Characterization of Adenylate Cyclase-Hemolysin Gene Duplication in a *Bordetella pertussis* Isolate

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We describe a clinical isolate of *Bordetella pertussis*, the agent responsible for whooping cough, composed of at least two clones harboring one or two copies of the *cya* locus encoding one of the major toxins, adenylate cyclase-hemolysin. No difference was observed between the two clones in murine and cellular models, probably due to the high instability of the *cya* locus duplication.

A detailed analysis of the etiologic agent of whooping cough, *Bordetella pertussis*, revealed large-scale chromosomal rearrangements (15, 16) probably due to the presence of numerous insertion sequences (IS) in the *B. pertussis* genome (12, 16). This genomic fluidity did not seem to affect genes encoding virulence factors composed of adhesins, such as filamentous hemagglutinin (FHA), fimbriae, and pertactin (PRN), and toxins, such as tracheal cytotoxin, pertussis toxin (PT), dermonecrotic toxin, and adenylate cyclase-hemolysin (AC-Hly). However, we describe here a *B. pertussis* clone producing an abnormally large amount of AC-Hly due to the duplication of the whole *cya* locus encoding the AC-Hly toxin (*cyaA* gene), the proteins required for its secretion (*cyaB*, *cyaD*, and *cyaE* genes), and the protein required to convert the inactive AC-Hly precursor into the active toxin (*cyaC* gene) (3, 10).

**Characterization of a highly hemolytic clone of the *B. pertussis* APV23a isolate.** The *B. pertussis* APV23a isolate displayed a mixture of phenotypically different colonies on Bordet-Gengou agar (BGA; Difco Laboratories, San Jose, Calif.) supplemented with 15% defibrinated sheep’s blood (Bio Merieux, Marcy l’Etoile, France). Two phenotypes, one highly hemolytic and unstable and one weakly hemolytic and very stable, were purified by subculturing. The DNA from the two clones was purified with a genomic tip (QIAGEN, Hilden, Germany), digested with either XbaI or SpeI, and analyzed by pulsed-field gel electrophoresis (PFGE) as previously described (11). The DNA profiles of the two purified clones obtained after digestion with XbaI were identical, with the exception of one high-molecular-weight fragment (Fig. 1A). The clone containing the 390-kb fragment was named 23ai, and the one presenting the 320-kb fragment was named 23am. The DNA profiles obtained after digestion with SpeI were also different, with the exception of one fragment of varied lengths: a 675-kb fragment in the 23ai clone and a 635-kb fragment in the 23am clone (Fig. 1B). PFGE analysis confirmed the plasmidic instability of the 23ai clone, which generated 23ai and 23am clones after subculturing, and the stability of the 23am clone, which never generated a 23ai clone.

It is now well established that hemolytic activity is carried out by the AC-Hly toxin, which also exhibits adenylate cyclase activity (3). Adenylate cyclase activity was measured directly, as described previously (9), on bacterial suspensions of 23ai and 23am clones grown on BGA. As expected, the 23ai clone exhibited higher adenylate cyclase activity (84 ± 31 nmol of cyclic AMP/min/ml) than the 23am clone (27 ± 8 nmol of cyclic AMP/min/ml). These data based on adenylate cyclase and hemolytic activities were confirmed at the level of protein production by using specific antisera and Western blotting as described previously (7). The concentrations of PT, FHA, and PRN were similar in 23ai and 23am bacterial suspensions, whereas the concentration of AC-Hly was higher in 23ai than in 23am (Fig. 1D).

**Duplication of the *cyaA* gene in the genome of the *B. pertussis* APV23a isolate.** To determine whether the phenotype of the 23ai clone was linked to the chromosomal size increase observed by PFGE analysis, we used the AC1-2 probe to hybridize the DNA fragments obtained after digestion with SpeI. This probe, specific to the 5′ end of the *cyaA* gene encoding AC-Hly, was amplified from the *B. pertussis* Tohama I strain (12, 15) with the primer pair formed by AC1 (5′-ATGCAGC AATCGCATCAGGCTGCTGTTAC-3′) and AC2 (5′-GC CGATCACCCGTGACCCTCGAAAT-3′) and was labeled with fluorogreen F-dUTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) by PCR using *Taq* polymerase (Amersham Biosciences). Southern blotting was performed as described previously (13). The hybridized DNA fragments were visualized by the enhanced chemiluminescence system (Amersham Biosciences). Both the 23ai- and 23am-specific fragments of 675 and 635 kb, respectively, hybridized, indicating that the *cyaA* gene is located in this fragment of varied lengths (Fig. 1C). To determine whether the 23ai clone harbored a *cyaA* gene duplication, we digested 23ai and 23am DNA with both SpeI and DraI, enzymes which cleave the chromosome outside the *cyaA* gene, according to the genome sequence (12). The AC1-2 probe hybridized a 270-kb fragment common to 23ai and 23am DNA products but also a 70-kb fragment present only in 23ai DNA products (Fig. 1D).
suggesting that the cyaA gene is duplicated in this clone.

To analyze more precisely the cyaA gene duplication, genomic DNA was digested with SmaI and Ascl, which also cleave outside of the probe sequences according to the chromosomal sequence (12), and hybridized with probes specific to each extremity of the cyaA gene, the AC1-2 and AC12-31 probes. The AC12-31 probe, which is specific to the 3'H11032 end of the cyaA gene, was amplified with the primers AC12 (5'H11032-AGGCAGGCGGGCCGTGA-3'H11032) and AC31 (5'H11032-GAGGGCTCGGACCTGCT-3'H11032) from B. pertussis Tohama I and labeled as described for the AC1-2 probe. As expected, in both the 23ai and 23am DNA products, AC1-2 hybridized a fragment of 10.2 kb for SmaI digests and one of 10.7 kb for Ascl digests, and AC12-31 hybridized a fragment of 13.8 kb for SmaI digests and one of 14.6 kb for Ascl digests. However, with the 23ai DNA products only, AC1-2 also hybridized fragments of 13.8 and 14.6 kb generated, respectively, by SmaI and Ascl digests. These results indicate that 23ai harbors two very similar or identical copies of the whole cyaA gene.

The CyaC probe, which hybridized almost the whole cyaC gene, was then used to determine whether the other genes of the cya locus were duplicated. This probe was amplified from Escherichia coli XL1 carrying pCAC3 (2) with the primers AC55 (5'CAGCGGGCGGTATCCGCCG-3') and AC56 (5'C CGTCGCCCAAGGGCCC-3'). Fragments of 10.2 and 10.7 kb that were generated, respectively, after SmaI and Ascl DNA digestion of both clones were hybridized. However, as before, a second fragment was also hybridized in the 23ai digestion products (13.8 and 14.6 kb for SmaI and Ascl digests, respectively), indicating that the duplication present in 23ai also contains the cyaC gene.

Considering the restriction sites that detected (Fig. 2C) and did not detect (data not shown) the duplication by Southern blotting and by comparison with the genome sequence, we constructed a partial map of the duplicated regions (Fig. 3). Both clones contained the cyaABDE operon and a cyaC gene similar to that of B. pertussis (Fig. 3). However, 23ai also contained a duplication of the five genes included in the cya locus bordered by a region of ~3 to ~4 kb upstream from cyaC and a region of ~9.5 to ~16 kb downstream from cyaE. This result is consistent with the higher production of AC-Hly by this clone. We hypothesize that IS481 is involved in the duplication of the cya locus, as this sequence is present on both sides of the cya locus (12, 14).

In vivo virulence of the two clones of the B. pertussis APV23a isolate. The AC-Hly toxin plays an important role in the pathogenesis of B. pertussis in the early stages of lung colonization (4, 5, 7, 17). Using the intranasal murine model described previously (7), we tested whether the 23ai clone was more virulent than the 23am clone in vivo. Female Swiss mice were purchased from Janvier (Le Genest-St.-Isle, France). All procedures involving animals were conducted in agreement with the Institut Pasteur animal care and use committee guidelines. Different amounts of bacteria were administered intranasally.
to groups of eight 4-week-old mice. The mean 50% lethal doses after 30 days were similar for 23ai (10^7 bacteria/mouse) and 23am (9 × 10^6 bacteria/mouse). However, all of the bacteria recovered from the lungs and the tracheas of mice 7 days after infection with the 23am clone exhibited the 23am phenotype and PFGE profile, whereas the bacteria recovered from the lungs and tracheas of the mice infected with the 23ai clone exhibited either the 23ai or the 23am phenotype and PFGE profiles (data not shown), confirming the high instability of the 23ai clone in vivo.

**Cytotoxicity towards macrophages and ability to invade HTE (human tracheal) cells of the two clones of the *B. pertussis* APV23a isolate.** We have previously shown that AC-Hly is the factor responsible for macrophage apoptosis (8). We thus analyzed the effect of cya locus duplication on cytotoxicity towards macrophages (J774A.1), as described previously (8). Cells were infected with the bacteria for 6 h at a bacterium/cell ratio of 100:1. Cytotoxicity was determined using the CytoTox96 assay (Promega), i.e., by measuring the amount of lactate dehydrogenase released by the cells after lysis. Both clones were equally cytotoxic (46% for clone 23ai versus 44% for clone 23am).

*B. pertussis* is not cytotoxic towards epithelial cells but possesses invasive properties (1). Since AC-Hly inhibits this invasive capacity (1), we compared the abilities of 23ai and 23am to invade HTE cells. As described previously (1), HTE cells were infected for 5 h at a bacterium/cell ratio of 100:1. Although 23ai produced more AC-Hly, no difference could be detected between its invasive capacity (approximately 1 bacterium/cell) and that of 23am or other *B. pertussis* isolates.

We describe here a *B. pertussis* clinical isolate that was composed of two clones, one of which harbors a duplication of the cya locus, which encodes AC-Hly, one of the major toxins expressed by *B. pertussis*. This phenomenon is rare but has also been observed in other clinical isolates (data not shown). Although AC-Hly plays an important role in the pathogenesis of *B. pertussis*, the clone producing more AC-Hly was not more virulent in the murine respiratory model or in a cellular model. However, this failure to demonstrate any difference could be due to the high instability of this clone in vivo and in vitro. It is possible that AC-Hly overexpression is necessary during human infection but not in vitro or that it is unstable in vitro or in the murine model. The murine model may not be the best model for comparing clones expressing different levels of AC-Hly. In fact, AC-Hly might have a higher affinity for the human
receptor CD11b/CD18 (6) than for the murine receptor (identities of 75 and 82%, respectively). It has also been shown that efficient secretion of AC-Hly requires an interaction with FHA (18). As the 23ai clone expresses more AC-Hly but not more FHA, it is possible that the excess of AC-Hly is not able to interact efficiently with host cells. In conclusion, further studies are required to determine whether this duplication plays a role during human infection or whether it is part of the adaptation of B. pertussis to humans. Precise clinical and molecular analyses of isolates collected from infected humans are especially important.

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