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Strong inhibition of fimbrial 3 subunit gene transcription by a novel downstream repressive element in *Bordetella pertussis*

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Abstract

The Bvg-regulated promoters for the fimbrial subunit genes *fim2* and *fim3* of *B. pertussis* behave differently from each other both *in vivo* and *in vitro*. *In vivo* P_{fim2} is significantly stronger than P_{fim3}, even though predictions based on the DNA sequences of BvgA binding motifs and core promoter elements would indicate the opposite. *In vitro* P_{fim3} demonstrated robust BvgA~P-dependent transcriptional activation, while none was seen with P_{fim2}. This apparent contradiction was investigated further. By swapping sequence elements we created a number of hybrid promoters and assayed their strength *in vivo*. We found that, while P_{fim3} promoter elements upstream of the +1 transcriptional start site do indeed direct Bvg-activated transcription more efficiently than those of P_{fim2}, the overall promoter strength of P_{fim3} *in vivo* is reduced due to sequences downstream of +1 that inhibit transcription more than 250-fold. This element, the DRE (downstream repressive element), was mapped to the 15 bp immediately downstream of the P_{fim3} +1. Placing the DRE in different promoter contexts indicated that its activity was not specific to *fim* promoters, or even to Bvg-regulated promoters. However it does appear to be specific to *Bordetella* species in that it did not function in *E. coli*.

Keywords

Bordetella pertussis; global virulence regulation; virulence gene promoters; promoter architecture; transcriptional activation

Introduction

Whooping cough caused by *Bordetella pertussis* is a highly contagious respiratory disease that is persistent in developing countries and reemerging in developed countries, despite well accepted vaccination programs. As with many Gram-negative bacterial pathogens, adherence of *B. pertussis* to the host respiratory tract is thought to play an important role in establishing and maintaining infection (Mooi *et al.*, 1992; Cotter *et al.*, 1998). Filamentous hemagglutinin (FHA) and fimbriae are two of the most well-recognized adhesins produced

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in *B. pertussis* (Relman *et al.*, 1989; Weiss & Goodwin, 1989) and have been included as antigen components in current acellular pertussis vaccines (Poolman & Hallander, 2007).

In *B. pertussis* the serologically distinct fimbriae 2 and fimbriae 3 are composed primarily of the major subunit proteins Fim2 and Fim3. Interestingly, while the chaperone and usher proteins FimB and FimC, and the minor subunit and tip adhesin FimD, are encoded in the same operon as filamentous hemagglutinin (Locht *et al.*, 1992; Willems *et al.*, 1992), the genes encoding the pilin major structural subunits are at widely separated chromosomal locations (Stibitz and Garletts, 1992). Each of these fimbrial subunits undergoes phase-variation at a rate much higher than random mutation. The mutational change leading to altered expression of *fim2* or *fim3* is the addition or deletion of one basepair within a monotonic stretch of Cs in the promoters P_{fim2} and P_{fim3}, presumably due to slipped-strand mispairing during replication (Willems *et al.*, 1990). We have previously determined an optimum length of this C-stretch to permit maximal transcription of *fim2* and *fim3* (12 and 15 Cs, respectively), as well as a normally silent *fimX*. In the latter case, inactivation was due to a much shorter C-stretch in its promoter that presumably could not switch on spontaneously by the insertion of one or small number of basepairs (Chen *et al.*, 2010).

Expression of virulence genes in *B. pertussis*, and in the related species *B. bronchiseptica* and *B. parapertussis*, is regulated globally by a two-component system comprising the membrane-spanning sensor histidine-kinase BvgS and the phospho-accepting response regulator BvgA (Weiss *et al.*, 1983; Aricó *et al.*, 1989; Stibitz and Yang, 1991). In standard culture media at 37°C BvgA, phosphorylated by BvgS, activates the transcription of multiple virulence genes (*fha*, *cya*, *ptx*, etc.) while it represses a separate group of genes known as *vrg*s (Knapp & Mekalanos, 1988). A third class, exemplified by the gene *bipA*, is maximally expressed under conditions intermediate between those resulting in maximal activation and repression. This is due to activation when levels of BvgA~P are low or moderate, and repression when they are high (Williams *et al.*, 2005). Unlike many other two-component sensor kinases, the ground state of BvgS appears to be “on”. Specific activators of BvgS have never been identified, and recent crystallographic evidence suggests an active conformation in the absence of ligands (Herrou *et al.*, 2010). However, the Bvg^- state, in which the BvgS kinase is inactive, can be induced by the addition of compounds including $MgSO_4$ or nicotinic acid or by culture at reduced temperatures (Lacey, 1960; Boulanger et al., 2013). This phenomenon is known as modulation.

Even among Bvg-activated genes, the kinetics of gene activation after a shift from Bvg^- to Bvg^+ mode are different, and depend on the affinity and/or number of BvgA~P binding sites. These features of promoter architecture dictate a given promoter’s response to the different levels of BvgA~P encountered following a shift (Scarlato *et al.*, 1991). For example, as shown in Fig. 1A, the early gene promoter P_{fha}, which has one strong primary and two weak secondary binding sites for BvgA~P (Boucher *et al.*, 2001a; Boucher *et al.*, 2003), is activated at the initial stages of induction when BvgA~P level is low; whereas the late gene promoter P_{ptx} that is activated by BvgA~P binding to six sites of moderate strength is activated hours later when high levels of BvgA~P have been reached (Scarlato *et al.*, 1991, Boulanger *et al.*, 2013). It has long been known that fimbrial production is ultimately controlled by BvgAS at two points. One is through the direct binding of BvgA~P to P_{fha}.

This in turn activates the transcription of P_{fha}, which co-transcribes, as part of the *fha* operon, the *fimBCD* genes required for pilus secretion, assembly, and function. The other control point, expression of the unlinked pilin structural genes *fim2* and *fim3*, was also shown over two decades ago to be ultimately BvgAS-regulated (Willems *et al.*, 1990). However, we have confirmed only recently that this regulation involves direct binding of BvgA~P to the *fim* promoters (Chen *et al.*, 2010). As summarized in Fig. 1B, each of these promoters has one primary, stronger, upstream binding site and one secondary, weaker, downstream binding site in a BvgA binding geometry generally similar to that of P_{fha}. However, unlike P_{fha}, in which the proximal secondary BvgA~P binding site is juxtaposed to the −35 region, the secondary binding site of the *fim* promoters is centered on the −35 region, which is contained within the C-stretch. Further study (Decker *et al.*, 2011) on the spatial interrelationships of BvgA~P, RNAP, and $P_{fim3\text{-}15C}$ promoter DNA, revealed that Region 4 of the sigma factor of RNAP retains its normal location relative to the −35 region of the promoter despite the highly non-consensus sequence (CCCCCC vs. the consensus TTGACA). We also previously demonstrated that the $^{-15}TG^{-14}$, present in P_{fim3} but not P_{fim2}, contributes significantly to P_{fim3} activity. The presence of this functional extended −10 motif in a promoter similar to P_{fim2} is inconsistent with the weaker transcriptional activity we observed from P_{fim3} *in vivo* (Chen *et al.*, 2010). Here we report that $P_{fim3\text{-}15C}$ promoter sequences down to and including the transcription start at +1 direct stronger transcription than their counterparts in $P_{fim2\text{-}12C}$. However, a repressive element (DRE), present immediately downstream of the transcription start, represses this strong $P_{fim3\text{-}15C}$ activity. Furthermore, inhibition of transcription by the DRE is not specific to Bvg-regulated promoters, but is specific to *Bordetella* species, suggesting that it may involve an intrinsic property of *Bordetella* RNAP or the involvement of a species-specific co-factor.

Results

P_{fim3} exhibits strong transcriptional activity *in vitro* but not *in vivo*

Previously, using promoter-luciferase fusions integrated as single copies at an arbitrary but constant location in the chromosome of *B. pertussis* strain BP536, we showed that the C-stretch-length-optimized *fim* promoters $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$ were activated directly by BvgA (Chen *et al.*, 2010). These promoter sequences demonstrated identical spacing of their main features, from the upstream primary BvgA binding sites to the core features of their inferred −35 regions (concentric with secondary BvgA binding sites), −10 regions, and transcription starts. Only P_{fim3} contains the extended −10 sequence $^{-15}TG^{-14}$, which has been shown to be required for robust $P_{fim3\text{-}15C}$ transcription (Chen *et al.*, 2010; Decker *et al.*, 2011; Fig. 1B for $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$). In this study, in order to understand the relationships between promoter structure and promoter activity, we compared $P_{fim2\text{-}12C}$, $P_{fim3\text{-}15C}$, and the known Bvg-regulated strong promoter P_{fha}. These promoters and all hybrids or variants thereof were examined in single copies of an “ectopic” promoter-luciferase fusion in *B. pertussis* BP536 as previously reported (Chen *et al.*, 2010). Relative promoter strengths are presented in Fig. 1D. As expected, luciferase activity was undetectable with the pSS3967 vector containing no promoter (control), or with the non-permissive shortened C-stretch promoters $P_{fim2\text{-}10C}$ and $P_{fim3\text{-}13C}$. With optimized C-stretch promoters, $P_{fim2\text{-}12C}$ was ~6-fold more active than $P_{fim3\text{-}15C}$, indicating that it is a stronger

promoter *in vivo*, consistent with our previous observation (Chen *et al*., 2010). Relative to the strong promoter P_{fha}, transcription from both $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$ was much weaker, with ~8-fold and ~47-fold decreased activity, respectively (Fig. 1D), suggesting that *fim* promoters, especially $P_{fim3\text{-}15C}$, are weaker than P_{fha}, one of the strongest *B. pertussis* promoters described to date.

In contrast, the levels of transcription observed from these promoters *in vitro* using *E. coli* RNAP and BvgA~P were quite different. While transcription from the optimized *fim3* promoter $P_{fim3\text{-}15C}$ was comparable to that from P_{fha}, (Chen *et al*., 2010; Decker *et al*., 2011), we were unable to detect transcription from $P_{fim2\text{-}12C}$ (data not shown), consistent with our observation that *in vivo* $P_{fim2\text{-}12C}$ is a weak promoter relative to P_{fha} (Fig. 1D). Taking together our observations from *in vivo* and *in vitro* studies, it was evident that $P_{fim3\text{-}15C}$ is highly competent at promoting transcription *in vitro*, but not *in vivo* in *B. pertussis*.

P_{fim3} possesses a downstream repressing element (DRE) that inhibits its transcription *in vivo* in *B. pertussis*

Given the disparity between the *in vivo* and *in vitro* transcriptional activities of $P_{fim3\text{-}15C}$, we speculated that it possesses sequence feature(s), not found in $P_{fim2\text{-}12C}$, that are either stimulatory *in vitro* or repressive *in vivo*, or that P_{fim2} possesses features with opposing activities. As shown in Fig. 1B, a sequence comparison of P_{fim3} vs P_{fim2} suggests two significant differences that could affect promoter activity. First, P_{fim3} appears to possess a primary BvgA-binding site of higher affinity than that of P_{fim2}. In both promoters, the upstream, primary, high-affinity BvgA-binding site is composed of two heptads, each directing the binding of one monomer of a BvgA-dimer. In both, the rightward, downstream heptad is nearly identical and very close to the consensus high-affinity sequence. However, the leftward, upstream heptad of P_{fim3} is predicted to be of higher affinity than that of P_{fim2} as determined by the application of an algorithm based on mutational studies (Boucher *et al*., 2001b, Merkel *et al*, 2003). This heptad in P_{fim3} has a moderately good score of −5.5, while that of P_{fim2} has a poor score of −12. The secondary binding sites of both promoters are nearly identical and coincide at 10 of 14 bp with the C-stretch. The second notable difference in the *fim2* vs *fim3* promoter sequences is within core promoter itself where P_{fim3} possesses an extended −10 $^{-15}$TG^{-14} region. This element is known to contribute to promoter activity in a number of systems, including P_{fim3} (Decker *et al*., 2011). Thus, two sequence features of P_{fim3} are predictive of higher promoter activity relative to $P_{fim2,}$ and are consistent with the higher level of $P_{fim3\text{-}15C}$ activity observed *in vitro*. The fact that these features fail to result in a stronger overall promoter *in vivo* is consistent with the hypothesized existence of an *in vivo* inhibitory sequence element unique to P_{fim3} in *B. pertussis*.

To map the positions of functional sequence differences, we constructed hybrid promoter fragments by swapping segments of $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$. We first constructed two hybrid promoters by swapping BvgA-binding regions, to create H1 containing the upstream BvgA binding regions of $P_{fim3\text{-}15C}$ together with the downstream core region of $P_{fim2\text{-}12C}$, and H2 with the complementary hybrid sequence (depicted in Fig. 1C). Readouts of transcriptional

activity by light production *in vivo* are shown in Fig. 1D. Pairwise comparisons of these results indicate that core promoter and/or downstream sequences are most important. For example, substitution of that region in $P_{fim3\text{-}15C}$ with the corresponding sequences from $P_{fim2\text{-}12C}$ results in a substantial increase (H1) whereas the complementary substitution to P_{fim2} results in a decrease (H2). This is in contrast to the lack of effects of BvgA binding site substitutions in that $P_{fim3\text{-}15C}$ and H2 have a similar level of activity, as do $P_{fim2\text{-}12C}$ and H1. Taken together these results indicate that key differences between the two promoters are downstream of the BvgA binding sites. It also indicates that the *fim2* promoter contains the more optimal combination of core promoter, transcriptional start, and downstream sequences, an observation that is inconsistent with the presence of the extended −10 element in P_{fim3}, but not P_{fim2}.

To functionally map relevant sequence features we constructed additional swaps of more specific sequence features. As shown in Figs. 2A and 2B, these were: 1) the T (in P_{fim2}) in the −35 region/secondary BvgA-binding site/C-stretch (H9 and H15), 2) the extended −10 element (H10 and H16), 3) the −10 element (H11 and H17), 4) downstream sequences (H7 and H13), 5) just the +1 initiating nucleotide (H19 and H25), and 6) +1 plus downstream sequences (H8 and H14). Although there were some changes in activity when upstream promoter sequences were swapped (as detailed below), the most dramatic differences arose when the sequences just downstream of +1 were exchanged. Addition of the P_{fim2} downstream sequences to P_{fim3} (H13 and H14) increased transcription of P_{fim3} over 250-fold (also seen in Fig. 1D for H13 activity relative to $P_{fim3\text{-}15c}$) while the reciprocal substitutions (H7 and H8) reduced transcription 50-fold (relative activity 0.02). The effect of these substitutions was not influenced significantly by the identity of the initiating nucleotide and substitution of the +1 nucleotide itself (H19 and H25) had only a three-fold effect in either direction. These results indicate that the downstream sequences of P_{fim3} are repressive or that those of P_{fim2} have a stimulatory effect. To address this question directly, we deleted the downstream sequences of P_{fim3} in an incremental manner. The adjacent pSS3967 vector sequence that replaces the deleted sequences in each construct is shown in faded gray with the exception of nucleotides identical to those in P_{fim3}, at a given position. As shown in Fig 2C, leaving either 10 or 15 bp of the native downstream sequence intact maintained a moderate repressive effect, while reduction to 0 or 5 bp had a profound stimulatory effect. We highlighted the first 10 bp (ACGCCAAGCA) in dark green to indicate its repressive effect observed in the deletion study. These results indicate that P_{fim3} harbors a repressive element, not that P_{fim2} harbors a stimulatory one. We have termed this element the DRE for downstream repressive element.

To accurately localize DRE, we swapped 5-bp incrementally, from both directions, of the +1 downstream regions of $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$, as depicted in Fig. 2D and 2E. Among the DRE swapped-in variants in $P_{fim2\text{-}12C}$, only H32-H35, containing at least 10 bp, in dark green, of the immediate downstream sequence of $P_{fim3\text{-}15C}$, nearly abolished $P_{fim2\text{-}12C}$ activity (Fig. 2D). As shown in Fig. 2E, of the reciprocal substitutions of P_{fim3}, only H28-H30 (Fig. 2E), retaining at least 15 bp, 5 of them marked in light green (CATGA), of the immediate downstream sequence of $P_{fim3\text{-}15C}$, maintained the repressive effect. These data

localize the DRE to the 15 bp (in both dark and light green) immediately downstream of the initiation nucleotide of P_{fim3}.

The profound changes observed with the DRE were not seen with other swaps. The "T" swaps in the C-stretch, H9 and H15, did not change promoter activity significantly, consistent with the previous finding of little or no influence of the sequence content of the C-stretch on promoter function (Chen et al., 2010). Results with the −10 and extended −10 were qualitatively as expected although the quantitative levels were unexpected. For example, while hybrid H10, in which P_{fim2} acquired the extended −10 sequence, showed a 23-fold increase in activity, in keeping with the importance of this element, the reciprocal exchange (H16) had only a three-fold negative effect on P_{fim3}. Similarly, the substitution of the −10 element of P_{fim2} with that of P_{fim3} (H11) resulted in a 14-fold reduction, suggesting that the latter was weaker, while the reciprocal exchange (H17) had no effect on P_{fim3}. These results suggest that function of the −10 region may not be entirely independent of the effects of the DRE (see below), and that the $P_{fim3\text{-}15C}$ −10 element (TATTCT) is somewhat weaker than the $P_{fim2\text{-}12C}$ −10 element (TAAGAT), despite their possession of four matches to the consensus of canonical −10 (TATAAT), including the most crucial nucleotides TA---T (Hook-Barnard & Hinton, 2007).

DRE represses promoter activity in a manner that is independent of BvgA-promoter binding and BvgAS regulation

$P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$, whose activity can be repressed by DRE, constitute a distinct subset of BvgA-activated promoters. These promoters contain a unique architectural feature in that the most downstream (secondary) BvgA-binding site is concentric with the position expected of a *bona fide* −35 element (see Fig. 1B). Binding of BvgA~P to this secondary binding site is essential for promoter activation, and our previous analyses suggest that productive contacts with RNAP are made by this BvgA dimer (Decker *et al.*, 2011). Specifically, we have demonstrated that, in an active ternary transcriptional complex, the alpha subunit C-terminal domain, Region 4 of sigma, and BvgA~P are all localized spatially to the same linear segment of promoter DNA, but to different faces of the helix. This architecture is in contrast to that of the BvgA-activated promoters of other genes including *fha, ptx, cya, prn* and *bvgR*, in which the most downstream secondary BvgA-binding site is juxtaposed to, but is not overlapping, the putative −35 element. Also, as shown recently, $P_{fim3\text{-}15C}$ plays host to BvgA and RNAP in a conformational complex different from that at P_{fhaB} (Decker *et al*., 2011). Given the discovery of the DRE in context of the unique architecture of the *fim* promoters, we sought to determine if its effects could be seen on other BvgA-regulated or non-BvgA-regulated promoters.

To this end, we tested the DRE repression effect on the BvgA-activated promoters P_{fha} and P_{ptx}. We constructed promoters in which 20 bp of the DRE-containing region (+1 to +21 of P_{fim3}), was placed immediately downstream of the transcription starts of P_{fha} and P_{ptx}. Compared to the wild type P_{ptx} and P_{fhaB} the resulting $P_{ptx\text{-}DRE}$ and $P_{fhaB\text{-}DRE}$ both showed dramatically reduced activity (Fig. 3). Effective DRE repression of other BvgA-activated promoters thus strongly suggests that the unusual *fim*-like promoter architecture apparently dictating atypical specific interactions between BvgA and RNAP is not required for DRE to

function as a repressive element. In addition, since the distance from the DRE to the positions of BvgA binding sites varied in these constructs, we speculated that BvgA-promoter binding is not required for DRE function, but rather that RNAP, without the transcriptional activator, interacts with DRE to block transcription. To test this hypothesis we used a BvgA independent promoter that is a mutational variant of the *gapA* promoter described by (Thouvenot *et al.*, 2004). This promoter variant, here termed P_{gapA*}, has consensus −35 and −10 elements as well as the extended −10 $^{-15}$TG^{-14} dinucleotide. As shown in Fig. 3, P_{gapA*} was active in *B. pertussis*, but in constructs where DRE sequences were placed in the same context as previously, i.e. directly downstream of the initiating nucleotide, activity was greatly reduced. This observation of similar DRE repression effects observed on both BvgA-dependent and BvgA-independent promoters demonstrates that the DRE does not require interaction with BvgA for it to manifest its repressive effects.

Although it was clear that specific BvgA-binding was not directly involved in DRE-mediated repression it was still possible that DRE repression was one of the many aspects globally regulated by BvgAS in *B. pertussis*. To address this we included the modulator $MgSO_4$ in growth media. In *B. pertussis* grown in modulating media, BvgA is present in its unphosphorylated form (Boulanger et al., 2013). It therefore cannot activate BvgA-dependent promoters. We first tested the effect of modulation on the DRE effect on a BvgA-dependent promoter. As shown in Fig. 4A and Fig. 4B, the weak transcription of $P_{fim3\text{-}15C}$ was totally abolished in the presence of $MgSO_4$ in wild type *B. pertussis* strain BP536, thus demonstrating its BvgA-dependent behavior. Removal of DRE from $P_{fim3\text{-}15C,}$ in hybrid promoter H13, did not affect the level of modulation, which remained complete, thus demonstrating that the DRE does not interfere with $MgSO_4$ modulation, nor is modulation required. However, in this case, we could not discern if the repressive effects of DRE were subject to BvgAS regulation. To address this question we tested the effect of modulation on DRE's ability to repress a BvgA-independent promoter. As shown Fig. 4D and Fig. 4E, P_{gapA*} activity in wild type *B. pertussis* BP536, as expected, is constitutive and independent of BvgA regulation, whereas $P_{gapA*\text{-}DRE}$ remained repressed in the presence of $MgSO_4$. In addition, we used a *bvgA* deletion *B. pertussis* strain BP1526 and observed the same patterns of activities of all the tested promoters as that in the modulated condition (Fig. 4C vs. Fig. 4B; Fig. 4F vs. Fig. 4E). The observed constitutive repression effect of DRE on P_{gapA*} suggests that it is unlikely that DRE-activity is regulated as part of the BvgAS global virulence regulon.

DRE function is conserved and specific to Bordetella species

It has been known that *B. pertussis* is different from its ancestor species *B. bronchiseptica* in many aspects, such as pertussis toxin production, utilizable growth conditions, and host range, due to the result of genomic changes (Parkhill *et al.*, 2003; Mattoo & Cherry, 2005 and ref. therein). Consequently, we compared sequences downstream of the transcriptional start of P_{fim3} among *Bordetella* species and found that the DREs are identical in sequence (data not shown). To check if the DRE is also functionally conserved, we tested the effect of DRE in *B. bronchiseptica* strain RB50 by using DRE-containing promoters integrated as promoter-*lux* fusions in the same way as in *B. pertussis* strain BP536. We found that both $P_{fim3\text{-}15C}$ and $P_{gapA*\text{-}DRE}$ are similarly repressed in both *B. pertussis* and *B. bronchiseptica*

(Fig. 5). Therefore, DRE is conserved not only in its sequence but also in its function within *Bordetella* species.

However, DRE was not functional when tested in *E. coli*. When we introduced P_{gapA*} and $P_{gapA*\text{-}DRE}$ into *E. coli* strain SM10 as plasmid-encoded luciferase-fusions, no repressive effect of the DRE was observed, with the difference being only ~1.9-fold. We did not use $P_{fim3\text{-}15C}$ for this test since it is not transcribed in *E. coli*. Failure to repress P_{gapA*} promoter activity in *E. coli* suggests that functioning of DRE involves a difference between the *Bordetella* and *E. coli* RNAPs, an unknown factor present only in *Bordetella* but not in *E. coli*, or both.

Discussion

The genetic organization of genes involved in fimbrial biosynthesis in *B. pertussis* is notable in two respects. First, while the genes coding for the chaperone, usher, and adhesin proteins are encoded within the same operon as the filamentous hemagglutinin (Willems et al., 1992; Locht et al., 1992), genes for the major fimbrial structural protein are at unlinked locations. Second, these genes, *fim2*, *fim3*, and *fimX*, are capable of undergoing independent phase-variation by changes in the length of a run of C residues in their promoters (Willems et al., 1990). While it has been known for sometime that fimbrial expression is ultimately regulated by the *bvgAS* locus, the demonstration that BvgA interacts directly with *fim* promoters is relatively recent (Chen et al., 2010; Decker et al., 2011). While the *fimX* promoter appears to be essentially silenced due to a too-short C-stretch, a comparison of the *fim2* and *fim3* promoters, each optimized in the length of their C-stretch for maximal expression, has revealed a large difference in promoter strength, with the *fim2* promoter exhibiting approximately 6-fold higher activity *in vivo*.

This discrepancy is puzzling for two reasons. One is that the *fim3* promoter is predicted, on the basis of its core promoter elements, to be the stronger promoter than *fim2* promoter. Although each promoter has a nearly identical, very poor, −35 region (CCCCCC or CCCTCC, due to its location within the C-stretch), the *fim3* promoter has the extended −10 motif consisting of a TG dinucleotide one basepair upstream of the −10 element. This element, combined with a strong −10, has been shown to compensate for the absence of a −35 element and to contribute to promoter strength when a −35 is present (reviewed in Hook-Barnard & Hinton, 2007). The −10 elements of the two promoters would both be expected to be moderately strong and roughly equal, since each contains the most highly conserved nucleotides in the pattern TA - - - T. Therefore, the *fim3* promoter, with its extended −10 $^{-15}$TG^{-14} dinucleotide, would be predicted to be a stronger promoter *in vivo*. However, the opposite was observed. A second puzzling discrepancy was revealed in *in vitro* transcription experiments. *In vitro*, the *fim3* promoter shows strong BvgA~P-dependent transcriptional activity, although none has been detected from the *fim2* promoter (Chen *et al.*, 2010; Decker *et al.*, 2011; Decker, unpublished).

To understand these discrepancies, we created a number of hybrid promoters, with key sequence segments exchanged between $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$, and measured their activities *in vivo*. Our results demonstrated that the 15 bp sequence just downstream of the

transcription start site of P_{fim3} (called here DRE), contains a repressive *cis*-element that significantly attenuates transcription *in vivo*. Furthermore, DRE activity is not BvgA-dependent, but it is dependent on some aspect specific to *Bordetella* since DRE inhibition was observed in both *B. pertussis* and *B. bronchiseptica*, but not in *E. coli*, *in vivo*. The apparent discrepancies cited in the previous paragraph can now be understood. Based only on core promoter elements and predicted BvgA binding strength, P_{fim2} should be weaker than P_{fim3}, accounting for the lack of detectable activity *in vitro. In vivo*, where the DRE inhibits transcription only of P_{fim3}, the opposite is true.

In other *in vitro* work using purified *E. coli* RNAP or *B. pertussis* RNAP, we have shown that the presence of the DRE results in a novel inhibitory transcription complex at P_{fim3} when non-phosphorylated BvgA is present, but not in the presence of BvgA~P (Boulanger et al., unpublished). These results might appear puzzling in light of the *in vivo* work here. However, the results of both studies are consistent with the idea that the DRE is an inhibitory sequence whose function is affected by as yet unknown conditions or factors *in vivo*. Furthermore, even though it is not yet clear how DRE inhibits transcription *in vivo* or forms an inhibitory complex *in vitro*, it is clear that the DRE has a dramatic affect on transcription, presumably at the step of initiation. Previously, work in *E. coli* has identified sequences just downstream of the +1 start that affect the promoter strength *in vitro* and *in vivo* (Kammerer *et al.*, 1986), but the mechanism of this action has not been determined. In addition, extensive work has characterized sequences immediately downstream of the transcription start site of some lambdoid phage promoters that are needed for σ-dependent pausing and the loading of an antitermination factor, such as λ Q protein (reviewed in Perdue & Roberts, 2011). However, the DRE sequence does not appear to share significant similarities with the sequences described in either of these systems. Thus, an investigation of DRE function *in vivo* and *in vitro* is expected to elucidate both the complex regulation of fimbrial gene expression and a novel mechanism of regulation during promoter clearance.

Experimental procedures

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB broth or on LB agar. *B. pertussis* and *B. bronchiseptica* strains were grown on BG agar (Chen *et al.*, 2010). The antibiotics used in LB for *E. coli* strains were ampicillin, 100 μg ml^{-1}; gentamicin, 5 μg ml^{-1}. The antibiotics used in BG agar for *B. pertussis* and *B. bronchiseptica* strains were streptomycin, 50 μg ml^{-1}; gentamicin, 10 μg ml^{-1}.

Plasmid and strain constructions

The promoters H1 - H40, which are hybrids of $P_{fim2\text{-}12C}$ and $P_{fim3\text{-}15C}$ were constructed using a variation of the method of Stemmer and Morris with the hybrid promoters cloned into the promoter assay vector pSS3967, between *EcoR*I and *Sal*I sites upstream of *luxCDABE* (Stemmer & Morris, 1992; Chen *et al.*, 2010). The generated hybrid sequences are depicted in Fig. 1B and Fig. 2ABDE. Plasmids pQC1440 to pQC1444 (Fig. 2C) are identical to pQC1157 (pSS3967::$P_{fim3\text{-}15C}$), except that the regions downstream were shortened by PCR amplification with different downstream primers. Plasmids pQC1840

(pSS3967::P_{ptx}) and pQC1843 (pSS3967::P_{fhaB}) contain PCR-generated *EcoR*I and *Sal*I promoter fragments of *ptx* and *fhaB*, respectively, in pSS3967. Plasmid pQC1631 contains P_{gapA*}, with the sequence tacggTTGACActgcgtaaggtttgTGtTATAATacaggcAacccc (−35 element, extended −10 motif, −10 element and +1 in uppercase), inserted as two complementary oligonucleotides in pSS3967. Subsequently, the +2 to +21 downstream regions of P_{ptx} and P_{fhaB}, and +2 to +6 downstream region of P_{gapA*} were replaced by the +2 to +21 downstream sequence of $P_{fim3\text{-}15C}$ (ACGCCAAGCACATGACGGCA), to generate plasmids pQC1841 (pSS3967::$P_{ptx\text{-}DRE}$), pQC1844 (pSS3967::$P_{fhaB\text{-}DRE}$), and pQC1761 (pSS3967::$P_{gapA*\text{-}DRE}$). *E. coli* DH5α was used as a host for cloning.

To introduce the promoter-*lux* fusions into *Bordetella*, the pSS3967-based plasmids were integrated, respectively, in single copy at an ectopic site in the chromosome of *B. pertussis* strain BP536 or BP1526, and *B. bronchiseptica* strain RB50 using *E. coli* strain SM10 as a donor in conjugation, as previously described (Chen et al., 2010).

In vivo luciferase activity assay

Bacterial strains harboring promoter-*lux* fusions in pSS3967 were streaked in sectors and grown at 37°C for 48 h for *B. pertussis* strain BP536, for 24 h for *B. bronchiseptica* strain RB50 on BG agar (streptomycin 50 μg ml^{-1}; gentamicin, 10 μg ml^{-1}), or for 24 h for *E. coli* strain SM10 on LB plate (ampicillin 100 μg ml^{-1}; gentamicin, 5 μg ml^{-1}). To modulate BvgAS-mediated regulation in *B. pertussis*, 50 mM $MgSO_4$ was included in the BG agar. The light output was revealed and analyzed as described previously (Chen et al., 2010). The data, averaged from at least 4 assays, were presented as a relative to the wild type promoter control strain or to the most luminescent strain on a given plate.

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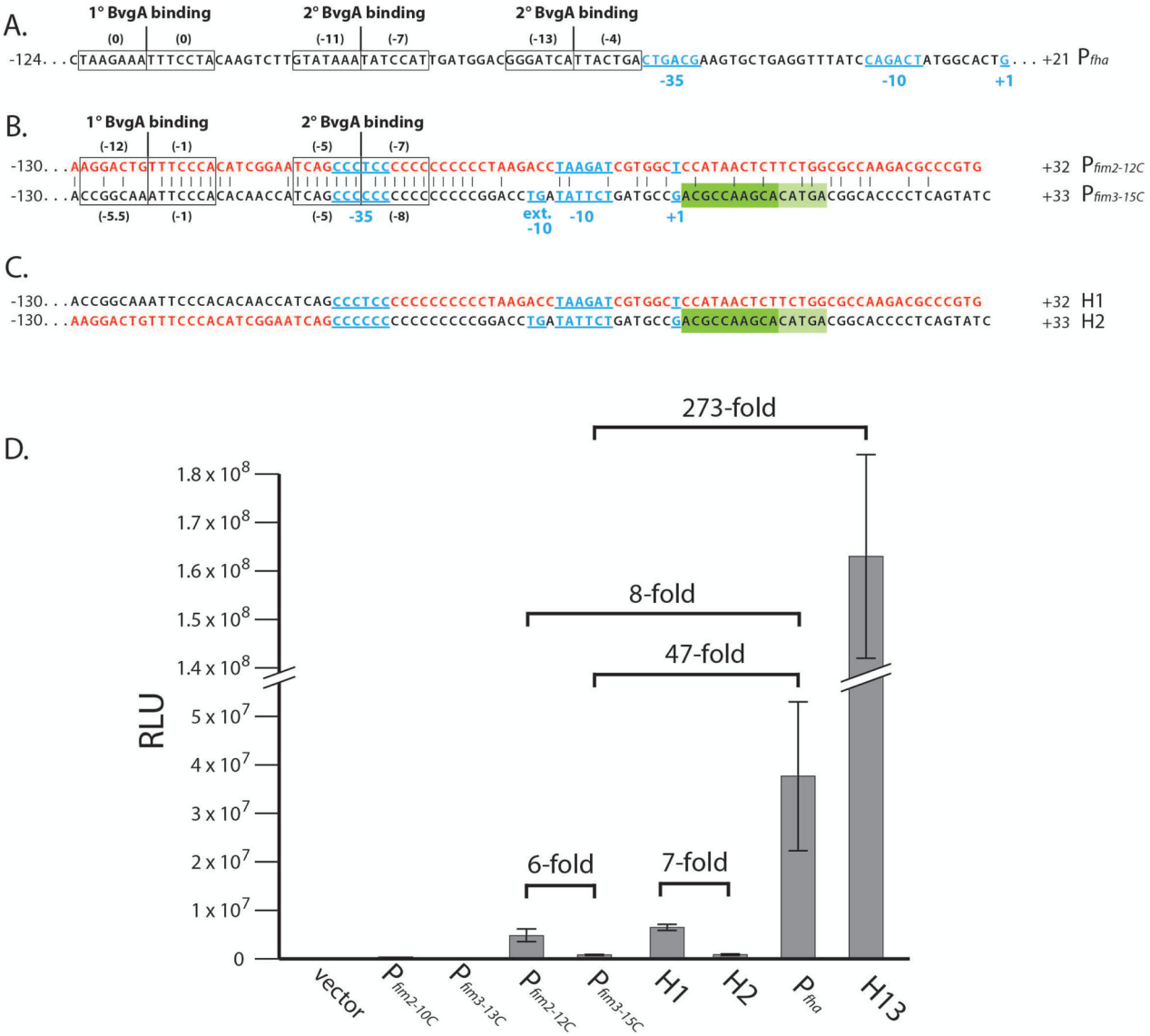

Fig. 1. $P_{fim3\text{-}15C}$ transcriptional activity in *B. pertussis*

(A). Sequence of P_{fha}. The −35 region, −10 element, and transcription start site +1 are in blue underlined. The numbers at each end indicate the extent of cloned fragments. The BvgA binding sites are boxed, with a vertical line to indicate the center, and with numbers in parentheses that denote scores of the individual half-sites using an algorithm to assess BvgA~P binding strength (Chen *et al.*, 2010, Merkel *et al.*, 2003).

(B). Sequence comparison of $P_{fim2\text{-}12C}$ (in red) and $P_{fim3\text{-}15C}$ (in black). Identical residues are denoted by vertical dashes. The −35 region, extended −10 $^{-15}$TG^{-14} motif, −10 element, and transcription start site +1 are in blue underlined. Positions +1 to +15 constituting the DRE are indicated by a dark and light green rectangles to indicate its repressive effect observed in the deletion or swapping analysis. The numbers at each end and the BvgA binding sites are indicated as in (A)

(C). Cloned hybrid sequence for H1 and H2 with colors as in (B) to indicate sequence source.

(D). *B. pertussis* strain BP536 harboring chromosomally integrated pSS3967 containing no insert (control), or different promoter constructs were grown on BG agar and analyzed for light production by luciferase as described in the Experimental Procedures. Values are given in arbitrary units (RLU for relative light units). Data averaged from at least four assays were used in the calculation of standard deviations as indicated by error bars.

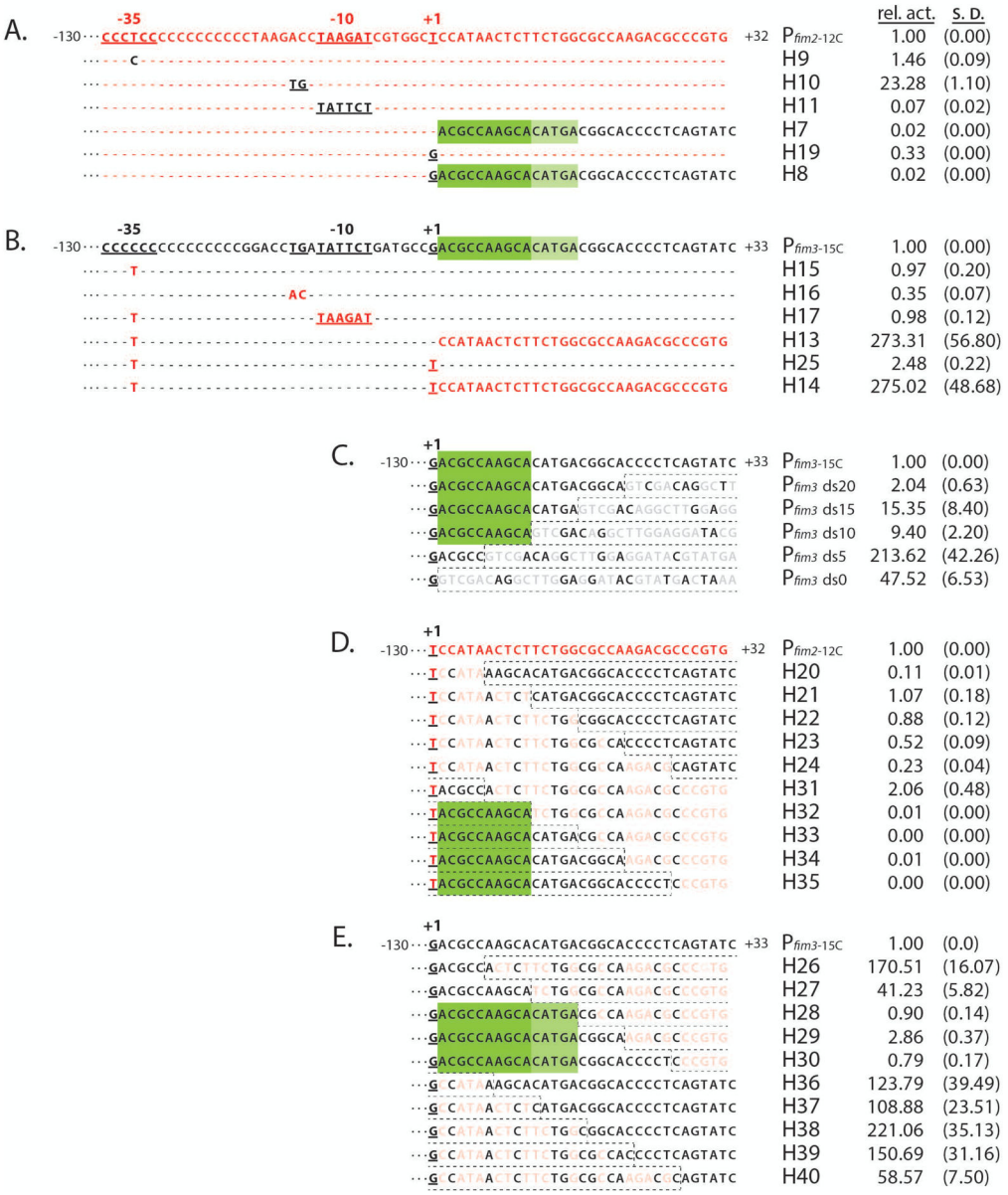

Fig. 2. DRE represses $P_{fim3\text{-}15C}$ transcription in *B. pertussis*

Transcriptional activities of *fim* hybrid promoters were assessed *in vivo*. Promoter fragments comprising sequences from −130 to +32 (P_{fim2}) or +33 (P_{fim3}) were cloned into the *luxCDABE* transcriptional vector pSS3967, the resulting constructs were inserted into the chromosome of BP536 at a constant, ectopic, location, and light output was measured as described in Experimental Procedures. Promoter activities normalized to the wild-type in each set are presented to the right, with values for standard deviation in parentheses. Based on the data in each panel, the inferred extent of the DRE is indicated by green rectangles (A & B). Relevant portions of the nucleotide sequences of the *fim2* promoter (A) and the *fim3* promoter (B) are presented with sequence elements exchanged to create hybrid

promoters indicated below and in contrasting color. Unchanged nucleotides are depicted by dashes.

(C). Deletion analysis of the downstream region of P_{fim3}. Downstream sequences were deleted to varying extents. Vector sequences substituting for promoter sequences are boxed by dashed lines. Nucleotides identical at a given position to those in the wildtype *fim3* promoter are shown in black, and those that are different are depicted in light gray.

(D & E). Hybrid promoters were derived by substituting, to varying extents, sequences downstream of +1 with those of the alternate promoter. Substituting sequences are boxed by dashed lines with those differing at a given position shaded out.

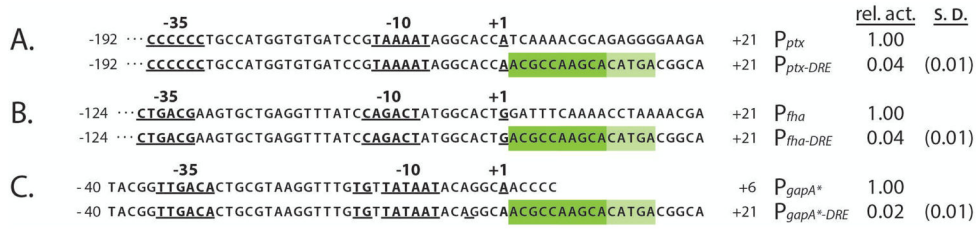

Fig. 3. Universal promoter repression by DRE in *B. pertussis*

Wild-type *ptx* (A), *fha* (B), and *gapA** (C) promoters were compared to those in which sequences downstream of +1 were substituted with those of P_{fim3}, which contain DRE. All promoters were assessed, and data are presented, as described for Fig. 2.

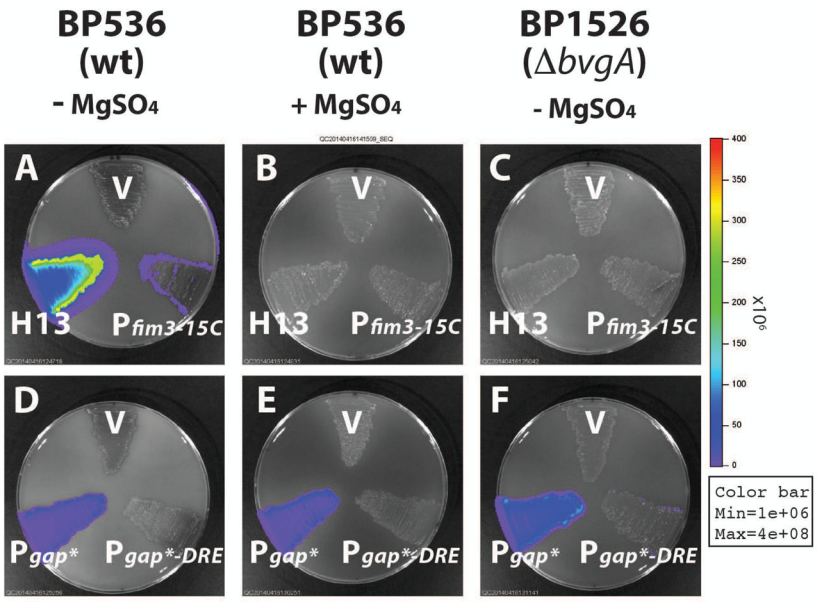

Fig. 4. BvgAS-independent repression by DRE in *B. pertussis*

The effects of DRE under different growth conditions and genetic backgrounds were determined by comparing $P_{fim3\text{-}15C}$ with its DRE-free variant H13 (A, B and C), and P_{gapA*} with its DRE-containing variant $P_{gapA*\text{-}DRE}$ (D, E and F). *B. pertussis* strains BP536 (wild type) were grown on BG agar supplemented with 50 mM $MgSO_4$ (B and E) or with no $MgSO_4$ added (A, and D). *B. pertussis* strains BP1526 (Δ*bvgA*) were grown on BG agar supplemented without 50 mM $MgSO_4$ (C and F). Light output was measured as described in Experimental Procedures. Error bars indicate standard deviation as calculated from the results of four independent assays.

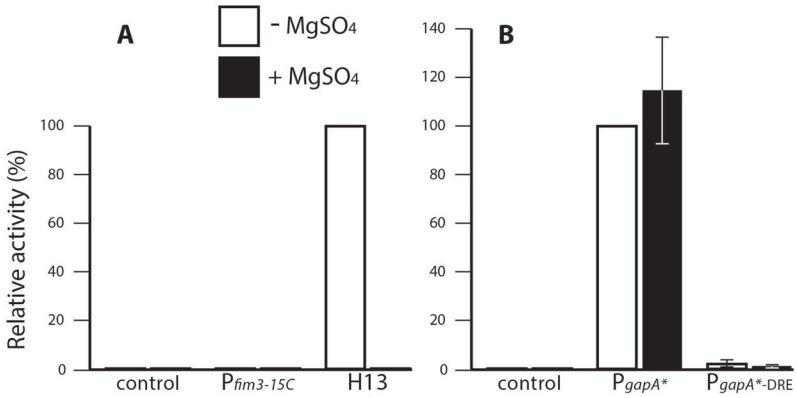

Fig. 5. Repression by DRE is observed in *Bordetella* species, but not in *E. coli in vivo*

Plasmids containing $P_{fim3\text{-}15C}$, $P_{fim3\text{-}15C}$ lacking DRE (H13), P_{gapA*}, or P_{gapA*} containing DRE ($P_{gapA\text{-}DRE}$) were introduced in single copy in the chromosome of *B. pertussis* strain BP536 and *B. bronchiseptica* strain RB50, or as plasmid multi-copies in *E. coli* strain SM10. The resulting strains were grown at 37°C on BG plates for *Bordetella* strains, or on LB plates for the *E. coli* strain, and analyzed for the light output as described in Experiment Procedures. The activity of the DRE-containing promoter (solid box, $P_{fim3\text{-}15C}$, $P_{gapA\text{-}DRE}$) is expressed as a percentage of that of the DRE-free promoter (open box, H13, P_{gapA}) in each species. Error bars indicate standard deviation as calculated from the results of four independent assays.

Table 1

Bacterial strains and plasmid used in this study

Strain or plasmid	Relevant features	Source or reference
E. coli		
DH5	High-efficiency transformation	Bethesda Research Laboratories
SM10	Mobilization of RK2 *oriT* plasmids	Simon *et al*. (1983)
B. pertussis		
Tohama I	Patient isolate	Kasuga, *et al*. (1954)
BP536	Tohama I, Str^R, Nal^R	Stibitz and Yang (1991)
BP1526	BP536, *fha-lacZ, ptx-phoA*, Δ*bvgA*-1215	Chen, *et al*. (2010)
Plasmids		
pSS3967	*luxCDABE* promoter assay vector	Chen, *et al*. (2010)
pQC1023	pSS3967:: $P_{fim2\text{-}10C}$	Chen, *et al*. (2010)
pQC1025	pSS3967:: $P_{fim2\text{-}12C}$	Chen, *et al*. (2010)
pSS4159	pSS3967:: $P_{fim3\text{-}13C}$	Chen, *et al*. (2010)
pQC1157	pSS3967:: $P_{fim3\text{-}15C}$	Chen, *et al*. (2010)
pHn	pSS3967:: hybrids ($P_{fim2\text{-}12C}$/$P_{fim3\text{-}15C}$)	This study
pQC1440	pSS3967:: $P_{fim3\text{-}15C\text{-}ds0}$	This study
pQC1441	pSS3967:: $P_{fim3\text{-}15C\text{-}ds5}$	This study
pQC1442	pSS3967:: $P_{fim3\text{-}15C\text{-}ds10}$	This study
pQC1443	pSS3967:: $P_{fim3\text{-}15C\text{-}ds15}$	This study
pQC1444	pSS3967:: $P_{fim3\text{-}15C\text{-}ds20}$	This study
pQC1840	pSS3967:: P_{ptx} [a]	This study
pQC1841	pSS3967:: $P_{ptx\text{-}DRE}$	This study
pQC1843	pSS3967:: P_{fha} [b]	This study
pQC1844	pSS3967:: $P_{fha\text{-}DRE}$	This study
pQC1631	pSS3967:: P_{gapA*}	This study
pQC1761	pSS3967:: $P_{gapA*\text{-}DRE}$	This study

[a] Cloned *ptx* fragment contains nucleotides 3988042 to 3988253 of the *B. pertussis Tohama* I genome and extends, relative to the start of transcription, from −192 to +21.

[b] Cloned *fhaB* fragment contains nucleotides 1968586 to 1968731 of the *B. pertussis Tohama* I genome and extends, relative to the start of transcription, from −124 to +21.