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Nitrogen Sources Inhibit Biofilm Formation by *Xanthomonas oryzae* pv. *oryzae* [S]

Youngseok Ham and Tae-Jong Kim*

Department of Forest Products and Biotechnology, College of Science and Technology, Kookmin University, Seoul 02707, Republic of Korea

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial blight, which results in severe economic damage to rice farms. *Xoo* produces biofilms for pathogenesis and survival both inside and outside the host. Biofilms, which are important virulence factors, play a key role in causing the symptoms of *Xoo* infection. In the present study, we investigated the nutritional conditions for biofilm formation by *Xoo*. Although *Xoo* biofilm formation may be initiated by interactions with the host, *Xoo* biofilm cannot mature without the support of favorable nutritional conditions. Nitrogen sources inhibited *Xoo* biofilm formation by overwhelming the positive effect that cell growth has on it. However, limited nutrients with low amino acid concentration supported biofilm formation by *Xoo* in the xylem sap rather than in the phloem sap of rice.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, biofilm, nitrogen source, rice, xylem

Introduction

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causative agent of bacterial blight in rice [1], which is the second most cultivated crop worldwide. Bacterial blight may cause losses of up to 32% of rice yields, depending on the rice cultivar and environmental conditions [2]. The natural habitat of *Xoo* has not yet been completely defined, but several potential habitats have been suggested [3, 4]. The two primary potential habitats of *Xoo* are seeds and the natural environment. It has been previously demonstrated that rice seeds may carry live *Xoo* cells [5]; however, whether *Xoo* carried by seeds causes bacterial blight is a subject of debate [6, 7]. In the natural environment, *Xoo* survival depends upon conditions surrounding the cells [8, 9]. Biofilm formation by *Xoo* is pivotal not only for the survival of the bacteria but also for its persistence in the environment.

A biofilm provides a protective niche for bacteria [10, 11]. Surrounded by a biofilm, bacteria change their gene expression and eventually become more resistant to hostile environmental conditions [12, 13]. *Xoo* is a good biofilm producer [14]; in addition to increased survivability, biofilm formation has been suggested to be involved in *Xoo* pathogenesis [15, 16]. *Xoo* enters rice by withdrawing guttation fluid [17, 18] or by traveling through wounds or sheath openings [18]. After reaching the xylem, *Xoo* propagates [19], activates rice defense systems [20], thickens the secondary wall [21], and increases cationic peroxidase levels [22]. Eventually, the aggregation of cells and their exopolysaccharides form a biofilm that blocks the rice vessels [23, 24]. In rice, *Xoo* preferentially forms a biofilm in the xylem rather than in the phloem. In order to understand *Xoo* pathogenesis and its biofilm formation in rice, it is essential to identify the nutritional conditions that promote biofilm formation by *Xoo*.

Materials and Methods

Bacterial Strain and Growth Conditions

X. oryzae pv. *oryzae* (KACC 10331) was used in the present study. The *Xoo* genome has been completely sequenced [25], making this strain a good model for further molecular biology studies. Minimal medium [26], 210 medium [27], nutrient broth (BD, Franklin Lakes, NJ), PS medium [28], SOC medium [29],

XOM2 medium [30], and MME medium [31] were prepared, according to previous studies or the manufacturer's instructions. The detailed compositions of media were listed in Tables S1 to S7. The amino acid compositions of xylem [32] and phloem [33] saps in XOM2 medium were made according to previous studies. The detailed compositions of media were listed in Tables S8 and S9, respectively.

Measuring Cell Numbers

For cell number analysis, *Xoo* was subcultured to an absorbance of 0.05 at 600 nm (Abs_{600}) in 100 ml of 210 medium in 250-ml baffled flasks and incubated at 28°C and 250 ×g for 48 h. The growth of *Xoo* was measured as Abs_{600}, and the colony-forming units (CFUs) were calculated using a conversion factor of 1.12×10^9 CFU/Abs_{600}.

Biofilm Formation and Quantitative Analysis Using Crystal Violet

Xoo stored as frozen stock at −80°C was grown on YGC agar plates (50 g/l glucose, 5 g/l yeast extract, 12.5 g/l $CaCO_3$, and 15 g/l agar) at 28°C for 48 h. Five milliliters of 210 medium was inoculated with *Xoo* and incubated at 28°C and 250 ×g for 48 h. For the biofilm assays, *Xoo* was subcultured to an Abs_{600} of 0.05 in 100 µl of each medium indicated above in 96-well polyvinyl chloride (PVC) microplates. Cultures were incubated at 28°C for 24 h. Biofilms were quantified via the crystal violet assay, as previously described [34]. After cell culture in 96-well PVC microplates, excess cells were removed and the wells were rinsed three times with double-distilled water. Each well was incubated with 100 µl of 1% crystal violet for 15 min at room temperature. The wells were then rinsed three times with double-distilled water, and 100 µl of 95% ethanol was then added to each well to solubilize the crystal violet. The plates were then incubated for an additional 15 min. An ELx800 microplate reader equipped with KC4 software (Bio-Tek Instruments, Inc., Winooski, VT) was used to measure the absorbance of each well at 595 nm. Uninoculated wells were used as blanks and were subtracted from each value to calculate the final absorbance value at 595 nm.

Results

Effects of Nutrient Availability on Biofilm Formation

Biofilm formation by *Xoo* was evaluated in seven reported media (Fig. 1A). Biofilm formation in complex nutrient-rich media, such as 210 medium, nutrient broth, PS medium, and SOC medium, was minimal; however, biofilm formation in the defined nutrient-limited media, such as XOM2 medium, minimal medium, and MME medium, was considerable. To verify the limitation of bacterial growth in the defined nutrient-limited media, the cell growth in seven reported media was evaluated (Fig. 1B). After 10 h of incubation, the cell density of the nutrient-rich medium was more than twice that in the nutrient-limited
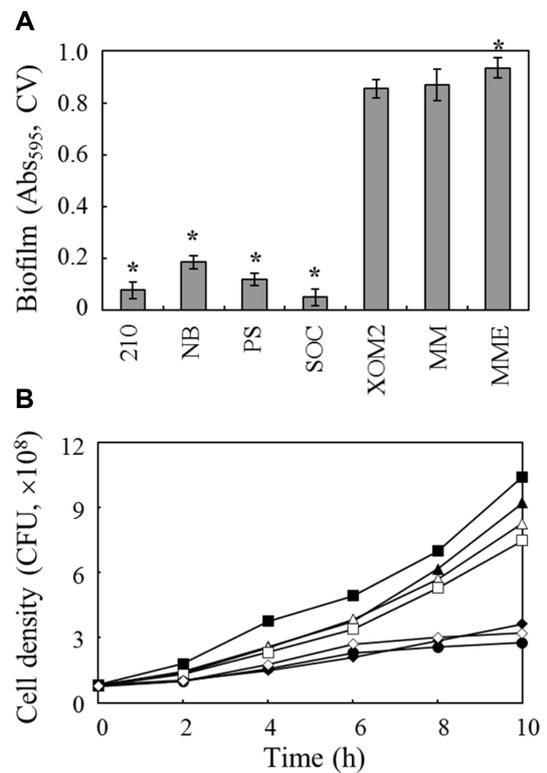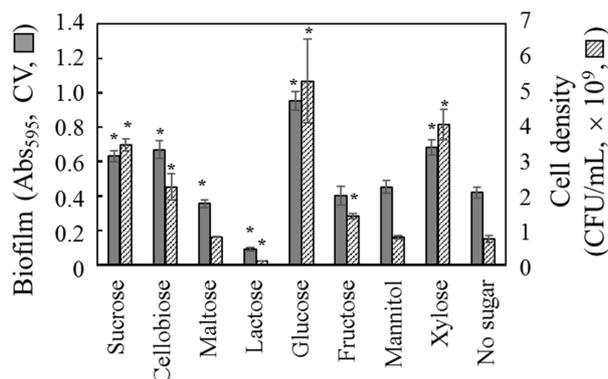

Fig. 1. Effect of media on *Xoo* biofilm formation and cell growth.

(**A**) *Xoo* biofilm formation. Cells were incubated in 96-well polyvinyl chloride microplates for 24 h. Biofilms were quantified using crystal violet (CV in the y-axis label). The media used were 210 medium (210), nutrient broth (NB), PS medium (PS), SOC medium (SOC), XOM2 medium (XOM2), minimal medium (MM), and MME medium (MME). The mean values showing a statistically significant difference in comparison with the value of XOM2 were denoted by * ($p < 0.05$). (**B**) *Xoo* cell growth. The colony-forming units were quantified using a conversion factor of 1.12×10^9 CFU/Abs_{600}. The media used were 210 medium (■), nutrient broth (□), PS medium (▲), SOC medium (△), XOM2 medium (◆), minimal media (◇), and MME medium (●). Data represent mean ± standard deviation from 6 independent experiments.

medium containing defined nutrients. These results, shown in Figs. 1A and 1B, suggested that growth-limiting conditions supported biofilm formation by *Xoo*.

Effect of Carbon Source on Biofilm Formation

In order to determine the nutrient component in the complex media that inhibited biofilm formation by *Xoo*, eight carbon sources, including those used in the tested complex media listed in Fig. 1, were tested (Fig. 2). The xylose in the XOM2 medium was substituted with the
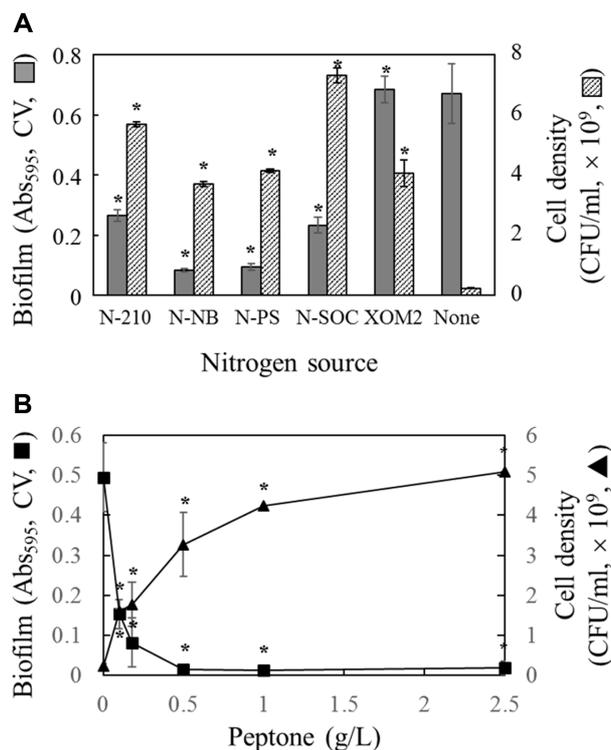
Fig. 2. Effect of carbon sources on *Xoo* biofilm formation (solid bars) and cell growth (hatched bars).

Cells were incubated in 96-well polyvinyl chloride microplates for 24 h with XOM2 medium in which xylose was replaced with 0.18% of the indicated carbon sources. The cell growth was measured independently by culturing cells in test tubes (hatched bars). The number of colony-forming units (CFU) was calculated using a conversion factor of 1.12×10^9 CFU/Abs_{600}. No sugar: no added carbon source. Data represent mean ± standard deviation from at least 10 independent experiments for biofilm and from at least 4 independent experiments for cell density. The mean values showing a statistically significant difference in comparison with the control value of 'No sugar' were denoted by * ($p < 0.05$).

tested carbon sources at the same concentration. Sucrose, cellobiose, and glucose supported cell growth, generating a cell density of more than 2×10^9 CFU/ml, and biofilm formation under these conditions was comparable to the original XOM2 medium containing xylose. In contrast, maltose, fructose, and mannitol did not support cell growth, and the biofilm formation was similar to that in XOM2 medium with no carbon source. Interestingly, lactose significantly inhibited both cell growth and biofilm formation. Among the carbon sources tested, glucose elicited maximum cell density and biofilm formation. This result showed that biofilm formation was positively correlated with cell growth as a function of the available carbon source. Therefore, this observation, presented in Fig. 2, contradicted the inverse correlation between the cell density and biofilm formation shown in Fig. 1.

Effect of Nitrogen Source on Biofilm Formation

Since the carbon source test did not show an inverse correlation between cell growth and biofilm formation, as presented in Fig. 1, the nitrogen sources were subsequently tested (Fig. 3). In the XOM2 medium, L-methionine (670 μM) and sodium L-(+)-glutamate monohydrate (10 mM) were replaced with the nitrogen sources of the four nutrient-rich

Fig. 3. Effect of nitrogen sources on *Xoo* biofilm formation and cell growth.

(**A**) Effect of nitrogen source on *Xoo* biofilm formation (solid bars) and cell growth (hatched bars). Cells were incubated in 96-well polyvinyl chloride microplates for 24 h with XOM2 medium in which L-methionine and sodium L-(+)-glutamate monohydrate were replaced with the indicated nitrogen sources. N-210: 8 g/l casein hydrolysate and 4 g/l yeast extract, N-NB: 3 g/l beef extract and 5 g/l peptone, N-PS: 10 g/l bacto-peptone, N-SOC: 20 g/l tryptone and 5 g/l yeast extract, XOM2: XOM2 medium alone, None: XOM medium without nitrogen sources. The cell growth was measured independently by culturing cells in test tubes (hatched bars). After measuring Abs_{600}, the number of colony-forming units (CFU) was calculated using a conversion factor of 1.12×10^9 CFU/Abs_{600}. The mean values showing a statistically significant difference in comparison with the control value of 'None' were denoted by * ($p < 0.05$). (**B**) Effect of peptone on *Xoo* biofilm formation (■) and cell growth (▲). L-methionine and sodium L-(+)-glutamate monohydrate in XOM2 medium were replaced with peptone at the same concentration. Data represent mean ± standard deviation from at least 5 independent experiments for biofilm and from 2 independent experiments for cell density. The mean values showing a statistically significant difference in comparison with the control value at 0 g/l were denoted by * ($p < 0.05$).

media tested. The tested nitrogen sources were 8.0 g/l casein hydrolysate and 4.0 g/l yeast extract from 210 medium (N-210 in Fig. 3A), 3.0 g/l beef extract and 5.0 g/l

peptone from nutrient broth (N-NB in Fig. 3A), 10.0 g/l peptone and 1.0 g/l monosodium glutamate from PS medium (N-PS in Fig. 3A), and 20.0 g/l tryptone and 5.0 g/l yeast extract from SOC medium (N-SOC in Fig. 3A). None of the XOM2 media with substituted nitrogen sources tested supported biofilm formation, but the cell growth remained at a similar or higher level when compared to the original XOM2 media (Fig. 3A). These results are consistent with the inverse correlation between cell growth and biofilm formation shown in Fig. 1.

Decreased biofilm formation was observed with nitrogen sources from nutrient broth and PS medium, and their common nitrogen source was peptone. Thus, to confirm the inverse correlation between biofilm formation and the available nitrogen sources, the concentration-dependent inhibition of biofilm formation was evaluated using peptone (Fig. 3B). Cells grew better as the peptone concentration increased, and less biofilm was formed. This result confirmed that biofilm formation was inhibited by the nitrogen source; both carbon and nitrogen sources supported the cell growth, but only nitrogen sources inhibited biofilm formation by *Xoo*.

Effect of Amino Acids and Ammonium Nitrate on Biofilm Formation

The nitrogen sources tested in Fig. 3 were made primarily with amino acids. Hence, the effect of each amino acid on *Xoo* biofilm formation was evaluated (Fig. 4). Of the 19 amino acids tested at 50 mM, 10 amino acids reduced biofilm formation by over 40% (Fig. 4A). At 100 mM, 12 of the 18 amino acids tested inhibited biofilm formation by over 68% (Fig. 4B) (100 mM tyrosine and tryptophan were not tested because of their poor solubility). Only five amino acids (arginine, aspartic acid, glutamic acid, lysine, and proline) supported cell growth at 100 mM (Fig. S2). These results showed that single amino acids were sufficient to inhibit *Xoo* biofilm formation.

The effect of ammonium nitrate on *Xoo* biofilm was also evaluated (Fig. 5). Like most amino acids, ammonium nitrate inhibited both biofilm formation and cell growth.

Biofilm Formation in Rice Sap Condition

In rice, *Xoo* forms a biofilm in the xylem rather than the phloem [23, 24]. We examined whether the preferential formation of *Xoo* biofilms in the xylem may be determined by the amino acids in rice (Fig. 6). To simulate the amino acid composition of xylem [32] and phloem [33] saps, L-methionine (670 μM) and sodium L-(+)-glutamate monohydrate (10 mM) were substituted in the XOM2
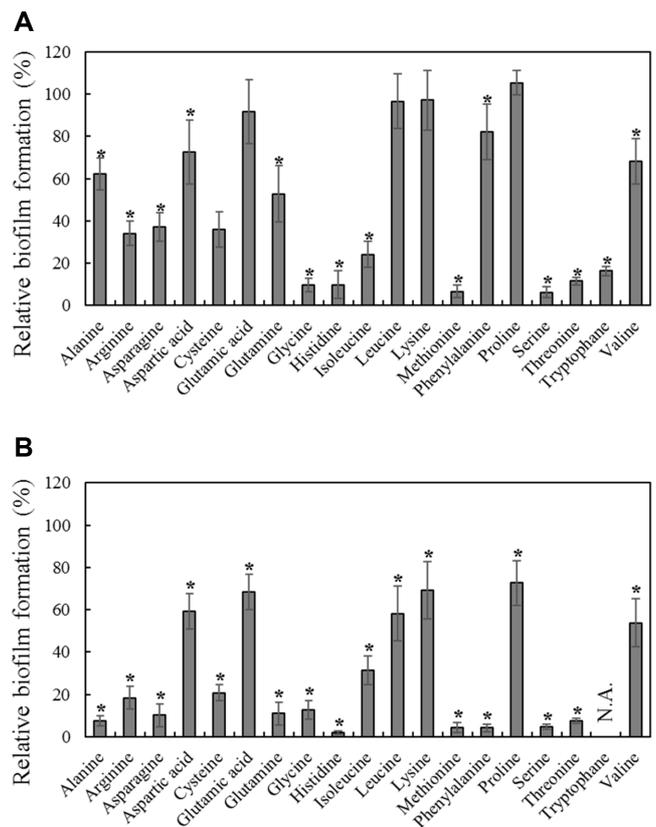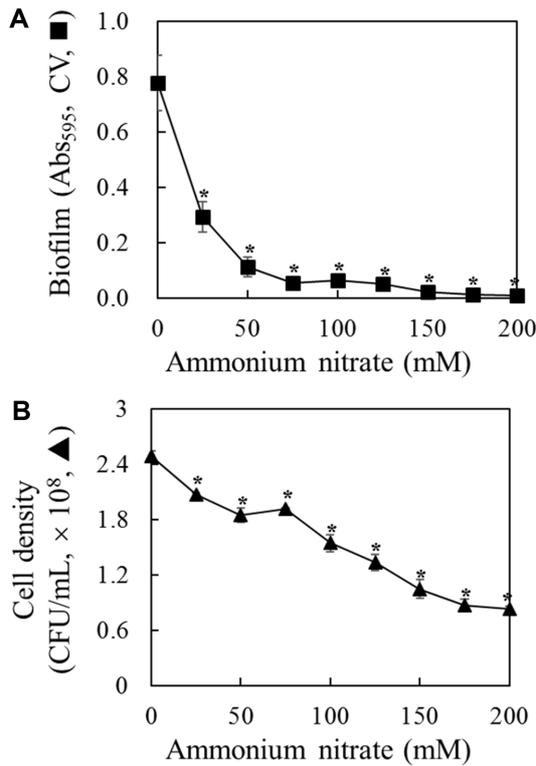

Fig. 4. Effect of amino acids on *Xoo* biofilm formation.
Cells were incubated in 96-well polyvinyl chloride microplates for 24 h with XOM2 medium in which L-methionine and sodium L-(+)-glutamate monohydrate were replaced with the indicated amino acid. Amino acids at 50 mM (**A**) and 100 mM (**B**) were tested. Biofilms were quantified using crystal violet (CV in the y-axis label). Data represent mean ± standard deviation from at least 5 independent experiments, except for proline, which was used in 3 independent experiments. The mean values showing a statistically significant difference in comparison with the control value without amino acid were denoted by * ($p < 0.05$).

medium. The amino acid composition of xylem sap (A.A. in xylem in Fig. 6) supported increased biofilm formation compared with the XOM2 medium (Fig. 6A), but reduced cell growth (Fig. 6B). However, the amino acid composition of phloem sap (A.A. in phloem in Fig. 6) showed the opposite effects, inhibiting biofilm formation (Fig. 6A) and supporting cell growth comparable to that in the XOM2 medium (Fig. 6B).
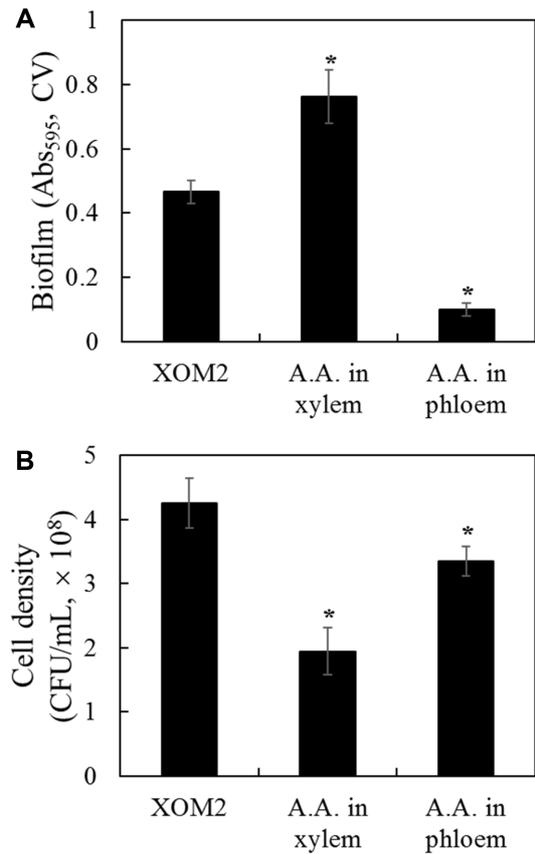
Discussion

Increased bacteria cell growth usually supports increased biofilm formation. However, biofilm formation by *Xoo*

Fig. 5. Effect of ammonium nitrate on *Xoo* biofilm formation (**A**) and cell growth (**B**).

Cells were incubated in 96-well polyvinyl chloride microplates for 24 h with XOM2 medium in which L-methionine and sodium L-(+)-glutamate monohydrate were replaced with ammonium nitrate. Biofilms were quantified using crystal violet (CV in the y-axis label; ■). Cell growth was measured independently by culturing cells in test tubes. The number of colony-forming units (CFU) was calculated using a conversion factor of 1.12×10^9 CFU/Abs$_{600}$ (▲). Data represent mean ± standard deviation from 10 independent experiments for biofilm and from 2 independent experiments for cell density. The mean values showing a statistically significant difference in comparison with the control value at 0 mM were denoted by * ($p < 0.05$).

Fig. 6. Effect of the amino acid composition of xylem and phloem saps on *Xoo* biofilm formation (**A**) and cell growth (**B**).

The amino acid mixtures, which were prepared according to previous studies on xylem sap (shown as A.A. in xylem) and phloem sap (shown as A.A. in phloem), substituted the equivalent amino acids in the XOM2 medium. Data represent mean ± standard deviation from at least 6 independent experiments. The mean values showing a statistically significant difference in comparison with the control value of XOM2 medium were denoted by * ($p < 0.05$).

increased in minimal defined media more so than in complex media, which favored cell growth. This inverse correlation between biofilm formation and cell growth suggested the existence of strong negative effectors of biofilm formation among the nutrient components in the complex media. The presence of a nitrogen source inhibited biofilm formation (Fig. 3) unlike the presence of carbon sources (Fig. 2). These results showed that the inhibitory effect of nitrogen sources on biofilm formation was enhanced by increasing concentration, regardless of cell growth. Additional experiments in the present study showed that environmental conditions, such as pH, temperature, and

salt, do not contribute to the inverse correlation between cell growth and biofilm formation (Fig. S3).

Amino acids inhibit biofilm formation in several bacteria. Previous studies have suggested that arginine inhibits biofilm formation by *Streptococcus mutans* [20, 35], *S. gordonii* [36], and oral bacterial communities [37, 38]. Other studies have suggested that glycine inhibits the biofilm formation of bacterial communities [39], and methionine and tryptophan inhibit biofilm formation by *Pseudomonas aeruginosa* [40, 41]. In addition, nitrite, which is an inorganic nitrogen source, has been shown to inhibit biofilm formation by *Staphylococcus aureus* and *S. epidermidis* [42].

Xoo produced a biofilm and blocked the plant vascular system [23, 24], which is one of the pathogenic mechanisms of *Xoo* [43]. The majority of the *Xoo* biofilm was detected in the xylem of rice leaves [23, 24]. The xylem sap of rice provides favorable conditions for biofilm formation by *Xoo*, with a concentration of 1 mM or less of each amino acid [32]. The limited cell growth of *Xoo* in xylem vessels [19] suggests that xylem sap is a nutrient-limited medium for *Xoo*. However, the phloem sap of rice is not an ideal medium for biofilm formation by *Xoo* because it contains 3–8% amino acids [33]. Our experimental results, presented in Fig. 6, showed that amino acids may be an important factor influencing biofilm formation by *Xoo* in xylem rather than phloem.

Exopolysaccharide is an important determinant of biofilm formation [44], and the exopolysaccharide of *Xoo* is an important virulence factor [45, 46]. When *Xoo* was treated with thyme oil, its exopolysaccharide production, biofilm formation, and virulence were simultaneously reduced [47]. We observed that XOM2 medium supported the production of exopolysaccharide, but PS medium did not (Fig. S4). This result confirmed the strong relationship between exopolysaccharide production and biofilm formation. Moreover, it suggests that the xylem sap of rice may promote the formation of *Xoo* exopolysaccharide, while the phloem sap may not. This inference may also provide an explanation of why *Xoo* selectively forms biofilms in xylem vessels. The results of the present study predict biofilm formation by *Xoo* in xylem sap as a result of nutritional conditions, excluding interaction with the host.

Among the cultivars of rice, some are resistant to *Xoo* pathogenesis, while others are susceptible. Resistance in rice cultivars was associated with the flow in the xylem vessel after infection, while *Xoo* formed a biofilm mass in the xylem vessel of the susceptible cultivars after infection [23]. Based on the results of the present study, differences between the nutritional compositions of the xylem sap of rice cultivars resistant to *Xoo* pathogenesis and those of rice cultivars susceptible to *Xoo* will confirm the importance of nutritional composition in the xylem sap for bacterial blight.

Rice paddies also provide an ideal environment for biofilm formation by *Xoo* because they provide limited nutrients [48]. This observation supports the hypothesis that the rice paddy and other natural environments are the origin of *Xoo* infection.

Based on the conditions that promote the formation of *Xoo* biofilms, the results of the present study suggest that the composition of the xylem sap and the natural habitat of rice are suitable for biofilm formation by this pathogen.

This suggestion is consistent with the location of *X. oryzae* found in previous studies [19].

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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