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Identification of an Atypical Zinc Metalloproteinase, ZmpC, from an Epidemic Conjunctivitis-causing Strain of *Streptococcus pneumoniae*

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Abstract

Streptococcus pneumoniae is a pathogen associated with a range of invasive and noninvasive infections. Despite the identification of the majority of virulence factors expressed by *S. pneumoniae*, knowledge of the strategies used by this bacterium to trigger infections, especially those originating at wet-surfaced epithelia, remains limited. In this regard, we recently reported a mechanism used by a nonencapsulated, epidemic conjunctivitis-causing strain of *S. pneumoniae* (strain SP168) to gain access into ocular surface epithelial cells. Mechanistically, strain SP168 secretes a zinc metalloproteinase, encoded by a truncated *zmpC* gene, to cleave off the ectodomain of a vital defense component – the membrane mucin MUC16 – from the apical glycocalyx barrier of ocular surface epithelial cells and, thereby invades underlying epithelial cells. Here, we compare the truncated SP168 ZmpC to its highly conserved archetype from *S. pneumoniae* serotype 4 (TIGR4), which has been linked to pneumococcal virulence in previous studies. Comparative nucleotide sequence analyses revealed that the *zmpC* gene corresponding to strain SP168 has two stretches of DNA deleted near its 5' end. A third 3 bp in-frame deletion, resulting in the elimination of an alanine residue, was found towards the middle segment of the SP168 *zmpC*. Closer examination of the primary structure revealed that the SP168 ZmpC lacks the canonical LPXTG motif – a signature typical of several surface proteins of gram-positive bacteria and of other pneumococcal zinc metalloproteinases. Surprisingly, *in vitro* assays performed using recombinant forms of ZmpC indicated that the truncated SP168 ZmpC induces more cleavage of the MUC16 ectodomain than its TIGR4 counterpart. This feature may help explain, in part, why *S. pneumoniae* strain SP168 is better equipped at abrogating the MUC16 glycocalyx barrier en route to causing epidemic conjunctivitis.

Keywords

Streptococcus pneumoniae; bacterial conjunctivitis; zinc metalloproteinases; mucins

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1. Introduction

The gram-positive pathogen *Streptococcus pneumoniae* or the pneumococcus is responsible for causing infections such as pneumonia, acute otitis media, conjunctivitis, and septicemia. While the inventory of virulence factors associated with pneumococcal disease is still being compiled, the most common ones to date include the capsule, the cell wall and cell wall polysaccharide, and pneumococcal proteins such as secreted proteases, pneumolysin, autolysin, and pneumococcal surface protein A (PspA) [1–3].

Depending on the reactivity of the capsular polysaccharide to different anti-capsular sera, most pneumococcal isolates can be categorized into one of 90+ distinct serotypes. Yet, a few pneumococcal strains exist that lack a detectable capsule and are thus rendered unable to react with typing sera. Such strains, referred to as nonencapsulated or nontypeable, have been frequently associated with large and sporadic outbreaks of conjunctivitis [4, 5], which involves inflammation of the mucus membrane covering the white region of the ocular surface and the inner surface of the eyelids. Previous studies have also shown that nonencapsulated strains of pneumococci exhibit enhanced binding patterns to epithelial cells [6, 7]. A straightforward explanation for this phenomenon is that, in the strains that lack a capsule, pneumococcal cell surface proteins required for adherence and colonization may be expressed or exposed to a greater extent. Typically, pneumococcal factors that aid in the binding process include adhesins such as phosphorylcholine (ChoP), and choline binding proteins [2]. Some studies have reported that the choline binding protein SpsA, or CbpA of pneumococci, interacts with the human polymeric Ig receptor (pIgR), which mediates attachment and internalization into mucosal epithelial cells [8, 9]. Other factors that have been reported to contribute to adherence, although not directly, include surface-associated enzymes such as neuraminidase (NanA), β -galactosidase (BgaA), and β -N-acetylglucosaminidase (StrH) [10], all of which contain the canonical LPXTG motif required for cell wall anchoring [11]. These enzymes catalyze the removal of terminal sugars anchored on glycoprotein and glycolipid molecules, which results in the unmasking of host surface receptors. Surface proteins such as pneumococcal adhesion and virulence A (PavA) and enolase (Eno) have been shown to bind to the extracellular matrix molecules, fibronectin and plasminogen, respectively [12, 13]; however, these interactions most likely involves exposure of the pneumococcus to the epithelial basement membrane [2]. A few pneumococcal strains express pili [14, 15] that are believed to bind to extracellular matrix proteins [16].

Binding, colonization, and subsequent invasion of host epithelial cells by the pneumococcus can be envisioned as interlinked, yet independent processes. Binding and colonization of the pneumococcus may not always be followed by invasion of host cells and establishment of infection. En route to gaining access to the epithelial surface and subsequently triggering infections such as conjunctivitis and pneumonia, the pneumococcus must first overcome an upper, loosely held mucus layer and an underlying glycocalyx coat that remains apically tethered to the epithelial surface. Both the mucus layer and the apical glycocalyx are predominantly composed of a class of heavily O-glycosylated proteins called mucins, which exist in secreted and membrane-associated forms. While secreted mucins, produced by goblet cells, make up the bulk of the upper mucus layer, which primarily functions in sweeping away trapped foreign material, the apical glycocalyx coat is comprised of membrane mucins (also referred to as cell surface mucins) that serve as the first physical barrier to prevent entry of pathogens and other noxious agents into underlying epithelial cells [17]. The distribution and abundance of membrane mucins vary across different epithelial surfaces. These molecules have long been thought to serve a crucial role in fending off pathogens; however, only a few studies addressing the defense properties of membrane mucins against pathogenic bacteria have been reported. Desouza M.M., *et al.* [18]

demonstrated that a strain of female *Muc1*^{-/-} mice (mucins in humans are designated as MUC and in mice as *Muc*) was shown to be more prone to chronic inflammation of the lower reproductive tract. In other studies, *Muc1*^{-/-} mice showed increased colonization and rapid passage of the pathogen *Campylobacter jejuni* across the gastrointestinal epithelial barrier [19, 20]. Recently, it was demonstrated that MUC16, one of the largest known membrane mucins with a molecular weight of > 2 MDa, is an important component of the apical glycocalyx barrier at the ocular surface that prevents binding of the pathogen *Staphylococcus aureus* to human corneal-limbal epithelial cells (HCLE) *in vitro* [21]. Despite these observations, our understanding of the factors and mechanisms employed by virulent pathogens to abrogate the membrane mucin barrier and invade epithelial cells still remains limited. In this regard, we recently reported a mechanism employed by a nontypeable, epidemic conjunctivitis-causing strain of *S. pneumoniae* (strain SP168) to invade ocular surface epithelial cells. *S. pneumoniae* strain SP168 secretes a zinc metalloproteinase, ZmpC, which selectively cleaves the ectodomain of the membrane mucin MUC16 from conjunctival and corneal epithelial cells, in turn, compromising the glycocalyx barrier function and promoting internalization of the pneumococcus [22]. Fortuitously, we discovered that the *zmpC* gene of strain SP168 is a truncated version, smaller than any of the known *S. pneumoniae zmpC* sequences by 723 bp. Work reported here was undertaken to do an initial characterization of the truncated SP168 ZmpC and compare its MUC16 sheddase ability to that of its homologue found in *S. pneumoniae* serotype 4 (TIGR4). Our results indicate that the truncated ZmpC from *S. pneumoniae* strain SP168 is different than all of its known homologues; it has two N-terminal stretches of in-frame deletions, one of which results in the loss of a hydrophobic region bearing the prototypical cell wall anchoring LPXTG motif. Furthermore, based on our *in vitro* cell culture-based assays, the truncated SP168 ZmpC induces more cleavage of the MUC16 ectodomain in comparison to its counterpart from strain TIGR4.

2. Materials and Methods

2.1. Pneumococcal strains and growth conditions

S. pneumoniae strain SP168 [7] and TIGR4 were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). These strains were routinely propagated on trypticase soy agar supplemented with 5% sheep blood. For growing liquid cultures, individual pneumococcal colonies were inoculated into Todd-Hewitt broth containing 5% w/v yeast extract. Liquid cultures were grown statically to an OD₆₀₀ of ~0.2 at 37°C in the presence of 5% CO₂.

2.2. Cell Culture

Telomerase-immortalized human corneal-limbal epithelial (HCLE) cells [22, 23] were cultured and grown to confluence in keratinocyte serum-free medium (Invitrogen) containing manufacturer recommended growth supplements, respectively. Cells were then switched to Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Cellgro) supplemented with 10% calf serum and 10 ng/mL epidermal growth factor for 7 days to promote differentiation as well as optimal membrane mucin production [23].

2.3. PCR amplification, DNA sequencing, and protein sequence alignments

Amplification of *zmpC* from strains SP168 and TIGR4 was performed using the primer set listed in Supplementary table 1. These primers were designed to anneal to the 5' and 3' ends of the coding region of the *zmpC* gene. Genomic DNA isolated from strains SP168 and TIGR4 served as templates in reactions. Amplified products were sequenced at the University of Maine DNA Sequencing Facility to confirm the identities of *zmpC*. BLAST searches were performed using the BLASTP program (<http://blast.ncbi.nlm.nih.gov/>).

Protein sequence alignments were performed using the ClustalX2 Alignment Tool version 2.0.11.

2.4. Cloning and recombinant ZmpC (rZmpC) expression

The *zmpC* genes corresponding to strains SP168 (accession: JQ396430, protein ID: AFD03115) and TIGR4 (genome accession: AE005672, protein ID: AAK74260) were cloned into the enzyme-free cloning vector pRham-C-his-kan (Lucigen Corp.), which places the *zmpC* gene under the control of the rhaP_{BAD} promoter and results in the expression of a C-terminal his₆ fusion of the ZmpC protein upon induction by the sugar L-rhamnose. Primers to amplify *zmpC* were designed following manufacturer's guidelines (primer set listed in Supplementary table 1). Briefly, 100 ng of amplified *zmpC* along with 25 ng of the pRham-C-his-kan vector were used to co-transform chemically competent *E. coli* cells (*E. coli* 10G from Lucigen Corp.). Transformants recovered from LB medium containing 30 $\mu\text{g/mL}$ kanamycin were subjected to plasmid DNA isolation procedures followed by restriction digestion and sequencing analyses for verifying the presence of the correct expression constructs. Correct clones were then grown overnight to saturation in LB liquid medium containing 30 $\mu\text{g/mL}$ kanamycin and 0.5% w/v glucose at 37°C with constant shaking. The addition of glucose to cultures ensures tight repression of *zmpC* (catabolite repression).

For expressing ZmpC, a 1:100 dilution of overnight grown cultures was used to inoculate 2L of LB medium + kanamycin, without any glucose. This culture was further grown to an OD₆₀₀ of ~0.4. At that point, L-rhamnose, at a final concentration of 0.2% v/v, was added to the flasks, which were then incubated further for an additional 4 h. Optimal ZmpC expression was pre-determined in pilot scale studies. After induction for 4 h, cells were harvested by centrifugation at 10,000 $\times$ g for 10 min at 4°C. The cell pellet was resuspended in 20 mL B-PER reagent (Thermo Scientific) containing 2 mg/mL lysozyme and 200 μL of a 1 mg/mL DNase I solution. The cell suspension was incubated on a rocker for 30 min and then centrifuged at 15,000 $\times$ g for 10 min at 4°C. The resulting supernatant was discarded and the cell pellet was resuspended in 20 mL of breaking buffer (50 mM Tris-HCl, pH 8.0, 5 mM 2-mercapoethanol) and sonicated on ice using a 9.5 mm disruption horn connected to a Branson model 450 sonifier set for 10 sec, three times, with 1 min cooling intervals in between. The sonicated pulp was then centrifuged at 15,000 $\times$ g for 10 min at 4°C and the supernatant discarded. The pellet fraction was resuspended in 30 mL of denaturing lysis buffer (100 mM NaH₂PO₄, 6 M guanidine-HCl, 10 mM Tris-HCl, pH 8.0) and incubated at room temperature (RT) for 60 min with constant stirring. The suspension was then centrifuged at 10,000 $\times$ g for 15 min at 4°C and the supernatant recovered was applied to 5 mL of Ni-NTA agarose resin that was pre-equilibrated in the denaturing lysis buffer. The slurry was incubated at RT for 1 h and then transferred to a Polyprep chromatography column (Bio-Rad). After collecting the flow-through fraction, unbound proteins were washed off the column beads using a series of wash buffers (buffer 1: 100 mM NaH₂PO₄, 6 M guanidine-HCl, 20 mM imidazole, 10 mM Tris-HCl, pH 8.0; buffer 2: 100 mM NaH₂PO₄, 8 M urea, 10 mM Tris-HCl, pH 8.0; buffer 3: 100 mM NaH₂PO₄, 8 M urea, 10 mM Tris-HCl, pH 6.3). Bound ZmpC-his₆ was eluted off the column using elution buffer (100 mM NaH₂PO₄, 8 M urea, 250 mM imidazole, 10 mM Tris-HCl, pH 6.3). The eluted ZmpC-his₆ was then dialyzed against 10 mM Tris-HCl, pH 8.0 in a 7000 MWCO dialysis cassette (Thermo Scientific) for 48 h, with replacement of the dialysis buffer every 12 h. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Scientific).

2.5. Biotinylation experiments and western blotting

Biotinylation experiments were performed as described previously [24]. Briefly, differentiated HCLE cells were exposed to 200 pmole of SP168 and TIGR4 rZmpC, diluted in DMEM/F12 medium, or DMEM/F12 medium alone as control, for 4 h at 37°C/5% CO₂. Immediately following exposure to rZmpC, the culture supernatants were harvested to assess the amount of cleaved MUC16 ectodomain. Then, biotinylation of cell surface proteins was carried out using the PinPoint Cell Surface Protein Isolation kit (Thermo Scientific). The amount of biotinylated MUC16 remaining on the surface of epithelial cells post treatment with rZmpC was analyzed by SDS-agarose gel electrophoresis and western blotting as described previously [22]. Detection of MUC16 using western blotting was done using the M11 antibody [25], which recognizes the MUC16 ectodomain and with HRP-tagged goat anti-mouse IgG₁ (Santa Cruz Biotechnology, sc-2969) as the secondary antibody. Western blots were developed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Band intensities corresponding to the MUC16 ectodomain on blots were measured using the Kodak dS 1D digital science software, version 2.02 (Kodak).

3. Results

3.1. The *zmpC* gene of strain SP168 has two stretches of DNA deleted near the 5' end of the coding region

Previously, we reported that the *zmpC* gene corresponding to strain SP168 is smaller than the one encountered in an *S. pneumoniae* strain belonging to serotype 11A [22]. In contrast to *zmpC* of *S. pneumoniae* serotype 11A and strain TIGR4, both of which are 5571 bp long and are near identical, our sequencing results revealed that the SP168 *zmpC* is comprised of 4848 bp. Further comparative analyses to all known *S. pneumoniae zmpC* sequences deposited in the GenBank database reveals in-frame deletions of two stretches of DNA, one 288 bp (96 aa) long and the other 432 bp (144 aa) long, near the 5' end of the coding region of *zmpC* in strain SP168 (Figure 1). Additionally, a small 3 bp in-frame deletion, resulting in the elimination of an alanine residue, is observed towards the middle segment of the gene. The translated amino acid sequence of SP168 ZmpC reveals an overall identity of 86% to the other known ZmpC sequences (Supplementary Figure 1). Upon closer examination and comparisons of the primary structures of the truncated SP168 ZmpC, we find the presence of several putative domains, which are also found across its homologues from other pneumococcal strains. These include 1) the YSIRK gram-positive signal peptide (protein family or pfam 04650), which is characteristic of several surface proteins found in *Streptococcus* and *Staphylococcus*, 2) a G5 domain (pfam 07501), which is found in a variety of extracellular proteins including proteases that cleave human IgA, and 3) peptidase_M26_N (pfam 05342) and peptidase_M26_C (pfam 07580) domains that are typical N and C termini signatures, respectively, found in IgA proteases of gram-positive bacteria (Figure 1). The truncated ZmpC also retains the highly conserved zinc-binding HEXXH motif, which is typical of most metalloproteinases (Supplementary Figure 1). Surprisingly, the LPXTG motif, which is a hallmark of surface proteins of gram-positive bacteria and is present near the N-terminus of all other known pneumococcal zinc metalloproteinases, is absent in the truncated SP168 ZmpC. As a reference for comparing the SP168 ZmpC in subsequent studies, we chose the ZmpC homologue in *S. pneumoniae* serotype 4 (strain TIGR4), which has been well-documented as a virulence factor [26, 27] and is near identical to all of the other known ZmpC sequences.

3.2. Recombinant ZmpC corresponding to strains SP168 and TIGR4 are expressed as functional proteins

Earlier it was reported that growth culture filtrates derived from ZmpC-expressing strains of *S. pneumoniae* are capable of cleaving the ectodomain of MUC16 from human corneal, conjunctival, and tracheal-bronchial epithelial cells [22]. It was also noted that the growth culture filtrate derived from strain SP168 induced MUC16 ectodomain shedding to a greater extent than that induced by a growth culture filtrate from another ZmpC-expressing strain, *S. pneumoniae* serotype 11A [22]. A similar result was observed when the filtered growth culture supernatants corresponding to strains SP168 and TIGR4 were compared for their MUC16 ectodomain shedding abilities (Figure 1C). However, one could argue that the differences in MUC16 cleaving abilities observed could be a result of differential ZmpC expression levels and/or variance in the amount of ZmpC that is secreted into the growth culture medium by the nonencapsulated strain SP168 as compared to the encapsulated serotype 11A. Moreover, the growth culture filtrate, which also contains other proteins, proteases, and metabolic waste material, may not represent the best medium for assessing the true MUC16 sheddase potential of ZmpC. To address these concerns and to better characterize the truncated SP168 ZmpC, we chose to express and purify a recombinant version of the SP168 ZmpC and compare it alongside a recombinant TIGR4 ZmpC. We cloned the SP168 and TIGR4 *zmpC* coding sequences into the pRham-C-his-kan vector, which results in a C-terminal polyhistidine (his₆) fusion with the corresponding proteins. The reason for choosing a C-terminal his₆ fusion is because the N-terminus of ZmpC harbors signal peptide and propeptide motifs, which is required for release of the mature ZmpC protease, and fusing a polyhistidine tag to that end would result in loss of the fused tag. After determining the optimal expression conditions, SP168 and TIGR4 rZmpC were purified using Ni-NTA affinity chromatography under denaturing-renaturing conditions. Differences in the molecular weights between the SP168 and TIGR4 rZmpC were reflected in their different migration patterns upon separation by SDS-PAGE (Figure 2A). Both recombinant ZmpC (rZmpC) forms were functional as assessed by their abilities to cleave the ectodomain of MUC16 from HCLE cells (Figure 2B).

3.3. SP168 ZmpC induces more MUC16 ectodomain cleavage than its TIGR4 homologue

To quantify the amount of residual MUC16 on the epithelial cell surface post cleavage by both rZmpC forms, we performed a biotinylation experiment on HCLE epithelial cells that were exposed to equimolar amounts of SP168 and TIGR4 rZmpC. Prior to proceeding with the biotinylation assay, the cell culture supernatants were harvested to examine the amounts of cleaved MUC16 protein. Results from the western blot analysis clearly show that SP168 ZmpC induces more cleavage of the MUC16 ectodomain from HCLE cells in comparison to its TIGR4 counterpart (Figure 3 A, B). Furthermore, analyses of biotinylated cell surface proteins reveals that the abundance of MUC16 on the surface of HCLE cells was reduced by ~90% and ~40% in the SP168 rZmpC- and TIGR4 rZmpC-exposed conditions, respectively (Figure 3 C, D). Similar results were observed in human conjunctival and tracheal-bronchial epithelial cells (data not shown). Altogether, results from the biotinylation experiments indicate that the SP168 ZmpC is more efficient at cleaving the MUC16 ectodomain than the TIGR4 ZmpC.

4. Discussion

Zinc metalloproteinases expressed by pneumococci have been implicated as virulence factors that contribute to the establishment and progression of pneumococcal disease. To date, four such proteinases have been identified in *S. pneumoniae*, and these include the products of the *iga*, *zmpB*, *zmpC*, and *zmpD* genes. The distribution of these metalloproteinase-encoding genes across pneumococcal strains is highly variable. For

instance, a scan of the published genomes of *Streptococcus pneumoniae* for the presence of *iga*, *zmpB*, *zmpC*, and *zmpD* indicates that strain G54 (accession: CP001015.1) harbors all four genes, strain TIGR4 (accession: AE005672.3) possesses three genes (*iga*, *zmpB*, and *zmpC*), and strain R6 (accession: AE007317.1) contains only two genes (*iga* and *zmpB*). This irregular distribution has also been confirmed by Camilli *et al.* [28], who reported that *iga* and *zmpB* were found in all of the 218 pneumococcal isolates that were screened for the presence of the metalloproteinase-encoding genes, while *zmpC* and *zmpD* could be detected in only a fraction of those isolates. In terms of demonstrating substrate specificity and assigning biological function, the IgA proteinase has been the most extensively studied. Its role in cleaving human immunoglobulin IgA1 in the hinge region [29] and promoting pneumococcal adherence and colonization of mucosal surfaces have been well documented [30]. The next-best-studied metalloproteinase appears to be ZmpC, which was recently shown to 1) cleave the human matrix metalloproteinase MMP-9 and contribute to virulence in experimental pneumonia in mice [26], 2) induce ectodomain shedding of the proteoglycan syndecan-1 in mammary epithelial cells [27], and 3) trigger cleavage of the membrane mucin MUC16 from human ocular surface and tracheal-bronchial epithelial cells [22]. Although the target of the ZmpB metalloproteinase has not yet been identified, its role in modulating inflammation and contributing to virulence in murine models of infection has been demonstrated [31, 32]. With regard to the ZmpD metalloproteinase, nothing is known about its substrate or function.

This manuscript describes the identification and initial characterization of a variant form of *zmpC* that we discovered in a nontypeable, epidemic conjunctivitis-causing strain of *S. pneumoniae* (strain SP168). The variant form is unique in that it is truncated and shorter than all known *zmpC* sequences by 723 bp. At first, this observation did not seem entirely unexpected since metalloproteinase-encoding genes are known to exhibit polymorphisms [32, 33] and have been grouped within a streptococcal gene pool that displays widespread sequence variability [26, 34]. This is especially evident in the case of the *zmpB*, *iga*, and *zmpD* genes. At the amino acid sequence level, the ZmpB and IgA proteinases from strains TIGR4, G54, and R6 exhibit 49–73% and 63–87% identity, respectively, among their homologues [26, 31]. *zmpD*, which is considered to be a duplicated gene residing in the *iga* locus [26, 28], is present only in strain G54 and shares a meager 26–27% identity at the amino acid level with the IgA proteinase from strains TIGR4, G54, and R6. In contrast to ZmpB, IgA, and ZmpD, the ZmpC metalloproteinase of strains TIGR4, G54, and AP200 (a clinical isolate belonging to serotype 11A, genome accession: CP002121) [35] appears to be relatively better conserved displaying an overall 99% identity. However, our discovery of a truncated form of ZmpC from *S. pneumoniae* strain SP168 represents a departure from all known ZmpC homologues in that 1) it exhibits an overall ~86% identity with its otherwise well-conserved counterparts from strains TIGR4, G54, and AP200 (Supplementary Figure 1) and 2) it lacks the characteristic cell wall-anchoring LPXTG motif, which is a signature of all known pneumococcal zinc metalloproteinases [11] as well as of other gram-positive surface proteins and is required for recognition and cleavage by sortases [36, 37]. The importance of the atypical, N-terminally located LPXTG motif of pneumococcal zinc metalloproteinases was elegantly demonstrated by Bender M. H. and Weiser J. N. [38] using the IgA proteinase as a model. In the case of IgA, deletion of the N-terminal region, which includes the LPXTG motif, was shown to cause a surface localization defect and an altered function of the enzyme. In contrast to the full length enzyme, which remains completely cell surface-associated [38, 39], the mutant IgA proteinase lacking the N-terminal region was found to be entirely secreted into the supernatant [38]. However, considering that both the full length TIGR4 ZmpC and the truncated SP168 ZmpC are proteinases secreted into the supernatant, the exact role of the LPXTG sequence in anchoring and/or secretion of ZmpC, remains to be determined. Aside from the deletions encountered within the N-terminus of the SP168 ZmpC, the remaining sequence was found to retain all other essential domains,

including the zinc-binding HEXXH motif, shared by its homologues from strains TIGR4, G54, and AP200 (Supplementary Figure 1).

Using recombinant versions, we compared the MUC16 ectodomain-cleaving ability of the truncated SP168 ZmpC alongside its full length counterpart from strain TIGR4. To account for their differences in molecular weights, equimolar amounts of both recombinant proteinases were used in *in vitro* assays. The biotinylation assay performed to assess the amount of MUC16 released as well as that remaining on the epithelial surface following cleavage by the SP168 and TIGR4 rZmpC revealed that, for equimolar amounts of both rZmpC used, MUC16 ectodomain cleavage was more pronounced in the SP168 rZmpC-exposed condition. Furthermore, the amount of MUC16 protein remaining on the surface of HCLE cells was reduced by close to 90% in the SP168 rZmpC-exposed condition as opposed to the ~40% reduction observed in the TIGR4 rZmpC-exposed condition. Clearly, these results indicate that the truncated *zmpC* gene in strain SP168 encodes a proteinase that is capable of inducing more MUC16 ectodomain cleavage, which is somewhat surprising, since insertions/deletions/point mutations within genes are typically associated with loss of function or reduced function of the translated gene products. The lack of a crystal structure for ZmpC, or for any of the remaining three metalloproteinases, precludes performing homology modeling/protein threading studies to make structure-based predictions and comment on the enhanced activity of the truncated ZmpC. Perhaps the N-terminal deletions within the SP168 ZmpC result in a smaller metalloproteinase that has better substrate (MUC16) accessibility and/or the active site within the truncated ZmpC provides a better pocket for binding of the MUC16 substrate.

In conclusion, findings from this study suggest that the nontypeable, epidemic conjunctivitis-causing strain of *Streptococcus pneumoniae* (strain SP168) has acquired a truncated variant of *zmpC* that encodes a more efficient metalloproteinase, as judged by its enhanced ability to cleave the membrane mucin MUC16 in *in vitro* cell culture-based assays. It also raises the possibility that *S. pneumoniae* strain SP168 may harbor other variant genes that encode virulence factors with enhanced functional profiles, which could provide the organism a selective advantage for triggering an infection such as epidemic conjunctivitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A variant form of a pneumococcal zinc metalloproteinase, ZmpC, has been identified.
- The truncated ZmpC lacks a canonical cell wall anchoring LPXTG motif.
- The truncated ZmpC was compared alongside its archetype from *S. pneumoniae* TIGR4.
- The variant ZmpC was found to induce more cleavage of the membrane mucin MUC16.

Figure 1. *S. pneumoniae* strain SP168 harbors a truncated *zmpC* gene

A) Two independent genomic DNA isolates (1 & 2) from *S. pneumoniae* strains SP168 and TIGR4 were used as templates in polymerase chain reactions along with *zmpC*-specific primers. Amplified products were resolved on a 1% agarose-TBE gel. DNA molecular weight markers are indicated in kilobase pairs on the left of the gel. **B)** Representation of the predicted domains within the SP168 and TIGR4 ZmpC proteins as determined by the Conserved Domain Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>). YS: YSIRK type signal peptide; G5: G5 domain; P_M26_N: M26 IgA1-specific Metallo-endopeptidase N-terminal region; G1: GLUG motif found in IgA1 endopeptidases; P_M26_C: M26 IgA1-specific Metallo-endopeptidase C-terminal region. Descriptions of these domains are included in the results section. The symbol Δ represents deletions within the SP168 ZmpC primary structure. Δ_{96} and Δ_{144} denote the deletion of 96 and 144 amino acids, respectively, while Δ_{ala} denotes the deletion of an alanine residue. **C)** A western blot, using the M11 antibody, on HCLE cell culture supernatants after a 4 h exposure to ZmpC-containing, growth culture filtrates derived from strains SP168 and TIGR4 to assess MUC16 ectodomain cleavage.

Figure 2. Recombinantly expressed SP168 and TIGR4 ZmpC are functional
 Recombinant SP168 and TIGR4 ZmpC (rZmpC) were purified under denaturing-renaturing conditions. Following purification, their function was assessed by exposing stratified HCLE cells to equimolar amounts (200 pmole) of both enzymes for 1 h and checking for cleavage of the MUC16 ectodomain. **A)** The expression and purity of the SP168 and TIGR4 rZmpC were monitored by running samples on a denaturing 7.5% polyacrylamide gel. The lanes labeled 'Uninduced' and 'Induced' represent fractions of *E. coli* clones harboring *zmpC*-expression constructs, before and after induction with L-rhamnose, respectively. Protein standards are indicated in kilodaltons (kDa) on the left of the gel. **B)** Western blot, using the

M11 antibody, showing that the purified SP168 and TIGR4 rZmpC cleave MUC16 from HCLE cells.

Figure 3. The surface abundance of MUC16 is drastically reduced in HCLE cells exposed to SP168 rZmpC

Biotinylation experiments were performed to estimate the amount of MUC16 remaining on the surface of HCLE cells following exposure of both cell types to equimolar amounts of SP168 and TIGR4 rZmpC. Western blots, using the M11 antibody, of released and residual fractions, along with corresponding quantitative analyses in HCLE (A-D). The MUC16 band intensities observed on the blots were within range of a standard curve (data not shown). The percentages of residual MUC16 in the SP168 and TIGR4 rZmpC-exposed conditions were calculated relative to the control condition, which was set to 100%. Error bars represent standard error of the mean. Statistical analyses were performed using the Student's t-test. * $P < 0.0001$.