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Genomics of *Clostridium botulinum* group III strains

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Abstract

In *Clostridium botulinum*, the characteristics of type C and D strains are quite different from other types, and they are classified as group III. They produce C2 binary toxin and C3 exoenzyme in addition to type C and D neurotoxins. Two different phages and many plasmids are identified in the organisms. The genes of neurotoxin and C3 exoenzyme are converted from toxigenic strains to non-toxigenic strains by the specific bacteriophages (phages), whereas, the C2 toxin gene is carried by large or small plasmids. Classification of type C and D strains has been in confusion because 1) antigenicity of type C and D neurotoxins is complex, 2) the cells produce two types of toxins, neurotoxin and C2 toxin, and 3) some non-toxigenic strains can be converted to produce C or D neurotoxin by the infection with phages. Until now, entire nucleotide sequences of cell chromosomes, phages, and plasmids have been determined. Since both genetic and protein-chemical analyses have been clarifying the above confusions, these data are reviewed historically.

Keywords: *Clostridium botulinum* group III; Neurotoxin; Progenitor toxin; C2 toxin; C3 exoenzyme; Phage conversion

1. Introduction

Clostridium botulinum and some other Clostridial strains produce poisonous botulinum neurotoxins (NTX). NTXs are classified into seven groups, types A to G, based on the antigenicity. Recently, production of new type of toxin, type H, was reported. On the other hand, *C. botulinum* cultures can be classified into three groups on the basis of their biochemical properties, cell wall composition, and DNA or RNA homologies [1,2]: (I) all of type A strains and the proteolytic strains of types B and F; (II) the non-proteolytic strains of types B and F and all type E strains; (III) type C and D strains. Type G strain is now considered as new species, *C. argentinense*. NTXs inhibit the release of acetylcholine at the neuromuscular junctions and synapses, and cause botulism in humans and animals. Molecular mass (Mr) of all the types of NTXs is approximately 150 kDa. NTXs associate with nontoxic components in cultures (under an acid condition), and become large complexes that are designated progenitor toxins (PTX)

Abbreviations: NTX, neurotoxin; L, light chain; Hn, N-terminal region of heavy chain; Hc, C-terminal region of heavy chain; PTX, progenitor toxin; HA, hemagglutinin; NTNH, nontoxic non-HA; Mr, molecular mass; ORF, open reading frame.

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[3]. PTXs are found in three forms with Mr of 900 kDa (19S, LL), 500 kDa (16S, L), and 300 kDa (12S, M); later, it became clear that actual Mr of L and LL PTXs is much larger than 500 kDa and 900 kDa. The M PTX is composed of an NTX and a nontoxic component having no hemagglutinin (HA) activity (designated nontoxic non-HA; NTNH). L and LL PTXs are formed by conjugation of M PTX with HAs, and LL PTX is a dimer of L PTX (these will be explained later). The type A strain produces three forms of PTXs (M, L, and LL), whereas types B, C, and D produce M and L PTXs. In alkaline conditions without gastric juice, PTXs dissociate into NTX(s) and nontoxic components.

Until now, entire nucleotide sequences of the genes of all 8 types of NTXs, and almost all types of PTXs as well as whole genome have been determined from representative strains. Usually, the genes of type A, B, E, and F NTXs exist on the cell chromosome, but on large plasmid in type G (and also in some type A, B, and F strains). In types C and D, toxin production is governed by bacteriophages (phages), and the toxin genes are transmitted from the toxigenic strains to the non-toxigenic indicator strains by the specific phages: phage conversion to toxigenicity [4–9]. We determined the whole base sequences of NTX and PTX genes, and a type C converting phage DNA in order to analyze the mechanism of this phenomenon, as well as the structure and function of toxins [10–16]. Some type C and D cultures produce C2 toxin and C3 exoenzyme in addition to C or D NTX (therefore, type C NTX sometimes described as C1 toxin).

In this chapter, the history of classification of type C and D strains has first been briefly reviewed because there was some confusion as to it, especially the definition of C2 toxin. Thereafter, the global genetics of group III strains, including genetic analyses of chromosomes, phages, and plasmids have been summarized with paying attention to their toxigenicity.

2. Materials and methods

2.1. Purification of NTXs and preparation of their antisera

The type C and D cells were cultured by cellophane tube procedure. The toxins were precipitated by ammonium sulfate, and then purified by successive column chromatographies at pH 8.0 (0.05 M Borax-0.1 M phosphate buffer) with Sephadex G-75, DEAE cellulose, QAE Sephadex A-50, and finally Sephadex G-200. The purified NTXs thus obtained were made into toxoids by Formalin, mixed with an equal volume of Freund's incomplete adjuvant, and then injected subcutaneously into rabbits [17,18].

2.2. Preparation of monoclonal antibodies

Hybridomas with secreting and non-secreting type of mouse myeloma cells were employed. BALB/c mice were immunized with toxoids. The spleen cells from immunized mice were fused by polyethylene glycol with myeloma cells, and selected by treatment with HAT (Hypoxanthine-Aminopterin-Thymidine) medium. The cell lines producing antibodies were then cloned by the limiting dilution method. Thereafter, the cloned cells were injected into BALB/c mice to obtain the ascites fluids containing high amounts of antibodies [19,20].

2.3. Toxin neutralization

Neutralization test of type C and D toxins by polyclonal and monoclonal antibodies were performed. The antibodies were diluted serially with 0.01 M phosphate buffered saline (PBS, pH 7.2) and mixed with an equal volume of 10 50% lethal doses of toxin per ml in the same buffer or 0.02 M phosphate buffer (pH 6.0) containing gelatin at 0.2% (gelatin buffer). After incubation at 37 °C for 2 h, 0.5 ml of the mixture was injected intraperitoneally into two to five mice. The mice were observed for 6 days, and the highest dilution which neutralized toxin was determined.[1]

2.4. Phage conversion to toxigenicity

Phage conversion to toxigenicity was performed by a similar procedure reported by Inoue and Iida [4,5]. The phages were induced from the toxigenic cultures by Mitomycin C treatment, and filtered through a membrane filter with pore size of 0.45 μm (Later, it became clear that some phages usually exist in the cultures of toxigenic strains without such induction). The filtrates (0.5 ml) were mixed with young cultures of non-toxigenic indicator strains in 2.5 ml of Lactalbumin-Yeast extract-Glucose (LYG) medium. After incubation at 37 °C for 3–4 h, 0.2 ml of the cultures were inoculated into 10–15 ml of cooked meat medium. After incubation at 37 °C for more than 2 days, the supernatants (0.5 ml) were injected into mice to check the toxin production. Plaque formation was performed by the procedure reported by Eklund et al. [6,7]. Eklund et al. found that the organisms carry large (named β phage) and small (named γ phage) phages, and toxin production is governed by a large β phage. Since the genes for NTX and the nontoxic components (NTNH and HA) that conjugate with NTX to form PTX are closely linked, all of these genes are usually concomitantly transmitted by the phages [13,14,21] (Fig. 2).

2.5. Sequencing of phage genome harboring NTX and C3 exoenzyme genes, and plasmid harboring C2 toxin gene, and in silico analysis

The phage c-st DNA was isolated from phage particles by pulsed-field gel electrophoresis (PFGE) as follows [14]. The phage c-st, which was isolated from a toxigenic type C strain C-Stockholm, was purified by successive three times plaque formation with the indicator strain, non-toxigenic type C strain (C)-AO2. Thereafter, its titer was increased by infection through the

[1] These procedures were performed more than 15 years ago. Recently, we have seldom done such a neutralization test. When we perform the neutralization test, the toxin-injected mice are observed only one to two days for their symptoms, and euthanasia is carried out, if necessary.