RESEARCH ARTICLE Indian Journal of Animal Research, Volume 54 Issue 7: 864-868 (July 2020)

Cloning and Expression of Twin-Arginine Translocation D Family Deoxyribonuclease of *Clostridium Chauvoei*

Aakanksha Tiwari, Saroj K. Dangi, Prasad Thomas, Viswas Konasagara Nagaleekar **10.18805/ijar.B-3855**

ABSTRACT

Clostridium chauvoei, an anaerobic bacterium reported worldwide, is responsible for Black Quarter, a dreadful disease of ruminants. This bacterium produces many toxins responsible for the pathogenesis of the disease. Except for the well-studied virulence factors such as *cctA,* flagellin andsialidase genes, the exact role of other toxins of *C. chauvoei* remains unknown. This necessitates studies on the activities of the *C. chauvoei* toxins and virulence. In the present study, Twin-Arginine Translocation D (TatD) family deoxyribonuclease of the bacterium was selected. The *tatD* gene *C. chauvoei* was amplified by PCR and cloned into p-Rham-N-His-SUMO-Kan expression vector, followed by transformation into the *E.cloni* 10G competent cells. Clones obtained were confirmed by colony PCR. These *tatD*clones were sequenced and analysed phylogenetically, which revealed the close relationship of *C. chauvoei* strain to *C. isatidis, C. saccharobutylicum, C. botulinum* and *C. taeniosporum* based on *tatD* sequence analysis. Upon induction of the clones with L-rhamnose, the protein expression was obtained at 42.3 kDa and the same was further confirmed by Western blotting.

Key words: Black quarter, *Clostridium chauvoei,* TatD deoxyribonuclease.

INTRODUCTION

Black Quarter, an acute, highly fatal disease of ruminants, is caused by *Clostridium chauvoei*. It has been reported in various species of animals and two human cases have also been reported (Nagano *et al.* 2008). Infected animal dies within 12 to 36 hrs after the appearance of the symptoms (Hatheway 1990). Spores remain viable in the soil for years together and hence act as the major source of infection (Sathish and Swaminathan 2008). The spores enter the animal's body and their germination induced by amino acids often coupled with potassium and sodium ions occurs upon muscle injury (Durre 2014).

It has been proposed that *C. chauvoei* produces various toxins, *viz*. alpha, beta, gamma and delta toxins (Moussa 1958). Further studies on *C. chauvoei* virulence factors confirmed sialidase and flagellin also as the contributors to the pathogenicity (Tamura *et al.* 1995). However, among these proposed toxins, the genes for nanA sialidase, hyaluronidase, cctA and flagellin only have been characterized (Tamura *et al.* 1984; Tamura *et al.* 1995; Kojima *et al.* 2000; Vilei *et al.* 2011; Frey *et al.* 2012; Dangi *et al.* 2017; Dangi *et al.* 2018), and the role of other toxins including beta toxin (deoxyribonuclease) and the existence of genes encoding these toxins is not known.

Deoxyribonucleases can degrade highly polymerized deoxyribonucleic acid. Production of deoxyribonuclease indicates the virulence mechanism in many bacteria including *Clostridium* (Koneman 1997). Earlier studies have proposed that *C. chauvoei* produces deoxyribonuclease (Carloni *et al.* 2005). Studies have also reported beta toxin as a heat-stable toxin, activated or inhibited by metal ions and chelating agents (Princewill and Oakley 1976; Hatheway 1990). They are responsible for nuclear breakdown of muscle cells and involved in the process of gangrenous myositis (Songer 1998; Cortiñas *et al.* 1999). However, no study has yet attributed specific gene responsible for deoxyribonuclase activity. Twin-arginine translocation family deoxyribonuclease (TatD) reported to be a cytoplasmic magnesium dependent deoxyribonuclease expressed by various bacterial species (Chen *et al.* 2014). TatD is detected almost exclusively in the cytoplasmic protein fraction suggesting that it is normally located in the cytoplasm (Wexler *et al.* 2000). In the present study, we expressed and characterized the gene for TatD family deoxyribonuclease (*tatD*) of *C. chauvoei.*

Division of Bacteriology and Mycology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243 122, Uttar Pradesh, India.

Corresponding Author: Aakanksha Tiwari, Division of Bacteriology and Mycology, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243 122, Uttar Pradesh, India.
Email: aakanksha2191@yahoo.in

How to cite this article: Tiwari, A., Dangi, S.K., Thomas, P. and Nagaleekar, V.K. (2020). Cloning and Expression of Twin-Arginine Translocation D Family Deoxyribonuclease of *Clostridium Chauvoei.* Indian Journal of Animal Research. 54(7): 864-868.

Submitted: 08-06-2019 **Accepted**: 09-08-2019 **Published**: 09-11-2019

MATERIALS AND METHODS

Bacterial strains and plasmids

E. cloni 10G competent cells and pRham-N-His SUMO Kan vector were procured from Lucigen Corporation, USA. *E. cloni* 10G cells were cultured in Luria Bertani broth or agar under aerobic conditions at 37°C. *Clostridium chauvoei* ATCC 10092 strain (ATCC, USA) was cultured in ATCC 2107

modified reinforced clostridial (MRC) medium (1% Tryptose, 1% Beef extract, 0.3% Yeast extract, 0.5% Dextrose, 0.5% NaCl, 0.1% Soluble Starch, 0.05% L-cystineHCl and 0.03% Sodium acetate, pH 7.2) at 37°C for 48 h under anaerobic conditions. The genomic DNA was isolated from the culture using Genomic DNA isolation kit (Thermo Scientific, USA) as per the manufacturer's protocol and used for species confirmation by PCR targeting the 16S-23S rDNA spacer region and *cctA* gene as reported earlier (Sasaki *et al.* 2000; Dangi *et al.* 2017; Kumar *et al.* 2018).

PCR amplification of *tatD* gene of C. chauvoei

PCR amplification of the *tatD* gene was carried out in a 25 µl reaction mixture consisting of 2.5 µl of 10X Taq Buffer, 0.5 µl $MgSO_4$ (25mM), 0.5 µl dNTPs (10mM), 0.5 µl of 10 pmoles/µl each of the primers (For- 5′-CGCGAACAGA TTCGA GGTGAAGGAAAATATTTAATTTTT-3′ and Rev- 5′-GTGGC GGCCGCTCTATTATATTCTA TTTTCAAGCAAGTC-3′), 0.25 µl Pfu polymerase: Taq Polymerase (1:15). To this, about 1.5 µl of genomic DNA was added and the PCR was performed in a thermocycler (Agilent Technologies, USA) with initial denaturation temperature of 94°C for 5 min followed by 34 cycles of denaturation of 94°C for 1 min, annealing temperature of 51°C for 1 min and extension of 72°C for 1min. Final extension was carried out at 72°C for 10 min. PCR product was analysed by agarose gel electrophoresis and gel extraction was carried out according to the manufacturer's protocol using the Mini Elute Gel extraction kit (Thermo Scientific, USA).

Cloning and sequencing of the *tatD* gene

Purified *tatD* PCR product was cloned into pRham-N-His SUMO Kan expression vector and transformation was performed according to the manufacturer's protocol (Lucigen, USA). Briefly, 25 ng of the vector DNA was added to the 100 ng of the purified PCR product, mixed gently and then the mixture was added to 40 µl of *E. cloni* 10G competent cells. The transformed product was plated onto LB agar plates containing 30 µg/ml Kanamycin and incubated at 37°C overnight. The recombinant clones obtained were screened and confirmed by colony PCR using the gene specific primers.

Recombinant plasmids were extracted from the positive clones and were sequenced at a custom DNA sequencing facility (Eurofins). Sequences were analysed using BLAST programme of NCBI (https://www.ncbi.nlm.nih.gov/) against the nucleotide database. A phylogenetic analysis of the nucleotide and protein sequences of the gene was also done using the MegaX software (Tamura *et al.* 2013).

Expression and protein purification of *TatD* deoxyribonuclease gene

The recombinant clones were subcultured and the protein expression was obtained by induction with 0.2% L-Rhamnose. The induction was done when the OD_{600} of the culture reached 0.4-0.6. The protein expression was confirmed by SDS-PAGE and Western Blot analysis. The recombinant TatD deoxyribonuclease expressed protein was produced in bulk in 500 ml of LB broth. Further, the polyhistidine (6X His) tagged fusion protein was purified under denaturing conditions in urea using Ni-NTA affinity chromatography. The purified protein was pooled in a dialysis membrane (Qiagen, Germany) and dialyzed against decreasing concentrations of urea (7 M to 0.5 M) and one litre phosphate buffered saline to remove urea. The protein concentration was determined by Bradford assay (Ramagli and Rodriguez 1985).

Western Blot for the detection of the His-tagged recombinant *TatD* deoxyribonuclease protein

The reactivity of the recombinant protein with the antiserum raised against the recombinant TatD protein was checked by Western blot analysis (Towbin *et al.* 1979). Protein was transferred to nitrocellulose membrane, blocked and then 1:200 dilution of primary antibody (Goat anti-chicken IgY) in blocking buffer (5% skimmed milk powder in PBS) was added and incubated at 37°C for 2 hrs. After washing 3 times with PBST (500 µl Tween-20 in 1000 ml PBS), 1:10,000 dilutions of secondary antibody (anti-chicken HRPO conjugate; Sigma, USA) in blocking buffer was added and kept at 37°C for 2 hrs. The blotted nitrocellulose membrane was developed using DAB solution.

RESULTS AND DISCUSSION

The beta toxin (deoxyribonuclease), among the proposed toxins of *C. chauvoei*, is postulated to be one of the major toxin responsible for the degradation of host DNA. Though previous reports showed deoxyribonuclease activity of the pathogen, no specific deoxyribonuclease protein has been identified yet. Complete genome sequence of *C. chauvoei* for a Switzerland (JF4335), German (12S0467) and ATCC 10092 (DSM 7528) strains are currently available (Falquet *et al.* 2013; Thomas *et al.* 2017). These studies have mostly targeted the genome composition, potential virulence factors, CRISPR elements, prophage composition and genetic divergence of the species (Frey and Falquet 2015; Thomas *et al.* 2017). As a preliminary step in identification of deoxyribonuclase protein, in the present study, we expressed and characterized TatD deoxyribonuclease of *C. chauvoei.*

First, we confirmed the species identity of *C. chauvoei* by16S-23S rDNA spacer region and *cctA* gene specific PCR, which revealed 522 bp and 983 bp target specific amplicons, respectively (Fig 1).

Next, the amplification of the *tatD* gene using the designed gene specific primers was obtained at the expected size of 780 bp (Fig 2). The purified PCR product was cloned into pRham-N-His SUMO Kan vector and transformed into *E. cloni* 10G competent cells. Four colonies were obtained, which were then confirmed by colony PCR. All of them gave amplification at the desired size of 780 bp with the gene specific primers.

Later on, plasmid was isolated from the *tatD* clones and sequenced. The sequence was submitted to NCBI

nucleotide sequence database and was assigned with accession number - MF177720. The *tatD* sequence was aligned with sequences from other closely related Clostridia and phylogenetic tree was constructed. The phylogenetic analysis of *tatD* sequences indicated the close relationship of *C. chauvoei* strains to the *C. isatidis,* an indigo reducing anaerobe, followed by *C. saccharobutylicum, C. botulinum* and *C. taeniosporum* (Fig 3).

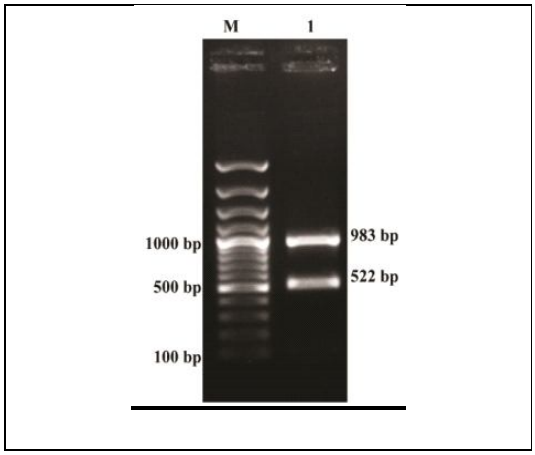

Fig 1: Confirmation of *C. chauvoei* (ATCC 10092) by PCR based on 16S-23S rDNA spacer gene and cctA gene. Lane M: 100 bp DNA ladder; Lane 1: PCR product.

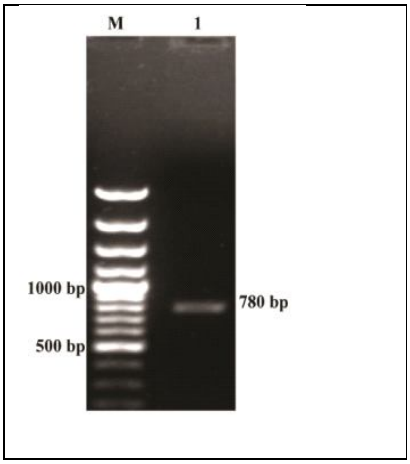

Fig 2: PCR amplification of *tatD*gene of *C. chauvoei.* Lane M: 100 bp DNA Ladder; Lane 1: *tatD*gene.

Further, the positive recombinant clones of *E. cloni* 10G cells were induced for protein expression and the clones showed the expression of protein at the expected size of 42.3 kDa, as analyzed by SDS-PAGE (Fig 4A). The TatD deoxyribonuclease protein was purified by Ni-NTA affinity chromatography and the eluted fraction of the protein was assessed by SDS-PAGEanalysis (Fig 4B). On Western Blotting, using anti-His antibodies, a specific band at 42.3 kDa size was obtained both in the expressed and the purified protein samples (Fig 4C).

The deoxyribonuclease activity of the organism is already evident (Chaudhuri and Singh 1992). *TatD* deoxyribonuclease is reported to be a cytoplasmic protein in bacteria having a magnesium-dependent deoxyribonuclease activity. The DNase enzyme is responsible for the hydrolytic cleavage of phosphodiester bonds in the DNA backbone, thereby causing the degradation of the DNA. The production of extracellular nuclease which degrades the DNA in *Clostridium* sp. has been observed (Timmis and Winkler 1973).

DNase production by *C. septicum* was detected previously and the molecular weight of the DNase of *C. septicum* was found out to be 45kDa. On comparing the DNase activity with the DNAse activity of other Clostridial species *C.septicum*inthe culture supernatant was observed to be having the strongest DNase activity (Swiatek *et al.* 1987). In case of *C. acetobutylicum,* it was observed that the maximum DNase activity was obtained in the cell-wall compartmentalised fraction which indicates that this protein in present outside the cytoplasmic membrane. This was detected by the examinig the DNA hydrolysis around the cells (Burchhardt and Dürre 1990). The presence of extracellular DNase in *C. botulinum* posed a problem in its DNA isolation by causing the degradation of the DNA (Hielm *et al.* 1998). A recent study reported TatD-like DNase (PfTatD) as a novel virulence factor of *Plasmodium* spp. The study also proved that PfTatD exhibits typical deoxyribonuclease activity, and its expression is higher in virulent parasites than in avirulent parasites. The mice immunized with recombinant

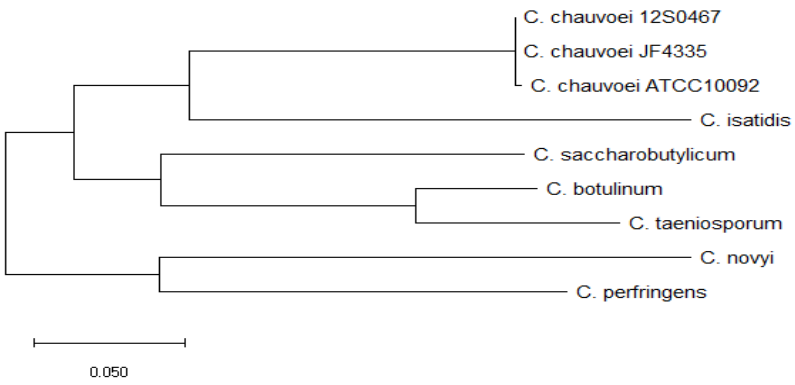

Fig 3: Phylogenetic analysis of the *tatD*gene nucleotide sequence.

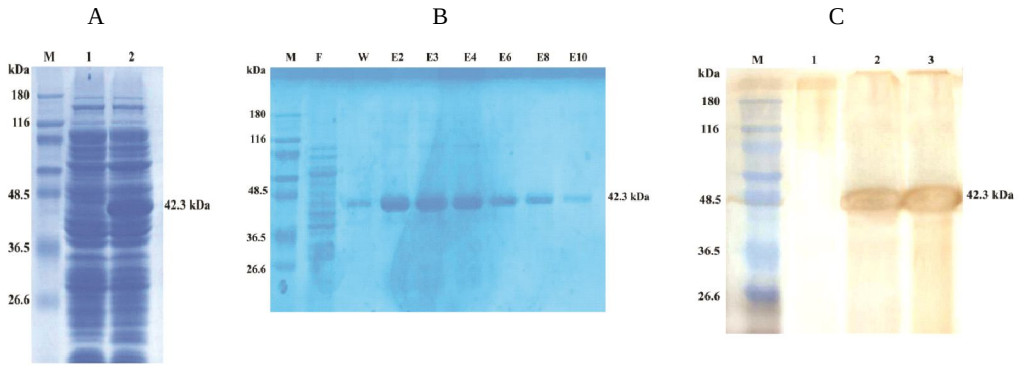

Fig 4: Expression of rTatD deoxyribonuclease. **A.**SDS-PAGE analysis of expressed recombinant TatD deoxyribonuclease.Lanes M: Prestained protein marker; 1: Uninduced cell lysate; 2: Overexpressed cloneupon Rhamnose induction.**B.** SDS-PAGE Profile of purified recombinant TatD deoxyribonuclease. Lanes M: Prestained protein marker; F: Flowthrough fraction; W: Wash fraction; E2 to E10: Different fractions of elutes. **C.**Confirmation of overexpression of rTatD deoxyribonuclease by Western blot. Lanes M: Prestained protein marker; 1: Uninduced cell lysate; 2: Induced cell lysate; 3: Purified rTatD deoxyribonuclease.

TatD exhibit increased immunity against lethal challenge (Chang *et al.* 2016).

CONCLUSION

To summarize, in the present study, the *tatD* gene was successfully cloned into pRham-N-His SUMO Kan expression vector and sequence of *tatD* was analyzed. Phylogenetic analysis of *tatD* gene showed that *C. chauvoei* strains were closely related to *C. isatidis, C. saccharobutylicum, C. botulinum* and *C. taeniosporum.* Upon induction of positive recombinant clones, the expression of TatD deoxyribonuclease was obtained at size of 42.3 kDa which was confirmed by SDS-PAGE and Western Blot analysis.

ACKNOWLEDGEMENT

We are thankful to National Agricultural Science Fund (NASF), ICAR, New Delhi (Grant No. NASF/ABA-5011/2015-16) for providing financial support and the Director, ICAR-Indian Veterinary Research Institute, Izatnagar for providing the necessary facilities.

REFERENCES

Burchhardt G and Dürre P. (1990). Isolation and characterization of DNase-deficient mutants of clostridium acetobutylicum. Current Microbiology. 21(5): 307-11.

Carloni, G.H., Bentancor, L.D. and De Torres, R.A. (2005). Deoxyribonuclease activity detection in clostridium chauvoei strains. Rev Argent Microbiol. 37(2): 87-8.

Chang Z, Jiang N, Zhang Y, Lu H, Yin J, Wahlgren M, Cheng X, Cao Y and Chen Q. (2016). The *TatD*-like DNase of Plasmodium is a virulence factor and a potential malaria vaccine candidate. Nat Commun. 7: 11537.

Chaudhuri P and Singh S D. (1992). Modified methods for simple and rapid detection of bacterial deoxyribonuclease production. Journal of Microbiological Methods. 16(4): 307-11.

Chen Y C, Li C L, Hsiao Y Y, Duh Y and Yuan H S. (2014). Structure and function of *TatD* exonuclease in DNA repair. Nucleic Acids Res. 42(16): 10776-85.

Cortiñas T I, Mattar M A and de Guzmán A M S. (1999). Alpha and beta toxin activities in local strains of *Clostridium chauvoei*. Anaerobe. 5(3-4): 297-99.

Dangi S K, Yadav P K, Mashooq M, Agarwal R K and Nagaleekar V K. (2018). Cloning and expression analysis of nagJ hyaluronidase gene of Clostridium chauvoei. Indian Journal of Animal Sciences. 88(3): 304-06.

Dangi S K, Yadav P K, Tiwari A and Nagaleekar V K. (2017). Cloning and sequence analysis of hyaluronoglucosaminidase (nagH) gene of clostridium chauvoei. Vet World. 10(9): 1104-07.

Durre P. (2014). Physiology and sporulation in clostridium. Microbiol Spectr. 2(4): 0010-2012.

Falquet L, Calderon-Copete S P and Frey J. (2013). Draft genome sequence of the virulent clostridium chauvoei Reference Strain JF4335. Genome Announc. 1(4): 00593-13.

Frey J and Falquet L. (2015). Patho-genetics of clostridium chauvoei. Res Microbiol. 166(4): 384-92.

Frey J, Johansson A, Burki S, Vilei E M and Redhead K. (2012). Cytotoxin CctA, a major virulence factor of clostridium chauvoei conferring protective immunity against myonecrosis. Vaccine. 30(37): 5500-5.

Hatheway C L. (1990). Toxigenic clostridia. Clinical microbiology reviews. 3(1): 66-98.

Hielm S, Björkroth J, Hyytiä E and Korkeala H. (1998). Genomic analysis of clostridium botulinum group II by pulsed-field gel electrophoresis. Applied and Environmental Microbiology. 64(2): 703-08.

Kojima A, Uchida I, Sekizaki T, Sasaki Y, Ogikubo Y, Kijima M and Tamura Y. (2000). Cloning and expression of a gene encoding the flagellin of clostridium chauvoei. Vet Microbiol. 76(4): 359-72.

Koneman E W. (1997). Color Atlas and Textbook of Diagnostic Microbiology. edn 5th. Lippincott, Philadelphia.

Kumar S, Mashooq M, Gandham R K, Alavandi S V and Nagaleekar V K. (2018). Characterization of quorum sensing system in Clostridium chauvoei. Anaerobe. 52: 92-99.

Moussa R S. (1958). Complexity of toxins from clostridium septicum and clostridium chauvoei. Journal of Bacteriology. 76(5): 538-45.

Nagano N, Isomine S, Kato H, Sasaki Y, Takahashi M, Sakaida K, Nagano Y and Arakawa Y. (2008). Human fulminant gas gangrene caused by *Clostridium chauvoei*. J Clin Microbiol. 46(4): 1545-7.

Princewill T J and Oakley C L. (1976). Deoxyribonucleases and hyaluronidases of Clostridium septicum and Clostridium chauvoei. III. Relationship between the two organisms. Med Lab Sci. 33(2): 10-118.

Ramagli L S and Rodriguez L V. (1985). Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis. 6(11): 559-63.

Sasaki Y, Yamamoto K, Kojima A, Tetsuka Y, Norimatsu M and Tamura Y. (2000). Rapid and direct detection of clostridium chauvoei by PCR of the 16S-23S rDNA spacer region and partial 23S rDNA sequences. J Vet Med Sci. 62(12): 1275-81.

Sathish S and Swaminathan K. (2008). Molecular characterization of the diversity of Clostridium chauvoei isolates collected from two bovine slaughterhouses: analysis of cross-contamination. Anaerobe. 14(3): 190-9.

Songer J G. (1998). Clostridial diseases of small ruminants. Vet Res. 29(3-4): 219-32.

Swiatek P J, Allen S D, Siders J A and Lee C H. (1987). DNase production by clostridium septicum. Journal of Clinical Microbiology. 25(2): 437-38.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 30(12): 2725-9.

Tamura Y, Kijima-Tanaka M, Aoki A, Ogikubo Y and Takahashi T. (1995). Reversible expression of motility and flagella in clostridium chauvoei and their relationship to virulence. Microbiology. 141(Pt 3): 605-10.

Tamura Y, Minamoto N and Tanaka S. (1984). Demonstration of protective antigen carried by flagella of clostridium chauvoei. Microbiol Immunol. 28(12): 1325-32.

Thomas P, Semmler T, Eichhorn I, Lubke-Becker A, Werckenthin C, Abdel-Glil M Y, Wieler L H, Neubauer H and Seyboldt C. (2017). First report of two complete clostridium chauvoei genome sequences and detailed in silico genome analysis. Infect Genet Evol. 54: 287-98.

Timmis K and Winkler U. (1973). Isolation of covalently closed circular deoxyribonucleic acid from bacteria which produce exocellular nuclease. Journal of Bacteriology. 113(1): 508-09.

Towbin H, Staehelin T and Gordon J. (1979). Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. Proceedings of the National Academy of Sciences of the United States of America. 76(9): 4350-54.

Vilei E M, Johansson A, Schlatter Y, Redhead K and Frey J. (2011). Genetic and functional characterization of the NanA sialidase from clostridium chauvoei. Veterinary Research. 42(1): 2.

Wexler M, Sargent F, Jack R L, Stanley N R, Bogsch E G, Robinson C, Berks B C and Palmer T. (2000). TatD is a cytoplasmic protein with DNase activity. No requirement for TatD family proteins in sec-independent protein export. J Biol Chem. 275(22): 16717-22.