

Highly Conserved Alpha-Toxin Sequences of Avian Isolates of *Clostridium perfringens*

Scott A. Sheedy,^{1,2} Aaron B. Ingham,¹ Julian I. Rood,² and Robert J. Moore^{1,2*}

Commonwealth Scientific and Industrial Research Organisation, Livestock Industries, Australian Animal Health Laboratory, Geelong 3220,¹ and Australian Research Council Centre for Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, Geelong 3800,² Australia

Received 12 August 2003/Returned for modification 13 September 2003/Accepted 4 November 2003

***Clostridium perfringens* causes necrotic enteritis in chickens, and alpha-toxin has been suggested to be a key virulence determinant. Analysis of the alpha-toxin of 25 chicken-derived *C. perfringens* strains demonstrated high homology to mammal-derived strains rather than to the only avian-derived *C. perfringens* alpha-toxin sequence reported previously.**

Clostridium perfringens is a widely distributed pathogen (6) commonly isolated from the environment and the gastrointestinal tract of birds and mammals (7, 24). *C. perfringens* isolates are classified into five types (A to E) according to the production of four major toxins (alpha, beta, epsilon, and iota) (11, 13). The alpha-toxin has been implicated in several diseases (18), including necrotic enteritis in chickens (3, 10). The alpha-toxin structural gene (*plc* or *cpa*) has been isolated from several strains of *C. perfringens* and characterized (4), and the encoded proteins were found to be highly conserved in all but one recently identified strain (8). This strain (SWCP) was isolated from a diseased swan. Justin et al. (8) found that the SWCP alpha-toxin had only 80% amino acid sequence identity to the other *C. perfringens* alpha-toxins and questioned if this difference in sequence was typical of all avian isolates. In this study, we examined the alpha-toxin sequences encoded by a range of isolates of *C. perfringens* derived from chickens to determine if the divergent SWCP alpha-toxin sequence is common in avian isolates.

The *C. perfringens* strains used in this study (Table 1) were isolated from chickens displaying clinical signs of necrotic enteritis (1). Genomic DNA was prepared as the template for PCR by boiling crude cells in water for 3 min. The PCR conditions and reaction concentrations were as described before (14). Two PCR products, together encompassing the complete *plc* gene, were amplified from each of the 25 strains and sequenced to determine the amino acid sequence of the encoded alpha-toxin (Fig. 1). In each isolate, the full-length sequence was predicted to have 398 amino acids. The toxins were all highly conserved in amino acid sequence (Fig. 2), and only five different alpha-toxin sequence types (I to V) were identified among the 25 isolates sampled from several different outbreaks of necrotic enteritis in different locations (Table 1). Each alpha-toxin gene was sequenced twice with independently generated templates to confirm that the changes were not due to sequencing or PCR errors. All the alpha-toxin

sequence types from the chicken isolates closely resembled that of the toxin from the human isolate, strain 13 (20), with greater than 98% identity, but differed considerably from the swan isolate (SWCP), with only 82 to 84% identity. The SWCP isolate has between 67 and 70 amino acid differences from the chicken isolates, and of all the changes in the SWCP sequence, only two amino acid changes are found in the field isolates reported here.

Sequence type I has only one amino acid difference from the strain 13 sequence and was the most common alpha-toxin found in the group sampled (Table 1). The threonine-to-alanine substitution is within the putative signal peptide sequence (21) and would not be present in the mature protein and therefore cannot affect the properties of the mature toxin (4). Alanine is also found in this position in the alpha-toxin signal sequence from strain NCTC 8237 (9). Sequence type II has two amino acid differences compared to the strain 13 sequence and includes the threonine-to-alanine change at position 13 and an isoleucine-to-valine substitution at position 373. Sequence type IV contains two amino acid changes, the threonine-to-alanine change (position 13) and a leucine-to-methionine alteration (position 54). The latter amino acid substitution is also seen in the alpha-toxin from *C. perfringens* strain 8-6 (19) and the phospholipase C from *Clostridium novyi* (22). The type V alpha-toxin sequence contains three amino acid substitutions compared to strain 13, the common threonine-to-alanine substitution at position 13, an aspartic acid-to-alanine change at position 202 (also found in the SWCP sequence), and an alanine-to-threonine substitution at position 205. The most distinct alpha-toxin sequence type seen in this study was type III. It contains six amino acid changes compared to the strain 13 alpha-toxin, including the isoleucine-to-valine substitution at position 373 and a methionine residue that replaces a lysine residue at position 54.

Overall, the amino acid differences detected in this study were minimal compared to the sequence differences observed between strains SWCP and 13. The differences that were found in the alpha-toxin sequences of the chicken isolates were all the result of single base substitutions and did not significantly alter the predicted physical properties of the encoded proteins (45.5 kDa, pI 5.58, and overall negative charge). Plating each of the

* Corresponding author. Mailing address: Commonwealth Scientific and Industrial Research Organisation Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria 3220, Australia. Phone: 61-3-52275000. Fax: 61-3-52275555. E-mail: Rob.Moore@csiro.au.

in mammalian isolates of *C. perfringens* but are significantly different from that of the SWCP isolate obtained from a diseased swan, the only bird-derived strain characterized previously. These results are encouraging for the development of diagnostic tests and vaccines for the control and treatment of *C. perfringens* infections of commercial chickens, as they signify that the vaccines and tests used for other *C. perfringens* infections may be able to be used in this host species.

We thank Pat Blackall (Queensland Department of Primary Industries), who supplied several strains of *C. perfringens*, Ambrosio Rubite and Peter Scott for facilitating access to poultry farms for sample collection, and Mark Ford (Commonwealth Scientific and Industrial Research Organisation, Livestock Industries) for animal handling and disease diagnosis.

The kind support of the Australian Rural Industries Research Development Corporation (RIRDC), through which this work was funded, is acknowledged.

REFERENCES

1. Al-Sheikhly, F., and R. B. Truscott. 1977. The pathology of necrotic enteritis of chickens following infusion of crude toxins of *Clostridium perfringens* into the duodenum. *Avian Dis.* **21**:241–255.
2. Awad, M. M., A. E. Bryant, D. L. Stevens, and J. I. Rood. 1995. Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. *Mol. Microbiol.* **15**:191–202.
3. Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992. Effects of *Eimeria brunetti* infection and dietary zinc on experimental induction of necrotic enteritis in broisoleucineciner chickens. *Avian Dis.* **36**:59–62.
4. Ginter, A., E. D. Williamson, F. Dessy, P. Coppe, H. Bullifent, A. Howells, and R. W. Titball. 1996. Molecular variation between the alpha-toxins from the type strain (NCTC 8237) and clinical isolates of *Clostridium perfringens* associated with disease in man and animals. *Microbiology* **142**:191–198.
5. Guillouard, I., T. Garnier, and S. T. Cole. 1996. Use of site-directed mutagenesis to probe structure-function relationships of alpha-toxin from *Clostridium perfringens*. *Infect. Immun.* **64**:2440–2444.
6. Hatheway, C. L. 1990. Toxigenic clostridia. *Clin. Microbiol. Rev.* **3**:66–98.
7. Hein, H., and L. Timms. 1972. Bacterial flora in the alimentary tract of chickens infected with *Eimeria brunetti* and in chickens immunized with *Eimeria maxima* and cross-infected with *Eimeria brunetti*. *Exp. Parasitol.* **31**:188–193.
8. Justin, N., N. Walker, H. L. Bullifent, G. Songer, D. M. Bueschel, H. Jost, C. Naylor, J. Miller, D. S. Moss, R. W. Titball, and A. K. Basak. 2002. The first strain of *Clostridium perfringens* isolated from an avian source has an alpha-toxin with divergent structural and kinetic properties. *Biochemistry* **41**:6253–6262.
9. Leslie, D., N. Fairweather, D. Pickard, G. Dougan, and M. Kehoe. 1989. Phospholipase C and haemolytic activities of *Clostridium perfringens* alpha-toxin cloned in *Escherichia coli*: sequence and homology with a *Bacillus cereus* phospholipase C. *Mol. Microbiol.* **3**:383–392.
10. Long, J. R., and R. B. Truscott. 1976. Necrotic enteritis in broisoleucineciner chickens. III. Reproduction of the disease. *Can. J. Comp. Med.* **40**:53–59.
11. MacLennan, J. D. 1962. The histotoxic clostridial infections of man. *Bacteriol. Rev.* **26**:177–276.
12. Mahony, D. E., and T. J. Moore. 1976. Stable L-forms of *Clostridium perfringens* and their growth on glass surfaces. *Can. J. Microbiol.* **22**:953–959.
13. McDonel, J. L. 1980. *Clostridium perfringens* toxins (type A, B, C, D, E). *Pharmacol. Ther.* **10**:617–655.
14. Meer, R. R., and J. G. Songer. 1997. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am. J. Vet. Res.* **58**:702–705.
15. Nagahama, M., T. Nakayama, K. Michiue, and J. Sakurai. 1997. Site-specific mutagenesis of *Clostridium perfringens* alpha-toxin: replacement of Asp-56, Asp-130, or Glu-152 causes loss of enzymatic and hemolytic activities. *Infect. Immun.* **65**:3489–3492.
16. Nagahama, M., Y. Okagawa, T. Nakayama, E. Nishioka, and J. Sakurai. 1995. Site-directed mutagenesis of histidine residues in *Clostridium perfringens* alpha-toxin. *J. Bacteriol.* **177**:1179–1185.
17. Naylor, C. E., J. T. Eaton, A. Howells, N. Justin, D. S. Moss, R. W. Titball, and A. K. Basak. 1998. Structure of the key toxin in gas gangrene. *Nat. Struct. Biol.* **5**:738–746.
18. Rood, J. I. 1998. Virulence genes of *Clostridium perfringens*. *Annu. Rev. Microbiol.* **52**:333–360.
19. Saint-Joanis, B., T. Garnier, and S. T. Cole. 1989. Gene cloning shows the alpha-toxin of *Clostridium perfringens* to contain both sphingomyelinase and lecithinase activities. *Mol. Gen. Genet.* **219**:453–460.
20. Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* **99**:996–1001.
21. Titball, R. W., S. E. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson, and D. C. Kelly. 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. *Infect. Immun.* **57**:367–376.
22. Tsutsui, K., J. Minami, O. Matsushita, S. Katayama, Y. Taniguchi, S. Nakamura, M. Nishioka, and A. Okabe. 1995. Phylogenetic analysis of phospholipase C genes from *Clostridium perfringens* types A to E and *Clostridium novyi*. *J. Bacteriol.* **177**:7164–7170.
23. Williamson, E. D., and R. W. Titball. 1993. A genetically engineered vaccine against the alpha-toxin of *Clostridium perfringens* protects mice against experimental gas gangrene. *Vaccine* **11**:1253–1258.
24. Willis, A. T. 1984. Treatment of anaerobic infections. *Scand. J. Gastroenterol. Suppl.* **90**:53–64.