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Extracellular vesicles of *Pseudomonas aeruginosa* downregulate pyruvate fermentation enzymes and inhibit the initial growth of *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus and *Pseudomonas aeruginosa* are well-known opportunistic pathogens that frequently coexist in chronic wounds and cystic fibrosis. The exoproducts of *P. aeruginosa* have been shown to affect the growth and pathogenicity of *S. aureus,* but the detailed mechanisms are not well understood. In this study, we investigated the effect of extracellular vesicles from *P. aeruginosa* (PaEVs) on the growth of *S. aureus*. We found that PaEVs inhibited the *S. aureus* growth independently of iron chelation and showed no bactericidal activity. This growth inhibitory effect was also observed with methicillin-resistant *S. aureus* but not with *Acinetobacter baumannii, Enterococcus faecalis, S.* Typhimurium, *E. coli, Listeria monocytogenes*, or *Candida albicans*, suggesting that the growth inhibitory effect of PaEVs is highly specific for *S. aureus*. To better understand the detailed mechanism, the difference in protein production of *S. aureus* between PaEV-treated and non-treated groups was further analyzed. The results revealed that lactate dehydrogenase 2 and formate acetyltransferase enzymes in the pyruvate fermentation pathway were significantly reduced after PaEV treatment. Likewise, the expression of *ldh2* gene for lactate dehydrogenase 2 and *pflB* gene for formate acetyltransferase in *S. aureus* was reduced by PaEV treatment. In addition, this inhibitory effect of PaEVs was abolished by supplementation with pyruvate or oxygen. These results suggest that PaEVs inhibit the growth of *S. aureus* by suppressing the pyruvate fermentation pathway. This study reported a mechanism of PaEVs in inhibiting *S. aureus* growth which may be important for better management of *S. aureus* and *P. aeruginosa* co-infections.

1. Introduction

Staphylococcus aureus is a gram-positive bacterium that frequently colonizes human skin and the mucous membrane of the nasal cavity (Oliveira et al., 2018; Ryu et al., 2014), whereas *Pseudomonas aeruginosa* is a gram-negative bacterium that can survive under a variety of environmental conditions (Crone et al., 2020). These bacteria have been known as opportunistic pathogens that cause a wide variety of infections including life-threatening diseases in the immunocompromised hosts (Krishna and Miller, 2012; Leong et al., 2022). *S. aureus* and *P. aeruginosa* commonly coexist in the lungs of cystic fibrosis patients (Fischer et al., 2021), and they are the predominant species found in polymicrobial infections of chronic wounds (Bessa et al., 2015). It has been suggested that the pathogenesis of *S. aureus* and *P. aeruginosa* co-infection is promoted, compared to individual infections (Serra et al., 2015). An in vivo study demonstrated that the epithelization of wounds

Abbreviations: PaEVs, Extracellular vesicles from *Pseudomonas aerugionsa*; Ldh2, Lactate dehydrogenase 2; PflB, Formate acetyltransferase; EVs, Extracellular vesicles; OD, Optical density; PBS, Phosphate-buffered saline; TSB, Tryptic soy broth; TSA, Tryptic soy agar; CFU, Colony-forming unit; RT-qPCR, Reverse-transcription quantitative PCR.

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infected with both *S. aureus* and *P. aeruginosa* is delayed (Pastar et al., 2013). Moreover, chronic *S. aureus* and *P. aeruginosa* coinfection in cystic fibrosis, especially by multidrug-resistant strains, is associated with high-rate mortality in patients (Maliniak et al., 2016).

S. aureus and *P. aeruginosa* have been reported to communicate with each other via extracellular substances to alter their pathogenicity. The extracellular proteins of *S. aureus* are shown to enhance growth and suppress autolysis of *P. aeruginosa* (Michelsen et al., 2014). On the other hand, the exoproducts of *P. aeruginosa* have been shown to promote the production of staphyloxanthin and enhance the virulence of *S. aureus* (Maliniak et al., 2016; Antonic et al., 2013). In addition, the exoproducts of *P. aeruginosa* enhanced the tobramycin resistance of *S. aureus* (Barraza and Whiteley, 2021).

P. aeruginosa can adapt to conditions that other organisms cannot tolerate because of low nutritional requirements (Silby et al., 2011). To potentiate its own survival, *P. aeruginosa* produces a large number of extracellular substances to outcompete other microorganisms. For example, small respiratory inhibitors such as hydrogen cyanide, pyocyanin and quinoline N-oxides target the electron transport chain and modulate the growth of other bacterial species (Biswas and Götz, 2022). Peptidoglycan hydrolases possess the capacity to hydrolyze peptidoglycans from other gram-positive and gram-negative bacteria (Kadurugamuwa and Beveridge, 1996). These extracellular substances affect a wide variety of microorganisms including *S. aureus* (Jayaseelan et al., 2014; Hoffman et al., 2006; Voggu et al., 2006). In particular, *P. aeruginosa* produces staphylolysin to cleave the glycyl-alanine and glycyl-glycine bonds of the pentaglycine crosslink in *S. aureus* peptidoglycan and induces lysis of *S. aureus* (Kessler et al., 1993). A study of in vitro interaction between *S. aureus* and *P. aeruginosa* demonstrated that the early adapted *P. aeruginosa* DK2 strains outcompete *S. aureus* during coculture on agar plates (Michelsen et al., 2014). On the other hand, the *P. aeruginosa* strains of DK2 clone lineage isolated from chronically infected cystic fibrosis patients showed a commensal-like interaction with *S. aureus*, where *S. aureus* growth was not inhibited by *P. aeruginosa* (Michelsen et al., 2014). This study suggests that *P. aeruginosa* has a complex interaction with *S. aureus* either competitive or cooperative, in which the exoproducts are key mediators. Therefore, understanding in the more detailed mechanism of this complex interaction should be helpful in the prevention or treatment of *P. aeruginosa* and *S. aureus* coinfections.

Among all exoproducts of *P. aeruginosa*, extracellular vesicles (EVs) are one of the most important mediators because the EVs are an enclosed lipid bilayer of outer-inner membrane containing several kinds of bioactive molecules (Jan, 2017). Based on the structure and properties, the EVs are able to protect the internal substances from degradative enzymes in the environment. In addition, the molecules localized on the surface of vesicles can facilitate EVs to reach specific targets. Therefore, the EVs are considered to be an efficient mediator for long-distance transport of the bioactive molecules between specific pathogens (Caruana and Walper, 2020).

In this study, we focused on the interaction between *S. aureus* and *P. aeruginosa* mediated by *P. aeruginosa*-derived EVs (PaEVs). We hypothesized that PaEVs facilitate direct interaction and deliver several exoproducts of *P. aeruginosa* to *S. aureus*. Based on the activities of several *P. aeruginosa* exoproducts, this study aims to investigate the effect of PaEVs on the growth of *S. aureus*. In addition, the production of *S. aureus* proteins after PaEV-treatment was also analyzed to elucidate the detailed mechanism of PaEVs that affects *S. aureus*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Methicillin-susceptible *S. aureus* ATCC1718, methicillin-resistant *S. aureus* ATCC1717, *P. aeruginosa* ATCC15692, *Acinetobacter baumannii* ATCC19606, *Enterococcus faecalis* ATCC700802, *Salmonella enterica* subsp. *enterica* serovar χ3306 (Sashinami et al., 2006), *E. coli* ATCC25922, *Listeria monocytogenes* 1b1684, and *Candida albicans* NBRC1385 were cultivated at 37°C for 16–24 h in tryptic soy broth (TSB; BD Bioscience, Sparks, MD) and maintained in tryptic soy agar (TSA, BD Bioscience). For experiments, bacterial and yeast cells were inoculated into TSB and cultured for 4–6 h to reach an exponential phase before use. The numbers of bacterial and yeast cells were adjusted in corresponding to optical density (OD) measurement at a wavelength of 600 nm. For PaEV purification, *P. aeruginosa* was cultivated in Brain Heart Infusion medium (BD Bioscience) for 8 h. After removal of bacterial cells by centrifugation at 5,000 × *g*, 4°C for 30 min twice, the supernatant was collected, filtrated through 0.45 μm filter (Merck Millipore Ltd, Tullagreen, Ireland), and stored at -80°C until use.

2.2. Purification of PaEVs

PaEVs in the culture supernatant of *P. aeruginosa* were harvested by ultracentrifugation at 100,000 × *g*, 4°C for 90 min using a Himac CP80WX Preparative Ultracentrifuge (HITACHI, Tokyo, Japan). After removal of the supernatant, the pellet was washed twice and suspended in ice-cold sterile phosphate-buffered saline (PBS). PaEVs were then separated from other damaged membranes, aggregated proteins and non-membranous proteins by step-gradient ultracentrifugation using OptiPrep™ Density Medium (Sigma Aldrich, St. Louis, MO). After centrifugation at 100,000 × *g*, 4°C for 16 h, the substances in all six fractions (F1-F6) were collected, diluted in PBS, and harvested by ultracentrifugation at 100,000 × *g*, 4°C for 3 h. The pellet from each fraction was washed twice and resuspended in a suitable volume of ice-cold sterile PBS. The suspensions were filtrated through 0.45 μm filter (Merck Millipore Ltd), and a portion of these suspensions was inoculated into TSB and TSA to check for bacterial contamination. No bacterial growth was found after incubation at 37°C for 48 h. The protein concentration of each fraction was measured by the Bradford protein assay. A transmission electron microscope (JEM-1230, JELO, Tokyo, Japan) with platinum blue staining (TI-blue staining kit: Nisshin EM Co. Ltd., Tokyo, Japan) was used to detect the presence of PaEVs in each fraction. Size distribution and concentration of PaEV particles were analyzed using qNano instrument (Izon Science, Oxford, United Kingdom) by following the manufacturer's instructions. Briefly, the purified PaEVs were diluted in Measurement Electrolytes (qNano reagent kit; Izon Science) containing 0.02% Tween 20 and analyzed with NP150 nanopore membranes in comparison with 200 nm calibration particles by running at 1.1 V. Data were analyzed using Izon Control Suite software version 3.3.2.2001.

2.3. Growth inhibition assay

Growth inhibition assay in *P. aeruginosa* culture supernatant and TSB supplemented with PaEVs was performed. To prepare *P. aeruginosa* supernatant, *P. aeruginosa* was cultured in TSB for 8 h, and the bacterial cells were removed from the culture supernatant by centrifugation and filtration as described above. The absence of bacterial cell contamination was confirmed by inoculating a portion of the supernatant into TSB and TSA and incubating at 37°C for 48 h. *S. aureus* as well as other bacterial and yeast cells were inoculated into *P. aeruginosa* culture supernatant or TSB containing 0–5 μg/mL purified PaEVs by adjusting to 5 × 10^3 CFU/mL and incubated at 37°C for 0–12 h. Growth of each microorganism was monitored by measuring OD at 600 nm and/or the microbial numbers were enumerated by plate count assay on TSA. The growth of PaEV-treated *S. aureus* in the presence of 50 and 100 μM $FeCl_3$ or 15 mM sodium pyruvate (FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) supplementation was also assessed by the same method as described above. For the effect of PaEVs under oxygen supplementation, *S. aureus* was cultured in the presence of 1 μg/mL PaEVs at 125 rpm shaking.

2.4. Bactericidal assays

S. aureus suspensions (OD_{600nm} = 1.0 in PBS or OD_{600nm} = 0.2 in 0.85% NaCl) were incubated with PaEVs at 37°C for 6 h under static condition. The bacterial numbers were then evaluated by plate count assay on TSA. Viability of *S. aureus* was observed using LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Waltham, MA). Live and dead cells stained with SYTO9 (green fluorescence) and propidium iodide (red fluorescence), respectively, were observed under a fluorescence microscope (KEYENCE, BZ-X700, Osaka, Japan). Proportions of live and dead cells were quantified by BZ-X Analyzer (KEYENCE). Moreover, the bacteriolytic activity of PaEVs was examined by detecting the release of genomic DNA from *S. aureus*. *S. aureus* (OD_{600nm} = 0.1 in sterile distilled water) was incubated at 37°C for 2 h with and without 1 μg/mL PaEVs. After removal of bacterial cells and/or cell debris by filtration through 0.22 μm filter, the lysate was directly used as a PCR template. The genomic DNA released into the lysate by lysostaphin (10 μg/mL) was used as a positive control. Partial of the housekeeping gene for 6- phosphofructokinase was amplified by PCR using *pfkA* primers (Table 1). The following thermal protocol was used: 95 °C for 5 min, followed by 30 cycles of amplification (95 °C for 40 s, 58 °C for 40 s and 72 °C for 40 s), and 72 °C for 5 min. The PCR product was analyzed by electrophoresis on 0.8% agarose gel.

2.5. Quantification assay of pyocyanin in PaEVs

Pyocyanin in PaEVs was measured by colorimetric assay as described by Essar et al., 1990 with some modifications. Briefly, PaEVs were diluted in PBS to a final concentration of 10 μg/mL. Chloroform (300 μL) was added to 500 μL of the PaEV samples. The samples were then vigorously vortexed and left at room temperature for 10 min to allow aqueous phase separation. The chloroform layer was carefully transferred to a new tube, and 160 μL of 0.2 N HCl was added to the chloroform fraction. After vigorous mixing, the samples were left for 10 min and centrifuged at 3,000 × *g* for 5 min. The upper layer (100 μL) was carefully transferred to a 96-well plate, and absorbance was measured at 520 nm using a microplate spectrophotometer (Multiskan Sky, Thermo Fisher Scientific, Waltham, MA). Pyocyanin in the cell-free supernatant of *P. aeruginosa* cultured for 48 h was used as a positive control, whereas the negative controls are PBS and non-culture TSB.

2.6. Proteomic analysis of PaEVs and S. aureus treated with PaEVs

Proteomic analysis of the purified PaEVs was performed by liquid chromatography-tandem mass spectrometry as described previously (Asano et al., 2021). Briefly, the proteins in PaEVs were precipitated with acetone, denatured with 50% trifluoroethanol, and reduced using 4 mM dithiothreitol. After free cysteine residues were alkylated, the proteins were digested with trypsin. The peptides were then desalted and separated by liquid chromatography. The obtained spectra were searched against *P. aeruginosa* ATCC15692 proteins (NCBI Genebank: U80962.1). The proteins that reached a 1% local false discovery rate were considered as positive identification. The proteomic data are available under accession numbers PXD036377 and JPST001829 for Proteome Xchang and jPOST Repository, respectively. The molecular weight and subcellular localization of each protein were predicted using Compute pI/Mw tool-ExPASY (https://web.expasy.org/compute_pi/) and PsortB v.3.0 (https://www.psort.prg/psortb/), respectively.

Table 1
Primer sequences used in this study.

Gene	Primer	Sequence
pfkA	Forward	5′-ATGTTCGTGCGTGACTTGAC-3′
	Reverse	5′-CAACGCATCAGTGAGGAATG-3′
ftsZ	Forward	5′-TGCTTATGAATATTACTGGTGG-3′
	Reverse	5′-TTTACGCTTGTTCCGAATCC-3′
ldh2	Forward	5′-ATCTGTAGGATCAAGCTATGCC-3′
	Reverse	5′-ACTGGTGAAGGACTGTGGAC-3′
pflB	Forward	5′-CATGTGGGATATGGACACGA-3′
	Reverse	5′-ACAAGCTGCTTTCGCCATAC-3′

2.7. Differential proteomic analysis of S. aureus after treatment with and without PaEVs

S. aureus (5×10^3 CFU/mL) was incubated with and without 1 μg/mL PaEVs for 6 h at 37°C under static condition, and the bacterial cells were lysed using 2% sodium dodecyl sulfate/7 M urea. Proteomic analysis of the *S. aureus* whole cell lysate was performed by liquid chromatography-tandem mass spectrometry as described above. The identified proteins were searched against *S. aureus* ATCC1718 (NCBI Genebank: NC_007795.1). In comparison with *S. aureus* without PaEV treatment, differential protein production in PaEV-treated *S. aureus* was assessed by quantitative proteomics using SWATH analysis. The proteomic data are available under accession number PXD036381 and JPST001830 for Proteome Xchang and jPOST Repository, respectively. The molecular weight and subcellular localization of each protein were predicted as described above.

2.8. RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

S. aureus was incubated with 1 μg/mL PaEVs for 6 h at 37°C under the static condition as described above. The bacterial cells were harvested at 6,000 × *g* for 10 min and immediately kept in -80°C until use. Total RNA from bacterial cells was extracted by TRIzol reagent (Ambion, Waltham, MA) according to the manufacturer's instruction. The contaminated genomic DNA was degraded with DNase I (Takara Bio, Shiga, Japan), and TRIzol extraction was performed again. cDNA was synthesized from 1 μg total RNA using random primer and M-MLV reverse transcriptase (Invitrogen, Waltham, MA). The expression of *ldh2* and *pflB* gene in *S. aureus* was measured by RT-qPCR using SYBR Green Supermixes (Bio-Rad, Richmond, CA). The expression of the housekeeping *ftsZ* gene (encoding for FtsZ cell division protein) was used as a reference for normalization (Sihto et al., 2014). Primer sequences are shown in Table 1. The following thermal protocol was used: 95 °C for 5 min, followed by 45 cycles of amplification (95 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s), and 72 °C for 5 min.

2.9. Statistical analysis

Statistical differences were analyzed using the method mentioned in each figure legend. A *P*-value less than 0.05 is considered statistically significant.

3. Results

3.1. P. aeruginosa culture supernatant affects S. aureus growth

The effect of *P. aeruginosa* exoproducts in the culture supernatant on the growth of *S. aureus* was first examined. *S. aureus* was inoculated in *P. aeruginosa* supernatant and incubated at 37°C under static condition. As shown in Fig. 1A, the growth of *S. aureus* in the *P. aeruginosa* supernatant was slower than that in the TSB control. From 2 to 12 h of incubation, the number of *S. aureus* in the *P. aeruginosa* supernatant was lower than that in the TSB control, and significant at 6, 8, and 12 h (Fig. 1B). This growth inhibitory effect of *P. aeruginosa* supernatant was also observed at 6 h of cultivation with other microorganisms including *E. faecalis*, *S.* Typhimurium, *E. coli*, L. *monocytogenes* and *C. albicans*, but not with *A. baumannii* (Fig. S1).

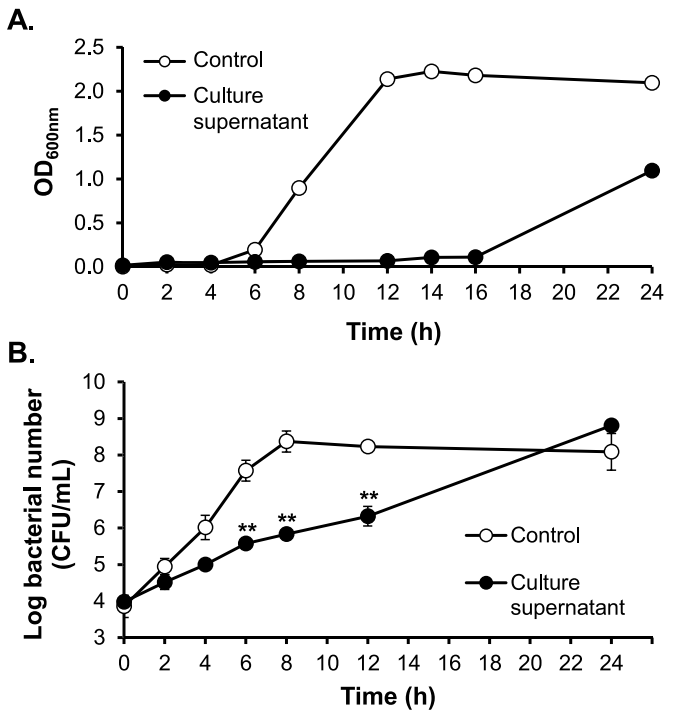

Fig. 1. Effect of *P. aeruginosa* culture supernatant on the growth of *S. aureus*. *P. aeruginosa* was cultured in TSB, and the culture supernatant was prepared as described in the Materials and Methods. *S. aureus* (5×10^3CFU/mL) was inoculated into the *P. aeruginosa* supernatant and incubated at 37°C under static condition. Cultivation of *S. aureus* in TSB under the same condition was used as control. At the indicated time points, (A) growth of *S. aureus* was monitored by OD_{600nm} measurement, and (B) the bacterial number was enumerated by plate count assay ($n = 6$ from 3 independent experiments). *P*-value was calculated using Mann-Whitney *U* test. **: $P < 0.01$.

3.2. Purification of PaEVs and identification of proteins in PaEVs

PaEVs were purified from the culture supernatant of *P. aeruginosa* by step-gradient ultracentrifugation. The substances in each fraction (F1-F6) were harvested and the protein concentration was determined. The highest protein yield was obtained in F5 followed by F6 and F4 (Table S1). The particles with enclosed-vesicle structure of PaEVs were detected in F4, F5 and F6 by negative-staining transmission electron microscopy (Fig. 2A, Fig. S2A). Due to the highest protein yield obtained from F5, the PaEVs in F5 were used for further experiments. By nanoparticle tracking analysis, the diameters of PaEVs were in a range of 90–140 nm with an average size at 115 ± 21.4 nm (Fig. S2B). The number of particles was 1.2×10^8 particles per 1 μg protein. Proteomic analysis of PaEVs from F5 was then performed, and a total of 57 proteins were identified (Table S2). These proteins included ribosomal proteins, ATP synthase subunits, outer membrane proteins, B-type flagellin and lipoproteins. In addition, several lytic enzymes such as endolytic peptidoglycan transglycosylase RlpA and protease LasA were also detected in the PaEVs.

3.3. Effect of PaEVs on the growth of S. aureus

The effect of the purified PaEVs (from F5) on the growth of *S. aureus* was further investigated. As shown in Fig. 2B, the growth of *S. aureus* in the presence of 1 μg/mL PaEVs was slower than that of the control. Especially at 4–12 h of cultivation, the number of *S. aureus* was significantly lower than that of the control, and the number of *S. aureus* was recovered at 24 h of cultivation. When the concentrations of PaEVs were varied, the inhibitory effect of PaEVs on *S. aureus* number at 6 h of cultivation was dose-dependent (Fig. 2C). The growth inhibitory effect was also found against methicillin-resistant *S. aureus* (Fig. 2D). However, PaEVs did not affect the number of *E. faecalis, S.* Typhimurium, *E. coli, L. monocytogenes* or *C. albicans* (Fig. S3), unlike the results obtained from the *P. aeruginosa* supernatant (Fig. S1). These results indicated that PaEVs tend to exert a specific effect on growth of *S. aureus*.

The reduction in the bacterial number of *S. aureus* after cultivation with PaEVs implies that PaEVs may exhibit bactericidal activity against *S. aureus* due to the presence of lytic enzymes. Therefore, the bactericidal activity of PaEVs was further assessed. Instead of cultivation in TSB, *S. aureus* was suspended in PBS or normal saline and incubated at 37°C in the presence of PaEVs. The results in Fig. 3A and 3B demonstrated that the bacterial number of *S. aureus* was not significantly decreased by PaEVs. The viability of *S. aureus* was also observed using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit. As shown in Fig. S4 and Fig. 3C, the ratio of live and dead cells of PaEV-treated *S. aureus* was not significantly different from the non-treated control. In addition, the bacteriolytic activity of PaEVs was investigated by using the lysate from PaEV-treated *S. aureus* directly as a template for PCR. As shown in Fig. 3D, a PCR product of the partial *pfkA* gene with a size of 169-bp was clearly amplified from lysostaphin-treated *S. aureus* lysate (lane 4) and purified *S. aureus* genomic DNA (lane 5) which are used as positive controls. On the other hand, no PCR product was obtained from PaEV-treated *S. aureus* lysate (lane 3), while a faint band at 169-bp appeared when non-PaEV-treated *S. aureus* lysate was used as a template (lane 2). Taking all together, the results suggested that PaEVs inhibit growth but not exhibit bactericidal or bacteriolytic activity against *S. aureus*.

3.4. Inhibitory effect of PaEVs on the growth of S. aureus is independent of pyocyanin and iron chelation

It is possible that pyocyanin in PaEVs may contribute to the inhibition of *S. aureus* growth. Therefore, the presence of pyocyanin in PaEVs was determined. By colorimetric assay, 0.43 μg/mL pyocyanin was detected in the *P. aeruginosa* culture supernatant. This correlates with the results reported by Park et al., 2015. However, by using the same method, no pyocyanin in 5 μg PaEVs could be detected as the absorbance values were comparable to the PBS negative control.

In addition to pyocyanin, PaEVs may contain iron-chelators such as *P. aeruginosa* quinolone signal (PQS) and inhibit *S. aureus* growth due to iron starvation. Therefore, we further investigated whether the iron-chelating activity is involved in the inhibitory effect of PaEVs on the growth of *S. aureus*. The growth inhibitory assay was performed by addition of 50 and 100 μM $FeCl_3$. Fig. S5 showed that iron supplementation did not recover the bacterial number of *S. aureus*. This result suggested that iron-chelating activity is not involved in the inhibitory effect of PaEVs on the growth of *S. aureus*.

3.5. PaEVs affect the expression of L-lactate dehydrogenase 2 and formate acetyltransferase in S. aureus

To explore more detail in the mechanism of PaEVs, a quantitative proteomic analysis of *S. aureus* treated with or without PaEVs was performed. Both PaEV-treated and non-treated *S. aureus* proteins were identified, and differential protein production was analyzed. From triplicate analysis, a total of 401 proteins were identified (data are available under PXD036381 and JPST001830 for Proteome Xchang and jPOST Repository, respectively). Among these proteins, the amount of 8 proteins in *S. aureus* was significantly reduced after PaEV treatment (Table 2). Importantly, L-lactate dehydrogenase 2 and formate acetyltransferase (with *P*-values less than 0.005) are pyruvate metabolism-related enzymes. L-Lactate dehydrogenase 2 synthesizes lactate from pyruvate, while formate acetyltransferase is involved in the reversible conversion of pyruvate to formate and acetyl-CoA (Ferreira et al., 2013). The level of these enzymes was approximately 2 and 4 times lower than that of the control with *P*-values of 0.00323 and 0.00337, respectively.

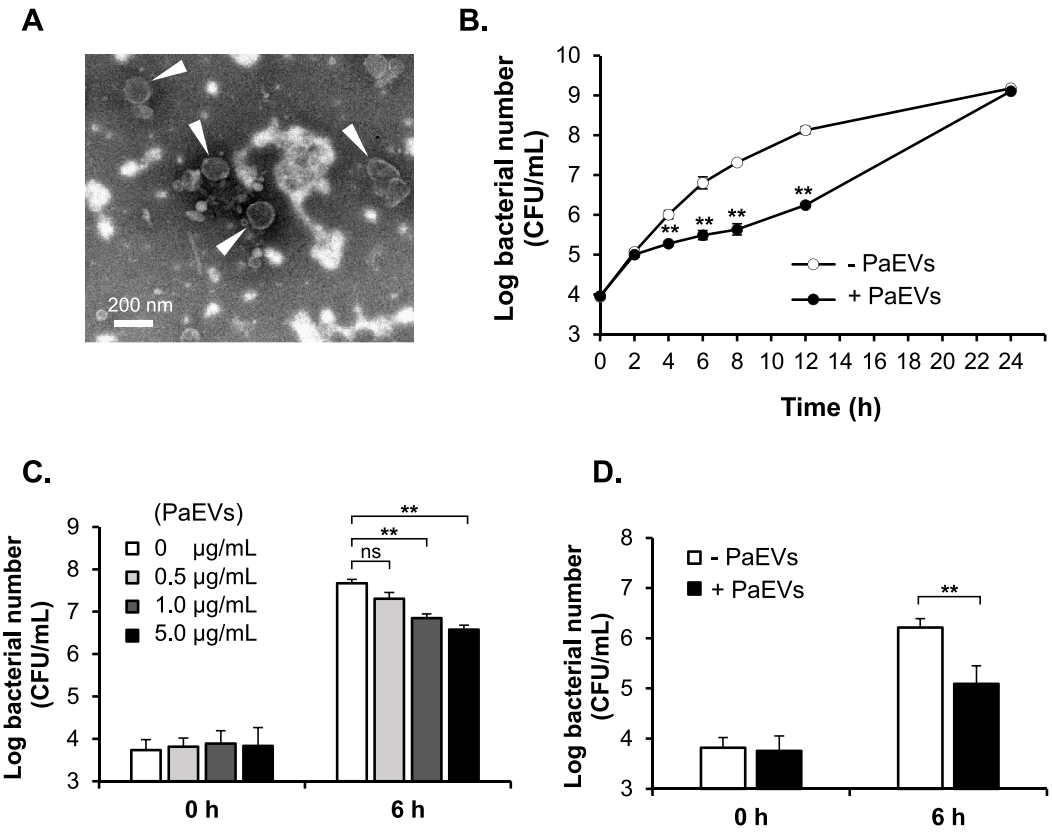

Fig. 2. Effect of PaEVs on the growth of *S. aureus* at 6 h of incubation. (A) PaEVs were purified by step gradient ultracentrifugation and detected in F5 after fractionation. Negative staining was performed and PaEVs (arrowheads) were observed under a transmission electron microscope. Scale bar=200 nm. (B) *S. aureus* was inoculated into TSB and incubated at 37°C under static condition with or without 1.0 μg/mL PaEVs. At 0–24 h of incubation, the bacterial number of *S. aureus* was enumerated by plate count assay ($n = 6$ from 2 independent experiments). *P*-value was calculated using Mann-Whitney *U* test. **: $P < 0.01$. (C) *S. aureus* was inoculated into TSB and incubated at 37°C under static condition with 0, 0.5, 1.0 and 5.0 μg/mL PaEVs. At 0 and 6 h of incubation, the bacterial number of *S. aureus* was enumerated by plate count assay ($n = 6$ from 3 independent experiments). *P*-value was calculated using Kruskal Wallis H-test with post hoc Mann-Whitney *U* test. ns: not significant, **: $P < 0.01$. (D) Methicillin-resistant *S. aureus* ATCC1717 was inoculated into TSB and incubated at 37°C with and without 1 μg/mL PaEVs. At 0 and 6 h of incubation, the number of *S. aureus* was enumerated by plate count assay ($n = 6$ from 2 independent experiments). *P*-value was calculated using Mann-Whitney *U* test. **: $P < 0.01$.

The effect of PaEVs on the expression of *ldh2* and *pflB* genes (encoding L-lactate dehydrogenase 2 and formate acetyltransferase, respectively) in *S. aureus* was further investigated by RT-qPCR. As shown in Fig. 4A and 4B, the relative mRNA expression of *ldh2* and *pflB* genes in *S. aureus* was significantly decreased after treatment with PaEVs. These results suggested that PaEVs suppress the expression of L-lactate dehydrogenase 2 and formate acetyltransferase that are related to pyruvate metabolism and may consequently inhibit the growth of *S. aureus*.

3.6. Pyruvate or oxygen supplementation abolishes the inhibitory effect of PaEVs on the growth of S. aureus

L-lactate dehydrogenase 2 and formate acetyltransferase are enzymes associated with the pyruvate fermentation pathway in *S. aureus* (Fuchs et al., 2007). Without oxygen supplementation, these enzymes convert pyruvate to lactate and formate, respectively. Therefore, we further examined whether pyruvate or oxygen supplementation alters the effect of PaEVs. As shown in Fig. 5A, the number of *S. aureus* treated with PaEVs recovered after supplementation with pyruvate. In addition, the cultivation of *S. aureus* under vigorous agitation abolished this growth inhibitory effect of PaEVs (Fig. 5B). These results suggested that PaEVs suppress a part of pyruvate fermentation in *S. aureus*.

4. Discussion

P. aeruginosa can adapt itself to survive under stress conditions and secrete several exoproducts to outcompete other microorganisms. However, it commonly coexists with *S. aureus* (Bessa et al., 2015). Therefore, we expected that *P. aeruginosa* and *S. aureus* would have some specific interactions, in particular via EVs. In the present study, the effect of PaEVs on the growth of *S. aureus* was focused on. Because PaEVs are released into the culture supernatant of *P. aeruginosa*, the growth of *S. aureus* in the culture supernatant was first monitored. From low residual nutrients in the *P. aeruginosa* culture supernatant and the activities of several exoproducts of *P. aeruginosa*, it was not surprising that the growth of *S. aureus* in the culture supernatant of *P. aeruginosa* was retarded. The growth of *S. aureus* recovered at 24 h of cultivation, suggesting that this retardation of *S. aureus* growth was found only in the early adaptation. At 6 h of incubation, the growth of a wide range of microorganisms including *E. faecalis, S.* Typhimurium, *E. coli, L. monocytogenes* and *C. albicans* was also inhibited in the *P. aeruginosa* supernatant. Interestingly, the growth inhibitory effect of *P. aeruginosa* supernatant was not observed in *A. baumannii*. The tolerant mechanism of *A. baumannii* against *P. aeruginosa* exoproducts is unknown. The possibility is that *A. baumannii* possesses metabolic flexibility for nutrient acquisition (Mortensen and Skaar, 2013) or the membrane structure of *A. baumannii* may be closely related to that of *P. aeruginosa* (Du et al., 2017).

PaEVs were then purified from *P. aeruginosa* supernatant and analyzed. The size of PaEVs purified in this study correlates with that of the previous reports (Choi et al., 2011). Li et al. (1998) have demonstrated that peptidoglycan hydrolases are detected in PaEVs, and these

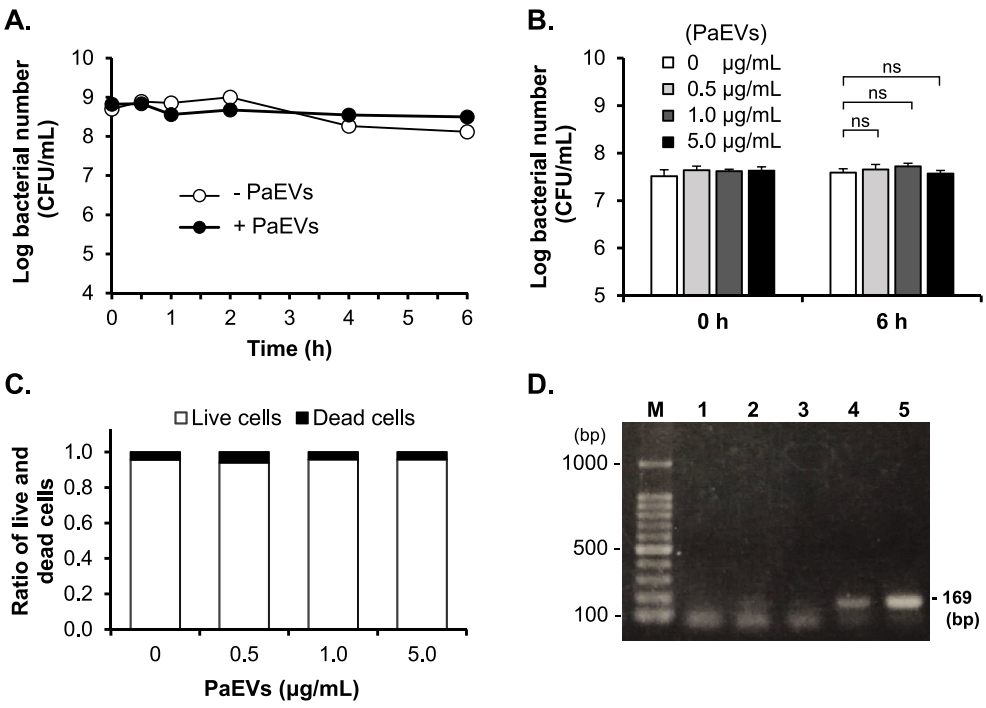

Fig. 3. Investigation of the bactericidal and bacteriolytic activity of PaEVs against *S. aureus*. (A) *S. aureus* suspension (OD_{600nm}=1.0 in PBS) was incubated at 37°C with or without 1.0 μg/mL PaEVs. At 0–6 h of incubation, the number of *S. aureus* was enumerated ($n = 3$). (B) *S. aureus* suspension (OD_{600nm}=0.2 in 0.85% NaCl) was incubated with 0, 0.5, 1.0 and 5.0 μg/mL PaEVs. At 0 and 6 h of incubation, the number of *S. aureus* was enumerated ($n = 4$ from 2 independent experiments). *P*-value was calculated using Kruskal Wallis H-test with post hoc Mann-Whitney *U* test. ns: not significant. (C) Viability of *S. aureus* after incubation with PaEVs. *S. aureus* suspension (OD_{600nm}=0.2 in 0.85% NaCl) was incubated at 37°C with 0, 0.5, 1.0 and 5.0 μg/mL PaEVs. At 6 h of incubation, live and dead cells were stained using LIVE/DEAD™ BacLight™ Bacterial Viability Kit and quantified using BZ-X Analyzer software from sixteen randomly selected images. (D) Bacteriolytic activity of PaEVs against *S. aureus*. *S. aureus* was incubated at 37°C for 6 h with or without 1 μg/mL PaEVs, and 10 μg/mL lysostaphin (as control). After removal of the bacterial cells and cell debris, 1 μL of the lysate was used as a template for PCR to amplify a partial *pfkA* gene. Lanes M: Marker, 1: negative control (use of distilled water as template), 2: *S. aureus* without PaEV treatment, 3: *S. aureus* treated with 1 μg/mL PaEVs, 4: *S. aureus* treated with lysostaphin, 5: positive control (use of isolated genomic DNA of *S. aureus* as template). The PCR product with a size of 169 bp was observed by agarose gel electrophoresis.

Table 2
Differential protein production in *S. aureus* after treatment with PaEVs for 6 h.

Protein name	MW (kDa)	Predicted localization	Fold Change	*P*-value*
L-Lactate dehydrogenase 2	34	Cytoplasm	0.46	0.003
Formate acetyltransferase	85	Cytoplasm	0.26	0.003
Thymidine kinase	22	Cytoplasm	0.29	0.008
Adenylosuccinate synthetase	48	Cytoplasm	0.48	0.014
Methionine aminopeptidase	28	Cytoplasm	0.50	0.016
L-Threonine dehydratase catabolic TdcB	37	Cytoplasmic membrane	0.27	0.033
Transcriptional regulatory protein SrrA	28	Cytoplasm	0.30	0.037
Pyridoxal 5′-phosphate synthase subunit PdxS	32	Cytoplasm	0.52	0.044

* In comparison to untreated control, proteins that show significant difference (*P*- value less than 0.05) are shown.

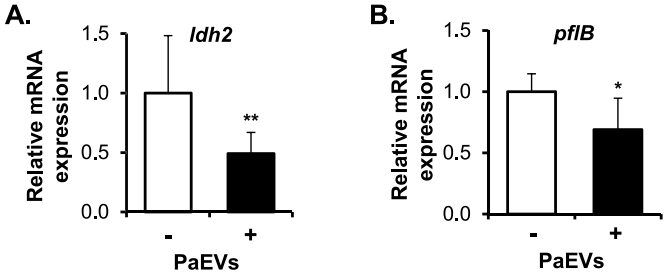

Fig. 4. Relative mRNA expression of *ldh2* and *pflB* genes in *S. aureus* after treatment with PaEVs. *S. aureus* was inoculated into TSB with or without 1 μg/mL PaEVs. After incubation for 6 h, bacterial cells were harvested, and total RNA was prepared. After cDNA was synthesized, relative mRNA expression of (A) *ldh2* and (B) *pflB* genes was evaluated by RT-qPCR and normalized to the housekeeping gene, *ftsZ* ($n = 9$ from 3 independent experiments). *P*-value was calculated using Mann-Whitney *U* test. *: $P < 0.05$, **: $P < 0.01$.

enzymes can hydrolyze peptidoglycans in both gram-positive and gram-negative bacteria (Kadurugamuwa and Beveridge, 1996). In addition, pyocyanin released from *P. aeruginosa* exhibits widely antibacterial activity (Jayaseelan et al., 2014). The presence of pyocyanin in PaEVs was evaluated by colorimetric method. However, no pyocyanin in 5 μg PaEVs was detected. The minimum detectable pyocyanin by the colorimetric method was further examined by using 2-time serial dilutions of the *P. aeruginosa* supernatant, and 5 μg PaEVs was predicted to contain less than 20 ng/mL of pyocyanin. A study by Noto et al., 2017 showed that 4 μg/mL pyocyanin reduced growth of *S. aureus*. Therefore, it is unlikely that pyocyanin less than 20 ng/mL in 5 μg PaEVs is the main factor responsible for the inhibitory effect of PaEVs on *S. aureus* growth. The proteomic analysis results demonstrated that endolytic peptidoglycan transglycosylase RlpA is included in the PaEVs. These factors may contribute to the antibacterial effect of PaEVs. Our results showed that the purified PaEVs inhibited *S. aureus* growth at the early phase of incubation in a dose-dependent manner, and this effect was also found in the methicillin-resistant *S. aureus* ATCC1717 (Fig. 2D). However, the PaEVs did not show antibacterial effect against several gram-positive and gram-negative bacteria such as *E. faecalis, S.* Typhimurium, *E. coli* and *L. monocytogenes*. These results indicated that the RlpA and/or pyocyanin may not be primarily associated with antibacterial effect of PaEVs against *S. aureus*.

According to the proteomic analysis (Table S2), LasA protease was detected in PaEVs. This enzyme has been reported to have staphylolytic activity and is well-known as a staphylolysin (Barequet et al., 2009). Based on this activity, we further assessed the bactericidal and bacteriolytic effects of PaEVs on *S. aureus*. However, the significant bactericidal and bacteriolytic effects of PaEVs against *S. aureus* could not be detected.

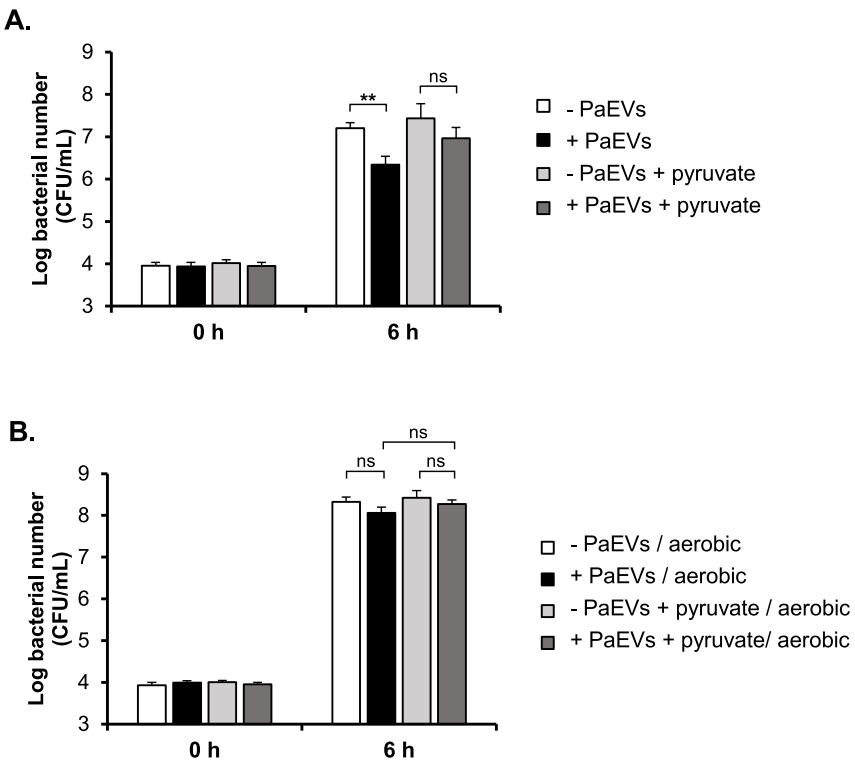

Fig. 5. Effect of PaEVs on the growth of *S. aureus* after supplementation with pyruvate and oxygen. (A) *S. aureus* was inoculated into TSB and incubated at 37°C under static condition with or without 1.0 μg/mL PaEVs, and supplemented with or without 15 mM pyruvate. (B) *S. aureus* was cultured in TSB under aerobic condition with or without 1.0 μg/mL PaEVs and 15 mM pyruvate. At 0 and 6 h of incubation, the number of *S. aureus* was enumerated by plate count assay ($n = 7$ from 3-independent experiments). *P*-value was calculated using Kruskal Wallis H-test with post hoc Mann-Whitney *U* test. ns: not significant, **: $P < 0.01$.

A previous study has demonstrated that the PQS plays an important role in inhibiting the growth of several bacteria (Qin et al., 2009). Especially, *S. aureus* growth and biofilm were strongly inhibited by PQS-containing PaEVs (Zhao et al., 2022). Toyofuku et al. (2010) have reported that the bacterial inhibition activity of PQS was restored in $FeCl_3$ supplemented medium, suggesting that the iron-chelating activity of PQS is involved in *S. aureus* growth inhibition. Although the presence of PQS molecules in PaEVs was not proved in our study, we further examined whether the inhibitory effect of PaEVs on the growth of *S. aureus* is caused by the iron-chelating activity of PQS. As shown in Fig. S5, iron supplementation was not recovered the inhibitory effect of PaEVs on the growth of *S. aureus*. Taken together, our results indicate that PaEVs act specifically on *S. aureus*, inhibiting growth rather than exhibiting bactericidal or bacteriolytic activity. In addition, this mechanism was found to be independent of the iron-chelating activity of PQS that has been shown in previous reports (Zhao et al., 2022; Toyofuku et al., 2010). These findings prompt us to further investigate in more detailed mechanisms of PaEVs on growth inhibition of *S. aureus*.

Differential protein production in *S. aureus* after treatment with or without PaEVs was then analyzed. The results in Table 2 showed that formate acetyltransferase had the highest decreasing fold change (3.8 times lower than that of the untreated control, with *P*-value = 0.00337). This enzyme is involved in the reversible conversion of pyruvate to formate and acetyl-CoA in the fermentation pathway of *S. aureus* (Ferreira et al., 2013). L-lactate dehydrogenase 2 is another enzyme that is involved in the pyruvate metabolism and synthesizes lactate from pyruvate when *S. aureus* is under anaerobic condition (Ferreira et al., 2013). We also found that not only protein level, but also mRNA expression of both *ldh2* and *pflB* genes in *S. aureus* was significantly reduced by PaEV-treatment. These results contradict the study of Filkins et al. (2015), which reported that *P. aeruginosa* promotes the expression of *pflB, ldh*, and *adh* genes in *S. aureus* (Filkins et al., 2015). These contradictory results may be due to the 2-heptyl-4-hydroxyquinoline N-oxide and siderophores, which are important for promoting the expression of fermentation-related genes in *S. aureus*, may not be associated with PaEVs. Troitzsch et al., 2021 showed that transcription of *Idh2* and *pflB* is regulated by catabolite control protein A (CcpA). However, the differential proteomic analysis between PaEV-treated and non-treated *S. aureus* demonstrated that CcpA in *S. aureus* was not significantly altered (0.7-fold change after PaEV treatment with *P*-value = 0.63). Therefore, the mechanism of how *ldh2* and *pflB* transcription are downregulated is unclear. A previous study has shown that pyruvate promotes the expression of *ldh2* and *pflB* genes when *S. aureus* is cultured under anaerobic condition (Fuchs et al., 2007). From this study, we hypothesized that some molecules in PaEVs may affect pyruvate, reduce the expression of *ldh2* and *pflB* genes and consequently inhibit the growth of *S. aureus*, especially when *S. aureus* is cultured under static condition. This hypothesis was proved by supplementing pyruvate and oxygen into the reaction of the inhibition assay. As shown in Fig. 5, both supplementations abolished the PaEV activity.

Since *P. aeruginosa* and *S. aureus* have complex interactions to regulate the growth and pathogenicity of each other, clear elucidation of the interaction mechanisms mediated by PaEVs will provide new strategies for the protection and/or treatment of their coinfections. The results obtained in this study are important for better management of *S. aureus* in the competitive stage of *P. aeruginosa* coinfections, not only antibiotic-susceptible strains but also antibiotic-resistant strains that become a major therapeutic problem worldwide. However, it should be noted that in vivo experiments should be conducted for further clinical applications. In addition, an investigation of the molecules including in PaEVs that specifically react to *S. aureus* is required.

5. Conclusions

In this study, we demonstrated that PaEVs specifically act on *S. aureus* to suppress the growth by affecting the enzymes in the pyruvate fermentation pathway. This growth inhibitory effect is independent of the iron-chelating activity of PQS but recovers after supplementation with pyruvate or oxygen.

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Ethical statement

N/A

CRediT authorship contribution statement

Takahito Ishiai: Data curation, Formal analysis, Funding acquisition, Investigation, Visualization, Writing – original draft. **Phawinee Subsomwong:** Conceptualization, Formal analysis, Funding acquisition, Project administration. **Kouj Narita:** Methodology, Resources. **Noriaki Kawai:** Methodology, Visualization. **Wei Teng:** Formal analysis. **Sachio Suzuki:** Validation. **Rojana Sukchawalit:** Writing – review & editing. **Akio Nakane:** Methodology, Resources, Funding acquisition, Writing – review & editing. **Krisana Asano:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100190.

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