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Short Communication

Plasticity of *Corynebacterium diphtheriae* pathogenicity islands revealed by PCR

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ABSTRACT. Despite the existence of a vaccine against diphtheria, this disease remains endemic and is reemerging in several regions due to many factors, including variations in genes coding for virulence factors. One common feature of virulence factors is their high concentration in pathogenicity islands (PAIs), very unstable regions acquired via horizontal gene transfer, which has lead to the emergence of various bacterial pathogens. The 13 putative PAIs in *Corynebacterium diphtheriae NCTC 13129* and the reemergence of this disease point to the great variability in the PAIs of this species, which may reflect on bacterial life style and physiological versatility. We investigated the relationships between the large number of PAIs in *C. diphtheriae* and the possible implications of their plasticity in virulence. The GenoFrag software was used to design primers to analyze the genome plasticity of two pathogenicity islands of the reference strain (PiCds 3 and 8) in 11 different strains. We found that PiCd 3 was absent in only two

strains, showing genes playing putative important roles in virulence and that only one strain harbored PiCd 8, due to its location in a putative "hotspot" for horizontal gene transfer events.

Key words: *Corynebacterium diphtheriae*; Pathogenicity islands; Genome plasticity; PCR; GenoFrag; Horizontal gene transfer

Since the development of a vaccine based on the inactivation of the diphtheria toxin (DT), coded by the *tox* gene, the cases of diphtheria over the world have decreased drastically (Popovic et al., 2000). However, despite the existence of immunization with DT vaccine, the disease remains endemic in several regions (Mattos-Guaraldi et al., 2003), and more than 150,000 new cases of diphtheria were reported in the former Soviet Union in the 1990s (Nakao et al., 1996; Popovic et al., 2000; Sharma et al., 2007).

Although the reasons for the reemergence of diphtheria in those countries are not clearly elucidated, there are hypotheses that consider several possibilities, including changes in biochemical properties due to potential variations in genes coding for virulence factors harbored by the pathogen *Corynebacterium diphtheriae*, such as *tox* and DT repressor (*dtxR*) (Nakao et al., 1996; Popovic et al., 2000; Mattos-Guaraldi et al., 2003). According to Nakao et al. (1996), variations in the *tox* gene could lead to inadequate immune response from vaccination with the toxoids originated from the vaccinal strain, *C. diphtheriae PW8*, in people who had contact with the new variant strains.

The toxoid-based nature of the vaccine along with the fact that the *tox* gene occurs in a pathogenicity island (PAI) that was acquired from a corynephage, may also be responsible for the emergence of toxigenic strains causing recurrent infectious processes, since PAIs are very unstable genomic regions, which leads to fast acquisition/loss of genes (Tumapa et al., 2008). Besides, PAIs are acquired by horizontal gene transfer (HGT) events and harbor genes coding for virulence factors in pathogenic bacteria, and therefore, they are involved in the emergence of several bacterial pathogenic strains (Karaolis et al., 1998; Kauser et al., 2004; Gal-Mor and Finlay, 2006).

All together, these statements, along with the presence of 13 putative PAIs in *C. diphtheriae NCTC 13129* and the appearance of nontoxigenic strains causing deaths, point to a great variability in the PAIs of this species. In fact, Iwaki et al. (2010) have demonstrated the deletion of 11 of the 13 PAIs in the non-toxigenic *C. diphtheriae* strain *ATCC 27010*, a standard strain used in several works. This high plasticity may reflect on bacterial life style and physiological versatility, and the results obtained through comparative genomic approaches can elucidate its importance to bacterial evolution (Dobrindt and Hacker, 2001).

The aim of the present study was to investigate the relationships between the great number of PAIs in *C. diphtheriae* and the possible implications of their plasticity on virulence.

The GenoFrag software was used to design primers to perform analysis based on the method plasticity of chromosome revealed by long range-polymerase chain reaction (PCR) (Ben et al., 2004, 2006). The software was pre-configured to design primers with 25 nucleotides and 60-65°C of melting temperature, keeping other parameters immutable. Primers that generated amplicons and overlapping regions of approximately 5.7 and 1.0 kb, respectively, were chosen to perform the analyses since this length satisfactorily covers the extent of the third putative pathogenicity island of *C. diphtheriae* (PiCd 3).

PiCd 3 was chosen as a positive control since it is harbored by *C. pseudotuberculosis*

(third putative pathogenicity island of *C. pseudotuberculosis* - PiCp 3), another pathogenic bacterium from the same genus, therefore making this PAI potentially important for virulence in the described genus. Besides, PiCd 3 and PiCd 5 seem to be more stable than the others, since they are the only PAIs harbored by *C. diphtheriae ATCC 27010* (Iwaki et al., 2010). PiCd 8 was chosen as a model since it is substituted by another PAI in *C. pseudotuberculosis* (PiCp 5) with a different gene composition, which points to a putative "hotspot" for HGT. Furthermore, this island is one of the 3 PAIs that are concurrently absent on the *C. diphtheriae* strains *ATCC 27010* and *PW8* (Iwaki et al., 2010). Table 1 shows primers used to amplify regions inside the PiCds 3 and 8, designed from the genome sequence of *C. diphtheriae NCTC 13129*.

Table 1. Name, annealing position and nucleotide sequence of the primers used to amplify regions inside PiCds 3 and 8.

Name	Start	Nucleotide sequence	Name	End	Nucleotide sequence
PiCd 3 1F	250200	5'-AACGTCGACTTACCGGCCCCAGAAC-3'	PiCd 3 1R	255934	5'-CTGGTTCTCACTGCGTGTGGGTCTG-3'
PiCd 8 1F	1680348	5'-CCTCCGTCTCGATCACGACGAAGGT-3'	PiCd 8 1R	1686105	5'-ACGGGAGACCGTCAACAGCTCGATG-3'
PiCd 8 2F	1685049	5'-CCCCAGCATATTGGCCGTTGATGGG-3'	PiCd 8 2R	1690786	5'-TCCAGTTGTCTCGCCGGGTAGAAGG-3'
PiCd 8 3F	1690054	5'-AGCCTAGGCTTCCCCAGAACTCACG-3'	PiCd 8 3R	1695784	5'-TCTGGTGGATGGGCTATTCCGGTGG-3'
PiCd 8 4F	1694713	5'-AGACTCCGGTGTCCCTCAACACACC-3'	PiCd 8 4R	1700440	5'-CTACCGCGATATGGCGAACACCTGG-3'

We used 11 *C. diphtheriae* strains: *ATCC 27010* and *ATCC 27012*, standard strains for virulence studies of non-toxigenic and toxigenic strains, respectively; *CDC E8392*, from the Centers for Disease Control collection, and 8 Brazilian strains, 2 of them isolated from respiratory diphtheria (*TR 241* and *VA01*) and 6 from invasive infections (*814*, *402* and *HC01-04*). *ATCC 27010* was used as control.

C. diphtheriae strains were maintained in brain heart infusion (BHI) broth at 37°C, with stirring. Afterwards, chromosomal DNA sequences were obtained using phenol-chloroform and 10% lysozyme (Sambrook et al., 1989) and DNA concentrations were determined spectrophotometrically and on 0.8% agarose gels. Finally, amplifications were carried out in an MJ Research thermocycler as follow: 0.5-2 µL DNA solution, 2X Mix FideliTaq USB and 1 µL 100 µM of each primer in a final volume of 25 µL (sterile Milli-Q water). The mixture was denatured at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 30 s, 60°C for 30 s and 72°C for 10 min, with a final extension of 72°C for 12 min.

Figure 1 shows amplification patterns using 1 set of primers for PiCd 3 and 4 sets of primers for PiCd 8. Nine of the 11 strains had PiCd 3 amplicons, corroborating the putative

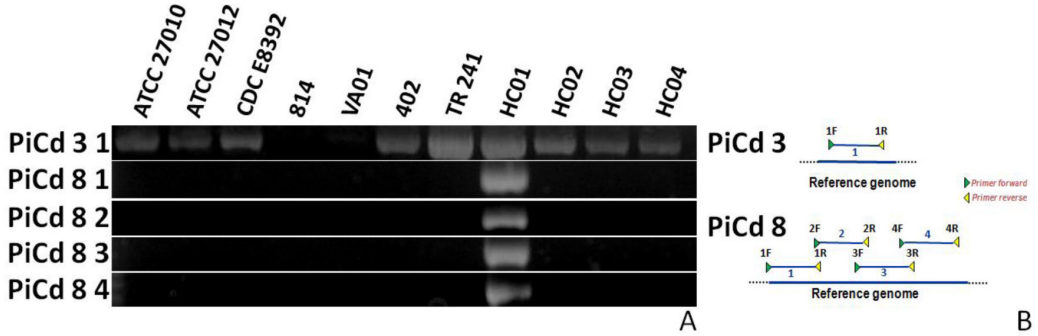

Figure 1. A. Electrophoresis gel exhibiting amplification patterns of PiCds 3 and 8. **B.** Graphic representation of primer annealing. PiCd 3 an PiCd 8 were amplified using 1 and 4 sets of primers, respectively.

importance of this island for the species and, possibly, for the genus *Corynebacterium*. Only strains 814 and VA01 did not have PiCd 3.

PiCd 3 harbors genes that play important roles in virulence. The *afuAB* operon, a *pitAB*-like operon, is responsible for acquiring iron from proteins, aiming to overcome the restricted availability of iron within hosts. As main characteristics, the *pitAB*-like transporters target proteins such as transferrin, hemin and ferritin and are commonly harbored by PAIs, and, due to their importance, they are frequently present in several copies, so the deletion of one operon will not affect virulence drastically (Brown et al., 2002).

PiCd 3 also harbors the *glpT* and *tcsS1-R1* genes. The *glpT* gene is responsible for mediating the transport of glycerol 3-phosphate across the membrane in bacteria and plays an important role during inorganic orthophosphate starvation (Enkavi and Tajkhorshid, 2010). Besides, the *glpT* gene is regulated by the *phoBR* genes, which show great similarity with the *tcsS1-R1* genes. In addition, the *pho* regulon is directly involved in the regulation of virulence genes of several bacteria, including an *Escherichia coli* strain pathogenic to pigs (Ishige et al., 2003; Pratt et al., 2010; Yoshida et al., 2010). Finally, the importance of the genes present in PiCd 3 corroborates its greater stability.

Unlike PiCd 3, PiCd 8 was present in only one of the *C. diphtheriae* strains analyzed, the *C. diphtheriae HC01*. This pattern may be due to the total absence of the PAI or due to insertions of great amounts of DNA inside each of the four regions of PiCd 8 analyzed, which could prevent the amplification of the fragments. Anyway, the two hypotheses point to the instability of PiCd 8 and corroborate the assignment of this region as a putative "hotspot" for HGT events.

PiCd 8 presents a high concentration of hypothetical and putative genes, a common feature of PAIs (Hsiao et al., 2005). On the other hand, the island harbors a GntR-like transcriptional regulator that plays a pivotal role in bacterial growth under carbon starvation. The *gntR* product acts in the presence of gluconate to negatively regulate gluconate permeases and kinases that are responsible for transporting and phosphorylating gluconate, respectively, in order to use it as an additional carbon source (Frunzke et al., 2008). However, although *gntR* is important in bacterial growth, imposing an obstacle for its deletion, it is present as three copies along the *C. diphtheriae* genome.

Finally, the high stability of PiCd 3 and the plasticity of PiCd 8 may provide valuable information about the lifestyle of *C. diphtheriae* making the studies of other pathogenicity islands in this organism a very important approach to gather data for the development of more effective vaccines and treatments for the medical disease known as Diphtheria.

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