

## Bovine $\beta$ -defensin gene family: opportunities to improve animal health?

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**Meade KG, Cormican P, Narciandi F, Lloyd A, O'Farrelly C.** Bovine  $\beta$ -defensin gene family: opportunities to improve animal health?. *Physiol Genomics* 46: 17–28, 2014. First published November 12, 2013; doi:10.1152/physiolgenomics.00085.2013.—Recent analysis of the bovine genome revealed an expanded suite of  $\beta$ -defensin genes that encode what are referred to as antimicrobial or host defense peptides (HDPs). Whereas primate genomes also encode  $\alpha$ - and  $\theta$ -defensins, the bovine genome contains only the  $\beta$ -defensin subfamily of HDPs.  $\beta$ -Defensins perform diverse functions that are critical to protection against pathogens but also in regulation of the immune response and reproduction. As the most comprehensively studied subclass of HDPs,  $\beta$ -defensins possess the widest taxonomic distribution, found in invertebrates as well as plants, indicating an ancient point of origin. Cross-species comparison of the genomic arrangement of  $\beta$ -defensin gene repertoire revealed them to vary in number among species presumably due to differences in pathogenic selective pressures but also genetic drift.  $\beta$ -Defensin genes exist in a single cluster in birds, but four gene clusters exist in dog, rat, mouse, and cow. In humans and chimpanzees, one of these clusters is split in two as a result of a primate-specific pericentric inversion producing five gene clusters. A cluster of  $\beta$ -defensin genes on bovine chromosome 13 has been recently characterized, and full genome sequencing has identified extensive gene copy number variation on chromosome 27. As a result, cattle have the most diverse repertoire of  $\beta$ -defensin genes so far identified, where four clusters contain at least 57 genes. This expansion of  $\beta$ -defensin HDPs may hold significant potential for combating infectious diseases and provides opportunities to harness their immunological and reproductive functions in commercial cattle populations.

$\beta$ -defensin; bovine; fertility; immunology

KEY TO SUCCESSFUL DEFENSE against pathogens is the constitutive and inducible expression of small effector molecules of the innate immune response known as host defense peptides (HDPs). Originally classified as antimicrobial peptides (AMPs), HDPs are natural antibiotics (43, 120, 160) and consist of three different subfamilies of peptides, classified according to their structure (80). Mammalian  $\alpha$ -defensins were originally found in macrophages from rabbit lung (118) and quickly thereafter in human neutrophils (45, 119). These molecules are also found in Paneth cells of the small intestine (100). Some primate genomes also contain theta ( $\theta$ )-defensins, which are lectins with broad-spectrum antiviral efficacy (79). Neither of these defensin subtypes are present in cattle (83), but it is a related family of small, cationic peptides subsequently found in bovine tracheal cells and neutrophils (33, 121), called  $\beta$ -defensins that are the focus of this review.

Traditionally,  $\beta$ -defensins were viewed as exclusively antimicrobial molecules providing front-line protection against pathogens. Their responsiveness to diverse bacterial, viral, and fungal infections supported this view (23, 88, 134). Several

mechanisms of antimicrobial action documented for  $\beta$ -defensins, including aggregation, pore formation, and prokaryotic membrane depolarization (111), have enabled these peptides to retain their potency against infectious agents throughout evolution (102). One of the first  $\beta$ -defensins to be discovered was bovine tracheal antimicrobial peptide (TAP) (33), which has since been associated with the early response to inflammation (23) and later shown to be expressed in the bovine mammary gland (109). As additional functions for  $\beta$ -defensins were determined (17, 151), a re-evaluation of the traditional view of them as exclusively antimicrobial agents occurred (15, 37).  $\beta$ -Defensins also have significant immunomodulatory functions (16) and serve as an important link between innate and adaptive immune responses (2, 105). Expression of  $\beta$ -defensins in the sterile environment of the developing embryo in a range of species (87, 91) suggests a potential role in development. Defensin-like genes in the Platypus genome encode major components of venom, suggesting an evolutionary link between antimicrobial and venom peptides (146). Further support for expanded  $\beta$ -defensin functions is witnessed in plants, where >300 defensin-like genes have been found in the Arabidopsis genome (129), and a specific cluster expressed in reproductive tissues are involved in male-female gamete interactions to facilitate fertilization (135). This latter finding con-

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curs with the strong evidence emerging for a similar role for these defensins in mammals, with hBD126 shown to regulate fertility in humans (139, 140).

#### $\beta$ -DEFENSIN GENE STRUCTURE, POSITIVE SELECTION, AND EVOLUTION

$\beta$ -Defensins are the ancestral gene from which  $\alpha$ -defensins arose in some mammals (150). However, their presence in marsupials, shows that  $\alpha$ -defensins must have been present very early in mammalian evolution and subsequently lost in most artiodactyls (83). The equine genome encodes a high number of functional  $\alpha$ -defensins (18, 19) that are not present in other members of this order studied to date (40). Only  $\beta$ -defensin genes have been found in birds (56, 84), and at least one  $\beta$ -defensin gene has been identified in all the vertebrate genomes so far sequenced, indicating that evolution of the gene family predates the divergence of fish from tetrapods (166). Recent sequencing of high-quality drafts of many more vertebrate genomes has facilitated a comparative genomics approach to characterize the  $\beta$ -defensin gene repertoire, and species-specific clades have been identified. Current estimates vary from 14  $\beta$ -defensin genes in chicken to 29 in pigs, 38 in dog, 33 in chimp, and 48 in mice and humans (27, 84, 101, 104, 117), although the final number will be subject to change as more genomes are sequenced and correctly annotated.

Most  $\beta$ -defensin genes have a characteristic two exon structure, the first of which encodes a prepropeptide with a hydrophobic leucine-rich signal sequence, while the mature peptide is encoded by the second exon (Fig. 1). Multiple gene duplication events and subsequent sequence diversification in the mammalian lineage has resulted in a large family of  $\beta$ -defensin peptides with diverse amino acid sequence but virtually identical

tertiary structure based on the characteristic intramolecular disulfide bonds (9). The specific C1-C5, C2-C4, and C3-C6 cysteine pairing, which is conserved across all  $\beta$ -defensins, indicates that the disulfide bonds are essential to the function of the molecule. Although human defensin derivatives with different cysteine pairings also have antimicrobial activity (63), the disulfide bonds maintain peptide stability by conferring resistance to proteolysis (20) and may also be important for the cell signaling and immunomodulatory functions of these molecules (125).

An unusually high degree of sequence variation in the mature peptide, even within closely related family members, implies extensive specialization and species-specific adaptation in  $\beta$ -defensins (122). Comparison of the rates of synonymous and nonsynonymous nucleotide substitution suggests that gene duplication was followed by a period of positive selection for diversification in the amino acid sequence of the mature peptide (60, 137), and this precipitated the emergence of multigene  $\beta$ -defensin loci in primates (123). Positively selected residues in the NH<sub>2</sub> terminus of the human BD4 defensin peptide indicate involvement in oligomerization of these peptides (14), which could form another prospect for selection pressure to shape the diversity of modern  $\beta$ -defensins. This rapid divergence among second exon sequences contrasts with relative stasis found in the sequences of the first exon. Interestingly, divergent selection pressures have been detected within rodent and primate lineages for residues within the prepropeptide region of  $\beta$ -defensins (124). Selection for noncoding regulatory regions in hBD103 in human populations from Asia has also recently been described (54), which is thought to be a response to selection pressure from influenza viruses in the region. This represents a new dimension to natural selection for expression levels of a particular defensin protein, as distinct from selection for additional gene copies.

Several hypotheses have been proposed to account for the natural selection pressures that drove the expansion of these genes in particular lineages [for review, see Yeaman and Yount (155)] including the following:

1) The common threat hypothesis: structural similarities and retention of these genes across very divergent species throughout the course of evolution suggests that gene expansion forms part of a common survival strategy;

2) The immunosaltation hypothesis: sudden emergence of many new life forms (during the Cambrian explosion, ~540 million years ago) drove the development of specialized immune systems in response to new pathogenic challenges;

3) The niche adaptation hypothesis: development of specialized anatomical and physiological niches created a requirement for equally specialized immune responses (e.g., rumen development).

Expansion of the  $\beta$ -defensin gene repertoire in multiple species in response to a common pathogenic threat supports the view of  $\beta$ -defensins as front-line effectors against pathogenic insults across multiple tissues exposed to microbes including tongue and epithelial cells, as well as in primary immune cells: bovine neutrophils (121) and macrophages (110). Extensive  $\beta$ -defensin gene copy number variation (CNV) has been shown in the human chromosome 8 cluster (orthologous to and syntenic with chromosome 27 in cattle), which influences transcript expression level (61).  $\beta$ -Defensin gene CNV has a documented association with disease resistance (58, 62); it is

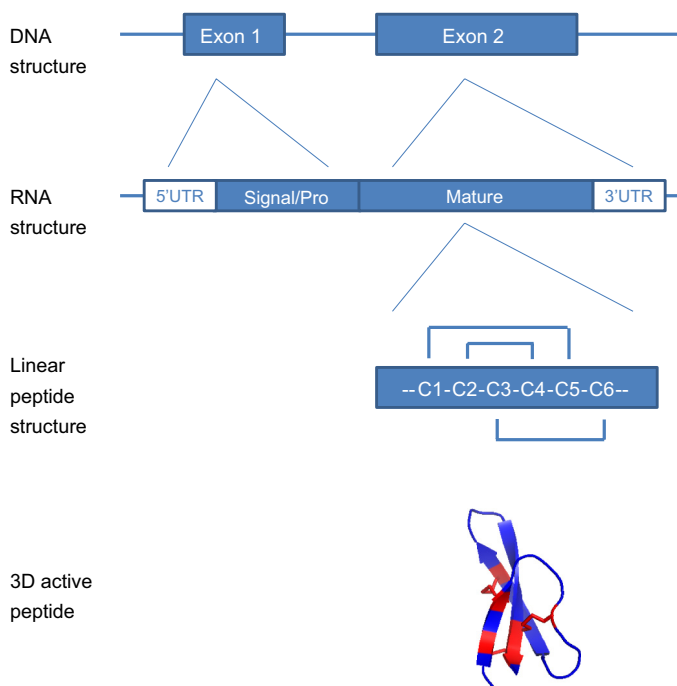


Fig. 1.  $\beta$ -Defensin gene structure encodes distinct sections of the transcribed RNA, and only the mature region of the RNA encodes the functional peptide. The signature disulfide bonding pattern of the active  $\beta$ -defensin peptide is also shown (cysteines and disulfide bonds colored red in 3D model).

hypothesized that increasing defensin gene-copy number contributes to increased susceptibility to inflammatory or autoimmune diseases but is protective against infectious disease (59). More recent work also fits this model, showing that lower defensin gene-copy number contributes to reduced HIV load as a result of altered efficacy of Th17 lymphocyte recruitment to the site of infection (53, 54). The multiple immunomodulatory functions now emerging for  $\beta$ -defensins (discussed below) also support the common threat hypothesis that expansion of these genes occurred in response to multiple infectious disease challenges encountered by cattle over the course of their evolution. Of course, emergence of defensins in common ancestors during the Cambrian explosion (immunosaltation hypothesis) does not preclude subsequent gain and loss of specific genes or gene clusters as a result of speciation in response to divergent selection pressures. Expression of  $\beta$ -defensin genes in the absence of infection, such as in the developing embryo (87, 91), is potentially supportive of neofunctionalization of some  $\beta$ -defensin gene family members. However, the suggestion that the evolution of the rumen led to a requirement for more sophisticated immune mechanisms to manage the interface between microbes and the animal host (38, 90) is one that has gained support and fits under the “niche adaptation hypothesis.” Rumen-specific defense mechanisms are important to ensure the balance between immune surveillance of the diverse gut microbiota (41) and the maintenance of the integrity of the gastrointestinal epithelial barrier (112). Bovine enteric  $\beta$ -defensin was originally found expressed in the small intestine and the colon (136). In sheep, maximal expression of *oBD1* and *oBD2*  $\beta$ -defensins was detected in the rumen during the first 6–8 wk of life and also in the digestive tract in the prenatal lamb (64, 91). This evidence would support a role for defensins in managing the microbial interface, especially during initial postnatal colonization of the intestine and rumen. It is tempting to speculate that in the absence of infection, lingual antimicrobial peptide (LAP) in bovine milk (67) may help regulate the microflora in the developing rumen of the postnatal calf, but this has not yet been established. A recent study showed downregulation of *LAP*, *TAP*, and *DEFB4A* (all chromosome 27  $\beta$ -defensins) expression in rumen epithelium in response to infusion with butyrate, a short chain fatty acid (7), suggesting a close association between dietary composition and HDP expression in the rumen.

Biological systems most affected by changes in the number and organization of genes in the cattle lineage include reproduction and immunity, as well as lactation and digestion (38). It is therefore likely that rather than a single event, multiple natural selection pressures have been exerted on the bovine genome over the course of evolution, leading the biological diversity in  $\beta$ -defensin gene repertoire now present. The divergent selection pressures (both positive and negative) detected between  $\beta$ -defensin sequences in rodents and primates (60, 124), as well as the diverse expression patterns in epithelial tissue and immune cells detected for the expanded chromosome 27 genes in cattle (23, 24, 26, 82, 134, 145), supports multiple events, which may include the development of the rumen. However, the chromosome 13 cluster of bovine  $\beta$ -defensin genes are preferentially expressed in reproductive tissues (97), indicating that their function is unlikely to be related to rumen development. It may be more likely that the herd structure in ruminants, which could promote rapid disease

transmission, is also a contributory cause of the selection pressure for expanded  $\beta$ -defensin repertoires in cattle. Duplication and subsequent diversification of  $\beta$ -defensin genes in response to a common threat as a possible consequence of species-specific or clade-specific pathogenic insults faced by individual species during the course of their evolution are supported by the human literature (54, 60) and is likely to be also true in cattle (104).

## $\beta$ -DEFENSIN GENE EXPANSION IN CATTLE

*Bos taurus* was one of the first species in which  $\beta$ -defensin-like molecules were discovered (33, 121, 136). Eighteen complete and partial bovine  $\beta$ -defensin (bBD) sequences were identified through a combination of genomic sequence analysis (109) and direct sequencing of isolated purified proteins from blood neutrophils (121). This number was noticeably smaller than the numbers of purportedly functional  $\beta$ -defensin genes identified in other species subsequently by both in silico and laboratory methods (27, 101). However, subsequent comprehensive bioinformatic search of the bovine genome identified 57 open reading frames with the characteristic six-cysteine spacing of the  $\beta$ -defensin family of genes (29).

Synteny analysis based on gene sequence conservation with the human genome (which has the most reliable annotation) shows four distinct clusters of  $\beta$ -defensin genes within the bovine genome. Located on chromosomes 8 (*cluster A*), 13 (*cluster B*), 23 (*cluster C*), and 27 (*cluster D*),  $\beta$ -defensin gene sequences within a cluster tend to be more similar to each other than to genes in other clusters within the same species.

### Syntenic Cluster A

Syntenic *cluster A* consists of four genes, namely bBD136, bBD131, bBD135, and bBD134, which span 93 kb on bovine chromosome 8 (Fig. 2A). This is a conserved mammalian cluster that with *cluster C* arose at one site early in mammalian evolution. This ancestral cluster subsequently split in two, ~180 million years ago (10) when eutheria separated from marsupials to generate *clusters A* and *C*. In contrast to *clusters B* and *D*, *A* and *C* show little evidence of gene gain or loss in any eutherian mammals for which genome sequence data is available (101). This cluster is conserved in human and dog (dog being the closest relative to bovine with a completely sequenced and well-annotated genome) but is missing from the recent analysis of defensin genes in the pig (*Sus scrofa*) genome (27). However, close genomic matches exist in pig (at least for bBD131 and bBD135) and are not yet annotated (unpublished data). While expression of these genes has not been investigated in cattle to date, gene expression in the reproductive tract has been detected in rat epididymis (101).

### Syntenic Cluster B

$\beta$ -Defensin *cluster B* is a relatively stable cluster in higher mammals with few gene gain or loss events disrupting the syntenic order and gene orientation established early in eutherian mammalian evolution (104). In the bovine genome, this newly discovered cluster spans 320 kb on chromosome 13 (29). Sixteen of these genes are conserved in a 1:1:1 orthologous relationship among human, dog, and bovine (Fig. 2B). Interestingly, the bovine genome appears to encode a number of relatively recent gene duplicates within this cluster com-



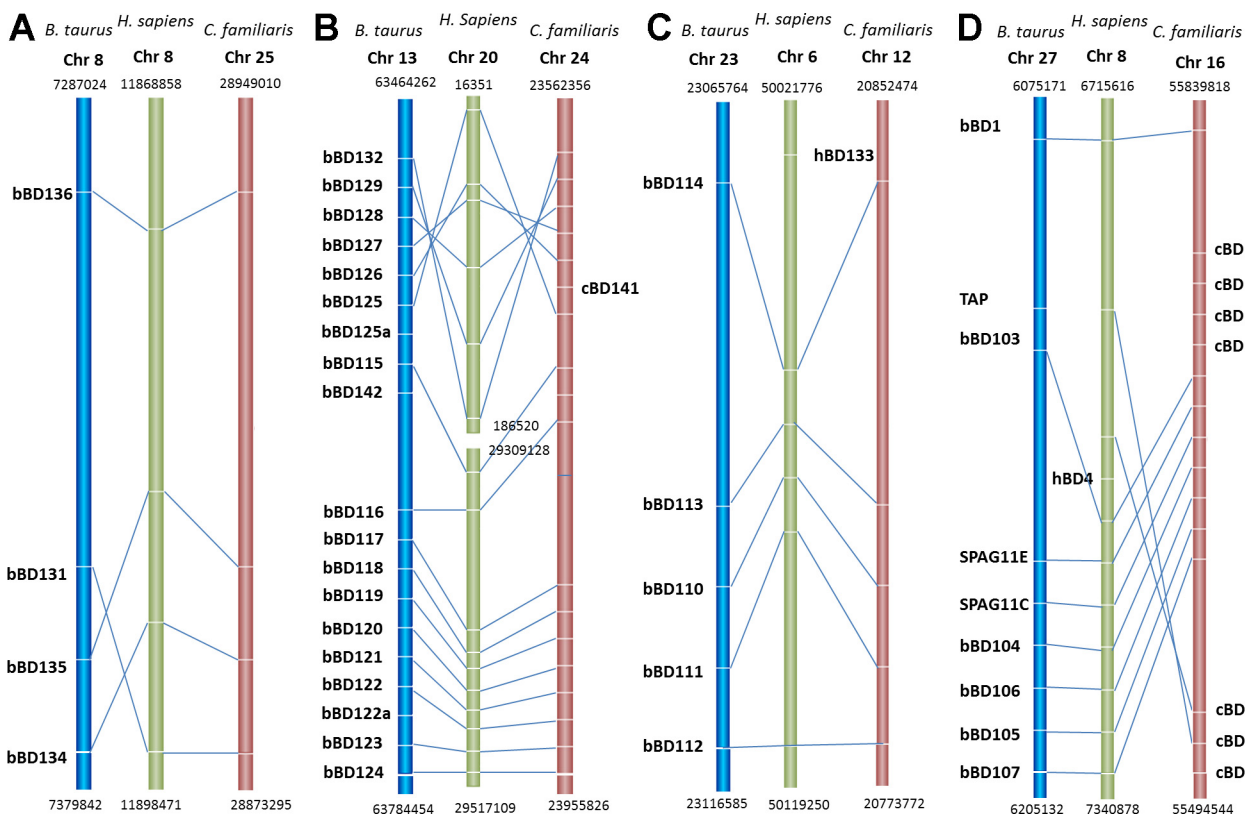


Fig. 2. Syntenic maps of bovine, human, and canine  $\beta$ -defensin genes in a direct 1:1 relationship with those in other species mapping to: bovine chromosome 8 (cluster A) (A), bovine chromosome 13 (cluster B) (B), bovine chromosome 23 (cluster C) (C), bovine chromosome 27 (cluster D) (D). Chromosomes are color coded according to species: *Bos taurus* in blue, *Homo sapiens* in green, and *Canis familiaris* in red.

pared with this gene cluster in other mammals. BBD122 and bBD125 are each represented by two genes, which bear a closer similarity to each other than to any other defensin in the bovine genome.

Early studies localized the expression of human  $\beta$ -defensins in this cluster to the testis, prostate (46, 162), as well as sertoli and seminal vesicle epithelial cells in primates (8). Subsequent cross-species analysis found predominant expression of these  $\beta$ -defensins in reproductive tissues of rodents (28, 68) and also demonstrated antimicrobial activity of human seminal plasma extracts. These  $\beta$ -defensins have since become known as HDPs of the male reproductive tract (50). Expression of the bovine orthologs of these genes was localized to the reproductive tract of healthy bulls, indicating constitutive expression in the absence of infection (97), although earlier in vitro assays showed significant antimicrobial activity of bBD123 against a range of bacterial species (29). A recent study of  $\beta$ -defensin gene expression in the pig has confirmed the expression profile detected earlier in cattle, showing predominant but not exclusive expression of the porcine homologs in mature as well as 2 wk old pig testis (27).

A potent reproductive role has been demonstrated for hBD126, a human ortholog in this gene cluster (138). Macaque sperm have been shown to be coated with  $\beta$ -defensin 126 (formerly ESP13.2) (141, 158), which confers a negative charge to the sperm so that the mutual repulsion between the negative charge of hBD126-coated sperm and the negatively charged cervical mucus enables sperm transmigration (140). Recent work has also shown that glycosylation of defensins on

the sperm surface contributes to charge-mediated passage of sperm through cervical mucus (159). Macaque  $\beta$ -defensin 126 protects the sperm from immune recognition by the female immune system (157) and also mediates the attachment of sperm to oviductal epithelium (139). Interestingly, significantly extended COOH-terminal amino acid tails have also been reported for some peptide members of the bovine chromosome 13 cluster (97), and a number of O-linked glycosylation sites were predicted in the tails of some  $\beta$ -defensins, particularly bBD125, bBD126, and bBD129. Although further experimental validation is required, this work supports a role for glycosylation mediating at least the reproductive functions of these genes in cattle.

#### Syntenic Cluster C

Cluster C contains five genes, bBD114, bBD113, bBD110, bBD111, and bBD112, which span 51 kb on bovine chromosome 23 (Fig. 2C). While nothing is yet known about the expression or function of these genes in cattle, their human orthologs are expressed in the reproductive tract (101) and also in epithelial cells (73). Interestingly, two members of syntenic cluster C (*DEFB111* and *DEFB113*) have recently been shown to be expressed in the epididymis of the boar (49).

#### Syntenic Cluster D

Syntenic cluster D represents the most ancient cluster that has been conserved throughout the evolution of mammals and is orthologous to the single defensin cluster found in both

The orthologous genes in this cluster in humans (chromosome 8) demonstrate extensive CNV, as discussed above. Although CNV in bovine  $\beta$ -defensin genes has not been comprehensively assessed, one recent study has found six of the bovine chromosome 27 defensins within one of the top 25 CNV regions in the genome (12). Not only do the copy numbers of DEFB1, DEFB4A, DEFB5, LAP, TAP, and BNBD10 genes vary between the *Bos indicus* and *B. taurus* animals sampled, but variation in mean copy number is also apparent among Angus, Holstein, and Hereford breeds (12). Using next-generation sequencing, Bickhart et al. (12) have shown that on average *B. taurus* genomes contain between 13 and 16 copies of each of these genes. Interestingly, but anecdotally the number of copies of each of the six  $\beta$ -defensin genes was lower in the single *B. indicus* animal sequenced (range 7–11 copies) compared with the taurine cattle, potentially resulting from divergent natural selection pressures since the most recent common ancestor shared by these bovine subspecies.

Regulation of  $\beta$ -defensin gene expression is incompletely understood, in part because new genes have only recently been

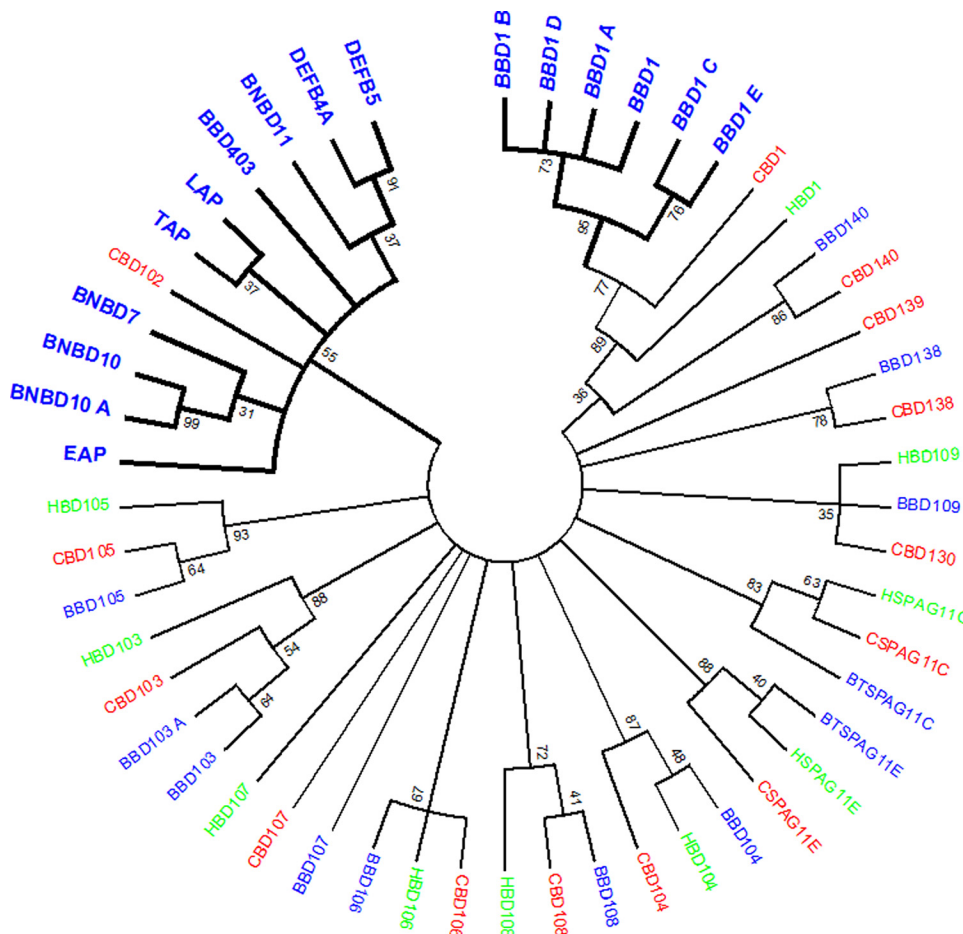


Fig. 3. Maximum likelihood derived phylogenetic tree of the bovine, human, and canine  $\beta$ -defensin genes mapping to bovine chromosome 27 (*cluster D*) showing the existence of family members expanded in the bovine genome (boldface) and also showing that copy number variants of bBD1 (bold-faced italics) are not identical. Branches with <30% bootstrap support have been collapsed. Gene names are color coded according to species: *B. taurus* in blue, *H. sapiens* in green, and *C. familiaris* in red.

discovered and correctly annotated in the majority of mammalian species. Produced mainly by mucosal epithelia, such as in the trachea (31) and intestine (64, 136),  $\beta$ -defensins are also expressed in immune cells including macrophages (110) and neutrophils. Early work showed that *TAP* expression was dramatically increased in tracheal epithelial cells upon stimulation with bacteria or 100 ng/ml bacterial lipopolysaccharide (LPS) (32), and was NF- $\kappa$ B mediated. These findings are in contrast to the decreased expression of *SPAG11E* and eight other defensin genes in rat epididymis in response to injections of LPS in vivo (22). The differences in the responses of these defensins may result from the different models and are likely due to the significantly higher dosage of LPS used in the latter study (50–400  $\mu$ g). However, it is also possible that promoter regions of defensin genes from the four clusters contain diverse transcription factor binding sites and are therefore under different regulatory control. A role for the transcription factor Oct-1 in regulating *TAP* expression has been demonstrated in bovine mammary epithelial cells (154), while a role for NOD2 (Card15), an intracellular pattern recognition receptor, has also been established for the induction of hBD2 via the transcription factor NF- $\kappa$ B (144). A recent study demonstrated that bacterial-induced defensin (*LAP*, *DEFB1*, and *DEFB4*) expression in bovine umbilical vein endothelial cells is regulated by the autocrine production of the cytokine, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (4).

Subsets of  $\beta$ -defensin genes may also be developmentally regulated, as they are expressed during sexual maturation in the rat (101). Cell type-specific growth factors and androgens are known to play an important role in sperm maturation within the epididymis and may also regulate defensin expression (74, 78). Recent work has shown hormonal regulation of rat epididymis-specific  $\beta$ -defensin 15 (163). Whereas expression started at day 15, peaked at 1 mo and remained stable in the mature animal, DEFB15 expression was reduced in castrated animals alongside declining testosterone levels, which was rapidly rescued in response to ex vivo androgen supplementation.

Expression of defensins in macrophages can also be induced by hypoxia, as hBD2 was shown to be significantly increased at low oxygen tension in response to *Mycobacterium tuberculosis* (98). The transcriptional regulator hypoxia inducible factor (HIF)-1 $\alpha$  was also upregulated, which has particular relevance as HIF-1 $\alpha$ -deficient mice, which fail to adapt to oxygen deprivation, are more susceptible to numerous bacterial infections (103). The recent discovery of  $\beta$ -defensin expression in both the testis and pituitary gland of fish (71) and in the murine brain (93) suggests other regulatory mechanisms for defensin expression have yet to be identified.

### $\beta$ -DEFENSIN ANTIMICROBIAL FUNCTIONS

As small cationic peptides,  $\beta$ -defensins are preferentially attracted to the negatively charged phospholipids that compose bacterial membranes. The amphipathic nature of defensins allows them to insert into the phospholipid membrane of pathogens to induce membrane depolarization (111) and to destroy the integrity of the cell wall (79). Comparisons of structure and functional changes demonstrated that activity on eukaryotic cells depends on overall hydrophobicity, whereas the antimicrobial efficacy was determined by distribution of positively charged amino acids and hydrophobic side chains

(75). Recent work has also demonstrated that defensins can target prokaryotes through specific lipid receptors to block and sequester lipids and thereby inhibit cell wall biosynthesis (113, 147). As a result of these multiple antimicrobial mechanisms, defensins are known to be active against gram positive and gram negative bacteria as well as fungi and enveloped viruses (131). Functional studies have been mainly performed in vitro, which can lead to inappropriate attribution of antimicrobial function in nonphysiological conditions (122), although their salt sensitivity may be concentration dependent (130). Relevantly, defensins have been found in uncharacteristically high concentrations in porcine tongue epithelium (20–100  $\mu$ g/ml) (128) and between 1 and 10 mg/ml in neutrophil granules (44, 81). Furthermore, some in vivo studies have verified their role in enhanced immunity during infection. Mice lacking  $\beta$ -defensin Defb1 have been shown to have delayed clearance of *Haemophilus influenzae* from the lung (94) and increased incidence of *Staphylococcus* species in the bladder (92). Expression of porcine  $\beta$ -defensin 1 (pBD1), an ortholog of human  $\beta$ -defensin-2, is associated with protection against respiratory disease; 500  $\mu$ g of pBD1 given at the time of challenge conferred protection against *Bordetella pertussis* in newborn piglets (36).

Although the antibacterial activity of many defensins is abolished at physiological concentrations of NaCl (150 mM) (95), low sodium concentrations are found in rat epididymis (58 mM in caput to 15 mM in cauda), indicating that they may also play a protective role in the reproductive tract (143). Furthermore, neutrophil elastase in semen is thought to activate defensins, and the titer of this enzyme increases during inflammation in the male reproductive tract (164, 165). When tested in vitro,  $\beta$ -defensins have shown efficacy against a broad range of pathogens that can invade the reproductive tract [including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*] at concentrations ranging from 10 to 50  $\mu$ g/ml (52, 115, 116). *E. coli* is a major cause of postpartum reproductive failure in cattle (126, 127), and bBD123, a novel bovine defensin, has shown very high efficacy against *E. coli* at a concentration range of 0.9  $\mu$ g/ml in vitro (29).

### $\beta$ -DEFENSIN IMMUNOMODULATORY FUNCTIONS

It is now well established that innate antimicrobial peptides, including defensins, have immunological roles in addition to their direct antimicrobial ones and emerging studies show that  $\beta$ -defensins can link the innate and adaptive arms of the immune response. Human  $\beta$ -defensin-3 (hBD3), for example, can induce expression of the costimulatory molecules CD80, CD86, and CD40 on monocytes and myeloid dendritic cells in a Toll-like receptor (TLR)-dependent manner. In studies with HEK cells engineered to express various TLRs, it was shown that activation of NF- $\kappa$ B by hBD3 depends on the expression of both TLR1 and TLR2 (42). Thus, TLR signaling is not restricted to recognition of microbial patterns but also can be initiated by host-derived defensin peptides (13). Recent work has shown that hBD3 can rapidly enter TLR4-stimulated macrophages to dampen the expression of proinflammatory genes (125). The cross talk between the immune and reproductive systems provided by endogenous HDP expression may play important functional roles in dampening the immune response



to foreign antigen such as sperm but also in regulating immune tolerance of an allogenic fetus during pregnancy (3).

At lower concentrations of 0.1–1  $\mu\text{g/ml}$ , the primary role of  $\beta$ -defensins may be to induce chemotaxis of immune cells to the site of infection (153). Striking similarities are emerging between defensins and traditional chemokines, including size, disulfide bonding, and cationic charge (35), which potentially account for the ability of defensins to recruit innate as well as adaptive immune cells. HBD2 and HBD3 has been shown to be chemotactic for a broad spectrum of leukocytes including neutrophils (99), CD4 memory T cells, macrophages (70), and immature dendritic cells (149). Their role as chemoattractants is mediated via both CCR2 and CCR6 receptors (108, 152). The upregulation of costimulatory molecules and dendritic cell maturation drive a robust Th1 type immune response, and so these molecules may also play additional important roles in the activation as well as regulation of an immune response to infection.

### $\beta$ -DEFENSINS AND IMPROVED ANIMAL HEALTH

Developments in molecular biological tools, including next-generation sequencing, are helping identify new clusters of  $\beta$ -defensin genes across genomes of farm animal species and annotate the specific genes that vary in copy number among breeds and individuals. They also offer an unprecedented window into understanding  $\beta$ -defensin gene expression and regulation across multiple tissues. Targeting regulation of  $\beta$ -defensin expression and function during diverse physiological events could have a significant impact on bovine health and reproduction (Fig. 4).

#### Alternatives to Antibiotics

Despite millions of years of exposure, microbes have not developed resistance against antimicrobial peptides, perhaps because they target components that are integral to bacterial structures (77). Native  $\beta$ -defensins are effective against many

pathogenic agents both in vitro and in vivo (36, 92, 94). Modification of the  $\beta$ -defensin structure-function relationship through targeted residue substitution (39) can generate peptides with additional immunomodulatory as well as enhanced antimicrobial efficacy (156). Often based on information garnered from positive selection studies (57, 86, 149), these strategies offer an opportunity to overcome problems associated with emerging antibiotic resistant bacterial strains. A recent study has shown that cattle are a reservoir for the emergence of human-pathogenic MRSA (132). In this regard, it is of interest that increasing the hydrophobicity of a bovine  $\beta$ -defensin 123-derived peptide had increased efficacy against MRSA with a 50% lethal dose of 3.91  $\mu\text{g/ml}$  in vitro (86). Human  $\alpha$ -defensins are also effective against trypanosomes (72, 85), and therefore bovine  $\beta$ -defensins could provide novel trypanocidal treatments or breeding targets for one of the most important zoonotic pathogens in the developing world. Murine  $\beta$ -defensin-3 has also shown to have potent antiviral effects against influenza virus, both in vitro and in vivo (69). The role of bovine  $\beta$ -defensin orthologs in resisting viral infections in cattle has not been investigated but may hold significant promise. It is also possible that the novel AMPs discovered as new genomes are completed may be useful to treat diseases in other species (48) and to meet emerging pathogenic challenges now and into the future (142).

#### Vaccine Adjuvants

$\beta$ -Defensins may prove useful as broad-spectrum adjuvants, which are required for improved vaccine design in cattle (1). Artificial induction of  $\beta$ -defensin-2 (mBD2) contributes to improved control *M. tuberculosis* infection in mice (107). It is suggested that  $\beta$ -defensin expression contributes to the establishment of a beneficial Th1 response via dendritic cell activation and increased expression of cytokines such as IFN- $\gamma$ , IL-12, and IL-6 (13, 106). Additionally, defensins facilitate the

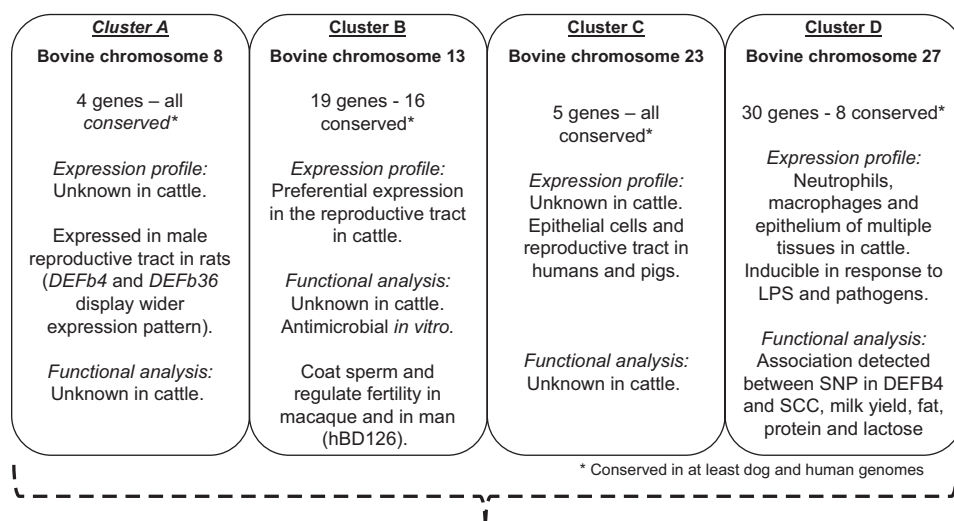


Fig. 4. Sequencing and annotation of multiple genomes across many species are facilitating a comparative immunology discovery and characterization of  $\beta$ -defensin gene repertoire within the bovine genome. Functional characterization has identified important roles for genes in 2 of the 4 clusters in reproduction and immunology. Important potential future research applications of these genes are also identified.

#### Potential Research Applications

- Alternative to antibiotics
- Adjuvants for improved vaccine design
- Dietary manipulation of  $\beta$ -defensin expression levels
- Breeding targets for higher fertility and disease resistance

efficient delivery of bound antigen to antigen presenting cells (76). In vivo murine models have shown that mBD2 promotes antitumor NK and beneficial T cell responses (89).

#### *Dietary Manipulation of Defensin Levels in Cattle*

Controlled induction of these natural antibiotics to protect against infections (51, 161) could hold significant promise for reducing the burden of infectious disease in livestock. Interestingly, upregulation of hBD1 and hBD2 by epithelial cells in response to short-chain fatty acids including acetate, propionate, and butyrate has recently been documented (11). In chickens, dietary supplementation with 0.1% butyrate led to a significant increase in defensin gene expression in the caecum and a concurrent 10-fold reduction in the *Salmonella enteritidis* titer (133). As these short chain fatty acids (including butyrate) are also by-products of ruminal digestion, this finding suggest that dietary supplementation may enhance immunity in cattle.

#### *Targets for Improved Cattle Breeding*

The observed gene duplication within the bovine  $\beta$ -defensin cluster has created a repertoire of phylogenetically diverse functional genes, which is substantially larger than that described for humans and mice. Single nucleotide polymorphisms (SNPs) in  $\beta$ -defensin genes have been advocated as potential markers for selection of avian resistance to *Salmonella* infection (55). A SNP in the bovine bBD103 gene (contained within the CNV region on chromosome 27) is associated with red coat color in cattle (34). The canine ortholog (cBD103) also binds with high affinity to a melanocortin receptor, which regulates pigment type-switching in dogs and in wolves (5, 21). While coat color may not be a breeding priority in cattle, SNPs and CNV in  $\beta$ -defensin genes involved in the innate immune response could be valuable for selecting animals with superior disease resistance. In humans, defensin gene CNV has been associated with differing susceptibility to Crohn's disease as well as psoriasis (59, 62). Although data in cattle are limited, upward of 13 gene copies are seen for some immune genes in various cattle breeds sequenced, including, for LAP, TAP, and DEFB5, genes that have been previously shown to be upregulated in response to infection, particularly in mammary, lung, and uterine tissues (23, 25, 30, 47, 82, 114, 134). In terms of production traits,  $\beta$ -defensin genotypes are also associated with reduced somatic cell count (SCC) in the Jersey breed of cattle (148). Studies in Holstein-Friesians supported this finding as polymorphisms in DEFB4A were associated with milk constituents (fat, protein, and lactose) as well as SCC (6).

In regard to the bovine chromosome 13 defensins, there is considerable evidence now that the orthologs of these genes regulate fertility in other species, including humans (139, 140, 157). Importantly a polymorphism found in the human  $\beta$ -defensin 126 gene has been correlated with lower fertility in a human cohort. Sperm from homozygous men exhibit lower levels of glycosylation and an 84% reduction in the rate of penetration of a hyaluronic acid gel, a surrogate for cervical mucus, compared with the other genotypes (138). In cattle, population genetic analysis showed significant SNP frequency differences in  $\beta$ -defensin genes 115, 117, 121, and 122 between Holstein-Friesian and the higher-fertility Norwegian Red breed (97). Combined with the corresponding expression of these genes in reproductive

tissues in this study, this suggests a potential role for these genes in the regulation of bovine fertility (96).

#### CONCLUSION

The gene content and organization of the bovine  $\beta$ -defensin loci are broadly similar to that of humans and mice; however, multiple duplication events have led to a marked expansion in the number of the chromosome 27  $\beta$ -defensin genes of the *B. taurus* genome. This cluster shows remarkable CNV with recent analyses with upward of 25 individual gene copies in some bovine genomes sequenced, as well as marked interanimal variation in gene number (12). Strong evolutionary pressures in this lineage have selected for the development of enlarged sets of multifunctional  $\beta$ -defensin genes. Because key roles are emerging for these genes in reproduction and immunity, genetic variation within these genes hold exciting potential for improved cattle breeding to improve bovine health and fertility.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

Author contributions: K.G.M., P.C., and C.O. conception and design of research; K.G.M., P.C., F.N., A.T.L., and C.O. interpreted results of experiments; K.G.M. drafted manuscript; K.G.M., P.C., A.T.L., and C.O. edited and revised manuscript; K.G.M. and C.O. approved final version of manuscript; F.N. prepared figures.

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