The BPI-like/PLUNC family proteins in cattle

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Abstract
Members of the protein family having similarity to BPI (bactericidal/permeability increasing protein) (the BPI-like proteins), also known as the PLUNC (palate, lung and nasal epithelium clone) family proteins, have been found in a range of mammals; however, those in species other than human or mouse have been relatively little characterized. Analysis of the BPI-like proteins in cattle presents unique opportunities to investigate the function of these proteins, as well as address their evolution and contribution to the distinct physiology of ruminants. The present review summarizes the current understanding of the nature of the BPI-like locus in cattle, including the duplications giving rise to the multiple BSP30 (bovine salivary protein 30 kDa) genes from an ancestral gene in common with the single PSP (parotid secretory protein) gene found in monogastric species. Current knowledge of the expression of the BPI-like proteins in cattle is also presented, including their pattern of expression among tissues, which illustrate their independent regulation at sites of high pathogen exposure, and the abundance of the BSP30 proteins in saliva and salivary tissues. Finally, investigations of the function of the BSP30 proteins are presented, including their antimicrobial, lipopolysaccharide-binding and bacterial aggregation activities. These results are discussed in relation to hypotheses regarding the physiological role of the BPI-like proteins in cattle, including the role they may play in host defence and the unique aspects of digestion in ruminants.

Introduction
Previous studies on the family of proteins from humans and mice with sequence similarity to BPI (bactericidal/permeability increasing protein), known as the BPI-like proteins or the PLUNC (plate lung and nasal epithelium clone) family proteins, have established a base of knowledge of their structure and biology, including their association with host defence [1–4]. The study of the BPI-like proteins in cattle has the potential to contribute significantly to our understanding of the function and biological roles of these proteins, as well as helping us to understand some of the distinct aspects of host–pathogen interaction in this species. The host defence system in cattle is of interest for a number of reasons, including changes associated with domestication and living in high-density populations, the role of cattle as a source of human pathogens [5] and the possible influence of the innate immune defence system in rumen fermentation. It has recently become apparent, through characterization of the bovine genome, that there has been significant expansion of the innate immune defence system in rumen fermentation. The first of the BPI-like proteins in cattle to be described was a protein that migrated with an apparent mass of approximately 30 kDa on SDS/PAGE gels that had been loaded with bovine saliva [7]. This protein, named ‘bovine salivary protein 30 kDa’ (BSP30), is one of the most abundant proteins in bovine saliva, with an estimated concentration of between 0.1 and 0.5 mg/ml, and was of interest initially because its abundance appeared to be correlated with the susceptibility of the cattle to pasture bloat, a metabolic disease of cattle characterized by a build-up of stable foam in the rumen that causes its distension and thereby respiratory distress [8].

Characterization of BPI-like family in cattle
The first of the BPI-like proteins in cattle to be described was a protein that migrated with an apparent mass of approximately 30 kDa on SDS/PAGE gels that had been loaded with bovine saliva [7]. This protein, named ‘bovine salivary protein 30 kDa’ (BSP30), is one of the most abundant proteins in bovine saliva, with an estimated concentration of between 0.1 and 0.5 mg/ml, and was of interest initially because its abundance appeared to be correlated with the susceptibility of the cattle to pasture bloat, a metabolic disease of cattle characterized by a build-up of stable foam in the rumen that causes its distension and thereby respiratory distress [8].

The protein was subsequently excised from a gel and subjected to Edman degradation amino acid sequencing to produce
a short stretch of sequence. A low-redundancy region of this was back-translated and an oligonucleotide mixture was synthesized, which was used as a probe to isolate and amplify two positive-hybridizing clones from a cDNA library made from bovine parotid salivary gland mRNA. The sequencing of these two cDNAs, named BSP30A and BSP30B, revealed the proteins to be very closely related to each other (over 80% amino acid sequence identity) and to have approximately 40% amino acid sequence identity with PSP from human and rodents. Phylogenetic analysis confirmed that the BSP30 proteins were the first characterized bovine members of the BPI-like protein family [9].

In the last few years, advances in genome sequencing have enabled a comprehensive characterization of the bovine BPI-like protein family. The first contiguous sequence of the complete BPI-like locus was produced by the selection of five bovine BACs (bacterial artificial chromosomes) that contained end-sequence alignment with the region of the human genome containing the BPI-like locus, and then subsequently fully sequencing their inserts [10]. This revealed the presence of 13 intact genes and one pseudogene. Phylogenetic analysis confirmed that nine of these are the bovine orthologues of human BPI-like proteins, whereas the remaining four (BSP30A, BSP30B and two other previously uncharacterized genes) are each paralogues of BSP30A, BSP30B and two other previously uncharacterized genes are each paralogues of human PSP/SPLUNC2. The two newly discovered genes were named BSP30C and BSP30D [10]. Alignment of the bovine genomic sequences with those of human and mouse revealed that each of the four BSP30 genes as well as the pseudogene are contained within discrete regions of high similarity, bounded by a high density of retrotransposon elements [10]. This is consistent with retrotransposon-mediated genomic duplication within the bovine lineage, giving rise to the multiple BSP30 genes from a single gene that was also ancestral to PSP/SPLUNC2. Analysis of the ratio of non-synonymous to synonymous differences in alignments of the BSP30 and PSP/SPLUNC2 sequences (substitution analysis) provides evidence that at least some of the BSP30 genes are under positive Darwinian selection, that is, there is evolutionary pressure driving the sequences apart. Taken together, these observations suggest that the BPI-like family is evolving new functions at a relatively high rate within the cattle lineage.

The completion of the sequencing and assembly of the entire bovine genome has revealed an additional gene-duplication event within the broader BPI-like protein family. BPI has sequence similarity to a number of well-characterized genes besides SPLUNC and LPLUNC (long PLUNC), including LBP (lipopolysaccharide-binding protein). The BPI and LBP genes are adjacent to each other in the bovine genome, just as they are in mouse and human. However, the bovine genome also contains a third gene that encodes an approximately 60 kDa protein with approximately equal amino acid sequence identity with both BPI and LBP (N.J. Maqbool and T.T. Wheeler, unpublished work). This gene, which we provisionally name BPI-2, is not present in either the mouse or human genome.

The BPI-2 gene is expressed, based on the presence of a number of ESTs (expressed sequence tags) encoding it that were found in public bovine EST sequence databases. However, the libraries containing these ESTs were made from mixtures of tissues, and thus it is not possible without further analyses to specify in which tissues it is expressed. The biochemical function, bioactivities and physiological role of the protein encoded by this gene remain to be explored.

Searches against recently sequenced sheep genome (preliminary BGI assembly, results not shown) shows that multiple BPI30-like proteins are also present in sheep. However, given the draft nature of the assembly, it is currently not possible to fully extract gene structure of this locus. We have also obtained some experimental evidence that one or more BSP30-like proteins exist in the saliva of sheep. A major 30 kDa protein band is visible on SDS/PAGE gels loaded with sheep saliva. Furthermore, this band is immunoreactive against antibodies raised against full-length BSP30A (Figure 1A). A BLAST search against sheep EST sequence databases revealed two separate but related cDNA sequences, each encoding a protein with greatest identity with BSP30A and BSP30B (Figure 1B). However, these sheep salivary proteins are not orthologous to the two bovine proteins, rather it appears that there may have been separate gene-duplication events within both the sheep and cattle lineages from a common ancestral gene. Further investigation is required to provide a complete picture of the evolution of these genes.

Figure 1 | BPI-like proteins in saliva from sheep
(A) Saliva was collected from sheep and subjected to SDS/PAGE, followed by staining of proteins with Coomassie Blue (left-hand panel) or Western blotting (WB) using rabbit polyclonal immunoglobulin raised against full-length recombinant BSP30A and which recognizes both BSP30A and BSP30B (right-hand panel). (B) Two distinct contiguous nucleotide sequences (contigs) assembled from sheep ESTs were obtained from the National Center for Biotechnology Information and the Institute for Genomic Research expressed sequence databases, and then translated to amino acid sequence. These were aligned with BSP30A, BSP30B and BSP30C amino acid sequences and a dendrogram constructed using the Lasergene software package (DNASTar).
Expression of the BPI-like family in cattle

The pattern of expression has been characterized of several members of the bovine BPI-like family. Most work has been focused on expression of the BSP30 genes. Overall, the pattern of expression of the BSP30A and BSP30B genes is similar to that of PSP/SPLUNC2 from other species [11–16]. Northern blots of RNA from a number of bovine tissues indicate that expression of these genes is restricted to the major salivary glands (parotid, submandibular, sublingual and buccal) [17]. These experiments also revealed that BSP30A and BSP30B are expressed independently of each other [17], an intriguing observation given that the two genes are very similar to each other in other respects. BSP30A mRNA is found at approximately the same abundance in the parotid glands of almost all animals tested, while in contrast, BSP30B mRNA is found at high abundance in only a proportion of animals [17]. There is very high concordance in abundance between the left and right parotid glands from a given animal. BSP30C was also found to be highly expressed in the major salivary glands, but unlike for BSP30A and BSP30B, moderate amounts of mRNA were also found in other tissues, including tonsil and abomasum, albeit at a more moderate level. Evidence for the expression of BSP30D was obtained only through RT (reverse transcription)–PCR, suggesting that it is expressed only at a low level. It was not possible to determine in which tissues it is expressed, since the RNA used for the analysis was a mixture of material from several tissues associated with the oral cavity and airways [10].

The expression of other members of the BPI-like family in cattle has also been investigated. RNA encoding SPLUNC1 was found in tissue derived from the lining of the trachea as well as the nasal cavity. In contrast, SPLUNC3-encoding mRNA was found at high abundance only in the tongue. LPLUNC1 was found to be expressed in trachea, nasal epithelium and in the parotid gland, whereas LPLUNC2 was expressed predominantly in the sublingual and buccal salivary glands, and the mucosa lining the mouth and the soft palate [10,17]. For the most part, these tissue distributions are similar to what has been reported for their homologues in humans and rodents [3,18,19] and underscores the previously made observation that each of the family members has a distinct pattern of expression among tissues of the oral cavity and airways.

Analyses at the protein level of the abundance of the bovine BPI-like proteins have focused on BSP30. Saliva from cattle has a protein profile that is quite distinct from human and rodent saliva. One distinguishing aspect of the profile is a very high abundance of BSP30A and BSP30B, which are easily visible on gels after Coomassie Blue staining (Figure 2A). This is in marked contrast with their parologue, PSP/SPLUNC2 in human saliva, in which the protein is only just abundant enough to be visible by Coomassie Blue staining, even on a two-dimensional gel (Figure 2B). Immunohistochemical analysis of bovine salivary tissue using antibodies recognizing both BSP30A and BSP30B has shown that these proteins are localized to the serous secretory cells (Figure 3). Antibodies raised against a region of high-sequence divergence between BSP30A and BSP30B have been used to show that BSP30B is present at high abundance in the saliva of almost all animals tested, while BSP30A is present at high abundance only in a proportion of animals. BSP30B migrates on electrophoretic gels with an apparent molecular mass 5 kDa greater than that of BSP30A. It is possible that this is due to post-translational modification such as glycosylation. This is supported by the observation that BSP30B appears to stain preferentially with the glycoprotein-specific stain Pro Q Emerald [20]. Further Western-blot analysis showed that, in cattle expressing BSP30B, the protein is present in the parotid, submandibular and buccal salivary glands, but that BSP30A is present only in the submandibular gland [20]. The absence of BSP30A in the parotids in this analysis is surprising, since BSP30A mRNA has been shown to be abundantly expressed in this gland in other analyses, and an explanation can only be speculated upon. The abundance and localization of BSP30C is only just beginning to be studied. Preliminary results suggest that BSP30C is present in bovine saliva, as might be expected from the mRNA studies described above, but a significant immunoreactive signal is observed in saliva from only a proportion of cattle in a population. Interestingly, there appears to be no significant concordance with the abundance of BSP30B (M.K. Broadhurst, M. Berg, B.J. Haigh and T.T. Wheeler, unpublished work). The abundance of BSP30C in

Figure 2 | Abundance of BSP30 in and PSP/SPLUNC2 in bovine and human saliva

(A) SDS/PAGE of bovine saliva from two Friesian–Holstein cows (lanes 1 and 2) followed by staining of proteins with Coomassie Blue (left-hand panel) or Western blotting (WB) using rabbit polyclonal immunoglobulin raised against full-length recombinant BSP30A, and which recognizes both BSP30A and BSP30B (right-hand panel). The BSP30A and BSP30B proteins are indicated. (B) Two-dimensional electrophoresis (2DE) of human saliva followed by staining for proteins with Coomassie Blue (upper panel) or Western blotting using rabbit polyclonal immunoglobulin raised against human PSP/SPLUNC2 (lower panel). The locations of the PSP isoforms are indicated by a box.
Immunohistochemical analysis of BSP30 proteins in bovine salivary tissues
Parotid, submandibular, sublingual and buccal salivary glands were recovered from a lactating Friesian–Holstein cow after slaughter, the tissues fixed and sections subjected to immunohistochemical analysis using rabbit polyclonal immunoglobulin raised against full-length recombinant BSP30A. This antibody recognizes both BSP30A and BSP30B, but not BSP30C. The immunoreactive signal (dark areas, with examples arrowed) was visualized with diaminobenzidine with nickel enhancement. The slides were counterstained with eosin and visualized at ×20 and ×200 magnification. Positive signals were observed in the serous acini and striated ducts (parotid), serous demilunes and striated ducts (submandibular), the striated ducts (sublingual) and in a patchy distribution among the serous acini (buccal).

Figure 3 | Immunohistochemical analysis of BSP30 proteins in bovine salivary tissues

Few results are available on the analysis of other members of the BPI-like protein family in cattle, thus one must infer their abundance in saliva and other airways secretions from the mRNA analyses reported above, and from studies on their orthologues in other species.

Function of BSP30A and BSP30B
The structural similarity of the BPI-like proteins to BPI and LBP, together with the well-documented potent antimicrobial activity of BPI [21] and the pro-inflammatory activity of LBP [22] suggests similar functions for the rest of the BPI-like protein family. However, to date only limited experimental evidence has been published that directly addresses this. It has been reported that human recombinant SPLUNC1 has antimicrobial activity [23], anti-biofilm activity [24], that human recombinant PSP/SLPUNC2 has antimicrobial activity [14,16], and that peptides derived from this latter protein have immunomodulatory properties [25]. However, the activity of the human BPI-like proteins isolated from natural sources is as yet unknown.

Bovine saliva, because of its plentiful supply and high abundance of BSP30 proteins, provides an opportunity to purify large amounts of at least one BPI-like protein to investigate its function. BSP30A and BSP30B have been purified separately from bovine saliva and their activities investigated alongside that of recombinant BSP30A and BSP30B [20]. Both saliva-purified and recombinant BSP30A and BSP30B inhibit the growth of the opportunistic human lung pathogen Pseudomonas aeruginosa with moderate potency, but not that of a range of other pathogens ([20], and B.J. Haigh, M. Callaghan and T.T. Wheeler, unpublished work). These results, which are in marked contrast with the extreme potency and broad range of other innate immune effector proteins such as the cathelicids and defensins [26], suggest that, despite their antimicrobial activities, the BSP30 proteins may not function primarily as microbicidal proteins.

Antimicrobial proteins may manifest their activity in a way other than by rendering pathogens non-viable. For example, they may reduce their virulence through preventing colonization on the mucosal surface. Furthermore, some proteins have antimicrobial activity through their ability to cause aggregation of pathogens so as to prevent rapid dissemination of microbes from a site of initial colonization. Preliminary investigations indicate that saliva-purified BSP30A and BSP30B both inhibit adhesion of Escherichia coli to an intestinal epithelial cell line in culture and that BSP30A also stimulates aggregation of Ps. aeruginosa and Candida albicans (M.K. Broadhurst, M. Callaghan and T.T. Wheeler, unpublished work). Aggregation activity is also observed with the human paralogue of the BSP30 proteins, PSP/SPLUNC2 ([27], and B.J. Haigh, M. Callaghan and T.T. Wheeler, unpublished work). While suggestive, the antimicrobial activity of these proteins has not yet been verified in whole animal models, and so their biological significance remains unresolved to date.

The aggregation activity of BSP30A suggests that it may act as a linker between microbial cells, in much the same way as immunoglobulins have agglutinating activity. BSP30 is predicted to possess only one hydrophobic pocket, which presumably acts as a ligand-binding site [10]. If this were the case, then it would seem likely that the aggregation activity of BSP30 is facilitated through dimerization. This possibility is supported by the observation in some purified preparations of an approximately 60 kDa BSP30 immunoreactive band (T.T. Wheeler, unpublished work). We have investigated the

bovine saliva relative to the other BSP30 proteins is as yet unknown.

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existence of BSP30 dimers by subjecting purified preparations of BSP30A and co-purified BSP30A and BSP30B to size-exclusion chromatography (M. Agnew, M.K. Broadhurst and T.T. Wheeler, unpublished work). Perhaps surprisingly, the results revealed clearly that all the immunoreactivity was eluted at 30 kDa, with no evidence for any significant dimerization. Thus the basis of the aggregation activity for BSP30A remains unexplained.

The immunomodulatory activity of saliva-purified and recombinant BSP30A and BSP30B have been investigated. Only recombinant BSP30A showed a moderate degree of LPS-binding activity, while the others showed no activity above that of a control protein, casein [20]. Consistent with this, BSP30A also stimulates LPS-induced pro-inflammatory signalling in a cultured macrophage cell line (B.J. Haigh and T.T. Wheeler, unpublished work). However, from the results published to date it is unclear as to whether the BSP30 proteins contribute significantly in mediating the inflammatory response to LPS in vivo. To date, no experiments have been reported addressing whether the BSP30 proteins modulate inflammatory signalling by interacting with other ligands.

**Biological role of the BPI-like proteins**

Despite the accumulation of data on the structure and expression of the bovine BPI-like proteins, and the circumstantial evidence for a role in host defence, little is known for certain about the role these proteins play in bovine physiology. For the BSP30 proteins, the results produced to date would appear to favour a role in pathogen recognition rather than as microbicidal effector proteins. Apart from BPI itself, of which there are numerous reports associating it with susceptibility to infection (e.g. [28–30]), studies on the BPI-like proteins from other species would appear to offer little further insight. Human SPLUNC1 has been associated with cystic fibrosis [31] and has been shown to suppress growth of *Mycoplasma pneumoniae* [23] and reduce biofilm formation of a lung pathogen in vitro [24], consistent with the idea that the BPI-like protein family contributes to host defence of the oral cavity and airways. However, direct evidence of a significant host fence role is still lacking.

The single most notable feature from studies of the bovine BPI-like proteins has been the extremely high abundance of the BSP30 proteins in bovine saliva, in contrast with its paralogue, SPLUNC2, in human saliva. This observation, coupled with the large volume of saliva entering the rumen (estimated to be approximately 100 litres per day for cattle [32]), suggests that the BSP30 proteins may play a role in rumen digestion. Rumination is a signature adaption of the ruminant lineage, serving to digest otherwise indigestible plant fibre through microbial fermentation in an anaerobic environment at neutral pH in a specialized fore stomach, namely the rumen. It is conceivable that the BSP30 proteins act within the rumen to promote a microbial ecological niche that is optimal for this fermentation by suppressing the growth of undesirable microbial species or perhaps facilitating the adherence of fibrolytic species to plant fibre. A role as a modulator of the rumen ecology could also provide an explanation for the association with bloat, if the BSP30 proteins were found to suppress rumen methanogens, since methane production is a significant factor in the aetiology of bloat. However, each of these possibilities remains to be experimentally verified.

**Conclusion**

The investigations on the bovine BPI-like proteins, and BSP30 in particular, support the idea that this family plays a role in host defence. Furthermore, they suggest that this protein family is evolving rapidly within the bovine lineage, including the acquisition of new physiological roles. The information produced to date addressing the function of the proteins leaves open many questions. These include the identity of the ligands with which, presumably, they interact, the microbial species whose prevalence they might modulate, the signalling pathways they might activate and the physiological situations in which they play a role. To date, most attention has been placed on their role in the oral cavity and airways, but it is also possible that they also contribute to host defence at other sites of high pathogen exposure, for example, the mammary gland, digestive tract or reproductive tract. Addressing these questions may lead to new insights into how the BPI-like proteins contribute to host defence, and lead to new strategies to deal with human infections and inflammatory diseases, as well as in livestock production.

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