The length of poly(C) stretch in the *Bordetella pertussis* P fim3 promoter determines the vag or vrg function of the fim3 gene

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Abstract

*Bordetella pertussis*, a human pathogenic bacterium, produces either one or two types of serologically distinct fimbriae, Fim2 and Fim3, as virulence factors. The expression of fim2 and fim3 is regulated by the BvgAS two-component system and the length of poly(C) stretches in P fim promoters. In the Bvg⁺ phase, *B. pertussis* virulence-activated genes (vags) are up-regulated and virulence-repressed genes (vrgs) are down-regulated. Previous studies have shown that fim2 is a vag, but there is no consensus on fim3 regulation. We examined the regulation of fimbrial expression in *B. pertussis* clinical isolates. Our findings indicate that fim2 is a vag, while fim3 is a vag when P fim3 poly(C)>13C, and a vrg when poly(C)≤13C. Although increased fim3 expression was observed in the Bvg⁻ phase in isolates with P fim3 poly(C)≤13C, Fim3 production was not detected, suggesting post-transcriptional regulation of fim3 expression. These findings provide an insight into the regulation of fimbrial expression in *B. pertussis*.

*Bordetella pertussis* is the causative agent of whooping cough, or pertussis, and produces several virulence factors, including pertussis toxin, filamentous haemagglutinin, pertactin and fimbriae (Fim). The expression and production of genes encoding these virulence factors are controlled by the BvgAS two-component regulatory system [1]. In the BvgAS-activated (Bvg⁺) phase, this system controls two distinct subsets of genes, which either induce (virulence-activated genes, vags) or repress (virulence-repressed genes, vrgs) their expression. The transcriptional state mediated by BvgAS can be negatively modulated by adding MgSO₄ or nicotinic acid, or by reducing the temperature to 25 °C; the ensuing transcriptional state corresponds to the Bvg-repressed (Bvg⁻) phase, when vrgs, and not vags, are activated.

Fimbriae are filamentous cell surface structures that play a role in bacterial adherence. *B. pertussis* produces either one or two types of serologically distinct fimbriae, Fim2 and Fim3 (22.5 and 22.0 kDa, respectively) [2, 3]. The genes encoding the major fimbrial subunits, fim2 and fim3, are located in different parts of the *B. pertussis* chromosome and their expression is Bvg-regulated [4]. In addition, fim2 and fim3 gene expression independently undergoes phase variation via slipped-strand mispairing within monotonic cytosine stretches in gene promoter regions [i.e. P fim poly(C)] [4]. It has also been shown that the initial transcribed sequence of fim3 contains a cis-regulatory element (the down-repressive element, DRE), whose presence significantly decreases fim3 gene expression in *B. pertussis* cells [5]. Additional minor subunits, FimB, FimC and FimD, are required for the biosynthesis of *B. pertussis* fimbriae; these proteins are a chaperone, an usher protein and a tip adhesin, respectively [3]. In contrast to major fimbrial subunit genes, the genes encoding these accessory proteins, fimBCD, are located in the same operon as filamentous haemagglutinin, and are also regulated by the BvgAS system, as vags [3, 6].

In previous studies, fim2 has been reported to be a vag; however, no consensus has been reached about the regulation of the fim3 gene, i.e. whether fim3 is a vag or vrg [7–9]. In the *B. pertussis* reference strain Tohama I and its derivative BPSM, fim3 was demonstrated to be a vrg that was strongly expressed in the Bvg⁻ phase [7, 9]. However, in a recently identified clinical isolate GMT-1, fim3 was shown to behave as a vag [7].

In the present study, selected *B. pertussis* clinical isolates, producing either Fim2 or Fim3, were evaluated using genetic, transcript and protein analyses to better understand the mechanism(s) of fimbrial gene expression and production in this bacterium.
The *B. pertussis* isolates used in this study are listed in Table 1. FDA460, producing both Fim2 and Fim3, was chosen as a calibrator control isolate. The Tohama I strain and its derivatives, BPSM and BLOW, were used to compare the results with those of other studies. Five additional isolates producing Fim2, and five isolates producing Fim3, were also evaluated. The *B. pertussis* isolates were initially grown on Bordet–Gengou agar (Difco, Franklin Lakes, NJ, USA) supplemented with 15% v/v defibrinated sheep blood at 36 °C for 4 d. The bacterial cells were suspended in modified Stainer–Scholte (mSS) medium and incubated at 37 °C with constant agitation. Three independent biological replicates were analysed for each tested isolate.

First, the length of the poly(C) stretches in the *fim2* and *fim3* promoter regions in 14 *B. pertussis* isolates were examined by DNA sequencing (Table 1). *B. pertussis* genomic DNA was extracted using the DNeasy tissue kit (Qiagen, USA) according to the manufacturer’s instructions. Phusion high-fidelity DNA polymerase (New England Biolabs France, Evry) was used for *Pfim2* and *Pfim3* poly(C) stretch analysis. The primer sets used for sequencing of the *fim2* and *fim3* genes and their promoter regions are listed in Table S1 (available with the online Supplementary Material). With respect to the *Pfim2* poly(C) lengths, the Fim2-producing isolates (Fim2 and Fim2/3 strains) harboured 12–15C, whereas the isolates that did not produce Fim2 (Fim3 strains) harboured 9–11C. Meanwhile, with respect to the *Pfim3* poly(C) lengths, the Fim2/3- and Fim3-producing isolates harboured 13–15C, whereas the Fim2 isolates only harboured 9–13C. These findings are in agreement with previous reports on *Pfim* poly(C) lengths [8, 10, 11]. As described in Table 1, Fim2 and Fim3 isolates were both shown to harbour 13C in the Pfim3 region. From the sequencing chromatograms, we observed that Tohama I (Fim2 isolate) mainly harbours 13C in Pfim3, but this strain does not produce Fim3. Therefore, Pfim3-13C is considered to have an inactive poly(C) length. Fim3 isolates were also shown to harbour 13C and 14C in Pfim3, but in contrast to Tohama I, these isolates produce Fim3. This can be explained based on the abundance ratio of Pfim3-14C (active poly(C) length), which is superior to that of 13C (inactive poly(C) length) in Fim3 isolates. The presence of a DRE sequence located just downstream of the *Pfim3* +1 start site was also examined. All of the tested isolates possessed this element, irrespective of Fim serotype.

Next, to verify the *fim2* and *fim3* gene expression levels in the *Bvg*+ and *Bvg*− phases, quantitative real-time RT-PCR (qRT-PCR) was performed. After overnight incubation in mSS medium, bacterial suspensions were used to initiate cultures in mSS medium or mSS medium supplemented with 50 mM MgSO4 (mSS+MgSO4). The initial OD650 of cultures was 0.2 and the cells were grown until the mid-log phase, OD650=1; 10 ml of the cultures was harvested by centrifugation and stored at −80 °C for subsequent RNA extraction. Total RNA was prepared by extraction with TRIzol reagent (Life Technologies France, Saint Aubin) according to the manufacturer’s instructions. *B. pertussis* FDA460 was used as a calibrator strain in all of the qRT-PCR experiments. The primer sequences and reaction conditions are described in Table S1. Changes in transcript levels were determined by the comparative CT method, using the actual efficiency values; statistical analyses were performed using a Student’s *t*-test [12]. Values of *P*<0.05 were considered to be statistically significant. The *fim2* gene was expressed in all

### Table 1. Characteristics of selected *Bordetella pertussis* strains and isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Year*</th>
<th>Fim serotype</th>
<th>Poly(C) length</th>
<th>Allele type</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td><em>Fim2</em></td>
<td><em>Fim3</em></td>
<td><em>fim2</em></td>
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<td>14–15C</td>
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<td>12–13C</td>
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<tr>
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<td>1999</td>
<td>3</td>
<td>11C</td>
<td>13–14C</td>
<td>A</td>
</tr>
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</table>

*Year of isolation.
†Tohama I derivative, streptomycin-resistant strain.
‡Derivative of the BPSM strain with the entire bvgA gene and the 5’ terminus of the bvgS gene deleted.
Fig. 1. Quantitative analysis of fim2 and fim3 mRNA levels, and Fim2 and Fim3 protein production in 14 *B. pertussis* strains and clinical isolates grown in mSS or mSS+MgSO₄ media. (a) fim2 and (b) fim3 mRNA levels were first calibrated against the recA gene, and then normalized to mRNA levels in *B. pertussis* reference strain FDA460. (c) Fim2 and (d) Fim3 production was measured by ELISA with biotinylated anti-Fim2 or anti-Fim3 monoclonal antibodies. Protein levels were determined by measuring A₄₅₀ and normalized to protein levels in the reference strain *B. pertussis* FDA460. Error bars represent standard deviations of biological replicates (n=3) and technical triplicates. Significance was determined using Student’s t-test. *P<0.05; **P<0.01; NS, not significant.
Fim2 and Fim2/3 isolates when the bacteria were grown in mSS medium (Bvg⁺ phase), but not when they were grown in mSS+MgSO₄ medium (Bvg⁻ phase) (Fig. 1a). Fim3 isolates did not express the fim2 gene in either growth medium (Fig. 1a). This observation confirmed that B. pertussis fim2 gene is a vrg [7, 9]. Meanwhile, the fim3 gene expression pattern was different: Fim2 isolates repressed the expression of the fim3 gene in the mSS medium, but not during growth in mSS+MgSO₄. Fim2/3 and Fim3 isolates expressed the fim3 gene when cultured in mSS medium, but the expression was significantly reduced during growth in mSS+MgSO₄. These findings indicated that the fim3 gene can act as a vrg in B. pertussis Fim2 isolates, and as a vrg in Fim2/3 and Fim3 isolates. This phenomenon was associated with the length of the Pfim3 poly(C) stretch in each isolate (Table 1). B. pertussis vrg genes are known to be repressed by BvgR protein in the Bvg⁺ phase, and activated by RisA in the Bvg⁻ phase [13–16]. Analysis of the B. pertussis Tohama I genome (GenBank accession no. BX470248) revealed that the fim3 coding region and the Pfim3 region contain putative binding sequences for BvgR (5'-CGCTG-3') and RisA (5'-AAATT-3'), respectively, as in the promoter regions of other vrgs [14, 15]; however, these sequences are not present in the fim2 and Pfim2 regions. These observations might explain the differences in the fim2 and fim3 gene expression profiles.

To determine whether Fim2 and Fim3 production is directly linked to fim2 and fim3 gene expression, Fim2 and Fim3 protein levels were measured in Bvg⁺ and Bvg⁻ phase cells (Fig. 1c, d). B. pertussis serotyping was performed by agglutination tests as previously described [17, 18], and ELISA was performed using anti-Fim2 and anti-Fim3 monoclonal antibodies (NIBSC, UK) [19]. For ELISA serotyping, aliquots of mid-log cultures (1 ml) in mSS or mSS+MgSO₄ were immediately heated at 56 °C for 1 h and used; monoclonal antibodies were labelled with biotin to allow detection using a biotin–streptavidin system (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). The biotinylated anti-Fim2 (1 : 100) and anti-Fim3 (1 : 50) antibodies were diluted with the blocking buffer. Heat-inactivated bacterial cells were diluted in PBS (1 : 400 for Fim2 detection and 1 : 100 for Fim3 detection) and 100 µl of diluted cells were added to Nunc Immunoplate Maxisorp (Thermo Scientific, Denmark) at 25 °C overnight. After washing and treatment with blocking buffer (10 v/v% FBS in PBS), biotinylated anti-Fim2 (1 : 100) and anti-Fim3 (1 : 50) antibodies were added to blocking buffer. After 1 h at 25 °C, the plates were washed and streptavidin–HRP and TMB substrate reagent (Thermo Fisher Scientific France, Villebon) were added. After 10 min, the reaction was stopped by adding 2M H₂SO₄. Then, A₄₅₀ was measured in the Multiskan FC microplate photometer (Thermo Fisher Scientific France, Villebon), using A₄₅₀ readings as a reference. Fim2 protein was detected in Fim2 isolates when they were cultured in mSS medium, but this production was significantly reduced during growth in mSS+MgSO₄ medium (Fig. 1c). Fim2 protein was not detected in Fim3 isolates, regardless of whether they were cultured in mSS or mSS+MgSO₄ media, as anticipated. Despite the fact that the Fim2 isolates expressed the fim3 gene in the Bvg⁺ phase (Fig. 1b), Fim3 protein production was not detected (Fig. 1d). We hypothesize that this discrepancy between gene expression and protein production might be due to the regulation of fimbrial accessory genes, fimBCD, known as BvgA-activated genes, i.e. vags [3, 6, 7, 9, 20, 21]. The fimbrial protein levels were measured by ELISA in this study and consequently we detected the fimbrial protein exposed to the cell surface. Thus, there are two possibilities: (1) Fim3 protein is not generated, or (2) Fim3

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**Fig. 2.** Hypothetical mechanisms of the regulation of fim2 and fim3 gene expression in B. pertussis. (a) fim2 gene expression and the Fim2 production mechanism. (b) fim3 gene expression and the Fim3 production mechanism. The thick lines are mechanisms demonstrated in this study; dashed lines are hypothetical mechanisms. The molecules that require further investigation to confirm their actual roles are shown in grey.
protein is generated but not secreted from the cell without FimBCD accessory proteins. Fim2/3 and Fim3 isolates produced Fim3 protein in the Bvg<sup>−</sup> phase, and Fim3 protein production in the Bvg<sup>+</sup> phase was reduced, in accordance with the fim3 gene expression pattern and in agreement with a vag pattern.

In conclusion, the current study confirmed that the <i>B. pertussis</i> fim2 gene acts as a vag in all of the tested isolates, while the fim3 gene can be a vag in Fim2/3 and Fim3 isolates, and a vrg in Fim2 isolates. As summarized in Fig. 2, the expression of the fim2 and fim3 genes, and the production of Fim proteins, might be closely associated with the length of P<sub>fim</sub> poly(C) stretches. We hypothesize that BvgR and RisA proteins regulate fim3 expression as for other vrgs when the length of the P<sub>fim</sub> poly(C) stretch is ≤13C (i.e. an isolate producing Fim2). However, Fim3 production does not take place, because fimbrial accessory proteins are not produced. These findings provide an insight into the regulatory mechanisms of <i>B. pertussis</i> fim3 gene expression and Fim3 protein production, resolving the contradictory findings of previous reports. Further investigations are required to verify this hypothesis, e.g. Western blotting against fimbrial accessory proteins, or a binding assay of BvgR or RisA to the putative binding sequences of fim3.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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