J. Gen. Appl. Microbiol., **67**, 59–66 (2021)
doi 10.2323/jgam.2020.07.001

jGAM

Full Paper

The cytidine repressor regulates the survival of *Pantoea agglomerans* YS19 under oxidative stress and sulfur starvation conditions

(Received December 30, 2019; Accepted July 2, 2020; J-STAGE Advance publication date: January 29, 2021)

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***Pantoea agglomerans* YS19 is a dominant endophytic bacterium isolated from rice, which is capable of promoting host plant growth by nitrogen-fixing and phytohormone secreting. We previously found that the cytidine repressor (CytR) protein conducts the regulation of indole signal in YS19. Here, we compared the whole-cell protein of the wild type YS19 and the Δ*cytR* mutant and subsequently identified one differential protein as alkyl hydroperoxide reductase subunit C related to oxidative stress and sulfur starvation tolerance. It was tested that *cytR* had a positive effect on the survival of YS19 under the oxidative stress and sulfur starvation conditions and this effect was inhibited by indole. To further understand the functional mode of indole in this regulation, we cloned the *cytR* promoter region (P_{cytR}) of YS19 and tested the effect of indole on P_{cytR} using *gfp* as a reporter gene. It was found that P_{cytR} can sense indole and significantly inhibit the expression of the downstream gene. This study provided a deeper understanding of the multiple function of *cytR* and expanded a new research direction of how indole participates in gene regulation.**

Key Words: AhpC; CytR; indole; *Pantoea agglomerans*; stress resistance

Introduction

Pantoea agglomerans is a gram-negative bacterium widely existing in environments (Feng et al., 2006). *P. agglomerans* YS19 strain is a dominant endophyte isolated from rice (*Oryza sativa*) cv. Yuefu (Yang et al., 1999), which cannot produce indole but senses exogenous indole to promote its symplasmata formation and stress-resistant capability (Yu et al., 2016). The cytidine repressor (CytR) is a lactose repressor (LacR) family member that mainly functions in regulation of the genes being involved in nucleotide metabolism and transport, such as Tsx, a specific nucleoside channel protein located on outer membrane (Gerlach et al., 1991), NupG, a nucleoside transport protein on plasma membrane (Henrik et al., 1995), and DeoABCD (Søgaard-Andersen et al., 1990a, b), Cdd (Holst et al., 1992), Udp (Brikun et al., 1996), proteins responsible for the catabolism of deoxynucleosides and nucleosides. Besides, CytR was also found to mediate the regulation of *rpoH* which encoded the heat shock factor σ^{32} in *Escherichia coli* (Kallipolitis and Valentin-Hansen, 1998), and to promote the natural competence of *Vibrio cholerae* (Antonova et al., 2012). We previously obtained a *cytR* mutant of YS19 and revealed that the expression of *cytR* was negatively regulated by indole and the presence of this gene was not conducive to the survival of the bacterium under carbon starvation conditions and also the endophytic colonization on the host rice, which made it seem to be a "negative survival factor" in YS19 (Jia et al., 2017).

Indole is a decomposition product of tryptophan (L-Trp) in many bacterial species. It regulates a variety of bacterial behaviors including pathogenicity, acid resistance, drug resistance, motility, and biofilm formation (Kim and Park, 2015; Lee et al., 2010). More interestingly, apart from the indole secretor population, the molecule also affects non-secretors (Lee et al., 2009a; Nikaido et al., 2008). Later, indole was also found to be abundant in animals and human intestinal tract (up to 0.1 to 1.0 mM) and to play an important role in micro-ecological systems in animals (Kim and Park, 2015). Up to now, an indole receptor protein has never been found, which has prompted the question "how do bacteria sense indole and regulate the expression of related genes".

In this study, we further investigated the function of *cytR*

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

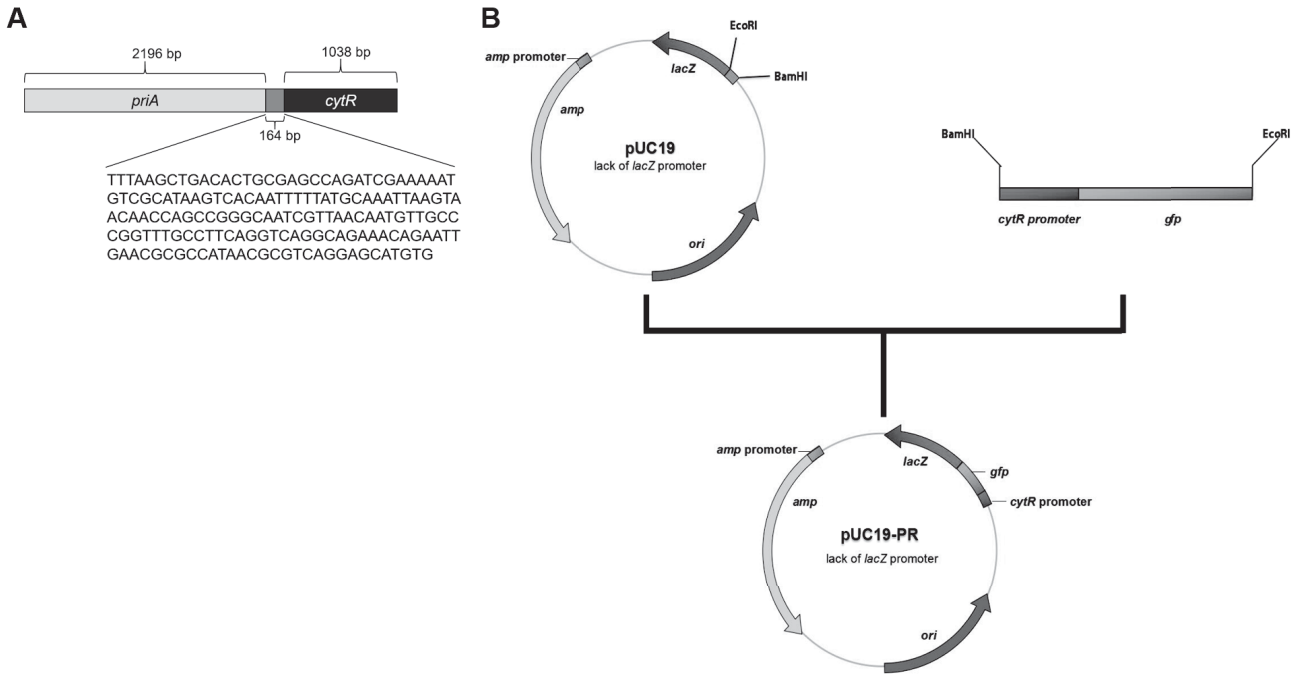

Fig. 1. Cloning strategy and construction of recombinant plasmid pUC19-PR for indole regulatory assays on P_{cytR}.

A. Dark gray indicates *cytR*, light gray indicates gene upstream of *cytR* and normal gray indicates the 164 bp promoter region of *cytR* whose sequence is shown. B. The SOEPCR products and pUC19 without *lacZ* promoter were digested and linked together.

and sought to pursue the significance of the existence of *cytR* gene. We have also given solid evidence to explain that even though *cytR* is not conducive to the survival of YS19 under carbon starvation and host rice association, it contributes to the bacterial survival under oxidative stress and sulfur starvation, where indole functioned as a suppressor signal of *cytR* through *cytR* promoter region.

Materials and Methods

Bacterial strains and cultivation methods. *P. agglomerans* YS19, a dominant endophytic bacterium isolated from rice cv. Yuefu (Yang et al., 1999), is preserved in our lab. The empty plasmid pUC19 and the recombinant plasmid pUC19-PR were introduced into the YS19, respectively, to result in the strains YS19/pUC and YS19/pUC-PR. Lacking the cytidine repressor gene, Δ*cytR* mutant is screened from YS19 by using mTn5 transposon mutagenesis. As described previously (Jia et al., 2017), the transposon insertion mutants were selected on LB agar plates containing kanamycin, rifampicin and X-gluc with, or without, indole (0.5 mM) and the GusA activity of the colony that showed an obvious color difference on two plates was measured to further confirm whether the mutation was generated, and then the mutational sites was identified by high-efficiency TAIL PCR (hiTAIL-PCR). The strains were routinely maintained in glycerol tubes at –20°C. Inocula of the bacteria were prepared by inoculating glycerol tube bacteria on LB plate and cultivating at 30°C for 16 h in a DH3600-type electric thermostat incubator (Tianjin Taisite Instrument Co., Ltd.). Then, the single colony was transferred to fresh LB liquid medium and grown on a THZ-C rotary shaker (2.67 rev s^{-1}, Suzhou Pei-Ying Experimental Equipment Co., Ltd.). For all liquid cultivations, the cells were transferred in 1% proportion (v/v). Bacteriological agar (1.5%, w/v) was added to LB medium when the preparation of a solid culture was required.

Construction of recombinant plasmid. As shown in Figs. 1A and B, the green fluorescent protein reporter gene (*gfp*) and the *cytR* promoter region (P_{cytR}) sequence of YS19 were cloned using plasmid pFAJ1819 (primers: *gfp*-f 5′-CATAACGCGTCAGGAGCATGTGATGAGTAA-AGGAGAAGAAC-3′; *gfp*-r 5′-CG**gaattc**TTATTTG-TATAGTTCATCCATGCCATGTGTAATCCCAG-3′) and genomic DNA of YS19 as the templates (primers: *cytRP*-f 5′-CGC**ggatcc**TTTAAGCTGACACTGCGAGCCA-3′; *cytRP*-r 5′-AGTTCTTCTCCTTTACTCATCACATGC-TCCTGACGCGTTATG-3′), respectively. And the PCR products from YS19 were fused with the *gfp* gene by splicing overlap extension PCR (SOEPCR). The SOEPCR primers were: forward *cytRP*-f (5′-CGC**ggatcc**TTT-AAGCTGACACTGCGAGCCA-3′) and reverse *gfp*-r (5′-C G **g a a t t c** T T A T T T G T A T A G T T C A T C C A T-GCCATGTGTAATCCCAG-3′). The bold lowercase letters indicate the restriction endonuclease recognition sites (*Bam*HI and *Eco*RI), respectively. The SOEPCR products and the multiple clone site region sequence in vector pUC19 without *lacZ* promoter (to exclude the influence of the *lacZ* promoter) were digested with FastDigest *Bam*HI and *Eco*RI (Thermo Scientific) and linked together using T4 DNA ligase (Fermentas, Shenzhen, China). The recombinant plasmid was named pUC-PR.

Whole-cell differential protein analysis and identification. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 15% polyacrylamide gel) was used to identify the

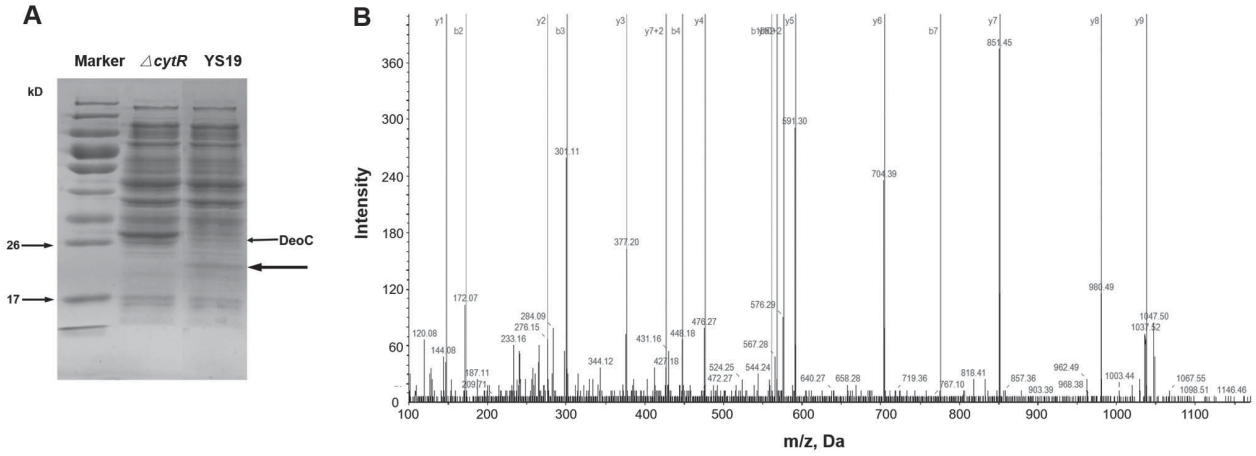

Fig. 2. Display and identification of differential proteins induced by *cytR* mutation.

A. Whole-cell proteins of YS19 and Δ*cytR* mutant that were cultivated in LB medium and sampled at 24 h were analyzed by SDS-PAGE. A differential protein band other than DeoC is indicated by the bold arrow. B. The peptide mass fingerprinting of the differential protein obtained by HPLC-MS was identified as AhpC by a search in SwissProt for *P. agglomerans*.

variation in protein expression in the cells of YS19 and the Δ*cytR* mutant. Bacterial cells were grown in LB liquid medium for 24 h, respectively. Adjusted to the same concentration (A_{600} = 0.20) with phosphate-buffered saline (PBS, 0.027% KH_2PO_4, 0.142% Na_2HPO_4, 0.8% NaCl, 0.02% KCl, pH7.4), the two cells suspension samples of equal volume (20 mL) were centrifuged at 10,625 × *g* for 20 min, washed with PBS 3 times, and finally resuspended with 2 ml PBS. To obtain whole-cell protein samples, the solution samples were disrupted in an ultrasonic cell grinder (SCIENTZ-IID Ningbo New Cheese Co., Ltd.) with 100 W power, 5 s ultrasound/5 s break for 30 min. After being centrifuged (10,625 × *g*, 4°C) for 20 min, 15 μL of the supernatant was mixed with an equal volume of SDS-PAGE loading buffer and heated in a boiling water bath for 10 min. SDS-PAGE was carried out at 80 V for the spacer gel and 120 V for the separation gel, and proteins were visualized by Coomassie brilliant blue staining. The protein bands of interest were excised and digested by trypsin (Shevchenko et al., 2006) and then a peptide mass spectrogram was produced by Q-Tof Ultima Global mass spectrometer (Waters, Milford, USA) and detected by MASCOT software. Swiss-Prot was used to search for matching proteins.

qRT-PCR analysis. YS19 and Δ*cytR* mutant were cultivated in LB medium with, or without, supplementary indole (final concentration 0.5 mM, a concentration whereby the bacterial growth is almost close to the natural growth state) (Jia et al., 2017), for 24 h, and 2 mL of the culture was taken for RNA extraction and reverse transcription. Bacterial total RNA was extracted using Trizol Reagent (TransGen Biotech, Beijing, China). RNA integrity was verified at a ratio of A_{260}/A_{280}0 to 1.80–2.00. cDNA was obtained by using TransScriptII One-Step gDNA Removal and cDNA Synthesis Super Mix (TransGen Biotech). PCR of reverse transcription was carried out at 25°C for 10 min, 42°C for 30 min, and 85°C for 5 min. The expression of the target gene was analyzed by the SYBR I labeling method, using an SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). *gyrB* was selected as a reference gene (Brady et al., 2008). The running protocol was as follows: 95°C for 2 min, 40 cycles of 95°C for 50 s, 60°C for 30 s and 72°C for 30 s. The primers used for qPCR were as follows: *gyrB*-f 5′-TGCAGTGGAACGATGGCTTC-3′, *gyrB*-r 5′-CGAGGAGAATTTGGGATCCG-3′; *ahp*C-f 5′-AAAACGGCGAATTCATCGAC-3′, *ahp*C-r 5′-CGCCCAGTTTCTGGAACTCT-3′.

Survival assays of bacteria under oxidative stress and sulfur starvation conditions. The oxidative stress resistance assay method was the same as previously described (Zheng et al., 2019). Bacterial cells were cultivated in LB liquid medium supplemented with, or without, indole (final concentration 0.5 mM) for 24 h. And then H_2O_2 were added (final concentration 1 mM, 10 mM and 100 mM) and further cultivated for 4 h. The cells were harvested for bacterial counting (colony forming units, CFU) on LB plates. The survival rate was defined as the number of CFU after the treatment divided by the number of CFU of the culture which did not receive a treatment by H_2O_2. All the experiments were performed in triplicate.

The sulfur starvation test method was the same as previously described (Jia et al., 2017). Bacterial cells were cultivated in M9 minimal medium (Sambrook et al., 1989) for 24 h, washed and adjusted to the same concentration (A_{600} = 1.00) with distilled water, transferred to 20 mL of M9 minimal medium with the sulfur source replaced (using $MgCl_2$ to replace $MgSO_4$) supplemented with, or without, indole (final concentration 0.5 mM), respectively, and were grown at 30°C on a THZ-C rotary shaker (2.67 rev s^{-1}, Suzhou Pei-Ying Experimental Equipment Co., Ltd.). The cultures harvested at 0 d, 4 d, 8 d and 12 d were diluted and spread on LB plates for bacterial counting. The survival rate was defined as the number of CFU at 0 d, 4 d, 8 d and 12 d divided by the number of CFU at 0 d. All the experiments were performed in triplicate.

Indole regulation assays to P_{cytR}. To test the regulating effect of indole on P_{cytR}, YS19/pUC-PR was cultivated in LB liquid medium with 0, or 0.5, mM indole added, while YS19/pUC was used as a negative control. Bacterial cultures sampled (1 mL) at 24 h were centrifuged, resuspended/washed three times with 1 mL sterilized PBS and the A_{600} value of samples were adjusted to 0.8. After filtering through a 200-mesh filter, fluorescence intensity of *gfp* expression was detected by a flow cytometry (excitation at 488 nm, Beckman Coulter, Inc.). All the experiments were performed in triplicate.

Statistical analysis. All the experiments were performed in triplicate. The statistical tests (standard deviations and *t*-distribution) used in the figures and description were carried out using Prism 5 (GraphPAD Software Inc.). *P* values <0.05 were considered significant.

Results

Mutation of cytR gene led to the decreased expression of the alkyl hydroperoxide reductase subunit C gene ahpC

Bacterial whole-cell proteins of YS19 and the Δ*cytR* mutant were analyzed by SDS-PAGE. On the gel, two differential bands stood out (Fig. 2A). The deoxyribose aldolase (DeoC, 27.7 kD), which was related to the survival of cells under carbon starvation, had been reported in our previous work (Jia et al., 2017). The expression of the other differential protein band around 21–23 kD was significantly higher in YS19 than that in the Δ*cytR* mutant. A mass spectrogram (Fig. 2B) of the target protein, obtained by HPLC-MS, identified it as the subunit C (22 kD) of alkyl hydroperoxide reductase (Ahp) (Smillie et al., 1992). Ahp consists of two subunits AhpC and AhpF (Smillie et al., 1992) and is reported to be related to oxidative stress (Seaver and Imlay, 2001) and sulfur starvation tolerance (Quadroni et al., 1996) in *E. coli*. The search of Swiss-Prot for *P. agglomerans* revealed that the number of peptides (95% confidence coefficient) was 57 and the sequence coverage (95% confidence coefficient) was 93.58%. Moreover, the molecular weight was consistent with the electrophoresis results. So, these data confirmed that *ahpC* is a downstream gene of the *cytR* regulation and the mutation of the *cytR* gene inhibited its expression.

The regulation of indole on ahpC expression was realized through cytR

We previously found that the expression of *cytR* can be regulated by indole (Jia et al., 2017). Then the question that indole, whether, or not, was involved in the regulation of *ahpC* expression became of relevance. We then tried to compare the expression of *ahpC* in YS19 wild type and Δ*cytR* mutant with, or without, indole added (0.5 mM). qPCR analysis revealed that the expression of *ahpC* in YS19 was significantly greater (1.89-fold change) than that in the Δ*cytR* mutant (Fig. 3). The difference was more obvious in the result of SDS-PAGE, probably because the amount of protein was not only related to the transcription level but, more importantly, it was also related to the translation level. And the addition of indole reduced the expression of *ahpC* in YS19 (1.38-fold change). However,

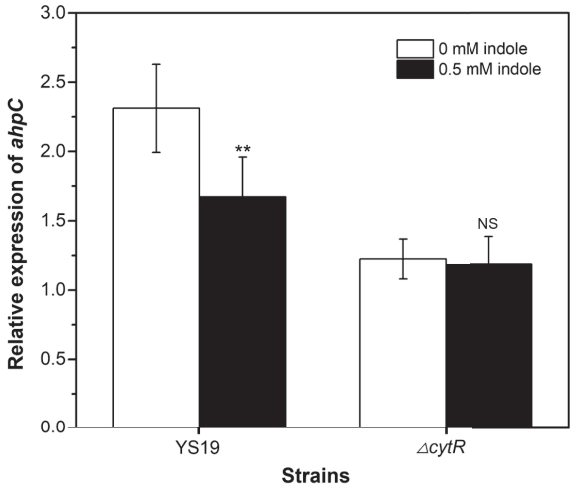

Fig. 3. The relative expression of *ahpC* in YS19 and Δ*cytR* mutant. Bacterial cells were cultivated in LB medium with or without supplementary indole (0.5 mM) for 24 h and the relative expression of the genes was analyzed by qRT-PCR. *gyrB* was used as a reference gene. Error bars represent standard deviations ($n = 3$). $^{**}P < 0.01$; NS, not significant compared to the control group.

the expression of *ahpC* in the Δ*cytR* mutant was not affected by indole, indicating that indole affected *ahpC* expression through *cytR*, rather than directly.

cytR played a significant role on the survival of the bacterium under oxidative stress and sulfur starvation conditions

Given that the mutation of the *cytR* gene affects the expression of *ahpC*, and that *ahpC* is related to oxidative stress (Seaver and Imlay, 2001) and sulfur starvation tolerance (Quadroni et al., 1996), one would conclude that the mutation of the *cytR* gene most likely results in a lowered resistance of the strain to these two stresses. As shown in Fig. 4A, under the treatment of 1 mM H_2O_2, without indole added, the survival rate of the wild type and Δ*cytR* mutant was 88.8% and 72.2%, respectively, the survival rate of the Δ*cytR* mutant decreased by 18.7% compared with the wild type. In the indole-added group, the survival rate of the wild type decreased by 14.9%. The difference was more obvious under the treatment of 10 mM H_2O_2, where the survival rate of the Δ*cytR* mutant decreased by 36.1% compared with the wild type without indole added, and the survival rate of the wild type decreased by 23.5% with indole added. For all the tests, the survival rate of the Δ*cytR* mutant was not affected by indole addition. When the concentration of H_2O_2 reached 100 mM, the damage might have exceeded the tolerance of the bacteria, and the survival rate of all four groups was very low and differences were not significantly obvious.

Under sulfur starvation conditions (Fig. 4B), at 12 d in the absence of indole, the survival rate of the wild type and Δ*cytR* mutant was 44.4% and 18.1%, respectively, i.e., the survival rate of the Δ*cytR* mutant decreased by 59.2% compared with the wild type. In the indole-added group, the survival rate of the wild type decreased by 23.2%, while the survival rate of the Δ*cytR* mutant was also not affected by indole addition. These results indicated that the pres-

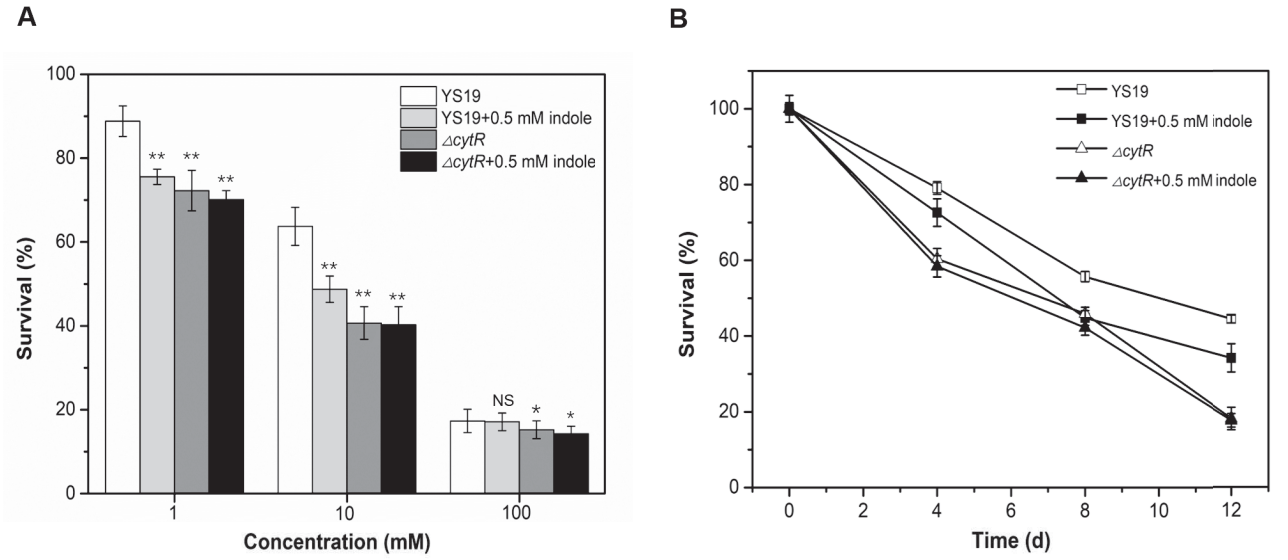

Fig. 4. The survival of YS19 and Δ*cytR* mutant under oxidative stress (A) and sulfur starvation (B) conditions.

A. YS19 and Δ*cytR* mutant were cultured in LB liquid medium for 24 h, and then H_2O_2 was added (final concentration 1 mM, 10 mM and 100 mM) followed by another 4 h of cultivation. B. Bacterial cells cultivated in M9 medium for 24 h were harvested, washed and incubated (1% inoculation) in the starvation menstruum (M9 medium without any sulfur source). Exogenous indole was added to a final concentration of 0.5 mM. The survival rates were detected by CFU method. Error bars represent standard deviations (n = 3). *P < 0.05; **P < 0.01; NS, not significant compared to the control group.

ence of *cytR* was crucial to the survival of YS19 under oxidative stress and sulfur-starvation conditions. Moreover, the addition of indole posed a greater threat to the survival of YS19 wild type and had little effect on the Δ*cytR* mutant. Namely, *cytR* had a positive effect on the survival of YS19 when faced with conditions of oxidative stress and sulfur starvation, and this effect was decreased by indole.

Indole carried out its inhibition of cytR expression through the promoter region of the gene

To explore how indole regulated *cytR*, we cloned P_{cytR} and detected its promoting effect on gene expression using *gfp* as a reporter. In the flow cytometry graph (Fig. 5), the dark gray part in the left column of each panel is the number of positive cells producing fluorescence, while the light gray part is the number of negative cells. It is shown that with 0.5 mM exogenous indole added, the positive cells producing green fluorescence decreased. The greater the fluorescence intensity, the closer the peak area is to the right, and so it could be seen that the peak area of fluorescence intensity was at the leftmost because YS19/pUC had no fluorescent expression (Fig. 5A), and the peak area shifted to the right because YS19/pUC-PR had fluorescent expression (Fig. 5B). Moreover, when exogenous indole was added, the peak area of fluorescence intensity of YS19/pUC-PR shifted to the left (from 70.8% to 34.1%) and the relative number of cells was decreasing (Figs. 5B and C). These results suggested that indole inhibited the expression of *cytR* through its promoter region.

Discussion

Under normal physiological conditions, *cytR* is involved in the uptake, utilization and recycling of nucleosides in the cells, which is important for the cells (Valentin-Hansen et al., 1996). However, our previous studies found that *cytR* was a negative factor for the survival of YS19 under carbon starvation conditions, and was not conducive to its colonization on rice (Jia et al., 2017). This study represents our further effort on the functions of *cytR* on stress resistance. Here, we analyzed the differential proteins between YS19 wild type and the Δ*cytR* mutant, and found that mutation in *cytR* decreased the expression on the alkyl hydroperoxide reductase subunit C gene *ahpC*, suggesting that *cytR* might promote its expression in a direct or indirect manner. The inhibition of *ahpC* expression by indole was not present under *cytR* mutation, indicating that regulation by indole was mediated by *cytR*.

AhpC can reduce H_2O_2, alkyl hydroperoxides, and organic peroxides into the corresponding alcohols (Bryk et al., 2002; Saikolappan et al., 2015; Seaver and Imlay, 2001). Interestingly, AhpC was previously reported to be regulated by a LysR family transcriptional regulator OxyR, in response to oxidative stress (Mongkolsuk and Helmann, 2002), and this study revealed that AhpC was regulated by a lactose repressor (LacR) family member CytR at both protein and mRNA levels, indicating there were at least two protein families related to its transcriptional regulation. In addition, AhpC is also significant to sulfur starvation in *E. coli* (Quadroni et al., 1996). When bacteria are subjected to oxidative stimulation from the external environment, the original redox homeostasis in the cell will be destroyed, resulting in oxidative damage to proteins, DNA, lipids and other biological macromolecules, and even cell apoptosis (Apel and Hirt, 2004; Droge, 2002; Kim and Holmes, 2012). Sulfur is one of the essential nutrients for the growth of all the living species (Koprivova and Kopriva, 2016) and sulfur-containing compounds such as glutathione (Scholz et al., 1989), H_2S (Mironov et al.,

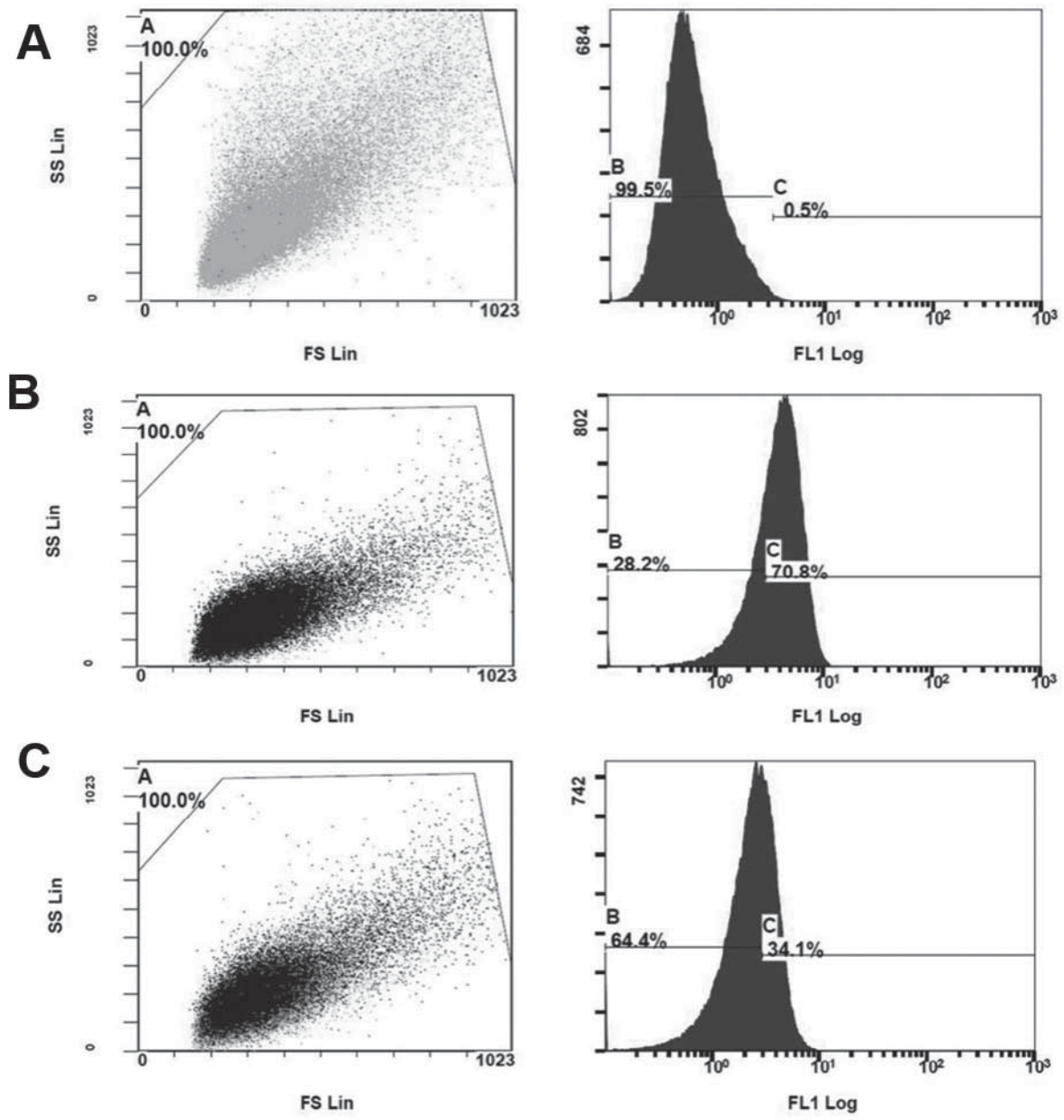

Fig. 5. Fluorescence intensity analysis of YS19/pUC and YS19/pUC-PR.

Bacteria cultivated in LB liquid medium for 24 h were detected by a flow cytometry at 488 nm excitation: YS19/pUC (A), YS19/pUC-PR (B, without indole added), YS19/pUC-PR (C, with 0.5 mM indole added). In the left panel, on the X-axis FS Lin is the forward angular scattering, which indicates the relative size and surface area of cells. On the Y-axis SS Lin is the lateral angular scattering, which represents the cell granularity and the relative complexity of cells. The lines represent the gate area set for detection and the capital A represents the percentage of cells in the area (100%). In the right panel, on the X-axis FL is the fluorescence intensity, and on the Y-axis the relative number of cells is given. The value of the abscissa corresponding to the intersection of the two lines below capital letters B and C is a set fluorescence intensity value, and B represents the percentage of cells with fluorescence intensity below this value while C represents the percentage of cells with fluorescence intensity above this value.

2017; Shatalin et al., 2011) and reactive sulfane sulfur (Ida et al., 2014) were of great significance to the antioxidation of bacteria. Here, we showed that *cytR* was conducive to the antioxidation and sulfur starvation resistance of *P. agglomerans* YS19. Obviously, there was a certain relevance between antioxidation and sulfur starvation resistance of bacteria, and we presumed that the antioxidant ability of the bacteria was reduced and was faced with a crisis of survival in the case of sulfur starvation, but *cytR* can help the bacteria resist the oxidation threat caused by the lack of sulfur, thereby helping the bacteria to survive under conditions of sulfur starvation. This leads to the new discovery regarding the function of *cytR* to helping bacteria against oxidative stress and sulfur starvation, even though it seems not to be beneficial to carbon starvation resistance and colonization on rice (Jia et al., 2017). Considering that oxidative stress and sulfur deficiency is also an adversity faced by rice endophytes, the CytR-conducted stress resistance ability of YS19 might be beneficial to its endophytic living.

Meanwhile, we also found that the positive regulatory factor *cytR* itself was inhibited by indole in YS19 wild type strain. One question is how indole is perceived by bacteria to participate in the regulation? It was once thought that indole can participate in the regulation of bacteria through SdiA (a transcriptional regulator of the LuxR protein family), which was once considered to be an indole protein receptor (Lee et al., 2007, 2009b). But this was refuted later (Sabag-Daigle et al., 2012). Since then, in-depth research on the issue has been lacking. This study has revealed that indole significantly inhibited the expression of the downstream reporter gene that controlled by P_{cytR}, implying that this promoter sequence played a novel role in the process of bacteria perceiving the indole signal and regulating gene expression, thereby opening up a new avenue for the study of indole signaling regulation.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31870090) and the National Key Research and Development Program of China (No. 2018YFA0901800).

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