Indole affects the formation of multicellular aggregate structures in *Pantoea agglomerans YS19*  

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**Introduction**

Endophytes are generally referred to as microbes symbiotically colonizing tissue spaces, or within the cells of plants, yet not causing apparent diseases to the hosts (Stone et al., 2000). The host plants provide safe habitats for the endophytes, while the latter promote the growth of the former via nitrogen fixation, phytohormone secretion and other effects (Feng et al., 2006; Morris and Monier, 2003). With adequate moisture and nutrients, microbes adhere to, and colonize on, the surfaces offered by host plants. These association sites are regarded as niches, where the endophytes usually form specific aggregate structures to function like tissues (Morris and Monier, 2003). In such niches, a series of interactions between the endophytes and the plants help the microbes to survive the environmental stresses, and these also generate beneficial effects for the host.

*Pantoea agglomerans* YS19 is an endophytic diazotrophic bacterium isolated from rice. As well as having the ability to form a biofilm, as do most bacteria, it is characterized by the formation of a unique multicellular aggregate structure called symplasmata. Indole is traditionally known as a metabolite of the amino acid tryptophan, which, however, has recently been shown to participate in various regulations of bacterial physiological processes, including stress resistance, quorum sensing and biofilm formation. Here, an indole signal was found to promote symplasmata formation, yet inhibit biofilm formation, indicating different regulatory pathways of indole in the construction of the two structures. However, symplasmata showed almost an equivalent stress-resistant capability, as compared with biofilms, for YS19 to confront acids, heavy metals (Cu²⁺), and UV treatments. Moreover, indole was tested to show a promoting effect on exopolysaccharides (EPS) production and an inhibition effect on the expression of an outer membrane protein OmpW. These results provide evidence for understanding the regulatory mechanisms of indole on such multicellular aggregates.

**Key Words:** biofilm; indole; *Pantoea agglomerans*; symplasmata
nal roles in quorum sensing, biofilm formation, etc. (Hu et al., 2010). For example, *Escherichia coli* SdiA is the sensor of AHLs (quorum sensing signals) produced by other bacteria, yet indole regulates SdiA-mediated transcription in the absence of AHLs (Lee et al., 2007). It has been determined that the addition of indole to a medium reduced the biofilm mass in a wild-type strain but had no effect on the sdiA mutant. Besides, it has been reported that the deletion of yliH and yceP (genes found to regulate indole transport) greatly enhanced biofilm formation in *E. coli* paralleling the reduction in extracellular and intracellular indole concentrations, while the addition of indole into the culture restored the wild-type biofilm phenotype (Domka et al., 2006). Indole has also been found to exhibit multiple regulatory effects on the expression of stress-related proteins, e.g., YmgB (Hha homologue), an important protein involved in biofilm formation and acid resistance in *E. coli* (Wood, 2009). In this paper, the effects of indole on the formation of symplasma and biofilm of the rice endophyte *P. agglomerans* YS19, and the potential regulation mechanisms of indole on these multicellular aggregate structures, are investigated.

**Materials and Methods**

**Bacterial strains and cultivation methods.** *P. agglomerans* YS19 was isolated as an endophytic diazotrophic bacterium from rice cv. Yuefu (Yang et al., 1998). *P. ananatis* YJ76 (database accession numbers of the 16S rDNA sequence in NCBI: KT957000) was isolated as an endophytic diazotrophic bacterium from rice cv. Yuefu in our laboratory, and *Bacillus subtilis* CGMCC 1.4255 has also been preserved. The bacterial strains were maintained routinely on Luria-Bertani (LB) agar (Sambrook et al., 1989). Inocula of the bacteria were prepared by inoculating a colony on Luria-Bertani (LB) agar (Tianjin Fuchen, China) after being stained with Safranine O (Chongqing, China) after being stained with Safranine O (Tianjin Fuchen, China). The quantification of symplasma was evaluated by their formation ratios (i.e., the percentages of the cells that were found in the aggregates) and also by their average sizes (the diameters of the aggregates) according to the statistical analysis of the bacterial-aggregating profiles on a blood counting chamber viewed under a microscope (Jiang et al., 2015). The biofilm formed on the 96-well PVC was assayed by detecting the $A_{540}$ value after staining with crystal violet as described by O’Toole and Kolter (1998), where the effect of indole on bacterial growth was also normalized by measuring the biomass of the planktonic cells. Symplasma and biofilm were harvested at 30 h.

**Detection of indole, kinetics of cell growth, symplasma and biofilm formation.** Kovac’s reagent was used to test the production of indole as described (Gabriel and Gadebusch, 1956). Considering the fact that YS19 forms multicellular symplasma in most of the growth phases (after 6 h of cultivation in LB medium (Feng et al., 2003)) and the formation of symplasma might decrease the optical density, even if the total cell number is equivalent, the optical density method or the colony-forming units (CFU) method are considered not suitable for bacterial growth measurement. A pre-established relationship among $A_{600}$, CFU and biomass (dry weight) at various growth times, presented in the supplementary material (Fig. S1), also supports the above concern. For example, according to Fig. S1, after 6 h of cultivation, the approximate stabilization of CFU (a bacterial colony can be formed by the growth of a single bacterial cell or a symplasmatum) and yet the fast increase of dry weight (which represents the absolute quantity of bacteria) suggest that the newborn cells of *P. agglomerans* YS19 are essentially participating in the construction of the gradually increasing symplasmata (probably both in size and in number). Therefore, the determination of the cell growth in this study was carried out by monitoring the dry weight of YS19 cells as described (Feng et al., 2003).

For symplasma formation, YS19 was cultivated in LB liquid medium and observed by a light microscope (GOIC, Chongqing, China) after being stained with Safranine O. The quantification of symplasma was evaluated by their formation ratios (i.e., the percentages of the cells that were found in the aggregates) and also by their average sizes (the diameters of the aggregates) according to the statistical analysis of the bacterial-aggregating profiles on a blood counting chamber viewed under a microscope (Jiang et al., 2015). The biofilm formed on the 96-well PVC was assayed by detecting the $A_{540}$ value after staining with crystal violet as described by O’Toole and Kolter (1998), where the effect of indole on bacterial growth was also normalized by measuring the biomass of the planktonic cells. Symplasma and biofilm were harvested at 30 h.

**Treatment of acid, heavy metal ion and ultra violet light on YS19.** The cultures of YS19 in the three states (planktonic cells, symplasma and biofilm) were used for the test. Symplasma and planktonic cells in the culture were separated using a G4 sand-core funnel (intercept size 4 µm, Changchun Glass Instrument Company, China) (Zhang et al., 2009). The initial cellular biomass of planktonic cells, symplasma and biofilm were adjusted to be the same. According to Fig. S1, or our previous work (Duan et al., 2007; Feng et al., 2003; Jiang et al., 2015) which shows the relationship between optical density and biomass (dry weight) of YS19, 1 unit of $A_{600}$ of YS19 approximately equals 0.36 g L$^{-1}$ biomass at 30 h of cultivation (here, the symplasma and biofilm used for stress-resistance were harvested at 30 h). In this test, the planktonic cells and symplasma were diluted to 0.01 g L$^{-1}$ according to their biomass. Biofilm was shaved off from the biofilm-covered cover slips, dispersed and $A_{600}$ was adjusted to 0.03, a value being approximately equivalent to 0.01 g L$^{-1}$. To examine the resistance to acid, the cultures of YS19 were treated at pH 3.5 (adjusted by 5 M HCl) and incubated on a rotary shaker for 4 h. For the heavy metal experiment, the cultures of YS19 were treated with 5 mM CuSO$_4$ and incubated on a rotary shaker for 4 h. For ultraviolet (UV) treatment, 50 µL of YS19 cultures or biofilm-covered cover slips were added to a culture dish containing 5 mL of sterilized water and exposed to UV light (30 W) at a distance of 30 cm for 30–120 s on a cleaning bench (SW-CJ-2D, Suzhou Zhijing, China). For the biofilm group, both sides of the cover slips were exposed. All the experiments were performed in triplicate and the survival of the bacterial cells was detected by the plate counting (CFU) method.

**Whole-cell protein analysis and identification.** To identify the bacterial cellular differential proteins induced by an indole signal, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide gel) was performed as described previously (Li et al., 2011). YS19 culture was transferred to two groups of fresh LB medium, one group of which was supplemented with indole (1.5 mM). After being cultivated for 10 h, 30 h and
Bacterial cultures (1 mL) were incubated overnight in LB medium and then transferred to 1.5 mL tubes, respectively (a), and then Kovac’s reagent (200 µL) was added (b), demonstrating that P. agglomerans YS19 produces no indole (unchanged color), and the positive control P. ananatis Y76 produced large amount of indole (rose-bengal color), which however is shown as a slightly darker color in the black & white publication scale.

50 h, respectively, the culture was adjusted to the same biomass (final volume 2 mL) and centrifuged at 13000 g for 2 min to remove the supernatant. The cells were resuspended in 200 µL of sterile water. 15 µL of the suspension was mixed with equivoluminal SDS-PAGE loading buffer and was heated in a boiling water bath for 15 min for obtaining the whole-cell protein samples. Electrophoresis was carried out at 80 V for spacer gel and 120 V for separation gel and visualized by Coomassie brilliant blue staining. Protein bands of interest were excised and digested by trypsin as described previously (Andrej et al., 2006). Peptide mass fingerprinting was produced by Waters Q-Tof Ultima Global mass spectrometer (USA) and detected by MASCOT. Swiss-Prot was used to search protein bands of interest were excised and digested by trypsin as described previously (Andrej et al., 2006). Peptide mass fingerprinting was produced by Waters Q-Tof Ultima Global mass spectrometer (USA) and detected by MASCOT. Swiss-Prot was used to search for matching proteins. Detection and purification of EPS. Bacterial EPS was detected by the Congo red (CR, 10 mg mL⁻¹) plate method as described previously (Ronald and Peter, 1982). After 12 h of cultivation, YS19 cells were spread on two CR plates, of which one was supplemented with 1.5 mM indole. The plates were grown at 30°C for 24 h to examine the red colouration. B. subtilis CGMCC 1.4255 was used as the positive control.

For the purification of EPS, ultrasonic and ice-cold ethanol extraction was used as described previously (Guibaud et al., 2008; Zhang et al., 2008). After 30 h of cultivation, 100 mL of YS19 culture was used for the EPS extraction. However, the volume of the culture that was supplemented with 1.5 mM indole was adjusted accordingly for insuring the same initial cellular biomass as the non-indole-supplementary ones. The cultures were centrifuged (6,000 g, 4°C) for 15 min, washed by 30 mL of sterile phosphate-buffered saline (PBS, g L⁻¹: NaCl 8.0, KCl 0.2, Na₂HPO₄ 1.15, KH₂PO₄ 0.2, pH 7.4) and resuspended in 10 mL of sterile NaCl (1 M). To separate EPS from the cells, the bacterial suspension was treated by ultrasound (40 W, 4°C) for 3 min and centrifuged (13000 g, 4°C) for 30 min to remove any insoluble substance. The supernatant was mixed with a double volume of ice-cold ethanol and incubated overnight at 4°C. After centrifuging (13000 g, 4°C) for 30 min, the precipitate was resuspended in 10 mL of sterile water. The Sevag method was used to remove the proteins (Sevag et al., 1938) until the solution had no absorption at 280 nm, and the remaining aqueous phase was then sufficiently dialyzed with water. The production of EPS was quantified by the phenol-sulfuric acid method (Masuko et al., 2005), using glucose as a standard.

Results

Indole promotes symplasmata yet inhibits biofilm formation in YS19

Kovac’s reaction proved that P. agglomerans YS19 produces no indole (Fig. 1). To study the regulatory roles of indole on the physiological behavior of YS19, the effects of indole on the bacterial growth kinetics, and the formation of multicellular aggregate structures, were first explored. Data presented in Fig. 2a indicates that the growth was promoted by low concentrations (0.25–1.0 mM) of indole, yet obviously inhibited and delayed by higher concentrations (1.5–2.0 mM) of indole.

Traditionally, the effects of indole on bacterial biofilm formation are not univocal. Both promotion (Lee et al., 2007; Sasaki-Imamura et al., 2010) and inhibition (Lee et
al., 2007) effects have been reported. In this study, the influence of indole on both symplasmata and biofilm formation was examined. Since P. agglomerans YS19 produces no indole, exogenous indole was supplemented to the cultures. Data presented in Figs. 2b and d indicate that indole-promoted symplasmata formation in the liquid culture, where the ratio and volume of symplasmata increased (the volume increase being more significant than that of the ratio) with increasing concentrations of indole after 30 h of cultivation. For example, the formation ratios of symplasmata in LB medium supplemented with 0 and 1.5 mM indole are 62.9% or 80.7%, respectively, while the average sizes (represented by the diameters of the aggregates) of the symplasmata in the two cultures are 1.8 µm and 6.4 µm, respectively. Nevertheless, there is a limit of the promotion effect when indole reaches certain high concentrations (1.0–1.5 mM).

However, the formation of biofilm exhibited a negative correlation with the concentrations of indole, as detected by the optical absorption ($A_{540}$) of the biofilm formed on polyvinylchloride (PVC) (Fig. 2c) upon cultivation in liquid LB medium. For example, the formation of biofilm on PVC in LB medium supplemented with 1.5 mM indole decreased by 80.8% as compared with that in the non-supplementary ones, where, however, the concentration of planktonic cells in the former culture only decreased by 19.08% compared with that in the latter, strongly suggesting that the inhibition of indole on biofilm formation was substantial while not an apparent effect of the bacterial growth inhibition.

**Symplasmata and biofilm show almost equivalently enhanced stress-resistant capabilities**

The minimum biocidal concentration of acid was first tested to show that no YS19 cells could survive the treatment at pH 3.0 for 4 h. Therefore, pH 3.5 was selected for the acid treatment. Compared with planktonic cells, the acid resistant abilities of symplasmata and biofilm were enhanced approximately 11 and 14 times, respectively (Fig. 3a). For the heavy metal resistance test, it was detected that 8.0 mM CuSO$_4$ was fatal to the bacterium during 4 h of treatment in LB medium. Therefore, 5.0 mM CuSO$_4$ was selected for the treatment. The viabilities of the symplasmata and biofilm were tested to be 2.3 and 2.0 times of the planktonic cells, respectively (Fig. 3a). Based on the stress-resistance data of YS19 against acid and CuSO$_4$, symplasmata and biofilm showed almost equivalent resistant capabilities to such chemicals.

The symplasmata and biofilm of YS19 also survived the exposure to UV light well (Fig. 3b). The survival rates of the two structures after the first 30 s of treatment were as follows: symplasmata 25.0%, biofilm 37.5%, as compared with planktonic cells 8.25%. However, the longer time the bacterium is exposed to UV light, the greater the resistant ability of the biofilm becomes compared with that of the symplasmata, e.g., after 60 s of treatment, the survival rate of the cells in biofilm still maintained 17.8%, eight times that of the planktonic cells (2.2%) and even nearly four times that of the symplasmata (4.6%). Considering the low penetrative property of UV light, both sides of the biofilm-bearing cover slips were irradiated for 60 s in this experiment, and the actual superiority of the biofilm in UV light-resistance might be even larger.

**Indole remarkably inhibits the expression of an outer membrane protein OmpW**

As shown on the gel of the SDS-PAGE analysis (Fig. 4a), the expression of many proteins changed remarkably due to indole induction, of which the most obvious one is the band located at about 23 kD. The protein was expressed normally at different growth times; nevertheless, it was almost not expressed when 1.5 mM indole was added. Peptide mass fingerprinting (Fig. 4b) of the differential protein obtained by HPLC-MS identified it as an outer membrane protein OmpW (23 kD) by searching Swiss-Prot of P. agglomerans. The sequences coverage is 85%. Also, the molecular weight is nicely consistent with the electrophoresis results. So it is confirmed that indole remarkably inhibits the expression of OmpW.

**Indole promotes the production of EPS**

The production of EPS in YS19 detected by the Congo
red-plate method revealed that YS19 colonies in indole supplementary (1.5 mM) culture and the positive control show an obvious red color (shown in thicker density in the black & white publication scale), while YS19 colonies in the non-indole-supplementary culture are almost unstained (Fig. 5a), indicating that the EPS secretion in YS19 was significantly promoted by indole. In the groups of YS19 with indole added and the positive control B. subtilis CGMCC 1.4255, the secretion of large scales of mucous EPS makes the size of the bacterial colonies seem much bigger. In contrast, without indole added, YS19 almost secretes no EPS and then is poor for Congo red dyeing, which makes the culture seem to be apparently weaker than the EPS-producing ones.

The quantitative measurement of the EPS production based on the phenol-sulfuric methods suggested that the EPS production of indole-supplementary (1.5 mM) YS19 culture increased by 62.2%, compared with the non-indole-supplementary YS19 control (Fig. 5b). Considering the fact that EPS is significant to bacterial aggregation, it implies that the promotion effect of indole on the production of EPS may be an explanation for the promoted symplasmata formation by the signal.

**Discussion**

Rice diazotrophic endophyte *P. agglomerans* YS19 was verified to contribute to plant growth (Feng et al., 2006) and its formation of symplasmata is a conducing factor for colonization and stress-resistance (Zhang et al., 2009; Zhang et al., 2010). Formed via aggregation of individual single cells (Duan et al., 2007), symplasmata, in turn, can be induced by adverse environmental stresses, implying their beneficial effects to the bacterial survival adaptation (Li et al., 2011). This paper represents our continued effort on investigating the signal regulatory profile of the structure by indole, an important signal molecule that has been of great interest recently.

The formation of biofilm is limited by the initial attachment of a cell onto the two-phase interfaces (Monroe, 2007), while a symplasmatum is always constructed by cell-cell contact and binding (Duan et al., 2007). The disparate pattern of aggregation implies that biofilm and symplasmata might have different formation mechanisms. This hypothesis is confirmed by the opposite effects of indole signals on their formation in YS19 which are discussed in this paper. Traditionally, indole is regarded as an ordinary metabolic intermediate in tryptophan catabolism. Primarily, tryptophanase (encoded by *tna*) catalyzes tryptophan to produce pyruvate ammonia and indole (Lee et al., 2007). However, indole was recently discovered to act as a signal molecule in bacterial population behavior (Hu et al., 2010; Lee et al., 2010). Previous works have suggested that the effects of indole on biofilm formation are complicated. For some bacteria, such as *E. coli*, biofilm formation is inhibited by indole (Lee et al., 2007). Interestingly, for some bacteria, such as *Vibrio cholerae* (Mueller et al., 2009) and *Pseudomonas aeruginosa* (Domka et al., 2006), indole promotes their biofilm formation. Mueller et al. suggested that the effects of indole may be strain specific (Di Martino et al., 2002; Mueller et al., 2009). As a non-indole-producing bacterium, the formation of biofilm in YS19 is inhibited by exogenous indole. Prior to the work reported here, no observations have been carried out on the regulatory effects of indole on symplasmata. Here, we have shown that symplasmata formation in YS19 is significantly promoted by indole, which is contrary to what is expected from traditional knowledge based on biofilms. The positive effect of indole on symplasmata formation and its negative effect on biofilm formation suggests that the two structures most likely have different formation mechanisms and regulation pathways,

**Fig. 4.** Display and identification of the differential proteins that are regulated by indole signals.

Whole-cell proteins of YS19 cultivated with (lanes 3, 5, 7) or without (lanes 2, 4, 6) supplementary indole were analyzed by SDS-PAGE. The cultures were harvested at 10 h (lanes 2, 3), 30 h (lanes 4, 5), 50 h (lanes 6, 7), respectively (a). The peptide mass fingerprinting of a differential protein (as indicated by the bold arrow in a) obtained by HPLC-MS was identified as an outer membrane protein OmpW by search SwissProt of *P. agglomerans* (b).

**Fig. 5.** The production of EPS detected by the Congo red-plate method (a) and the phenol-sulfuric method (b).

Streak culture of *P. agglomerans* YS19 was performed on LB plates with, or without, supplementary indole (1.5 mM) for 24 h. *B. subtilis* CGMCC 1.4255 was used as a positive control (a). EPS was extracted by ultrasonic and ice-cold ethanol treatments and quantified by the phenol-sulfuric acid method. The EPS production in the indole supplementary (1.5 mM) culture was increased a lot, compared with that in the non-supplementary ones. Error bars represent standard deviations (n = 3) (b).
i.e., the cell aggregates in symplasmata differ from those in biofilms.

Although the two aggregate structures possess different construction mechanisms, they showed a similar, much stronger, stress-resistant ability than planktonic cells. Symplasmata and biofilms are highly specialized and independent microbial aggregates, especially in their different gene expression profiles (Li et al., 2011; Stewart and Franklin, 2008) and also their viscous matrices surround structures (Costerton et al., 1995; Zhang et al., 2010). These genes include those which encode certain special matrices (e.g., lipoprotein and extracellular polysaccharide) to envelope the aggregates and protect the inner cells efficiently. Teitzel and Parsek (2003) treated biofilms with heavy metal ions and found that only cells at the bottom of the biofilm survive. Elasri and Miller (1999) proved that few UV rays penetrated the outer layer of the biofilm. In addition, extracellular matrices can prevent damage to cells inside a biofilm, brought about by dryness, by increasing water-holding effects (Decho, 2000). Similar to the case of a biofilm, previous electronic microscopic studies on P. agglomerans, including our own, have revealed that a thick matrix of glycoproteins coats the symplasmata (Achouak et al., 1994; Li et al., 2011; Zhang et al., 2010). Moreover, a poor distribution of nutrients induced by cell-aggregating causes the inner cells to metabolize at lowered rates and, however, be more resistant to stresses. Besides serving as a barrier to protect the inner cells against toxic substances, the matrix fibers also decrease the defense reaction of the host, which is extremely significant for the endophytic association of YS19 with the host rice plant (Zhang et al., 2010).

Our results show that indole indeed regulates both biofilm and symplasmata formation by affecting cellular out membrane EPS and proteins. More specifically, indole inhibits the expression of the outer membrane protein OmpW of YS19, which is a kind of minor porin. It was reported that calcium-induced biofilm formation is related to the expression of OmpW in Pseudoalteromonas sp. 1398 (Andrés et al., 2012). The oprG (ompW homologue) mutant of Ps. aeruginosa formed biofilms with a modified structure and a lower biovolume than the wild type strain. Interestingly, when E. coli aggregates to form a biofilm, the oxidative stress gene soxS is activated and then the SoxS will positively regulate the ompW gene (Beloin et al., 2004; Gil et al., 2009). Therefore, in turn, the inhibition of the biofilm formation may inhibit the expression of ompW. This study also suggested the synchronization of indole inhibiting both biofilm formation and the expression of ompW. Based on these observations, it is reasonable to infer that the regulatory pathway of indole in the biofilm formation of YS19 might also be concerned with OmpW.

EPS is considered to be one of the main components for cell-cell adhesion. Although our previous work has proved that YS19 produces little EPS during symplasmata formation in LB medium (Zhang et al., 2010), this study revealed that indole not only promotes symplasmata formation, but also greatly promotes the production of EPS. Despite the fact that the exact role of EPS needs further exploration, this study has suggested that the production of EPS and symplasmata formation are related. As we know, EPS is essential for aggregate construction in some bacteria, of which mutants unable to synthesize EPS are unable to form biofilms (Allison and Sutherland, 1987; Watnick and Kolter, 1999). Interestingly, in this research, indole was observed to promote EPS production yet inhibit biofilm formation. We extrapolate that indole affects some factors (such as OmpW and YmgB (Lee et al., 2007)) to inhibit biofilm formation and other factors such as EPS to promote biofilm formation, which results in the impression of an overall effect of inhibition.

The formation of symplasmata or biofilm in YS19 is an adaptive mechanism for the tolerance of various unfavorable living conditions. Thus, the highly-adaptive characteristic of YS19 is one of the reasons why it is a dominant strain of the widely-planted rice cultivar Yuefu. Symplasmata are observed in all tissues in planta (Zhang et al., 2010) as well as on solid and in liquid media (Feng et al., 2003), and biofilm is always found on the two-phase interfaces of the cultures, which are complementary structures of symplasmata formed by YS19, and perhaps by other strains in P. agglomerans, in realizing a successful environmental life. Clearly, their regulation by environmental indole signals have promoted such an adaptive process.

Supplementary Materials

Fig. S1. The relationship among $A_{600}$ (□), colony-forming units (CFU) (△) and biomass (○) of YS19 at various growth times when it was cultivated in LB medium.

Error bars represent standard deviations ($n = 3$).

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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