Two putative zinc metalloproteases contribute to the virulence of *Clostridium perfringens* strains that cause avian necrotic enteritis

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Abstract. Two putative zinc metalloproteases encoded by *Clostridium perfringens* have been implicated in the pathogenesis of necrotic enteritis, an economically significant poultry disease that is caused by this anaerobic bacterium. These proteases have ~64% amino acid identity and are encoded by the *zmpA* and *zmpB* genes. We screened 83 *C. perfringens* isolates by PCR for the presence of these genes. The first gene, *zmpB*, is chromosomally located and was present in all screened strains of *C. perfringens*, regardless of their origin and virulence. The second gene, *zmpA*, is plasmid-borne and was only found in isolates derived from chickens with necrotic enteritis. We describe the generation of insertionally inactivated mutants of both *zmpA* and *zmpB* in a virulent *C. perfringens* isolate. For each mutant, a significant ($p < 0.001$) reduction in virulence was observed in a chicken necrotic enteritis disease model. Examples of each mutant strain were characterized by whole genome sequencing, which showed that there were a few off-site mutations with the potential to affect the virulence of these strains. To confirm the importance of these genes, independently derived *zmpA* and *zmpB* mutants were constructed in different virulent *C. perfringens* isolates and shown to have reduced virulence in the experimental disease induction model. A *zmpA*–*zmpB* double mutant also was generated and shown to have significantly reduced virulence, to the same extent as the respective single mutants. Our results provide evidence that both putative zinc metalloproteases play an important role in disease pathogenesis.

Key words: *Clostridium perfringens*; metalloproteases; mucin; necrotic enteritis, poultry.

Introduction

Necrotic enteritis imposes a heavy economic burden on poultry producers and has been estimated to cost the world poultry industry U.S.\$6 billion a year.³⁹ The impact of necrotic enteritis has led to a surge in research on this disease⁴ and on the causative bacterium, *Clostridium perfringens* type G.³¹

The pore-forming toxin, NetB, is recognized as a major virulence factor, but there is still much to be learned about the pathogenesis of this complex, multifactorial disease.^{12,30} Few bacterial factors, other than NetB, have been shown to contribute to disease. A putative fimbrial operon has been shown to have a role in the adherence, colonization, and necrotic enteritis pathogenesis of *C. perfringens*.^{40,41} It has also been suggested that 2 zinc metalloproteases may have some role in the pathogenesis of necrotic enteritis.^{15,19}

These 2 putative proteases belong to a family of M60-like/PF13402 domain-containing proteins²⁵ that carry a classical HEXXH gluzincin-like motif that is responsible for zinc-binding and acts as the catalytic core.¹⁰ The proteins are usually secreted and are generally produced by bacteria that reside on or in the mucosal surfaces of their animal hosts. The proteins often also have accessory domains that have binding specificity for eukaryotic extracellular matrix molecules,

specifically mucin, the primary constituent glycoprotein of mucosa.^{23–26,28} These M60-like proteases have been shown to degrade mucin and have been implicated in the disease processes of pathogens, especially enteric pathogens.^{23,24,28} Of the 2 putative zinc metalloproteases of this family implicated in necrotic enteritis, one, designated ZmpB, has been shown to be mucolytic.²⁶

The ~190 kDa zinc metalloproteases from *C. perfringens* have been studied previously in the context of necrotic enteritis,^{15–17} and it was shown that there was an immune response to ZmpB after chickens were exposed to virulent *C. perfringens* strains.¹⁵ In subsequent vaccination trials, immune

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responses to this antigen were shown to be partially protective, and a role for the protease in disease was postulated.^{16,17} However, induction of a protective immune response does not necessarily indicate involvement in pathogenesis; for example, alpha-toxin can induce a degree of immune protection, but there is no evidence that it is involved in the pathogenesis of necrotic enteritis.⁶

Little relevant *C. perfringens* genomic data were available at the time of these earlier studies; therefore, it was not apparent that the virulent *C. perfringens* isolates generally carried 2 homologs of the protease, as has subsequently been shown for other virulent necrotic enteritis-causing strains of *C. perfringens*.²⁰ The *zmpB* gene is located on the chromosome, and a homologous but distinct gene, referred to here as *zmpA*, is located within a plasmid-encoded region, NELoc-1.¹⁹ This conserved plasmid region also encodes the NetB toxin. It was not clear from these previous studies which zinc metalloprotease (they have 64% amino sequence identity) may be involved in the disease process, or if both (or neither) proteins have a role in disease.^{15,19}

We report herein the prevalence of both *zmpA* and *zmpB* in *C. perfringens* isolates from different sources. We constructed several *zmpA* and *zmpB* mutants as well as a double mutant in necrotic enteritis-causing *C. perfringens* type G strains and determined their virulence in a chicken necrotic enteritis disease induction model. Our results showed that both putative zinc metalloproteases play an important role in disease pathogenesis.

Materials and methods

Bacterial strains and growth conditions

The growth conditions of strains used in our study (Table 1) were identical to those described previously,⁴¹ except that the brain–heart infusion (BHI) medium did not contain soluble starch. Growth curves were generated by inoculating 20 mL of BHI broth with 200 μ L of an overnight culture and growth was monitored over 8 h, with 1-mL aliquots being taken every 2 h and the optical density at 600 nm determined. Growth curves were performed in triplicate.

PCR for detection of *zmpA* and *zmpB* and for Sanger sequencing

Screening for *zmpB* and *zmpA* used primer combinations 1 and 2, respectively (Supplementary Table 1). PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 20 s, 50°C for 20 s, and 75°C for 1 min per kb of product size. The templates for PCR were generated from supernatants of boiled cell preparations. PCR primer combinations 3–9 were used for suicide and TargeTron vector construction or analysis, described below, and combinations 10–14 were used to produce amplicons for Sanger sequencing (Supplementary Table 1).

Targeted mutagenesis of *zmpA* and *zmpB*

The pALK1 vector was used as before^{11,41} for the generation of the suicide vector pBWP4. A 2,156-bp *SpeI* fragment of the region downstream of *zmpA* was generated by primer combination 3 (Supplementary Table 1), using EHE-NE18 genomic DNA as a template, and cloned into the *SpeI* site of pALK1, resulting in pBWP3. Similarly, a 2,535-bp *BclI/NheI* digested fragment of the region upstream of *zmpA*, generated by primer combination 4, was cloned into the *BamHI/NheI* sites of pBWP3, resulting in the final suicide vector, pBWP4. Successful mutagenesis using this construct resulted in 4,530 bp of *zmpA* being replaced with a 973-bp fragment containing the *catP* cassette, which encodes chloramphenicol and thiamphenicol resistance.³⁴ After electroporation³² of pBWP4 into the target strain, colonies that grew on tryptose sulfite cycloserine (TSC) agar with thiamphenicol were selected for preliminary confirmation via PCR. Primer combination 5, which was designed to prime outside the genomic regions cloned into pBWP4, was used to show the expected reduction of the size of the amplified product from 9,456 bp to 5,917 bp in the mutants. Primer combinations 6 and 7 were then used to show that the *catP* cassette had inserted within *zmpA*.

The insertionally inactivated *zmpB* mutants were generated using TargeTron technology as before.^{5,41} The *plc*-targeted vector pJIR3562 was retargeted to *zmpB* using a *BsrGI/HindIII* fragment of the product of primer combination 8 cloned into a *BsrGI/HindIII* digestion of pJIR3562, resulting in pBWP5. The resultant plasmid was introduced into the target strain by electroporation and the transformants plated onto TSC agar with thiamphenicol overnight and plated onto TSC agar with erythromycin; erythromycin resistance becoming active only upon genomic insertion. Colonies were screened using primer combination 9 for successful insertion of the erythromycin resistance gene into *zmpB*. The strain was cured of pBWP5 via a single passage overnight in BHI broth.

High-throughput pyrosequencing

Strains EHE-NE18*zmpB* and EHE-NE18*zmpA1* were subjected to whole genome sequencing (454 FLX genome sequencer; Roche, Hawthorn, Victoria, Australia) using a 3-kb paired-end protocol with Titanium chemistry, with average depths of coverage for the sequencing of 31.6 $\times$ and 27.8 $\times$, respectively.

The resultant sequences from the 2 mutants were compared to the wild-type EHE-NE18 genome (gsMapper of the Newbler package v.2.6; Roche). Predicted differences were collated and then filtered based upon the parameters that any variation must be present in ≥ 10 reads, and that the variation must be supported by $> 75\%$ of reads covering that sequence. The sequence differences that were found to be either non-

Table 1. Prevalence of *zmpA* and *zmpB* among *Clostridium perfringens* isolates.

Strain	Source	Reference	Strain	Source	Reference
NAG-NE1	Australia, chicken with NE	33	CN1797	Type C, 1947	BWC
EHE-NE3	Australia, chicken with NE	33	JIR4036 D13	Belgium, cattle	41
EHE-NE4	Australia, chicken with NE	33	CN2065	UK, sheep	BWC
EHE-NE5	Australia, chicken with NE	33	CN3706	UK, cattle	BWC
EHE-NE7	Australia, chicken with NE	33	CN3686	UK, pig	BWC
EHE-NE9	Australia, chicken with NE	33	CN394	UK, pig	BWC
EHE-NE13	Australia, chicken with NE	33	JIR100	Australia, pig, 1982	18
EHE-NE14	Australia, chicken with NE	33	44025C03	Belgium, bovine	41
EHE-NE15	Australia, chicken with NE	33	#77885	Country unknown, goat	41
EHE-NE16	Australia, chicken with NE	33	SM101	Human food poisoning type F	42
EHE-NE17	Australia, chicken with NE	33	CW459	USA, pig	29
EHE-NE18	Australia, chicken with NE	33	Strain 13	Human gas gangrene	22
EHE-NE20	Australia, chicken with NE	11	PA1	Australia, healthy chicken	40
EHE-NE21	Australia, chicken with NE	11	PA2	Australia, healthy chicken	41
EHE-NE22	Australia, chicken with NE	11	PA3	Australia, healthy chicken	41
NAG-NE23	Australia, chicken with NE	33	PA4	Australia, healthy chicken	41
NAG-NE24	Australia, chicken with NE	33	PA5	Australia, healthy chicken	41
NAG-NE25	Australia, chicken with NE	13	PBS1	Australia, healthy chicken	41
UNK-NE30	Australia, chicken with NE	13	PBS2	Australia, healthy chicken	41
NAG-NE31	Australia, chicken with NE	13	PBS3	Australia, healthy chicken	41
NAG-NE32	Australia, chicken with NE	13	PBS4	Australia, healthy chicken	41
BER-NE33	Australia, chicken with NE	13	PBS5	Australia, healthy chicken	40
SOM-NE34	Australia, chicken with NE	13	PBD1	Australia, healthy chicken	40
SOM-NE35	Australia, chicken with NE	13	PBD2	Australia, healthy chicken	41

(continued)

Table 1. (continued)

Strain	Source	<i>netB</i>	<i>zmpB</i>	<i>zmpA</i>	Reference	Strain	Source	<i>netB</i>	<i>zmpB</i>	<i>zmpA</i>	Reference
WER-NE36	Australia, chicken with NE	+	+	+	35	PBD3	Australia, healthy chicken	-	+	-	41
TAM-NE42	Australia, chicken with NE	-	+	+	Current study	PBD4	Australia, healthy chicken	-	+	-	41
JGS4102	USA, turkey with NE	+	+	+	27	PBD5	Australia, healthy chicken	-	+	-	41
JGS4120	USA, chicken with NE	-	+	-	7	PC1	Australia, healthy chicken	-	+	-	41
JGS4122	USA, chicken with NE	+	+	+	Songer,* pers. com., 2006	PC2	Australia, healthy chicken	-	+	-	41
JGS4125	USA, chicken with NE	+	+	+	Songer,* pers. com., 2006	PC3	Australia, healthy chicken	-	+	-	41
200302.1.1.Ba	Denmark, chicken with NE	+	+	+	13	PC4	Australia, healthy chicken	-	+	-	41
99.63206.34	Denmark, chicken with NE	+	+	+	13	PC5	Australia, healthy chicken	-	+	-	40
Strain 48	Belgium, chicken with NE	-	+	-	9	M1	Australia, healthy chicken	-	+	-	40
Strain 56	Belgium, chicken with NE	+	+	+	9	M2	Australia, healthy chicken	-	+	-	41
Strain 67	Belgium, chicken with NE	+	+	+	9	M3	Australia, healthy chicken	-	+	-	41
W1319	Australia, chicken with NE	-	+	+	33	M4	Australia, healthy chicken	-	+	-	41
ATCC 13124	Human gas gangrene	-	+	-	8	M5	Australia, healthy chicken	-	+	-	41
CNI884	Type B	NT	+	-	BWC	LP1	Australia, healthy chicken	-	+	-	40
CN2109	Gas gangrene, type C	NT	+	-	BWC	LP2	Australia, healthy chicken	-	+	-	41
CN462	Goat, type D, 1942	NT	+	-	BWC	LP3	Australia, healthy chicken	-	+	-	41
ATCC27324	Japan, type e	NT	+	-	36	LP4	Australia, healthy chicken	-	+	-	41
ATCC10543	Type A, 1948	NT	+	-	3						

BWC = Burroughs Wellcome Collection; NE = necrotic enteritis; NT = not tested.

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synonymous, or had the potential to disrupt promoter sequences, were confirmed via Sanger sequencing.

Necrotic enteritis disease induction model

A necrotic enteritis induction protocol was used as described previously.¹⁴ Briefly, unvaccinated 1-d-old Ross 308 birds were fed an antibiotic-free commercial starter feed. Animals were housed in 1.5 × 1.5-m pens with 3-cm deep wood shavings. On day 15, a wheat-based feed containing 50% (w/w) fishmeal was introduced. Feed mixed with a *C. perfringens* culture was fed to the birds on days 21 and 22, twice on each day. Birds were euthanized on day 23, and the mucosal surface of the intestines was examined for lesions of necrotic enteritis. Disease severity, on a scale of 0–6, was scored as described previously.¹¹ Control animals had the same feed mixed with sterile broth. The assay was done in duplicate with 10–12 birds per group.

Ethics statement

All animal experiments were undertaken with the approval and oversight of the CSIRO Australian Animal Health Laboratory Animal Ethics Committee (Approval AEC 1517) and in accordance with national (Australian Code for the Care and Use of Animals for Scientific Purposes, 2013) and state (Victorian Prevention of Cruelty to Animals Act 1986 and Part 4 of the Prevention of Cruelty to Animals Regulations 2008) legislation.

Statistical analysis

All statistical analysis was undertaken using Prism v.5.02 (GraphPad Software, San Diego, CA). The nonparametric Kruskal–Wallis test with Dunn post-test was used to statistically compare disease outcome results across all groups.

Results

Prevalence of *zmpA* and *zmpB*

We screened 83 isolates of *C. perfringens* from Australia, United States, Belgium, Denmark, Japan, and the UK by PCR for the presence of *zmpA* and *zmpB*. Most of these strains were isolated from diseased or healthy chickens, many were NetB-positive, *C. perfringens* type G. Other strains included isolates from a variety of livestock species and humans, including representatives of each of the *C. perfringens* toxinotypes (Table 1). All strains were found to carry *zmpB*, regardless of their origin, whereas *zmpA* was found exclusively among strains isolated from birds suffering from necrotic enteritis (34 of 36 positive for *zmpA*).

The *zmpA* and *zmpB* single and double mutants display reduced virulence

Given that *zmpA* was specific to isolates from chickens suffering necrotic enteritis, we initially targeted this gene for

mutagenesis in the virulent isolate EHE-NE18. Two independently derived isogenic *zmpA* mutants (EHE-NE18*zmpA1* and EHE-NE18*zmpA2*) were generated by allelic exchange, replacing ~4.5 kb of *zmpA* with a *catP* cassette.⁴ These mutants were confirmed by PCR as described above.

To investigate if *zmpA* plays a role in virulence, the 2 independent mutants were tested for their ability to cause disease in a chicken necrotic enteritis induction model. In 2 independent trials, the wild-type strain caused disease in 68% of experimentally inoculated birds. By contrast, the isogenic *zmpA1* and *zmpA2* mutants caused disease in 30% and 23% of birds, respectively (Fig. 1A). The average disease score was 2.74 ± 1.74 (disease data presented as mean $\pm$ standard deviation) in wild-type infected birds, and 0.83 ± 1.37 and 0.52 ± 1.04 in birds infected with the *zmpA1* and *zmpA2* mutants, respectively. The difference in severity of disease between the wild-type strain and the isogenic mutants was statistically significant ($p < 0.001$).

Given that there was a significant reduction in virulence, but not complete abolition, we postulated that the *zmpB* gene may have provided partial complementation of the *zmpA* mutants. Therefore, a *zmpA–zmpB* double mutant was constructed in strain EHE-NE18. Because this process required a different antibiotic resistance marker, we utilized TargeTron technology (MilliporeSigma, Burlington, MA) to insertionally inactivate the *zmpB* gene with the erythromycin resistance gene, *ermB*. However, given the nature of the TargeTron mutagenesis system, in which thiamphenicol is used for selection of the carrier plasmid that is subsequently lost after mutagenesis, it was necessary for selection purposes to generate a *zmpB* mutant and then to use that strain for the subsequent generation of the *zmpA–zmpB* double mutant. The *zmpB* mutant (EHE-NE18*zmpB*) was generated by TargeTron mutagenesis and was then used to generate the double mutant, EHE-NE18*zmpA**zmpB*, by allelic exchange.

Both the *zmpB* mutant and its isogenic *zmpA–zmpB* double mutant were tested in the chicken disease model. The wild-type caused disease in 90% of infected animals with an average disease score of 3.15 ± 1.31 ; the *zmpB* mutant and its isogenic *zmpA–zmpB* double mutant caused disease in 19% (average disease score 0.43 ± 0.93) and 20% (average disease score 0.45 ± 0.94) of infected birds, respectively (Fig. 1B). There was a statistically significant ($p < 0.001$) reduction in disease incidence induced by both mutants, compared to the wild-type strain, indicating that the mutations reduced the virulence of the resultant strains.

Evidence that the phenotypes of the mutants were the result of the targeted mutations

We made numerous attempts to clone the wild-type *zmpA* and *zmpB* genes into the *E. coli–C. perfringens* shuttle vectors pJIR1457²¹ and pJIR751,² so that the mutations could be complemented. However, we were unable to generate full-length, mutation-free clones of either gene. This result was

Figure 1. Both *zmpA* and *zmpB* are required for virulence in EHE-NE18. **A.** The virulence of the wild-type EHE-NE18 strain and 2 independent *zmpA* mutants. **B.** The virulence of the EHE-NE18 wild-type, a *zmpB* mutant, and a *zmpA*-*zmpB* double mutant. The symbols represent the lesion severity for individual birds ($n=20$ – 23 birds for each group), and the dotted line represents the mean lesion severity of that group. Error bars represent standard error of the mean. Lesion severity is scored as: 0 = no gross lesions; 1 = thin or friable walls; 2 = focal necrosis or ulceration (1–5 foci); 3 = focal necrosis or ulceration (6–15 foci); 4 = focal necrosis or ulceration (≥ 16 foci); 5 = patches of necrosis 2–3 cm long; 6 = diffuse necrosis typical of field cases.¹¹ Medium control groups were inoculated with sterile broth only. Statistical analysis (Kruskal–Wallis with Dunn post-test) showed a significant difference ($p < 0.001$) between the positive control and other groups as denoted by an asterisk (*). Data represent combined results from 2 independent trials.

not entirely unexpected given that other researchers^{16,17} found that the *zmpB* gene was difficult to clone in its entirety. Accordingly, we used a different strategy to confirm that the inactivation of the *zmpA* and *zmpB* genes was responsible for the observed phenotypic changes in the mutants.

Growth assays of the wild-type and the *zmpB* and *zmpA*1 mutants were performed (Supplementary Fig. 1) to demonstrate that mutation of *zmpA* and/or *zmpB* did not alter the growth rate, and thereby negatively impact virulence. No statistically significant difference in growth rate was observed.

To confirm that the phenotypic changes resulted from the mutations, independent *zmpA* and *zmpB* mutants were constructed in different *C. perfringens* G strains. These strains were WER-NE36, another Australian virulent isolate, and strain 56, a virulent Belgian strain, both of which have the *zmpA* and *zmpB* genes. Mutants of *zmpA* were generated in both strains, and a *zmpB* mutant of strain 56 was obtained. Despite numerous attempts, no *zmpB* mutant could be generated in WER-NE36, for unknown reasons. These newly generated mutants were tested for virulence in the chicken disease model. The WER-NE36 wild-type caused disease in 80% of infected animals (average disease score 2.80 ± 1.67), whereas the isogenic *zmpA* mutant caused disease in only 29% of infected birds (average disease score 0.76 ± 1.30). Infection with the wild-type strain 56 resulted in disease in 85% of birds (average disease score 2.80 ± 1.44); the *zmpB* and *zmpA* mutants caused disease in 29% and 5% of birds (average disease scores 0.81 ± 1.25 and 0.09 ± 0.43 , respectively). All 3 mutants displayed significantly reduced ($p < 0.001$) virulence compared to their respective wild-type strains (Fig. 2).

In addition, the whole genomes of both the EHE-NE18*zmpB* and EHE-NE18*zmpA*1 mutants were sequenced

to enable comparison to the parent strain (GenBank BioProject PRJNA341531; BioSample SAMN05725818). The objective was to confirm the site of mutagenesis and to identify any secondary mutations that could potentially affect the virulence phenotype. From the draft assemblies of the genomes of the *zmpA* and *zmpB* mutants, putative genomic changes were identified and then further interrogated by a combination of manual inspection of the sequence data and additional Sanger sequencing.

In both mutant strains, 2 single-nucleotide polymorphisms (SNPs) were identified (Supplementary Table 2). In the *zmpB* mutant, apart from the ~ 1.8 -kb insertion of the *ermB* cassette within *zmpB*, both SNPs led to conserved amino acid changes. One mutation was in a gene predicted to encode a methyltransferase and changed a leucine residue to an isoleucine residue, and the second mutation was in a gene predicted to encode an alcohol dehydrogenase, changing a glutamate to an aspartate residue.

In the *zmpA*1 mutant, apart from the ~ 4.5 -kb deletion within *zmpA* and the insertion of the *catP* cassette,¹¹ there were 2 SNPs in open reading frames; one of which resulted in an alanine to serine substitution in a gene that encoded a predicted queuine tRNA-ribosyltransferase, a protein not likely to have any role in necrotic enteritis virulence, with no previous studies having identified it as a possible virulence factor for any clostridial diseases. The second SNP also resulted in an amino acid change from alanine to serine in a predicted sigma-54-dependent regulatory gene. This gene, which we will refer to by its *C. perfringens* strain 13 ortholog, *cpe2358*, appears to encode an atypical response regulator, based on sequence similarity and BLAST analysis.¹ The analysis of the predicted protein showed that it had a

The occurrence in 2 independently derived protease mutants of 2 off-target mutations that potentially affected the same gene, *cpe2358*, was unexpected. Either what was observed was an improbable chance event, which seems unlikely, or the inactivation of either of these proteases exerted a selective pressure to mutate or change the expression or activity of *cpe2358*. To decipher which was the more likely scenario, we amplified, using several overlapping PCRs (Supplementary Table 1), the upstream region and coding region of *cpe2358* and subjected the amplicons to

Because it was not possible to clone either *zmpA* or *zmpB* as intact expressed genes suitable for complementation, we used 2 different approaches to show that the reduction in virulence in the various mutants was the result of the targeted

mutation. First, mutants were generated in different strains and examined for their effect on virulence. Irrespective of the parent strain, all of the *zmp* mutations, in 7 independently derived mutants, had the same effect—attenuation of the ability to cause avian necrotic enteritis. Second, genomic sequencing confirmed the presence of the expected mutations, as well as screening for off-target effects. Of the 5 secondary mutations that were in putative genes or promoter regions (4 SNPs and 1 additional insertion) in the 2 mutants that were sequenced, 2 were conserved amino acid changes. Of the remaining mutations, one was not considered likely to influence virulence, being an alanine to serine substitution in a putative queuine tRNA-ribosyltransferase. Although it cannot be absolutely ruled out that these mutations may be influencing the virulence phenotype of these mutants, it seems unlikely, given that both sequenced protease mutants had a similar level of virulence yet contained different SNPs.

The *zmpA* mutation in EHE-NE18 significantly reduced virulence but did not totally abolish the ability to cause disease. The presence of a SNP in *cpe2358* in EHE-NE18*zmpA1* was deemed unlikely to be affecting virulence given that EHE-NE18*zmpA2* lacked any such mutations in *cpe2358* but caused a near identical level of disease. We hypothesized that *zmpB* may partially complement the *zmpA* mutant. Therefore, a *zmpA*–*zmpB* double mutant was generated and tested; however, it had the same level of virulence attenuation as the *zmpA* mutant. Taken on its own, this result would suggest that there was no partial complementation by *ZmpB* and that this protein did not play a role in disease. However, the EHE-NE18*zmpB* mutant was also attenuated for virulence, to an extent comparable to the *zmpA* mutants and the double mutant. This result was confirmed by the construction and analysis of a *zmpB* mutant in strain 56. The only source of doubt raised about the potential role of *ZmpB* in virulence was the unintended TargeTron insertion upstream of *cpe2358* in the genome sequence of EHE-NE18*zmpB*. Also supporting the conclusion that *ZmpB* plays a role in disease were the virulence data generated for the strain 56 *zmpB* mutant that lacked any mutation in *cpe2358* or its upstream region and produced disease levels equivalent to that of EHE-NE18*zmpB*.

Although it has been shown that *ZmpB* can degrade mucin, its exact affinity, in the context of the different types of mucin within the chicken intestinal mucosa, remains unknown, as does the precise target(s) of *ZmpA*. If the 2 proteins have different targets, we may have expected to see a cumulative impact upon virulence in the double mutant. Given the 64% amino acid sequence identity of *ZmpA* and *ZmpB*, it is possible that they have similar substrates and may be acting in a similar manner during necrotic enteritis. If so, it is difficult to understand why mutation of either gene would attenuate virulence so significantly and why only *zmpA* is preferentially associated with strains of *C. perfringens* isolated from diseased chickens. Perhaps the 2 putative proteases are involved in the same virulence-enhancing catabolic process, and loss of either protease is sufficient to halt

the process, resulting in the observed reduction in virulence. What is clear from our experimental data is that both *ZmpA* and *ZmpB* are required for full virulence in the avian disease model, and further studies are required to determine the mode of action of these putative proteases and their precise role in the disease process.

We demonstrated that the zinc metalloprotease-encoding genes *zmpA* and *zmpB* are involved in necrotic enteritis in chickens. Together with the identification of a putative fimbrial operon that is required for virulence,⁴¹ it is clear that the pathogenesis of necrotic enteritis is a complex process that involves much more than the ability to produce the essential extracellular toxin, NetB. It is important that future studies focus on how these virulence factors, and potentially others that remain to be discovered, interact with each other and with the host to enable *C. perfringens* to cause avian necrotic enteritis.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with regards to the research, authorship, and/or publication of this article.

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Supplementary material

Supplementary material for this article is available online.

References

1. Altschul SF, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402.
2. Bannam TL, Rood JI. *Clostridium perfringens*-*Escherichia coli* shuttle vectors that carry single antibiotic resistance determinants. *Plasmid* 1993;29:233–235.
3. Boyd MJ, et al. The growth requirements of *Clostridium perfringens* (welchii) BP6K. *J Biol Chem* 1948;174:1013–1025.
4. Broom LJ. Necrotic enteritis; current knowledge and diet-related mitigation. *Worlds Poult Sci* 2017;73:281–292.
5. Chen Y, et al. Construction of an alpha toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. *Appl Environ Microbiol* 2005;71:7542–7547.
6. Cooper KK, et al. Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with *Clostridium perfringens*. *Vet Microbiol* 2009;133:92–97.

7. Cooper KK, Songer JG. Virulence of *Clostridium perfringens* in an experimental model of poultry necrotic enteritis. *Vet Microbiol* 2010;142:323–328.
8. Eastoe JE, Long JE. The effect of nisin on the growth of cells and spores of *Clostridium welchii* in gelatine. *J Appl Microbiol* 1959;22:1–7.
9. Gholamiandekhordi AR, et al. Molecular and phenotypic characterization of *Clostridium perfringens* isolates from poultry flocks with different disease status. *Vet Microbiol* 2006;113:143–152.
10. Hooper NM. Families of zinc metalloproteases. *FEBS Lett* 1994;354:1–6.
11. Keyburn AL, et al. Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. *Infect Immun* 2006;74:6496–6500.
12. Keyburn AL, et al. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *PLoS Pathog* 2008;4:e26.
13. Keyburn AL, et al. Association between avian necrotic enteritis and *Clostridium perfringens* strains expressing NetB toxin. *Vet Res* 2010;41:21.
14. Keyburn AL, et al. Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis. *Vet Res* 2013;44:54.
15. Kulkarni RR, et al. *Clostridium perfringens* antigens recognized by broiler chickens immune to necrotic enteritis. *Clin Vaccine Immunol* 2006;13:1358–1362.
16. Kulkarni RR, et al. Oral immunization of broiler chickens against necrotic enteritis with an attenuated *Salmonella* vaccine vector expressing *Clostridium perfringens* antigens. *Vaccine* 2008;26:4194–4203.
17. Kulkarni RR, et al. A live oral recombinant *Salmonella enterica* serovar Typhimurium vaccine expressing *Clostridium perfringens* antigens confers protection against necrotic enteritis in broiler chickens. *Clin Vaccine Immunol* 2009;17:205–214.
18. Abraham LJ, et al. Hybridization analysis of the class P tetracycline resistance determinant from the *Clostridium perfringens* R-plasmid, pCW3. *Plasmid* 1988;19:113–120.
19. Lepp D, et al. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. *PLoS One* 2010;5:e10795.
20. Lepp D, et al. Identification of accessory genome regions in poultry *Clostridium perfringens* isolates carrying the *netB* plasmid. *J Bacteriol* 2013;195:1152–1166.
21. Lyras D, Rood JI. Conjugative transfer of RP4-oriT shuttle vectors from *Escherichia coli* to *Clostridium perfringens*. *Plasmid* 1998;39:160–164.
22. Mahony DE, Moore TI. Stable L-forms of *Clostridium perfringens* and their growth on glass surfaces. *Can J Microbiol* 1976;22:953–959.
23. Martens EC, et al. Interactions of commensal and pathogenic microorganisms with the intestinal mucosal barrier. *Nat Rev Microbiol* 2018;16:457–470.
24. Belousov MV, et al. M60-like metalloprotease domain of the *Escherichia coli* YghJ protein forms amyloid fibrils. *PLoS One* 2018;13:e0191317.
25. Nakjang S, et al. A novel extracellular metalloprotease domain shared by animal host-associated mutualistic and pathogenic microbes. *PLoS One* 2012;7:e30287.
26. Noach I, et al. Recognition of protein-linked glycans as a determinant of peptidase activity. *Proc Natl Acad Sci U S A* 2017;114:E679–E688.
27. Paredes-Sabja D, et al. *Clostridium perfringens* tpeL is expressed during sporulation. *Microb Pathog* 2011;51:384–388.
28. Tapader R, et al. YghJ, the secreted metalloprotease of pathogenic *E. coli* induces hemorrhagic fluid accumulation in mouse ileal loop. *Microb Pathog* 2017;105:96–99.
29. Rood JI, et al. Isolation and characterization of multiply antibiotic-resistant *Clostridium perfringens* strains from porcine feces. *Antimicrob Agents Chemother* 1978;13:871–880.
30. Rood JI, et al. NetB and necrotic enteritis: the hole movable story. *Avian Pathol* 2016;45:295–301.
31. Rood JI, et al. Expansion of the *Clostridium perfringens* toxin-based typing scheme. *Anaerobe* 2018;53:5–10.
32. Scott PT, Rood JI. Electroporation-mediated transformation of lysostaphin-treated *Clostridium perfringens*. *Gene* 1989;82:327–333.
33. Sheedy SA, et al. Highly conserved alpha-toxin sequences of avian isolates of *Clostridium perfringens*. *J Clin Microbiol* 2004;42:1345–1347.
34. Sloan J, et al. Construction of a sequenced *Clostridium perfringens*-*Escherichia coli* shuttle plasmid. *Plasmid* 1992;27:207–219.
35. Stanley D, et al. Changes in the caecal microflora of chickens following *Clostridium perfringens* challenge to induce necrotic enteritis. *Vet Microbiol* 2012;159:155–162.
36. Stiles BG, Wilkins TD. *Clostridium perfringens* iota toxin: synergism between two proteins. *Toxicon* 1986;24:767–773.
37. Sridharan S, et al. The HPr proteins from the thermophile *Bacillus stearothermophilus* can form domain-swapped dimers. *J Mol Biol* 2005;346:919–931.
38. Taylor BL, Zhulin IB. PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* 1999;63:479–506.
39. Wade B, Keyburn AL. The true cost of necrotic enteritis. *World Poult* 2015;31:16–17.
40. Wade B, et al. Binding of *Clostridium perfringens* to collagen correlates with the ability to cause necrotic enteritis in chickens. *Vet Microbiol* 2015;180:299–303.
41. Wade B, et al. The adherent abilities of *Clostridium perfringens* strains are critical for the pathogenesis of avian necrotic enteritis. *Vet Microbiol* 2016;197:53–61.
42. Zhao Y, Melville SB. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *J Bacteriol* 1998;180:136–142.