High antimicrobial activity of lactoferricin-expressing
Bacillus subtilis strains

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Summary
The lactoferricin expressed in Bacillus subtilis is relatively low in yield, making it hard to apply in industrial settings. We constructed a six tandem repeat of lactoferricin cDNA driven by promoter PtrnQ. After transformation, two transformants P245 and P263 possessing a stable inheritance of plasmid and high expression of lactoferricin were selected. The bactericidal activities, 1 μl of aliquot of a total 5.5 ml of solution extracted from 5 ml of cultured P245 and P263, were equivalent to the efficacy of 238.25 and 322.7 ng of Ampicillin against Escherichia coli, respectively, and 366.4 and 452.52 ng of Ampicillin against Staphylococcus epidermidis respectively. These extracts were able to kill an Ampicillin-resistant E. coli strain. The bactericidal activities of P245 and P263 equivalent to the efficacy of Tetracycline against Vibrio parahaemolyticus and V. alginolyticus were also determined. Moreover, the bactericidal activities of P245 and P263 were 168.04 and 249.94 ng of Ampicillin against Edwardsiella tarda, respectively, and 219.7 and 252.43 ng of Tetracycline against Streptococcus iniae respectively. Interestingly, the survival rate of E. tarda-infected tilapia fry fed the P263 extract displayed a significantly greater than that of the fried control strain. Collectively, these B. subtilis transgenic strains are highly promising for use in animal husbandry during a disease outbreak.

Introduction
Antibiotics have been used for medical treatment purposes and combating pathogens common in animals and fish. For example, in the aquaculture industry, highly intensive culture systems are favoured to improve efficiency, yet such systems are vulnerable to viruses, bacteria and parasites with consequences ranging from mortality to financial losses. This has resulted in an extraordinary reliance on antibiotic drugs, which, in turn, can induce pathogenic resistance (Moreno et al., 2006; Lai et al., 2011), again causing even more severe impacts on fish cultivated in hatcheries (de la Fuente-Núñez et al., 2013). Depending on their administration, the use of antibiotics can also result in contaminated soil and water sources (Hsiao and Chen, 2015). For example, about 75% of administered antibiotic drugs will eventually be released into the natural aquatic environment (Lalumera et al., 2004). Although the risk of antibiotics in the environment to humans has not been fully evaluated, antibiotics are well known to cause safety problems during pregnancy (de Tejada, 2014). Furthermore, genotoxic antibiotic residues have been detected in hospital wastewater (Hartmann et al., 1998). Therefore, finding a viable alternative antibacterial protein with efficacy against bacteria, but without side effects, becomes an important issue.

Nowadays, more than 880 antimicrobial peptides (AMPs) have been discovered or predicted (Brogden, 2005a), such as cytokines (Kaplan et al., 2017), chemokines (Yang et al., 2003), neuropeptides (Kowalska et al., 2002; Brogden et al., 2005b), peptide hormones (Allaker and Kapas, 2003; Ganz, 2006) and derivatives of some large molecular weight proteins (Liepke et al., 2003). The AMP bovine lactoferrin is an iron-binding glycoprotein with 80 kDa from the transferrin family. It is widely found in mammalian secretions, such as breast milk, saliva, tears and pancreatic juice (Caccavo et al., 2002). Additionally, it can be obtained in granules of granular white blood cells (PMNs) (Cramer...
et al., 1985). The antimicrobial capacity of lactoferrin has been widely studied since it was discovered to have the ability to kill microorganisms (Reiter and Oram, 1967). Apart from antimicrobial capacity (Stephens et al., 1980; Rainard, 1988), lactoferrin not only displays antifungal activity (Kirkpatrick et al., 1971) and antiviral ability (Valenti and Antonini, 2005) but also suppresses inflammation (Elass et al., 2002). An antimicrobial short peptide fragment of about 24–25 amino acids, named lactoferricin, is cleaved at the N-terminus of lactoferrin by pepsin. It shows antibacterial efficacy approximately 12-fold over that of undegraded lactoferrin (Bellamy et al., 1992a). The secondary structure of lactoferricin is changed from original alpha helix to twisted beta sheet after separation from intact lactoferrin (Hwang et al., 1998), allowing lactoferricin to form an amphiphile with a positively charged hydrophilic region and a hydrophobic area at two opposite ends of the peptide (Gifford et al., 2005). Interestingly, unlike bovine lactoferrin that contains five potential N-glycosylation sites at Asn233, 281, 368, 476 and 545 (Karav et al., 2017), lactoferricin is unlikely that the bactericidal function of lactoferricin might be affected by glycosylation modification since it is a small peptide derived from the 17–41 amino acid residues of lactoferrin.

Lee et al. (2019) reported that lactoferricin-producing Bacillus subtilis transgenic strains not only have a preventive effect on Edwardsiella tarda-infected tilapia fry in vivo but also show antimicrobial ability on different types of pathogens, such as Escherichia coli, Staphylococcus epidermidis and Vibrio parahaemolyticus in vitro. However, to improve the application value of B. subtilis transgenic strains, it is necessary to increase the expression level of recombinant lactoferricin in the bacteria. Generally, several strategies can be employed to increase the expression of foreign proteins in bacterial hosts, including increasing plasmid copy numbers in the host, using appropriate ribosomal binding sites and applying stronger promoters (Makrides, 1996; Baneyx, 1999).

Promoter P43 is commonly used to express exogenous proteins in B. subtilis, as developed by Wang and Doi (1984). However, Song et al. (2016) used GFP intensity as a screening criterion of promoter strength. Screened from the promoters of 83 genes of B. subtilis, the expression level of the PtrnQ promoter of the tRNA gene encoding tRNA with Arginine-corresponding codon can reach 4.53 times as compared to that of P43. Therefore, based on the difference of expression level, we replaced P43, originally used in the construction of plasmid, with the more powerful PtrnQ promoter to increase the expression of recombinant lactoferricin in B. subtilis transgenic strains, thereby enhancing the antibacterial capacity of the transgenic strains and reducing the cost of application.

Results

Plasmid, gene transfer rate and transformant screening

The 8 kb of expression plasmid pPtrnQ-6LFB-GFP (Fig. 1A) was constructed. It contained promoter PtrnQ, six tandem repeats (6LFB) of the N-terminal fragment of lactoferricin fused with GFP reporter, and anti-Ampicillin and anti-Tetracycline resistance genes, which allow this plasmid to shuttle between B. subtilis and E. coli. In total, we performed electroporation 65 times. For each gene transfer, 1.3 × 10⁸ competent cells were applied, resulting in 10–25 colonies grown on Tetracycline-selective agar plates. The average transferal rate was 1.9 × 10⁻⁵–7.6 × 10⁻⁶. Finally, we obtained about 800 B. subtilis colonies for further PCR screening. As described in the Experimental procedures section, three steps were performed to pick up putative transformants. We found 480 PCR-positive clones out of these 800 colonies in the first step. Thirty colonies exhibiting positive antimicrobial activity were obtained from the second step, and two transfectants, P245 and P263, were finally selected after we examined the stable inheritance of plasmid for several passages.

Using PCR to detect plasmid DNA transferred into the host organism B. subtilis

To further confirm that exogenous plasmid PtmQ-6LFB-GFP had been electroporated in transfectants P245 and P263, we performed PCR analysis in more detail. Colonies of P245 and P263 grown on the agar plate after 10 passages were directly used as templates to amplify the coding region of recombinant GFP cDNA with the forward primer GFP-NheI and reverse primer GFP-HindIII (Fig. 1A). The expected molecular size of recombinant GFP fragments after PCR amplification was 700 bp. No DNA fragment was amplified from host cell B. subtilis WB800, which served as a negative control since it contained no plasmid (Fig. 1B). However, a PCR product with molecular mass of about 700 bp in size was shown in the DNA extracted from colonies P245 and P263 (Fig. 1B). The size of this DNA fragment was exactly the same as that of the PCR product amplified from plasmid pPtmQ-6LFB-GFP, and it served as a positive control (Fig. 1B).

Copy number of plasmid harbourd by the transgenic strains

To identify the plasmid copy number in transgenic B. subtilis strains P245 and P263, we used DIG-labelled GFP cDNA fragment as a probe to hybridize with the plasmids contained in the transgenic P245 and P263 strains using the Dot blot analysis method. Plasmid
pPtrnQ-6LFB-GFP served as a positive control, while the *B. subtilis* WB800 strain with no plasmid served as a negative control. Cell lysates were extracted from strains P245 and P263 when their absorbance optical density reached 1.

VisionWorks software was used to analyze the signal intensities of three known concentrations of plasmid DNA in the positive control group; thus, the signals obtained from Dot blot analysis were quantified. As shown in Fig. 2A, 20 ng of plasmid DNA showed the equivalent of $2 \times 10^3$ units; 10 ng of plasmid DNA exhibited the equivalent of $1.16 \times 10^4$ units; and 5 ng of plasmid DNA displayed the equivalent of $6.38 \times 10^3$ units. Plasmid DNA was contained in 10, 5 and 2.5 μl of extracts out of bacterial liquid solution from the P245 strain at $1.7 \times 10^3$ units (Fig. 2B). Similarly, plasmid DNA was contained in 10, 5 and 2.5 μl of extracts out of bacterial liquid solution from the P263 strain at $1 \times 10^6$ CFU ml$^{-1}$, which represented $8.76 \times 10^3$, $3 \times 10^3$ and $5.99 \times 10^2$ units respectively.

The signal intensities of three known concentrations of plasmid were converted into the trendline to obtain the linear regression equation (LRE) of $y = 900.94x + 2162.9$ with $R^2$ equal to 0.997 (Fig. 2C). The Dot blot signal intensities of the plasmids contained in the 10 μl extracts of transgenic P245 and P263 strains were located in the range of the LR trendline. Therefore, we could obtain plasmid concentrations of 7.32 and 6.66 ng respectively. Meanwhile, the molecular weight of pPtrnQ-6LFB-GFP plasmid was 2.47 $\times$ 10$^6$ kDa, and the transgenic P245 and P263 strains each contained $1.7 \times 10^6$ CFU ml$^{-1}$ and $1 \times 10^6$ CFU ml$^{-1}$ respectively. Based on these parameters, the copy numbers of plasmid per cell of transgenic *B. subtilis* P245 and P263 strains were 1050 and 1626 respectively.

Using Western blot to analyze the recombinant lactoferricin produced by the transgenic strains

After confirming that the exogenous plasmid DNA had been harboured by transgenic P245 and P263 strains, we went further to examine whether the recombinant lactoferricin fused with GFP reporter was expressed in these two transformants using polyclonal antisemur against GFP protein. We used Coomassie blue staining to identify the total proteins extracted from the transgenic *B. subtilis* strains. A specific band with molecular weight of 44 kDa appeared in the extracts of P245 and P263, but not the extract of host cell *B. subtilis* WT800 strain (Fig. 3A). Furthermore, based on Western blot analysis, a positive hybridization band with an approximate MW of 44 kDa was detected in the total proteins extracted from the transgenic P245 and P263 strains. In contrast, no signal was detected in the host cell *B. subtilis* WB800 strain (Fig. 3B). Since the molecular weight of this hybridization-positive band was similar to that of the extra band shown on SDS-PAGE, we strongly suggest that the band with MW of 44 kDa is a recombinant lactoferricin fused with GFP reporter. Taken together, we conclude that the recombinant lactoferricin was expressed intracellularly in transgenic strains P245 and P263.

Bactericidal activity of recombinant lactoferricin produced by transgenic *B. subtilis* against common pathogens

After confirming that the transgenic *B. subtilis* P245 and P263 strains could express recombinant lactoferricin protein, we used an agar well diffusion assay to determine whether these two transgenic strains displayed bactericidal activity against *E. coli*, as a representative of Gram-negative bacteria and *S. epidermidis*, as a representative Gram-positive bacteria. The extracts from these strains...
were individually added into the wells on agar plate confluent with common pathogens, such as *E. coli* and *S. epidermidis*. The well containing Ampicillin exhibited an inhibition zone and thus served as a positive control, while the extract from *B. subtilis* WB800, which did not exhibit an inhibition zone, served as a negative control (Fig. 4). After measuring different dosages of Ampicillin with concentrations of 1, 1.5 and 3 µg, an average inhibition zone of 2.15, 3.61 and 5.84 mm, respectively, appeared on the *E. coli*-confluent plate (Fig. 4A and B). To understand the relationship between dose versus inhibition zone, the three doses and corresponding inhibition zones were converted into the trendline to obtain an LRE, as \( y = 1.762x + 0.637 \) with \( R^2 \) equal to 0.9741 (Fig. 4C). Interestingly, no inhibition zone was observed from the extract from the transgenic P263 strain without pepsin treatment, suggesting that the non-pepsin-digested six tandem repeats of recombinant lactoferrin do not have antibacterial activity. In contrast, the pepsin-treated extracts from transgenic P245 and P263 strains displayed an average inhibition zone of 2.74 and 3.48 mm respectively. After substituting an average inhibition zone of 4 times diluted extracts of P245 and P263 into the equation, antibacterial potency against *E. coli* with the equivalence of 1.19 and 1.61 µg of Ampicillin was shown respectively. This result was divided by the amount of extract (20 µl), multiplied back four times to compensate for the quadruple dilution of the working extracts and combined with the calculation of cell density of transgenic P245 and P263 strains, which was \( 1.7 \times 10^8 \) and \( 1 \times 10^8 \) CFU ml\(^{-1} \) respectively. When the OD\(_{600}\) reached 1, the bactericidal activity of 1 µl out of a total 5.5 ml of solution extracted from 5 ml of cultured P245 strain against *E. coli* was approximately equivalent to the potency of 238.25 ng of Ampicillin, while that of transgenic P263 strain was equivalent to 322.7 ng of Ampicillin.

We further confirmed whether the extracts from transgenic strains contained bactericidal activity against Gram-positive bacteria, such as *S. epidermidis*. No
inhibition zone was observed from the extract of B. subtilis WB800 or the extract of transgenic P263 without pepsin treatment (Fig. 4D and E), which suggested that the non-pepsin-digested six tandem repeats of recombinant lactoferricin had no antibacterial activity. Three dosages of Ampicillin with concentrations of 0.75, 1.50 and 3.00 \( \text{lg} \) showed an average inhibition zone of 1.69, 3.61 and 5.84 mm respectively. The LRE was \( y = 1.5443x + 0.7725 \) with \( R^2 \) equal to 0.9678 (Fig. 4F). The extracts from transgenic P245 and P263 strains displayed an average inhibition zone of 3.60 and 4.27 mm, respectively (Fig. 4D and E), suggesting that the antibacterial potency against S. epidermidis was equivalent to 1.83 and 2.26 \( \mu \text{g} \) of Ampicillin. Therefore, we concluded that the antimicrobial activity of P245 and P263 per \( \mu \text{l} \) from 5.5 ml of cultured solution against S. epidermidis was equivalent to 366.4 and 452.52 \( \mu \text{g} \) of Ampicillin respectively.

**Bactericidal activity of recombinant lactoferricin produced by transgenic strains against the Ampicillin-resistant E. coli strain, euryhaline pathogens and halophilic pathogens**

Similar to the strategy, we examined whether this extract of transgenic strain could kill an Ampicillin-resistant E. coli strain. Since this strain is susceptible to Tetracycline, we used Tetracycline served as a positive control on the agar well diffusion assay, while Ampicillin with a total amount of 2 \( \mu \text{g} \) to serve as a negative control. The results showed that Tetracycline with concentrations of 0.25, 1.00 and 1.50 \( \mu \text{g} \) exhibited an average inhibition zone of 3.78, 5.28 and 6.00 mm, respectively, on the agar plate confluent with Ampicillin-resistant E. coli strain (Fig. S1A). The extracts from transgenic P245 and P263 strains after pepsin treatment displayed an average inhibition zone of 5.58 and 5.88 mm respectively (Fig. S1B). Based on the calculation from LRE (Table 1), the bactericidal activity of P245 and P263 against this pathogen was equivalent to 1.25 and 1.38 \( \mu \text{g} \) of Tetracycline (Fig. S1C) respectively. Therefore, the bactericidal activity of extracts per \( \mu \text{l} \) from cultured P245 and P263 against Ampicillin-resistant E. coli strain was equivalent to the potency of 250.39 and 276.76 \( \mu \text{g} \) of Tetracycline respectively.

Next, we carried out another antimicrobial test on common euryhaline pathogens, namely E. tarda and S. iniae. Similar to the strategy described above, different dosages of Ampicillin with concentrations of 0.5, 1.0 and 1.5 \( \mu \text{g} \) were measured and showed an average inhibition zone of 3.04, 4.46 and 5.75 mm, respectively, on the E. tarda-confluent plate (Fig. S2A and B). The pepsin-treated extracts from transgenic P245 and P263 strains displayed an average inhibition zone of 3.85 and 4.56 mm respectively (Fig. S2B). Based on the calculation from LRE shown in Table 1, the antibacterial potency against E. tarda was equivalent to 0.84 and 1.25 \( \mu \text{g} \) of Ampicillin (Fig. S2C) respectively. Thus, the bactericidal activity of extracts per \( \mu \text{l} \) from the prepared P245 strain against E. tarda was equivalent to the potency of 168.04 \( \mu \text{g} \) of Ampicillin, while that of P263 strain was equivalent to 249.94 \( \mu \text{g} \) of Ampicillin.

For S. iniae, we used Tetracycline as the positive control. Three dosages of Tetracycline with concentrations...
Fig. 4. Bactericidal activity of recombinant lactoferricin produced by transgenic *B. subtilis* against common pathogens. (A, D) Using agar diffusion assay to examine the antibacterial efficacy of extracts from transgenic strains P245 and P263 (*n* = 6). Cell lysate was added into the well on agar plate confluent with common pathogen (A) *E. coli* (Gram-negative) or (B) *S. epidermidis* (Gram-positive). Ampicillin (Amp) with different concentrations, as indicated, served as a positive control for calculating the potency of recombinant protein produced by transgenic strains. Negative controls: well filled with water (control), pepsin-digested extracts from *B. subtilis* WB800 (WT) and non-pepsin-digested extracts from *B. subtilis* P263 (indicated by P263’). Experimental groups: the pepsin-treated extracts from transgenic strains P245 and P263 against (A) *E. coli* and (D) *S. epidermidis*. (B, E) The size of each inhibition zone shown in Figures A and D was measured and presented as a bar chart. (C, F) The regression equation was obtained based on three known dosages of Ampicillin that corresponded to their inhibition zones. Antibacterial potency equivalent to Ampicillin of extracts from P245 and P263 against *E. coli* and *S. epidermidis* was determined.

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calculation from LRE shown in Table 1, the bactericidal extract against *V. alginolyticus* line with concentrations of 0.25, 0.75 and 1.50 mm respectively (Fig. S3D and E). The extracts equivalent to 192.81 ng of Tetracycline (Fig. S3C). was equivalent to the potency of *V. parahaemolyticus* from P245 and P263 strains displayed an average inhibi-

*LRE represented the relationship between antibiotic dose versus inhibition zone shown on the agar plate confluent with each patho-
gen as indicated. Amp, Ampicillin; *R*², Coefficient of determination; Tet, Tetracycline. of 0.50, 1.00 and 1.50 μg showed an average inhibition zone of 5.28, 6.54 and 7.48 mm on tryptic soy medium mixed with 3% defibrinated sheep blood respectively (Fig. S2D). The pepsin-treated extracts from transgenic P245 and P263 strains displayed an average inhibition zone of 6.65 and 7.01 mm respectively (Fig. S4D and E). Based on the calculation from LRE shown in Table 1, the antibacterial potency of P245 and P263 against *S. iniae* was equivalent to 1.10 and 1.26 μg of Tetracycline (Fig. S2F) respectively. Therefore, the bac-
tericidal activity of extracts from cultured P245 strain against *S. iniae* per μl was equivalent to the potency of 219.70 ng of Tetracycline, while that of P263 strain was equivalent to 252.43 ng of it.

We also carried out another antimicrobial activity experiment using extracts of P245 and P263 strains against halophilic pathogens, such as *V. parahaemolyticus* and *V. alginolyticus*. Tetracycline with concentrations of 0.25, 0.75 and 1.50 μg exhibited an average inhibition zone of 4.16, 5.86 and 6.82 mm, respectively, on the *V. parahaemolyticus*-confluent plate (Fig. S3). The extracts from P245 and P263 strains after pepsin treatment displayed an average inhibition zone of 5.73 mm and 5.88 mm respectively (Fig. S3A and B). Based on calculation from LRE shown in Table 1, the bactericidal activity per μl extract from P245 strain against *V. parahaemolyticus* was equivalent to the potency of 178.42 ng of Tetracycline, while that of P263 strain was equivalent to 192.81 ng of Tetracycline (Fig. S3C).

We continued to define the bactericidal activity of the extract against *V. alginolyticus* and found that Tetracycline with concentrations of 0.25, 0.75 and 1.50 μg showed an average inhibition zone of 2.49, 4.43 and 5.71 mm respectively (Fig. S3D and E). The extracts from P245 and P263 strains displayed an average inhibition zone of 5.44 and 5.58 mm, respectively, on the *V. alginolyticus*-confluent plate (Fig. S3D and E). Based on calculation from LRE shown in Table 1, the bactericidal activity of P245 and P263 against *V. alginolyticus* was equivalent to 1.32 and 1.38 μg of Tetracycline (Fig. S3F) respectively. Therefore, the bactericidal activity of 1 μl extract from P245 strain against *V. alginolyticus* was equivalent to the potency of 264.25 ng of Tetracycline, while that of P263 strain was equivalent to 275.73 ng of Tetracycline.

**In vivo pathogen challenge test**

*E. tarda* is a typical freshwater and seawater pathogen commonly found in aquaculture fish. The typical symptoms of *E. tarda*-infected fish are gastrointestinal-related pathogenesis, such as aggravation, internal bleeding and stomach disintegration, which causes high mortality in aquaculture ponds. Since transgenic *B. subtilis* strain P263 exhibited the highest bactericidal activity, as shown above the agar diffusion assay, we selected this strain for *in vivo* pathogen challenge. Meanwhile, although tilapia are commonly cultured in freshwater, sometimes tilapia are also cultured in seawater for better taste quality of muscle. Accordingly, it would be more practical to know whether the antibacterial efficacy of P263 extracts against *E. tarda* differed between tilapia cultured in freshwater and seawater. Therefore, we performed an *in vivo* system to determine the effect of feeding extracts from P263 strain on the survival rate of *E. tarda*-infected tilapia fry both in freshwater and seawater environments.

After 5 h of immersion in the seawater without containing pathogen, the fry in this mock group remained alive and healthy (Fig. 5A). In contrast, the survival rate was gradually decreased in the *E. tarda*-infected group fed with *B. subtilis* WB800 strain, which served as a negative control. The survival rate of this group even could reach 0% at 26 days post-*E. tarda* infection (dpi) (Fig. 5A). However, the average survival rate of *E. tarda*-infected fry fed with transgenic strain P263 expressing lactoferricin was 77% at 26 dpi. We also noticed that all dead fish from each group exhibited gastrointestinal-related pathogenesis caused by *E. tarda* infection.

The survival rate of *E. tarda*-infected tilapia fry fed with the *B. subtilis* control strain was rapidly decreased in the freshwater environment (Fig. 5B). For example, the survival rates were reduced to 55 and 43% (*n* = 40) at 6 and 9 dpi. In contrast, the survival rate of *E. tarda*-infected tilapia fry fed with the extracts from transgenic strain P263 exhibited a gradual decrease to 80% at 9 dpi and maintained this level until the end of the trial at 26 dpi, suggesting that fish fry fed with the extract from the lactoferricin-containing transgenic strain P263 displayed a significantly greater survival rate compared to that of the fry-fed control strain.

Previously, Lee et al. (2019) reported that transgenic *B. subtilis* strain T13 and T1 could produce recombina-

Table 1. The linear regression equation (LRE) of each pathogen displayed on agar well diffusion assay.

| The pathogen confluent on plate | Antibiotic used | LRE | *R*² |
|---------------------------------|-----------------|-----|-----|
| Ampicillin-resistant *E. coli* strain | Tet | *y* = 2.225x + 2.7961 | 0.961 |
| *E. tarda* | Amp | *y* = 1.7338x + 2.3933 | 0.9532 |
| *S. iniae* | Tet | *y* = 2.2x + 4.2333 | 0.993 |
| *V. parahaemolyticus* | Tet | *y* = 2.0604x + 3.8953 | 0.925 |
| *V. alginolyticus* | Tet | *y* = 2.5086x + 2.1215 | 0.946 |

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Fig. 5. In vivo study to determine the effect of bactericidal activity directed by recombinant lactoferricin produced by B. subtilis strains to defend against pathogen infection in fish fry.

A. Survival rate fish fry kept in seawater.
B. Survival rate fish fry kept in freshwater. Tilapia fry fed with wild-type (WT) strain, and transgenic strains T13 and P263 were tested with E. tarda. Transgenic T13 and P263 strains were transformed cells expressing recombinant lactoferricin. Tilapia fry immersed in freshwater and seawater without E. tarda served as a mock group. Sample size for each assessment group was 40. The cumulative average survival rate was recorded every three days from the day the tilapia fry (n = 40) was fed B. subtilis and immersed in water containing E. tarda. Data are presented as an average from two independent trials. (A) Transgenic strains T13 and P263, WT and mock groups in seawater, and (B) transgenic strains T13, P263, WT and mock groups in freshwater.

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high competence of bactericidal activity. They found that *E. tarda*-infected tilapia fry fed with the T13 extracts showed a better survival rate compared to feeding with T1 extracts. Thus, for a more refined comparison, we wanted to compare the protective efficacy between these two lactoferricin-containing transgenic strains, P263 strain in this study and T13 from the previous study. The results demonstrated that the survival rate of *E. tarda*-infected tilapia fry fed with the P263 extract was 15-17% greater than that fry fed with T13 extract. Therefore, we concluded that the survival rate of tilapia fry fed with extracts from transgenic *B. subtilis* strains was increased after bacterial infection both in sweater and freshwater environment.

**Discussion**

*The extracts from lactoferricin-containing transgenic B. subtilis strains are able to effectively kill a broad spectrum of bacterial pathogens*

Lactoferricin has been reported to possess inhibitory activity against microorganisms, including Gram-negative bacteria, Gram-positive bacteria (Bellamy et al., 1992b), yeast, epidermal fungi (Dermatophytes), filamentous fungi (Bellamy et al., 1994), parasites (Tanaka et al., 1995; Omata et al., 2001) and viruses (Siciliano et al., 1999; Berkhout et al., 2002; Di Biase et al., 2003). In this study, we obtained consistent results that support the conclusion reported by Gifford et al. (2005) since we found that the extracts from transgenic *B. subtilis* strains P245 and P263 that express recombinant lactoferricin can effectively kill a variety of bacteria, including Gram-positive, such as *S. epidermidis* and *S. iniae*, and Gram-negative, such as *E. coli*, *E. tarda*, *V. alginolyticus* and *V. parahaemolyticus*. More specifically, extracts from transgenic *B. subtilis* strains P245 and P263 can kill *E. coli* and *S. epidermidis* that grow in a general environment, *E. tarda* and *S. iniae* that grow in a wide dilution of saline environment, and *V. alginolyticus* and *V. parahaemolyticus* that grow in a strictly high saline environment. Therefore, these six genera of bacteria represent a large part of the bacterial species and waterbody environments, indicating that the recombinant lactoferricin showed a wide range of bacteriostatic activity. Therefore, these lactoferricin-producing transgenic *B. subtilis* strains with broad bacteriostatic activity may potentially provide a versatile alternative for application in the aquaculture industry because different species of fish and shellfish are cultivated in various water environments under different salinities. Moreover, the application of these transgenic strains may drastically reduce the widespread use of antibiotics in the aquaculture field. More importantly, recombinant lactoferricin is a biodegradable protein; thus, it will not remain and accumulate in the environment like antibiotics, mitigating the impact on food chain accumulation of environmental residues.

*The bactericidal activities of transgenic strains using promoter PtrnQ are dramatically improved*

Whereas the transgenic *B. subtilis* T1 and T13 strains obtained from the previous work of Lee et al. (2019) used P43 promoter, the transgenic *B. subtilis* P245 and P263 strains selected from this study used PtrnQ promoter to drive the expression of recombinant lactoferricin. The results demonstrated that the transgenic P245 and P263 strains were superior to transgenic T1 and T13 strains in terms of bactericidal activity against *E. coli* (Table 2). To rule out the possible effect of plasmid copy number, we compared transgenic P245 and T1 since these two strains harboured around 1000 plasmid copies. Unexpectedly, the bactericidal activity of P245 strain against *E. coli* displayed about four-fold greater bactericidal activity than that of T1 strain. Additionally, the bactericidal activity of P245 against pathogens, such as *S. epidermidis* and *E. tarda*, was about two-fold higher than that of T1 (Table 2). On the other hand, we noticed that six tandem repeats of lactoferricin cDNA, which is a functional domain constructed in the expression plasmid, had transferred into the P245 strain, while only three tandem repeats of lactoferricin cDNA exist in the T1 strain. However, the bactericidal activity of the extract from the P245 strain improved significantly over that of the T1 strain by more than two-fold (Table 2), suggesting that the PtrnQ promoter exerts much stronger effect on lactoferricin expressed in *B. subtilis*. The data presented here were consistent with the conclusion reported by Song et al. (2016) who demonstrated that promoter PtrnQ could significantly improve the expression of exogenous genes in host cell *B. subtilis*. Nevertheless, we noticed that the general conclusion we made in this study was completely based on the above observation. Since the main purpose of this manuscript was to describe the high antimicrobial activity of the lactoferricin-expressing *B. subtilis* transgenic strains we screened, it was reasonable to expect that some bacterial strains having especially high antibacterial competence would be screened based on some unknown biological characteristics, even without following the general rules.

*Genetic engineering design protects lactoferricin-expressing host cells from being killed by the lactoferricin produced intracellularly*

It has been reported that lactoferricin has a bactericidal effect on *B. subtilis* cells (Bellamy et al., 1992b); it is critical to prevent transgenic *B. subtilis* strains from being
Table 2. The bactericidal activities of extracts from transgenic *B. subtilis* strains equivalent to the efficacious doses of antibiotic against different pathogens.

| Strain | Promoter | Tandem repeats | Copy number of plasmid | Pathogen | Antibacterial activity equivalent to antibiotic potency | Copy number of plasmid |
|--------|----------|----------------|------------------------|-----------|--------------------------------------------------------|------------------------|
|        | PtmQ     | 6x LFB         | 1050                   | V. parahaemolyticus | 238(Amp) 323(Amp) 56(Amp) 53(Amp) | 178(Tet) 250(Tet) 44(Tet) 26(Tet) |
| P245   | PtmQ     | 6x LFB         | 1626                   | E. coli | 238(Amp) 323(Amp) 56(Amp) 53(Amp) | 178(Tet) 250(Tet) 44(Tet) 26(Tet) |
| P263   | P43      | 6x LFB         | 931                    | S. epidermidis | 366(Amp) 453(Amp) 154(Amp) 130(Amp) | 252(Tet) 44(Tet) 33(Amp) |
|        | P43      | 3x LFB         | 647                    | E. coli (Ampicillin resistance) | 250(Tet) 277(Tet) ND ND | 252(Tet) 44(Tet) 33(Amp) |
|        | P43      | 3x LFB         | ND                     | E. tarda | 168(Amp) 250(Amp) 79(Amp) 33(Amp) | 193(Tet) ND ND |
|        | P43      | 3x LFB         | ND                     | S. iniae | 229(Tet) 252(Tet) ND ND | 193(Tet) ND ND |
|        | P43      | 3x LFB         | ND                     | V. paraaemolyticus | 178(Tet) 193(Tet) 44(Tet) 26(Tet) | 178(Tet) 193(Tet) 44(Tet) 26(Tet) |
|        | P43      | 3x LFB         | ND                     | V. alginolyticus | 264(Tet) 276(Tet) ND ND | 264(Tet) 276(Tet) ND ND |

ND, not determined.

a. Transgenic *B. subtilis* strains obtained from this study.
b. Transgenic *B. subtilis* strains previously published by Lee et al. (2019).
c. Tandem repeats of cDNA-encoding lactoferricin in expression vector.
d. An average copy number of plasmid per cell.
e. Antibacterial activity of extracts per µl was equivalent to the potency of Ampicillin (Amp) or Tetracycline (Tet) in ng.

Table 2 highlights the bactericidal activities of extracts from transgenic *B. subtilis* strains. The results indicate that the bactericidal activity driven by the P263 strain is equivalent to the potency of Ampicillin (Amp) or Tetracycline (Tet) in ng.

We also noticed that the growth rate of the transgenic *B. subtilis* strain was slower than that of the wild-type WB800 strain during cultivation. After 18 h of cultivation, the WB800 strain generally grew to OD_{600}, reaching 2.6, while the transgenic strains grew to OD_{600}, reaching 0.7 to 0.8 only, showing the different growth curve between nontransgenic and transgenic strains (Fig. S4). The phenomenon that delays the normal growth of the transgenic strain might be caused by the production of a large amount of recombinant protein. Transgenic *B. subtilis* strain growth results found in the previous study of Lee et al. (2019) showed a similar trend as well.

**Correlation between plasmid copy number and bacteriostatic activity in the transgenic *B. subtilis* strains**

In this study, we found that the bactericidal activities of transformants screened after electroporation were different, even though they contained the same expression vector. Dot blot results revealed the plasmid copy number in the transgenic *B. subtilis* strains we screened. The P263 strain contains 1626 copies per cell, while the P245 strain contains 1050 copies. Based on the agar well diffusion assay, we found that the P263 strain exhibits a higher bactericidal activity than that of the P245 strain. It can therefore be concluded that plasmid copy number correlates with antibacterial potency. However, we did not conclude any certain proportional relationship between plasmid copy number and bacteriostatic activity. For example, although P263 contains 1.5-fold higher plasmid copies compared to P245, the bacteriostatic activity driven by *B. subtilis* P263 strain is not 1.5 times greater than that of the P245 strain. Clearly, the plasmid copy number in the transgenic *B. subtilis* strains is not linearly related to bactericidal activity. Therefore, we propose that the plasmid copy number is only one of the factors, but not the sole one, that impacts antibacterial potency in the transgenic *B. subtilis* strain.
GFP expression was not expressed in the transformants

The expression plasmid used to generate the transgenic strains of B. subtilis was pTmQ-6LFB-GFP, which contains six tandem repeats of lactoferrin fused with GFP reporter. The original idea was to take advantage of GFP expressed in the colonies as a selection marker. When this construct was transformed, the GFP reporter could not be observed in the transformants, including P263 and P245, even though the sequence of GFP cDNA fragment was correct using DNA sequencing and the recombinant lactoferrin-fused GFP was detected by Western blot analysis using antiserum against GFP in the transformants. To explain this, we speculate that the GFP motif might be hindered or blocked somehow within the structure of entire recombinant protein in this construct. Solving this problem will be a priority as we conduct future experiments.

Potential applications

Environmental crises arising from the excessive use of antibiotic drugs have prompted researchers to seek new antibacterial strategies, such as the use of probiotics in aquaculture, which inhibits the onset of bacterial infection (Kuebutornye et al., 2019). Newaj-Fyzul et al. (2007) reported that it takes 14 days to induce immune response against Aeromonas sp. in rainbow trout after having been fed $1 \times 10^7$ B. subtilis wild-type strain per gram of feed as a probiotic supplement. When $1 \times 10^7$ B. subtilis cells were fed tilapia per gram of feed for 58 days, Aly et al. (2008) also reported antibacterial effects against A. hydrophila and P. fluorescens. Additionally, Tseng et al. (2009) demonstrated that white shrimp could display antimicrobial effect against V. alginolyticus after having been fed with $1 \times 10^5$–$1 \times 10^8$ B. subtilis cells per kilogram of feed for 98 days. In sum, the aquatic organisms fed with wild-type B. subtilis as probiotics have shown a higher survival rate against the invasion of bacterial pathogens. However, we noticed that the above method of feeding probiotics to suppress various pathogens is completely dependent on the induction of immune response in animals, which means that it commonly takes 2 weeks to 3 months to achieve complete protection. Nevertheless, this strategy might be hard to implement in the case of acute disease outbreak. Therefore, the application of transgenic B. subtilis P245 and P263 strains generated by this study will be a good alternative not only because they are probiotic, but also because they contain the recombinant antibacterial peptide, lactoferrin, which is able to kill a broad spectrum of pathogens directly and immediately. For example, we demonstrated that the extracts from transgenic P245 and P263 strains were able to kill the Ampicillin-resistant E. coli strain, which supports the conclusion reported by Flores-Vilasenor et al. (2010) who pointed out that lactoferrin protein has bactericidal activity against Methicillin-resistant Staphylococcus aureus and E. coli. Interestingly, the extracts from P245 and P263 strains displayed a high bactericidal activity against S. iniae, which is a highly infectious pathogen between humans and fish. This evidence is of great informative for fish farmers who supply raw fish for reducing the risk of human infection. Moreover, we also found that the extracts from the previous T13 strain did not exhibit the antibacterial capacity for V. alginolyticus (data not shown), while the extracts from P245 and P263 strains did.

Quite recently, Lee et al. (2021) reported that the antibacterial activity driven by the transgenic B. subtilis strains expressing recombinant CiMAM, which is an AMP originated from marine chordate, displays a higher competence against halophilic pathogens specifically. In this study, based on the results obtained from the bactericidal activity, the equivalent to the efficacious dose of Tetracycline against marine pathogen V. parahaemolyticus of CiMAM-expressing C117 strain (Lee et al., 2021) and lactoferrin-expressing P263 strain (in this study) was 47 and 193 ng, respectively, suggesting that transgenic B. subtilis P263 strain exhibited a stronger bactericidal activity in seawater environment than that of CiMAM-expressing C117 strain. Therefore, we conclude that the use of transgenic B. subtilis P245 and P263 strains able to produce large amounts of AMPs can both effectively kill pathogenic bacteria and mitigate the excessive use of the antibiotics, thereby reducing their harmful effects on the aquatic environment, including the elimination of antibiotic-resistant bacterial strains generated in fish and shellfish farm.

Lastly, we observed that few surviving tilapia fry could exhibit gastroenteritis-related pathogenesis a couple of weeks after the in vivo pathogen challenge test. Thus, although most E. tarda had been killed by the P263 extracts, the survival of E. tarda in these few surviving fry after stop feeding P263 extracts still had a chance to continuously proliferate, eventually reaching the number of bacteria sufficient to cause pathogenesis of tilapia fry. Therefore, continuous feeding of P263 extracts for fish might be recommended if necessary.

Taken together, we strongly suggest that the transgenic B. subtilis strain P263 generated by this study shows high promise for use in aquaculture and animal husbandry to prevent animals from bacterial infection during a disease outbreak.

Experimental procedures

Bacterial species and culture conditions

The host cell used in this study was B. subtilis WB800 strain (Liew et al., 2012), a gift from Dr. Sui-Lam Wong.
Department of Biological Science, University of Calgary, Canada. It contains the deletion of eight extracellular proteases, including nprE, aprE, epr, bpr, mpr, nprB, dpr and wprA genes (Wu et al., 2002). The pathogens used as targets for bactericidal testing in an agar well diffusion assay were E. coli, antibiotic-resistant E. coli strain (BCRC 13B0202), E. tarda (BCRC 16702), S. epidermidis (BCRC 15245), S. iniae, V. parahaemolyticus (BCRC 12866) and V. alginolyticus. E. coli and E. tarda were cultured in LB medium, while S. epidermidis was cultured in nutrient broth medium, and V. parahaemolyticus and V. alginolyticus were cultured in Tryptic soy medium with 2.5% NaCl. S. iniae was cultured in Todd Hewitt broth medium. They were all incubated at 37°C for 18 to 24 h.

Construction of expression vector, gene transfer, PCR detection, dot blot analysis, protein analysis by SDS-PAGE and Western blot analysis

All these detailed protocols were described in the Appendix S1: Experimental procedures.

In vitro study of bactericidal activity of B. subtilis extract: agar well diffusion

Five millilitre of bacterial culture grown at OD_{600} = 1 was suspended in 0.5 ml synthetic gastric juice (150 mM of HCl, 15 mM of KCl and 0.5 mg ml^{-1} of pepsin) (Atkins, 1998), adjusted to pH 2.0 and mixed in a shaker under a rotation speed of 150 rpm min^{-1} at 37°C for 10 h. Since the enzymatic activity of pepsin is inactive at pH 6.5 or higher (Johnston, et al., 2007), the pH value was readjusted back to 7 and centrifuged to remove the precipitate. The supernatant was saved and served as the extract for testing bactericidal activity. If the extract was obtained from bacterial culture resuspended in synthetic gastric juice without containing pepsin, it served as one of the negative control groups. The agar plates were confluent with test pathogens, such as E. coli, antibiotic-resistant E. coli strain (BCRC 13B0202), E. tarda, S. epidermidis, S. iniae, V. parahaemolyticus and V. alginolyticus. When test pathogens grown at OD_{600} reached 0.5, 50 μl of them were separately added into culture medium containing 1.5% agar containing 50°C. The extract and three different dosages of Ampicillin/Tetracycline in a total volume of 20 μl were then added separately into the well on the agar plate. After the plates were cultured at 37°C for 8–10 h, the size of the inhibition zone shown on the gel was measured by the shortest distance from the edge of the agar well to the edge of the inhibition ring. The data of inhibition zone size (in mm) were averaged from six replicate plates and presented as a bar chart. Three known dosages of Ampicillin/Tetracycline and their corresponding sizes of inhibition zone were presented on the x-y scatter diagram respectively. Therefore, the LRE was determined based on the linear trend line drawn on the graph. In the experimental group, 20 μl taken from one-fourth dilution of extracts from transgenic lines P245 and P263 was individually loaded on the well to determine the size of the inhibition zone. After these data were fitted in the LRE, the equivalent concentrations of antibiotic potency of the extracts were finally obtained.

In the bactericidal activity test against S. iniae, we used a trypsic soy medium mixed with 3% defibrinated sheep blood to carry out the experiment, and the measurement was conducted after culturing for 24 h. Tetracycline served as a positive control. Finally, the above result was divided by the amount of extract (20 μl), multiplied four times and combined with the calculation of cell density of transgenic P245 and P263 strains. We then obtained bactericidal activity of 1 μl out of a total 5.5 ml of solution extracted from cultured transgenic B. subtilis strains against pathogens equivalent to the potency of Tetracycline/Ampicillin.

In vivo study of bactericidal activity of B. subtilis extract: tilapia fry challenged by E. tarda infection

The protocol and treatment of tilapia fish were reviewed and approved by the Institutional Animal Care and Use Committee, Mackay Medical College, Taiwan, with approval number MMC-A1060012. All institutional and national guidelines for the care and use of laboratory animals were followed. If the in vivo test the methodology used for tilapia fry cultured in freshwater, we followed the procedures previously described by Lee et al. (2019) except that (1) around 2700 outdoor tilapia fry (Oreochromis sp.) with an average body length of 24 mm was obtained from a local fish hatchery farm, Chi-Feng Aquaculture Company, Ping-Tung, Taiwan; and (2) these fish fry were adapted in the aquarium tank in an indoor wet laboratory for three weeks. If the in vivo test for tilapia fry cultured in seawater, 3000 fry with body lengths of 22 mm were adapted in an aquarium tank (30 × 25 × 25 cm, 18.75 l of water) indoors for one month. Fish fry was gradually adjusted to salt environment through a 5% increase of seawater every week. After four weeks, the fish would have adjusted to an environment of approximately 20% seawater and grown to have body lengths of 28–29 mm. These fish were then separated into eight individual aquarium tanks containing 40 fish each. Fish were divided into four groups. The first and second groups were fed extracts from B. subtilis transgenic strain T13 (Lee et al., 2019) and P263, respectively; the third group was fed B. subtilis WB800 (control), and the last one was a mock group in

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which the treated fish were not challenged with pathogen. Each experimental group was duplicated. In total, 320 tilapia fry of equal length were studied.

*B. subtilis* strains were cultured until OD600 of 1 was reached. The cells were then collected, centrifuged and resuspended in 3 ml of synthetic gastric juice containing 10% pepsin (pH = 2) and incubated at 18–22°C for 10 h. Fifty microlitres of this cell extract was then taken and fed to each fry through a feeding tube. Fish remained in their original aquariums for 1 h before performing the pathogen challenge test. We cultured *E. tarda*, the test pathogen of this *in vivo* study, in a 50 ml of liquid medium until reaching a cell density of 1 × 10^12 CFU ml^-1 based on the number of viable cells counted from agar plates. Fifty millilitres of cultured medium was then divided by two (25 ml of each), followed by centrifugation. The concentrated bacteria from 25 ml (25 × 10^12 CFU) were resuspended in 1 ml of liquid medium and diluted in 1000 ml of seawater or freshwater, which then transferred into a small tank. The cell extract-fed fish fry from each group was immersed in this 1000 ml of tank containing *E. tarda* with a final density of 2.5 × 10^10 CFU ml^-1 at 22–24°C. After a period of infection lasting 5 h, fry was returned to their original tanks (18.75 l) where their day-by-day survival rate was recorded until the fish from the control group had all died, representing the end of the trial.

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**Conflict of interest**

The authors declare no financial or commercial conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Bactericidal activity of recombinant lactoferricin produced by transgenic strains against Ampicillin-resistant E. coli strain. (A) Using an agar diffusion assay to examine the anti-bacterial efficacy of extracts from transgenic B. subtilis strains P245 and P263. Cell lysate was added into the wells on agar plate confluent with Ampicillin (Amp)-resistant E. coli strain (BCRC number: 13B0202). Tetracycline (Tet) served as positive control for calculating the antibacterial potency of recombinant protein; Negative controls: wells filled with water (control), pepsin-digested extracts from B. subtilis WB800 (WT) and non-pepsin-digested extracts from B. subtilis P263 (263'). Experimental groups: experimental groups: pepsin-treated extracts from B. subtilis WB800 (WT) and/or non-pepsin-digested extracts from B. subtilis P263 (263'); Experimental groups: pepsin-treated extracts from transgenic B. subtilis P245 and P263 strains (n = 6). (A, B, C) The size of each inhibition zone shown in Figures A and D was measured and presented as a bar chart. (C) The Regression Equation was obtained and antibacterial potency equivalent to Ampicillin and tetracycline of extracts from P245 and P263 against E. coli Ampicillin-resistant strain was determined.

Fig. S2. Bactericidal activity of recombinant lactoferricin produced by transgenic B. subtilis against euryhaline pathogens. Agar diffusion assay as described in Fig. 4, except for the agar plate confluent with euryhaline pathogens (A) E. tarda and (D) S. iniae. Ampicillin (Amp) or Tetracycline (Tet) served as positive control for calculating the antibacterial potency of recombinant protein; Negative controls: well filled with water (control), pepsin-digested extracts from B. subtilis WB800 (WT) and/or non-pepsin-digested extracts from B. subtilis P263 (263'); Experimental groups: pepsin-treated extracts from transgenic B. subtilis P245 and P263 strains (n = 6). (B, E) The size of each inhibition zone shown in Figures A and D was presented as a bar chart. (C, F) The Regression Equation was obtained and antibacterial potency equivalent to Ampicillin and tetracycline of extracts from P245 and P263 against E. tarda and S. iniae was determined.

Fig. S3. Bactericidal activity of recombinant lactoferricin produced by transgenic strains against halophile pathogens. A strategy similar to that described in Figs S1 and S2, except for agar plate confluent with halophile pathogens (A) V. parahaemolyticus and (D) V. alginolyticus. Positive control, negative controls and experimental groups were described previous figures. Regression Equation was obtained and antibacterial potency equivalent to Tetracycline of extracts from P245 and P263 against (A) V. parahaemolyticus and (D) V. alginolyticus was determined (n = 6).

Fig. S4. The growth curves of the B. subtilis strains. The growth curves of (A) WB800, (B) transgenic P245 strain and (C) transgenic P263 strain were determined individually when they were cultured in LB medium at 37°C for the duration time (in h) as indicated. Experimental procedures.