The DNases of pathogenic Lancefield streptococci

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Abstract

DNases are abundant among the pathogenic streptococci, with most species harbouring genes for at least one. Despite their prevalence, however, the role for these extracellular enzymes is still relatively unclear. The DNases of the Lancefield group A Streptococcus, S. pyogenes are the best characterized, with a total of eight DNase genes identified so far. Six are known to be associated with integrated prophages. Two are chromosomally encoded, and one of these is cell-wall anchored. Homologues of both prophage-associated and chromosomally encoded S. pyogenes DNases have been identified in other streptococcal species, as well as other unique DNases. A major role identified for streptococcal DNases appears to be in the destruction of extracellular traps produced by immune cells, such as neutrophils, to ensnare bacteria and kill them. These traps are composed primarily of DNA which can be degraded by the secreted and cell-wall-anchored streptococcal DNases. DNases can also reduce TLR-9 signalling to dampen the immune response and produce cytotoxic deoxyadenosine to limit phagocytosis. Upper respiratory tract infection models of S. pyogenes have identified a role for DNases in potentiating infection and transmission, possibly by limiting the immune response or through some other unknown mechanism. Streptococcal DNases may also be involved in interacting with other microbial communities through communication, bacterial killing and disruption of competitive biofilms, or control of their own biofilm production. The contribution of DNases to pathogenesis may therefore be wide ranging and extend beyond direct interference with the host immune response.

INTRODUCTION

A number of clinically significant eukaryotic and prokaryotic micro-organisms produce DNases as virulence factors. These include Gram-positive bacterial pathogens such as Staphylococcus aureus and streptococcal species such as Streptococcus pyogenes [1, 2]. Gram-negative pathogens such as Helicobacter pylori and Shigella flexneri, in addition to members of the genera Salmonella and Yersinia, also implement these enzymes in a similar manner [3, 4]. Further, the opportunistic fungal pathogens Candida albicans and Cryptococcus neoformans and the malarial parasites of the genus Plasmodium are also known to utilize DNases in pathogenesis [5–7].

The genus Streptococcus comprises a multitude of obligate and opportunistic pathogens of both humans and animals [8]. A classification system identified by Rebecca Lancefield in the 1930s is still used to classify the beta-haemolytic streptococci based on their type specific carbohydrate antigen [8]. S. pyogenes, the Lancefield group A Streptococcus (GAS) is a major human pathogen responsible for a diversity of clinical manifestations and considerable global disease burden exceeding 700 million infections per annum [9]. Clinical manifestations include superficial infections such as pharyngitis, non-bullous impetigo and scarlet fever in addition to potentially lethal invasive manifestations such as streptococcal toxic shock syndrome, necrotizing fasciitis and puerperal sepsis [10]. Furthermore, S. pyogenes is associated with serious post-infectious sequelae, notably post-streptococcal glomerulonephritis and rheumatic fever [11]. Streptococcus agalactiae, the Lancefield group B Streptococcus (GBS) is another major human pathogen and, although present as a commensal in the gastrointestinal and genitourinary tract, is a leading cause of neonatal morbidity and mortality worldwide, often associated with neonatal meningitis, pneumonia and sepsis [12]. Streptococcus dysgalactiae subsp. equisimilis (SDSE) can carry the Lancefield group C or G antigens and has only relatively recently been recognized as a major human pathogen, increasing in incidence and prevalence [13]. The spectrum of symptoms associated with SDSE infection are similar to those observed with S. pyogenes, indeed the two species share many virulence factors and significant DNA sequence similarity [13]. Other Lancefield group C streptococci include Streptococcus equi subsp. equi (hereafter referred to as S. equi) and S. equi subsp. zooepidemicus (hereafter referred
to as *S. zooepidemicus*). *S. equi* is almost exclusively a pathogen of horses and is believed to be descended from an ancestral strain of *S. zooepidemicus*, which will readily colonize and infect humans in addition to a vast spectrum of domestic and livestock animals. Both species share in excess of 80% DNA sequence similarity with *S. pyogenes* [14].

The ability for Lancefield streptococci to cause a wide range of disease may be due to an extensive arsenal of virulence factors. Some of these factors, which include DNases, are associated with mobile genetic elements and can transmit between strains and even species. The function of DNases during pathogenesis is still relatively unclear and the potential for DNases to be virulence factors has only recently been explored.

**DNASE HISTORY AND NOMENCLATURE**

Historically, it was believed that *S. pyogenes* produced only four DNases and these were serologically classified as DNase A, DNase B, DNase C and DNase D. Anti-DNase B titres have been used as a serological biomarker of streptococcal infection and post-streptococcal immune sequelae since at least the 1970s [15, 16]. However, the identity of DNase B would not be truly known for over a decade when it was demonstrated that the chromosomally encoded DNase *spdB* or mitogenic factor (*mfn*; then thought to be a streptococcal superantigen) was in fact DNase B [17]. It would be many years subsequent to the initial use of DNase B in the clinical laboratory before it was established that these enzymes could contribute to the pathogenesis of *S. pyogenes* [2]. DNase C is now known to be Spd3, and DNase D has been identified as Sda2 (SdaD2) [2], however, the identity of DNase A in the original serological system is not currently known.

Since their discovery, the classification, nomenclature and role of the DNases found in streptococci has been a confusing topic. With advances in molecular biology and the application of whole genome sequencing, it is now apparent that a number of variants exist for the majority of DNases and there is homology between DNases of different streptococcal species.

**GENETIC IDENTIFICATION AND CLASSIFICATION OF STREPTOCOCCAL DNASES**

**DNases of the Lancefield GAS**

The DNases of GAS are by far the best characterized and currently eight have been identified; *spnA*, *spdB*, *sda1*, *sda2*, *spd1*, *spd3*, *spd4* and *sdh*. Both *spnA* and *spdB* are encoded on the chromosome and have been shown to be common to all *S. pyogenes* isolates tested, existing as different alleles related to the *emm* genotype of the isolate [18, 19]. We confirmed the presence of both *spnA* and *spdB* in all available completed *S. pyogenes* genomes (NCBI, *n*=54) representing 25 different *emm* genotypes. *SpnA* is the only *S. pyogenes* DNase to be identified that is cell-wall anchored via an LPXTG motif [19].

The other six *S. pyogenes* DNases are associated with prophages or prophage-like elements (Table 1). *S. pyogenes* has a close evolutionary relationship with temperate bacteriophage as most strains are polylysogenized, and prophage and prophage-like elements account for −10% of the *S. pyogenes* genome [20, 21]. Bacteriophages are transmissible between hosts, carrying genes for bacterial virulence factors; the streptococcal superantigens, DNases and a secreted phospholipase. Different bacteriophages may carry the same virulence factor and each factor may exist as a different allele, as has been shown for the streptococcal superantigens [22]. By mining the 54 available genomes of *S. pyogenes* we identified that at least two different DNA sequence alleles exist for each of the six prophage-associated DNases (Table 1). The most prolific prophage-associated DNase genes were *spd1* and *spd3*. The other four prophage-associated DNase genes were restricted to isolates belonging to only two–five different *emm* genotypes, which may be reflective of host-specificity or functionality of the associated prophage. Twelve isolates did not carry any prophage-associated DNases and they belonged to the *emm* genotypes *emm*44, 59, 71, 82, 83, 89 and 101. Although other *emm*89 isolates did carry prophage-associated DNases, two *emm*89 isolates (H293 and MGAS23530) have been shown to not have any prophage elements integrated into their chromosomes, and therefore only carry chromosomal DNase genes [23, 24].

**DNases of other streptococci**

Literature search and BLAST analyses identified DNases in other streptococcal species that are similar to those found in *S. pyogenes*. Despite the prevalence of *spd1* and *spd3* among *S. pyogenes* isolates, we only identified three potential homologues of either of these genes (Fig. 1). Homologues of *spd1* were identified by BLAST analysis (sharing ≥90% identity over 75% or more of the sequence length) in isolates of *S. iniae* and *S. porcinus*, pathogens of fish and swine respectively, although they may also cause disease in humans. A third gene, similar to *spd1* was identified in *S. zooepidemicus* MGCS1056, and is one of three inferred DNases in this genome termed *sdzA* (the *spd1* homologue), *sdzB* and *sdzD* [25]. The gene *sdzA* was unique to MGCS1056 but the other two DNases were identified in the other three *S. zooepidemicus* genomes and the two genomes of the closely related equine pathogen *S. equi*. Alleles of the *sdzD* gene cluster with *sda* genes of *S. pyogenes* and SDSE, suggesting they are homologous. Although in *S. pyogenes*, *sda* is associated with prophages, no such elements were identified associated with the *sda*-like genes of *S. zooepidemicus* [25], *S. equi* or SDSE. This was also the case for the third DNase gene of *S. zooepidemicus* and *S. equi*, *sdzB*, although it shares some similarity to the *S. pyogenes* chromosomally encoded *spdB* as well as the prophage-associated *spd4*. Recently, two further DNases were identified in *S. zooepidemicus* that are cell-wall anchored, termed ENuc and 5Nuc [26]. The *enuc* alleles
identified in both *S. zoopneumoniae* and *S. equi* clustered with other identified cell-surface anchored DNases (Fig. 1) that include *spa*-like alleles from SDSE. The SDSE *spa-*like alleles all have a cell-wall anchor motif, except *spa*-like carried by the SDSE strain GGS_124, which has a truncation mutation resulting in the loss of the far C-terminal region including the LPXTG anchor motif. Other cell-surface-anchored DNases have been identified include the *S. sanguinis* cell-wall-anchored nuclease, SWAN (*Sanguinis Wall Anchored Nuclease*) and the *S. suis* SsnA. The *spa* gene of *S. suis* shows homology to *spa*-like alleles from SDSE. The SDSE strain GGS_124 and associated with a DNase gene [32]. The prophage is most closely related to a prophage element found in *S. pyogenes*, *NCTC8198* although this prophage is not associated with any virulence factors, the prophage element in *S. pyogenes* carries a mutation that would truncate the protein. These findings suggest an exchange of prophages and associated virulence factors between *S. pyogenes* and SDSE.

Table 1. Prophage-associated DNases in *S. pyogenes* and their alleles

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*Allele based on nucleotide sequence of the entire coding region.

confirmed DNase activity [30]. It seems likely that other DNases exist in *S. agalactiae* but have yet to be identified.

We did identify two prophage-associated DNases in *SDSE*. One was in strain GGS_124 and associated with a prophage element that shares ~90 % identity to prophage 315.3 from *S. pyogenes* *emm*3 genome MGAS315 [31]. Although in *S. pyogenes*, the prophage 315.3 is associated with the DNase *spd4*, the gene found in GGS_124 (SDSE167_1285, SDSE *sdn*) is 100 % identical to a different prophage-associated *S. pyogenes* DNase, *sda*. (Fig. 1). Another SDSE strain, 167 also has a prophage-like element associated with a DNase gene [32]. The prophage is most closely related to a prophage element found in *emm*1 NCTC8198 *S. pyogenes*, although this prophage is not associated with any virulence factors, the prophage element in the SDSE strain 167 is associated with an *sda*-like gene (SDEG_1103, SDSE *sda*). However, in this strain, the gene carries a mutation that would truncate the protein. These findings suggest an exchange of prophages and associated virulence factors between *S. pyogenes* and SDSE.
Fig. 1. Phylogenetic analysis of streptococcal DNases. Full length coding regions of each identified and potential DNase gene were aligned using MUSCLE and a neighbour-joining tree created. Bootstrap values greater than 80% are shown on branches. Multiple alleles were compared for all prophage-associated *S. pyogenes* DNase genes (red) but only single representative alleles for the chromosomal...
THE ROLE OF DNASES

Immune evasion

Originally it was thought that DNases facilitated dissemination of streptococci through tissue planes in the human host by liquefying purulent exudate produced during infection [33]. It has also been speculated that in reducing the viscosity of the microenvironment, DNases expedite transmission of progeny phage particles between bacterial hosts, potentially conferring a selection advantage to both bacteriophage and bacterium [34]. Although this may still be the case, a recently described role for DNases is in the evasive strategy implemented by *S. pyogenes* to prevent neutrophil activation and degradation of neutrophil extracellular traps (NETs) (Fig. 2) [35]. NETs are composed of chromatin, histones, proteolytic enzymes and other peptides, and produced by neutrophils on degranulation whereupon they bind to invading micro-organisms by charge interaction [36]. Once entrapped, secreted cationic antimicrobial peptides attack the offending agent and neutralize virulence factors. Similar extracellular trap structures have been described in association with mast cells [37] and eosinophils [38], all of which can be degraded by DNases [39].

By secreting DNases, such as Sda1, *S. pyogenes* is able to escape these bactericidal traps by degrading their chromatin backbone, thus surviving and spreading (Fig. 2) [2, 35]. The ENuc and 5Nuc DNases of *S. zooepidemicus* also have the capacity to degrade NETs, both synergistically and alone, and enabled *S. zooepidemicus* to spread systemically in a murine model of infection [26]. The *S. agalacutiae* DNase NucA is also able to degrade NETs and its loss results in reduced virulence [30].

Cell-wall-anchored DNases have also been associated with NET degradation. The first description of a cell-wall-located DNase was the discovery of SsnA of *S. suis* [40]. *S. pyogenes*, further to secreting extracellular DNases, is also able to implement the cell-wall-anchored DNase SpnA to escape these traps [41]. SWAN of *S. sanguinis*, an opportunistic periodontal pathogen, has been shown to degrade NETs [42]. *S. pneumoniae* also produces a cell-wall-located nuclease, EndA, which is capable of degrading NETs [43] and a

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**Fig. 2.** Streptococcal DNases can degrade neutrophil extracellular traps (NETs). NETs are composed primarily of DNA (blue strands) associated with histones and other antibacterial factors (yellow circles). Bacteria can be ensnared in the DNA traps and killed by the associated factors (left-hand figure). Streptococcal secreted and cell-anchored DNases (indicated as scissors) degrade NETs, allowing the bacteria to escape and prevent killing (right-hand figure).

DNases spnA, spnB and s5nA are shown (blue). Alleles for *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) DNase genes were determined from five completed genomes; AC-2713 (NC_019042.1), GGS_124 (AP010935.1), RE378 (AP011114.1), 167 (AP012976.1), ATCC12394 (CP002215.1). Alleles for *Streptococcus zooepidemicus* (SESZ) DNase genes (green) were determined from four completed genomes; H70 (FM204884.1), CY (CP006770.1), ATCC35246 (CP002904.1), MGCS10565 (CP001129.1). Alleles for *Streptococcus equi* (SESE) DNase genes (green) were determined from two completed genomes; ATCC39806 (CP021972.1), 6047 (FM204883.1). Other DNase genes comprise endAsuis (SSU1009) and ssnA (SSU1760) from *S. suis* strain P1/7 (AM946016.1), swan (SSA_1750) from *S. sanguinis* SK36 (CP000387.1), spnA (K710_1281) from *S. iniae* SF1 (CP005941.1), spd1 (STRPO_1639) from *S. porcinus* str. Jelinkova 176 (AEUU02000001.1), group B *Streptococcus* (GBS) nuc (gbs0661) from *S. agalactiae* NEM316 (AL732656.1).
homologue of this enzyme, EndAsuis, can be found in S. suis. EndAsuis is reported to increase survival in NETs and is produced in addition to the aforementioned secreted nuclease [29]. Although it is unclear why streptococci might implement both cell-wall-anchored and secreted DNases, they may provide necessary localized DNase activity in the immediate environment as well as more wide-spread activity [44]. S. pyogenes remained attenuated following deletion of the cell-wall-anchored DNase SpnA, despite complementation with a secreted form of the enzyme [44]. SpnA may also have an additional role in pathogenesis that is not related to its enzymatic activity [44] and this may well extend to other streptococcal cell-wall-anchored DNases.

Another method by which S. pyogenes is able to evade innate immunity is by degrading its own nucleic acids. Indeed, depolymerization of bacterial DNA by DNases has been shown to prevent killing of S. pyogenes by reducing TLR-9 signalling and subsequent recognition of unmethylated CpG-rich DNA by macrophages [45]. The ENuc and SNuc DNases of S. zooepidemicus too have a dual action against the innate immune system. Both nucleases degrade extracellular traps but also possess 5’-nucleotidase activity and produce cytotoxic deoxyadenosine as a substrate, which impedes phagocytosis by macrophages [26]. A similar mechanism was also identified in S. pyogenes whereby a cell-wall-anchored 5’ nucleotidase (SSnA) acted synergistically with the DNase SpnA to cleave NETs and generate the cytotoxic deoxyadenosine [46]. SSnA and SpnA are closely related to SNuc and ENuc of S. zooepidemicus, respectively (Fig. 1). Other streptococcal species also express 5’ nucleotidases and so similar mechanisms may exist for DNase synergy.

Nutrient scavenging

Elimination of DNA during infection may also have an indirect impact on pathogenesis, serving more than one purpose. For certain strains of S. pyogenes, nucleic acid derivatives are essential for growth. Indeed, efforts to standardize a laboratory method for sulphonamide sensitivity testing in the 1940s were hindered by this necessity [47]. Scavenging nucleic acids during infection or colonization by implementing DNases may therefore provide nutrients during both colonization and infection. In addition, extracellular trap formation by neutrophils and mast cells ultimately leads to death of these phagocytes [39], and could provide further nutrients for the bacteria. This may also explain why S. pyogenes and other streptococci possess both secreted and cell-wall-anchored DNases, to retain DNase activity close to the bacterium for nutrition.

Role of DNases during infection

Experimental infection data obtained using a genotype emm1 strain with three DNases revealed that sequential inactivation of these genes, most importantly SdaD2, significantly impeded the capacity of the strain to establish pharyngeal infection in cynomolgus macaques [2]. Similarly, the acquisition of the Spd1 DNase by ST15 emm3 S. pyogenes was associated with increased nasal and airborne shedding in a murine nasopharyngeal infection model [48]. While not inherently more invasive, nor more lethal, the emm3 strains that had acquired Spd1 were found to be over-represented in an upsurge of disease, and their emergence was coincident with a dramatic but transient spike in invasive emm3 disease in the UK [49]. The exact role DNases play during upper-respiratory tract infection has yet to be elucidated but these findings support the potential for DNases to contribute directly to infection. DNases have also been shown to contribute to the disease progression in murine skin and soft tissue infections [2, 19, 35] and the Galleria mellonella model of invasive disease [44], which may be due to their role in preventing NET-mediated killing as well as some other as yet unidentified role [44].

Bacterial competition and communication

Spd1 is also reported to have RNase activity [50]. A number of secreted eukaryotic RNases are known to be bactericidal; human RNases, such as the eosinophil cationic protein (also known as RNase 3) and the keratinocyte-derived RNase 7 have been shown to play an important role in innate immunity and defence against both Gram-positive and Gram-negative pathogens, by attacking the bacterial cell wall [51–53]. It may be the case, therefore, that the duality of some of the streptococcal DNases in their ability to also degrade RNA, may serve a similar offensive purpose. Microbial RNases have also been reported to have the capacity to damage eukaryotic cells both directly and indirectly of their ribonucleolytic activity, targeting various cellular components, leading to altered gene expression, cellular dysfunction and cell death [54].

The contact-dependent growth inhibition (CDI) toxin of Yersinia kristensenii was recently identified as a novel bacterial RNase of the RNase A superfamily with a key role in bacterial competition and growth [55]. The RNase activity of streptococcal DNases may therefore also serve to mediate cell–cell interactions within and between bacterial species, coordinating microbial communities such as those observed in non-sterile sites and biofilm.

It is also possible that these enzymes may be used by streptococci to compete with commensal micro-organisms in non-sterile sites, such as in the nasopharynx or on the skin. Indeed, nucleic acids are a fundamental component of many microbial biofilms [56], and streptococcal DNases may be able to effectively eliminate biofilms formed by other bacteria or regulate the formation of their own biofilm.

The EndA nuclease of S. pneumoniae plays a role in immune evasion, virulence and competence [43, 57]. EndA degrades double-stranded DNA to single-stranded DNA during transformation for the purposes of uptake and recombination [57, 58]. Although no such mechanism has been described in S. pyogenes, it is possible that streptococcal DNases could reduce the potential for competing
bacterial cells in the environment to be transformed by degrading extracellular DNA.

**SUMMARY**

The prevalence of DNases suggests an important role in the biology of many streptococci, particularly *S. pyogenes*, with at least two being found in all strains tested. With the increased use of whole genome sequencing it may be that more DNases are identified and it will be important to maintain a consistent classification system across streptococcal species, similar to that proposed for the streptococcal superantigens [22].

It has been demonstrated experimentally that acquisition of prophage-associated DNases does not necessarily increase the virulence of a strain, and the genetic background of the bacterial host may play a role [59]. Although this could also be dependent on the types of virulence assays used and the sensitivity of both *in vitro* and *in vivo* disease models which are required to build a more complete picture of how DNases function. Indeed, DNase production has been shown previously to require interaction with eukaryotic cells or induction by other external triggers [34].

Both chromosomally encoded and prophage-associated DNases have also been shown to be under the control of the extensive regulatory systems used by streptococci. This includes the control of virulence system (CovR/S or CsrR/S), which negatively regulates *sda*2 but positively regulates *spdB* in M1 strains [60] and Rgg, which negatively controls *spdB* and *spd3* [61]. Other regulators such as PerR, Ihk/Irr and CodY have also been shown to influence expression of DNases [62–64]. Further work is required to fully understand the complex regulation of DNase expression, which could also be influenced by genotype and associated prophage.

Interestingly, Walker *et al.* demonstrated that *sda1* (*sda*D2) expression during disease is essential for *emm*1 strains and places a selective pressure upon CovR/S to mutate, not only to *de-repress* *sda1* expression but to *down-regulate* the protease SpeB which degrades Sda1 [65]. Sda1 can therefore influence the infection potential of isolates not only through direct means of protection against NETs, but also by indirectly promoting the development of ‘hyper-virulent’ CovR/S mutant strains.

The contribution of DNases to bacterial colonization and infection may be extensive. The main focus of DNase research so far has been on the destruction of NETs, however there are other potential roles for DNases that may facilitate infection and warrant further research.

**References**


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