From saprophytic to toxigenic clostridia, a complex evolution based on multiple diverse genetic transfers and/or rearrangements

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Editorial

From saprophytic to toxigenic clostridia, a complex evolution based on multiple diverse genetic transfers and/or rearrangements☆

The genus *Clostridium*, the definition of which is based on four criteria – rod-shaped, spore-forming, Gram-positive and an obligate anaerobic bacteria – comprises an extremely vast and heterogeneous group of bacteria. More than 150 species of clostridial species have been described. Most of them are fermentative bacteria involved in many biochemical transformations of various substrates, and are not associated with pathological processes. However, a few clostridial species, around 15 of them, produce highly potent toxins known to be responsible for severe diseases in man and animals, and are crucial biological threats.

The first evidence of bacterial life in strict anaerobic conditions was provided by Louis Pasteur (1861). He described a microbe that produced butyric acid and butanol and was unable to grow in the presence of oxygen. He named this microorganism *Vibrion butyrique* due to the major fermentation product, butyrate, and to the fact that it was a mobile rod. Pasteur introduced the term "anaerobic" to define the mode of life without free oxygen. A. Trecul (1867) first used the name "*Clostridium*", a diminutive form (kloth-ster) of the Greek word "kloth", to designate a small spindle; this was then latinized into *Clostridium*, corresponding to the shape of the microorganism in its sporulating form. Adam Prazmowski (1880) distinguished straight rods containing a spore (*Clostridium*) from the sporulated curved rods (*Vibrio*) and proposed the name *Clostridium butyricum* for Pasteur's *V. butyrique*, which was found to be identical to the *Bacillus amylobacter* of Van Tieghem. Thereby, the term *Clostridium* was based on the morphology of the bacteria containing a spore, but not on their physiology. The metabolic distinction between *Bacillus* (aerobic) and *Clostridium* (anaerobic) was introduced later in 1922, by the Committee on Classification of the Society of American Bacteriologists [2].

Pasteur's preparation was probably not a pure culture. *B. amylobacter* of Van Tieghem, renamed *C. butyricum* by Prazmowski, was the first isolated *Clostridium* strain and is considered as the type strain of the genus *Clostridium*.

The first pathogenic *Clostridium* isolated in pure culture and identified as producing a toxin was *Clostridium tetani*, by Kitassato in 1889. Then Achalme (1891) obtained the first culture of *Clostridium perfringens* from a case of rheumatism case. This bacterium was further characterized by Welch and Nuttal (1892), who initially designated it as *Bacillus aerogenes capsulatus* and showed the importance of this pathogen in gas gangrenes. Bull and Pritchett (1917) found that the cause of death was not related to blood invasion by these bacteria, but to a poisonous substance produced during bacterial growth in the tissues. McFarlane and Knight (1941) showed that the lethal and hemolytic "α-factor" was lecithinase C. It was the first bacterial toxin identified as exhibiting enzymatic activity, and the first described mode of action of a protein toxin. In 1895, van Emmingen identified the causative agent of botulism, *Bacillus botulinum*, later renamed *Clostridium botulinum*, which he isolated from ham and from a victim of a severe botulism outbreak in Belgium. *Clostridium difficile* was first identified as a resident bacteria of the colonic microflora of newborns by Hall and O'Toole in 1935. Although *C. difficile* was reported to be a toxin producer by Snyder in 1937, it was recognized as an important enteropathogen several decades later, starting with the original observation by Larson et al., in 1977 of a cytotoxin in the stool of a patient with pseudomembranous colitis (rev in [1]). In the meantime, additional toxigenic Clostridia, as well as solventogenic Clostridia, have been characterized.

Since the first historical descriptions of clostridial species, an increasing number of techniques and methods have been used to further characterize these bacteria and their metabolism products, including toxins and virulence factors. Among them, whole genome sequencing is one of the most powerful approaches for gaining access to a global understanding of the potential properties of bacteria. However, many genes are still assigned to unknown functions, and gene expression appears to result from ever more complex and diverse regulation networks. In addition, whole genome sequencing has evidenced genetic relatedness and gene exchanges between bacteria, thus leading to a better understanding of bacterial evolution and adaptation to specific environments. Among more than 150 clostridial species described until now, clostridia of medical and industrial interest have been the most thoroughly investigated. This special issue focuses on recent genetic advances in pathogenic clostridia.

☆ A special issue dedicated to the memory of Dr. Thoru Shimizu, University of Kanazawa, Japan.

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The main habitat of clostridia is the environment, where they can survive due to their spores. Sporulation and germination are more complex processes in clostridia than in *Bacillus*, which are also sporulating bacteria but with an aerobic or aero-anaerobic metabolism. While starvation of carbohydrates is the main signal inducing sporulation in *Bacillus*, clostridia require a sufficient level of energy and use a more complex and as yet only partially defined signaling pathway for sporulation. These aspects, which are important not only for understanding physiological mechanisms of this bacterial group but also for developing efficient preventive measures in hospital hygiene and the food industry, are discussed in two reviews [17,22].

An intriguing question is how, among the numerous clostridia which are environmental bacteria, a few species (about 15) have acquired the capacity to produce the most potent toxins known – and to which purpose? *Clostridium* species are fermentative bacteria which have adapted themselves to diverse ecological niches through specific enzymatic equipment enabling utilization of various substrates. Some clostridial species are autotrophic and obtain their energy from inorganic chemicals, but many others use organic polymers as substrates such as polysaccharides (cellulose, starch, pectin, etc.), proteins, and peptides [4]. Thereby, they secrete diverse hydrolytic enzymes that degrade high molecular weight substrates in the surrounding microenvironment of the bacteria and then absorb, through numerous transporters, breakdown monomers required for their metabolism. Clostridia have an essential role in the environment in recycling of various compounds, mainly organic molecules. Indeed, clostridia are the main bacteria involved in animal cadaver decomposition and plant degradation. Most pathogenic clostridia are defective in amino acid biosynthesis pathways and hydrolyze proteins or peptides, then absorbing amino acids. Based on the mechanism of action, most bacterial toxins can be divided into pore-forming toxins and toxins active through an enzymatic activity. Genetic and structural analyses support the notion that bacterial toxins have evolved from ancestor genes that are also at the origin of genes for regular structural and/or functional components of bacteria. Indeed, enzymatically active toxins probably derived from ancestral hydrolytic enzymes or enzyme precursors. A likely evolution mechanism might include mutations in enzyme ancestor gene(s) leading to modification of substrate specificity, from non-essential to highly critical molecules in cell survival or physiology, resulting in the emergence of a novel enzymatic toxin or toxin domain. Subsequent combination or fusion of the new enzyme domain with a delivery system able to transport and internalize the catalytic domain into specific target cells might yield highly potent toxin(s) [19]. For example, clostridial neurotoxins retain the proteolysis enzymatic site of zinc-dependent metalloprotease, which is common to many collagenases and proteases produced by various clostridia like *Clostridium histolyticum* and other bacteria. However, in contrast to collagenases and proteases used by clostridia for utilization of a wide range of protein substrates, clostridial neurotoxins have acquired a binding domain specific of a neuronal cell surface receptor that mediates their entry into only certain neurons, and highly specific proteolytic activity toward SNARE proteins, thus leading to specific inhibition of neurotransmitter release [9,19]. Interestingly, the two main types of clostridial neurotoxins, botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT), have followed distinct evolution pathways, albeit structurally and functionally closely related. BoNTs have sustained numerous genetic rearrangements (mutations, duplication, recombination), resulting in multiple BoNT types and subtypes, whereas only a unique TeNT sequence has been reported until now. Similarly, the genome background of BoNT-producing clostridia is extremely diverse, and only restricted genetic diversity is observed in *C. tetani* strains (see [5,6,20,21]).

Genetic variability in toxin genes and genomic background is also prevalent in other pathogenic clostridia, such as *C. perfringens*, *C. difficile* and *Clostridium sordellii* (see [10,12,13,23]). However, toxin genes are not all equally submitted to genetic variation. For example, in *C. perfringens*, the genes of enterotoxin, epsilon, beta and iota toxins are highly conserved, whereas other toxin genes, like those of alpha and beta2 toxins, exhibit variable sequences [10,19]. This raises the question of selective pressures and environmental factors that trigger or control the genetic variability of these pathogens, but differently according to the *Clostridium* species. Genomic variation might possibly correspond to bacterial adaptation to a new ecological niche, for example, from an environmental site to a host compartment such as the digestive tract or deep tissue subsequently to a wound, which are the most accessible sites for environmental bacteria. But why do certain pathogenic clostridia exploit such extended genetic variation? If the human digestive tract is the preferred host ecological niche for *C. difficile*, what is the significance of the numerous genetic variants of this opportunistic pathogen? Does genetic diversity represent specific adaptation so as to counterbalance multiple human microbiota, which represent the first line of defense against intestinal colonization? And what is the role of toxin gene variation in this adaptation process?

Genomic variability in clostridia is also mediated by horizontal gene transfer between intra- and inter-*Clostridium* species, and possibly other bacterial species or eukaryotic cells [15]. Indeed, mobile genetic elements, including plasmids, phages and transposons, are common in the genomes of pathogenic clostridia and are involved in mobilization of toxin genes between bacteria. The digestive tract, which harbors the densest population of bacteria, is a favorable ecosystem for genetic exchanges between bacteria. Thus, pathogenic clostridia, which colonize the intestinal tract, are those which show the greatest genetic variability. Different genetic supports and mechanisms are used by distinct pathogenic clostridia. Plasmids are the preferred support for toxin genes and subsequent genetic transfer and rearrangements in *C. perfringens* and, to some extent in *C. botulinum*, whereas in *C. difficile*, toxin genes are chromosomally located and are submitted to intense genetic variation. These aspects are discussed in [12,13,16,20,21].

It is not clear whether acquisition of toxin genes through horizontal gene transfer or gene evolution provides selective advantages to environmental bacteria such as clostridia. Clostridia are adapted to survive and develop in various environmental niches. Indeed, production of potent toxins leading to host death provides an abundant source of organic materials enabling massive growth of clostridia in cadavers, but bacterial multiplication in the intestinal tract throughout the life of the animal would result in greater *Clostridium* development over a longer period of time. Diseases due to toxigenic clostridia are an accidental connection between a host and an environmental bacterium which synthesizes a specific toxic compound, rather than resulting from bacterial strategy to invade an organism and subsequently survive in this new environment sheltered from host defenses.

But why is toxin synthesis by clostridia such a strongly regulated process? Toxin gene regulation was intensively analyzed in *C. perfringens*. Although some mechanisms of toxin gene regulation are conserved in toxigenic clostridia, distinct species such as *C. botulinum*, *C. difficile*, *C. sordellii* and *C. tetani* have developed specific regulatory pathways apparently triggered by different environmental factors, as discussed in [3,7,8,18]. Do clostridial toxins and their different regulation systems, coupled with specific environmental signals, play a role in circumventing the barrier effect of resident microbiota to colonize the host digestive tract, which is the most common organism site invaded by clostridia?

Intestinal and tissue colonization by clostridia also involves additional virulence factors like membrane proteins and other factors for attachment to various substrates. Whole genome sequencing is a powerful tool for revealing potential toxin and virulence factor genes and for estimating the potential pathogenicity of a *Clostridium* species or isolate. For example, genome sequencing of *C. tetani* led to identification of an array of cell-surface-associated proteins related to adhesins [4]. Recent genome sequencing of *Clostridium chauvoei*, the agent of severe myonecrosis in cattle, shed light on potential toxins and metabolic pathways possibly involved in pathogenesis due to this microorganism [11]. A major step was achieved, via whole genome sequencing, in unraveling the genetic evolution of pathogenic clostridia from saprophytic ancestors by highlighting phylogenetic relatedness and DNA exchanges, as well as deciphering putative virulence factors in addition to already known toxin genes. However, functional analysis of each gene possibly involved in pathogenicity, and its regulation network, remain to be performed. While clostridia are potent pathogens, they also provide efficient therapeutic tools, such as spores in transport systems for therapeutic molecules in the treatment of tumors [14], and toxins like BoNT in dystonia therapy.

Conflict of interest

None.

Acknowledgments

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