Genetic analysis of *Streptococcus uberis* plasminogen activators

Philip N. Ward & James A. Leigh

*Institute for Animal Health, Compton Laboratory, Compton, Berkshire, RG20 7NN, UK*

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**Background & objectives:** Streptococci produce a diverse range of secreted plasminogen activators capable of converting mammalian plasminogen to plasmin in a species-specific manner. In all examples to date, the host animal’s plasminogen and that of a number of additional species have been shown to interact with these molecules leading to the conclusion that the pathogenesis of streptococci is in some way dependent upon activation of host plasminogen. PauA was the first plasminogen activator described from *Streptococcus uberis*, a pathogen frequently isolated from cases of bovine mastitis. Recently, a second *S. uberis* plasminogen activator (PauB) was identified from a Danish mastitis isolate. Interestingly, the *pauB* open reading frame occupied the locus normally filled by *pauA*. In the present study a genetic screen of streptococcal and field isolates frequently associated with mastitis was undertaken to assess the distribution, chromosomal location and sequence variation of these putative virulence factors.

**Methods:** Southern analysis of a diverse panel of streptococci and additional bacterial isolates frequently associated with bovine mastitis was performed using *pauA* and *pauB* probes. Sequence variation of PauA was assessed at the protein level following nucleotide sequence analysis of *pauA* alleles amplified from isolates picked from different geographical locations.

**Results:** We observed plasminogen activators to be universally distributed amongst *S. uberis*. A *pauA* allele was identified in all but one strain of *S. uberis*. This strain had a *pauB* allele substituted for *pauA* at the same locus. The remarkably low level of sequence variation demonstrated by PauA was further restricted to a limited number of residues within the molecule.

**Interpretation & conclusion:** The high prevalence of PauA alleles in field isolates of *S. uberis* supported the observation that plasminogen activators are likely to confer an advantage with respect to colonization and growth. The findings of the present study support the theory that PauA plays a critical role in the pathogenesis of *S. uberis*.

**Key words** Bovine - mastitis - PauA - PauB - plasminogen activator - *Streptococcus uberis*

The role of mammalian plasminogen activators and plasmin in fibrinolysis, tissue remodelling, cellular migration and metastasis has long been established. The broad spectrum serine protease plasmin is formed following cleavage of the zymogen precursor plasminogen by host activators tissue plasminogen activator and urokinase. Numerous pathogenic bacteria have evolved mechanisms to take advantage of this protease activity and bind plasmin(ogen) at the cell surface. In addition to being able to bind plasmin(ogen) at the cell surface, a select group of pathogens including many streptococci and *Staphylococcus aureus* produce a diverse range of secreted plasminogen activators capable of converting mammalian plasminogen to plasmin in a species-specific manner. Currently, six different streptococcal molecules have been described that fulfil this function: the classical streptokinase (SK) family common to most group A, C and G streptococci of human origin, further plasminogen activators (Psk and Esk) identified from porcine and equine isolates of *S. equisimilis* respectively.
molecular weight plasminogen activator (PadA) from \textit{S. dysgalactiae}\textsuperscript{4} and two plasminogen activators (PauA and PauB) from \textit{S. uberis}\textsuperscript{5,6}. In all examples to date, the host animal’s plasminogen and that of a limited number of additional species have been shown to interact with these molecules leading to the conclusion that the pathogenesis of streptococci is in some way dependent upon activation of host plasminogen. With respect to the world-wide involvement of \textit{S. uberis} in the pathogenesis of bovine mastitis, the role of plasminogen activators PauA and PauB has yet to be defined. Previous studies suggested a widespread but not absolute distribution of PauA among field isolates of \textit{S. uberis}, however where PauA was absent, an alternative plasminogen activator (PauB) was found to be present\textsuperscript{7,8}. There are confusing reports in the literature regarding the invasive/adherent nature of the pathogen within the mammary gland and the role of plasmin(ogen) in the establishment of foci of infection\textsuperscript{9,10}. An alternative role for cell surface-associated plasmin(ogen) has been proposed from a nutritional standpoint. \textit{S. uberis} lacks demonstrable secreted protease activity, which renders this auxotrophic bacterium at a distinct disadvantage when grown in milk. Plasmin(ogen) localised at the cell surface is thought likely to facilitate the procurement of essential amino acids from complex milk proteins and peptides\textsuperscript{11}.

The present study was undertaken to assess the distribution, chromosomal location and sequence variation of plasminogen activators PauA and PauB from \textit{Streptococcus uberis}.

**Material & Methods**

**ORF assignments:** Open reading frame (ORF) assignments at the \textit{PauA} locus of \textit{S. uberis} strain 0140J were made using genomic sequence data from The Wellcome Trust Sanger Institute\textsuperscript{12}. The \textit{PauA} locus was scanned for predicted open reading frames using Artemis version 4.0 (Genome Research Ltd, The Sanger Centre, UK). Putative assignments for each ORF were made using “\texttt{tblastn}” (http://www.ncbi.nlm.nih.gov/).

**Southern analysis:** Genomic DNA was prepared using a previously described method\textsuperscript{13} from overnight cultures grown at 37°C. Two units of HindIII restriction endonuclease (Life Technologies, USA) were used to digest 5μg of each genomic DNA preparation as directed by the manufacturer. Southern analysis was conducted using standard procedures\textsuperscript{14}. Digoxygenin-labelled probes were prepared by amplification of \textit{pauA} (forward primer: 5'-AATAACCGGTATGATTCCGACTAC, reverse primer: 5'-AAAATTTACTCGAGACTTCCTTTAAGG) and \textit{pauB} (forward primer: 5'-CAAAGTGGCCCATGGCTTCAAAAAGAG, reverse primer: 5'-CAGCTTTATTTCGGATCTCAGTATTGC) using a DIG PCR labelling mix (Roche, Germany).

**PCR amplification and sequence determination of \textit{pauA} alleles:** The \textit{pauA} alleles were amplified and sequenced using flanking primers located in \textit{hexA} (5'-GAGATTCCCTCTCTAGATCA) and \textit{orf1} (5'-GGGCTGACGATCCGTTAAAAATGACATTAT) from a panel of UK mastitis isolates selected from 8 different locations. Amplified DNA was prepared for sequencing using DNA Purification Kit II spin columns (Thermo-Hybaid, UK) and sequence determination was performed in both directions by Cytomyx, UK.

**Results**

**ORF assignments for the plasminogen activator locus of \textit{S. uberis} 0140J:** A contiguous stretch of genomic DNA containing the \textit{pauA} allele was identified within the assemblies listed from the \textit{S. uberis} strain 0140J sequencing project currently being undertaken at the Wellcome Trust Sanger Institute, UK\textsuperscript{12}. Open reading frames were identified in the sequence flanking the \textit{pauA} allele and assignments made for each putative gene based upon tBlastn results (Fig. 1). The \textit{pauA} allele was flanked by DNA mismatch repair homologues \textit{hexA} and \textit{hexB} as previously reported\textsuperscript{8}. These ORFs in turn were flanked by putative 2 DNA helicases, a group A

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{\textit{pauA} locus – \textit{S. uberis} 0140J.}
\end{figure}
streptococcal (GAS) integral membrane protein homologue and a putative 3-methyl-adenine DNA glycosylase I homologue. Further assignments were made for putative arginyl tRNA synthetase and repressor genes and a competence/damage inducible protein homologue from S. pneumoniae.

The abundance of PauA and PauB in field isolates of S. uberis: A screen of Danish streptococcal field isolates of bovine origin from 20 different farms was undertaken by Southern analysis using pauA and pauB coding sequence probes (Fig. 2) and PCR (data not shown) to assess the distribution and chromosomal location of these putative virulence factors. The pauA allele was found on genomic DNA fragments of conserved size (arrows in Fig. 2) in all 20 Danish S. uberis isolates tested. Conversely, the pauB allele was only identified in the previously reported Danish isolate SK880 (data not shown) which lacked the pauA allele.

The abundance of pauA and pauB homologues in other streptococci and other relevant species: A wider panel of streptococci and other species commonly associated with bovine mastitis was also screened by Southern analysis to determine the presence or otherwise of homologous ORFs. The pauA allele was found to be restricted to, but universally present in S. uberis except in one strain (SK880) where pauB was present (Results not shown).

Sequence conservation of PauA and its geographic distribution: PauA sequence data were derived from a panel of S. uberis isolated from 8 different UK farms. The translation product of each allele sequenced was determined and compared with those already lodged on the GenBank nucleotide database. Multiple sequence alignment identified the positions within the coding sequence where heterogeneity was evident (Table). Variation that affected coding sequence was restricted to only 7 amino acids out of a possible 261. A novel combination of these 7 residues was identified (allele type 6) which was found in two discrete UK isolates. No evidence of geographical partitioning of strains was found between isolates from Europe and USA.

![Fig. 2. Southern analysis of Danish S. uberis (numbered 1-20) isolated from 20 different locations. Panels were probed with pauA pauB probes respectively. Arrows indicate PauA specific bands. Tracks A and B represent the respective control strains S. uberis 0140J and SK880.](image)

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Discussion

PauA was the first plasminogen activator described from *S. uberis*, a pathogen frequently isolated from cases of bovine mastitis. Recently a second *S. uberis* plasminogen activator (PauB) was identified from a Danish mastitis isolate. Interestingly, the PauB open reading frame occupied the locus normally filled by PauA, between putative DNA mismatch repair genes *hexA* and *hexB/orfI*. The chromosomal location of the *S. uberis* plasminogen activators differs significantly from the situation observed previously for the classical streptokinase locus. The locus encoding streptokinase in most group A, C and G streptococci was reported to be flanked by *lrp*, encoding a leucine rich protein, *abc*, an ATP binding cassette protein and *rel*, encoding a homologue of *E. coli* *relA* and *spoT* genes which moderate levels of guanosine 5',3' polyphosphate during nutritional stress. In contrast, the same locus in *S. uberis* was devoid of any plasminogen activator allele.

However, PauA is located elsewhere in the *S. uberis* chromosome, between *hexA* and *hexB* genes which display homology to the highly prevalent *mutS* and *mutL* DNA mismatch repair genes. Further analysis of the *S. uberis* plasminogen activator locus in this study identified further additional genes which indicated that this locus is primarily dedicated toward DNA replication and repair functions. The absence of any bacteriophage-like sequences or insertion elements in these loci raise interesting questions as to the origin and possible mechanisms of transfer of streptococcal plasminogen activators between species that are not naturally competent.

The very high prevalence of *pauA* alleles in field isolates collected from various European locations supported the observation that plasminogen activators are likely to confer an advantage with respect to colonization and growth. There remains only one *S. uberis* isolate which lacks the *pauA* allele, and this strain (SK880) is the only isolate identified with the *pauB* allele. Intriguingly, the *pauA* allele appeared to be restricted solely to *S. uberis* and was not detected in other streptococci or other bacteria commonly associated with bovine mastitis despite their occupation of the same environmental niche. In this respect, it is tempting to suggest that the streptococcal plasminogen activators are finely tailored to suit the specific requirements for the range of hosts and disease scenarios encountered across this diverse family.

Analysis of strains gathered from a variety of locations within the UK enabled comparisons to be drawn with *pauA* alleles derived from other European or North American sources. A very high level of amino acid sequence conservation was apparent, with the identification of only one further coding variant (type 6) which represented a different combination of amino acids at the 7 positions previously reported to vary within the 261 residues of the mature polypeptide. These observations further support the theory that *pauA* fulfils a critical role in the pathogenesis of *S. uberis*.

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References


12. Genome sequence of *Streptococcus uberis* strain 0140J. http://www.sanger.ac.uk/Projects/S_uberis.


*Reprint requests:* Dr Philip N. Ward, Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire RG 20 7HN, UK e-mail: phil.ward@bbsrc.ac.uk