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Two Novel Duck Antibacterial Peptides, Avian β-Defensins 9 and 10, with Antimicrobial Activity

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Two novel avian β-defensins (AvBDs) isolated from duck liver were characterized and their homologies with other AvBDs were analyzed. They were shown to be duck AvBD9 and AvBD10. The mRNA expression of the two genes was analyzed in 17 different tissues from 1–28-day-old ducks. AvBD9 was differentially expressed in the tissues, with especially high levels of expression in liver, kidney, crop, and trachea, whereas AvBD10 was only expressed in the liver and kidney of ducks at all the ages investigated. We produced and purified GST-tagged recombinant AvBD9 and AvBD10 by expressing the two genes in *Escherichia coli*. Both recombinant proteins exhibited antimicrobial activity against several bacterial strains. The results revealed that both recombinant proteins retained their antimicrobial activities against *Staphylococcus aureus* under a range of different temperatures (-70°C - 100°C) and pH values (pH 3–12).

Keywords: Antimicrobial peptides, duck, avian β-defensins, antimicrobial activity

Defensins have been identified in a variety of species, ranging from plants and insects to mammals, including humans [5–6, 14, 21, 24]. Defensins are characterized by the presence of six to eight conserved cysteine residues, and all defensins are structurally related in that they contain three to four intramolecular disulfide bonds and two to three antiparallel β-sheets, in the presence or absence of a β-helix. These peptides are broadly divided into the following five subfamilies, based on the spacing patterns of their cysteine residues: plant, invertebrate, and α-, β-, and θ-defensins [5–6, 14, 21, 24]. Of the three defensin subfamilies found in humans and other mammals (α-, β-, and θ-defensins), only β-defensins have been found in birds [19, 22]. Avian β-defensins play an important role in the innate immune system. Since avian heterophils lack superoxide ions and myeloperoxidase, they are more dependent on nonoxidative mechanisms, including the actions of lysozymes, cationic proteins, and peptides [8].

A new standardized nomenclature for avian β-defensins has recently been proposed [16], and all avian β-defensins have now been assigned gene names using the term avian β-defensin (AvBD). We have used this nomenclature throughout this paper. Fourteen AvBDs, previously known as gallinacins (Gals), have been described in chickens (*gallinacins*) [3, 9, 11]. Two β-defensins, AvBD103a and AvBD103b (previously known as spheniscin-1 and spheniscin-2, respectively) have been isolated from the gastrointestinal tract cells of King Penguins (*Aptenodytes patagonicus*) [25]. In addition, four ostrich (*Struthio camelus*) AvBDs (previously known as ostricacins), AvBD1, AvBD2, AvBD7, and AvBD8, have been purified from ostrich leukocytes [23, 28]. Most of these β-defensins exhibit antimicrobial activity against a wide range of pathogens, including bacteria and fungi [3–4, 11, 15, 17, 20, 23, 25, 27, 28].

However, there is little available information on duck AvBDs and their roles in the immune system of ducks. In the present study, we cloned and sequenced two novel duck AvBD genes, AvBD9 (previously known as duck β-defensin-6-like antimicrobial peptide) and AvBD10, from duck livers. The two genes were found to be differentially expressed in many tissues of 1-, 7-, 14-, 21-, and 28-day-old ducks. In addition, GST-tagged recombinant duck AvBD9 and AvBD10 proteins (rAvBD9 and rAvBD10) were successfully expressed in *Escherichia coli*, and their antimicrobial activities were investigated.

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Materials and Methods

RNA Extraction, Reverse Transcriptase-Polymerase Chain Reaction (RT–PCR) Amplification, and Sequencing

We extracted total cellular RNA from 200 μl of liver tissue fluid obtained from a 21-day-old specific pathogen-free (SPF) Peking duck, using TRIzol reagent (Invitrogen, Beijing, China), according to the manufacturer's instructions. RT–PCR was performed using primers designed internally on the basis of the coding sequences of chicken AvBDs (previously known as Gals) [15] and duck β-actin, as previously described [17]. The primers used were as follows: AvBD9, 5'-ATGAGAATCCTTTTCTTCCTTGTTGC-3' (forward) and 5'-TTAGGAGCTAGGTGCCCATTTGCAGC-3' (reverse); AvBD10, 5'-CTGTTCTCCTCTTCCTCTTCCAG-3' (forward), 5'-AATCTTG GCACAGCAGTTTAACA-3' (reverse); duck β-actin, 5'-CATCGCT GACAGGATGCAGAAGGAG-3' (forward) and 5'-TGATCCACAT CTGCTGCTGGTAG-3' (reverse). The PCR protocol was as follows: an initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and polymerization at 72°C for 1 min. The final polymerization step was performed at 72°C for 10 min. The PCR products were cloned into the pMD18-T vector (Takara, Japan) to confirm amplification, followed by sequencing of the recombinant plasmids.

The two duck AvBD sequences have been assigned GenBank accession numbers EF431957 (AvBD9) and EU833478 (AvBD10).

Sequence Analysis of Duck AvBD Genes

The sequences of the two duck AvBDs were compared with the sequences of most other known AvBDs using the Basic Local Alignment Search Tool (BLAST). Multiple alignments and phylogenetic analyses were performed using the Clustal V routine of the MegAlign program provided in the DNAStar package (Windows 4.05, DNAStar, Madison, WI, U.S.A.) [10].

Expression of Duck AvBD9 and AvBD10 mRNA in Different Tissues

The SPF Peking ducks used in this experiment were supplied by the National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Fifteen ducks were used and tissue samples were collected from three ducks at each of 1, 7, 14, 21, and 28 days old. The samples were analyzed individually. The tissues sampled included skin, tongue, esophagus, trachea, crop, glandular stomach, muscular stomach, breast muscle, small intestine, lung, liver, kidney, heart, spleen, bone marrow, thymus, and bursa of Fabricius. All the samples were rinsed, immediately dissected, and squeezed between a Whatman filter to remove excess blood. They were then rinsed in cold sterile saline, snap-frozen in liquid nitrogen, and stored at −70°C until further use. Total RNA extraction, cDNA synthesis, and RT–PCR were performed as described above. Seven μl of the PCR products was analyzed on 2.0% agarose gels. All assays were performed in duplicate.

Protein Expression and Purification

DNA fragments encoding the duck AvBD9 and AvBD10 genes were amplified by PCR using the following primers: AvBD9, 5'-GAATTCATGGCTGTTCTCTTCTTCCTC-3' (forward) and 5'-GTC GACTTAGGAGCTAGGTGCCCATTTGCAGC-3' (reverse); AvBD10, 5'-GGATCCATGGCTGTTCTCCTCTTCCTC-3' (forward), and 5'-GCCAAGATTCCGGCGCAGTAGCGGCCGC-3' (reverse). The PCR products containing the duck AvBD9 coding sequence, flanked by EcoRI and SalI restriction sites, and containing the duck AvBD10 coding sequence, flanked by BamHI and SalI restriction sites, were purified, ligated into the pMD18-T vector, and sequenced as described above. The duck AvBD9 gene contained in the above plasmid was inserted into the pGEX-6p-1 vector between the EcoRI and SalI restriction sites. The resultant plasmid was designated as duck rAvBD9-pGEX and sequenced again. Similarly, the duck AvBD10 gene contained in the above plasmid was inserted into the pGEX-6p-1 vector between the BamHI and SalI restriction sites. The resultant plasmid was designated as duck rAvBD10-pGEX and sequenced again.

The constructs confirmed to contain the fusion genes were transformed in competent *E. coli* BL21(DE3) cells. The GST-tagged proteins were induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified, as described previously [17], using a protein purification and refolding kit (No. 70123-3; Novagen, Germany), according to the manufacturer's instructions. Briefly, the induced culture was harvested by centrifugation at 6,500 ×*g* for 15 min at 4°C, and the supernatant was removed and discarded. Then, the cell pellet was weighted and resuspended in 1× inclusion body (IB) wash buffer (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100) and sonicated after a 15-min incubation at 30°C with lysozyme. The inclusion bodies were collected by centrifugation at 10,000 ×*g* for 10 min. Then, they were resuspended in 1× IB solubilization buffer supplemented with 0.3% *N*-lauroylsarcosine. The supernatant containing the fusion proteins were filtered through a cellulose acetate filtration membrane with a pore size of 0.45 μm and then passed through an affinity chromatography column of glutathione Sepharose 4B (Amersham) equilibrated with PBST (PBS+1% Triton X-100). The column was washed with 6 bed volumes of PBS to remove contaminating proteins. The GST-tagged recombinant proteins were then eluted with 10 ml of 50 mM of Tris-HCl buffer containing 10 mM reduced glutathione, pH 8.0. The fusion proteins were concentrated using Centricon Microconcentrators (Millipore, Beijing, China) with a molecular mass cutoff of 10 kDa. The fusion proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and the protein concentrations were determined by analysis of the SDS–PAGE gel images and by Bradford's method [1, 20].

Antimicrobial Activities and Minimal Inhibitory Concentrations (MICs)

Zone-of-inhibition assays were performed to test the antimicrobial activities of rAvBD9 and rAvBD10 against the following bacterial strains: *E. coli* BL21(DE3), *Bacillus cereus* (ATCC 9193), *Staphylococcus aureus* (ATCC 29213), *Pasteurella multocida* (ATCC 6529), and *Salmonella choleraesuis* (CVCC 2140), as previously described [17].

Their antimicrobial activities were also investigated using colony-counting assays [26]. All bacterial strains were maintained in Luria–Bertani (LB) medium at 37°C. Bacterial strains were cultured to mid-logarithmic phase by transferring 100 μl of stationary-phase suspension into LB medium followed by incubation and shaking for 4 h at 37°C. Mid-logarithmic phase cultures were centrifuged for 10 min at 4°C at 900 ×*g*, and the bacterial pellets were diluted in minimal medium (LB medium diluted 1,000-fold in distilled water for bacterial pellets). Initial concentrations of bacteria were determined by measuring the optical density at 620 nm. To determine cell

viability, 100 μl of 10-fold serial dilutions in PBS (pH 7.4) were transferred on to Trypticase soy agar (TSA; Oxoid Limited) plates, and colonies were counted after 24 h of incubation. Final dilutions were prepared in minimal LB media to reach a cell density of 2.5×10^8 colony forming units (CFU)/ml.

At first, kill-curve studies were performed to determine the incubation period. One hundred μl of diluted bacterial culture (mid-logarithmic phase diluted 1,000-fold in LB medium) was mixed with 100 μl of 400 μg/ml rAvBD9 or rAvBD10 (final concentrations 200 μg/ml) and anaerobically incubated at 37°C. At various time points (0, 1, 2, 2.5, and 3 h), a 50-μl bacterial suspension aliquot was taken and diluted 10–1,000-fold in LB medium, of which 100 μl was plated on TSA medium. The number of CFU was counted after overnight incubation at 37°C. As a negative control, the bacterial suspension was incubated with 50 μl of minimal LB medium. On the basis of the results of kill-curve studies, 100 μl of diluted bacterial culture (mid-logarithmic phase diluted 1,000-fold in LB medium) was mixed with 100 μl of 0 to 1,000 μg/ml rAvBD9 or rAvBD10 (final concentration, 0, 25, 50, 100, 200, and 500 μg/ml) in polypropylene microtiter plates and preincubated for 2 h at conditions suited to the investigated strain. After a 2-h incubation period, it was further diluted 100- to 10^6-fold in minimal medium, and transferred onto TSA plates, and colonies were counted after 24 h of incubation. For each antimicrobial activity assay, PBS (pH 7.4) was used as the negative control. All assays were performed in duplicate.

In addition, the MICs of the recombinant proteins were determined using a liquid growth inhibition assay, as previously described [2, 17]. A stock solution of recombinant protein was serially diluted with 2-fold amounts of PBS (pH 7.4) and 0.2% BSA as the negative control (0–100 μg/ml). Aliquots (10 μl) from each dilution were transferred to a 96-well polypropylene microtiter plate, and each well was inoculated with 100 μl of a suspension of mid-log bacteria (10^6 CFU/ml) in poor broth [1% tryptone, 0.5% NaCl (w/v), pH 7.5]. The culture was grown for 24 h with vigorous shaking at 37°C, and bacterial growth was evaluated by measuring the culture absorbance at 490 nm using a microplate reader. Growth inhibition was defined as the lowest concentration of peptide that reduced growth by >90%. All assays were performed in triplicate.

Temperature and pH Stability

One hundred μl of rAvBD9 or rAvBD10 was incubated at −70°C, −20°C, 4°C, 20°C, 40°C, 60°C, or 100°C for 30 min, and at pH 3, 5, 7, 10, or 12 for 30 min. The antimicrobial activities of all the samples were tested against *S. aureus* by colony-counting assay immediately after. Phosphate-buffered saline (PBS) at pH 3, 5, 7, 10, and 12 was used as a negative control for the pH stability assay. All assays were performed in duplicate.

RESULTS

Analysis of the Two Duck AvBDs Revealed That They were Duck AvBD9 and AvBD10

We cloned and sequenced two genes isolated from duck liver tissues and compared the sequences with those of published AvBDs. The complete nucleotide sequence of one of the genes contained a 204 bp open reading frame (ORF) encoding 67 amino acids, which shared 100% amino acid homology with chicken AvBD9 (previously known as Gal-6 [15]). The other gene fragment showed the highest percentage of amino acid homology (85.5%) with chicken AvBD10 (previously known as Gal-8 [15]). Furthermore, alignment of these two duck AvBDs revealed that, in common with other AvBDs, these AvBDs shared conserved regions with other avian peptides, including three paired cysteine disulfide bridges (Fig. 1A). These six conserved residues have been designated as the "β-defensins core motif," which is an essential structural element of β-defensins [9, 27]. Hence, we designated the two novel AvBDs as duck AvBD9 and AvBD10, respectively.

A phylogenetic tree was constructed based on our two duck AvBDs and the other AvBD amino acid sequences, and the results placed our two duck AvBDs in clusters with chicken AvBD9 and AvBD10, respectively (Fig. 1B). Hence we designated the two novel AvBDs as duck AvBD9 and AvBD10, respectively. Duck AvBD9 shared 100% nucleotide homology with its chicken analog. However, duck AvBD10 and chicken AvBD10 only shared 85.5% amino acid homology.

mRNA Distribution Studies Proved That Duck AvBD9 and AvBD10 had Different Expression Patterns in Duck Tissues

RT–PCR was used to analyze the expression patterns of AvBD9 and AvBD10 in duck tissues, using mRNA isolated from 17 different types of tissue from healthy SPF ducks aged 1, 7, 14, 21, and 28 days (Fig. 2). As shown in Fig. 2A, AvBD9 was highly expressed in all the tissues except the skin, glandular stomach, and muscular stomach of the ducks investigated at different ages. In 1-day-old SPF ducks, AvBD9 mRNA was highly expressed in the liver and kidney and moderately expressed in the trachea, breast muscle, spleen, bone marrow, thymus, and bursa of Fabricius. This expression pattern differed from that in 7-day-old SPF ducks. The highest AvBD9 mRNA expression levels in 7-day-old ducks were noted in the liver and kidney tissues, whereas low AvBD9 mRNA expression was detectable in a small number of tissues, including breast muscle, small intestine, lung, and thymus tissue. In 14-day-old ducks, the highest AvBD9 mRNA expression was found in the trachea, crop, small intestine, liver, and kidney tissues, with moderate expression in the tongue, esophagus, breast muscle, lung, heart, spleen, bone marrow, thymus, and bursa of Fabricius. As in the 14-day-old ducks, both 21- and 28-day-old ducks exhibited high tissue expression of AvBD9. Thus, AvBD9 was highly expressed in the liver and kidney and moderately expressed in the other tissues, except for the skin, glandular stomach, and muscular stomach.

In contrast to the gene expression patterns of duck AvBD9, duck AvBD10 mRNA was only highly expressed in the liver and kidney in ducks at all ages (Fig. 2B).

A

MRILFFLVAVLFFLFQAAPAYSQED-ADTLACRQSHGSCSFVACRAPSVDIGTCRGGKLKCCKWAPSS chickenAvBD9

MRILFFLVAVLFFLFQAAPAYSQED-ADTLACRQSHGSCSFVACRAPSVDIGTCRGGKLKCCKWAPSS duck AvBD9

MKILCLLFAVLLFLFQAAPGSAD**PLFPDTV**ACR**T**QGNFCRAGACPPTFT**IS**GQCHGGLLNCCAKIPAQ chicken AvBD10

---------VLLFLFQAAPGSAD**APFADTAA**CR**S**QGNFCRAGACPPTF**AASGS**CHGGLLNCCAK------ duck AvBD10

B

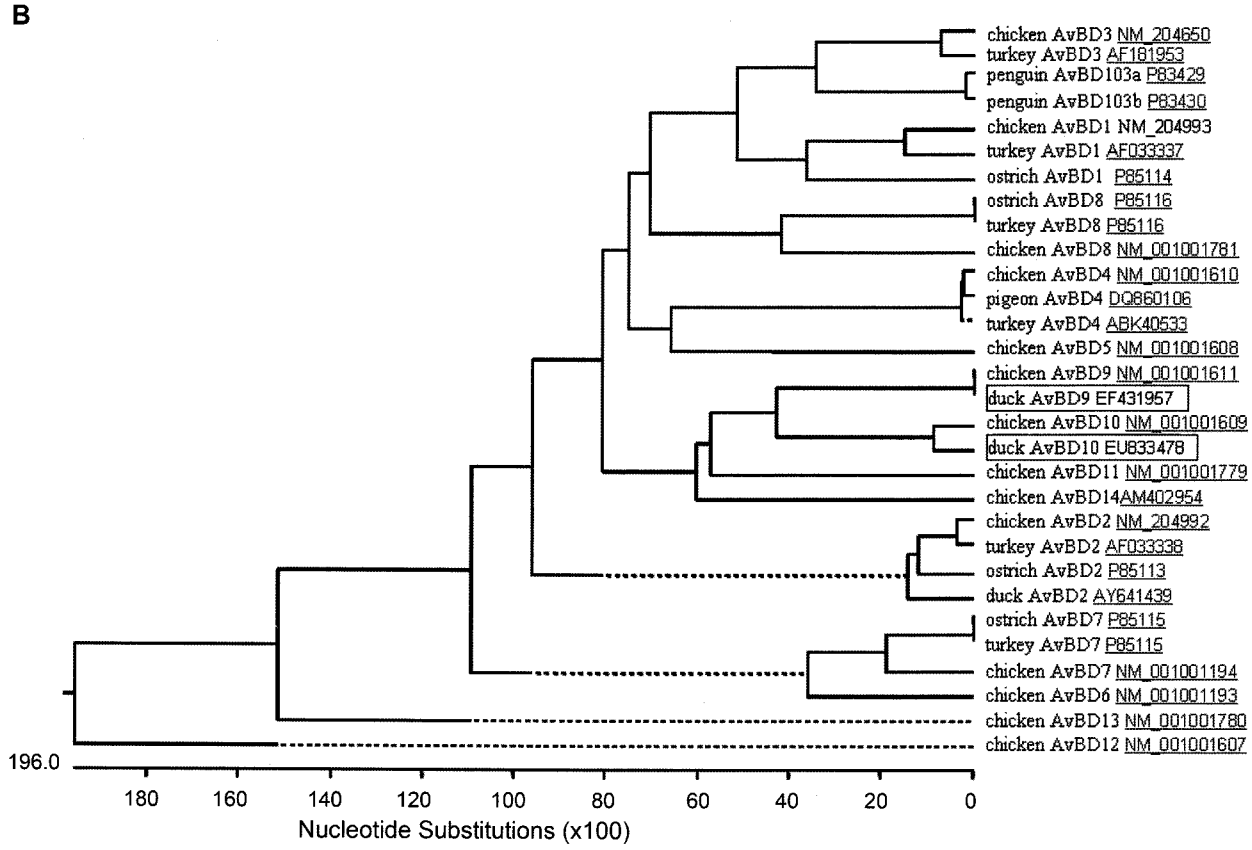

Fig. 1. Deduced amino acid sequences and phylogenetic relationships of duck avian β-defensins (AvBDs).
A. Alignment of the two duck AvBDs and the corresponding peptides reported by Lynn *et al.* [15] [accession numbers: chicken AvBD9 (previous known as gallinacin-6), AY534894, and AvBD10 (previous known as gallinacin-8), AY534898)]. **B**. Phylogenetic relationships, based on the sequences of the two duck AvBDs and other AvBDs, using the MEGALIGN program DNAStar with Clustal V method [10]. *Note*; The six cysteines of the AvBD motifs are underlined. The amino acids that differ between chicken and duck AvBD10 are in bold. The two duck AvBDs that were sequenced in the present study are in the box. "-" indicates no identical or conserved residues observed. AvBD9, avian β-defensin 9. AvBD10, avian β-defensin 10.

Production and Characterization of GST-tagged Recombinant Duck AvBD9 and AvBD10

High levels of GST-tagged rAvBD9 and rAvBD10 expressions were noted after induction with 0.6 mM IPTG for 5 h (Fig. 3A), and the production of rAvBD9 and rAvBD10 (both molecular mass, 32 kDa) accounted for approximately 38.7% and 40% of the total proteins, respectively. We also found that both the recombinant proteins were produced as inclusion bodies in the cells (Fig. 3B). The two recombinant proteins were purified, and both purified recombinant proteins were visualized as single bands on SDS–PAGE gels (Fig. 3B).

The antimicrobial activity of the two GST-tagged recombinant peptides (antimicrobial potency of chicken AvBDs has been shown previously not to be altered by the presence of a GST tag [17]) was tested against five bacteria strains including *E. coli, B. cereus, S. aureus, P. multocida,* and *S. choleraesuis*, using two different assays. Both assays produced similar results. A very distinct zone of inhibition appeared around the treated well following the addition of 50 μl of purified rAvBD9 or rAvBD10 at a concentration of 1 μg/μl (Fig. 4A). In contrast, no zone of inhibition was observed around untreated wells, suggesting that both

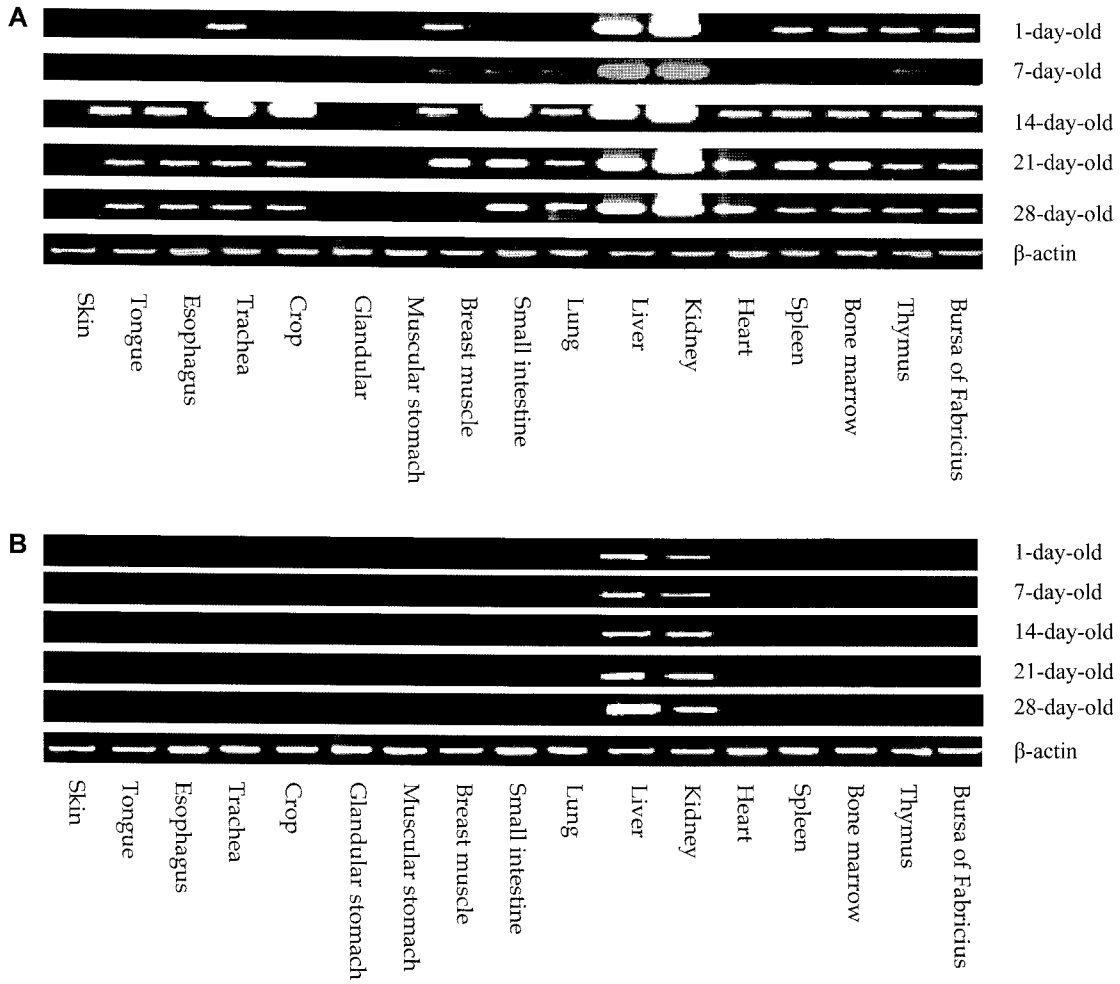

Fig. 2. Expressions of AvBD9 and AvBD10 mRNA in various tissues in 1-, 7-, 14-, 21-, and 28-day-old ducks.
All assays were performed in duplicate. **A**. Expression of duck AvBD9 mRNA. **B**. Expression of duck AvBD10 mRNA. AvBD9, avian β-defensin 9. AvBD10, avian β-defensin 10.

rAvBD9 and rAvBD10 possessed antibacterial activity against these five bacterial strains. The dose-dependent survival of the recombinant protein-treated bacteria was tested in colony-counting assays. Both recombinant proteins showed similar antibacterial activities (Fig. 4B and 4C). A rapid decline in surviving cells was observed for *B. cereus*, *S. aureus*, and *P. multocida*. A slower decline was observed for *E. coli* and *S. choleraesuis*. *E. coli* and *S. choleraesuis* cells were only partially eradicated at 500 μg/ml rAvBD9 (Fig. 4B) or rAvBD10 (Fig. 4C). Additionally, the MICs of these two recombinant proteins were determined using a liquid growth inhibition assay (Table 1). The results showed that both recombinant proteins could inhibit the growth of *B. cereus* at low concentrations, of *S. aureus* at medium concentrations, and of *P. multocida* at high concentrations. However, they were less effective against *E. coli* and *S. choleraesuis*.

Both recombinant proteins (final concentrations, 200 μg/ml) retained their microbicidal activities against *S. aureus* cells following incubation at different temperatures, ranging from −70°C–100°C (Fig. 5A), or following acid–alkali treatment at pH values ranging from pH 3–12 (Fig. 5B), indicating that the cell killing activities of both recombinant proteins were temperature and pH independent over these ranges.

DISCUSSION

To identify novel defensin genes in the duck, two sets of primers were designed on the basis of the coding sequences of chicken AvBD9 and AvBD10, respectively. Fortunately, two novel duck AvBDs, designed as duck AvBD9 and AvBD10, were identified in the duck liver using these two sets of primers. Alignment of the two peptides with respective peptides from chicken revealed a conservation of the signal sequence at the N-terminus and the characteristic six-cysteine defensin motif at the C-terminus (Fig. 1A). Consistent with the fact that all β-defensins are a group of

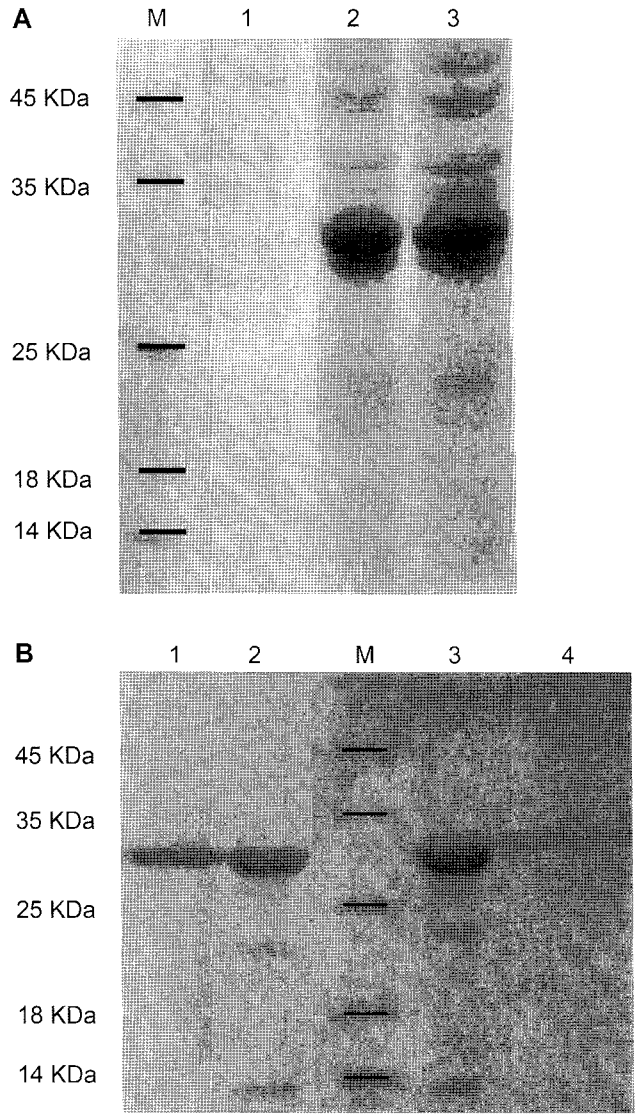

Fig. 3. SDS–PAGE analysis of GST-tagged recombinant duck AvBD9 and duck AvBD10 fusion proteins expressed in the *E. coli* BL21(DE3) cells.

A. Recombinant duck AvBD9 and duck AvBD10 fusion proteins. Lane M, protein molecular mass marker; Lane 1, total protein containing AvBD9, extracted from BL21(DE3) cells without IPTG induction; Lane 2, total protein containing AvBD9, extracted from BL21(DE3) cells 5 h after induction with IPTG; Lane 3, total protein containing AvBD10, extracted from BL21(DE3) cells 5 h after induction with IPTG. **B**. Recombinant duck AvBD9 and duck AvBD10 purified fusion protein. Lane M, protein molecular mass marker; Lane 1, purified AvBD9 after induction with IPTG; Lane 2, inclusion body of AvBD9; Lane 3, inclusion body of AvBD10; Lane 4, purified AvBD10 after induction with IPTG. AvBD9, avian β-defensin 9. AvBD10, avian β-defensin 10. IPTG, isopropyl-β-D-thiogalactoside.

secreted molecules in response to infections, the signal sequences of these β-defensins are hydrophobic and rich in leucines. In addition, the mature C-terminal sequences are all positively charged owing to the presence of excess arginines and lysines.

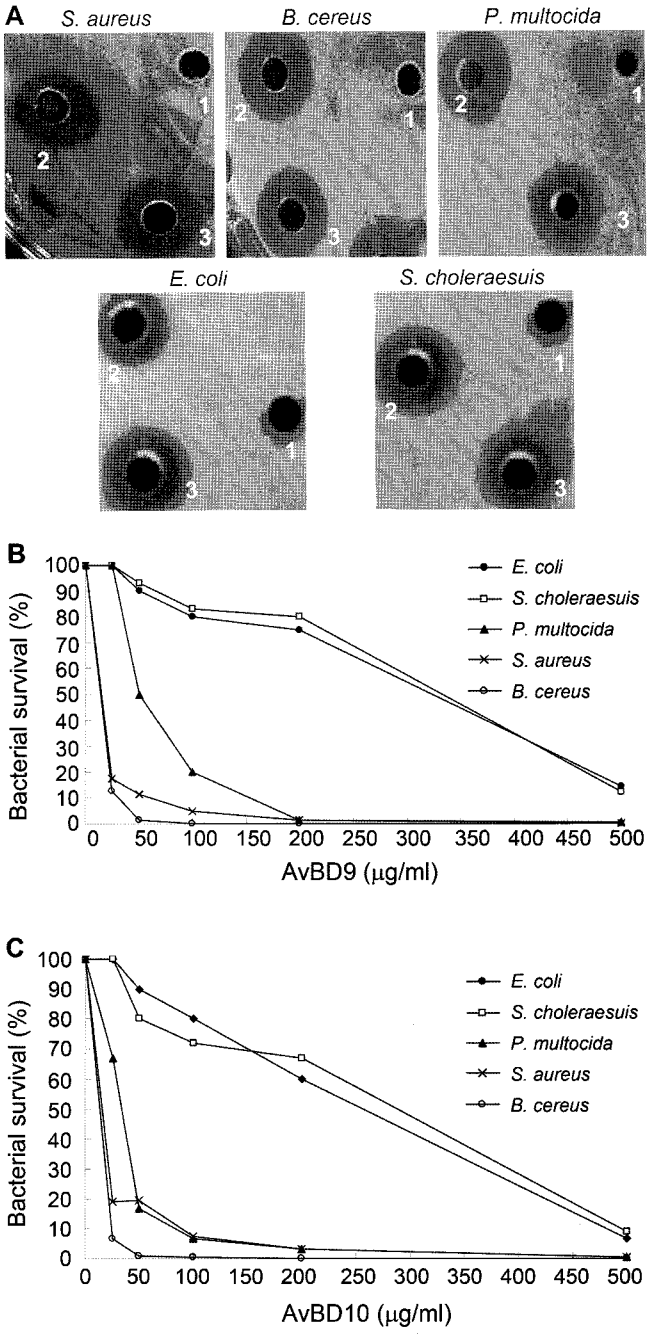

Fig. 4. Antimicrobial activity of GST-tagged recombinant duck AvBD9 and duck AvBD10 against *S. aureus*, *B. cereus*, *P. multocida*, *E. coli*, and *S. choleraesuis* bacteria.

A. Zone-of-inhibition assay. Zone 1: Control treated with 50 μl of PBS. Zone 2: Treatment with 50 μl of purified recombinant duck AvBD9 (1.0 μg/μl). Zone 3: Treatment with 50 μl of purified recombinant duck AvBD10 (1.0 μg/μl). All assays were performed in duplicate. AvBD9, avian β-defensin 9. AvBD10, avian β-defensin 10. **B**. Antimicrobial activity of recombinant duck AvBD9 conducted by colony-counting assay. **C**. Antimicrobial activity of recombinant duck AvBD10 conducted by colony-counting assay. The number of colony forming units (CFU) surviving from a 2 h incubation with recombinant duck AvBD9 and AvBD10 are presented relative to that of PBS control (%). All kill-curve studies were performed in duplicate and data points are the averages. AvBD9, avian β-defensin 9. AvBD10, avian β-defensin 10.

Table 1. Minimal growth inhibitory concentration (MIC) of recombinant duck AvBD9 and AvBD10 (means±SD).

Microorganism	MIC[a] (μg/ml)	
	AvBD9	AvBD10
E. coli	400.5±10.50	313.4±16.22
S. aureus	28.3±5.22	20±3.12
B. cereus	7.4±0.12	5.3±0.25
P. multocida	114.5±8.52	78.6±6.34
S. choleraesuis	>400	>400

[a]All assays were performed in triplicate.
AvBD9, avian β-defensin 9.
AvBD10, avian β-defensin 10.

As found for ostrich AvBD7 and turkey AvBD7, and ostrich AvBD8 and turkey AvBD8 (Fig. 1B), duck AvBD9 shared 100% nucleotide homology with its chicken analog. However, duck AvBD10 and chicken AvBD10 only shared 85.5% amino acid homology. This relatively low level of amino acid homology has also been observed between other avian species, such as chicken AvBD1 and turkey AvBD1, chicken AvBD2 and turkey AvBD2, chicken AvBD3 and turkey AvBD3, and chicken AvBD4 and turkey AvBD4 (Fig. 1B). This is thought to be due to positive Darwinian selection resulting from specific evolutionary pressures exerted by pathogenic microbial flora in different animal species [7, 12, 13, 27]. Since β-defensin sequences are very short, positive selection can cause significant diversification between species at the amino acid level during the course of evolution [13].

AvBDs mRNAs have been shown to be widely and differentially expressed in avian tissues [11, 15, 17–18, 22–23, 25–28]. Two independent studies have previously reported consistent mRNA expression patterns for AvBD9 and AvBD10 in chickens [15, 26]. Our results in ducks were partially consistent with the results reported for chickens. Variations in AvBD9 expression levels between chickens and ducks suggest that its upregulation and inducibility are tissue specific.

With the exception of the glandular and muscular stomach, moderate to high AvBD9 expression was observed throughout the digestive tract and respiratory systems of the 14- to 28-day-old ducks. These results suggest that the duck digestive tract and respiratory system only develop completely after 14 days. The crop is an extension of the esophagus in which food can be stored for up to 24 h, and is well developed in gallinaceous birds. The fact that AvBD9 was expressed in the crop of ducks suggests that it may play an important role in the defense responses mediated by the crop tissue. These results agree with those of a previous report on AvBD9 obtained from chickens [15]. Weak AvBD9 expression was noted in the small intestine of 1-day-old ducks, but mRNA levels had increased by 7 days. The variation in AvBD9 mRNA expression levels

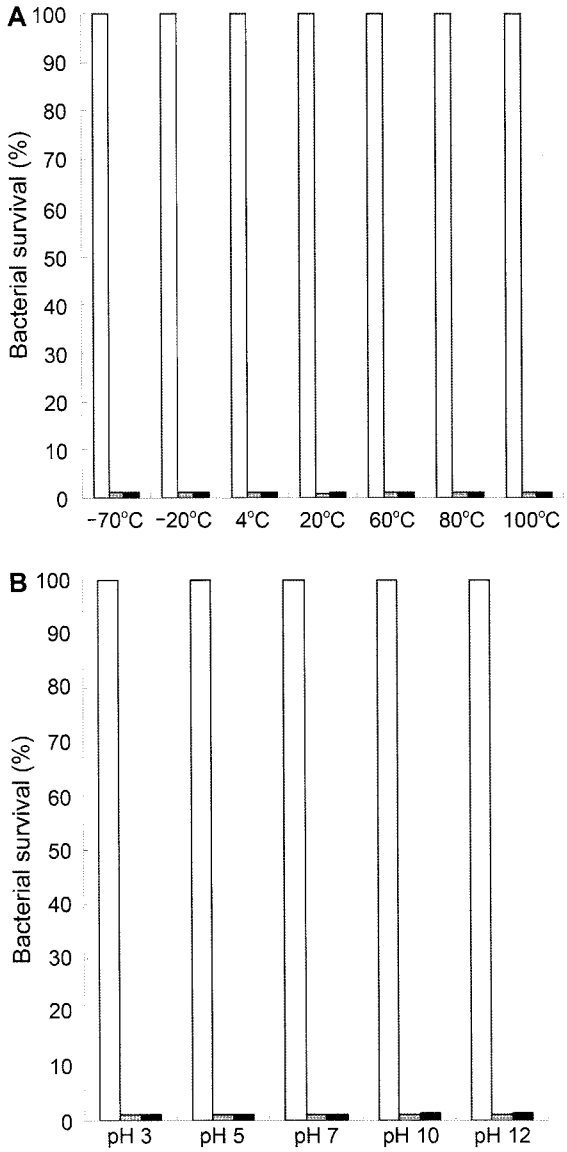

Fig. 5. Antimicrobial activity of GST-tagged recombinant duck AvBD9 and duck AvBD10 against *S. aureus* cells at different temperatures and pH values.

A. Incubation of recombinant duck AvBD9 (black bars) or AvBD10 (gray bars) at −20°C, 4°C, 20°C, 40°C, 60°C, and 100°C for 30 min. Control: Control treated with PBS (white bars). **B.** Incubation of the recombinant duck AvBD9 (black bars) or AvBD10 (gray bars) at pH 3, 4, 5, 7, 10, and 12 for 30 min, respectively. Control: Control treated with PBS (white bars) at pH 3, 4, 5, 7, 10, and 12, respectively. The number of colony-forming units (CFU) surviving from a 2 h incubation with recombinant duck AvBD9 and AvBD10 are presented relative to that of PBS control (%). All kill-curve studies were performed in duplicate and data points are the averages. AvBD9, avian β-defensin 9. AvBD10, avian β-defensin 10.

observed in the small intestine of ducks of different ages suggests that AvBD9 may be developmentally regulated. The gastrointestinal tract is particularly susceptible to infection, since it is constantly exposed to a broad range of potential pathogens. The presence of an efficient host defense system that involves the production of AvBDs by

the epithelial cells of the intestinal mucosa is critical. These findings suggest that AvBDs, including AvBD9, may play an important role in infections of the small intestine in chickens and ducks.

In the present study, high AvBD9 and AvBD10 mRNA expression levels were found in the liver. Similarly, moderate to high mRNA expressions of AvBD8, AvBD9, and AvBD10 have been found in the liver of chickens [11, 15, 27]; this suggests that AvBDs might play an important role in the liver during systemic infections. In the present study, high AvBD9 and AvBD10 expressions were also found in the kidney of ducks of all ages, consistent with the findings reported for other AvBDs in chickens [11, 15, 17, 27]. The high mRNA levels noted for multiple AvBDs in the kidneys of both ducks and chickens suggest that these AvBDs might play a similar role in the protection of the avian urogenital tract.

Most AvBDs, both naturally occurring [3, 9] and chemically synthesized [11, 26], or produced by recombinant expression [17, 18, 26], have been shown to possess antimicrobial activities against a wide range of pathogens, including bacteria and fungi. In agreement with previous studies, the present study showed that both duck rAvBD9 and rAvBD10 exhibited strong bactericidal activities against *B. cereus, S. aureus,* and *P. multocida,* and low activities against *E. coli* and *S. choleraesuis.* Contrary to our expectations, both the recombinant proteins retained their antimicrobial activities against *S. aureus* under different temperatures and pHs. We repeated these experiments several times in order to verify the results. van Dijk *et al.* [26] also found that the antimicrobial activity of chemically synthesized chicken AvBD9 against *E. coli.* was pH independent (range, 5.5–7.0). These results explain those of a previous report, suggesting that synthetic King Penguin AvBD103b retained its microbicidal activity *in vivo,* within the stomach environment, thus protecting the bird against microorganisms involved in the degradation of food [25].

In conclusion, two novel antimicrobial peptides, known as duck AvBD9 and 10, were identified and characterized from duck tissues. The two antimicrobial peptides are classified as β-defensins, characterized by the six cysteine residues and its pairings. The two genes have been shown to be expressed widely in tissues of ducks. The recombinant peptides were effective against bacterial cells and retained stability under different temperatures (range, −70°C to 100°C) and pH values (range, 3 to 12). Ducks are an economically important species, and although chicken AvBDs have been well studied over the past 20 years, few studies have investigated duck AvBDs. The identification of homologous AvBDs in ducks in the current study will facilitate investigations into their innate immune response. Moreover, the identified AvBDs have the potential to be exploited for the development of new therapeutic agents to treat economically significant duck diseases, and may have applications as natural alternatives to artificial antibiotics that are commonly fed to ducks, and that are currently arousing increasing public concern.

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