Display of Multimeric Antimicrobial Peptides on the
*Escherichia coli* Cell Surface and Its Application as Whole-Cell Antibiotics

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Abstract

Concerns over the increasing emergence of antibiotic-resistant pathogenic microorganisms due to the overuse of antibiotics and the lack of effective antibiotics for livestock have prompted efforts to develop alternatives to conventional antibiotics. Antimicrobial peptides (AMPs) with a broad-spectrum activity and rapid killing, along with low opportunity for development of resistance, represent one of the promising novel alternatives. Their high production cost and cytotoxicity, however, limit the use of AMPs as effective antibiotic agents to livestock. To overcome these problems, we developed potent antimicrobial *Escherichia coli* displaying multimeric AMPs on the cell surface so that the AMP multimers can be converted into active AMP monomers by the pepsin in the stomach of livestock. Buf IIIb, a strong AMP without cytotoxicity, was expressed on the surface of *E. coli* as Lpp-OmpA-fused tandem multimers with a pepsin substrate residue, leucine, at the C-terminus of each monomer. The AMP multimers were successfully converted into active AMPs upon pepsin cleavage, and the liberated Buf IIIb-L monomers inhibited the growth of two major oral infectious pathogens of livestock, *Salmonella enteritidis* and *Listeria monocytogenes*. Live antimicrobial microorganisms developed in this study may represent the most effective means of providing potent AMPs to livestock, and have a great impact on controlling over pathogenic microorganisms in the livestock production.

Introduction

Modern livestock production systems have continually used antibiotics and antimicrobial compounds to either prevent and treat infectious diseases or improve weight gain and feed utilization in animals. In fact, in-feed administration of non-therapeutic doses of antibiotics was found to increase the performance of growing livestock [1]. However, the indiscriminate non-therapeutic use of antibiotics in the livestock production has a negative impact not only on livestock but also on public health and food safety, as it promotes the rapid development of multidrug-resistant bacteria that do not respond to current antibiotics, thus further endangering human lives [2–5]. These antibiotic-resistant bacterial strains and associated genes have the potential to impart their resistance traits to disease-causing bacteria. Humans acquire these resistant bacteria either through direct contact with infected livestock or contaminated food or water. Therefore, many countries have banned the administration of conventional antibiotics (at non-therapeutic doses), as feed additives to livestock [6,7]. The rapid increase in the number of antibiotic-resistant pathogenic microorganisms due to antibiotic overuse as well as the limited number and low availability of effective antibiotics for livestock has led to numerous researchers focus on the development of alternatives to conventional antibiotics [8–10].

Antimicrobial peptides (AMPs) have been considered as one of the most promising alternative antibiotics because of their strong antimicrobial activity and microorganisms eradication with a little opportunity of developing resistance. AMPs are produced by all classes of life, and play key roles in primary host defense against infection by pathogenic microorganisms. While commonly prescribed antibiotics operate on specific intracellular targets, AMPs physically compromise bacterial membrane integrity by disrupting essential components within the cells, thereby causing bacteria difficult to develop resistance [11,12]. Despite their potential as alternative antibiotics, the use of AMPs in the livestock has a limitation due to their cytotoxicity and high production cost. Owing to their membrane lytic mechanism, several AMPs represent toxicity towards eukaryotic cells at higher concentrations [11]. Moreover, chemical synthesis of AMPs on a large scale and in a pure enough form to be used for livestock is extremely expensive. The production of recombinant AMPs is also expensive because the host cell needs to be protected against the potent action of the peptides. Thus, the livestock industry has not shown a great deal of interest in developing antibiotic alternatives based on AMPs.

Buf IIIb (RVVRQWPGRVVRVRRVVR), a potent cell-penetrating AMP which does not cause damage to mammalian host cells up to 200 μg/ml, is a promising candidate for alternative.
In this study, we developed the most cost effective means of providing AMPs to livestock by consisting rapid-acting and potent antimicrobial Escherichia coli displaying multimeric Buf IIIb on the cell surface. Buf IIIb was expressed as Lpp-OmpA-fused tandem multimers on the surface of E. coli, with a pepsin substrate residue at the C-termini of each monomer [14–16]. Upon cleavage by pepsin, the Lpp-OmpA-fused tandem multimers displayed on the surface of E. coli were converted into active AMP monomers, and the liberated Buf IIIb-L monomers inhibited the growth of selective major oral infectious pathogens of livestock.

Materials and Methods

Bacterial strains, plasmids, and enzymes

E. coli XL1-Blue (Stratagene, La Jolla, CA, USA) was used as a host for sub cloning, and E. coli BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) was used for gene expression. E. coli cells were grown in Luria-Bertani (LB) medium at 37°C, and ampicillin (50 μg/ml) was added for the growth of plasmid-containing cells. The pGEM-T easy vector (Promega, Madison, WI, USA) was used for sub cloning and multimerization of the Buf IIIb-L gene, and pET21c (Novagen, Madison, WI, USA) for the expression of the Lpp-OmpA-multimeric Buf IIIb-L fusion (LO-Bn) proteins. Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Taq polymerase and porcine pepsin were purchased from Takara (Otsu, Japan) and Sigma (St. Louis, MO, USA), respectively. All enzymes were used according to the recommendations of suppliers. All recombinant DNA techniques were performed as described by Sambrook and Russell [17].

Peptide synthesis

Peptides used in this work (Table 1) were chemically synthesized on a Milligen 9050 peptide synthesizer (Anygen, Kwangju, Korea). Synthesized peptides were purified to over 88% by reversed-phase high-pressure liquid chromatography (HPLC) on a Delta-Pak C18 column (3.9 mm x 300 mm, Waters, Milford, MA, USA). The peptide content of lyophilized samples was determined by quantitative amino acid analysis with a Pico-tag system on a Beckman Coulter, Fullerton, CA, USA.

Antimicrobial activity

The antimicrobial activity of each peptide was determined against eight representative microorganisms, including Gram-positive and Gram-negative bacteria and fungi, using the broth micro dilution assay as described by Park et al. with a slight modification [18]. Briefly, mid-logarithmic phase cells were diluted to 1 x 10^5 cfu/ml in 10 mM sodium phosphate buffer (NAPB), pH 7.4. Each well of 96-well micro titer plates (Costar, Cambridge, MA, USA) was filled with 90 μl of the diluted cell suspension and 10 μl of serially diluted peptide samples. After incubation for 3 h, fresh medium (trypticase soy broth for bacteria and Saboraud’ medium for fungi) was added to the mixture and incubated at 37°C (bacteria) or 30°C (fungi) for an additional 12 h. Inhibition of growth was determined by measuring the absorbance at 620 nm with a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). The lowest concentration of peptide that completely inhibited growth was defined as the ‘minimal inhibitory concentration’ (MIC). The MIC values were calculated as an average of two independent experiments performed in triplicate.

Hemolysis and in vitro cytotoxicity assays

Hemolytic activity was assayed as described by Jang et al. [13]. The percentage of hemolysis was calculated using the following equation: Hemolysis (%) = (A100 – A0)/(A100 – A0) x 100, where A100 is the absorbance at 567 nm of the sample, A100 is the absorbance of completely lysed human red blood cells (RBGs) in 0.2% Triton X-100, and A0 is the absorbance of zero hemolysis.

To analyze in vitro cytotoxic activity, HaCaT keratinocytes were cultured in 96-well plates (10^3 cells/well) in Dulbecco’s modified eagle medium (DMEM) with 10% FBS. After 24 h of incubation, cells were treated with each peptide (0–400 μg/ml) and incubated for another 24 h. Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using the CellTiter 96® Non-radioactive Cell Proliferation assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The percentage of cell viability was determined using the following equation: Viability (%) = (A100 – A0)/(A100 – A0) x 100, where A0 is the absorbance of at 570 nm of the sample, A0 is the absorbance of control (no peptide addition), and A0 is the background absorbance. Each experiment was performed in triplicate, and repeated at least three times independently.

Construction of expression vectors containing Lpp-OmpA-multimeric Buf IIIb-L fusion genes

The overall scheme for the construction of the expression vectors is illustrated in Fig. 1. The gene encoding the Buf IIIb-L was synthesized using the following deoxyoligonucleotides (oligos): 5’-GGATCCCATATGGGTCGTGGTCGTTCGCC GTGTTGTTCG-3’ and 5’-GGATCAGTCTCTAAGGCTGACGAAAGCAGAACGCAGCAGA-3’ (restriction sites NdeI, BglII, and BamHI are indicated in the oligos as bold, underlined, and italic, respectively). The two oligos were annealed by PCR and ligated into the linear vector with pMBT-B1. The DNA fragment encoding the Buf IIIb-L monomer was isolated from pMBT-B1 after digestion with BglII and BamHI, and cloned into pMBT-B1, generating pMBT-B2 containing a Buf IIIb-L dimer. These steps were repeated for the construction of tandem multimers of the Buf IIIb-L gene, generating pMBT-Bn (n = number of Buf IIIb-L monomers).

To construct Lpp-OmpA-multimeric Buf IIIb-L fusion genes, an anchor protein encoding the chimeric Lpp-OmpA gene consisting of the signal sequence and the first nine amino acids of lipoprotein (Lpp), residues 46-159 of outer membrane protein OmpA, and His tag (histidine 6-mer), was amplified from E. coli chromosomal DNA using recombinant PCR with the two primer pairs, 5’-CCTGTTGACATATGGGTCGTGGTCGTTCGCC GTGTTGTTCG-3’ (NdeI site indicated as bold)/5’-GGAAACGCCGGTG-3’ and 5’-TTCTCCGGTGTTTGCTGGCGGTGTTG-3’ and 5’-CGGATCCTGTTGACATATGGGTCGTGGTCGTTCGCC GTGTTGTTCG-3’ (BamHI site, His tag, AGAAACGCCGGTG-3’).
and BbsI site are indicated as bold, underlined, and italic, respectively). The PCR fragment containing Lpp-OmpA gene was ligated into pGEM-T vector to produce pLpp-OmpA. Into the pLpp-OmpA digested with BbsI, the BbsI-FokI fragment carrying the multimeric Buf IIIb-L gene isolated from pMBT-B1, generating pMBT-B2. These steps were repeated for the construction of tandem multimers of the Buf IIIb-L gene, generating pMBT-Bn (n = number of Buf IIIb-L genes). The BbsI and FokI fragments of pMBT-Bn were cloned into pLpp-OmpA digested with BbsI, generating pLpp-OmpA-Bn. The Ndel and BamHI fragments of pLpp-OmpA-Bn were then ligated into the expression vector pET21c digested with the same enzymes, generating pLO-Bn (n = 0, 1, 2, and 3). The arrows, open triangles and closed triangles indicate BbsI cleavage sites and FokI cleavage sites, respectively.

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Figure 1. Schematic representation of the construction of Lpp-OmpA-multimeric Buf IIIb-L fusion genes. A monomeric Buf IIIb-L gene was dimerized by; (1) excision of the monomeric Buf IIIb-L insert by digestion with BbsI and FokI, (2) isolation of the fragment, (3) cloning into the original pMBT-B1, vector digested with BbsI, generating pMBT-B2. These steps were repeated for the construction of tandem multimers of the Buf IIIb-L gene, generating pMBT-Bn (n = number of Buf IIIb-L genes). The BbsI and FokI fragments of pMBT-Bn were cloned into pLpp-OmpA digested with BbsI, generating pLpp-OmpA-Bn. The Ndel and BamHI fragments of pLpp-OmpA-Bn were then ligated into the expression vector pET21c digested with the same enzymes, generating pLO-Bn (n = 0, 1, 2, and 3). The arrows, open triangles and closed triangles indicate BbsI cleavage sites and FokI cleavage sites, respectively.

Expression of Lpp-OmpA-multimeric Buf IIIb-L fusion genes in E. coli

E. coli BL21 (DE3) cells were transformed with expression vectors containing the Lpp-OmpA-multimeric Buf IIIb-L fusion genes. Each transformant harboring pLO-Bn (n = 0, 1, 2, and 3) was inoculated into 3 ml of LB supplemented with ampicillin (50 µg/ml), and grown at 37°C for 9 to 12 h. Each culture was then diluted (1:100) into fresh medium and grown at 37°C. At an OD600 = 0.6, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM for the induction of fusion genes. The cells were harvested 4 h after induction by centrifugation at 6,000 × g for 10 min at 4°C, and lysed by sonication (6 × 30 s, B. Braun instruments, Allentown, PA, USA) on ice. The amount of LO-Bn fusion proteins in the whole cell lysates was determined by quantifying the protein bands in each lane of 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels by densitometry at 600 nm (Bio/Profile image analysis software; Bio-1D, Vilber Lourmat, France). The presence of LO-Bn fusion proteins in the whole cell lysates was also confirmed by Western blot using anti-His antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
Fractionation of outer membrane proteins

One hundred ml of each culture broth was centrifuged at 6,000 × g for 10 min at 4°C, and the cell pellets were washed with 25 mM Tris-HCl buffer (pH 8.0), followed by centrifugation at 6,000 × g for 10 min at 4°C. The cell pellets (1 × 10^11 each recombinant cells) were then resuspended in 25 mM Tris-HCl buffer (pH 8.0) containing 50 µg/ml of lysozyme, incubated for 1 h on ice; cells were then disrupted by sonicating. The lysates were centrifuged at 10,000 × g for 15 min at 4°C to remove any unbroken cells, and centrifuged again at 115,000 × g for 1 h at 4°C to separate the membrane and soluble fraction. The membrane pellets were then resuspended with phosphate-buffered saline (PBS) containing 0.01 mM MgCl₂ and 2% Triton X-100 for solubilization of the inner membrane. After incubation for 30 min at room temperature, the insoluble pellets containing outer membrane proteins were obtained by centrifugation at 115,000 × g for 1 h at 4°C. Outer membrane protein extracts were obtained by washing the insoluble pellets with 25 mM Tris-HCl buffer (pH 8.0), followed by resuspending in 20 µl of 2% SDS buffer, and analyzed by using 10% SDS-polyacrylamide gel electrophoresis [14,17,19,20]. The LO-Bn fusion proteins in the outer membrane protein extracts were quantified by measuring the protein bands of SDS-polyacrylamide gel by densitometry at 600 nm, according to the manufacturer’s protocol (Bio/Profile Image Analysis Software; Bio-1D, Vilber Lourmat, France). In brief, the intensity of each protein band was measured as the integrated volume of pixels (with linear dimensions x and y in millimeters and the z axis as the relative absorbance) associated with each Coomassie blue-stained band, and the amount of LO-Bn proteins in the outer membrane protein extracts was calculated by multiplying the ratio of the intensity of the LO-Bn fusion protein band over the sum of intensities of all protein bands in the same lane of a SDS gel by the total proteins determined above.

Cleavage of the surface-displayed LO-Bn fusion proteins by pepsin

For pepsin digestion, the cell pellets (1 × 10^11 each recombinant cells) were resuspended in 1 ml of simulated gastric fluid (SGF; 0.7% (v/v) HCl, 0.2% (v/v) NaCl, and 1000 U of pepsin), and incubated at 37°C for 1 h. The reactions were then stopped by adding 1 ml of 0.7% (v/v) NaOH, 0.2% (v/v) NaCl, and pepsin-digested peptides in the supernatants were recovered by centrifugation at 13,000 × g for 15 min at 4°C and analyzed using SDS-polyacrylamide gel electrophoresis. The digested peptides were then applied to a Resource 15S cation exchange column (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). The bound peptides were eluted by applying a linear 0 to 1 M NaCl gradient in elution buffer (20 mM 2-[N-morpholino] ethanesulfonic acid, pH 6.0 with 1 M NaCl) and concentrated by lyophilization. The lyophilized peptides were further purified by reversed-phase HPLC on a Delta-Pak C₁₈ column (3.9 mm × 300 mm, Waters) using a linear elution gradient of 0 to 50% acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) at 1 ml/min for 1 h. Synthetic Buf IIIb-L (0-400 µg/ml) was used as control for quantification of the purified recombinant Buf IIIb-L monomer. A calibration curve was constructed by plotting average peak area obtained by HPLC versus concentration of the synthetic peptide. The calibration curve showed excellent linearity (r² > 0.999) over the concentration range investigated: Y = 4.6457 X + 13.174, where Y is the peak-area obtained by HPLC and X is the concentration of the peptide.

Measurement of susceptibility of oral infectious pathogens to the pepsin-digested peptide mixtures obtained from E. coli with surface-expressed LO-Bn fusion proteins

Antimicrobial activities of the pepsin-digested peptide mixtures obtained from E. coli cells with surface-expressed LO-Bn fusion proteins were tested against two oral infectious pathogens, Salmonella enteritidis (ATCC 13076) and Listeria monocytogenes (ATCC 15313) as described above except using 10 µl of the 20-fold diluted peptide mixtures obtained from 1 × 10^11 E. coli cells with surface-expressed LO-Bn fusion proteins instead of synthetic peptide. Growth of cells was determined by measuring absorbance at 620 nm with a Model 550 Microplate Reader (Bio-Rad). The percentage of viability was determined as follows: Viability (%) = (A₁₀₀ − A₀)/(A₁₀₀ − A₀) × 100, where A₁₀₀ is the absorbance of the sample, A₀ is the absorbance of cells without any treatment, and A₀ is the absorbance of cells treated with MIC value of synthetic Buf IIIb-L.

Results

Buf IIIb derivatives with a pepsin substrate residue at the C-terminus

Earlier experiments disclosed that Buf IIIb exhibits potent antimicrobial activity via targeting intracellular components, such as DNA in microorganisms, and does not cause damage to mammalian cells at the same time, resulting in a 7-fold improvement in the therapeutic index, compared to its parent antimicrobial peptide, buforin IIb [13]. In this study, we made Buf IIIb derivatives by adding a pepsin substrate residue (L, F, or Y) at the C-terminus of Buf IIIb, thus making them to be released from multimers by pepsin cleavage (Table 1). Significantly, Buf IIIb itself was not cleaved by pepsin, as assessed using PeptideMass software tools on the Expert Protein Analysis System (ExPASy). As shown in Table 2, the addition of pepsin substrate residue (L, F, or Y) at the C-terminus of Buf IIIb lead to a ~2-fold decrease in MIC (0.5–4 µg/ml) compared to the parent peptide Buf IIIb (0.5–2 µg/ml). Among the Buf IIIb derivatives, Buf IIIb-F and Buf IIIb-Y lysed 7.5% and 4.0% of human RBCs (Fig. 2 A) and killed 27% and 24% of HaCaT keratinocytes at 400 µg/ml (Fig. 2 B). On the other hand, Buf IIIb-L, like the parent peptide Buf IIIb, was completely inactive against human RBCs and killed less than 10% of HaCaT keratinocytes at 400 µg/ml. Therefore, Buf IIIb-L was selected for the construction of tandem multimers for E. coli cell surface display.
Construction and expression of Lpp-OmpA-multimeric Buf IIIb-L fusion genes

To express Buf IIIb-L as tandem multimers on the surface of E. coli, Buf IIIb-L gene was multimerized and subsequently fused with the gene encoding an anchor protein, Lpp-OmpA (Fig. 1). The multimerization of Buf IIIb-L gene was performed as described by Kim et al. using two class IIS enzymes, BbsI and FokI [21]. The clones, pMBT-B1, -B2, and B3, each containing 1, 2, and 3 copies of Buf IIIb-L gene, respectively, were selected. The BbsI and FokI fragment of these clones were then cloned into pLpp-OmpA that has an chimeric Lpp-OmpA gene consisting of the signal sequence and the first nine amino acids of lipoprotein (Lpp), residues 46-159 of outer membrane protein OmpA, and His tag (histidine 6-mer), producing pLpp-OmpA-Bn (n = number of a Buf IIIb-L, 0, 1, 2, and 3). The number of Buf IIIb-L genes cloned in pLpp-OmpA-Bn was determined by digesting with NotI, whose sites flank each multimer (Fig. 3A). The DNA fragment of Lpp-OmpA-multimeric Buf IIIb-L was then isolated after digestion with NotI and BamHI from pLpp-OmpA-Bn, and ligated into the expression vector pET21c, generating pLO-Bn. The successful expression of LO-Bn fusion proteins were verified by SDS-polyacrylamide gel electrophoresis and Western blot analysis (Fig. 3B, C).

Confirmation of LO-Bn fusion proteins displayed on the cell surface

The goal of our study was to express LO-Bn fusion proteins on the surface of E. coli to make it as an antimicrobial microorganism. Therefore, we first determined whether LO-Bn fusion proteins were successfully displayed on the surface of E. coli. To determine...
induced with IPTG, indicating the presence of LO-Bn fusion proteins on the surface (Fig. 4B–D). Fluorescence of cells harboring pLO-Bn, which express only Lpp-OmpA anchor proteins, was relatively weak compared to the others (Fig. 4A). On the other hand, no fluorescence signal was detected when the same cells were not induced by IPTG (Fig. 4E–H).

We also fractionated the outer membrane proteins and inclusion bodies from the cells harboring pLO-Bn (n = 0, 1, 2, and 3) and analyzed them by SDS-polyacrylamide gel electrophoresis (Fig. 5I). The bands corresponding to each LO-Bn fusion protein (13.1, 15.85, 18.60, and 21.35 kDa, respectively) were detected in the outer membrane protein extracts and inclusion body extracts obtained from the cells harboring pLO-Bn. The ratio of surface-displayed LO-Bn fusion proteins to the total expressed LO-Bn fusion proteins increased as the number of Buf IIIb-L monomer attached to Lpp-OmpA anchor protein increased. 88% of the total expressed LO-B3 fusion proteins were displayed on the cell surface, while the percentage of successfully displayed LO-B2, LO-B1, and Lpp-OmpA were 57%, 28%, and 3%, respectively (Table 3 and Fig. 4J).

Cleavage of the surface-displayed LO-Bn fusion proteins by pepsin
To gain the antimicrobial activity, free Buf IIIb-L monomers should be released from the surface-displayed LO-Bn fusion proteins by pepsin-mediated cleavage. To test if pepsin can cleave the fusion protein in vivo, the cell pellets (1×10^11 each recombinant cells) were resuspended in simulated gastric fluid, and incubated at 37°C for 1 h. As shown in Fig. 5A, pepsin cleaved the fusion proteins and generated a band corresponding to Buf IIIb-L monomers. The digested peptides were further purified by cationic exchange chromatography and RP-HPLC. The HPLC chromatograms of the recombinant Buf IIIb-L monomers purified from the pepsin-digested peptide mixture showed the same retention time with that of synthetic Buf IIIb-L (Fig. 5B). The amounts of the purified recombinant Buf IIIb-L monomer obtained from E. coli with surface-expressed LO-Bn fusion proteins were calculated using the calibration curve constructed by plotting the average peak area obtained by HPLC versus the concentration of the synthetic Buf IIIb-L (Fig. 5B). The amounts of the purified recombinant Buf IIIb-L monomer obtained from E. coli with surface-expressed LO-Bn fusion proteins were calculated using the bioactivity assay described in Materials and Methods. The BioAssay demonstrated that pepsin digested peptide mixtures obtained from 1×10^11 E. coli cells with surface-expressed LO-B1, LO-B2, and LO-B3 fusion proteins, respectively (Table 3). The recombinant Buf IIIb-L monomers exhibited identical antimicrobial activity to chemically synthesized Buf IIIb-L against eight representative microorganisms (data not shown) and two oral infectious pathogens of livestock S. enteritidis (4 μg/ml of MIC) and L. monocytogenes (2 μg/ml of MIC).

Susceptibility of oral infectious pathogens to the pepsin-digested peptide mixtures obtained from E. coli with surface-expressed LO-Bn fusion proteins
To verify the effectiveness of using E. coli with surface-expressed LO-Bn fusion proteins as antimicrobial microorganism in the stomach of livestock against oral infectious pathogens, we measured the susceptibility of oral infectious pathogens to the pepsin-digested peptide mixtures obtained from 1×10^11 E. coli cells with surface-expressed LO-B1, LO-B2, and LO-B3 fusion proteins. As shown in Fig. 6, the viabilities of S. enteritidis and L. monocytogenes were significantly reduced by the pepsin-digested peptide mixtures (diluted 20-fold) obtained from E. coli with surface-expressed LO-B1 fusion proteins (30.9% and 39%, respectively). The pepsin-digested mixture obtained from E. coli with surface-expressed LO-

Figure 3. Confirmation of Lpp-OmpA-multimeric Buf IIIb-L fusion genes and the expression of LO-Bn, fusion proteins. (A) The number of Buf IIIb-L genes cloned in pLpp-OmpA-Bn was determined by digesting with NotI, whose sites flank each multimer. Lanes 1–4 represent NotI-digested pLpp-OmpA-B0, -B1, -B2, and -B3, which contains 0, 1, 2, or 3 copies of the Buf IIIb-L gene, respectively. Lane M represents size markers. [Image of Arrows indicating bands corresponding to each LO-Bn fusion protein (13.1, 15.85, 18.60, and 21.35 kDa, respectively).] (B) Total cell proteins were analyzed using SDS-PAGE. Lanes 1–4 represent total cell proteins from E. coli harboring pLO-B0, pLO-B1, pLO-B2, and pLO-B3, respectively. Lane M represents molecular weight markers. The closed triangles indicate the LO-Bn fusion proteins expressed from each clone. (C) The presence of LO-Bn fusion proteins in the total cell proteins from each clone was confirmed by Western blot using anti-His antibody. The bands corresponding to each LO-Bn fusion proteins (n = 0, 1, 2, and 3) were detected in lanes 1–4 (13.1, 15.85, 18.60, and 21.35 kDa, respectively). doi:10.1371/journal.pone.0058997.g003

the precise localization of LO-Bn fusion proteins, immunofluorescence labeling of cells was performed by incubation with anti-His-FITC antibody. As shown in Fig. 4, E. coli cells harboring pLO-B1, -LO-B2, or -LO-B3 were observed as solid fluorescent rods when
B3, which contained approximately 3-fold amounts of recombinant monomeric Buf IIIb-L than LO-B1 (Table 3), almost completely inhibited the growth of both bacteria. On the other hand, the pepsin-digested mixtures obtained from E. coli without surface-expressed proteins or E. coli with surface-expressed Lpp-OmpA did not show any inhibitory effect.

Discussion

Antibiotics have been used as therapeutics and prophylactic treatment to control a variety of bacterial infection clearance in the livestock production for more than 60 years. Many types of antibiotics have also been fed at non-therapeutic dosage in the livestock production to increase productivity and feed consumption of animals. However, the use of antibiotics in the livestock production, 100 to 1000 times in terms of annual quantities that in the human population, has been suspected as a major contributor to the emergence of antibiotic-resistant bacteria [22]. Thus many countries have banned the administration of conventional antibiotics (at non-therapeutic doses), as feed additives to livestock [6,7]. This situation has spurred herculean efforts to develop alternative antibiotics for livestock. AMPs have been regarded as a potential solution to the worldwide emergence and rapid horizontal spread of antibiotic-resistant traits in bacteria of human and veterinary clinical significance [23]. The livestock industry, however, has not shown a great deal of interest in developing alternative antibiotics base on AMPs. The main reason is undoubtedly the high cost of manufacturing peptides on a large scale and in a pure form to be used for livestock.

Here we developed the most cost effective means of providing AMPs to livestock by consisting rapid-acting and potent antimicrobial E. coli displaying multimeric Buf IIIb-L, a potent AMP without cytotoxicity, on the cell surface, and showed the effectiveness of using this antimicrobial E. coli as a whole cell antibiotics in the stomach of livestock against oral infectious pathogens. To make a live antimicrobial microorganism, a two-
Figure 5. Pepsin cleavage of the surface-displayed LO-Bn fusion proteins. (A) SDS-PAGE analysis of the pepsin-digested mixtures. Lanes 1–4 represent total cell proteins, and lanes 5–8 represent the pepsin-digested mixtures from *E. coli* BL21 (DE3) harboring pLO-B0, -B1, -B2, and -B3, respectively. Lane 9 represents the synthetic Buf IIIb-L, and lane M represents molecular weight markers. The closed triangle indicates a band corresponding to Buf IIIb-L monomer. (B) HPLC-chromatograms of the synthetic Buf IIIb-L (a) and the purified recombinant Buf IIIb-L monomers from the pepsin-digested mixtures from *E. coli* BL21 (DE3) harboring pLO-B0, -B1, -B2, and -B3, respectively (b–d). The arrow indicates a peak corresponding to Buf IIIb-L monomer.

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The amounts of the LO-Bn fusion proteins and the HPLC-purified recombinant Buf IIIb-L monomers obtained from $1 \times 10^{11}$ cells of *E. coli*.

| Strain                        | Total proteins (µg) | Total expressed LO-Bn (µg) | Surface-displayed LO-Bn (µg) | Buf IIIb-L (µg) |
|-------------------------------|---------------------|-----------------------------|-----------------------------|-----------------|
| *E. coli* harboring pLO-B1    | 37800               | 5818                        | 1629                        | 85.58           |
| *E. coli* harboring pLO-B2    | 38065               | 4058                        | 2313                        | 94.42           |
| *E. coli* harboring pLO-B3    | 32886               | 3631                        | 3195                        | 251.93          |

*a* Total protein concentration was determined by BCA protein assay using bovine serum albumin as a standard.

*b* The amounts of the total expressed LO-Bn fusion proteins and the surface displayed LO-Bn fusion proteins were determined by quantifying the protein bands of SDS-polyacrylamide gels by densitometry at 600 nm as described in Materials and Methods.

*c* The amounts of the HPLC-purified recombinant Buf IIIb-L monomers were calculated using the calibration curve constructed by plotting the average peak area obtained by HPLC versus the concentration of the synthetic Buf IIIb-L peptide.

As expected, the Lpp-OmpA-multimeric Buf IIIb-L (LO-Bn) fusion proteins did not affect the growth of host *E. coli*, and successfully displayed on the cell surface (Fig. 4). Upon cleavage by pepsin, the fusion proteins displayed on the surface of *E. coli* were converted into active AMP monomers, and the liberated Buf IIIb-L monomers inhibited the growth of *S. enteritidis* and *L. monocytogenes* (Figs. 5 and 6). These data indicate that the *E. coli* with surface-expressed LO-Bn fusion proteins may be used as whole-cell antibiotics in the stomach of livestock against oral infectious pathogens. Importantly, the liberated Buf IIIb-L may act only in the stomach and not affect the normal flora in the intestine, because the peptides will be degraded by trypsin or chymotrypsin there. In fact, Buf IIIb-L was almost completely digested by 30-min incubation with trypsin and chymotrypsin *in vitro*, and lost antimicrobial activities (Fig. S1 and Table S1 in File S1). We are now evaluating the effectiveness of *E. coli* with surface-expressed LO-Bn as whole-cell antibiotics *in vivo* using mouse model infected orally with either $1 \times 10^8$ CFU of *L. monocytogenes* or *S. enteritidis* [27]. Preliminary results showed that mice treated with $1 \times 10^{11}$ *E. coli* with surface-expressed LO-Bn, when administrated orally 2 h after pathogen infection, survived longer than PBS-treated control mice (data not shown). This increase in survival rate was not seen with the same number of *E. coli* with surface-expressed LO-B1, probably because there was not enough amount of recombinant monomeric Buf IIIb-L to be effective against pathogens in the $1 \times 10^{11}$ cells. In fact, LO-B1 produces approximately 3-fold amounts of recombinant monomeric Buf IIIb-L than LO-B1. The amounts of the HPLC purified recombinant Buf IIIb-L obtained from $1 \times 10^{11}$ *E. coli* with surface-expressed LO-B3 and LO-B1 were 251.93 µg and 85.58 µg, respectively (Table 3). In other words, we could use smaller number of *E. coli* with surface-expressed LO-B3 than *E. coli* with surface-expressed LO-B1 to get the same effect, which is important, because *E. coli* in itself might cause infectious disease. Usually, *E. coli* forms a beneficial symbiotic relationship with its...
host and plays important roles in promoting the stability of the luminal microbial flora and in maintaining normal intestinal homeostasis [20]. However, immune-suppressed host, or when the gastrointestinal barriers are damaged, some E. coli can cause infectious disease [29]. Therefore, ongoing studies are aimed to surface-display tandem multimeric AMPs on GRAS (Generally Recognized As Safe) strains such as Lactobacillus [30].

Overall, our novel strategy using microorganism with AMPs displayed on the cell surface as whole-cell antibiotics may represent the most effective means of providing potent AMPs to livestock, and have a great impact on controlling over pathogenic microorganisms in the livestock production.

Supporting Information

File S1 Figure S1. Protease digestion of synthetic BufIIIb-L. 2 μg of Buf IIIb-L was incubated with pepsin

References

1. Moore PR, Eynon A, Luckey TD, McCoy E, Elvichjem CA, et al. (1946) Use of sulfasuxidine, streptomycin, and streptomycin in nutritional studies with the chick. J Biol Chem 165: 437–441.
2. Cossy J (2002) Antimicrobial resistance in Canada. CMAJ 167: 805–809.
3. Funk JA, Lejune JT, Winum TK, Rajala-Schultz PJ (2006) The effect of subtherapeutic chlorotetracline on antimicrobial resistance in the fecal flora of swine. Microb Drug Resist 12: 210–218.
4. Inglis GD, McAllister TA, Busz HW, Yanke LJ, Merck DW, et al. (2003) Effects of subtherapeutic administration of antimicrobial agents to beef cattle on the prevalence of antimicrobial resistance in Campylobacter jejuni and Campylobacter hyointestinalis. Appl Environ Microbiol 71: 3872–3881.
5. Barton MD (2000) Antibiotic use in animal feed and its impact on human health. Nutr Res Rev 13: 279–299.
6. Casewell M, Friis C, Marco E, McMullin P, Phillips I (2003) The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J Antimicrob Chemother 52: 159–161.
7. Castanon JI (2007) History of the use of antibiotic as growth promoters in European poultry feeds. Poul Sci 86: 2466–2471.
8. Neu HC (1992) The crisis in antibiotic resistance. Science 257: 1064–1073.
9. Castanon JI (2007) History of the use of antibiotic as growth promoters in European poultry feeds. Poul Sci 86: 2466–2471.
10. Lloyd DH (2012) Alternatives to conventional antimicrobial drugs: a review of future prospects. Vet Dermatol 23: 299–304.
11. Mookherjee N, Hancock RE (2007) Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. Cell Mol Life Sci 64: 922–933.
12. Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, et al. (2007) An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol 25: 463–472.
13. Jang SA, Kim H, Lee JY, Shin JR, Cho JH, et al. (2012) Mechanism of action and specificity of antimicrobial peptides designed based on buforin IIb. Peptides 34: 203–208.
14. Francisco JA, Earhart CF, Georgiou G (1992) Transport and anchoring of beta-lactamase to the external surface of Escherichia coli. Proc Natl Acad Sci U S A 89: 2715–2717.
15. Stathopoulos C, Georgiou G, Earhart CF (1996) Characterization of Escherichia coli expressing an LppOmpA (46–159)-ProA fusion protein localized in the outer membrane. Appl Microbiol Biotechnol 45: 112–119.
16. Lee SY, Cho JH, Xu Z (2003) Microbial cell-surface display. Trends Biotechnol 21: 45–52.
17. Sambrook JDWR (2001) Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold spring Harbor, NY.
18. Park IY, Cho JH, Kim KS, Kim YB, Kim MN, et al. (2004) Helix stability confers salt resistance upon helical antimicrobial peptides. J Biol Chem 279: 13096–13091.
19. Lee SH, Choe H, Park SJ, Lee SY, Park BC (2004) Display of bacterial lipase on the Escherichia coli cell surface by using FurA, as anchoring motif and use of the enzyme in enantioselective biocatalysis. Appl Environ Microbiol 70: 5074–5080.
20. Wang AA, Mulchandani A, Chen W (2002) Specific adhesion to cellulose and hydrolysis of organophosphorus nerve agents by a genetically engineered Escherichia coli strain with a surface-expressed cellulose-binding domain and organophosphorus hydrolase. Appl Environ Microbiol 68: 1604–1609.
21. Kim JM, Jang SA, Yu BJ, Sung BH, Cho JH, et al. (2008) High-level expression of an antimicrobial peptide histomin as a natural form by multimerization and furin-mediated cleavage. Appl Microbiol Biotechnol 78: 123–130.
22. Khachatourians GG (1998) Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. CMAJ 159: 1129–1136.
23. Ingham AB, Moore RJ (2007) Recombinant production of antimicrobial peptides in heterologous microbial systems. Biotechnol Appl Biochem 47: 1–9.
24. Merrell DS, Camilli A (2002) Acid tolerance of gastrointestinal pathogens. Curr Opin Microbiol 5: 51–55.
25. Nivula CP, Bogomolnaya LM, Andrews-Polymenis HL (2008) A comparison of cecal colonization of Salmonella enterica serotype Typhimurium in white leghorn chicks and Salmonella-resistant mice. BMC Microbiol 8: 182.
26. Melton-Wint JA, Rafekhi SM, Pornoy DA, Bakardjiev AI (2012) Oral infection with signature-tagged Listeria monocytogenes reveals organ-specific growth and dissemination routes in guinea pigs. Infect Immun 80: 720–732.
27. Strandberg KL, Richards SM, Tamayo R, Reeves LT, Gunn JS (2012) An altered immune response, but not individual cationic antimicrobial peptides, is associated with the oral attenuation of AraA-deficient Salmonella enterica serovar Typhimurium in mice. PLoS One 7: e95088.
28. Yan F, Polk DB (2004) Commensal bacteria in the gut: learning who our friends are. Curr Opin Gastroenterol 20: 565–571.
29. Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic Escherichia coli. Nat Rev Microbiol 2: 123–140.
30. Burdock GA, Carabin IG (2004) Generally recognized as safe (GRAS): history and description. Toxicol Lett 150: 3–18.