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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 fry-fed 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

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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, 1986), lactoferrin not only displays antifungal ability (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 undigested 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, 1.3×10^8 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 transformants, 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 PtrnQ-6LFB-GFP had been electroporated in transformants 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 pPtrnQ-6LFB-GFP, and it served as a positive control (Fig. 1B).

Copy number of plasmid harboured 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

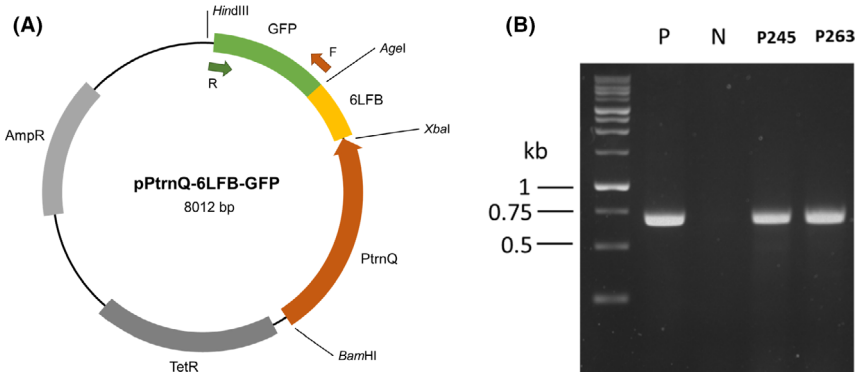

Fig. 1. Using PCR to detect of the existence of plasmid pPtrnQ-6LFB-GFP in the host cell *B. subtilis* after transformation.
A. Schematic map of the plasmid pPtrnQ-6LFB-GFP containing a PtrnQ promoter, six tandem repeats of lactoferricin cDNA (6LFB) and a GFP reporter gene. Primer sets used to determine the existence of recombinant GFP fragment shown as arrow.
B. The PCR-amplified product, which was obtained from plasmid pPtrnQ-6LFB-GFP, served as a positive control (P); *B. subtilis* WT (WB800) strain, which served as a negative control (N); and *B. subtilis* transgenic strains P245 and P263.

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 2×10^4 units; 10 ng of plasmid DNA exhibited the equivalent of 1.16×10^4 units; and 5 ng of plasmid DNA displayed the equivalent of 6.38×10^3 units. Plasmid DNA was contained in 10, 5 and 2.5 μl of extracts out of bacterial liquid solution from the P245 strain at 1.7×10^8 CFU ml^{-1}, which represented 8.76×10^3, 3×10^3 and 5.99×10^2 units respectively. Similarly, plasmid DNA was contained in 10, 5 and 2.5 μl of extracts out of bacterial liquid solution from the P263 strain at 1×10^8 CFU ml^{-1}, which represented 8.16×10^3, 4.24×10^3 and 1.79×10^3 units (Fig. 2B).

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 2.47×10^6 kDa, and the transgenic P245 and P263 strains each contained 1.7×10^8 CFU ml^{-1} and 1×10^8 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 antiserum 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

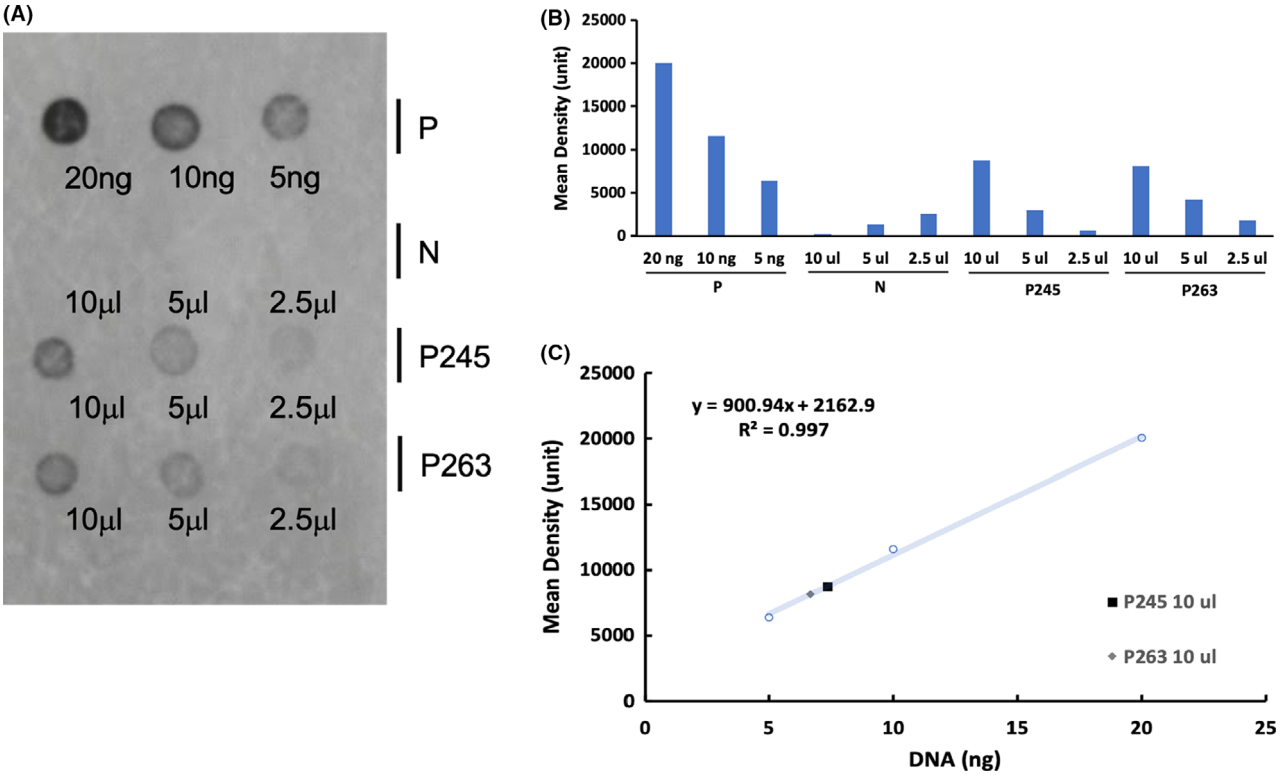

Fig. 2. Using Dot blot to analyze the copy number of plasmid contained in the transgenic *B. subtilis* strains.
A. Dot blot analysis. Plasmid pPtrnQ-6LFB-GFP, which was adjusted to the concentrations, as indicated, served as a positive control (P). Plasmid, which was extracted from three different volumes, as indicated, of cultured *B. subtilis* WB800, served as a negative control (N). Plasmid extracted from three different volumes, as indicated, of cultured *B. subtilis* transgenic strains P245 and P263, was prepared from bacterial culture of 1.7×10^8 CFU ml^{-1} and 1×10^8 CFU ml^{-1} respectively ($n = 3$).
B. The signal intensity shown in panel (A) was analyzed by VisionWorks software, transforming the mean density value into the bar chart.
C. The linear regression equation was based on the signal intensity of three known concentrations of the positive control. Based on this equation, the total weight of plasmids extracted from 10 ml of P245 was 7.32 ng, resulting in 1050 copies per cell, while the total weight of plasmids extracted from P263 was 6.66 ng, resulting in 1626 copies per cell.

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 lactoferricin 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 1.7×10^8 and 1×10^8 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

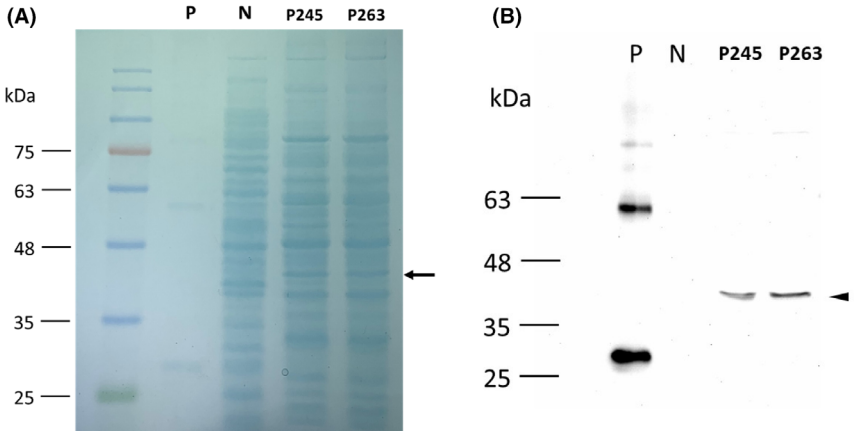

Fig. 3. Western blot analysis of recombinant lactoferricin expressed in the transgenic *B. subtilis* strains.
A. Proteins analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. GFP reporter protein served as a positive control (P). Total proteins extracted from *B. subtilis* WB800 strain (negative control; N) and from transgenic strains P245 and P263 (experimental groups) were analyzed. The recombinant protein produced by transgenic strains was detected, as indicated by the arrow.
B. Western blot analysis using polyclonal antibody against GFP. GFP reporter protein served as a positive control (P). Total proteins extracted from *B. subtilis* WB800 strain (negative control; N) and from transgenic strains P245 and P263 (experimental groups) were analyzed. A positive signal with molecular weight of 44 kDa (indicated by arrowhead) was shown on the gel, which was the recombinant lactoferricin produced by transgenic strains of *B. subtilis*.

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 μg 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 μg of Ampicillin. Therefore, we concluded that the antimicrobial activity of P245 and P263 per μl from 5.5 ml of cultured solution against *S. epidermidis* was equivalent to 366.4 and 452.52 ng 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 μg to serve as a negative control. The results showed that Tetracycline with concentrations of 0.25, 1.00 and 1.50 μg 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 μg of Tetracycline (Fig. S1C) respectively. Therefore, the bactericidal activity of extracts per μl from cultured P245 and P263 against Ampicillin-resistant *E. coli* strain was equivalent to the potency of 250.39 and 276.76 ng 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 μg 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 μg of Ampicillin (Fig. S2C) respectively. Thus, the bactericidal activity of extracts per μl from the prepared P245 strain against *E. tarda* was equivalent to the potency of 168.04 ng of Ampicillin, while that of P263 strain was equivalent to 249.94 ng of Ampicillin.

For *S. iniae*, we used Tetracycline as the positive control. Three dosages of Tetracycline with concentrations

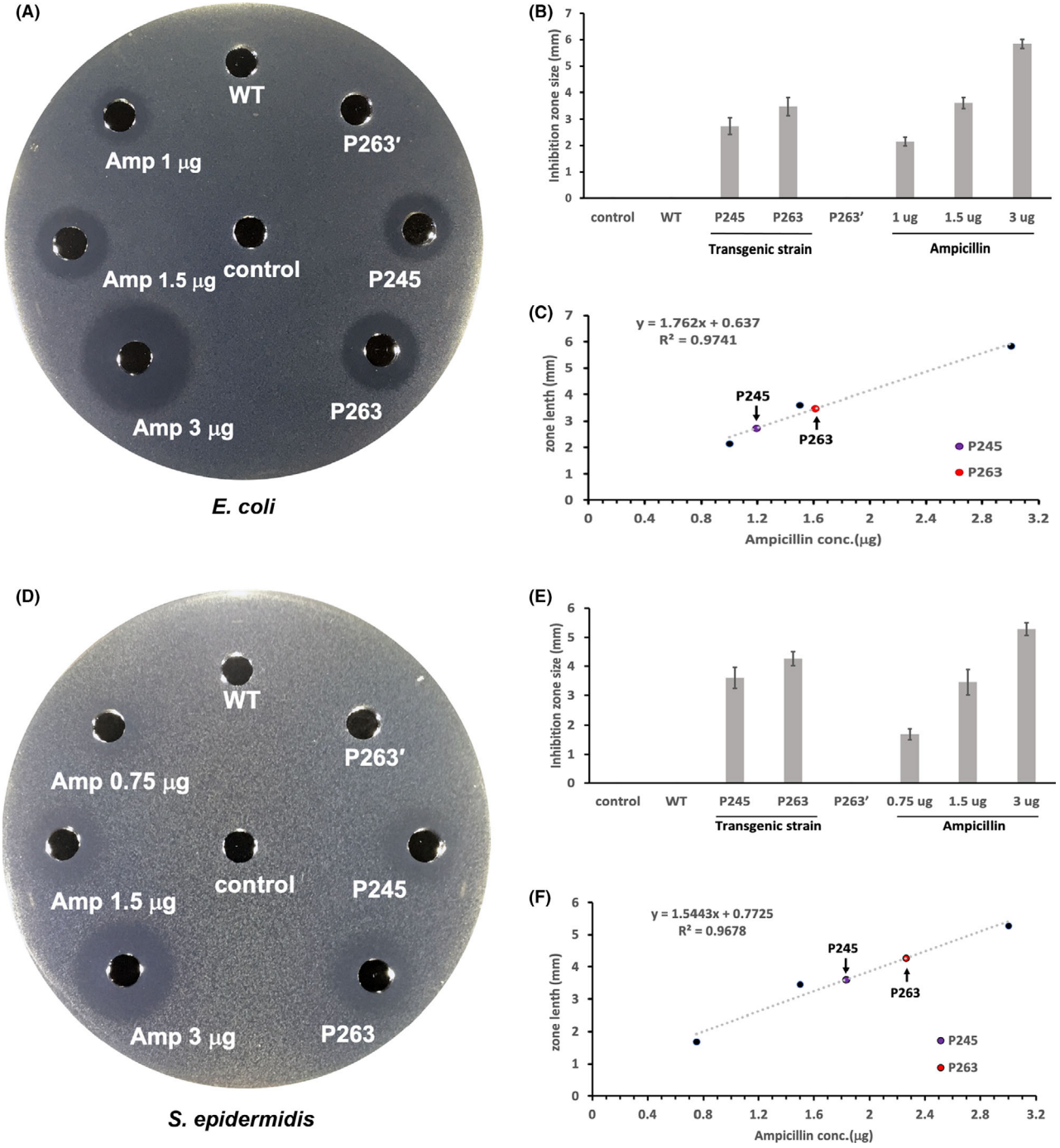

Fig. 4. Bactericidal activity of recombinant lactoferricin produced by transgenic *B. subtilis* against common pathogens. (A, D) Using agar diffusion assay to examine the antibacterial efficacy of extracts from transgenic strains P245 and P263 (n = 6). Cell lysate was added into the well on agar plate 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 263′). 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.

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

LRE represented the relationship between antibiotic dose versus inhibition zone shown on the agar plate confluent with each pathogen as indicated. Amp, Ampicilin; 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 bactericidal 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 gastroenteritis-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* pathogenic 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 gastroenteritis-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* strains T13 and T1 could produce recombinant lactoferricin driven by P43 promoter and exhibit a

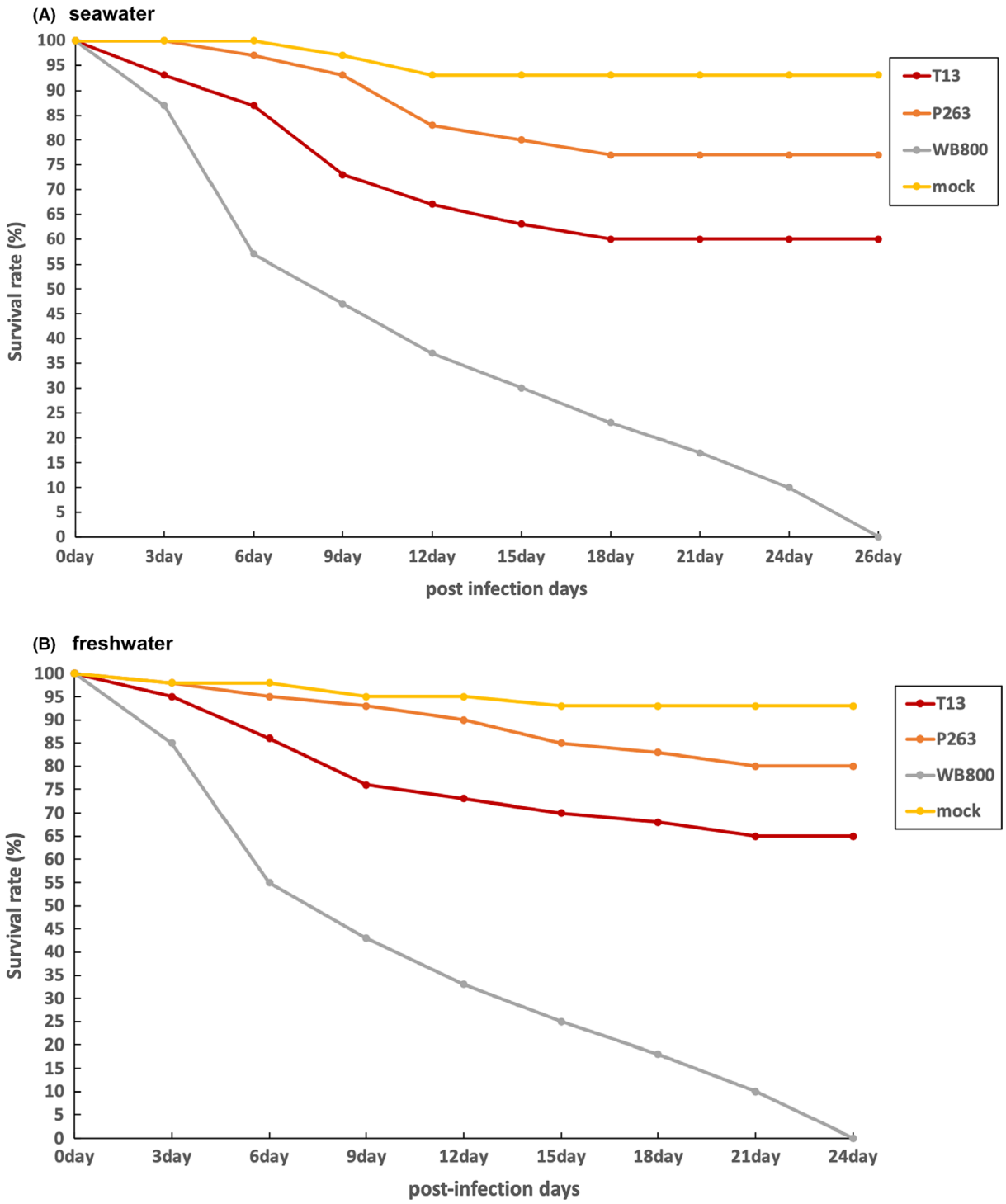

Fig. 5. *In vivo* study to determine the effect of bactericidal activity directed by recombinant lactoferricin produced by *B. subtilis* strains to defend against pathogen infection in fish fry.
A. Survival rate fish fry kept in seawater.
B. Survival rate fish fry kept in freshwater. Tilapia fry fed 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.

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	P245[a]	P263[a]	T1[b]	T13[b]
Promoter	PtrnQ	PtrnQ	P43	P43
Tandem repeats[c]	6x LFB	6x LFB	3x LFB	3x LFB
Copy number[d] of plasmid	1050	1626	931	647
Pathogen	Antibacterial activity equivalent to antibiotic potency[e]			
E. coli	$238_{(Amp)}$	$323_{(Amp)}$	$56_{(Amp)}$	$53_{(Amp)}$
S. epidermidis	$366_{(Amp)}$	$453_{(Amp)}$	$154_{(Amp)}$	$130_{(Amp)}$
E. coli (Ampicillin resistance)	$250_{(Tet)}$	$277_{(Tet)}$	ND	ND
E. tarda	$168_{(Amp)}$	$250_{(Amp)}$	$79_{(Amp)}$	$33_{(Amp)}$
S. iniae	$220_{(Tet)}$	$252_{(Tet)}$	ND	ND
V. parahaemolyticus	$178_{(Tet)}$	$193_{(Tet)}$	$44_{(Tet)}$	$26_{(Tet)}$
V. alginolyticus	$264_{(Tet)}$	$276_{(Tet)}$	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.

killed by the recombinant lactoferricin produced by itself. Transformants could not survive or grow on the plate if not protected from lactoferricin produced intracellularly. To solve this difficulty and also improve the efficacy of antimicrobial activity produced by transgenic *B. subtilis*, we engineered a specific concatener in the expression vector, which contains a six tandem repeat of lactoferricin cDNAs separated by a phenylalanine codon between each cDNA. Unlike the single lactoferricin molecule, the resultant prototype of recombinant lactoferricin fused by six tandem repeats fails to exhibit antimicrobial activity. However, the six tandem repeats of recombinant lactoferricin carry a pepsin recognition site (phenylalanine) in each segment, thus releasing single lactoferricin after having been digested by pepsin produced from an animal's stomach after consumption. By only carrying a pepsin recognition site, this novel DNA engineering prevents the recombinant peptide from killing the host cells. This was evident since no bactericidal activity was displayed in the extracts from the transgenic *B. subtilis* P263 strain without synthetic gastric juice treatment, according to the agar well diffusion assay, as shown in Fig. 4. The expression vector designed for the production of recombinant lactoferricin basically followed that described in Lee *et al*. (2019), except for employing a stronger promoter, PtrnQ, in this study. This study also supports the results reported by Lee *et al*. (2019) who demonstrated that the inactive form of six tandem repeated recombinant lactoferricin becomes an active and functional lactoferricin after passing through the stomach of tilapia fry.

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 bactericidal activity. For example, although P263 contains 1.5-fold higher plasmid copies compared to P245, the bactericidal 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 pPtrnQ-6LFB-GFP, which contains six tandem repeats of lactoferricin 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 lactoferricin-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 1×10^7 *B. subtilis* wild-type strain per gram of feed as a probiotic supplement. When 1×10^7 *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 1×10^6–1×10^8 *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, lactoferricin, 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-Villaseñor *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 lactoferricin-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, ⊿vpr 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 $O.D._{600}$ reached 0.5, 50 μl of them were separately added into culture medium containing 1.5% agar cooling at 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. Thereafter, 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 tryptic 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

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 OD_{600} 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.

References

Allaker, R.P., and Kapas, S. (2003) Adrenomedullin and mucosal defense: interaction between host and microorganism. *Regul Pept* **112:** 147–152.

Aly, S.M., Abdel-Galil Ahmed, Y., Abdel-Aziz Ghareeb, A., and Mohamed, M.F. (2008) Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. *Fish Shellfish Immunol* **25:** 128–136.

Atkins, T. W. (1998) Biodegradation of poly(ethylene adipate) microcapsules in physiological media. *Biomaterials* **19:** 61–67.

Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol* **10:** 411–421.

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K., and Tomita, M. (1992a) Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J Appl Microbiol* **73:** 472–479.

Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. (1992b) Identification of the bactericidal domain of lactoferrin. *Biochim Biophys Acta Protein Struct Mol Enzymol* **1121:** 130–136.

Bellamy, W., Yamauchi, K., Wakabayashi, H., Takase, M., Takakura, N., Shimamura, S., and Tomita, M. (1994) Antifungal properties of lactoferricin B, a peptide derived from the N-terminal region of bovine lactoferrin. *Lett Appl Microbiol* **18:** 230–233.

Berkhout, B., van Wamel, J.L., Beljaars, L., Meijer, D.K., Visser, S., and Floris, R. (2002) Characterization of the anti-HIV effects of native lactoferrin and other milk proteins and protein-derived peptides. *Antiviral Res* **55:** 341–355.

Brogden, K.A. (2005a) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3:** 238–250.

Brogden, K.A., Guthmiller, J.M., Salzet, M., and Zasloff, M. (2005b) The nervous system and innate immunity: the neuropeptide connection. *Nat Immunol* **6:** 558–564.

Caccavo, D., Pellegrino, N.M., Altamuram, M., Rigon, A., Amati, L., Amoroso, A., and Jirillo, E. (2002) Antimicrobial and immunoregulatory functions of lactoferrin and its potential therapeutic application. *J Endotoxin Res* **8:** 403–417.

Cramer, E., Pryzwansky, K.B., Villeval, J.L., Testa, U., and Breton-Gorius, J. (1985) Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood* **65:** 423–432.

Di Biase, A.M., Pietrantoni, A., Tinari, A., Siciliano, R., Valenti, P., Antonini, G., *et al.* (2003) Heparin-interacting sites of bovine lactoferrin are involved in anti-adenovirus activity. *J Med Virol* **64:** 495–502.

Elass, E., Masson, M., Mazurier, J., and Legrand, D. (2002) Lactoferrin inhibits the lipopolysaccharide-induced expression and proteoglycan-binding ability of interleukin-8 in human endothelial cells. *Infect Immun* **70:** 1860–1866.

Flores-Villaseñor, H., Canizalez-Román, A., Reyes-Lopez, M., Nazmi, K., de la Garza, M., Zazueta-Beltrán, J., *et al.* (2010) Bactericidal effect of bovine lactoferrin, LFcin, LFampin and LFchimera on antibiotic-resistant

Staphylococcus aureus and *Escherichia coli*. *Biometals* **23:** 569–578.

de la Fuente-Núñez, C., Reffuveille, F., Fernández, L., and Hancock, R.E. (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* **16:** 580–589.

Ganz, T. (2006) Hepcidin–a peptide hormone at the interface of innate immunity and iron metabolism. *Curr Top Microbiol Immunol* **306:** 183–198.

Gifford, J.L., Hunter, H.N., and Vogel, H.J. (2005) Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol Life Sci* **62:** 2588–2598.

Hartmann, A., Alder, A.C., Koller, T., and Widmer, R.M. (1998) Identification of fluoroquinolone antibiotics as the main source of umuC genotoxicity in native hospital wastewater. *Environ Toxicol Chem* **17:** 377–382.

Hsiao, P.C., and Chen, W.R. (2015) Technical review of the challenges of determining the emerging contaminants in a soil matrix: using antibiotics as an example. *J Soil Groundwater Remed* **2:** 141–168.

Hwang, P.M., Zhou, N., Shan, X., Arrowsmith, C.H., and Vogel, H.J. (1998) Three-Dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from Bovine lactoferrin. *Biochemistry* **37:** 4288–4298.

Johnston, N., Dettmar, P.W., Bishwokarma, B., Lively, M.O., and Koufman, J.A. (2007) Activity/stability of human pepsin: implications for reflux attributed laryngeal disease. *Laryngoscope* **117:** 1036–1039.

Kaplan, A., Lee, M.W., Wolf, A.J., Limon, J.J., Becker, C.A., Ding, M., *et al*. (2017) Direct antimicrobial activity of IFN-β. *J Immunol* **198:** 4036–4045.

Karav, S., German, J., Rouquie, C., Le Parc, A., and Barile, D. (2017) Studying lactoferrin N-glycosylation. *Int J Mol Sci* **18:** 870.

Kirkpatrick, C.H., Green, I., Rich, R.R., and Schade, A.L. (1971) Inhibition of growth of *Candida albicans* by iron-unsaturated lactoferrin: Relation to host-defense mechanisms in chronic mucocutaneous candidiasis. *J Infect Dis* **124:** 539–544.

Kowalska, K., Carr, D.B., and Lipkowski, A.W. (2002) Direct antimicrobial properties of substance P. *Life Sci* **71:** 747–750.

Kuebutornye, F.K.A., Abarike, E.D., and Lu, Y. (2019) A review on the application of *Bacillus* as probiotics in aquaculture. *Fish Shellfish Immunol* **87:** 820–828.

Lai, C.C., Wang, C.Y., Chu, C.C., Tan, C.K., Lu, C.L., Lee, Y.C., *et al*. (2011) Correlation between antibiotic consumption and resistance of Gram-negative bacteria causing healthcare-associated infections at a university hospital in Taiwan from 2000 to 2009. *J Antimicrob Chemother* **66:** 1374–1382.

Lalumera, G.M., Calamari, D., Galli, P., Castiglioni, S., Crosa, G., and Fanelli, R. (2004) Preliminary investigation on the environmental occurrence and effects of antibiotics used in aquaculture in Italy. *Chemosphere* **54:** 661–668.

Lee, B.C., Hung, C.W., Lin, C.Y., Shin, C.H., and Tsai, H.J. (2019) Oral administration of transgenic biosafe microorganism containing antimicrobial peptide enhances the survival of tilapia fry infected bacterial pathogen. *Fish Shellfish Immunol* **95:** 606–616.

Lee, B.C., Tsai, J.C., Lin, C.Y., Hung, C.W., Sheu, J.C., and Tsai, H.J. (2021) Using *Bacillus subtilis* as a host cell to express an antimicrobial peptide from the marine chordate *Ciona intestinalis*. *Mar Drugs* **19:** 1–19.

Liepke, C., Baxmann, S., Heine, C., Breithaupt, N., Ständker, L., and Forssmann, W.G. (2003) Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides. *J Chromatogr B* **791:** 345–356.

Liew, P.X., Wang, C.L., and Wong, S.L. (2012) Functional characterization and localization of a *Bacillus subtilis* sortase and its substrate and use of this sortase system to covalently anchor a heterologous protein to the *B. subtilis* cell wall for surface display. *J Bacteriol* **194:** 161–175.

Lu, Y.P., Zhang, C., Lv, F.X., Bie, X.M., and Lu, Z.X. (2012) Study on the electro-transformation conditions of improving transformation efficiency for *Bacillus subtilis*. *Lett Appl Microbiol* **55:** 9–14.

Makrides, S.C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev* **60:** 512–538.

Meddeb-Mouelhi, F., Dulcey, C., and Beauregard, M. (2012) High transformation efficiency of *Bacillus subtilis* with integrative DNA using glycine betaine as osmoprotectant. *Anal Biochem* **424:** 127–129.

Moreno, E., Prats, G., Sabaté, M., Pérez, T., Johnson, J.R., and Andreu, A. (2006) Quinolone, fluoroquinolone and trimethoprim/sulfamethoxazole resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli*. *J Antimicrob Chemother* **57:** 204–211.

Newaj-Fyzul, A., Adesiyun, A.A., Mutani, A., Ramsubhag, A., Brunt, J., and Austin, B. (2007) *Bacillus subtilis* AB1 controls Aeromonas infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *J Appl Microbiol* **103:** 1699–1706.

Omata, Y., Satake, M., Maeda, R., Saito, A., Shimazaki, K., Yamauchi, K., *et al*. (2001) Reduction of the infectivity of *Toxoplasma gondii* and *Eimeria stiedai sporozoites* by treatment with bovine lactoferricin. *J Vet Med Sci* **63:** 187–190.

Rainard, P. (1986) Bacteriostatic activity of bovine milk lactoferrin against mastitic bacteria. *Vet Microbiol* **11:** 387–392.

Reiter, B., and Oram, J.D. (1967) Bacterial inhibitors in milk and other biological fluids. *Nature* **216:** 328–330.

Siciliano, R., Rega, B., Marchetti, M., Seganti, L., Antonini, G., and Valenti, P. (1999) Bovine lactoferrin peptidic fragments involved in inhibition of herpes simplex virus Type 1 infection. *Biochem Biophys Res Commun* **264:** 19–23.

Song, Y., Nikoloff, J.M., Fu, G., Chen, J., Li, Q., Xie, N., *et al*. (2016) Promoter screening from *Bacillus subtilis* in various conditions hunting for synthetic biology and industrial applications. *PLoS One* **11:** e0158447.

Stephens, S., Dolby, J.M., Montreuil, J., and Spik, G. (1980) Differences in inhibition of the growth of commensal and enteropathogenic strains of *Escherichia coli* by lactotransferrin and secretory immunoglobulin A isolated from human milk. *Immunology* **41:** 597–603.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Yamauchi, K., Takase, M., *et al*. (1995) *Toxoplasma gondii:* parasiticidal effects of bovine lactoferricin against parasites. *Exp Parasitol* **81:** 614–617.

de Tejada, B.M. (2014) Antibiotic use and misuse during pregnancy and delivery: benefits and risks. *Int J Environ Res Public Health* **11:** 7993–8009.

Tseng, D.Y., Ho, P.L., Huang, S.Y., Cheng, S.C., Shiu, Y.L., Chiu, C.S., and Liu, C.H. (2009) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. *Fish Shellfish Immunol* **26:** 339–344.

Valenti, P., and Antonini, G. (2005) Lactoferrin: an important host defense against microbial and viral attack. *Cell Mol Life Sci* **62:** 2576–2587.

Wang, P.Z., and Doi, R.H. (1984) Overlapping promoters transcribed by *Bacillus subtilis* sigma 55 and sigma 37 polymerase holoenzymes during growth and stationary phase. *J Biol Chem* **259:** 8619–8625.

Wu, S.C., Yeung, J.C., Duan, Y., Ye, R., Szarka, S.J., Habibi, H.R., and Wong, S.L. (2002) Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl Environ Microbiol* **68:** 3261–3269.

Yang, D., Chen, Q., Hoover, D.M., Staley, P., Tucker, K.D., Lubkowski, J., and Oppenheim, J.J. (2003) Many chemokines including CCL20/MIP-3α display antimicrobial activity. *J Leukoc Biol* **74:** 448–455.

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) with different concentrations, as indicated, served as positive control for calculating the potency of recombinant protein produced by transgenic strains. Negative controls were 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: after extracts were treated with pepsin, the antimicrobial capabilities driven by extracts from P245 and P263 strains against *E. coli* Ampicillin-resistant strain were determined (n = 6). (B) The size of each inhibition zone shown in Figure A was measured and presented as a bar chart. The size was determined by the shortest distance between the edge of agar well to the edge of inhibition zone. (C) The Linear Regression Equation (LRE) was obtained based on three known dosages of Tetracycline corresponding to their respective inhibition zones. Antibacterial potency equivalent to 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 euryhaline 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.

Appendix S1. Experimental procedures.

