μL Lightning Cloning Master Mix (2×). The reaction was performed at 50 °C for 30 min, then the products were transferred into DH5α competent cells. PCR verification was used to select the positive clones (Fig. 2b), then bacteria cultivation and plasmid extraction were used for digestion and sequencing (Fig. 2c). The constructed plasmid was named as pCRISPomyces-2-sgRNA-LR.

AZC_2928 gene knockout using CRISPR/Cas9

A total of 8 μL pCRISPomyces-2-sgRNA-LR was firstly transformed into *A. caulinodans* ORS571 competent cells by electroporation. Then, the transformed bacteria were incubated in TY liquid medium with 10% glycerol for 8 h, and finally plated on the TY solid medium which contain apramycin, ampicillin, and tetracycline. After 2 days of culture, the colonies were selected by PCR identification using AZC_2928-Mid-F and AZC_2928-Mid-R; the positive colonies were sequenced by Sangon Biotech Co., Ltd.

Construction of complementation strains

A. caulinodans ORS571 genome was used as template while *AZC_2928*-F and *AZC_2928*-R were used as primers for amplifying *AZC_2928* (Fig. 3a). Both PCR products and

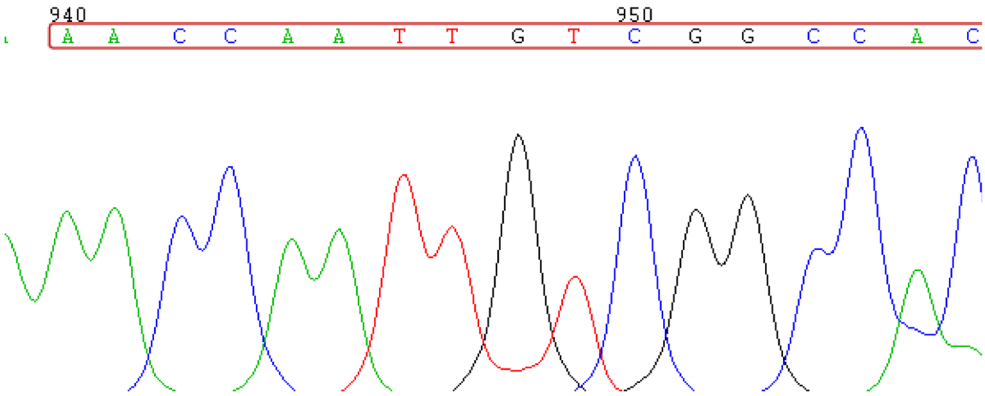

Fig. 1 The sequence of recombinant plasmid with sgRNA

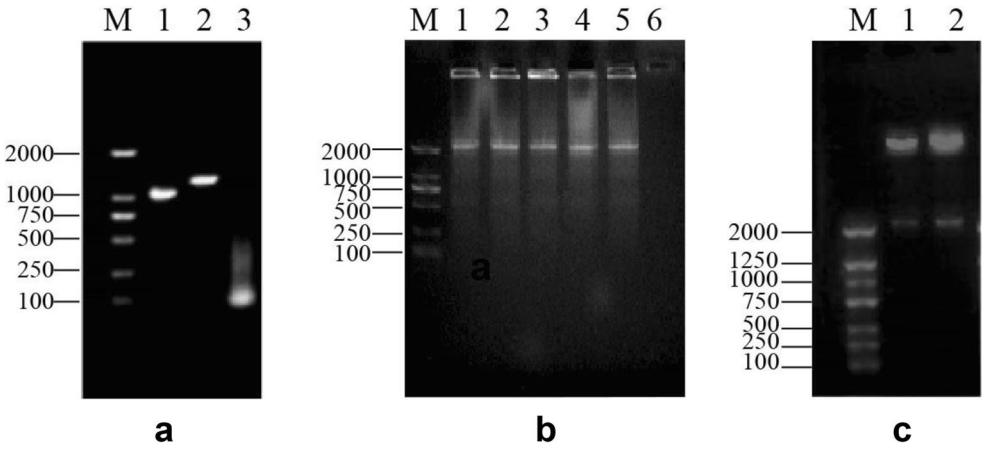

Fig. 2 The plasmid construction with *AZC_2928* gene homologous arms. **a** M: DL2000; 1: left homologous arm; 2: right homologous arm; 3: control. **b** Homologous arm recombinant plasmid PCR verification, M:DL2000; 1–5: Positive clone; 6: control. **c** pCRISPomyces-2-sgRNA-LR *Xba*I digestion, M: 125 bp DNA ladder; 1–2: Positive clone

pBBR1MCS-5 were digested by *Hin*d III and *Bam*H I at 37 °C for 4 h, followed by 1% agarose gel electrophoresis separated and purified, then added T4 ligase and buffer to connect purified *AZC_2928* with linearized pBBR1MCS5 at 22 °C for 3 h. The ligated products were transformed into DH5α competent cells, cultured on LB solid medium with gentamicin. Then, positive clones were screened and selected by PCR (Fig. 3b); positive clones were double digested and sequenced (Fig. 3c). Correct sequencing clones were expanded and extracted for next electricity transformation into △*AZC_2928* mutant electroporation-competent cells, cultured in TY liquid medium with no antibiotic for 8 h, then 100 μL was taken and spread on TY solid medium with gentamicin, ampicillin, and tetracycline. After 36 h of culture at 28 °C, the positive clones were selected via PCR, and then stored in 50% glycerol for later experiments.

Chemotaxis assay

The chemotaxis assay was performed according to a previous report (Liu et al. 2017). Single CIRSPR/Cas9 editing mutant colony and its wild-type were cultured in L3 liquid medium for overnight, respectively (Liu et al. 2017). The colonies were washed with sterilized water for 3 times, followed by chemotaxis buffer to dilute the colonies to achieve the same concentration ($OD_{600} = 0.6$). According to bioinformatics analysis, *AZC_2928* encodes putative lysine 2,3-aminomutase. Lysine 2,3-aminomutase is a radical SAM enzyme and this enzyme convers the amino acid lysine to beta-lysine (Frey 1993). In order to compare the chemotactic responses to different lysine concentrations, 1 μL aliquots of cell suspensions of the wild-type and genome editing strains were inoculated on L3 plates with 0.3% agar containing 10 mM or 20 mM L-lysine, respectively. The inoculated plates were cultured at 37 °C for 5 days.

Quantitative capillary chemotaxis assay

The quantitative capillary chemotaxis assay was performed according to a previous report (Liu et al. 2017). Single genome editing and wild-type colonies were selected and

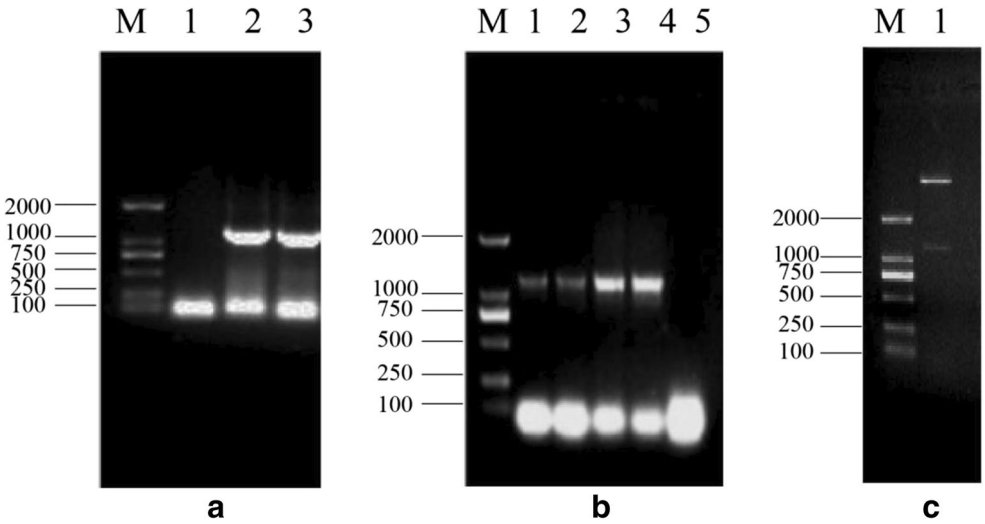

Fig. 3 Construction of complementation strains. **a** M: DL2000; 1: control; 2–3: *AZC_2928* gene. **b** *AZC_2928* recombinant plasmid PCR verification, M: DL2000; 1–4: Positive clone; 5: control. **c** AZC_*2928* recombinant plasmid double digestion, M: DL2000; 1: Positive clone

cultured in TY liquid medium for overnight, respectively. The cultures were washed by chemotaxis buffer for 3 times, and then diluted to a concentration of $OD_{600} = 0.08$. Equal amount of wild-type and genome editing mutants were selected, respectively. Then, 200 μL mixtures were added into 96-well plates. The open ends of the capillaries containing chemotaxis buffer with 10 mM or 20 mM lysine and the other ends sealed by Vaseline were inserted into the wells for 1-h incubation. The treated portion of the capillaries were rinsed with sterile water for 6–8 times; the sealed ends were broken, and the rest of the mixtures were transferred into 1.5-mL sterilized centrifuge tubes, which were loaded with 1 mL sterilized water. Thirty microliter diluents was coated on the TY solid medium in addition to 50 μL/mL tetracycline and ampicillin, and then the inoculated plate was cultivated at 37 °C for 36 h. Finally, the cells on the plate were counted and the genome editing mutants or wild-type was distinguished via PCR with *AZC_2928*-LF and *AZC_2928*-RR primer pairs.

Biofilm formation assay

The biofilm formation assay was performed according to a previous report (Liu et al. 2017). Single genome editing mutant or wild-type colony was selected and cultured in TY liquid medium overnight for OD600 = 1.2; the cultures were washed and adjusted the optical density to 0.6 with TY liquid medium. Three hundred microliter cultures was inoculated into 3 mL TY liquid medium which were loaded into glass tubes. The glass tubes were cultured at 37 °C for 3 days. The cultures were pipetted out of the tubes carefully. The tubes were washed with distilled water for 3 times gently, then added 3 mL 0.25% crystal violet (CV) into each tube; the tubes were cultured at 37 °C for 1 h. The CV was removed, and the tubes were washed with distilled water for 3 times. The glass tubes were placed at 37 °C for drying, and then photographed. Three milliliter acetic acid was used to dissolve the "CV ring" and the dissolved mixture was used to measure optical density at 570 nm.

Nodulation assay

The genome editing mutant strains were used to infect the natural host *S. rostrata* for nodulation initiation and development test. Sterilized *S. rostrata* seeds were treated as previous report (Lamouche et al. 2019). The germinated seeds were transplanted into a 9-cm-diameter mushroom bag containing two-thirds of the sterilized vermiculite. When the seedling grew into the stage of two fully opened leaves, the genome editing mutant strains and the wild-type strains were inoculated separately; the same amount of sterile water was sprayed on the roots of *S. rostrata* as control, repeating three times for each treatment.

Results

A. caulinodans ORS571 AZC_2928 sequence analysis

A. caulinodans ORS571 *AZC_2928* has highest amino acid sequence similarity to lysine 2,3-aminomutase in *Bradyrhizobium oligotrophicum* S58. Thus, we named *AZC_2928* as a lysine 2,3-aminomutase. We further compared *AZC_2928* amino acid sequence with the amino acid sequences of 12 other lysine 2,3-aminomutases by ClustalW. Figure 4 shows the phylogenetic tree and the evolutionary relationship of lysine 2,3-aminomutase amino acid sequence in various organisms or strains using MEGA. *A. caulinodans* ORS571 lysine 2,3-aminomutase *AZC_2928* has a 73% similarity with *Bradyrhizobium oligotrophicum* S58, its similarity is higher than *Prosthecochloris vibrioformis* and has a closer evolutionary relationship.

Based on the domain analysis, the identified lysine 2,3-aminomutase had not any conservative domain and signal peptide; all lysine 2,3-aminomutase had 3 transmembrane motifs and 5 domains with a molecular weight of 39.99 KD and theoretical pI 6.73 hydrophobic stable protein. Figure 5 shows the predicted protein structure of AZC_2928 by SWISS-MODEL, which could clearly show that it is not only alpha helices and beta sheets but also irregular crimps. According to CDD database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and KEGG (https://www.kegg.jp), lysine 2,3-aminomutase is a member of the "AdoMet radical" (radical SAM) family. It contains pyridoxal phosphate and a [4Fe-4S] cluster and binds an exchangeable S-adenosyl-L-methionine molecule. Activity in vitro requires a strong reductant such as dithionite and strictly anaerobic conditions. A 5′-deoxyadenosyl radical is generated during the reaction cycle by reductive cleavage of S-adenosyl-L-methionine, mediated by the iron-sulfur cluster. S-Adenosyl-L-methionine is regenerated at the end of the reaction.

Genome editing efficiency of AZC_2928 gene

After 36 h of culture, the genome editing of *A. caulinodans* ORS571 colonies were screened by PCR amplification of the

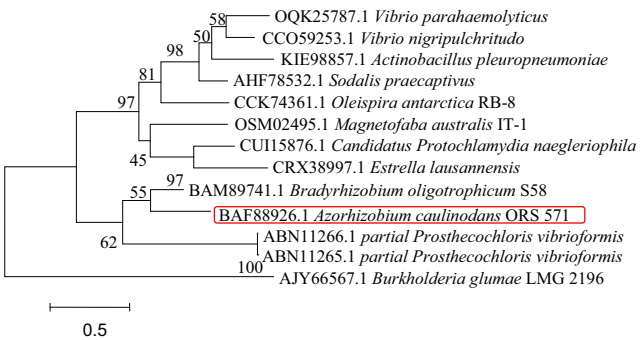

Fig. 4 Phylogenetic relationships of lysine 2,3-aminomutase

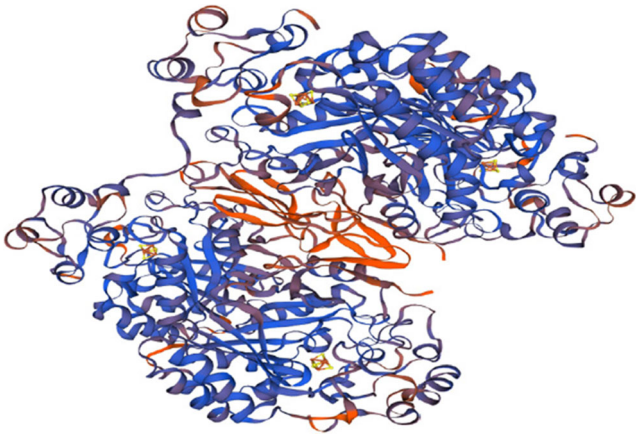

Fig. 5 Predicted protein structure prediction of AZC_2928 by SWISS-MODEL

genome editing site. Because CRISPR/Cas9 cut some fragments from the original genome sequences, the genome editing events had shorter PCR products than the non-editing wild-type. Based on the PCR results, the genome editing efficiency was 34%. Figure 6 shows several examples for genome editing in *AZC_2928* gene. The genome editing strains were named as *A. caulinodans* Δ*AZC_2928*/ORS571.

Genome editing of AZC_2928 gene enhanced chemotaxis but not motility in *A. caulinodans* ORS571

To determine the role of *AZC_2928* gene in *A. caulinodans* ORS571, we used the genome editing mutants *A. caulinodans* Δ*AZC_2928*/ORS571 to test the chemotaxis of *A. caulinodans* ORS571. Our results show that *AZC_2928* deletion mutants significantly enhanced chemotaxis about 144% compared with the non-editing wild-type (Fig. 7). In this study, the wild-type had very weak chemotaxis. The chemotactic ability of *A. caulinodans* ORS571 is affected by the concentrations of lysine in the medium. We tested two different concentrations of lysine (10 mM and 20 mM); no matter which concentrations, genome editing strains significantly enhanced *A. caulinodans* ORS571 chemotaxis (Fig. 7).

Capillary quantification assays were employed to further study the chemotaxis and motility of genome editing mutants and the wild-types. The total number of colonies was affected by lysine concentration, and the number of colonies at 10 mM lysine was much higher than that at 20 mM (Fig. 8). However, the motility of the bacteria was not affected by the genome editing (Fig. 9). Combined with plate chemotaxis assay, we could conclude that *AZC_2928* was an important regulatory gene in chemotaxis, but not in the motility.

▵AZC_2928 mutants enhanced biofilm formation

Biofilm is an important phenotype for many functions, including the relationship between nitrogen-fixing bacteria and host plants. We also performed a 96-well plate quantification test to verify whether the deletion of the *AZC_2928* gene would affect biofilm formation. As a result, the amount of biofilm formation of the CRISPR/Cas9 genome editing mutant strains was higher than that of the wild-type (Fig. 10). This suggests that the *AZC_2928* gene can regulate the formation of *A. caulinodans* ORS571 biofilm during growth.

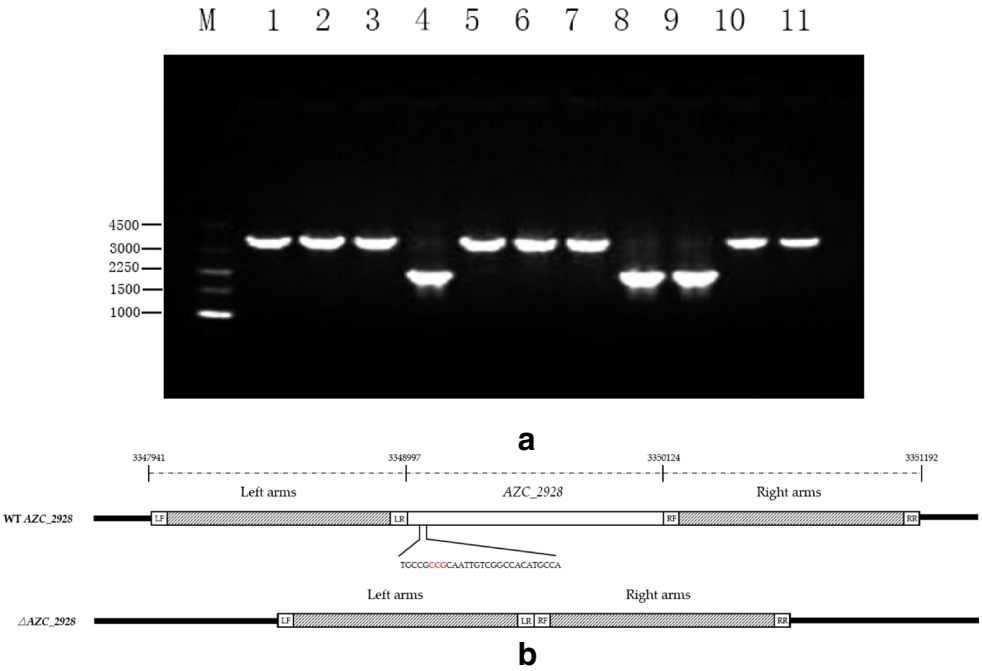

Fig. 6 The verification results of AZC_2928 gene knockout. (A) PCR verification results of *AZC_2928* gene knockout. M: 250 bp DNA ladder; 1–3, 5–7, 10–11: Knockout failure; 4, 8–9: Knockout successfully. (B) The nucleic acid sequences of *AZC_2928* wild-type and ΔAZC_2928

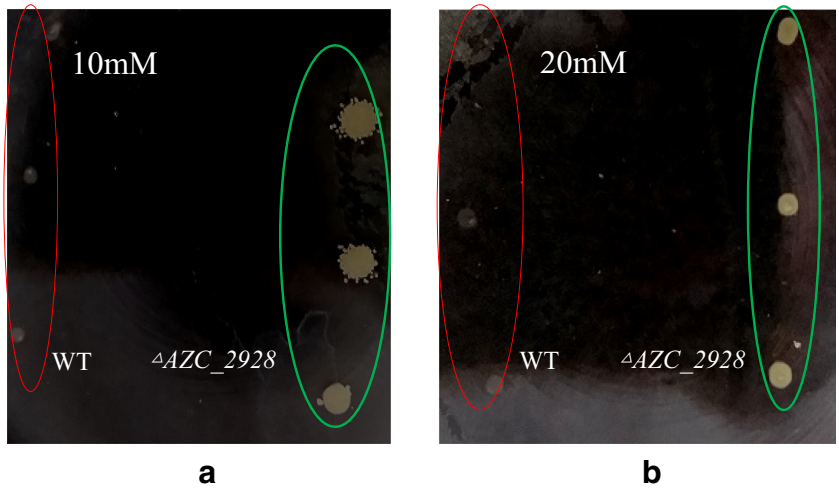

Fig. 7 Soft agar chemotaxis assay with different concentration lysine. **a** Red oval showed chemotactic behaviors of the *A. caulinodans* ORS571 (WT) treated with 10 mM lysine; green oval showed *ΔAZC_2928* mutants behaviors. **b** Red oval showed chemotactic behaviors of the *A. caulinodans* ORS571 (WT) treated with 20 mM lysine; green oval showed *ΔAZC_2928* mutants' behaviors

Growth curve of AZC-2928 wild-type and genome editing mutants

Growth curve shows that the growth rate of *AZC_2928* genome editing mutants were significantly lower than the wild-type, *A. caulinodans* ORS571; the growth rate of pBBRMCS-5-mediated gene complementation of *A. caulinodans* ORS571 is lower than the wild-type but higher than *AZC_2928* genome editing mutants. This suggests that the growth rate of pBBRMCS-5-mediated gene complementation of *A. caulinodans* ORS571 is caused by the complementation plasmid with *AZC_2928* (Fig. 11). In other words, *AZC_2928* can affect the basal metabolism of wild-type. According to bioinformatics analysis, *AZC_2928* coding lysine 2,3-aminomutase is involved in the metabolic pathway of lysine. The metabolic pathway of amino acids is crucial for the growth and metabolism of bacteria.

Nodulation competition

It seems that there was no difference in nodulation initiation and development after 2 weeks of *A. caulinodans* ORS571 infection between *AZC_2928* genome editing mutants and their wild-type (Fig. 12). This means that knockout of *AZC_2928* gene did not affect *A. caulinodans* ORS571 inducing nodulation and nodulation development.

Discussion

CRISP/Cas9 is a new gene/genome editing technology that provides a powerful research platform for molecular biology and biochemistry. Compared with ZFNs (Bibikova et al. 2002; Dreier et al. 2005) and TALENs (Hockemeyer et al. 2011; Huang et al. 2011), the advantages of the CRISPR/Cas9 system include simple design, short base pairing (20 bp) targeting gene (Gaj et al. 2013; Hwang et al. 2013; Mali et al. 2013; Cong et al. 2013; Ding et al. 2013; Ramalingam et al. 2013), short experimental cycle, and higher efficiency. Conversely, ZFNs and TALENs are more expensive and have lower editing efficiency. CRISPR/Cas9 is widely used for genome editing of many species, including both model and important species, such as *Caenorhabditis elegans* (Friedland et al. 2013), human (Mali et al. 2013),

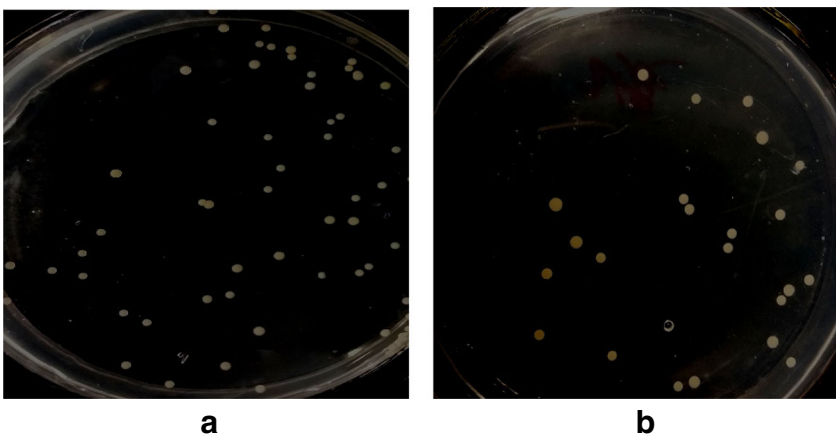

Fig. 8 Quantitative capillary chemotaxis assay of WT and *ΔAZC_2928* mutants. **a** TY solid plate was used for colony counting of quantitative capillary chemotaxis assay with 10 mM lysine. **b** Colony counting of quantitative capillary chemotaxis assay with 20 mM lysine

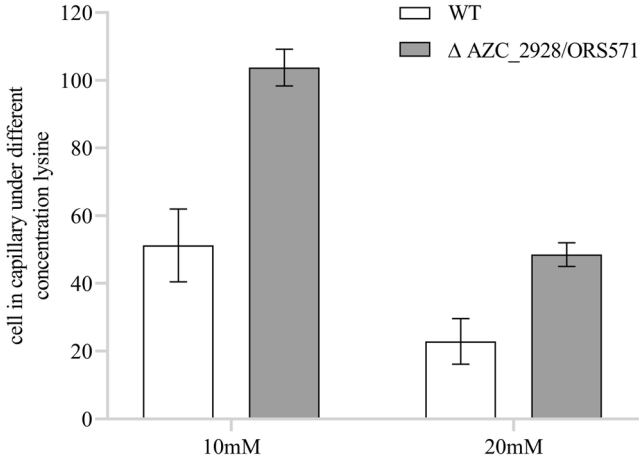

Fig. 9 Cell counting of WT and *ΔAZC_2928* mutants in capillary with different concentration lysine

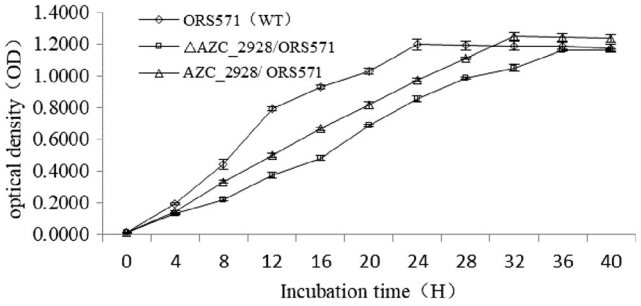

Fig. 11 The growth curve of *A. caulinodans* ORS571 genome knockout mutants and its wild-type

Saccharomyces cerevisiae (DiCarlo et al. 2013), fruit fly (Gratz et al. 2013), and zebrafish (Chang et al. 2013). With time going, more and more researchers have discovered that CRISPR technology has a serious shortcoming, such as off-target effects (Guilinger et al. 2014). Therefore, reducing the off-target effects has become a hot topic in the field of genome editing. The specificity of the CRISPR/Cas9 genome editing system is determined by the 10–12 bp base pairing near the PAM region in the sgRNA, while the recognition of the mispairing target sites far from the 8–10 bp base sequence of the PAM sequence is not significant (Jiang et al. 2013; Jinek et al. 2012; Cong et al. 2013). The length of sgRNA is also associated with the specificity. The shortening of the 20 nt target sequence of sgRNA to 17 nt or the addition of two guanine nucleotides at the 5′-end of sgRNA can increase the binding specificity of sgRNA, reduce the off-target effect, and increase the genome editing efficiency (Fu et al. 2014; Cho et al. 2014).

There has been little research on the *AZC_2928* gene of *A. caulinodans* ORS571. In this study, we employed the homologous recombination (HR) mechanism; the fusion of upstream and downstream homology arms of AZC_2928 was constructed on the knockout plasmid, which can help to delete the entire AZC_2928 gene quickly and accurately. The editing efficiency is about 34%. For further research, we could take some measures to improve editing efficiency, for instance, improving the specificity of sgRNA sequence, applying double-nicking strategy (Shen et al. 2014: Ahmad et al. 2020), and fCas9 system strategy (Guilinger et al. 2014). It is worth noting that knockout vector removal, which was used in this study as knockout vector pCRISPomyces-2, is temperature-sensitive plasmid (the plasmid could be cleaned under 37–39 °C culture). The plasmid cleaned could reduce the toxic effects.

In the chemotaxis assay, the chemotaxis of the genome editing mutants was significantly enhanced compared with the wild-type. The results indicated that the CRISPR/Cas9 genome editing mutants played an important inhibitory role in decontamination. Meanwhile, capillary quantification assays were used to further identify the chemotaxis and motility of the genome editing mutants as well as the wild-type strains. It showed that whether wild-type or genome editing mutants, their motility is unaffected; combined with plate chemotaxis assay, we could draw a conclusion that *AZC_2928* is an important regulatory gene in chemotaxis, but not in motility. In Kakkanat's study (Kakkanat et al. 2017), YjeA also acts as a Lys-2,3-aminomutase to modify the elongation factor EF-P at the post-translational levels in *E. coli*, and lysine residue 34 (Lys34) of EF-P was YjeA post-translational modification results in an increase in the affinity of EF-P with ribosomes and thus an increase in protein expression levels. Flagellin, as

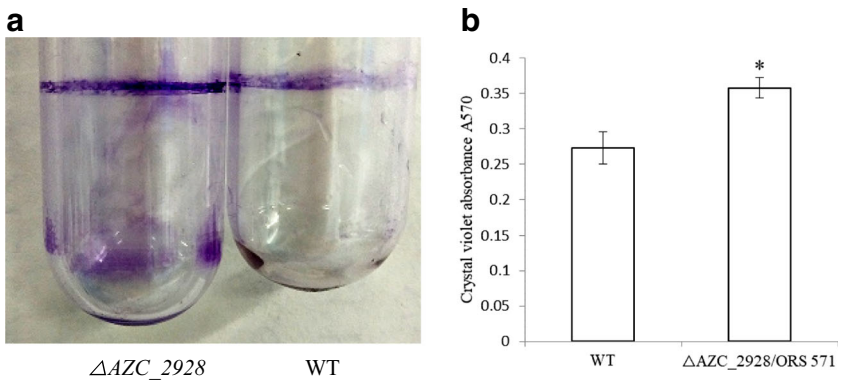

Fig. 10 *ΔAZC_2928* mutants enhanced biofilm formation. **a** WT and *ΔAZC_2928* mutant biofilm formation treated with crystal violet. **b** Biofilm formation was quantified treated with crystal violet staining method. Asterisks (*) indicate statistically significant differences ($P < 0.05$) between the wild-type and *ΔAZC_2928* mutants

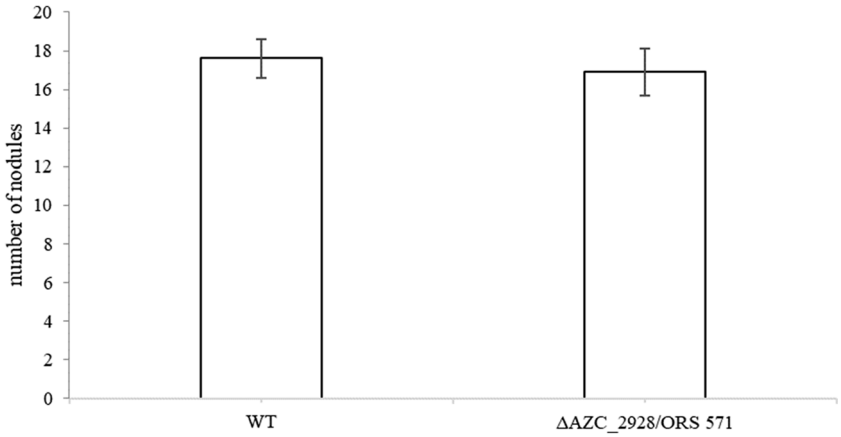

Fig. 12 Number of nodules of *S. rostrata* infected by mutants and its wild-type

a key protein affecting bacterial motility, is naturally regulated. Additionally, the defect of EF-P modification actually enhanced the exercise capacity of EC958. In a report by Hayrapetyan and colleagues (Hayrapetyan et al. 2015), a serotype opposite phenotype against the YjeA mutant has been reported, and mutations in these genes result in impaired exercise capacity.

The formation of biofilms is related to the colonization of rhizobium (Hölscher et al. 2015). Biofilm formation tests showed that *AZC_2928* CRISPR/Cas9 genome editing mutants lead to increasing biofilm formation, which is different from other large number of microorganisms. This might be one of the reasons why *A. caulinodans* ORS571 has specific parasitism in *S. rostrata*. Biofilm enhancement could improve the strain pressure against adverse environments. Sigma 54 is a pleiotropic regulator of growth, carbohydrate metabolism, motility, biofilm formation, and toxin production (Hayrapetyan et al. 2015). Lysine 2,3-aminomutase, which directs the transcription of the kamA operon, is controlled by the Sigma 54 factor and is activated through the Sigma 54-dependent transcriptional regulator KamR (Zhang et al. 2014b). All the research results show that lysine 2,3-aminomutase might make an influence in motility and biofilm formation.

There is no significant difference in the number of stem tumors and nodules between the CRISPR/Cas9 genome editing strains and the wild-type strains. The result shows that AZC_*2928* CRISPR/Cas9 genome editing mutants could not influence the number of nodules on *S. rostrata*; this result could be related to motility of *A. caulinodans* ORS571. Capillary quantification assays show no significant difference in the motility of the wild-type and the CRISPR/Cas9 genome editing mutant strains.

AZC_2928 can affect the basal metabolism of wild-type. According to bioinformatics analysis, *AZC_2928* putative encoding lysine 2,3-aminomutase is involved in the metabolic pathway of lysine. The metabolic pathway of amino acids is crucial in the growth and metabolism of bacteria. We also analyzed the *A. caulinodans* ORS571 genome and found that *AZC_2928* is related to redox and might interact with *AZC_2931* (putative molybdenum cofactor cytidylyl transferase, related to nitrogen fixation). The study could provide a basic research background for symbiotic nitrogen fixation.

Taken together, our results show that *AZC_2928* plays an extremely important role in regulating the formation of chemotaxis, biofilm, and the motility but not in nodulation of *A. caulinodans* ORS571.

Author contributions HL was the principal investigator and took primary responsibility for the paper. HL, XP, and XW conceived and designed the experiments. XW, SL, TL, JW, SQ, YL, JZ, and SO performed the experiments. HL, BZ, XP, and XW analyzed the data. XW wrote the paper and prepared the figures. XP, BZ, SO, and HL reviewed and revised the manuscript. All the authors read and approved the final manuscript.

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