Characterization of \textit{Bordetella pertussis} clinical isolates that do not express the tracheal colonization factor

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\textbf{Keywords}

\textit{Bordetella pertussis}; TcfA; gene loss; multiple locus sequence typing; multiple-locus variable-number of tandem repeat analysis.

\textbf{Abstract}

\textit{Bordetella pertussis}, the causative agent of whooping cough, has remained endemic and there is a resurgence in some countries despite vaccination. \textit{Bordetella pertussis} produces a wide range of virulence factors which are assumed to play an important role in infection and transmission, including tracheal colonization factor (TcfA). Here we show that clinical isolates belonging to distinct lineages may lose their ability to produce TcfA. Irreversible and reversible loss occurred, respectively, by recombination between repeats leading to deletion of the \textit{tcfA} gene and by mutations in a polymorphic G-track. These phenomena may reflect adaptation to distinct niches.

\section*{Introduction}

\textit{Bordetella pertussis} and \textit{Bordetella parapertussis} are the causative agents of whooping cough, or pertussis, a highly contagious disease of the human upper respiratory tract (Mattoo & Cherry, 2005). \textit{Bordetella pertussis} is generally isolated from whooping cough cases with higher frequencies (70–95\%) compared to \textit{B. parapertussis}. The closely related species \textit{Bordetella bronchiseptica} infects a broad range of mammalian species, including humans (Diaratopoulos \textit{et al.}, 2005). Since the 1950s, effective vaccines against pertussis have been available and their introduction has led to a dramatic decrease in pertussis world-wide. However, despite more than 50 years of vaccination, whooping cough remains an endemic disease in many countries, with outbreaks occurring every 3–5 years. Globally, 48.5 million cases of pertussis are recorded with as many as 295 000 deaths, mainly among children (Crowcroft & Britto, 2002). \textit{Bordetella pertussis} produces a large number of virulence factors including tracheal colonization factor A (TcfA). TcfA is a member of the autotransporter family, which is able to direct its own transport across the outer membrane of the bacterium (Henderson & Nataro, 2001). During routine surveillance, we observed several \textit{B. pertussis} strains isolated from children in which the \textit{tcfA} gene was not detected by PCR. Here we characterize these and other \textit{tcfA} mutants and show that they arise by two different mechanisms. Expression of \textit{tcfA} was assessed with antibodies raised against peptides. Further, we show that the \textit{tcfA} mutations are found in epidemiologically unrelated isolates, suggesting that inactivation of the \textit{tcfA} gene occurred independently in different strains.

\section*{Materials and methods}

\textbf{Bacterial strains and culture conditions}

The characteristics of \textit{B. pertussis} isolates used in this study are listed in Table 1. The strains were grown on Bordet Gengou (Becton, Dickinson and Company, Franklin Lakes, NJ) agar supplemented with 15\% sheep blood and incubated for 3–5 days at 35 °C. All strains were isolated from pertussis patients. The age of the Belgian patients was 1 month for B2774, B3099 and B3100, 3 months for B2772, B2775 and B3098 and 5 years for B2773. Based on the age of the patients, we presume that three patients were unvaccinated, three patients were incompletely vaccinated and one
Table 1. Characteristics of Bordetella pertussis strains used in this study

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Country of origin</th>
<th>Year of isolation</th>
<th>MLVA type</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1998</td>
<td>36</td>
<td>1</td>
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<td>B2773</td>
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</tr>
<tr>
<td>B2774</td>
<td>Belgium</td>
<td>2001</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>B2775</td>
<td>Belgium</td>
<td>2002</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
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<td>3</td>
</tr>
<tr>
<td>B3099</td>
<td>Belgium</td>
<td>2005</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>B3100</td>
<td>Belgium</td>
<td>2006</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>B1191</td>
<td>USA</td>
<td>1993</td>
<td>27</td>
<td>5</td>
</tr>
<tr>
<td>B3008</td>
<td>Belgium</td>
<td>2006</td>
<td>27</td>
<td>4</td>
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<tr>
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</tbody>
</table>

MLVA, multiple-locus variable-number of tandem repeat analysis; ST, sequence type obtained by MLST; ND, not determined.

patient was fully vaccinated. No patient information was available from the Dutch and American strains.

PCR and sequencing

Chromosomal DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), following the instructions for Gram-negative bacteria. To sequence tcfA, the gene was amplified by PCR. Primers used for PCR and sequencing are listed in Table 2. PCR was performed by adding 1 μL DNA to 19 μL of a PCR mix consisting of 50% HotStarTaq Mastermix (Qiagen, Westburg), 1 M betaine (Sigma Aldrich), 10 pmol of PCR primer tcfA-F, 10 pmol of primer tcfA-R and 3 μL H₂O. Thermal cycling was as follows: 15 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C. A final extension was performed for 10 min at 72 °C. The PCR products were purified using ExoSap-IT (USB, Cleveland, OH), according to the manufacturer’s instructions, and sequenced with the same forward and reversed primers used for amplification. Sequence reactions were performed with an ABI Prism Big Dye terminator reaction kit and the reactions were analyzed with a model 3700 ABI DNA Sequencer (Perkin-Elmer Applied Biosystems). A DNA region containing tcfA and 1500 bases upstream and downstream sequences was amplified as follows. Chromosomal DNA 2 μL was added to 18 μL of a PCR mix consisted of 50% HotstarTaq Mastermix (Qiagen, Westburg), 5% dimethyl sulphoxide (ICN), 10 pmol of PCR primer tcfA-all F, 10 pmol of primer tcfA-all R and 5 μL H₂O. Thermal cycling was as follows: 15 min at 95 °C, 30 cycles of 10 s at 95 °C, 1 min at 58 °C, 5 min at 72 °C. A final extension was performed for 10 min at 72 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer’s instructions. After sequencing with tcfA-all sp1 F–sp 2 R, tcfA sequences were compared with the tcfA sequence of the B. pertussis Tohama I strain, GenBank accession number NC_02929. Primers used for sequencing are listed in Table 2.

Southern blot hybridization

Chromosomal DNA was purified using the Wizard Genomic DNA Purification Kit (Promega) and was digested with the restriction enzyme ClaI (Roche Diagnostics). B0613 was used as tcfA-positive strain. Southern blotting and hybridization with a biotin-labeled oligonucleotide probe was performed as described previously (Schouls et al., 2003). The tcfA probe for hybridization is listed in Table 2.

Peptide synthesis and immunization

Polyclonal rabbit anti-TcfA sera were raised at the Eurogentec facility (Eurogentec, Seraing, Belgium). New Zealand white rabbits (n = 2) were immunized with a mixture of two 16-mer synthetic tcfA peptides (CSLEDFKRSLQESAPS and CERGDDAGPKPPEGEG) coupled to KLH.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Bacterial cells were collected and separated on SDS-PAGE as described (Laemmli, 1970). Samples were mixed with loading buffer and boiled for 5 min before loading.
onto 4–20% Tris-glycine polyacrylamide gels (Pierce, Rockford, IL).

Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Buckinghamshire, UK), air-dried and blocked with 5% (w/v) Protifar (Nutricia, Zoetermeer, the Netherlands) and 0.05% (w/v) Tween20 (Merck, USA) in 0.08 M Tris HCl/1.5 M NaCl buffer pH = 7.3–7.5 and subsequently incubated for 1 h with polyclonal rabbit anti-TcfA sera. Membranes were washed three times for 10 min with Tris/NaCl buffer containing 0.05% (w/v) Tween20 and incubated for 30 min with goat anti-rabbit IgG conjugated to horseradish peroxidase (van Loo et al., 2002) (Pierce, Rockford, IL). Membranes were washed three times for 10 min with Tris/NaCl buffer containing 0.05% Tween20 (w/v) and as substrate, SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) was used. Signals were detected using the LAS–3000 Imaging System (Fujifilm, USA).

**Multiple locus variable-number of tandem repeat analysis (MLVA)**

For MLVA, the number of tandem repeats in six loci (VNTR1, VNTR3a, VNTR3b, VNTR4, VNTR5 and VNTR6) was determined (Schouls et al., 2004) (Table 3). MLVA profiles were clustered as described previously (Schouls et al., 2004).

**Multiple locus sequence typing (MLST)**

MLST was performed using seven genes known to be polymorphic in *B. pertussis* (van Loo et al., 2002) (Diatavopoulos et al., 2005). Four genes (*ptxA, ptxC, prn, fim2* and *fim3*) code for surface proteins, and two genes (*adk* and *tyrB*) code for housekeeping genes. Primers used are indicated in Table 2. For *prn*, the two regions harbouring repeats were sequenced, as previous research has indicated that variation is essentially limited to these regions (van Loo et al., 2002).

**Results**

**The tcfA gene is absent from epidemiologically unrelated Belgian *B. pertussis* clinical isolates**

To detect shifts in the bacterial population, clinical isolates in Belgium and the Netherlands are characterized with respect to a number of genes coding for surface proteins, including *tcfA* (van Loo et al., 2002) (De Schutter et al., 2003). In the period 1998–2002, four Belgian clinical isolates were observed in which *tcfA* was not detected with PCR. The primers used for PCR were positioned inside the *tcfA* gene, suggesting imperfect fit of the primers or absence of one or both primer binding sites. To investigate these possibilities, a PCR was performed with primers located outside the *tcfA* gene (primers *tcfA*-all F and *tcfA*-all R, Fig. 1, Table 2). With these primers, PCR fragments of 3 and 5 kb were found in the Belgian strains and the Tohama I strain, respectively (not shown). The latter strain is known to contain an intact *tcfA* gene (van Loo et al., 2002). These results indicated the presence of a 2-kb deletion in the region harbouring *tcfA* in Belgian isolates. As the *tcfA* gene comprises 1944 bases, this suggested that the complete gene was deleted. To confirm the absence of the *tcfA* gene, a Southern blot was performed using an oligonucleotide derived from the 5′-end of the *tcfA* gene (*tcfA*-probe) (Table 2). When chromosomal DNA was digested with ClaI, the probe revealed a 5-kbp band in B0613, which contains *tcfA*2. However, no hybridizing bands were detected in the Belgian strains (B2272, B2773, B2774 and B2775) (Fig. 2). To identify the boundaries of the deletion, the 3-kb PCR fragment derived from the isolates was sequenced. All four strains revealed the same sequence. Comparison with the Tohama I strain revealed a deletion encompassing 2326 bases. The deletion was located between two direct repeats of 539 bases, suggesting it was the result of homologous recombination (Fig. 1). To confirm the absence of the *tcfA* product, Western blotting was performed with rabbit anti-TcfA sera. A 60-kDa protein was detected in strains with an intact *tcfA* gene (B1831 and B1834), but not

<table>
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<tr>
<th>Strain</th>
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<th>VNTR3A</th>
<th>VNTR3B</th>
<th>VNTR4</th>
<th>VNTR5</th>
<th>VNTR6</th>
<th>MLVA type</th>
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<td>9</td>
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</tr>
<tr>
<td>B2934</td>
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<td>0</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>130</td>
</tr>
</tbody>
</table>

MLVA, multiple-locus variable-number of tandem repeat analysis.
in the four Belgian strains in which the gene was deleted (Fig. 3).

**tcfA Mutant strains are not confined to Belgium**

In our strain collection, 10 additional isolates were found which harboured mutations in the tcfA gene, seven from Belgium, two from the Netherlands and one from the USA (van Loo et al., 2002; and this work). One Dutch strain (B3008, isolated in 1980) contained the same deletion as the four Belgian strains. The three Belgian strains (B3098, B3099 and B3100, isolated in 2002, 2005 and 2006, respectively) and the strain from the USA (B1191, isolated in 1993) contained an insertion (tcfA5) and the second Dutch strain (B2934, isolated in 1984) contained a deletion of a G (tcfA9) in a polymeric G-track located in tcfA, resulting in a frame-shift and premature termination of translation (Fig. 1) (van Loo et al., 2002). The absence of TcfA in strains containing mutations in tcfA was confirmed by Western blotting (Fig. 3).

**tcfA Mutations are observed in distinct B. pertussis lineages**

To determine whether tcfA mutants arise in particular lineages, the MLVA and MLST types of the 10 strains were determined. Seven different MLVA types were found (Tables 1 and 3). A minimum spanning tree revealed that the tcfA mutations occurred in different B. pertussis lineages (Fig. 4). The two Dutch strains with MLVA types 92 and 130, were closely related according to the MLVA analysis, differing in

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**Fig. 1.** Overview of the *Bordetella pertussis* chromosomal region harbouring tcfA. The deletion found in some strains is indicated and corresponds to position 1 264 096–1 266 422 in Tohama I (Parkhill et al., 2003). The positions of the polymorphic G-track and the primers used for amplification are indicated. HP, hypothetical protein.

**Fig. 2.** Detection of tcfA in *Bordetella pertussis* strains. Chromosomal DNA was digested with Clal and hybridized to a tcfA-specific probe. The numbers on the left refer to base pair sizes of marker DNA fragments (MF).

**Fig. 3.** Detection of TcfA with specific antibodies. Western blotting was performed with polyclonal rabbit anti-TcfA antibodies. B2775 and B3099 are representatives of strains missing the complete tcfA gene (∆tcfA) or carrying a frame-shift mutation (tcfA5). The numbers on the right refer to the molecular mass of protein markers in kDa.
one locus, although they were isolated in different years. Six Belgian strains were found to differ in one or two loci and one Belgian strain, with MLVA type 70, was found to differ in at least three loci and was most closely related to the two Dutch strains with MLVA type 130 and 92. MLST was performed using seven genes known to be polymorphic in B. pertussis (van Loo et al., 2002). Six sequence types (STs) could be distinguished. The seven Belgian strains comprised four distinct sequence types: ST1 (four strains), ST2 (one strain), ST3 (one strain) and ST4 (one strain). The two Dutch strains revealed the same ST (ST6), whereas the strain from the USA belonged to ST 5 (Tables 1 and 4). Thus both the MLVA and MLST results suggest that the tcfA mutations occurred in distinct lineages.

Discussion

In this study, 10 epidemiologically unrelated B. pertussis tcfA mutants were characterized. Two classes of tcfA mutants were observed. In the first class, the tcfA gene was deleted, whereas the second class contained frame shifts in the tcfA gene. Five strains, four isolated in Belgium and one in the Netherlands, contained an identical deletion of 2326 bases, which removed the whole tcfA gene. The deletion was located between two direct repeats of 539 bases. The two repeats comprise the 5′-ends of bapB and tcfA, respectively, and code for a conserved C-terminal region, called the β-barrel required for transport across the bacterial cell wall (Fig. 1) (Henderson & Nataro, 2001). Thus the proximity of bapB and tcfA facilitates recombination between the conserved 5′-ends of these genes, resulting in deletion of tcfA. Five strains, isolated in Belgium, the USA and the Netherlands, belonged to the second class of mutants and harboured a single base insertion and deletion, respectively, in a homopolymeric G-track, resulting in a translational frame shift. Regions with reiterated bases are known to be hotspots for small insertions or deletions due to transient misalignment during replication (Streisinger & Owen, 1985). Such
regions are found in the promoter region of *B. pertussis* fimbrial genes and also in several structural genes (Willems *et al.*, 1990; Parkhill *et al.*, 2003) and allow phase variation, i.e. the reversible, random, switching between expression and nonexpression of genes. The *B. pertussis* *tcfA* mutants have been found in Belgium, the Netherlands, the USA (this work, van Loo *et al.*, 2002) and in the UK (Packard *et al.*, 2004) and in different years. Furthermore, they were present in distinct *B. pertussis* lineages. Finally, two mechanisms of *tcfA* inactivation were observed. This indicates that inactivation of *tcfA* has occurred independently in different strains. As yet we do not know whether the *tcfA* mutants are transmissible and able to persist in the human population. *tcfA* mutants are less well able to colonize mice, suggesting that *tcfA* is important for strain fitness, at least in the mouse model (Finn & Stevens, 1995; Chen *et al.*, 1998). However, *tcfA* is not expressed by *B. parapertussis* and *B. bronchiseptica* (Finn & Stevens, 1995), suggesting that its loss may be compensated by other genes. The evolution of *B. pertussis* has been accompanied by substantial gene loss and 9.4% of its genes are pseudogenes (Parkhill *et al.*, 2003). It is conceivable that the inactivation or loss of *tcfA* reflects the ongoing reduction of the *B. pertussis* genome. Alternatively, temporary loss of *tcfA* expression may confer an advantage during particular stages in the transmission cycle of *B. pertussis*. The loss of *tcfA* expression may be related to immunological pressure. For example, in all three countries where the TcfA mutants were observed, whole cell vaccines, which contain *tcfA*, have been used for more than 40 years. Compared to other *B. pertussis* surface proteins, TcfA is highly polymorphic (van Loo *et al.*, 2002), suggesting that an immune response targeting TcfA has a significant effect on bacterial fitness. Under such conditions, (temporary) loss of *tcfA* may be beneficial. Previous studies have suggested an important role for other *B. pertussis* surface antigens in adapting to vaccination with whole cell vaccines (King *et al.*, 2001). In the last decade, whole cell pertussis vaccines have been replaced by acellular vaccines which induce high antibody levels against a limited number *B. pertussis* proteins and this may result in new adaptations. Clearly, it is important to continue to monitor changes in the *B. pertussis* population.

**References**


