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Full Paper

Extracellular DNA enhances the formation and stability of symplasmata in *Pantoea agglomerans* YS19

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Jing Zheng, Yifan Xia, Qi Liu, Xinyu He, Jiajia Yu, and Yongjun Feng*

School of Life Science, Beijing Institute of Technology, Beijing, China

Extracellular DNA (eDNA) is an important polymeric substance that plays essential roles in cell aggregation and nutrient provision for the sessile bacteria. eDNA in bacterial biofilms was extensively studied. Here we found that eDNA also exists in symplasmata, a bacterial cell aggregate, which is different to a biofilm, in the rice enophyte *Pantoea agglomerans* YS19. We found that exogenous eDNA enhanced the formation and stability of symplasmata significantly, and that, exogenous eDNA also improved the stress resistance and colonization ability of the bacterium on host rice. These results strongly indicate novel roles of the eDNA in *Pantoea agglomerans* YS19, showing its special relation to the stress-resistance and endophyte-host association of the strain.

Key Words: extracellular DNA; *Pantoea agglomerans*; stress resistance; symplasmata

Introduction

Extracellular DNA is known as cellular free DNA that generally exists in the extracellular environment (Böckelmann et al., 2006). Release and persistence of eDNA in the environments (such as soils, waters, etc.) have been observed as a general phenomenon in microbial organisms (Jakubovics et al., 2013; Levy-Booth et al., 2007; Pietramellara et al., 2009). As an important component of extracellular polymeric substances, eDNA plays essential roles in cell aggregation and nutrient provision for the bacteria. For example, significant effects of eDNA on biofilm formation were observed in both Gram-positive (*Staphylococcus aureus*, *S. pneumonia*, *Bacillus cereus*, *Listeria monocytogenes*, etc.) and Gram-negative (*Pseudomonas aeruginosa*, etc.) bacteria (Karatan and Watnick, 2009; Lappann et al., 2010; Spoering and Gilmore, 2006). In *S. mutans*, eDNA released from cell death influences the formation and maturation of biofilm significantly (Liao et al., 2014; Perry et al., 2009; Petersen et al., 2005; Wenderska et al., 2012).

Pantoea agglomerans YS19 is an endophytic diazotrophic bacterium isolated from rice. Our previous studies demonstrated that YS19 can form a unique multicellular symplastmata, structurally maintained by uniquely tight cell-cell bindings. Unlike biofilm, symplastmata can be formed by cell aggregation in cultures without other surfaces to adhere to (Feng et al., 2003). The symplasmata initially formed in the exponential growth phase after a disperse cell growth period (approximately 6 h) in LB medium (Feng et al., 2003). In rice, the existing form of YS19 is almost entirely symplasmata (Zhang et al., 2010), and it is beneficial to the endophyte for these aggregates to occupy all of the micro-ecological niches of the plant (Li et al., 2017; Morris and Monier, 2003). eDNA existing in biofilm has been proven to play important physiological roles. However, there are no reports in the literature concerning the roles of eDNA in symplasmata. Here, YS19 was selected as the model strain for such a study, and eDNA-induced stress-resistance and colonization have also been explored.

Materials and Methods

Bacterial strains and cultivation methods. *P. agglomerans* YS19 as an endophytic diazotrophic bacterium was isolated from rice (Cv. Yuefu) (Feng et al., 2003). The bacterium was routinely maintained at 4°C on LB agar (Sambrook et al., 1989). A colony was inoculated into 20 mL of LB liquid medium for 12 h to obtain the inocula. The cells in the inocula were transferred (1%, v/v) to 150 mL LB liquid medium in 500-mL shake flasks, and the

*Corresponding author: Yongjun Feng, School of Life Science, Beijing Institute of Technology, 5# Zhongguancun Nandajie, Beijing 100081, People's Republic of China.
Tel: +86 10 81736973 Fax: +86 10 68915956 E-mail: fengyj@bit.edu.cn

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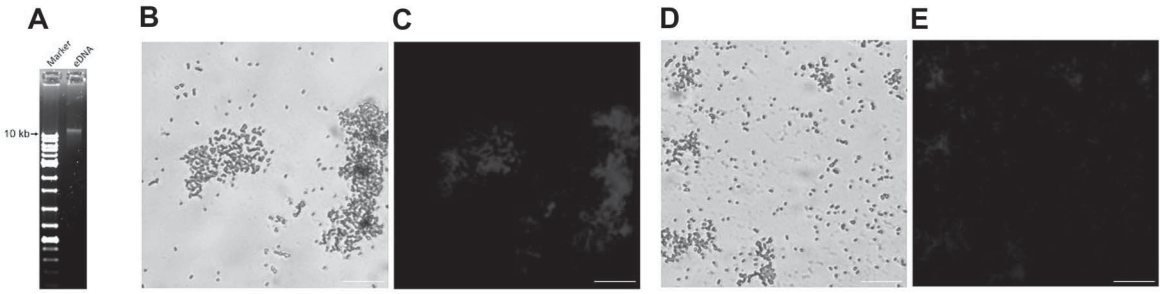

Fig. 1. YS19 produces eDNA, and eDNA mainly exists in symplasmata.

Agarose (1.0%) gel electrophoresis showing the purified eDNA (A). CLSM observation of EB-stained YS19 cells grown for 30 h is shown in B and C, while a DNase I-treated sample is shown in D and E. The symplasmata (cell aggregates) showed red fluorescence in fluorescent field channel (C, E) was compared with that in the bright field channel (B, D). Original magnifications, ×1 K; bar = 10 μm.

cells were routinely cultured on a rotary shaker (2.67 rev s^{-1}) at 30°C. When the solid medium was required, bacteriological agar (1.5% w/v) was added to the LB medium.

eDNA extraction and detection. Culture samples harvested at 30 h were centrifuged at 10,000 *g* at 4°C for 3 min. Under such a centrifugation, the eDNA will be disassembled from the cell surfaces and dispersed into the supernatant (Lappann et al., 2010). The supernatant was filtered with a 0.2 μm filter and transferred to a fresh Eppendorf tube, and NaCl (final concentration 0.25 M) was added to the tube. Then 2:1 volume of ethanol was added to precipitate the eDNA and the mixture was maintained at 4°C for 15 min. After a centrifugation (12,000 *g*) at 4°C for 6 min, the eDNA was collected and dissolved in TE buffer (Lappann et al., 2010). The eDNA was further purified by the phenol-chloroform method as previously described by Nur et al. (Nur et al., 2013). For eDNA production, the filtered supernatants were treated using the AccuGreen™ High Sensitivity dsDNA Quantization Kits (Biotium, USA) according to the manufacturer's instructions. Then, the samples were determined using a Thermo Fisher Qubit® 3.0 fluorometer (Ex/Em 502/523 nm).

eDNA staining and confocal laser scanning microscope (CLSM) observations. To observe the presence of eDNA on the extracellular matrix of YS19, the bacterium was cultivated for 30 h. A part of the samples was treated with DNase I (10 mg mL^{-1}, Sigma Aldrich) at 37°C for 1 h, then 10 μL of broth was transferred to one-well polystyrene chamber slides and blended with 1 μL ethidium bromide (EB, 5 μg mL^{-1}) at room temperature for 10 min followed by an examination under a Leica HCX PL APO CS 100 oil immersion objective on a Zeiss LSM510 META confocal system. The fluorescence of EB was excited at 543 nm, and the samples were observed in a bright field and dark fluorescent field, respectively. In a dark fluorescent field, the eDNA-enveloped, or dead, cells were observed having the colour red (Conover et al., 2011).

Cell growth curves, symplasmata formation, and sress resistance assays. For cell growth curve assay, bacterial cells were cultivated in LB liquid medium with certain supplementary concentrations of genomic DNA (0–1.5 μg mL^{-1}) extracted from YS19 with TIANamp Bacteria DNA Kit (TIANGEN Biotech) as exogenous eDNA. The cultures were sampled at different growth times (0–60 h), and cell growth and biomass were measured as described in our previous studies (Feng et al., 2003).

For symplasmata formation assay, bacterial cells were cultivated in LB liquid medium with 1.0 μg mL^{-1} of YS19 genome DNA as the exogenous eDNA. The cultures were sampled at different growth times (6–60 h), and stained by Safranine O (Beijing solarbio, China) for the assays of symplasmata formation, which was evaluated by formation ratio, (i.e., proportion of cells which formed the symplasmata) and average size (i.e., diameter of the symplasmata) according to the statistical analysis of the bacterial aggregating profiles on a blood counting chamber viewed under an light microscope (BK6000, Chongqing Optec Instrument Co., Ltd., China) as described previously (Jiang et al., 2015).

For stress resistance assay, bacterial cells were cultivated in LB liquid medium supplemented with 1.0 μg mL^{-1} of YS19 genome DNA as the exogenous eDNA. Cultures at three typical time points 6 h (in the exponential phase), 30 h (in the stable phase), and 48 h (in the decline phase) were chosen for the following chemical treatments. Chemicals were added ($CuSO_4$, final concentration 4.0 mM; or acid, final pH 4.5) and further cultivated for 4 h, respectively. Then, the cells were diluted and spread on the LB plates for bacterial counting (colony forming units, CFU). The survival rate was defined as the number of CFU after the treatment divided by the number of CFU prior to treatment. All the experiments were performed in triplicate.

DNase I treatment. To study the role of eDNA in YS19 symplasmata stability, the bacterium was cultivated in LB liquid medium for a certain time, then the cultures were treated with DNase I (10 mg mL^{-1}, Sigma Aldrich) and incubated at 37°C for 1 h. For excluding other effects beside the enzymatic function of DNase I, the heat-inactivated DNase I (1 h at 75°C) was used as a control. Then statistical analysis of the symplasmata formation ratio and average size were carried out. All the experiments were performed in triplicate.

Plant seeds, bacterial inoculation, and cultivation. Rice seeds (Cv. Yuefu) were kindly provided by Dr. Longzhi Han (Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China). For avoiding

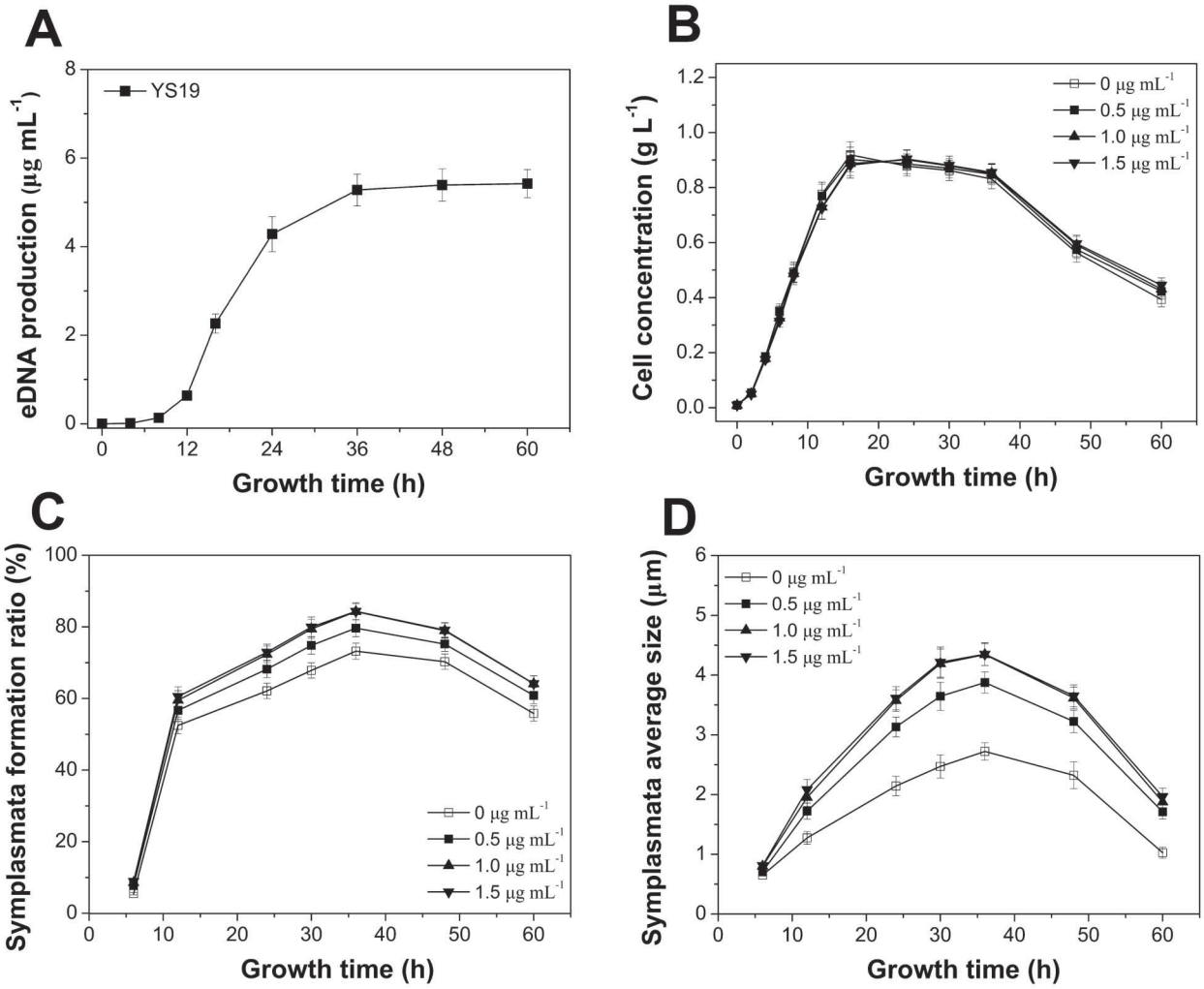

Fig. 2. The production of eDNA and the effects of eDNA on the growth and symplasmata formation of YS19.

The production of the eDNA was determined using a Thermo Fisher Qubit® 3.0 fluorometer (A). Bacterial cells cultivated in LB liquid medium with certain concentrations of supplementary eDNA (0–1.5 μg mL^{-1}) were sampled at different time points (0–60 h) for growth curve (B), symplasmata formation ratio (C), and size (D) assay. Error bars represent standard deviations (n = 3).

undetectable biotic and abiotic effects of environmental factors, gnotobiotic cultivation was selected. The rice seeds were gently dehulled to avoid injury. After immersion in sterile deionized water for 48 h at 25°C the seeds were subjected to a series of sterilization processes as described previously (Feng et al., 2010). Then, the seeds were inoculated with YS19 and randomly divided into four groups (20 seeds per group). Two groups were cultivated in semisolid EPA (endophyte-plant association) medium supplemented with 1.0 μg mL^{-1} genomic DNA; the remaining two groups were cultivated in semisolid EPA medium as controls. EPA medium was prepared as previously described (Feng et al., 2010). For the measurement of colonization, 10-day-cultivated rice seedlings were gently pulled out from the medium to ensure integrity using sterilized tweezers, then washed with sterilized deionized water to ensure that there was no adhesive agar. The samples (whole plant, root and shoot) were weighed and mashed to a fine suspension in sterilized PBS for a bacterial count on the LB plates.

Statistics. The sample variances of two comparative test groups were calculated to provide a basis for the assumption of equality of the SD of colonization number for both populations. It was then necessary to use the t-distribution with d.f. $n_1 + n_2 - 2$. 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 (1995) for details).

Results

Assay of eDNA existence

P. agglomerans YS19 eDNA was purified by the phenol-chloroform method as previous described at an ice-cold temperature. According to the agarose gel electrophoresis (Fig. 1A), YS19 cells produced eDNA during the growth. To explore the distribution of eDNA, YS19 cells were stained and observed by a CLSM. It was found that in a dark fluorescent field the cells in symplasmata (cell aggregates, Figs. 1B and C) showed obvious red fluorescence. However, when the culture was treated by DNase I, the symplasmata almost diminished, and at the same time, the red fluorescence was also weakened (Figs. 1D

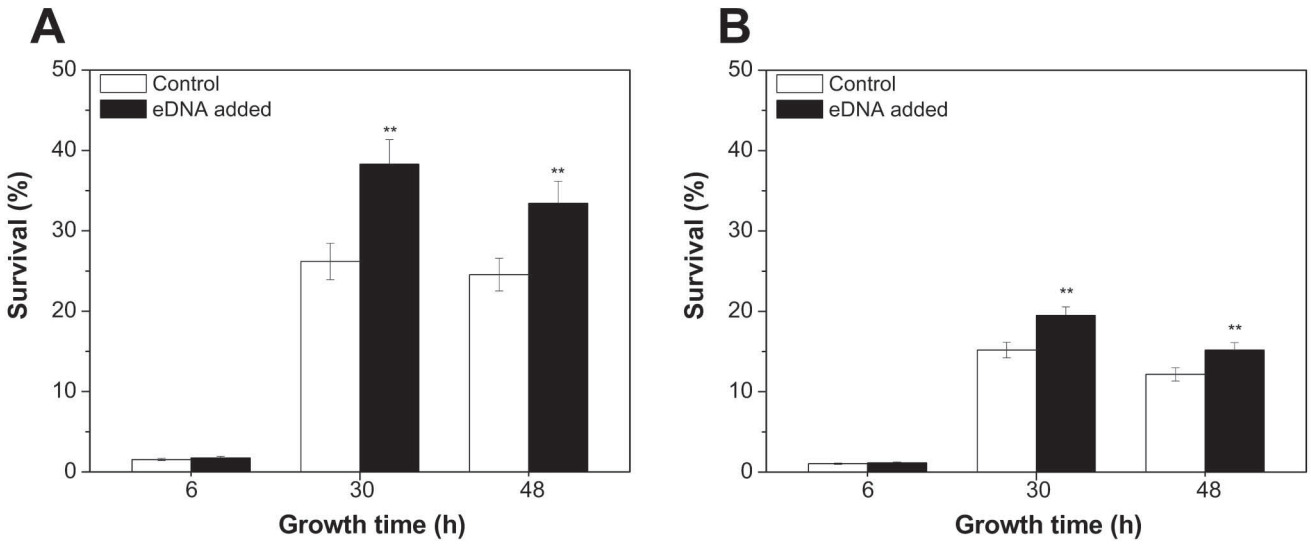

Fig. 3. Effects of eDNA on the resistance ability of YS19 to $CuSO_4$ and acid.

Bacterial cells were cultivated in LB liquid medium without (white), or with 1.0 μg mL^{-1} eDNA (black), added. Cells sampled at three typical time points (6, 30, 48 h) were used for chemical treatment with $CuSO_4$ (A) and acid (B). Error bars represent standard deviations (n = 3), $^{**}P < 0.01$ compared to control group.

and E). These results indicate that the eDNA gathers around the symplasmata of YS19 and demonstrated a significant role regarding the stability of the aggregates.

Assay of bacterial growth and symplasmata formation

The eDNA production and its influence on the bacterial growth curve were measured. At the stationary phase (30 h of cultivation) in LB medium, YS19 approximately produced 5.3 μg mL^{-1} endogenous eDNA. The cell growth curves at 0.5–1.5 μg mL^{-1} of exogenous eDNA were almost close to that of the control, suggesting that proper concentrations of exogenous eDNA has no obvious effect on the bacterial growth (Fig. 2B). It was also found that the symplasmata formation in YS19 was initiated at the exponential growth stage (approximately 6 h), and reached the maximum in the later of the stationary stage (approximately 36 h), and them decomposed gradually in the decline phase, regardless whether eDNA was added or not (Figs. 2C and D). Meanwhile, it can be seen from Figs. 2C and D, that symplasmata formation (ratio and size) was significantly increased by the exogenous eDNA, and that the higher the concentration of eDNA added, the greater the promoting effect observed. As an example, at 36 h in the groups supplemented with 0.5, 1.0 and 1.5 μg mL^{-1} of eDNA, the symplasmata formation ratio increased by 8.8, 15.1, and 15.2% ($P < 0.05$), respectively, meanwhile the symplasmata average sizes increased by 42.4, 59.6, and 59.9% ($P < 0.01$), respectively, compared to the control (formation ratio 73.2%, average size 2.7 μm). As we can see from Figs. 2C and D, the promoting effect at concentrations higher than 1.0 μg mL^{-1} was not more obvious, and, therefore, this concentration was selected for the following biological effects detection.

Assay of stress resistance

YS19 cells cultivated in LB liquid medium were harvested at three time points (6, 30, 48 h), and collected for treatment by $CuSO_4$, or acid. After 4 h of treatment, the survivals were detected and compared (Fig. 3). For the cells harvested at 30 h and 48 h, the survival rate in eDNA supplementary groups increased significantly, i.e., by 13.6, 46.2 and 36.1% for $CuSO_4$ treated group, or 11.4, 28.3 and 24.9% for the acid treated group, compared that of the control, respectively.

Assay of DNase I treatment

DNase I treatment gives the reverse positive evidence of the roles of eDNA on symplasmata formation and stability. For the cultures sampled at 6 h, 30 h and 48 h, the symplasmata formation ratios in the groups treated with DNase I decreased by 76.8, 9.3, and 16.8%, respectively, meanwhile the symplasmata average sizes decreased by 7.8, 51.7, and 60.6%, compared with the control, respectively (Figs. 4A and B), indicating that the symplasmata were degraded partially. At the three time points, the resistance ability of the cells to $CuSO_4$, or acid, was also decreased in the DNase I treated groups compared with that of the control (Figs. 4C and D). For the cells harvested at 6 h, 30 h and 48 h, the survival rates in DNase I supplemented groups decreased, i.e., by 8.1, 50.6 and 60.2% for $CuSO_4$ treated group or 4.9, 32.3 and 39.4% for the acid treated group, compared with that of the control, respectively. These results further explained that eDNA plays an important role on the symplasmata formation and stability.

Assay of colonization

In rice tissues, the cells of YS19 are almost all aggregated into symplasmata. This structure is very beneficial to the adaptation of the bacterium in endophyte-host association (Kaplan et al., 2011; Zhang et al., 2010). Considering the fact that exogenous eDNA promotes the formation of symplasmata, we explored the impacts of exogenous eDNA on the colonization ability of YS19 on the

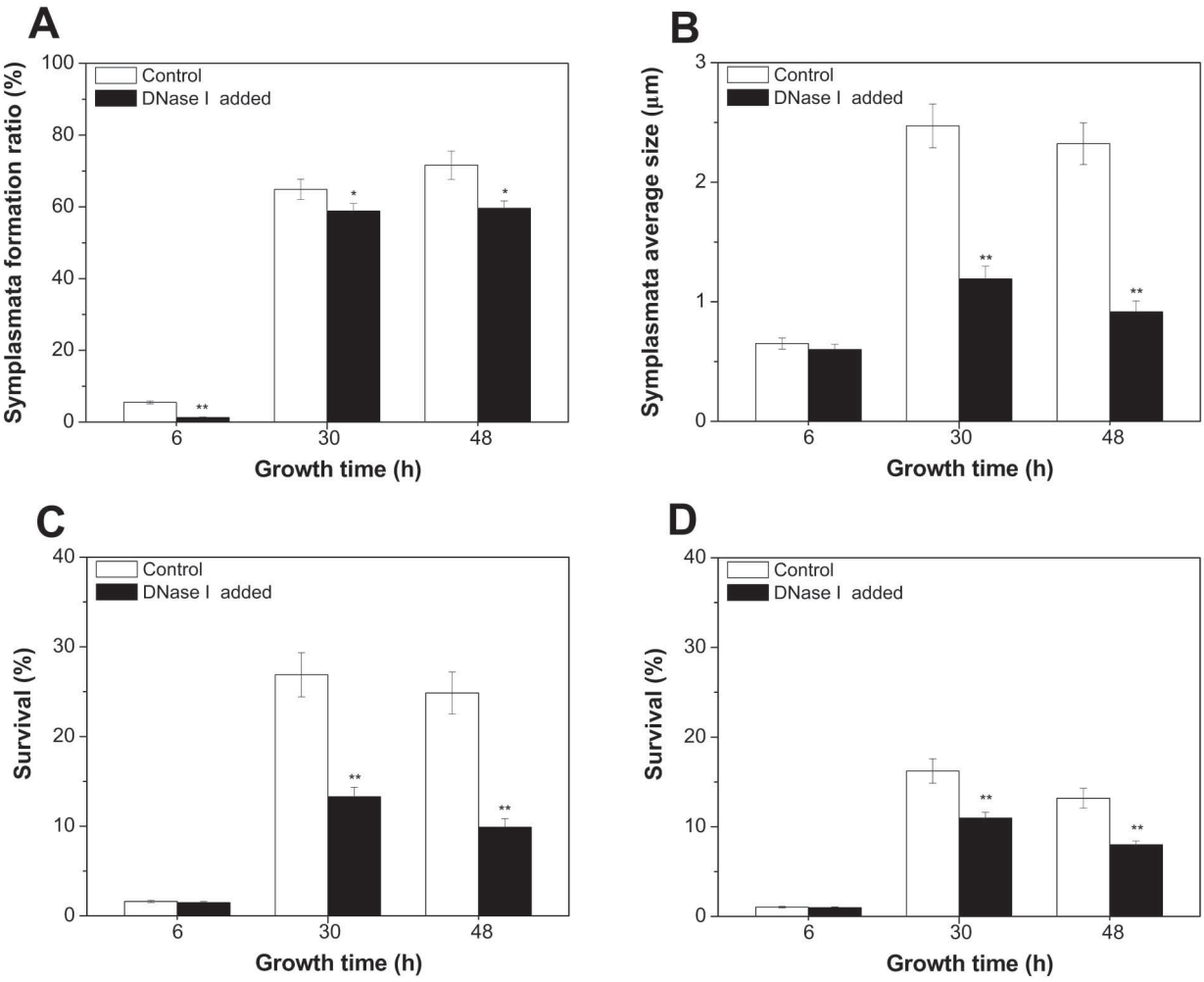

Fig. 4. Effect of DNase I on the symplasmata stability of YS19.

The cultures harvested at specific time points after inoculation (6, 30 and 48 h) were treated with DNase I (black). For the control groups (white), bacterial cells were also treated with heat-inactivated DNase I. Then the samples were used for the assay of symplasmata formation ratio (A), size (B), and resistance ability to $CuSO_4$ (C) and acid (D). Error bars represent standard deviations (n = 3), $^{**}P < 0.01$, $^{*}P < 0.05$ compared to the control groups.

Table 1. The enhancement of eDNA on colonization number of YS19 on gnotobiotically cultivated rice seedlings on EPA medium.

Treatment of seeds*	Whole plant (CFU mg^{-1})**	Roots (CFU mg^{-1})**	Shoots (CFU mg^{-1})**
Rice + YS19	1.1×10^4 A	1.4×10^4 A	1.3×10^4 A
Rice + YS19 + eDNA	1.9×10^4 B	2.5×10^4 B	2.3×10^4 B

*The seedlings (20 per group) were cultivated for 10 days as described under Materials and methods.
**Different letters in the column of colonization number represent significant differences among treatments according to t-test ($P < 0.05$).

host rice plant, and found that eDNA increased the colonization ability of this endophyte. As shown in Table 1, the colonization number of YS19 in the whole plant was 1.2×10^4 CFU mg^{-1}, which was improved by 76.9% after the addition of the eDNA (1.0 μg mL^{-1}). The colonization number in both the roots and shoots is increased.

Discussion

P. agglomerans is reported as being widespread in the environment and is most frequently associated with soil, air, water, plants, animal, and occasionally humans (Feng et al., 2010). Remaining in planktonic states or forming multicellular aggregates are two main life styles of the bacterium in niches (Achouak et al., 1994). As one of the most important multicellular aggregates, symplasmata formed by *P. agglomerans* are the significant structures for bacteria to adapt survival and association with the host. Here, *P. agglomerans* YS19 was tested to have the ability to produce eDNA, and the eDNA mainly exists in

symplasmata.

DNA molecules have a wide range of sources, which are released by normal apoptotic cells or abnormal death cells, or even by the normal living cells (Das et al., 2013). On a primary sequence, eDNA cannot distinguish from genome DNA (Regev-Yochay et al., 2006; Zheng et al., 2011). Therefore, in studying the role of eDNA in biofilm, adding genomic DNA was often used to simulate exogenous eDNA (Harmsen et al., 2010; Lappann et al., 2010; Nur et al., 2013), which was tested to promote the development of biofilm (Lappann et al., 2010). In this study, we also chose adding genomic DNA as exogenous eDNA.

Endophytic bacteria living in the natural environment or colonizing inside the host are often restricted by stresses of heavy metals, pH changes, temperature changes, starvation, high osmotic pressure, etc. (Ryan et al., 2008). The stresses from heavy metals or pH changes are often faced by endophytes (Zheng et al., 2017). In our previous work, the symplasmata of YS19 have been tested to enhance the stress-resistant ability to these chemicals ($CuSO_4$ and acid) in comparison with the planktonic cells (Yu et al., 2016). So, $CuSO_4$ and acid are selected for the stress-resistant experiments. Symplasmata which have a tighter cell-cell binding than other bacterial aggregates, such as biofilms, are considered to be a great asset for bacteria to survive from fluctuant environments (Li et al., 2012). This work found that eDNA significantly enhanced the resistance ability to the two adversities. Remarkably, the effect of eDNA on the stress resistance closely paralleled its effect on the formation of symplasmata. Therefore, eDNA might increase the formation of symplasmata in YS19 and finally help the bacterium to cope with the stress. It is reported that in biofilm formation eDNA played an important role (Das et al., 2010; Kaplan et al., 2011), and is essential for biofilm structure and stability (Qin et al., 2007). DNase I treatment experiment provided the strongest evidence that eDNA plays an important role in symplasmata stability, and, furthermore, helps the bacterium to cope with the stresses. These results indicate the novel roles of eDNA in symplasmata, implying a potential relation between stress-resistance regulation and eDNA production.

Inside a plant, symplasmata, which, comprises lots of tightly binded individual cells, have a lot of advantages, such as coordinating cells in metabolism, growth, and propagation (Jiang et al., 2015), promoting the process of occupying all the microcosmic niches (Duan et al., 2007; Li et al., 2017; Morris and Monier, 2003; Zhang et al., 2010). Considering the results, the fact that eDNA increases symplasmata formation and stability, eDNA may enhance the colonization of YS19 on host rice. Our eDNA supplementary experiment in the endophyte-host plant association system confirmed this inference.

This study has clearly revealed the novel promotion roles of eDNA in symplasmata formation and stability, and also in endophytic colonization, which shows its special relation to the endophyte-host association of the strain. Further exploration on the regulation mechanism of eDNA involved in symplasmata formation and colonization of YS19 might shed light on research into bacterial endophytic symbiosis living.

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