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Review

The *Bordetella pertussis* model of exquisite gene control by the global transcription factor BvgA

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Bordetella pertussis causes whooping cough, an infectious disease that is reemerging despite widespread vaccination. A more complete understanding of *B. pertussis* pathogenic mechanisms will involve unravelling the regulation of its impressive arsenal of virulence factors. Here we review the action of the *B. pertussis* response regulator BvgA in the context of what is known about bacterial RNA polymerase and various modes of transcription activation. At most virulence gene promoters, multiple dimers of phosphorylated BvgA (BvgA~P) bind upstream of the core promoter sequence, using a combination of high- and low-affinity sites that fill through cooperativity. Activation by BvgA~P is typically mediated by a novel form of class I/II mechanisms, but two virulence genes, *fim2* and *fim3*, which encode serologically distinct fimbrial subunits, are regulated using a previously unrecognized RNA polymerase/activator architecture. In addition, the *fim* genes undergo phase variation because of an extended cytosine (C) tract within the promoter sequences that is subject to slipped-strand mispairing during replication. These sophisticated systems of regulation demonstrate one aspect whereby *B. pertussis*, which is highly clonal and lacks the extensive genetic diversity observed in many other bacterial pathogens, has been highly successful as an obligate human pathogen.

Introduction

Bordetella pertussis, the causative agent of whooping cough (pertussis), was first isolated from an infected person in 1906 (Bordet & Gengou, 1906). The small Gram-negative aerobic coccobacillus is an obligate human pathogen and thus has no known environmental reservoir. Vaccination programs have contributed to a substantial decrease in pertussis incidence, from ~260 000 cases in the USA in 1934 to ~1000 cases in 1976 (CDC, 1995). However, the increase in incidence since the early 1980s – including 27 550 cases in 2010 (CDC, 2011) – makes pertussis the most prevalent vaccine-preventable disease in industrialized countries (Mooi *et al.*, 2009).

Whooping cough is resurging in countries with a historically low incidence attributable to high vaccine uptake (CDC, 1995; de Melker *et al.*, 2000; Kerr & Matthews, 2000). The hallmark of this resurgence is a shift in prevalence from young children to adolescents and adults (discussed by Halperin, 2007). Multiple explanations have been offered for the reemergence of pertussis. These include an increased awareness of the disease, improved laboratory diagnostic tools, suboptimal vaccines and decreased vaccination coverage in parts of the world (Gangarosa *et al.*, 1998).

At first glance, the persistence of pertussis despite intense vaccination efforts is unexpected because *B. pertussis* is highly clonal and lacks the genetic diversity of many other pathogens (Caro *et al.*, 2006; Diavatopoulos *et al.*, 2005; Parkhill *et al.*, 2003; van Loo *et al.*, 2002). Differences between *B. pertussis* clinical isolates are mainly due to differential expression of genes for surface-expressed proteins, mutations in genes for secreted proteins and gene reduction mediated by insertion sequence elements (Brinig *et al.*, 2006; Caro *et al.*, 2006; Heikkinen *et al.*, 2007). In fact, among the major *Bordetella* subspecies, including *Bordetella parapertussis*, which causes a typically milder respiratory disease in humans, and *Bordetella bronchiseptica*, which infects many four-legged mammals, as well as *B. pertussis*, phenotypic differences have not been attributed to pathogenicity islands, plasmids, transposable elements or insertions from phage genomes. This

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finding distinguishes *Bordetella* from *Salmonella* and *Vibrio* species (reviewed by Cotter & DiRita, 2000). Thus, in *Bordetella*, the virulence regulon is differentially expressed in the different subspecies, yielding bacteria with very different niches and lifestyles (reviewed by Cotter & DiRita, 2000; Mattoo *et al.*, 2001).

Effective pathogenesis involves tightly coordinated regulation of virulence factors in response to environmental cues (Dorman, 1995; Marteyn *et al.*, 2010; Rhen & Dorman, 2005; Swanson & Hammer, 2000). For example, disturbing the usual pattern of virulence gene expression in *Bordetella* can significantly reduce colonization in a mouse model (Akerley *et al.*, 1995; Kinnear *et al.*, 2001). Furthermore, *B. pertussis* regulation employs a sophisticated repertoire to provide phenotypic diversity within highly clonal, genetically homogeneous bacterial populations. Hence, in addition to its role in global health and infectious disease, *B. pertussis* provides a valuable model in the laboratory to investigate regulation across all bacterial species. Here, we discuss regulation of the *B. pertussis* virulence genes, highlighting the promoters for *fhaB*, encoding filamentous haemagglutinin, and the *fim* genes, encoding fimbriae. We emphasize how the control of these genes differs from other well-characterized systems of bacterial activation and discuss the role of these regulatory mechanisms in the context of *B. pertussis* virulence.

The two-component system BvgAS regulates virulence genes in *B. pertussis*

To sense relevant cues in the external environment and transduce these signals into intracellular responses such as changes in gene expression, bacteria frequently utilize two-component systems. These systems typically consist of a sensor kinase (SK) and a response regulator (RR), which functions as a DNA-binding transcriptional activator (reviewed by Stock *et al.*, 2000). The SK includes a sensing domain, situated in the bacterial periplasm, connected via a transmembrane segment to a kinase domain, located inside the cell. The SK is thought to transmit the signal to the cell interior via a conformational change within the protein, which affects the efficiency of ATP-dependent autophosphorylation of one SK molecule by its homodimer partner. However, the exact molecular mechanism remains elusive. The RR activator then catalyses its own phosphorylation with the phosphate group donated by its cognate SK or by a small molecule phosphodonor (acetyl phosphate, imidazole phosphate or phosphoramidate, among others). Because these signalling mechanisms are quite rare in eukaryotes (Galperin, 2010), two-component systems are potential targets for antimicrobial therapies.

In the genus *Bordetella*, the primary two-component system involved in virulence gene regulation consists of the sensor kinase BvgS and the response regulator BvgA. BvgS is a 'hybrid' SK, which has three phosphorylation sites in three distinct domains that mediate a phosphorelay (Fig. 1a) (Uhl & Miller, 1996). Phosphorylated BvgA (BvgA~P) binds to different virulence gene promoters in different binding patterns, discussed below, to activate transcription. BvgAS controls the expression of over 100 virulence genes (Bootsma *et al.*, 2002) and the BvgS and BvgA sequences are almost invariant among *B. pertussis* strains and clinical isolates (Herrou *et al.*, 2009).

In 1960, Lacey reported different antigenic properties for three distinct modes of *B. pertussis* and chose the term 'modulation' to describe the transition between modes (Lacey, 1960). Today, we understand that the three modes described by Lacey correspond to what are now known as the Bvg^+, Bvg^- and Bvg^i (intermediate) phases (Fig. 1c). The Bvg^+ phase, characterized by expression of all BvgA-activated adhesins and toxins, is required for virulence (Cotter & Miller, 1994). This phase is manifested under 'non-modulating' environmental conditions that are conducive to phosphorylation of BvgS, such as growth near 37 °C, the temperature in the respiratory tract of the human host. The Bvg^i phase, in which the BvgAS system is not fully induced, may have a role in transmission by aerosol route or the initial stages of infection.

'Modulating' conditions [growth at lower temperatures (25 °C) or the presence of nicotinic acid or magnesium sulfate ($MgSO_4$) in the growth medium] result in a reduction of BvgS activity, leading to the Bvg^- phase. The role of the Bvg^- phase is not yet understood, but it is clearly not required for virulence in animal models of infection (García San Miguel *et al.*, 1998). However, inappropriate expression of the Bvg^- phase *in vivo* is actually detrimental to successful infection (Merkel *et al.*, 1998). It has been hypothesized to be important for intracellular uptake and persistence, or transmission between mammalian hosts (Herrou *et al.*, 2009; Locht *et al.*, 2001). In *B. bronchiseptica*, the Bvg^- phase is adapted for survival under conditions of extreme nutrient deprivation (Cotter & Miller, 1994). Alternatively, it may be an evolutionary remnant from an ancestor that occupied an environmental niche (Gerlach *et al.*, 2001; von Wintzingerode *et al.*, 2001).

Temporal gene regulation by BvgA~P coordinates the expression of proteins needed for virulence

The differential binding to the regulatory regions of different virulence genes constitutes a simple yet highly effective system by which regulation by BvgA~P can result in varied kinetics of virulence factor expression. Virulence genes whose products are presumably involved early in pathogenesis – such as surface-expressed adhesin proteins and BvgA itself – are activated almost immediately upon a shift to permissive growth conditions (37 °C) (Scarlato *et al.*, 1991) (Fig. 1c). This rapid transcriptional response is thought to arise from the presence of high-affinity BvgA~P binding sites located upstream of the early virulence gene promoters (Fig. 1b).

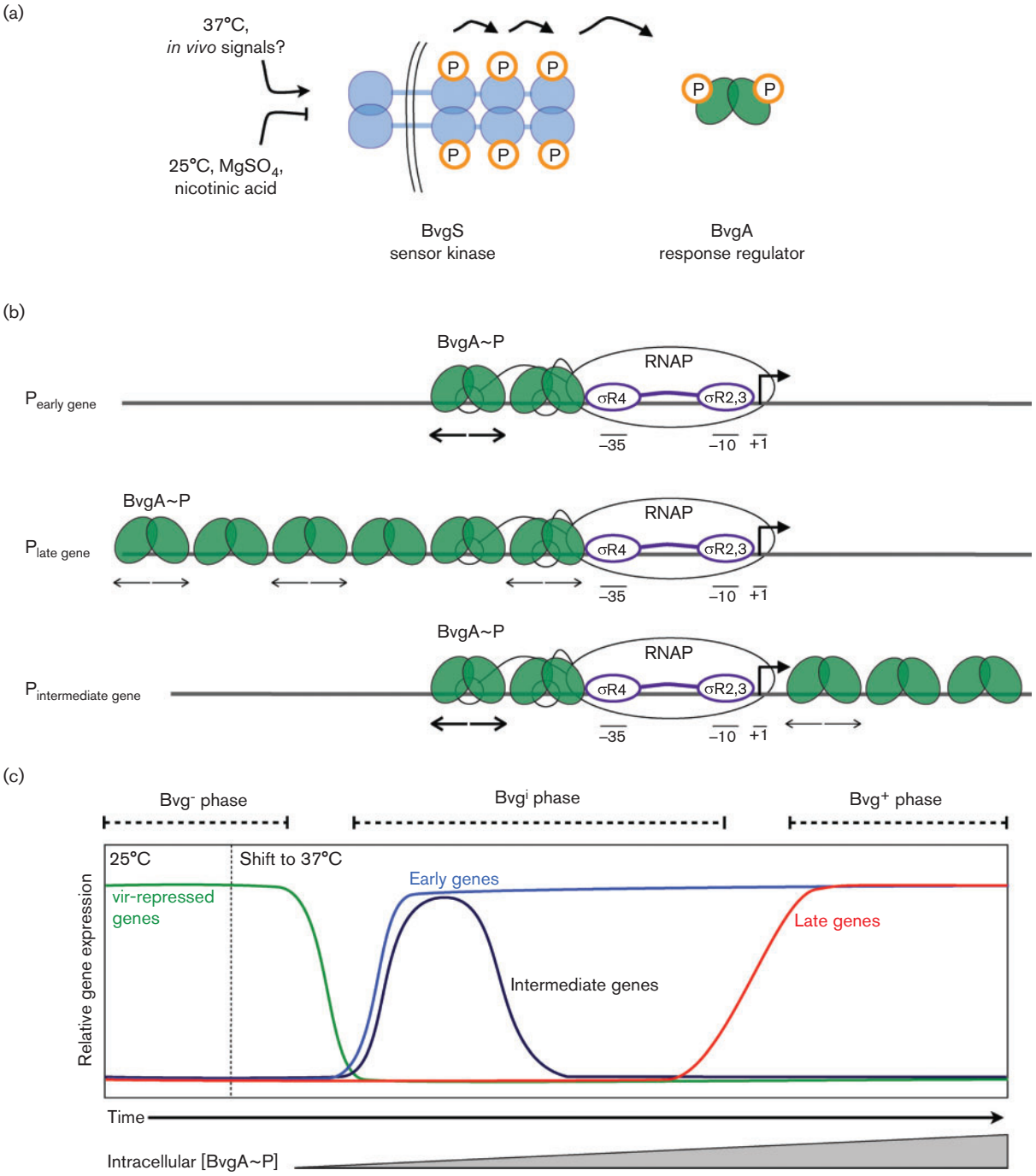

Fig. 1. The BvgAS system and temporal gene regulation. (a) The BvgS sensor kinase is anchored in the inner membrane. Activating signals trigger BvgS autophosphorylation, initiating a phosphorelay that results in phosphorylation of the BvgA response regulator. (b) Phosphorylated BvgA (BvgA~P) binds to virulence gene promoters to regulate activity. BvgA binding sites are marked with inverted arrows (thick lines, high-affinity sites; thin lines, low-affinity sites). At those promoters where the αCTD subunit positions have been identified, αCTD binds to the same region of DNA as the promoter-proximal BvgA~P dimers (Boucher *et al.*, 2003; Decker *et al.*, 2011). (c) Schematic illustrating temporal gene regulation by BvgAS. BvgA~P activates its own expression, so intracellular BvgA~P concentration increases with time at 37 °C (*x*-axis labels). The Bvg^- phase is characterized by expression of the *vir*-repressed genes; Bvg^i by expression of the early and intermediate genes; Bvg^+ by expression of the early and late genes, whose products are required for virulence.

Virulence genes whose products are thought to play a role later in pathogenesis – such as toxins and their secretion systems – remain transcriptionally inactive until the intracellular concentration of BvgA~P increases to a level sufficient to fill the low-affinity BvgA~P sites upstream of the late virulence gene promoters (discussed by Cotter & Jones, 2003) (Fig. 1b, c). In addition, an intermediate gene has been described (*bipA*) in which moderate levels of BvgA~P activate expression, but high levels repress expression (Williams *et al.*, 2005) (Fig. 1b, c). Furthermore, BvgA acts indirectly as a negative effector through its regulation of the BvgR protein, which negatively regulates a set of genes encoding outer membrane and secreted proteins (Merkel *et al.*, 2003).

The *B. pertussis* σ factor is an essential component of the transcription machinery

B. pertussis RNA polymerase (RNAP), like that of other bacteria, consists of an enzymic core ($\alpha_1\alpha_2\beta\beta'\omega$) plus a σ factor required to direct core to the promoter region at the start of a gene. Bacteria typically have multiple σ factors, which direct RNAP to different classes of genes based on the cell's needs (reviewed by Gruber & Gross, 2003).

In primary σ factors, which are responsible for most transcription during exponential growth, four conserved regions (regions 1–4) mediate interactions between σ and the core and/or promoter specificity (reviewed by Hook-Barnard & Hinton, 2007). Regions 2, 3 and 4 bind to specific recognition sites in the promoter DNA: the −10 element ($^{-12}$TATAAT^{-7}), an extended –10 element ($^{-15}$TG^{-14}) and the −35 element ($^{-35}$TTGACA^{-30}), respectively. Typically, two out of three such sequence elements are sufficient for recognition. In addition, the α subunit C-terminal domains (αCTDs) may also directly contact the DNA through AT-rich 'UP' elements, usually located between –40 and –60.

In *B. pertussis*, the primary σ is termed σ^{80}. Its counterpart in *Escherichia coli* is the well-studied σ^{70}. σ^{80} is 71 % similar and 55 % identical to *E. coli* σ^{70}, and it has been shown that several known promoters are active using RNAP reconstituted with either σ factor (Baxter *et al.*, 2006; Boucher *et al.*, 1997; Decker *et al.*, 2011; Steffen & Ullmann, 1998). Furthermore, within σ region 4, a portion of σ known to play a major role in gene regulation, σ^{70} and σ^{80} are 84 % similar and 73 % identical. Thus, it is reasonable to extrapolate much of the detailed characterization of σ^{70} to σ^{80}.

One discrepancy between *E. coli* σ^{70} and *B. pertussis* σ^{80} resides in the N-terminal region where σ^{80} has a positively charged 'region P' (residues 1 to ~150). Such a domain is present in many pathogenic bacteria but absent in *E. coli* σ^{70} (Yang *et al.*, 2010). In *Helicobacter pylori*, region P binds polyphosphate under conditions of limited nutrients, and mutations that eliminate this interaction lead to accelerated cell death during starvation. However, how and if this binding regulates gene expression is not yet known.

Bacterial transcription activation occurs through a variety of mechanisms

A promoter with 'perfect' elements does not equal a perfect promoter; such a construct cannot be regulated and, therefore, could be detrimental to an adaptive organism. Consequently, regulated promoters, such as those that drive expression of *B. pertussis* virulence genes, contain a complement of core promoter sequence elements that is suboptimal and then use DNA-binding factors to activate RNAP under the appropriate conditions.

Transcription initiation proceeds through multiple steps (Kontur *et al.*, 2008; Saecker *et al.*, 2002; reviewed by Hook-Barnard & Hinton, 2007), from an initial closed complex (RP_C), in which the DNA is fully double-stranded, to an open complex (RP_O), in which polymerase has isomerized and the DNA has bent, opened and descended into the active site (Gries *et al.*, 2010). Consequently, there are multiple points at which an activator might function. Several classes of activators, distinguished by their varying mechanisms, have been described.

Bacterial class I activators bind to promoter DNA upstream of the –35 element (typically near –60) and directly interact with the RNAP αCTDs (Fig. 2a). It is thought that this interaction, by stimulating initial binding of RNAP, helps to recruit the enzyme to the promoter (reviewed by Gourse *et al.*, 2000). In contrast, class II activators bind to promoter DNA adjacent to or overlapping the –35 element (Fig. 2b). At this location, they are positioned to interact with the DNA-recognition helix within σ^{70} region 4 and/or α subunits CTDs or NTDs (Dove *et al.*, 2003). These activators can also help recruitment and/or accelerate rate-limiting steps in the formation of RP_O (Barnard *et al.*, 2004; Browning & Busby, 2004; Lawson *et al.*, 2004). Interestingly, among class II activators, there is variation in the precise binding site and orientation of the activator, discussed below. In addition, some promoters use a combination of class I and class II (Fig. 2c).

Mechanisms of some other transcription factors bear little resemblance to class I or class II activation. For example, MerR activates expression of the mercuric ion operons by binding to the unusually long spacer region between the –10 and –35 core promoter elements (Fig. 2d). Its binding contorts the DNA, effectively shortening the spacer and creating a functional promoter for RNAP (Hobman *et al.*, 2005; Watanabe *et al.*, 2008).

Another differing mechanism is σ appropriation, by which a class of bacteriophage T4 promoters are activated and host promoters are silenced (reviewed by Hinton, 2010). In this system, a small T4 protein, AsiA, binds tightly to *E. coli* σ^{70} region 4 and structurally remodels it to preclude binding to the –35 element of *E. coli* promoters (Fig. 2e) (Lambert *et al.*, 2004). The remodelled σ factor is correctly

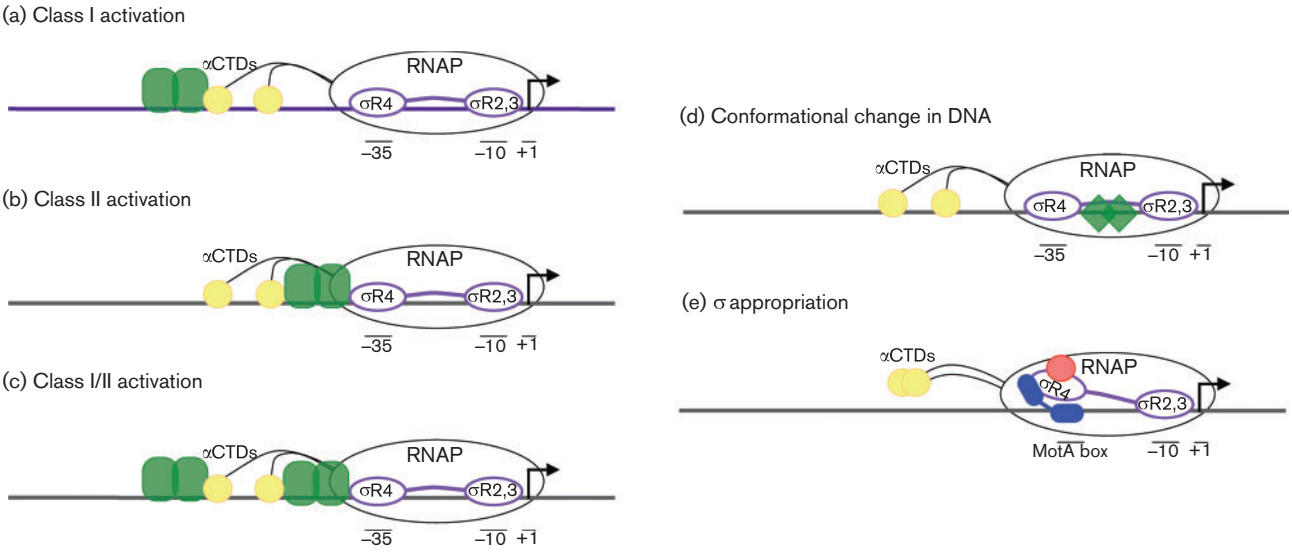

Fig. 2. Characterized mechanisms of prokaryotic gene activation. Purple, σ; yellow, αCTD; green, specified activator. (a) Class I activators bind upstream of the core promoter elements and interact with the RNAP αCTD subunit(s). (b) Class II activators bind closer to the core promoter elements, adjacent to or overlapping the −35 element, and interact with σ region 4 and/or αCTD. (c) Combination class I/II activation relies on independent interactions between an upstream activator and αCTD, and a downstream activator and σ region 4 and/or αCTD. (d) The MerR activator causes a conformational change in the alignment of the −10 and −35 elements that allows promoter recognition and gene activation. (e) During activation by σ appropriation, bacteriophage T4 proteins AsiA (red) and MotA (blue) interact with σ^{70} region 4 to redirect RNAP from host *E. coli* promoters to T4 middle promoters, which contain a MotA box sequence centred at −30.

positioned to interact with another T4 protein, MotA, which redirects transcription activity to T4's own middle promoters via recognition of a specific site at –30. Thus, this system works by replacing σ region 4 specificity for one sequence with the activator's specificity for a different sequence.

BvgA~P-regulated promoters have a characteristic architecture

At typical *B. pertussis* early promoters, such as those for the genes *fha* and *bipA*, a head-to-head dimer of BvgA~P binds to a primary binding site [inverted heptads with consensus sequence $(^{T}/_{A})$TTC$(^{C}/_{T})$TA typically located $\geqslant$60 base pairs upstream of a virulence gene promoter; Fig. 1b; Boucher *et al.*, 1997; Roy & Falkow, 1991], and additional dimers of BvgA~P bind to adjacent, secondary binding sites in a cooperative manner that can be relatively independent of the DNA sequence (Boucher & Stibitz, 1995; Boucher *et al.*, 2001; Marques & Carbonetti, 1997). Other promoters, such as the late promoters driving *ptx* and *cya* expression, utilize a consortium of binding sites with poorer matches to the consensus sequence. These sites, acting cooperatively, are filled and stimulate transcription only at higher intracellular concentrations of BvgA~P.

A structure of BvgA has not yet been obtained. Consequently, the DNA-binding domain of BvgA has been conventionally modelled on the response regulator NarL because of the sequence similarity between the two proteins (Boucher *et al.*, 2003). The binding behaviour of BvgA has been revealed by studies using BvgA~P modified at single residues with the cleavage reagent Fe-BABE (Boucher *et al.*, 2003). The details of this binding are entirely consistent with the X-ray crystal structure of NarL bound to its DNA site (Proulx *et al.*, 2002). However, the activity of BvgA seems to differ from that of NarL. For example, NarL does not detectably bind DNA unless it is phosphorylated (Proulx *et al.*, 2002). In contrast, unphosphorylated BvgA has been shown to bind DNA (Boucher *et al.*, 1994, 1997; Karimova *et al.*, 1996; Zu *et al.*, 1996; K. B. Decker, unpublished data). In addition, BvgA~P binds to virulence gene promoters with greater affinity and in at least one case, in a different binding pattern than does BvgA (Boucher *et al.*, 1994, 1997; Boucher & Stibitz, 1995; Karimova *et al.*, 1996; Steffen *et al.*, 1996; Zu *et al.*, 1996; K. B. Decker, unpublished data).

For most of the *B. pertussis* virulence gene promoters, the binding site for the downstream-most BvgA~P dimer is located near the –35 region of the promoter, in a position to interact with σ and/or αCTD, as in class II activation, while the upstream binding sites could interact with the other αCTD, as in class I (Boucher & Stibitz, 1995; Boucher *et al.*, 1997, 2001; Karimova *et al.*, 1996; Karimova & Ullmann, 1997; Kinnear *et al.*, 1999; Merkel *et al.*, 2003; Zu *et al.*, 1996). Consistent with a combination class I/II

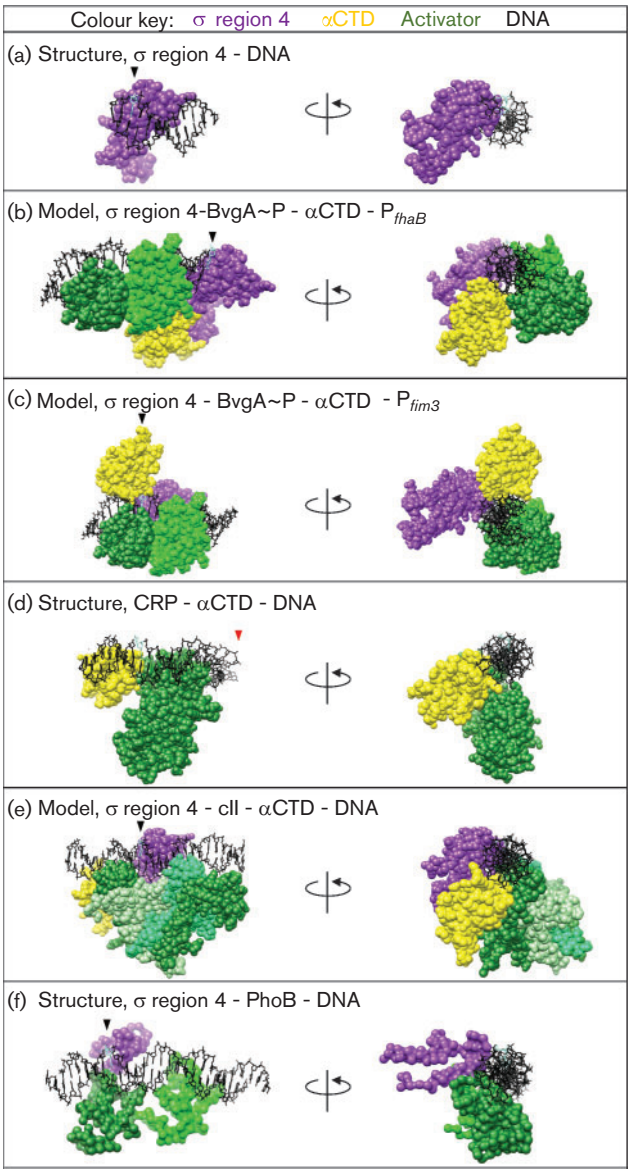

Fig. 3. The protein conformation at BvgA~P-activated P_{fim3} differs from that at characterized class II promoters. Purple, σ region 4; yellow, αCTD; green, specified activator. For (a)–(c) and (e)–(f), the −35 nontemplate strand nucleotide (nt) is shaded cyan and marked with a carat (left panel) and oriented in the 12 o'clock position (as viewed in the right panel). For (d), the −56 nontemplate strand nt is shaded cyan and oriented in the 12 o'clock position; because the −56 nt is ~two helical turns upstream from the −35 nt in B-form DNA, the complex in (d) is similarly oriented to (a)–(c) and (e)–(f). The red carat in (d) marks nt position −41.5. (a) σ Region 4 bound to −35 region DNA, as a reference for (b)–(f) (Campbell *et al.*, 2002); PDB no. 1KU7. (b) BvgA~P activation at P_{fhaB}: σ region 4, αCTD, BvgA~P dimer bound to its promoter-proximal site; modelled on the work of Boucher *et al.* (2003). (c) BvgA~P activation at P_{fim3}: σ region 4, αCTD, BvgA~P dimer bound to its promoter proximal site, overlapping the −35 region DNA; modelled on work of Decker *et al.* (2011). (d) Class II activation by CRP at *gal*P1: a dimer of CRP centred at −41.5 interacts with an upstream-bound αCTD. Only the upstream monomer of CRP is shown; σ region 4 is not shown as it was not part of the crystallized complex. Structure taken from Benoff *et al.* (2002); PDB no. 1LB2. (e) Class II activation by cII at P_{RE}: σ region 4 (aa 461–599), αCTD, cII tetramer. Model from Jain *et al.* (2005; gift from S. Darst, Rockefeller University). (f) Class II activation by PhoB at P_{pstS}: σ region 4 (aa 533–613), PhoB dimer. The β-flap tip helix, crystallized as a chimera with σ region 4, is not shown. Structure from Blanco *et al.* (2011); PDB no. 3T72.

mechanism (Fig. 2c), BvgA~P activation at P_{fhaB} requires residues within αCTD (Boucher *et al.*, 1997) and σ region 4 (Decker *et al.*, 2011).

Despite these similarities with class I/II promoters, the architecture of RNAP/BvgA~P at P_{fhaB} is not like other characterized class II activators. The molecular structures and models in Fig. 3 illustrate this point. The left panels in Fig. 3(b–f) depict activator/αCTD/σ region 4 complexes along the promoter DNA (upstream to downstream) whereas the right panels depict an end-on view, in which σ region 4 (when shown) is kept in the same orientation. In Fig. 3(a), σ region 4 can be seen at the −35 element of host promoter DNA. For the promoters at which region 4 is shown, this position remains generally the same, positioned at the −35 region of the promoter (compare the purple region 4 in the right panels).

In contrast, the relative positions of αCTD and the activator are not constant at these various promoters. At P_{fhaB} (Fig. 3b), each α contacts the same region of DNA as a BvgA dimer, but on a different helical face (Boucher *et al.*, 2003). This is unlike previously characterized class I/II systems (reviewed by Barnard *et al.*, 2004), such as the CRP dimer/αCTD/*gal*P1 structure (Fig. 3d) (Benoff *et al.*, 2002) or the modelled structure of λ cII dimer/σ region 4/αCTD/P_{RE} (Fig. 3e) (Jain *et al.*, 2005), in which the activator and αCTD are adjacent to one another (compare the different locations of the yellow αCTD at P_{fhaB} in the left panel of Fig. 3b versus its locations in the left panels of Fig. 3d and e). Thus, the RNAP/BvgA~P complex at P_{fhaB} represents a new twist on class I/II activation. Furthermore, as discussed in detail below, recent evidence indicates that BvgA~P activation at the promoters for the *fim* genes (P_{fim2} and P_{fim3}) involves an even more radical departure from the typical class II architecture.

The requirements for BvgA~P activation seem to involve more than just proximity between activator and polymerase. The positions of the proteins around the faces of the DNA double-helix also appear to be important. DNA mutations that disrupt the pattern of multiple sites along one face of the DNA double-helix are deleterious to promoter activity. However, mutations which remove an entire binding site yet maintain the dimer positions still allow promoter activity (Boucher *et al.*, 2001; Marques &

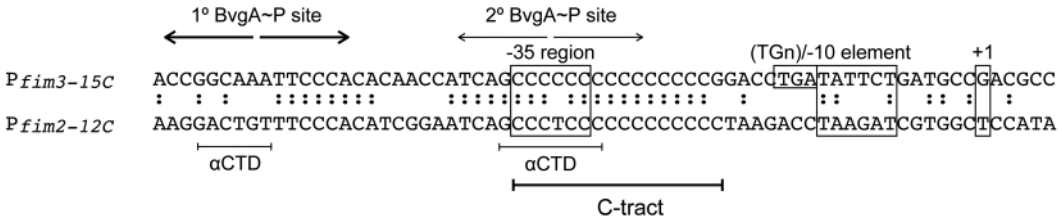

Fig. 4. Promoter architecture of P_{fim3} and P_{fim2}. The −35 region, extended −10 (TGn) element, −10 element and +1 transcription start site are outlined in boxes. BvgA~P binding sites are marked with inverted arrows: thick line, higher-affinity primary site; thin line, lower-affinity secondary site (Chen *et al.*, 2010). The positions at which the αCTD subunits are observed to bind at P_{fim3} are marked (Decker *et al.*, 2011). The C-tract, which can vary in length in each promoter, is marked. Sequence identity between *fim* paralogues is marked with two dots.

Carbonetti, 1997). The orientation of BvgA~P dimers along the same face of the double-helix may be important for cooperative binding of activators to the promoter or for the correct BvgA~P-RNAP interaction. Consistent with this idea is the effect of different lengths of homopolymeric tracts in the promoter regions of the *fim* genes, described below. Changing the lengths of these promoter regions, which alters the spacing between promoter elements as well as relative orientation of one bound protein to another around the double-helix, has dramatic effects on promoter activity (Chen *et al.*, 2010; Riboli *et al.*, 1991; Willems *et al.*, 1990).

Phase variation in the *fim* genes generates phenotypic diversity within a population

The *fim* promoters are emerging as instructive models for multiple levels of gene regulation that together create a fine-tuned response to the environment and ensure the success of a multicellular population. *fim2* and *fim3* encode subunits of the long serrated fimbriae, serotypes 2 and 3, respectively (Heck *et al.*, 1996; Mooi *et al.*, 1987). Fimbriae (also called pili) allow *B. pertussis* to adhere to host cells and are required for efficient establishment of tracheal colonization and persistence in mouse and rat models (Geuijen *et al.*, 1997; Mooi *et al.*, 1992).

Expression of the *fim* genes is regulated at multiple levels – as part of the BvgAS regulon and at the level of the individual gene through phase variation (Heikkinen *et al.*, 2008; Willems *et al.*, 1990). Phase variation is a phenomenon that allows expression of a given factor to switch between 'on' or 'off' states at a rate greater than that of random mutation, frequently affecting fimbriae, flagella, outer-membrane proteins and lipopolysaccharide components in Gram-negative bacteria (reviewed by Dybvig, 1993; Henderson *et al.*, 1999). Phase variation can occur by DNA inversion, recombination, differential methylation or, as in the case of the *fim* genes, by slipped-strand mispairing resulting in alteration of the length of a repetitive sequence in the regulatory or coding regions of a gene (Seifert & So, 1988; Streisinger & Owen, 1985).

The *fim2* and *fim3* promoters each contain a homopolymeric tract of cytosines ('C-tract') overlapping the −35 region, and each promoter can be activated by BvgA~P only when the C-tract is of a permissive length (Fig. 4) (Chen *et al.*, 2010; Willems *et al.*, 1990). This tract does not contain sequence-specific information, but instead appears to function as a spacer between transcription activation machinery bound to different elements of the promoter (Chen *et al.*, 2010). Because C-tract regulation of one *fim* promoter operates independently from the other, cells can express any combination of *fim* proteins on their surface, contributing to phenotypic diversity within a population. Interestingly, a *B. pertussis* gene with sequence homology to *fim3* and *fim2* has an extremely truncated promoter C-tract: 7 Cs compared with 15 Cs in the active form of *fim3*. This gene, called *fimX*, appears to be transcriptionally silent due to the deletion in the C-tract (Chen *et al.*, 2010; Willems *et al.*, 1990). Moreover, the shorter homopolymeric tract limits the amount of slipped-strand mispairing that is likely to occur, making a reversion to activity by addition of Cs highly improbable. Why *B. pertussis* has maintained an intact copy of this supposedly 'silent' gene remains unclear.

Phase variation by slipped-strand mispairing can be considered a 'programmed' random event: the insertion or deletion of nucleotides is stochastic, but the frequency with which it occurs increases for homopolymeric tracts of increasing length (Streisinger & Owen, 1985). One benefit of phase variation is that it allows an organism to create diversity in an otherwise clonal population – a valuable trait for *B. pertussis* which has unusually poor genomic diversity for a pathogen (Gogol *et al.*, 2007). Phenotypic diversity among surface-exposed adhesins is no doubt important for evading the host surveillance system and may be important to ensure some bacteria are poised to move to a new environment through detachment and shedding (Dybvig, 1993; Henderson *et al.*, 1999).

Notably, the *bvgS* open reading frame also contains a C-tract, which is susceptible in certain strain backgrounds to slipped-strand mispairing during replication (Levinson & Gutman, 1987); insertion or deletion of a C yields a

truncated, nonfunctional BvgS protein (Stibitz *et al.*, 1989). As a result, virulence genes that are normally activated by the BvgAS system are not expressed, rendering them avirulent (Stibitz *et al.*, 1989). However, the biological relevance of this system during an infection is not known.

The molecular mechanism of *fim* activation is complex and elegant

Besides the ability to undergo phase variation, the *fim* promoters are unusual because the position of the downstream BvgA~P binding site surrounds the −35 region of the promoter DNA (Chen *et al.*, 2010) (Fig. 4). Recent work has sought to define the interaction between the activators and RNAP within this unusual architecture (Decker *et al.*, 2011). Surprisingly, although σ region 4 is not required for BvgA~P activation of P_{fim3}, it is still located on or near its usual position at the –35 region of P_{fim3}, despite the fact that this region consists of the monotonic C-tract. In addition, the αCTD subunits of RNAP bind to the same regions of the DNA as the BvgA~P dimers, and in the same configuration relative to BvgA as shown for P_{fhaB}. This arrangement places σ region 4, one αCTD and a BvgA~P dimer at the −35 region of P_{fim3}, suggesting that the proteins bind to different faces of the same stretch of promoter DNA.

A speculative model of the protein arrangement at P_{fim3} consists of the three subunits arranged around the DNA double helix in a conformation that likely depends on specific protein–protein interactions, since the DNA lacks specific sequence information to direct the protein position (Decker *et al.*, 2011) (Fig. 3c). This model explains the exquisite control over regulation by the length of the C-tract. The insertion or deletion of one C would alter the

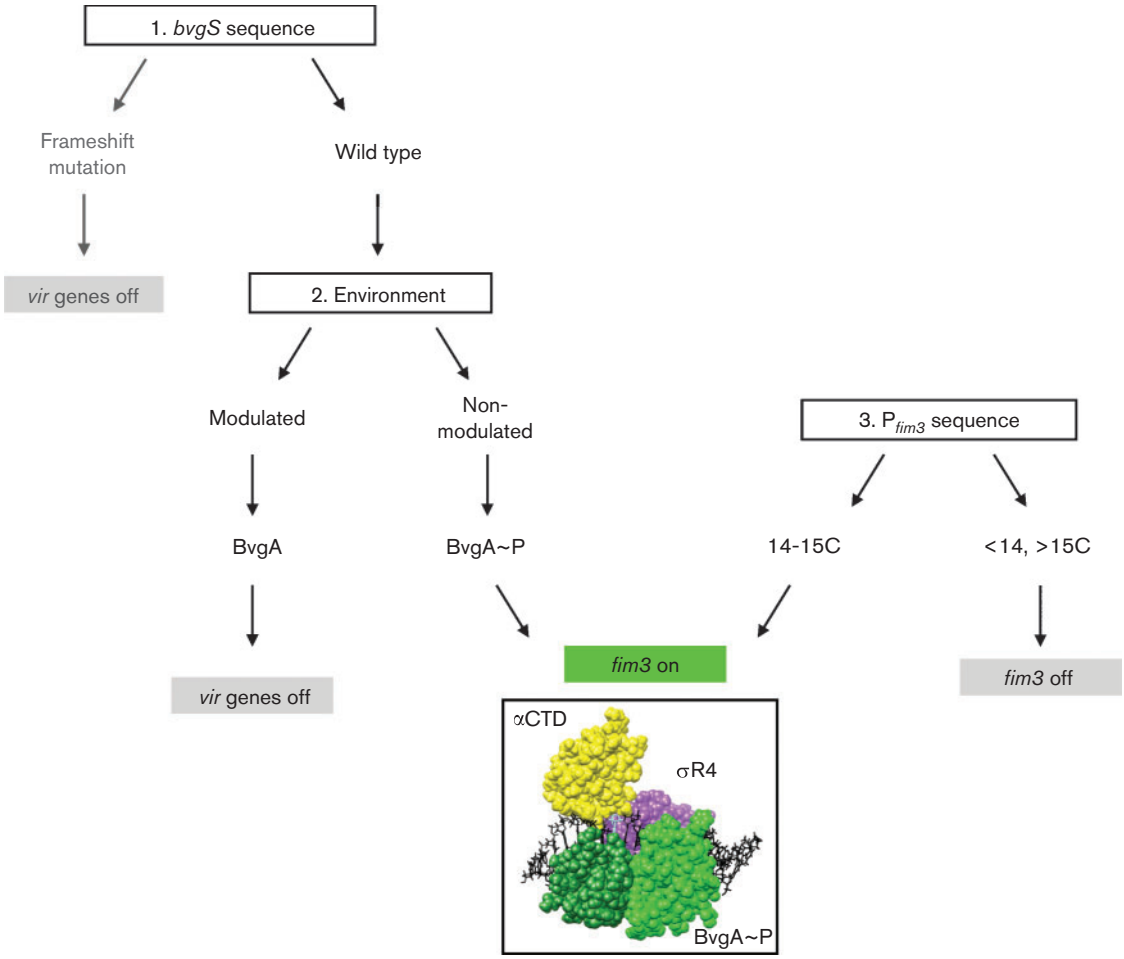

Fig. 5. Multiple independent regulatory mechanisms ensure appropriate virulence gene expression and create phenotypic diversity in a *Bordetella* population. The *bvgS* sequence, environmental temperature and the length of the *fim* promoter C-tract together determine *fim* activity. Modulating conditions include lower temperature (25 °C) or the presence of $MgSO_4$ or nicotinic acid; non-modulating conditions include growth at 37 °C. How frameshifting within *bvgS* contributes to pertussis infection is not clear.

orientation of the protein subunits around the double helix and thus should disrupt the correct positioning needed for activation.

The protein arrangement in BvgA~P activation of the *fim3* promoter offers an architecture that differs even more dramatically from that seen at typical class II promoters, or even at P_{fhaB}. This is because the αCTD within the −35 region of P_{fim3} is positioned nearly a helical turn farther downstream than has been seen previously. Furthermore, for class II activators like CRP at *gal*P1 (Fig. 3d) (Benoff *et al.*, 2002) or PhoB at *pho* box DNA (Fig. 3f) (Blanco *et al.*, 2011), the activator is poised to interact with a common set of region 4 residues (discussed by Bonocora *et al.*, 2008). However, the particular positioning of BvgA~P at P_{fim3} means that these residues are not available for a region 4/activator interaction. Finally, despite the fact that the BvgA~P site includes the −35 region for the promoter, just as the binding site of the T4 MotA activator includes this portion of the DNA, the *fim* promoter activation complex is completely different from that formed by σ region 4 with the bacteriophage T4 proteins AsiA and MotA (reviewed by Hinton, 2010). Thus, activation at the *fim* promoters provides another example of how σ region 4 can be utilized in an activation system.

Conclusions

A mechanistic understanding of the activation of *B. pertussis* virulence genes is vital given the reemergence of the pathogen and the fact that some acellular vaccines directly and exclusively target virulence factors of the bacterium, including Fim2 and Fim3 (Bouchez *et al.*, 2008, 2009; Geier & Geier, 2002). In addition, evidence from clinical studies may suggest that the Fim antigens are perhaps being subjected to immune selection due to vaccine-induced and natural-antibody-driven adaptation (Gogol *et al.*, 2007; Tsang *et al.*, 2004). The sophisticated controls used by *B. pertussis* to regulate virulence genes (differential binding of the BvgA~P activator to the promoters, different RNAP/activator architectures and phase variation by programmed mutation) demonstrate how a pathogen that is highly clonal and lacks the genetic diversity of many other pathogens can be quite successful as an obligate human pathogen (Fig. 5). A more complete understanding of the virulence factors and their regulation, and the host immune response is essential to develop the next generation of pertussis vaccines and treatments.

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