Poly(A) polymerase I participates in the indole regulatory pathway of *Pantoea agglomerans* YS19

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**Abstract**

*Pantoea agglomerans* YS19 is a preponderant endophytic bacterium isolated from rice. It is characterized by the formation of symplasmata, a type of multicellular aggregate structure, contributing to a strong stress resistance and specific adaptation of YS19 in endophyte–host associations. Indole is an important signal molecule in intra- or interspecies relationships, regulating a variety of bacterial behaviours such as cell aggregation and stress resistance; however, the regulatory mechanism remains an ongoing area of investigation. This study selected YS19 as a model strain to construct a mutant library, utilizing the mTn5 transposon mutagenesis method, thus obtaining a positive mutant with an indole-inhibited mutation gene. Via thermal asymmetric interlaced PCR, the mutational site was identified as the gene of *pcnB*, which encodes the poly(A) polymerase I to catalyse the polyadenylation of RNAs. The full length of the *pcnB* sequence was 1332 bp, and phylogenetic analysis revealed that *pcnB* is extremely conserved among strains of *P. agglomerans*. The expression of the gene was significantly inhibited (by 36.6% as detected via quantitative PCR) by indole (0.5 mM). Many physiological behaviours of YS19 were affected by this mutation: the cell decay rate in the post-stationary growth phase was promoted, symplasmata formation and motility were inhibited in the late stationary growth phase and the colonization ability and growth-promoting effect of YS19 on the host plant were also inhibited. This study discusses the indole regulatory pathways from the point of RNA post-transcriptional modification, thus enriching our knowledge of polyadenylation and expanding current research ideas of indole regulation.

**INTRODUCTION**

To confront diverse environmental stresses, bacteria have evolved numerous signal regulatory mechanisms of which indole-based regulation has attracted increasing attention over recent years. A study in 2010 indicated that at least 85 bacterial species had been demonstrated to produce large quantities of indole, including both Gram-positive and negative bacteria [1]. In the conventional sense, indole is an intermediate of the tryptophan catabolism, in which tryptophanase encoded by the gene *tnaA* reduces tryptophan to pyruvate, ammonia and indole in a reversible reaction. Numerous genes regulate this process, such as *aroP*, *tnaB*, *trpE*, *tnaC*, *trpL*, *acrEF* and *mtr* in *Escherichia coli* [2]. During the last decade, increasing numbers of studies have proved that indole is more than a metabolite of amino acid: it influences many types of bacterial behaviours including pathogenicity [3] and stress resistance [4]. Moreover, indole-based regulation shows differences between strains. For cell aggregation, both promoting and inhibiting effects of indole have been observed [5]. Besides acting on producers, indole also works in interspecies. For example, indole is not produced by *Pseudomonas aeruginosa* or *Salmonella enterica*, but it has been shown to reduce the virulence of the former [6] and to promote the drug resistance of the latter [7]. To date, the regulatory mechanism of indole remains under investigation.

*Pantoea agglomerans* YS19, isolated from rice (*Oryza sativa*) cv. Yuefu, is a type of preponderant endophytic bacterium with a strong nitrogen-fixing activity, phytohormone-producing ability and plant growth-promoting effect [8]. YS19 is characterized by the formation of symplasmata, a type of multicellular aggregate structure formed by individual cells that tightly bind together [9]. Symplasmata is the main form of existence for YS19 in host rice tissues, not only bestowing the strong stress resistance [10] but also contributing to the specific adaptation of host association [11].

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**Keywords**: *Pantoea agglomerans* YS19; indole; poly(A) polymerase; symplasmata; Tn5 transposon.

**Abbreviations**: hiTAIL-PCR, high-efficiency thermal asymmetric interlaced PCR; PAP I, poly(A) polymerase I; qPCR, quantitative PCR.

The GenBank accession number for the nucleotide sequence of *pcnB* is KT270476.1.

Two supplementary figures and four supplementary tables are available with the online Supplementary Material.
YS19 does not produce indole, yet it senses exogenous indole to regulate many biological behaviours including the promotion of symplastma formation [12]. This study selected YS19 as a model strain to construct a mutant library, using the mTn5 transposon mutagenesis method. From the mutant library, the pcnB mutant that lacks the poly(A) polymerase I (PAP I) to catalyse the polyadenylation of RNAs was obtained. Our results revealed that indole inhibits the expression of PAP I, through which multiple physiological behaviours of the bacterium are affected, revealing multiple regulatory effects of the indole signal and also a regulatory pathway of the bacterium.

**METHODS**

**Bacterial strains and cultivation methods**

*P. agglomerans* YS19 was isolated as an endophytic bacterium from rice cv. Yuefu [13]. The rifampicin-resistant mutant YS19 (YS19-Rp<sup>5</sup>) was screened and preserved in our laboratory. *E. coli* SM10Apir (pFAJ1819, Km<sup>1</sup>) was kindly provided by Professor Michiels (from the F. A. Janssens Laboratory of Genetics, K. U. Leuven). The pcnB mutant of YS19 (Ap<sub>cnb</sub>) was screened from the mTn5 mutant library of YS19 and constructed via biparental conjugations between YS19-Rp<sup>5</sup> with SM10Apir. The bacteria were routinely maintained on LB agar [14]. Inocula of the bacteria were prepared by inoculating a colony into 20 ml of LB liquid medium and harvesting after 12 h of cultivation. The cells were transferred (1 %, v/v) to 20 ml medium in 50 ml shake flasks. Cultures were routinely grown at 30 °C, through which multiple physiological behaviours of the bacterium are affected, revealing multiple regulatory effects of the indole signal and also a regulatory pathway of the bacterium.

**Constructing the mTn5 mutant library of YS19**

The mTn5 mutant library of YS19 was constructed via trans-conjugation and random insertion mutation [15]: LB cultures of YS19-Rp<sup>5</sup> and SM10Apir were centrifuged at 10 000 g for 1 min for the collection of the cells, washed twice and resuspended via MgSO<sub>4</sub> solution (10 mM). They were mixed (1:1, v/v) and dripped on to the filter paper (6×6 mm) in the centre of an LB plate and incubated at room temperature for 2 h, and then at 30 °C for 24 h to allow trans-conjugation. The filter paper was taken out via a pair of sterilized tweezers and washed with 5 ml of MgSO<sub>4</sub> solution, then 100 µl of the sample was spread on a screening plate containing kanamycin, rifampicin and X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide, Sigma) (20 µg ml<sup>–1</sup>). The plate was incubated at 30 °C for 72 h for a coloration examination (blue X-Gluc hydrolysis) [16], using SM10Apir cells or YS19-Rp<sup>5</sup> cells as positive or negative controls, respectively.

**Screening for positive mutants regulated by indole**

Colonies showing blue colour were inoculated on a control plate containing X-Gluc and a testing plate containing both X-Gluc and indole (0.5 mM) (Sigma) and were incubated at 30 °C for 24 h. If a colony on both plates showed obvious colour difference, the GusA activity would be measured and compared via pNPG (p-nitrophenyl-β-D-galactoside) as a substrate as described by Michiels et al. [17]: Overnight cultivated cells were transferred (1 %, v/v) to 20 ml LB medium as two groups, one of which was supplemented with indole. After cultivation for 12 h, the culture (1 ml) was centrifuged at 10 000 g for 1 min, washed with sodium phosphate buffer (100 mM) and resuspended in GusA buffer (100 mM sodium phosphate buffer, 5 ml; 100 mM KCl, 1 ml; 10 mM MgSO<sub>4</sub>, 1 ml; β-mercaptoethanol, 35 µl; deionized water, 3 ml; lysozyme, 20 mg) and was further incubated at 37 °C for 30 min. Then, 8 µl of Triton-X (10 %) was added and incubated on ice for 5 min subsequent to vortexing for 10 s; 80 µl of pNPG (Sigma, 10 g l<sup>–1</sup>) was mixed with the solution and incubated in a 37 °C water bath for 15 min. The reaction was stopped by adding 300 µl Na<sub>2</sub>CO<sub>3</sub> (1 M). The supernatant was collected after centrifugation at 6000 g for 10 min, of which 150 µl was added into the 96-well plates and the A<sub>405</sub> values were measured via a microplate reader (MK3, Thermo). A Miller unit (U) represents the pNP production over 1 min. All measurements were obtained in triplicate.

**Determination of transposon insertion site**

The sequence of the mutational site was identified by high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) as previously described [18]. The genomic DNA was extracted via a genomic DNA extraction kit (TIANGEN Biotech). Three specific primers (gusA-1, gusA-2 and gusA-3) were designed according to the sequence of gusA on pFAJ1819. Four degenerate primers (LAD1-1, LAD1-2, LAD1-3 and LAD1-4) and one specific primer (AC1) were identical to what Liu and Chen described [18]. Primers, PCR systems and thermal conditions for hiTAIL-PCR are shown in Tables S1–S3 (available in the online Supplementary Material), respectively. The target band was recovered with an Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 (TAKARA), cloned into the pMD18-T vector (TAKARA) and transformed into *E. coli* DH5α. The plasmid was extracted via a Mini Plasmid Kit (TIANGEN Biotech) and sequenced by the Shanghai Sangon Company.

**Amplification of the pcnB gene in YS19**

The mutational site of YS19 was identified as the pcnB gene and the identification data are presented in Results. Genomic DNA of YS19 was used as a template to amplify the pcnB gene via PCR using primers pcnB-5′ (5′-CTT CCT CGG ACA GCC GGT TTC AG-3′) and pcnB-3′ (5′-AGA AAA TCG GGC TGA TCG GGC G-3′), designed according to upstream and downstream of pcnB gene in the genome of *P. agglomerans* Tx10. PCR products were verified with agarose gel electrophoresis (1 %), purified with DNA agarose gel recovery kit (Thermo) and then sequenced. The pcnB sequence was edited via the Chromas Program, version 1.43 (Conor McCarthy; Griffith University, Brisbane, Queensland, Australia). Closely related pcnB sequences were
obtained from the GenBank database by BLAST search (http://www.ncbi.nlm.nih.gov/). Multiple alignments were performed with the programme CLUSTAL_X [19]. A phylogenetic tree was constructed using the neighbour-joining method, using MEGA version 5 [20]. In each case, bootstrap values were based on 1000 replications [21]. The amino acid sequence was translated and analysed using the programme DNA MAN (www.lynon.com).

**Kinetics of cell growth and symplasmata formation**

Considering the fact that YS19 forms multicellular symplasmata in most of its growth phases (after 6 h of cultivation in LB medium) and that the formation of symplasmata might decrease optical density even for equivalent total cell numbers, neither the optical density method nor the c.f.u. method is suitable for bacterial growth measurement. Therefore, all determination of cell growth in this study was carried out by monitoring the biomass (dry weight) as previously described [12, 22]. For symplasmata formation, bacterial samples were observed using a light microscope (BX51, OLYMPUS, Japan) subsequent to Safranine O staining (Tianjin Fuchen, China). Symplasmata formation was evaluated using formation ratios (i.e. the percentages of cells that were found in the aggregates) as well as their average sizes (i.e. the diameters of the aggregates). This was done according to the statistical analysis of the bacterial aggregating profiles on a blood counting chamber viewed under a microscope, as previously described [23]. All experiments were performed in triplicate, and the results are expressed as mean values: all SD values were less than 5 %.

To exclude any polar effects produced by the genetic insertion, information about neighbouring genes in the genome was also evaluated by detection of their expression profiles.

**Motility assays**

The effects of pcnB mutants on the motility of YS19 were evaluated as previously described [24]: A sterile needle was used to lightly touch an overnight culture of YS19 wild-type or ΔpcnB and the needle was gently pinned into the middle of either a swimming plate (LB, 0.3 % bacteriological agar) or a swarming plate (LB, 5 g l⁻¹ glucose, 0.5 % bacteriological agar). Motility rates were identified after incubation at 30 °C for 20 h. The diameters of the swimming/swarming haloes (observed as a turbid zone, in millimetres) were measured from the point of inoculation. The results presented are the means of at least three independent experiments.

**Quantitative PCR analysis of pcnB and gusA transcription**

Overnight-incubated YS19 wild-type and ΔpcnB cells were transferred into LB liquid medium with or without supplementary indole (0.5 mM), respectively, and cultivated at 30 °C. Bacterial cells were sampled at different intervals for total RNA extraction using Trizol Reagent (TransGen Biotech). The RNA concentration was measured using an UV spectrophotometer (NanoDrop 2000, Thermo) and adjusted to 1000 ng µl⁻¹. cDNA was obtained by TranscriptII 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 (i) pcnB in the wild-type strain, (ii) gusA gene in the ΔpcnB mutant strain and (iii) neighbouring genes of pcnB in both strains was analysed by the SYBR I labelling method, using the SYBR FAST qPCR Kit (Kapa Biosystems). Quantitative PCR (qPCR) was carried out at 90 °C for 5 min at the first step, followed by 40 cycles at 90 °C for 30 s and 60 °C for 1 min. Specificity of the PCR products was confirmed by melting curve analysis followed by verification of the amplicon length on 1.5 % agarose gels stained with SYBR I. Primers used for qPCR are presented in Table S4, using the gyrB gene as a reference.

**Plant seeds, bacterial inoculation and cultivation**

Rice seeds (O. sativa) of cv. Yuefu were kindly provided by Dr L. Han (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences). Gnotobiotic cultivation was selected to avoid the undetectable effects of environmental factors (both biotic and abiotic). The rice seeds were gently dehulled and immersed in deionized water for 48 h at 25 °C, followed by a series of sterilization processes as previously described [8]. The thoroughly sterilized seeds were randomly divided into six groups (20 seeds per group). Two groups were inoculated with YS19 wild-type cells and two groups were inoculated with ΔpcnB cells; the remaining two groups did not receive any bacterial inoculation and were used as controls. One of each of the two groups was supplemented with indole (0.5 mM). The cells of YS19 wild-type and ΔpcnB used for the inoculation were cultivated in LB medium and harvested at an exponential growth stage. Bacterial inoculation and cultivation of the rice seeds were carried out as previously described [8]. After 21 days of cultivation, the seedlings were gently pulled out together with the roots from the medium using a pair of sterilized tweezers, then washed with sterilized deionized water to ensure that there was no contamination of the agar from the rhizosphere of the roots, and were blotted with a filter paper to remove all residual water for 2 s. Shoot and root were separated by a blade at the junction of root and stem, and fresh weight was measured immediately. Seedlings were mashed to a fine suspension in sterilized PBS and spread on LB plates for a bacterial count.

**Statistics**

The sample variances of two comparative test groups were calculated to provide a basis for the assumption of equality of the SD of seedling weights for both populations. It was then necessary to use the t-distribution with d.f. n₁ + n₂ - 2. To test for differences between both population means, the pooled estimate of the common population variance was utilized (for the complete operational process, please refer to Aczel [25] for details).
RESULTS

An indole-regulated mutant was obtained and the mutation site is the pcnB gene

The regulation of indole on the unknown genes was characterized by the reporter gene gusA. The GusA activities of colonies showing blue colour were measured and compared on LB medium with or without supplementary indole. From the mutant library, a mutant with an indole-negatively regulated reporter gene was obtained (Fig. S1). The GusA activity of the mutant was 17.29 U in the indole supplementary medium, which was decreased by 32.6 % compared to that of the non-indole control (25.67 U). Via hiTAIL-PCR, DNA sequencing and BLAST on NCBI, the transposon insertion site was identified as the pcnB gene (Fig. 1a), which encodes the enzyme PAP I to catalyse the polyadenylation of RNAs on the 3’-OH end.

The pcnB gene of YS19 was amplified and shows extreme conservation in species of P. agglomerans

To confirm the validity of the mutational site, PCR amplification of the pcnB gene was performed using genomic DNA of YS19 as the template. The gene pcnB was successfully amplified (Fig. 1b) and sequenced, revealing a total length of 1332 bp (GenBank No. KT270476.1). A phylogenetic tree of pcnB was constructed via the neighbour-joining method (Fig. 2a) and suggests that the gene is extremely conserved in species of P. agglomerans, indicating its potential significance for the survival adaptability of the strains in the species. Since no crystal structure of bacterial PAP I is available and even as high as 77.2 % compared to that of the distantly related E. coli (Fig. 2b), further confirming that this enzyme is highly conserved in the bacterial kingdom.

The mutation of pcnB inhibited symplasmata formation and promoted the decay rate of YS19 in the late stationary growth phase

To study the regulatory roles of PAP I, the effects of pcnB mutation on bacterial growth kinetics and symplasmata formation were first explored. According to the bacterial growth curve presented in Fig. 3(a), the mutation of pcnB did not affect the growth of the bacterium during its exponential growth phase, but it promoted the decay rate during the late stationary growth phase. The cell concentration of the mutant (0.51 g l⁻¹) decreased by 23.9 % in comparison to that of the wild-type (0.67 g l⁻¹) at 36 h. After 40 h of cultivation, the symplasmata formation ratio and average sizes in the mutant were 14.2 % and 1.1 µm, which were only 19.6 and 37.9 % of that of the wild-type (72.6 %, 2.9 µm), respectively (Fig. 3b–d). These data suggest that although PAP I is not essential for normal growth, it most likely played an important regulatory role in bacteria confronting environmental stresses (here, a nutrient limitation) by affecting symplasmata formation. Low concentrations of indole promoted symplasmata formation: e.g. with 0.5 mM supplementary indole, the symplasmata formation ratio at 40 h in the wild-type and the mutant were increased by 36.9 and 110 %, respectively (Fig. 3b, c). However, even under the promotion of indole, both formation ratio and size of the symplasmata in the mutant were still far from that of the wild-type, also suggesting a significant role of the PAP I protein in the indole regulatory pathway.

The mutation of pcnB inhibited the motility of YS19 cells

Motilities are considered to be extremely important to cell population behaviours, especially for cellular aggregation [27, 28]. Here, the effects of pcnB mutation on motility, i.e. swimming (Fig. 4a, b) and swarming (Fig. 4a, c) of YS19, were explored. Swimming is a type of surface translocation produced through the action of flagella [29]. On the swimming plates, diameters of swimming haloes in the wild-type supplemented with exogenous indole were evidently smaller than in the control group, indicating that indole mediated inhibition of the swimming ability (Fig. 4a). The pcnB mutation also inhibited the swimming ability, which decreased by 15.0 % in comparison to the wild-type (Fig. 4a, b). A similar inhibiting effect of indole on the swimming of ΔpcnB was also observed (Fig. 4a). The swimming abilities of wild-type and mutant decreased by 65.0 and 70.6 % via 1.0 mM indole, respectively (Fig. 4b).

Unlike swimming, swarming is a type of group behaviour that depends not only on the action of flagella, but is also closely related to the interaction of bacterial cells. The micromorphological pattern is highly organized in whirls and bands [29]. On swarming plates, the pcnB mutation

Fig. 1. Determination of transposon insertion site within the YS19 genome. (a) Determination of transposon insertion site in the positive mutant using hiTAIL-PCR. In the primary PCR, a clear single target band of approximately 2000 bp was obtained with LAD4 primer. LAD1–4 represents four degenerate primers. (b) PCR amplification of the mutant gene using the template of YS19 genome. M represents the molecular marker.
inhibited the swarming ability of YS19. The diameters of swarming haloes in the mutant decreased by 15.4% in comparison to the wild-type (Fig. 4a, c). Interestingly, the influence of indole on swarming revealed a concentration effect. For example, 0.5 mM of indole significantly promoted the swarming ability, and the diameters of swarming haloes in the wild-type and the mutant were increased by 22.9 and 36.5%, respectively (Fig. 4a, c). Based on the importance of Pantoea agglomerans strain DAPP-PG734 (NZ_JNAO1000011.1) in this study, the authors conducted genetic analysis of pcnB in YS19. (a) The phylogenetic tree of pcnB shows the relationship of YS19 with seven other strains of P. agglomerans and also a number of representations of closely related genera. Numbers at the nodes indicate the bootstrap values on neighbour-joining analysis of 1000 resampled data sets. Only bootstrap values above 50% are shown. The bar represents sequence divergence. The GenBank accession numbers for nucleotide sequence data are shown in parentheses. Outgroup: E. coli K-12 sub-str. MG1655. (b) Alignment of the amino acid sequences of pcnB encoding proteins in YS19 and E. coli (WP_001344171.1), P. agglomerans RIT273 and P. agglomerans MP2 using DNAMAN analysis. Grey shading represents residue conservation = 100%. The consensus residues are shown at the bottom of each comparing column.
motility inhibition to stabilize cell aggregates and optimize resource investment [28], we suggest that the swimming inhibition of indole (0.5 mM) ensured the stability of a highly organized micromorphological pattern, which is beneficial to the interaction of cells and their swarming ability. However, when the concentration of indole reached 1.0 mM, the motility of YS19 cells was excessively suppressed and the overall advantage could not manifest; therefore, the swarming ability was inhibited.

Indole inhibited the expression of pcnB in YS19

To further confirm the negative regulatory effect of indole on pcnB, we explored the expression of pcnB in LB medium with or without supplementary indole via qPCR. The primers were specific and amplification efficiency was 100%. As shown in Fig. 5(a), the relative expression of pcnB was increased with growth time and reached a maximum at 40 h. Consistent with GusA activity, indole significantly inhibited the pcnB transcription. For example, the relative expression of pcnB in the indole supplementary (0.5 mM) group at 12 h decreased by 36.6 % (Fig. 5a). These data confirm that PAP I participated in the regulatory pathways of indole and that indole inhibited the expression of PAP I. However, data presented in Fig. 5a also show that indole promoted the expression of pcnB at 50 h, which is somewhat
unexpected but can be explained via convincing analysis, considering the facts (i) that when the bacterium was cultivated for 40 h, it entered the decay phase (Fig. 3a, or also see Ref. [22]), and this bacterial decay dramatically decreased the expression of \( \text{pcnB} \) (at 50 h in Fig. 5a); and (ii) that indole delayed the growth phases of YS19 [12], i.e. the decay rate of the bacterial cells slowed down via supplementary indole in the medium. Such effects overcome the inhibition of \( \text{pcnB} \) expression by indole and manifest an apparent accumulation of the expression.

We suspect that indole has a similar regulatory effect on the relative expression of \( \text{gusA} \) in the \( \Delta \text{pcnB} \) mutant, if our understanding of the specific identification of the mutant and the regulation of \( \text{pcnB} \) by indole is correct. In essence, qPCR examination revealed a similar regulatory effect of indole on the relative expression of \( \text{gusA} \) in the mutant (Fig. 5b). Remarkably, the trend of the inhibition ratio of indole on the relative expression of \( \text{gusA} \) was highly consistent with that of \( \text{pcnB} \) (Fig. 5c), providing the strongest evidence to confirm the results.

The mutation of \( \text{pcnB} \) severely affected the colonization and growth-promoting effect of YS19 on host rice plants

The colonization of YS19 in rice tissues is almost all as symplasmata, which are specific structures for the adaptation of the bacterium in an endophyte–host association [11]. Given the fact that the mutation of \( \text{pcnB} \) inhibits both symplasmata formation and motility of YS19, it might also influence YS19–host rice association. Data presented in Fig. 6a suggest that, in the rice groups that did not receive bacterial inoculation, the average fresh weight of the 21-day cultivated rice seedlings supplemented with indole (0.5 mM) was 52.15 mg, compared to 55.62 mg for the control; thus, no significant differences were detected between both groups. However, the fresh weight of roots of the rice seedlings in the indole supplementary group decreased from 20.32 to 15.53 mg, suggesting that indole affects the growth of roots of rice seedlings on EPA semi-solid medium.

Compared to non-endophyte-inoculated seedlings, the average fresh weight of the YS19 wild-type-inoculated (75.72 mg) and \( \Delta \text{pcnB} \) mutant-inoculated (66.82 mg) rice seedlings was increased by 36.1 and 20.1 % (Fig. 6a), respectively. This indicates that the mutation of \( \text{pcnB} \) significantly inhibited the growth-promoting effect of YS19 on the cultivated rice seedlings. In such an endophyte–rice system, the effect of indole on plant growth promotion of YS19 wild-type and \( \Delta \text{pcnB} \) mutant was somehow complicated. The average fresh weights of the shoots of rice seedlings were increased by 27.2 and 21.6 %, respectively (Fig. 6a); however, due to the inhibition of indole itself on the growth of the roots, the fresh weights of the whole plants were not significantly affected.

Fig. 4. Effect of \( \text{pcnB} \) gene mutation on swimming (a, b) and swarming (a, c) abilities of YS19. Swimming/swarming haloes formed on the swimming or swarming plates are shown in (a) (bar=10 mm). The diameters of the haloes were measured and compared in (b) or (c), respectively. Error bars represent \( \text{SD} \) values (\( n=3 \)). Data with different letters are significantly different at \( P<0.05 \).
Furthermore, the colonization ability of YS19 was also inhibited by the mutation of \( \text{pcnB} \). Compared to the wild-type, the total colonization number of the mutant was decreased by 16.6\%, namely from \( 9.06 \times 10^4 \) c.f.u. mg\(^{-1}\) of the former to \( 7.56 \times 10^4 \) c.f.u. mg\(^{-1}\) of the latter (Fig. 6b).

Regardless of the \( \text{pcnB} \) mutation, indole improved the colonization ability of YS19 by 34.7\% for the wild-type and by 25.0\% for the \( \Delta \text{pcnB} \) mutant (Fig. 6b).

qPCR analysis of \( \text{pcnB} \) neighbouring genes revealed that the genetic insertion did not produce any polar effects and that the observed phenotypes are only due to disruption of \( \text{pcnB} \) and not of any other neighbouring genes (Fig. S2).

**DISCUSSION**

Symplasmata formed by \( P. \) agglomerans YS19 is a type of structure that is significant for bacterial adaptive survival and host association. The formation and regulatory mechanisms of these are key entry points to gain understanding of bacterial aggregation. As an important signal molecule, indole participates in the regulation of many bacterial physiological processes. In this study, we screened a \( \text{pcnB} \) mutant...
from the mTn5 mutation library of YS19 and revealed that the PAP I encoded by pcnB participates in the regulatory pathways of indole. Furthermore, its expression is down-regulated by indole.

PAP I catalyses the polyadenylation of RNAs, which is a post-transcriptional event that involves the addition of untemplated adenosine residues to the 3’ ends of RNA substrates [30]. The structural gene pcnB for PAP I was first discovered in E. coli in 1992 [31], challenging the idea that RNA polyadenylation would be the exclusive domain of eukaryotes. Many studies suggested that the polyadenylation of RNA is widespread in bacteria, such as E. coli, Bacillus subtilis [32] and Streptomyces coelicolor [33]. However, the proteins responsible for polyadenylation in these species are still not determined, except for E. coli [30]. Here, we successfully amplified the pcnB gene of YS19 and constructed a phylogenetic tree of pcnB, where no discrimination was observed among this P. agglomerans species. P. agglomerans is considered to be one of the most widely distributed bacterial species that successfully inhabits various environments, with extremely diverse genetic and physiological characteristics [22]. The high conservation of the pcnB gene in P. agglomerans strains implies the significance of polyadenylation for their adaptive survival in different habitats.

Polyadenylation occurs not only on mRNA and sRNA during their degradation [30] but also on tRNA to control the quality of the target [34], showing a destabilizing effect on the RNAs of a global level. Therefore, the expression of PAP I must be strictly regulated via transcriptional and translational controls [30], even by specific localization or modification of a protein [35], pcnB expression is closely related to bacterial growth rate and regulated by factors responsible for transcriptional modulation in confronting various nutritional conditions [36]. Since indole is a catabolism intermediate product of tryptophan, described as the most complicated of the 20 types of amino acids for protein synthesis and with a catabolism with the highest severity of the nutritional conditions, there is certain inevitability and conviction that indole regulates the expression of PAP I, as a signalling molecule in a stress (starvation) environment.

When the global regulatory system mediated by PAP I is destroyed by insertion mutation, many physiological behaviours of YS19 are affected as a result. Although PAP I is not essential for bacterial exponential growth, the decay rate is promoted in the late stationary growth phase while growth is in a nutrition-limited condition. The formation ratio and average size of symplasmata significantly decreased in the mutant, which might be one reason for their accelerated decay. This is because symplasmata, as a type of uniquely tight cell–cell binding aggregate, bestows the strong stress resistance ability of YS19, which has been repeatedly revealed during previous studies [9, 12, 22]. It is clear that pcnB, which is independent of indole exposure, impacts certain phenotypes in YS19; however, our results also demonstrate that the pcnB mutant strain still responds to indole in a manner similar to that of the wild-type. This suggests that indole-induced regulation is very complicated and that it might also play a central role in other pathways beyond the PAP I pathway.

Bacterial aggregation has been suggested to be influenced by increased cell mobility [37]. Because the mutation of pcnB inhibits the swimming and swarming abilities of YS19, the decrease in symplasmata formation in the mutant is most likely driven by such suppression. Maes et al. [38] reported that the PAP I mutation in E. coli resulted in an alteration of the cell membrane composition caused by GlmS (glucosamine–6-P synthase) accumulation, consequently affecting the motility of the mutant. Evidently, such a type of PAP I regulation is significant in bacterial chemotaxis and also in the adaptive ability to survive detrimental environments (here: a nutritional limitation).

Remarkably, the plant growth-promoting effect and the colonization ability were also decreased in the pcnB mutant. Since YS19 colonizes on rice are almost all formed symplasmata [11], with hundreds of individual cells tightly bound together, these types of aggregate provoke numerous advantages in occupying the whole micro-ecological niches within plants, which had been suggested in many studies [9, 39]. The suppression of symplasmata formation via pcnB mutation significantly influenced the association between the strain and the plant. Indole significantly improves the colonization of YS19 on the host, yet such a promotion is noticeably weakened by the mutation of pcnB, providing the strongest evidence to support that such an indole-mediated signal regulation is effective in enhancing the adaptability and survivability of YS19 in rice tissues, in which PAP I plays an important role. Simultaneously, it has been demonstrated that indole inhibits the growth of rice roots, which may be explained by the fact that indole mimics phytohormones, such as indole-3-actic acid, to interfere with their functions. Since the roots of plants are more sensitive to hormones compared to stems and leaves [40], the growth of the roots might be inhibited by such a hormonal mimic at the same concentration.

In summary, the results of this study indicate PAP I as a key protein in the indole regulatory pathways of YS19, by which indole realizes its regulatory roles on many physiological and biochemical behaviours of the bacterium. Remarkably, despite the pcnB mutation, the addition of indole still affected certain performance parameters (inhibiting motility, while promoting colonization) of the mutant, implying that PAP I-conducted regulation is not the sole pathway induced by indole. Other indole-induced pathways in the mutant might also have improved the performance. Future studies should focus on the precise role of PAP I and its relation to other collateral pathways, which are also stimulated by indole.

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Conflicts of interest
The authors declare no substantial financial or commercial conflicts of interest with the current work or its publication.

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