ORIGINAL ARTICLE

Antimicrobial effect of *Bacillus licheniformis* HN-5 bacitracin A on rice pathogen *Pantoea ananatis*

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Abstract *Pantoea ananatis* is a plant pathogenic bacterium that severely impacts rice. In spite of its worldwide prevalence, limited studies have been conducted so far on the control of *P. ananatis*. Bacitracin A is a non-ribosomal peptide antibiotic with strong antibacterial activity produced by *Bacillus licheniformis* strain HN-5. We investigated the mechanisms of action underlying the biocontrol and bactericidal efficacy of bacitracin A against *P. ananatis*. Fluorescence microscopy and bacterial cell viability analyses revealed that the median effective concentration of bacitracin A against *P. ananatis* was 9.10 μg ml^{-1}. Scanning and transmission electron microscopy showed that bacitracin A damaged the cell wall and membrane of *P. ananatis*. Quantitative real-time PCR indicated that the transcriptional expression of *ftsZ*, *glmS*, and *gumD*, which are involved in cell division, cell-wall biosynthesis, and extracellular polymeric substance biosynthesis, respectively, was upregulated at 12 h and significantly downregulated at 24 h after bacitracin A treatment in *P. ananatis*. Bacitracin A caused cell leakage and changes to membrane permeability in *P. ananatis*, supporting its use as a natural biocontrol agent for *P. ananatis*.

Introduction

Rice (*Oryza sativa*) is a staple in the diet of more than three billion individuals worldwide, sustaining the nutritional needs of an ever-expanding global population (Skamnioti et al. 2009). *Pantoea ananatis* is a diverse species that occupies various unusual ecological niches and causes diseases such as sheath rot, grain discoloration, stem necrosis, and leaf blight in rice (Cother et al. 2004; Choi et al. 2012; Shyntum et al. 2015). *P. ananatis* affects cultivated and wild crops and trees, including rice, corn, onions, honeydew melons, and pineapples, and can cause bacteremia in humans (Coutinho et al. 2009; Kido et al. 2010; Maayer et al. 2014; Shyntum et al. 2015). In 2004, *P. ananatis* was classified as a rice pathogen in Australia (Cother et al. 2004). South Korea issued the same decree in 2010 (Choi et al. 2012). Furthermore, in 2008, a new variant of leaf blight in rice caused by *P. ananatis* was reported in India (Mondal et al. 2011). Current methods of controlling *P. ananatis* primarily involve the application of copper-based pesticides. However, the accumulation of such chemical

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pesticides in crops results in long-term environmental damage and the development of pesticide-resistant pathogens, thus limiting their applications (Zeriouh et al. 2011; Silva et al. 2012). Therefore, a cost-effective, convenient, and environment-friendly method of *P. ananatis* eradication is urgently needed. Biological control agents are increasing in popularity owing to their eco-friendliness, versatility, and potential to successfully control disease (Fravel 2005).

The intrinsic attributes of certain microorganisms can be manipulated and used to manage plant diseases (Zeriouh et al. 2011). *Bacillus* spp. are gram-positive rod-shaped endospore-forming organisms, which may be obligate aerobes or facultative anaerobes. In the traditional taxonomy system, *B. subtilis* encompasses multiple closely related species such as *B. licheniformis*, *B. subtilis*, and *B. amyloliquefaciens* (Wang et al. 2007; Borshchevskaya et al. 2013). Biopeptides primarily interact with fungal plasma membrane components (Magnet-Dana et al. 1994; Straus et al. 2006; Romero et al. 2007; Jin et al. 2020). Bacitracin is an important non-ribosomal peptide antibiotic produced by certain strains of *B. subtilis* and *B. licheniformis*. It is a potent antibiotic against gram-positive and certain gram-negative bacteria. The bacitracin synthetase gene cluster in *B. licheniformis* is composed of the *bacABC* operon and *bacT*, which encode non-ribosomal peptide synthetase and thioesterase, respectively (Konz et al. 1997; Wang et al. 2017). *B. licheniformis* is used for the industrial production of bacitracin, which is widely used as an additive in livestock feed (Schallmey et al. 2004). The mechanisms underlying the effects of bacitracin in reducing infectious diseases in livestock have been previously investigated (Hancock 2005). However, whether this antimicrobial efficacy of bacitracin A in humans and livestock translates to its potential for the biocontrol of plant diseases has not yet been elucidated. Thus, we conducted this study to evaluate the potential of bacitracin A as a biocontrol agent for *P. ananatis* and determine the mechanisms underlying its antibacterial activity.

Material and methods

Microorganisms and plants

Pantoea ananatis strain DZ-12 (stored at China General Microbiological Culture Collection Center,

ID CGMCC 1.13877) was obtained from professor Gao (Nan Jing Agriculture University, China) and cultured in nutrient broth (NB) medium. *B. licheniformis* strain HN-5 (Genebank ID No. MK648261.1) was isolated from soil collected from Hainan island on April 18, 2015, and stored at Hainan University, China until use. It was then cultured in Luria-broth (LB) medium. All microorganism strains were stored at −80 °C in an appropriate medium with 40% glycerol. Rice (cultivar IR24) which is grown to the tillering stage, was used in this study. The rice was grown in autoclaved soil in 1 l plastic pots (nine plants per pot) under glasshouse conditions of temperature 26 ± 1 °C, RH $70 \pm 5\%$, and ambient light (L:D 12:12).

Isolation and identification of bacitracin A

Bacillus licheniformis strain HN-5 was cultured in LB medium at 28 °C for 48 h with 180 rpm shaking. The culture (20 l) was reduced in vacuo to approximately 1 l at 60 °C. The metabolites in the concentrated medium were extracted thrice (3×1 l) using *n*-butyl alcohol at 28 °C. The extracts were combined, dried, and concentrated in vacuo. The obtained *n*-butyl alcohol extract (3.84 g) was separated into ten fractions on a silica gel column (200–300 mesh) using sequential gradient elution, first with 100% chloroform ($CHCl_3$), followed by a mixture of $CHCl_3$/methanol (MeOH) (100:1, 50:1, 25:1, 15:1, 10:1, 5:1, 2:1, 1:1 v/v), and finally 100% MeOH. The resulting fractions were combined according to their thin-layer chromatographic profiles on silica gel GF254 (Marine Chemical Industry Factory, Qingdao, China). Based on a bioassay using the paper disk method, the fractions were incubated at 28 °C for 24 h to test their ability to inhibit *P. ananatis* (Zuo et al. 2014). Then, 560 mg of Fraction 2 was used for chromatography and separated using Sephadex LH-20 (Merck, Darmstadt, Germany) and a silica gel column with $CHCl_3$/MeOH (2:1 v/v) to yield bacitracin A (234.25 mg). The bacitracin A sample was purified using high-performance liquid chromatography (HPLC), and the composition of the high-grade bacitracin A was analyzed by LC-MS (Pavli et al. 2001). Standard bacitracin A was purchased from Wako (FUJIFILM Wako Pure Chemical Corporation, Chuo-Ku Osaka, Japan).

Antibacterial assay

Pantoea ananatis was cultured for 24 h in NB medium at 28 °C. Then, 10 μl of bacitracin A (5 μg ml^{-1}) filtered through 0.22 μm Millipore membranes was added to a paper disk (6 mm diameter) and placed on an NB agar plate inoculated with *P. ananatis*. The plates were incubated at 28 °C for 48 h, and the diameters of the zones of inhibition were measured (Zuo et al. 2014). Six NB agar plates inoculated with *P. ananatis* were treated with either ultrapure water or bacitracin A, and the assays were performed independently and repeated thrice.

LIVE/DEAD BacLight bacterial viability staining

A viability assay was performed using the LIVE/DEAD BacLight bacterial viability staining kit L7012 (Invitrogen, Molecular Probes, USA) and observed using fluorescence microscopy, as previously described (Wu et al. 2015). The kit contains a green-fluorescent SYTO 9 stain and a red fluorescent propidium iodide (PI) stain used for two-color fluorescence staining. This assay was used to test the membrane integrity of *P. ananatis* upon exposure to bacitracin A. Live bacteria with intact membranes show green fluorescence, whereas bacteria with damaged membranes show red fluorescence. *P. ananatis* cells (1 ml at a concentration of 10^7–10^8 CFU ml^{-1}) were incubated with bacitracin A (final concentration 10, 40, 80, 181.82, and 333.33 μg ml^{-1}) for 24 h, centrifuged at $1000 \times g$ for 10 min, and then resuspended in 10 mM sodium phosphate buffer (pH 7.4). Further, 10 μl of the molecular probes, prepared as per the manufacturer's guidelines, were added to the suspension and incubated for 15 min at 25 °C in the dark. The number of cells was observed using an Olympus BX43 microscope with cellSens standard software (Tokyo, Japan). The bactericidal activity of bacitracin A was calculated using the following equation: percentage survival (%) = (1−treatment/control) × 100, where treatment indicates the cell densities of *P. ananatis* treated with bacitracin A and the control is the *P. ananatis* cell density without any treatment (Kim et al. 2007). Six sample suspensions inoculated with *P. ananatis* were treated with either water or bacitracin A, and the assays were repeated thrice, independently. The MIC$_{50}$ value of bacitracin A was obtained from the sigmoidal inhibition curves fitted by profit regression analysis (SPSS 19.0).

Microstructural and ultrastructural observations

The effects of bacitracin A on the microstructure and ultrastructure of *P. ananatis* were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as previously described (Zeriouh et al. 2011). Briefly, *P. ananatis* cells were harvested at a concentration of 10^7–10^8 CFU ml^{-1} after preparation for 48 h at 28°C and then incubated with 9.10 μg ml^{-1} (final concentration) bacitracin A for 12 h. The samples were centrifuged ($8000 \times g$ for 10 min at 4 °C.) and prefixed with 2.5% glutaraldehyde. The fixed cells were then washed thrice with 100 mM phosphate-buffered saline (PBS) for 10 min and dehydrated using an ethanol gradient. For SEM analysis, the samples were coated with gold and analyzed using Hitachi S-3000N SEM (Hitachi, Japan). For TEM analysis, the cells were post-fixed for 3 h in 1% osmium tetroxide, embedded in Epon 812, sectioned with an ultramicrotome, and examined under a Hitachi H-600 TEM (Jin et al. 2017). Six sample suspensions inoculated with *P. ananatis* were treated with either water or bacitracin A, and the analyses were repeated thrice, independently.

RNA extraction and qRT-PCR

RNAseq analysis (data not included) and microscopy were used to predict the cell membranes and the sites of cell division that may be targeted by bacitracin A. To this end, we designed and synthesized primers for glucosamine-6-phosphate synthase and cell division as well as the EPS biosynthesis gene primers for *P. ananatis*. The primers were designed using Primer 5.0 based on the genome of *P. ananatis* strain DZ-12 (GenBank: NZ_CM012203.1; Table 1). Primer specificity was confirmed by agarose gel electrophoresis and melting curve analysis. The expression of three target genes (*gumD*, *ftsZ*, and *glmS*) of *P. ananatis* was monitored by qRT-PCR using 16S rRNA as the internal reference (Wu et al. 2015). *P. ananatis* was exposed to bacitracin A (final concentration of 9.10 μg ml^{-1}) for 12 and 24 h. Untreated *P. ananatis* culture was used as a control. Total RNA of *P. ananatis* was extracted using a Bacterial RNA Kit (Omega Biotek, USA) according to the manufacturer's instructions,

Table 1 Real-time PCR analysis of three oligonucleotide DNA primers (*gumD, ftsZ,* and *glmS*) in *Pantoea ananatis* cells in response to bacitracin A treatment.

Target gene*	Primers sequence (5´-3´)
gumD	F:TTGAGGATCACACCTTTAATCTCA
	R:ATAACAAATCTGGCCTGTCAAATG
ftsZ	F:AGAAAAAAAAGTCGCGAAGC
	R:AAACAGCTGATTAATACGTCCC
glmS	F:TTTGAAACCAAAGGATTCA
	R:AGAAATTTTTGAAACCGCGCT
16S rRNA	F:TTACTCTGCGGCTGCTTCT
	R:ATGCGCCATCGTAAGAGTG

*gumD the gene involved in extracellular polymeric substance biosynthesis, *ftsZ* the gene involved in cell division, *glmS* the gene involved in cell-wall biosynthesis, *16S rRNA* the internal reference gene

and the complementary DNA (cDNA) was synthesized using the Prime Script RT-PCR kit (TaKaRa, Japan). A reaction volume of 20 µl, including SYBR Premix EX Taq (TaKaRa), was used for the qRT-PCR assay, which was performed on an ABI Prism 7500 system (Applied Biosystems Inc, Foster City, CA, USA). The cycling conditions consisted of a hot-start activation step for 14 s at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Six samples, suspensions in test tubes inoculated with *P. ananatis*, were treated with water or bacitracin A, and the experiments were performed independently in triplicate. The data were normalized according to the $2^{-\triangle\triangle CT}$ method (Livak et al. 2001).

Pathogenicity test on rice plants

A pathogenicity assay was conducted in a glasshouse at 25–28 °C as previously described (Qian et al. 2013). In brief, *P. ananatis* was cultivated in NB medium at 28 °C and mixed at 200 r min^{-1} for 24 h. One hour before inoculation with the bacterial pathogen, rice (IR24) leaves were sprayed with 9.10 µg ml^{-1} bacitracin A or water (control). To determine the length of lesions due to *P. ananatis*, two-month-old rice plants were inoculated with a suspension of 10^{7}–10^{8} CFU ml^{-1} *P. ananatis* by the spraying method. Disease symptoms were recorded after incubation for 15 days, and the protective rate of bacitracin A was calculated using the following equation: protective

rate (%) = (1−treatment/control) × 100, where treatment and control are lesion lengths with and without bacitracin A, respectively (Wu et al. 2015). For each test, 270 plants (rice IR24) were treated with either water or bacitracin A, and the experiment was performed independently in triplicate.

Statistical analysis

Statistical analyses were performed using SPSS (version 19.0; SPSS Inc). Data were analyzed using ANOVA, followed by a Fisher least significant difference test ($p < 0.05$). MIC$_{50}$ values were calculated using probit regression. Each treatment had three biological replicates. All data were expressed as means ± SD.

Results

Purification and structure elucidation of bacitracin A

We purified the active substance, designated as compound 1. Compound 1 appeared as a single peak when evaluated by HPLC, confirming the presence of a single compound. The retention time of compound 1 was 24.043 min at 254 nm (Suleiman et al. 2017). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed on a triple quadrupole mass spectrometer using the positive electrospray ionization (ESI) mode. Composition analysis by LC-MS showed that compound 1 and standard bacitracin A had nearly the same compositions. The ESI-MS result for compound 1 matched that for bacitracin-1422 Da (Suleiman et al. 2017; Choi et al. 2017).

Assessment of bactericidal activity of bacitracin A

For the agar disk diffusion assays, MeOH, control, *n*-butyl alcohol extract (5 µg ml^{-1}), and bacitracin A (5 µg ml^{-1}) samples were added to disks inoculated with *P. ananatis* and achieved 0, 0, 11.930 ± 0.003, and 20.328 ± 0.046 mm zones of inhibition, respectively. To accurately determine the bacteriostatic effect of bacitracin A, we labeled the *P. ananatis* cells using a LIVE/DEAD BacLight bacterial viability staining kit. This was followed by fluorescence microscopy to determine the bacteriostatic effect of bacitracin A

(Table 2). *P. ananatis* cell staining revealed stable, low levels of red fluorescent cells and high levels of green fluorescent cells, indicating many live intact cells. Following exposure to 10 μg ml^{-1} (final concentration) bacitracin A for 24 h, the proportion of red fluorescent cells increased to 48.49 ± 0.15 % ($p < 0.05$). Exposure to 40 μg ml^{-1} (final concentration) bacitracin A increased the proportion of red fluorescent cells to 75.26 ± 0.15 % owing to the accumulation of the red probe in the damaged *P. ananatis* membranes ($p < 0.05$). The cells were intensely stained with the red probe upon exposure to 333.33 μg ml^{-1} (final concentration) bacitracin A. Severe damage to the cell membranes was more evident in the cells exposed to higher concentrations of bacitracin A. Bacitracin A showed strong antibacterial activity against *P. ananatis*, with an MIC$_{50}$ value of 9.10 ± 0.18 μg ml^{-1} at 24 h ($F_{4,10} = 2654.97$; $p < 0.01$).

Scanning and transmission electron microscopy

SEM and TEM analyses revealed that bacitracin A damaged the cell walls and membranes of *P. ananatis*, resulting in the leakage of intracellular components and forming an empty space. The untreated cells had a normal, plump, rod-shaped appearance with smooth intact surfaces (Fig. 1a), whereas the cells treated with bacitracin A were loose, distorted, and heavily disrupted (Fig. 1b). TEM indicated that the untreated cells displayed normal prokaryotic morphology with intact cell membranes and a uniformly distributed cytoplasm (Fig. 1c). On the contrary, the cells exposed to bacitracin A displayed distinct damage with the formation of deep pits on the cell surface, condensed cytoplasm, and lysis of organelles and chromosomes (Fig. 1d). Lysed cells and empty vesicles were visible owing to plasmolysis and the efflux of the intracellular components.

Effect of bacitracin A on *P. ananatis* gene expression

The *ftsZ* gene is involved in cell division. Glucosamine-6-phosphate synthase is encoded by *glmS*, which is important in peptidoglycan biosynthesis, and *gumD*, which is in the gum operon, is responsible for extracellular polysaccharide (EPS) biosynthesis and required for virulence. Therefore, *ftsZ*, *glmS*, and *gumD* were assessed to investigate the effects of bacitracin A on *P. ananatis* gene expression. The qRT-PCR analysis revealed that *ftsZ*, *glmS*, and *gumD* exhibited higher transcript expression levels (1.15-, 1.75-, and 1.38-fold higher, respectively) than the control upon exposure to bacitracin A at 12 h (Fig. 2). However, compared to the control, *ftsZ*, *glmS*, and *gumD* transcripts were approximately 0.49-, 0.78-, and 0.58-fold less abundant, respectively, at 24 h. After 12 and 24 h, significant differences were observed between the untreated controls and the samples treated with bacitracin A (Fig. 2).

Biological control of rice diseases caused by *P. ananatis*

We examined whether bacitracin A protects rice (cultivar IR24) against *P. ananatis* infection. The virulence of *P. ananatis* in the infected rice plants treated with bacitracin A was significantly lower than

Table 2 MIC$_{50}$ quantification analysis by fluorescence microscope images of the viability of *Pantoea ananatis* cells after exposure to bacitracin A for 24 h

Final concentration of bacitracin A (μg ml^{-1})	Inhibition rate $\pm$ SD (%)	MIC$_{50}$* $\pm$ SD (μg ml^{-1})
10.00	48.49e $\pm$ 0.15	9.10 $\pm$ 0.18
40.00	75.26d $\pm$ 0.15	
80.00	79.67c $\pm$ 0.30	
181.82	83.03b $\pm$ 0.60	
333.33	90.78a $\pm$ 0.98	

* MIC$_{50}$ is the 50% minimal inhibitory concentration at 24 h. The MIC$_{50}$ value was obtained from the sigmoidal inhibition curves fitted by probit regression analysis (SPSS 19.0). The number of total, live (green), and dead (red) cells was counted in ten different microscopic fields. The values indicate the percentages of live and dead cells in the suspensions. Different letters indicate significantly different between values ($p < 0.05$)
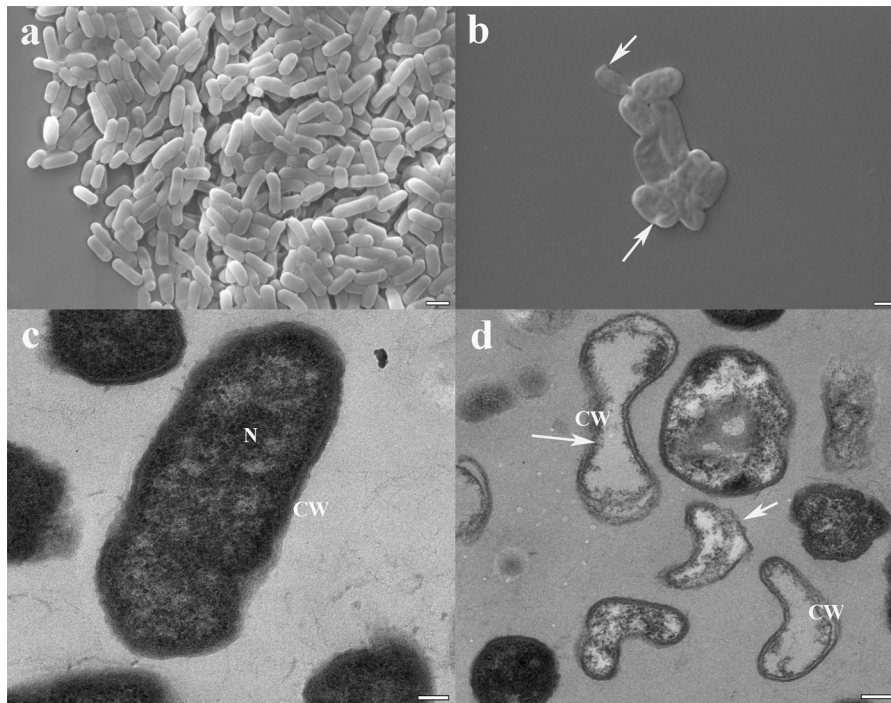
Fig. 1 Electron micrographs of the microstructure and ultra-structure of the *Pantoea ananatis* cells upon exposure to 9.10 µgml^{-1} (final concentration) bacitracin A for 24 h. **a** Untreated control *P. ananatis* cells determined by SEM; bars: 1 µm; **b** *P. ananatis* cells exposed to 9.10 µg ml^{-1} (final concentration) bacitracin A for 24 h as determined by SEM; bars: 1 µm;

c Untreated control *P. ananatis* cells determined by TEM; bars: 0.2 µm. **d** *P. ananatis* exposed to 9.10 µg ml^{-1} (final concentration) bacitracin A for 24 h as determined by TEM; bars: 0.2 µm. N: nucleus, CW: cell wall. The arrows point to bacitracin A damaged cell wall and membrane of *P. ananatis*

that in the untreated controls, and lesion length and disease severity were remarkably reduced. Lesion length was significantly shorter in the cells treated with bacitracin A than in the untreated controls (12.03 ± 1.12b and 17.86 ± 1.21a cm, respectively (p < 0.05). The protective rates of bacitracin A were 49.94 ± 1.56%.

Discussion

Bacillus spp. protect against plant pathogens through their antimicrobial properties (Ongena et al. 2005; Stein et al. 2005; Romero et al. 2007). Certain strains of *B. subtilis* produce broad-spectrum antifungals and can control cucurbit powdery mildew (Romero et al. 2007). *B. subtilis* can generate a wide variety of antibiotics, which are essential for the biocontrol of bacteria and fungi (Moyne et al. 2001; Ongena et al. 2008; Nasir et al. 2012). Bacitracin is a mixture of closely related compounds containing at least ten

distinct dodecapeptides that differ by one or two amino acids. The most active and abundant of these peptides is bacitracin A (Morris 1994). Few studies have evaluated whether bacitracin inhibits gram-negative phytopathogens. We found that bacitracin A, isolated from *B. licheniformis* strain HN-5, was a remarkable growth inhibitor of *P. ananatis* and has potential as a bactericidal agent for use in the biological control of plant diseases.

Hydrophobic groups on antimicrobial peptides interact with phospholipids and cholesterol in the bacterial cell membranes, allowing them to enter the cell (Carrillo et al. 2003; Makovitzki et al. 2006; Ciesiołka et al. 2014; Oh et al. 2018). Bacitracin A is particularly active against gram-positive bacteria. It inhibits bacterial cell wall biosynthesis and forms a tight ternary complex with C55-isoprenyl pyrophosphate. C55-isoprenyl pyrophosphate acts as a membrane-associated carrier for bacteria during the synthesis of the repeating subunits of peptidoglycan. Blockage of its recycling is fatal to cells (Pollock et al.
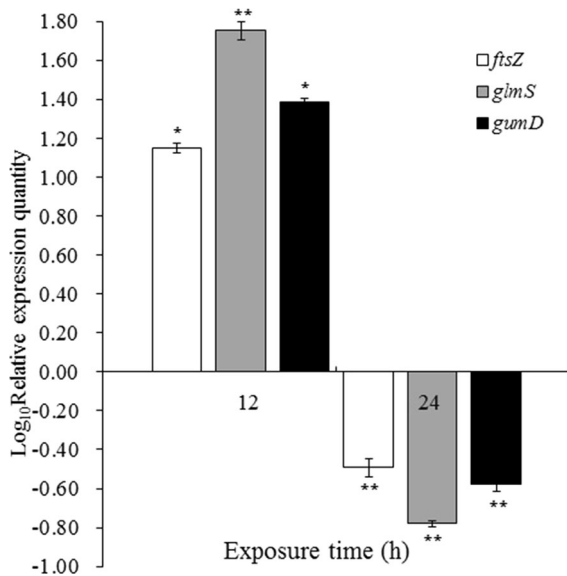
Fig. 2 Expression analysis of *ftsZ*, *glmS*, and *gumD* in *Pantoea ananatis* cells upon exposure to 9.10 μg ml^{-1} (final concentration) bacitracin A for 12 and 24 h. The values were normalized to the levels of *16S rRNA*, which is a housekeeping gene, as an internal reference gene. The Expression of *ftsZ*, *glmS*, and *gumD* gene in treated with bacitracin A compared with untreated control *P. ananatis* cells are significantly different. The y-axis represents mean expression values ± SD relative to the control. The "*" and "**" means significantly different according to Fisher's least-significant difference tests at $p < 0.05$ and $p < 0.01$, respectively

1994). Divalent metal cations from the bacitracin A-C55-isoprenyl pyrophosphate complex block the recycling of C55-isoprenyl pyrophosphate. This is fatal to the cell (Storm et al. 1973; Wang et al. 2017). The amino acid building blocks of bacitracin A are L-Ile$_1$, L-Cys$_2$, L-Leu$_3$, D-Glu$_4$, L-Ile$_5$, L-Lys$_6$, D-Orn$_7$, L-Ile$_8$, D-Phe$_9$, L-His$_{10}$, D-Asp$_{11}$, and L-Asn$_{12}$ (Drablos et al. 1999). The amino acid structure of bacitracin A contains an amino-terminal linear pentapeptide moiety with an isoleucine-cysteine thiazoline ring and a C-terminal heptapeptide ring, in which the free α-carboxy group of the C-terminal Asn is bound to the ε-amino group of lysine (Stone et al. 1971). Based on the structure and ultrastructural analysis of bacitracin A, we hypothesized that the isoleucine ring of bacitracin A binds to the fat-soluble phospholipids in the cell wall of the bacteria and penetrates the cell membrane in a fashion comparable to a balloon pierced with a needle. Owing to this mechanism of action along with its amphiphilic nature, there is a low probability of pathogens becoming resistant to bacitracin A. This is proved by the antagonistic effects of bacitracin against the newly identified rice pathogen owing to its amphiphilic nature.

Similar to previous findings by Wu et al. (2015), we found that, in *P. ananatis,* bacitracin stress regulates the gene expression of *ftsZ* and *glmS*, which are involved in cell division and biosynthesis, respectively. Bacitracin may prevent cell membrane repair and induce the production of EPS in *P. ananatis* cells. The decreased levels of *ftsZ* and *glmS* may be attributed to the high levels of dead *P. ananatis* cells caused by the severe cell wall damage. EPS is an important virulence factor that protects pathogens from host defenses (Torres et al. 2006; Yun et al. 2006; Li and Wang 2011). Downregulation of *gumD* by bacitracin may have resulted in EPS reduction and the attenuation of *P. ananatis* virulence. We plan to further evaluate mRNA and protein expression in our future studies.

To the best of our knowledge, this is the first study to perform microscopy and qRT-PCR to clarify the mechanisms underlying the antibacterial effects of bacitracin A against gram-negative phytopathogens. Bacitracin A caused severe ultrastructural damage to *P. ananatis* cell walls and membranes revealed by the examination of changes in the morphology of *P. ananatis*. Bacterial inhibition by bacitracin can be partially explained by its ability to cause cell leakage and impede the exchange of materials through the cell membrane owing to changes in membrane permeability.

Bacitracin A, isolated from *B. licheniformis* strain HN-5, exerted strong bactericidal activity against *P. ananatis* and showed potential as a natural biological control of this rice pathogen. Our results improved the current understanding of the mechanisms underlying the antibacterial biocontrol elicited by bacitracin A.

Compliance with ethical standards

Conflict of interest All authors declare that there is no conflict of interest in this work.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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