e-mail: clooft@tierzucht.uni-kiel.de

Genomic identification of bovine beta-defensin genes and their expression in mammary gland tissue

Susanne Roosen, C. Looft and E. Kalm

Institute of Animal Breeding and Husbandry, Christian-Albrechts-University, Olshausenstr. 40, 24098 Kiel, Germany

Abstract

20 bovine BACs were isolated and analyed. From these BACs 21 β-defensin genes have been identified, of which seven genes and four pseudogenes are novel. These genes are named D*EFB401–DEFB405*, *LAP-like* and *DEFB300*, the pseudogenes are named *EBDP*, *EBDP2*, *DEFB302P* and *DEFB303P*. *DEFB401* is expressed in the bovine udder. *DEFB300*, *DEFB302P* and *DEFB303P* represent a new subfamliy of β-defensin genes in cattle because of their homology to human β-defensin-103. Furthermore, the genomic sequences of *BNBD3* and *BNBD7–BNBD11* and the already published genes *TAP*, *DEFB1*, *BNBD4* and *BNBD12/13* were identified. The order of these genes on the chromosome are presented by contig-assembly. Two contigs spanning a region of approximatly 310 kb and 387 kb were constructed.

The analysis of β-defensin gene expression in the bovine healthy and infected udder revealed the existance of *LAP*-, *TAP*-, *DEFB1*-, *BNBD3*-,*BNBD9*-, *BNBD12*- and *DEFB401*-mRNA. The abundance of β-defensin mRNA and the inducibility of some genes gives first evidence that β-defensins may play an important role in local host defense.

Introduction

Mastitis is the most important disease affecting dairy cattle and causes significant economic losses (Bartlett and Van Wijk, 1991). It is caused mainly by environmental factors but is influenced by genetic components (Lund et al., 1999). The assumption that β-defensin genes may be a factor of inborn resistance to mastitis and may be considered as candidate genes, respectively, is due to the fact that β-defensins are part of the innate immune system of vertebrates, insects and plants. They are multifunctional peptides, having an antimicrobial activity against bacteria, viruses and fungi. Apart from their antimicrobial activities they have a function as signal molecules in the immune system and are chemoattractants immune cells (Risso, 2000; Yang et al., 1999).

Defensins are expressed in epithelial and mucosal tissue of most organs, in neutrophils and in non-epithelial tissue like muscle in a variety of species. The first β-defensin identified in cattle was *TAP* (Diamond et al. 1991), a peptide with antimicrobial activity isolated from bovine tracheal mucosa. Selsted et al. (1993) isolated and characterized 13 antimicrobial peptides from bovine neutrophils (BNBD1–13) and Schonwetter et al. (1995) proved inducible expression of bovine *LAP*-gene (lingual antimicrobial peptide) in tongue epithelium at sites of infection or injury. Tarver et al. (1998) isolated another β-defensin (EBD) from bovine enteric epithelial cells.

To date, the expression of defensin-genes in the bovine udder has not been further investigated. We therefore examined the expression of β-defensin-genes in bovine mammary gland tissue by RT-PCR. Apart from the analysis of gene-expression in the bovine udder, the aim of this study is the genomic characterization of bovine β-defensin genes.

Materials and Methods

Isolation and characterization of BACs. The genomic characterization of bovine β-defensin genes was accomplished by analysing defensin-gene containing BACs. BACs were isolated by screening the bovine BAC library RZPD number 750 (Zhu et al., 1999) and 754 (Buitkamp et al., 2000) of Ressourcenzentrum für Genomforschung Berlin (http://www.rzpd.de/). Screening has been done by PCR-amplification using primers based on consensus-sequences of published β-defensin sequences. BAC-DNA was prepared according to methods described by R.E.A.L.® Prep 96 Handbook (Qiagen). BACs were characterized by digestion with *Not*I (BioLabs) and PFGE. PCR-products from BACs have been amplified using primers established on cDNA-consensus sequences. PCR-products were analyzed by electrophoresis on an 1.5% agarose gel, products of the appropriate size were extracted and purified using the MinElute PCR purification Kit (Qiagen). Ligation and transformation of PCR-products was performed using the TOPO TA Cloning® Kit for sequencing, Version J (Invitrogen) according to the protocol supplied by the manufacturer. Plasmid preparation was performed with NucleoSpin® Plasmid Kit (Macherey und Nagel) according to the protocol "Isolation of plasmid DNA from *E. coli*". Purified Plasmid-DNA was sequenced from both strands with ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction, Version 3.1 (Perkin Elmer) and primers supplied by the manufacturer.

Identification of *DEFB300*, *DEFB302P* and *DEFB303P*. PCR-products from BACs were amplified using primers established on a EST-sequence which is homologous to *DEFB103*. PCR-products were analyzed, extracted, purified and sequenced as described previously. All nucleotide sequences have been submitted to EMBL Nucleotide Sequence Database (Stoesser et al., 2003; http://www.ebi.ac.uk/embl/).

Tissue collection, RNA extraction and RT-PCR. Tissue samples of the mammary gland of 9 different lactating and non-lactating cows with different clinical findings concerning mastitis were taken immediatly after slaughter. Tissue samples were frozen on dry ice and stored at –80 °C. Total RNA was isolated from 200 mg tissue using TriPure™ Isolation Reagent (Boehringer Mannheim). Synthesis of complementary first strand DNA was performed with reverse transcriptase (SuperSkript™ II, Invitrogen) and a gene specific primer with tail (5'-*TCGAGGTCGACGGTATC*CAAAATTTATTCTGG-3', the gene specific sequence is underlined, the sequence of the tail is in italics) derived from KS-primer (Stratagene). Complete cDNA was amplified using defensin-consensus-primers. Ligation into the pGEM®-T vector (Promega) and transformation into *E. coli* was carried out following the instructions of the manufacturer. Plasmids were prepared using NucleoSpin® Plasmid Kit (Macherey und Nagel). Purified Plasmid-DNA was sequenced from both strands with ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction, Version 3.0 (Perkin Elmer) and primers supplied by Promega.

Contig-assembly. Overlapping BACs were identified using BAC-endsequencing in addition to a PCR-strategy, determination of insert-length by *Not*I-digestion and information of subcloning-results.

Results

Genomic characterization. 20 BACs were identified by PCR-screening of two different bovine BAC libraries. From these BACs, 21 β-defensin genes were isolated. 17 of these genes are orthologous to *DEFB4* and therefore represent a subfamily of defensin-genes. *TAP*, *DEFB1*, *BNBD4* and *BNBD12/13* were identified, of which the genomic and peptide sequences are already published. *BNBD3* and *BNBD7–BNBD11* are published as peptides but not as genomic sequences. Seven genes and four pseudogenes had not been identified until

now. These novel genes are termed *DEFB401–DEFB405*, *LAP-like* and *EBD-P* as well as *EBD-P2*. They are highly homologous to published bovine β-defensins (53 % – 97 % similarity) and to human β-defensin 4 (34 % – 41 % similarity). Another subfamily of defensin-genes is represented by one gene, named *DEFB300*, and two pseudogenes, named *DEFB302P* and *DEFB302P* with respect to their orthology to human *DEFB103* (similarity 74 %). *DEFB302P* and *DEFB302P* are putative pseudogenes.

Analysis of expression in mammary gland tissue. Analysis of cDNA-clones obtained from RT-PCR of mammary-gland extracted mRNA revealed the expression of several β-defensin genes in the bovine udder. Table 1 shows the clinical mastitis findings of lactating or non-lactating cows and the results of gene expression. mRNA of *LAP*, *TAP*, *EBD*, *BNBD3*, *-9* and *-12* was identified using the BLAST search, whereas one cDNA-sequence shows homology to the novel *DEFB401*. It is evident that *LAP* is expressed constitutively in healthy and affected tissue and an isoform of *TAP* (Acc.-no. P25068) is expressed in healthy lactating tissue. mRNA of *EBD* is existing in affected lactating and non-lactating tissue, pointing towards an inducible expression in the mammary gland. Furthermore, mRNA of neutrophil β-defensins (*BNBD3*, *BNBD9*, *BNBD12*) has been isolated from infected and healthy tissue.

Table 1. Udder tissue samples with clinical findings, lactational status and identified mRNA.

Cow No.	lactational status	clinical findings	identified mRNA
1	juvenile tissue	healthy	*LAP*
2	lactating	healthy	*LAP*, *TAP*
3	lactating	purulent mastitis caused by *Actinomyces pyogenes*	*LAP*, *BNBD9*
4	lactating	purulent mixed infection	*LAP*
5	lactating	purulent mastitis caused by *Actinom. pyogenes,* partly necrotic	*BNBD9*, *DEFB401*, *BNBD12*
6	non-lactating	subclinical mastitis	*LAP*, *DEFB1*, *BNBD3*
7	lactating	bloody mixed infection	*DEFB1*
8	lactating	healthy udder half	*LAP*, *BNBD3*
″	″	affected udder half, mixed infection	*LAP*, *BNBD9*
9	lactating	purulent mixed infection	*LAP*

Contig-assembly. Two contigs were constructed by identification of overlapping inserts. The contigs are shown in Fig. 1 and Fig. 2. Contig A spans a region of approximatly 310 kb and contig B of 387 kb. The order of genes presented on the chromosome implicates the duplication of *TAP* and *DEFB300.*

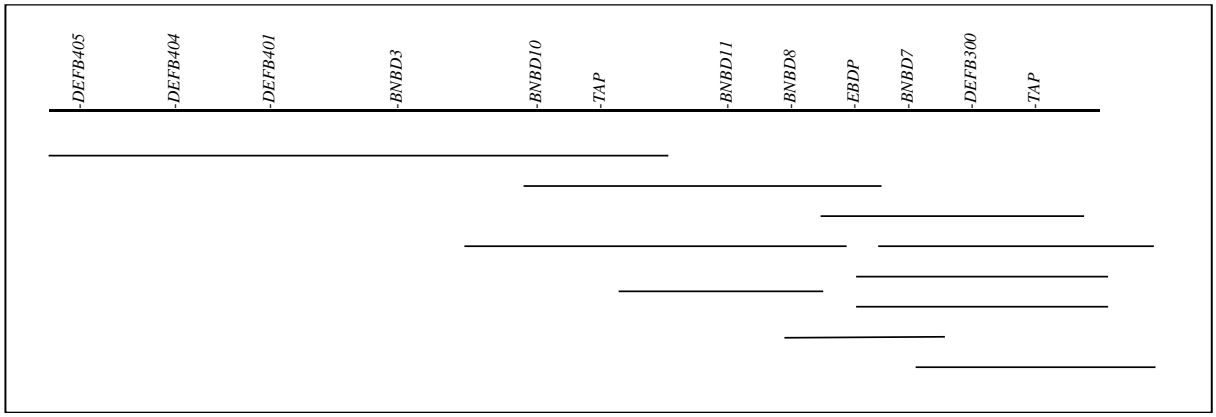

Fig. 1. Contig A. The bold horizontal line represents the chromosomal section with gene-loci. The lower lines show the relative arrangement of BAC-inserts.

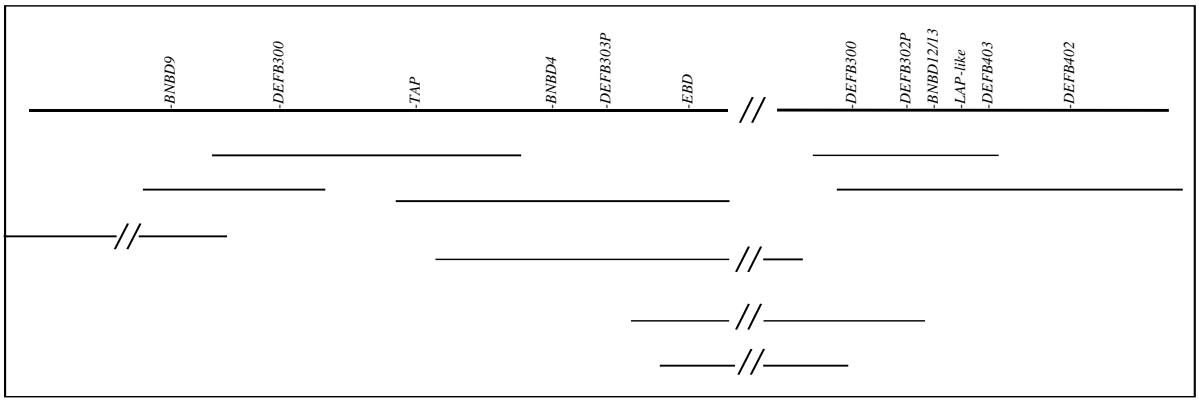

Fig. 2. Contig B. The bold horizontal line represents the chromosomal section with gene-loci. The lower lines show the relative arrangement of BAC-inserts.

Discussion

In this project we hypothezised that β-defensin genes may be candidate genes for genetic resistance to mastitis. By functional and structural genome analysis the activity of β-defensin genes against causal agents of mastitis and their role in the inborn resistance have to be characterized. We analysed the expression of β-defensin genes in the bovine udder with different clinical findings. The results show that defensin-genes are expressed in the bovine udder, indicating that they play a role in local host defense. The expression of *LAP* and *TAP* seems to be constitutive because it takes place in non-affected tissue, but the obviously inducible expression of *DEFB1* and *DEFB401* and the immigration of neutrophil defensin-mRNA (*BNBD3*, *BNBD9* and *BNBD12*) from myelopoetic cells to infected areas provide an indication of the relevance of β-defensins during udder infections. The lactational status of the cow does not influence the expression. The fact that mRNA of neutrophil defensins has been isolated requires further investigation, because the expression of neutrophil defensin-genes takes place in bone marrow and synthesis is complete at the time of release of the mature neutrophil into the peripheral circulation (Yount et al. 1999).

By genomic characterization of 20 BACs 21 defensin-genes have been isolated. Altogether, we identified seven novel genes and four putative pseudogenes. The novel genes show the conserved six cysteine-residues and the typical two exon-intron structure. Six novel genes (*DEFB401-DEFB405, LAP-like)* and two pseudogenes (*EBDP*, *EBDP2*) show homology to human β-defensin-4, whereas one gene (*DEFB300*) and two pseudogenes (*DEFB302P, DEFB303P*) share similarity with human β-defensin-103, presenting a new subgroub of defensin-genes unknown in cattle up to now. The *EBD*-pseudogenes are putative pseudogenes because they have no intron, but the exons share 100% identity to *DEFB1*. *DEFB302P* and *DEFB303P* have a point mutation at the third position of the start-codon, making it unable to work. By sequencing *BNBD3* and *BNBD7–BNBD11,* which are published as peptides, the genomic sequences of these genes are made available.

The order of genes on the chromosome is shown by construction of two contig. Contig A represents the arrangement of 12 genes with an average distance of 26 kb. In this section, *TAP* is duplicated and has two different loci. Contig B is also representing the arrangement of 12 genes with an average distance of 33 kb. *TAP* is existing at a third loci and *DEFB300* is duplicated, too. Duplication of genes max occur if a gene-product is needed in large amounts (Rooney 2002). The hypothesis of gene-duplication is supported by Hollox et al. (2003), who demonstrated that human *DEFB4*, *DEFB103* and *DEFB104* have several copies.

In summary, the findings of this project support the hypothesis that β-defensin genes may be candidate genes for inborn resistance to mastitis. The fact that β-defensin genes are abundant

in mammary gland tissue encourage further investigations, in which the gene-regulation has to be characterized by real-time PCR and cell culture. On the basis of genomic characterization, a gene-assisted or marker-assisted selection of mastitis resistance should be established and included in breeding goals.

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