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The BvgASR virulence regulon of *Bordetella pertussis*

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The BvgAS two-component system of *Bordetella pertussis* directly activates the expression of a large number of virulence genes in an environmentally responsive manner. The Bvg^+ mode also promotes the expression of the phosphodiesterase BvgR, which turns off the expression of another set of genes, the *vrg*s, by reducing levels of c-di-GMP. Increased levels of c-di-GMP in the Bvg^- mode are required, together with the phosphorylated response regulator protein RisA∼P, to activate *vrg* expression. Phosphorylation of RisA requires RisK, a non-co-operonic sensor kinase, but not its co-operonic sensor kinase RisS which is truncated in *B. pertussis* but intact in the ancestral *B. bronchiseptica*. The loss of RisS during evolution of *B. pertussis* led to the ability to express the *vrg*s, potentially enhancing aerosol transmission of *B. pertussis*.

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Introduction

Like many bacterial pathogens, the *Bordetellae*, encompassing the human pathogen *Bordetella pertussis* and the veterinary pathogen *B. bronchiseptica*, regulate their virulence potential according to environmental conditions. Also like many other pathogens, these species have a complementary set of capabilities that is regulated in an inverse fashion, relative to the virulence genes. Both arms are controlled by the regulatory locus *bvgASR*. Activation of virulence genes is governed by the two-component system BvgAS, while control of the other arm, the Bvg-repressed genes, or *vrg*s, is controlled by the action of BvgR, a c-di-GMP phosphodiesterase, itself a Bvg-activated gene, or *vag* (Figure 1).

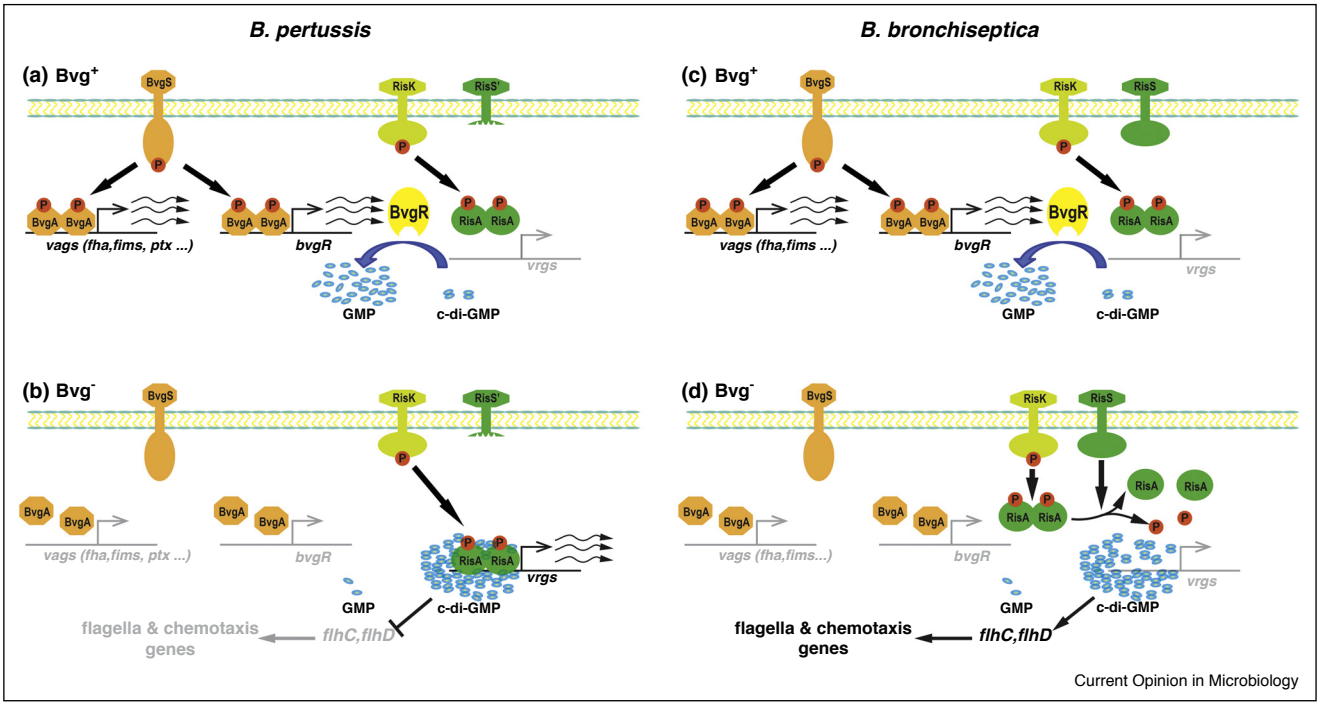
The BvgAS two-component system

BvgAS represents the apex of a regulatory system in *Bordetellae*. BvgA is fairly typical for its class, comprising an N-terminal response regulator domain and a C-terminal DNA binding domain of the LuxR/NtrC/FixJ family. The sensor kinase BvgS is less typical, although not unique, being representative of a class of sensor kinases. BvgS comprises, in order from periplasmic to cytoplasmic, tandem periplasmic Venus Fly Trap (VFT) domains, a transmembrane segment, a PAS domain, a histidine kinase module of the HisK-A type (comprising dimerization and phosphotransfer (DHp) and catalytic ATP-binding (CA) domains), a response-regulator domain, and a histidine phosphotransfer (Hpt) domain. These function in a phosphorelay with BvgA as the terminal phosphate acceptor [1–3]. A salient feature of two-component sensor kinases is the modulation of their activity in response to environmental signals. In the case of the *Bordetellae*, and due, we now understand, to BvgS, this was first described by Lacey and termed antigenic modulation [4]. Of the many different compounds that Lacey examined for their modulatory activity, $MgSO_4$ and nicotinic acid have been the ones used almost exclusively in *in vitro* laboratory growth studies. The signals to which *Bordetellae* may respond in nature remain unknown. While many sensor kinases have a ground state in which they are inactive, and increase their kinase activity in response to chemical or other signals, BvgS is somewhat unique in that its ground state is the active mode, and the effect of modulators is to reduce its kinase activity. Recent structural, genetic, and biochemical studies indicate that, in the Bvg^+ mode, nonspecific ligands are bound to sites within the 'trap'. Binding of the modulator nicotinic acid causes extensive conformational changes that, through their effects on the transmembrane helices, ultimately place BvgS into a phosphatase rather than a kinase mode [3]. The BvgS-active mode, in which virulence genes are activated, is called the Bvg^+ mode and the BvgS-inactive mode, experienced during modulation, the Bvg^- mode. Although the term 'phase' is often used in place of 'mode', we prefer the latter term because transition between the two states is due to regulatory, not genetic, changes.

BvgA∼P binding

Phosphorylated BvgA (BvgA∼P) activates genes encoding virulence factors. These include adhesins, such as filamentous hemagglutinin (*fha*), pertactin (*prn*) and fimbriae (*fim2*, *fim3*, *fimX*); toxins, such as pertussis toxin (*ptx*) and adenylate cyclase toxin (*cya*); and other immune evasion systems, such as BrkA (*brkA*) (*Bordetella* resistance to killing), which mediates complement resistance [1]. For all promoters that have been examined by *in vitro* transcription studies, BvgA∼P has been found to be the only activator required for their expression. These

Figure 1



Bvg-controlled gene regulation in *Bordetellae*. **(a)** and **(c)** Under normal growth conditions (Bvg$^+$ mode), the two-component sensor kinase BvgS and the cognate response regulator BvgA are in the default 'on' state and directly activate the transcription of *vag*s, including *bvgR*, *bvgA, fha*, *fim*s and, in *B. pertussis*, *ptx*. The level of c-di-GMP is low in the presence of the phosphodiesterase BvgR. RisA∼P, even though phosphorylated by cross talk from RisK, is, therefore, unable to activate transcription of *vrg*s, [36••]. The gene for the RisA co-operonic sensor kinase family protein RisS has been truncated due to a frameshift mutation in *B. pertussis* (RisS') during its evolution from a common ancestor more similar to *B. bronchiseptica*. **(b)** In *B. pertussis*, in the Bvg$^-$ mode, the *vag*-encoded BvgR is not produced and c-di-GMP levels are increased. RisA∼P, in the presence of this higher level of c-di-GMP, can activate the transcription of *vrgs* [36••] and transcription of flagella and chemotaxis genes is repressed, likely due to effects on expression of the regulatory genes *flhC* and *flhD* [23••]. **(d)** In *B. bronchiseptica*, in the Bvg$^-$ mode, intact RisS acts as a phosphatase, reducing levels of RisA∼P (Chen *et al.*, unpublished). Unphosphorylated RisA is unable to activate transcription of *vrg*s, even in the presence of accumulated c-di-GMP. This condition allows the expression of the regulators *flhC*, *flhD* along with genes for flagella and chemotaxis [43,44].

include those for *fha* [5–8], *bvgA* [6], *ptx* [6], *cya* [6], *fim* [8,9•], *prn* [10], *bvgR* [11], *bipA* [12] and *brpL* [13•]. Detailed mechanisms of BvgA-activation have been most extensively studied using the *fha* promoter as a model system. Affinity cleavage reagents generated by labeling of BvgA with Fe-BABE have been powerful tools. Their application has revealed the following aspects that are true for most BvgA-regulated promoters (exceptions noted).

1 BvgA binds as head-to-head dimers upstream of the core promoter elements of regulated promoters
2 Multiple BvgA dimers bind to this region, with a spacing of 22 bp 'on center', apparently stabilized by inter-dimer interactions. An exception is the *cya* promoter at which the upstream three dimers follow this rule, but the most promoter proximal dimer is separated from them by 6 bp.
3 BvgA-binding extends up to and abuts the −35 region. Exceptions include the BvgA promoter in which the most promoter proximal BvgA dimer is separated by one helical turn from the −35 region, and the *fim2*, *fim3*, and *fimX* promoters, in which the most promoter proximal BvgA binding site is co-centric with the −35 region.
4 A dimer of BvgA binds to an inverted heptad binding site.
5 The most high-affinity BvgA-binding sites are found in the *fha* and *bipA* promoters and represent optimal binding sites. Systematic mutational alterations to the *fha* primary binding site only decreased activity.
6 A pattern of primary and secondary binding is generally seen, with the most upstream, primary binding sites, typically being of highest affinity, and the more downstream, promoter proximal sites being of reduced affinity.

Classes of Bvg-activated promoters

Although BvgA∼P appears to be the sole activator required for transcription, promoters differ in their responsiveness to the modulators. Scarlato *et al.* first reported this phenomenon in terms of time-to-

expression following a shift from a non-permissive to a permissive temperature [14]. This temporal program of expression has also been shown to occur *in vivo* in mice inoculated with Bvg$^-$ mode *B. pertussis* [15] and can also manifest as differential sensitivity to modulation when a range of modulator concentrations is assessed under steady-state growth conditions [16]. Early genes, such as *fha*, are expressed shortly after a shift, and are relatively insensitive to modulation. Late genes, such as *ptx* and *cya*, are expressed much later and can be down regulated by lower concentrations of modulators. Both manifestations are indicative of levels of BvgA∼P, which rise following activation of the *bvgAS* promoter [14,17]. Promoter architecture, although consistent with the general rules described above, is varied, as shown in Figure 2. Early promoters have the highest-affinity binding sites while the late promotor P*ptx* has a higher number of lower-affinity BvgA-binding sites. The late promoter P*cya* is more complicated, manifesting a mix of medium and low affinity sites and deviating from the general rule of BvgA spacing, as described above. The promoter for *bipA* (*Bordetella* intermediate phase) is activated as an early gene via high-affinity binding of BvgA∼P but is repressed at higher concentrations of BvgA∼P due to occupation of lower affinity repressive sites downstream. This leads to maximal expression under conditions between Bvg$^+$ and Bvg$^-$ modes, also called the Bvg^i mode (intermediate) [12].

Mechanisms of BvgA-activation

The juxtaposition of BvgA binding to the −35 regions of activated promoters suggests interaction with the sigma subunit of RNA polymerase (RNAP) similar to that observed at Class II activated promoters [18,19]. However, a unique feature of these promoters is the mode of binding of the C-terminal domain of the RNAP alpha subunit (alpha-CTD). The previously studied activator CAP in *Escherichia coli*, when activating a Class II promoter, directs binding of alpha-CTD upstream of the CAP binding site, and to the same face of the DNA helix as CAP itself, in a tandem arrangement. BvgA on the other hand dictates binding of alpha-CTD to a different face of the DNA helix within the same segment bound by BvgA∼P. This unique configuration was first demonstrated at the *fha* promoter [20••], but has also been shown to occur at the *fim3* promoter [21••]. The latter case is even more remarkable because at this promoter, and within the same region, BvgA is also interacting with the RNAP sigma subunit [21••]. These observations suggest that BvgA-regulated promoters are most like Class II promoters in their mechanisms of activation. An exception is the promoter for *bvgAS* itself, in which BvgA and alpha-CTD binding take place a full helical turn upstream of the core promoter elements. This configuration is more reminiscent of a Class I promoter.

Bvg-repressed genes in *B. pertussis*

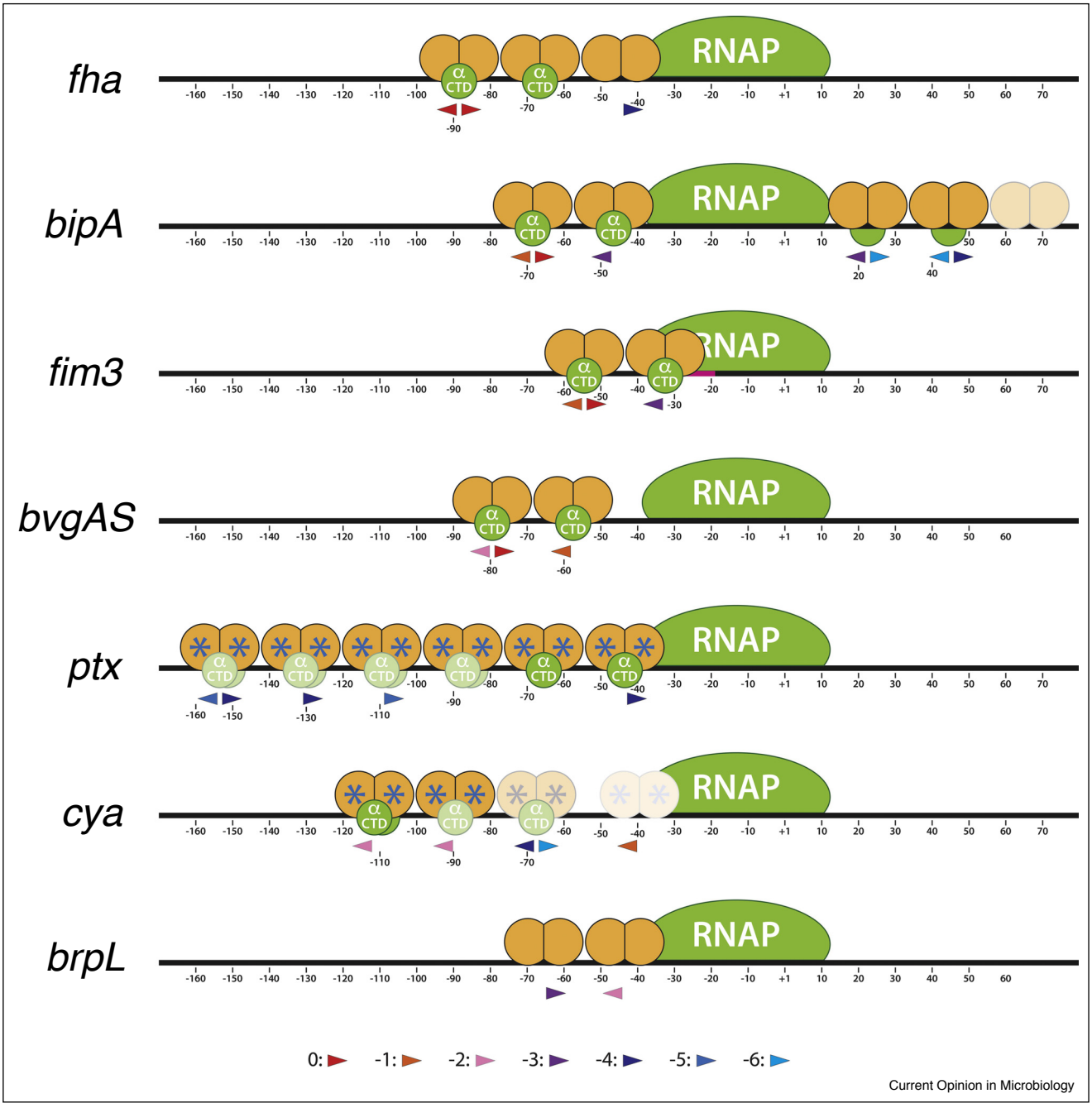
In *B. pertussis*, Bvg-repressed genes were first identified by Tn*phoA* insertions that displayed inverse regulation vis-à-vis virulence genes [22]. These genes were named *vrg*s (*vir* repressed genes) taking into account the then current name of the *bvg* locus, *vir*. Of these, *vrg*-6, *vrg*-18, *vrg*-24, and *vrg*-73 have been studied the most, although their specific function remains unknown. More recently, global transcriptomic analyses have greatly expanded this group of genes. These have included analyses by microarray [23••,24,25], and most recently by RNA-seq [13•]. In these studies, although strains, growth media and the cut-offs for *vrg* recognition (transcription in the Bvg$^-$ mode versus that in the Bvg$^+$ mode) have varied, all have confirmed the *vrg*-like expression pattern of the classical *vrg*s (*vrg*-6, *vrg*-18, *vrg*-24, and *vrg*-73) and genes for capsular polysaccharide biosynthesis, whose expression results in capsule production mainly in the Bvg$^-$ mode [26]. A large number of Bvg-repressed genes are involved in metabolic pathways. In multiple animal studies, the ability of *B. pertussis* to express this class of genes has been demonstrated not to contribute to virulence, and, in fact, to be detrimental if their expression is not repressed *in vivo* [27–29]. In searching for a role, their possible involvement in host-to-host transmission has often been invoked. A number of researchers are currently pursuing rigorous tests of this hypothesis.

A new Bvg-repressed promoter, BRP, was recently discovered upstream of the *fim3* gene, which encodes the serotype-3 fimbrial subunit Fim3 [30]. This discovery explained the repeated observations, from transcriptomic studies, that the *fim3* gene behaved as a *vrg* in *B. pertussis* [13•,23••,25], even though its promoter, like the promoters of the other *fim* genes, *fim2* and *fimX*, behaved in a BvgA-activated manner when examined in an isolated context [9•]. The purpose of the BRP is unclear. There is an open reading frame, *vrgX*, downstream of BRP in *B. pertussis*, but *vrgX* is not present in *Bordetella bronchiseptica* [30]. A deletion of a 62 bp segment of very high GC-content apparently occurred in the evolutionary lineage leading from a *B. bronchiseptica*-like ancestor to *B. pertussis*. The effect of this deletion is both to create the *vrgX* orf and to allow transcription to proceed into the *fim3* gene. Although this transcription from BRP can result in translation of both the VrgX and Fim3 proteins, as detected by *lacZ* translational fusions, stable protein in both cases is undetectable (see Figure 3). While the roles of BRP and VrgX, if any, remain to be elucidated, BRP has been a very useful tool for studying mechanisms of *vrg*-regulation.

Molecular mechanisms of *vrg*-regulation

Unlike the Bvg-activated genes, whose regulatory mechanisms have been actively studied for more than three decades, mechanisms of *vrg*-regulation are less well understood. Previous genetic studies indicated that the

Figure 2



Promoter architecture of Bvg-activated genes. BvgA binding positions determined by BvgA-FeBABE cleavage are shown. Data for *fha*, *bipA*, *fim3*, and *brpL* have been previously reported [9•,12,13•,20••]. Assignations for *bvgAS*, *ptx*, and *cya* are based on unpublished data (P.E. Boucher *et al.*, unpublished data). Coordinates expressed relative to the transcriptional start site are given below the black bar representing promoter DNA. BvgA-binding sites identified by BvgA-FeBABE analysis were scored according to an algorithm based on systematic mutational analysis of the P*fha* high-affinity primary binding site [46]. A perfect score by this method is zero with increasingly negative numbers indicating increasingly lower predicted binding affinity. Any sites with a score of −6 or higher are represented by arrowheads colored according to the scale given below. Binding sites with lower occupancy according to the BvgA-FeBABE analysis are indicated by lower opacity of the symbols for BvgA or alpha-CTD. Alpha-CTD binding was demonstrated by FeBABE labeling of this moiety at the 276 and 301 positions in reconstituted RNA polymerase, as described in published data for *fha* and *fim3* promoters [20••,21••]. Alpha-CTD assignations for the remaining promoters are based on unpublished data (P. E. Boucher *et al.*, unpublished data). This analysis has not been performed on the *brpL* promoter. Cases where alpha-CTD binding is depicted behind the black line, such as with upstream binding sites in the *ptx* and *cya* promoters, indicate that the relative positions of the 276 and 302 cleavages were reversed relative to promoter polarity. This is interpreted to indicate that the alpha-CTD is bound to a different face of the helix, but in an identical fashion vis-à-vis the BvgA dimer directing its binding. Asterisks labeling BvgA in the *ptx* and *cya* diagrams indicate

regulation of classical *vrgs* involves at least two gene products, BvgR as a negative regulator in the Bvg^+ mode [31,32] and RisA as an activator in the Bvg^- mode [33,34]. At the time of its discovery, the DNA sequence of the *bvgR* gene showed only that it belonged to a family of conserved genes of unknown function. In fact, *bvgR* was the first gene of this family for which any function was reported. We now know that this family of genes encodes EAL-domain proteins, which are c-di-GMP phosphodiesterases capable of degrading c-di-GMP and reducing its intracellular concentration [35]. The combination of BvgR's negative effect on *vrg* expression and its predicted negative effect on c-di-GMP levels leads to the logical conclusion that c-di-GMP is a positive factor influencing *vrg* expression (Figure 1).

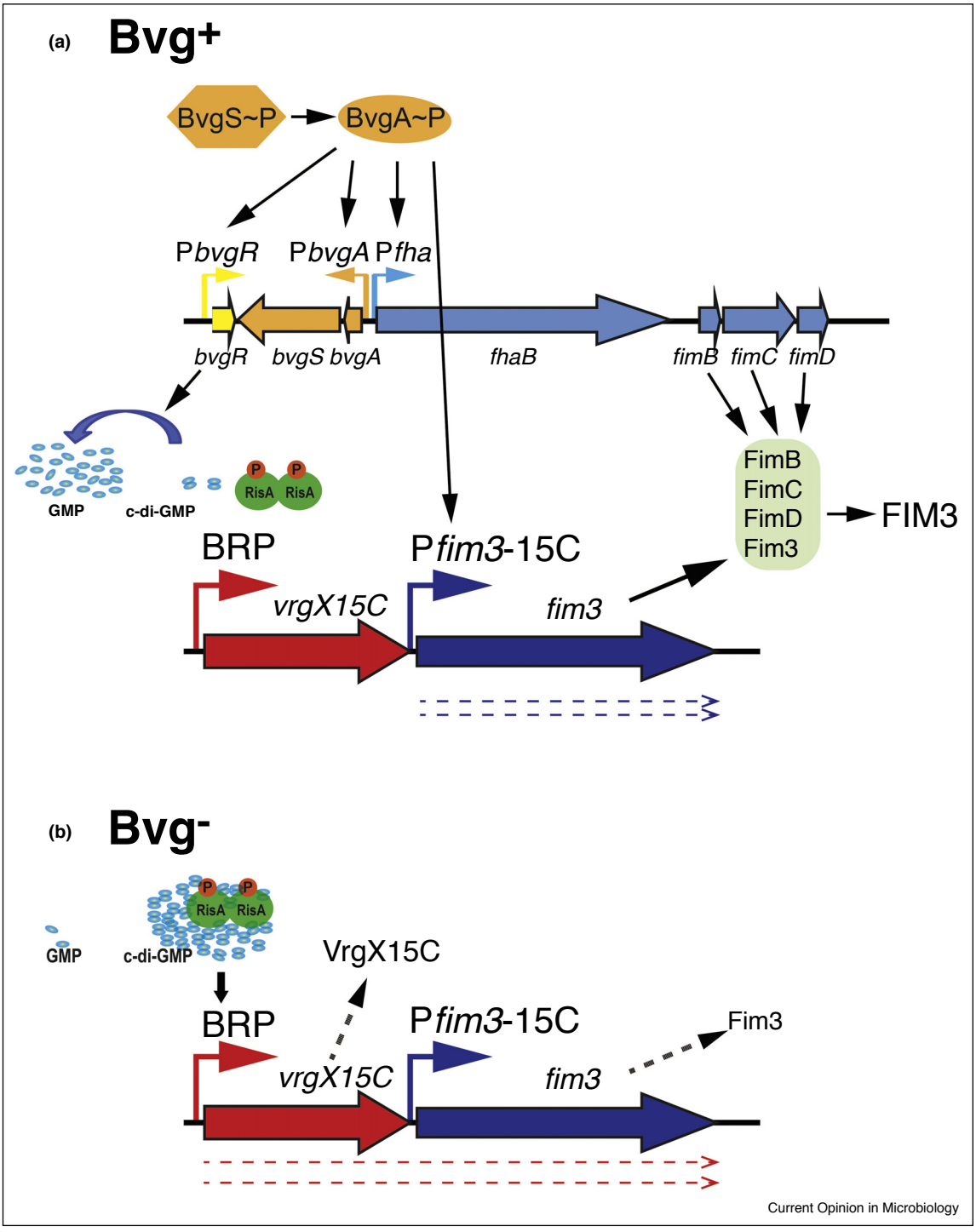
Much genetic evidence supports a role for the two-component response regulator RisA (an OmpR homologue) as a transcriptional activator of the *vrg* genes [23••,33,34,36••]. However, in all *B. pertussis* strains sequenced to-date, the companion *risS* gene, present in an operon with *risA*, and predicted to otherwise encode an EnvZ-like sensor kinase, is inactivated by a frameshift mutation upstream of the codon for the conserved histidine residue [34,37]. It had, therefore, been unclear whether RisA was in fact phosphorylated *in vivo*, and if so, whether phosphorylation was required for its ability to activate transcription of *vrg*s. Recently, these issues have been substantially clarified. A combination of genetic manipulation and the use of PhosTag^{TM} gel electrophoresis allowed Chen *et al.* [36••] to demonstrate, in *B. pertussis*, that RisA is in fact phosphorylated *in vivo*, that phosphorylation is required for its action, and that the degree of phosphorylation does not change in response to modulation. Interestingly, it is not phosphorylation *per se* that is required because mutation of the key phospho-accepting aspartate residue to a glutamic acid residue results in an active protein [36••]. A current working model, depicted in Figure 1, is that the actual cytoplasmic signal that this system responds to is c-di-GMP, whose levels are controlled by BvgR, itself the product of a Bvg-activated gene, sensitive to modulation. The actual kinase of RisA was found, independently by two groups, to be the product of the orf BP3223, and has been named RisK. Chen *et al.* [36••] identified it by systematic mutation of all sixteen predicted genes for histidine kinases. Only mutation of BP3223 led to loss of expression of a BRP-*lux* transcriptional fusion. Coutte *et al.* [23••] employed bioinformatics analyses, based on co-evolution of amino acid pairs in response regulators and their cognate histidine kinases, to identify the same gene. This group also performed extensive whole genome transcriptomic analyses of different mutant *B. pertussis* strains harboring Δ*risK*, Δ*risA*, Δ*bvgR*, $risA^{D60N}$ (phosphorylation inactivating) or $risA^{D60E}$ (phosphorylation mimicking) alleles. These studies have highlighted the complexity of RisA-controlled gene expression and have grouped genes into at least six clusters based on their requirement for non-phosphorylated RisA or phosphorylated RisA, with or without BvgR (affecting c-di-GMP levels). According to their study, both c-di-GMP (assessed by modulation with $MgSO_4$ or use of a Δ*bvgR* strain) and RisA∼P (by using $risA^{D60N}$ or Δ*risK* strains) are required for activation of almost all *vrg*s, including classical *vrg*s, capsular polysaccharide biosynthesis genes and others (Cluster 6). The same two factors, c-di-GMP and RisA∼P, appear necessary for repression of genes for flagella and chemotaxis (Cluster 4). A model for this repression, presented in Figure 1b, hypothesizes the existence of a negative regulator that is itself regulated like a *vrg*, and which is responsible for transcriptional repression of flagella and chemotaxis genes, potentially via effects on the two transcriptional activators, *flhC* and *flhD*. Interestingly, the Δ*risA* strain, but not the $risA^{D60N}$ strain, displayed a growth defect [36••] and Coutte *et al.* identified a set of genes down regulated in the deletion strain but not in the $risA^{D60N}$ strain. Further studies will be required to determine the role that these genes may play in overall fitness.

The role of the Bvg-repressed genes in *B. pertussis* evolution

Phylogenetic studies have suggested that the acutely virulent obligate human pathogen *B. pertussis* evolved from a chronically colonizing animal pathogen of the *B. bronchiseptica* lineage [37–39]. In the Bvg^+ mode, the two species produce a nearly identical set of known virulence factors, which are required for colonization and infection in both species [27,29,40], with the exception of pertussis toxin and tracheal colonization factor (reviewed in Ref. [41]). Expression of Bvg-repressed genes, on the other hand, has diverged in a systematic way. The classical *vrg*s identified in *B. pertussis*, while present in *B. bronchiseptica*, are expressed at a lower level and are up regulated to a lesser degree in the Bvg^- mode. Flagellar motility, on the other hand, is a Bvg-repressed trait in *B. bronchiseptica* but not in *B. pertussis*. [24,42–45]. It is significant that, while RisA is a central positive regulator of Bvg-repressed genes, its co-operonic histidine kinase, RisS, which is inactivated in *B. pertussis* lineages, is intact in *B. bronchiseptica*. Recent studies in our lab indicate that RisS actually functions as a phosphatase of RisA∼P (see Figure 1d). Inactivation of *risS* in *B. bronchiseptica* increases RisA∼P levels in the Bvg^- mode, leading to the expression of classical *vrg*s and repression of flagellar and chemotaxis genes. Conversely,

(Figure 2 Legend Continued) the use of a BvgA mutant that binds these promoters with higher affinity. The use of the mutant was necessitated by the reduced solubility of BvgA-FeBABE combined with the need for higher concentrations to promote binding.

Figure 3



Regulation of serotype 3 fimbriae production in *B. pertussis*. **(a)** In the Bvg$^+$ mode, *vag*s are expressed due to binding of BvgA∼P. The *fim3* promoter, if in a permissive configuration, such as P*fim3*-15C, is transcribed. This allows production of the Fim3 major fimbrial subunit, which is assembled to form serotype 3 fimbriae (FIM3) in the presence of the fimbrial assembly apparatus, chaperone FimB, usher FimC and the fimbrial tip protein FimD, whose genes are under the control of the Bvg-activated P*fha*. A Bvg-repressed promoter, BRP, located ∼400 bp upstream of P*fim3* transcriptional start, is repressed in the Bvg$^+$ mode. **(b)** In the Bvg$^-$ mode, *vag*s are silenced and *vrg*s, such as BRP, are expressed under the control of RisA∼P whose activity is influenced by c-di-GMP. Although BRP dictates the transcription and translation of downstream orfs, *vrgX15C* and *fim3*, both products are unstable (indicated by dashed lines). The instability of Fim3 is thought to be due to the absence of the fimbrial assembly apparatus in the Bvg$^-$ mode. [30].

in *B. pertussis*, reinstating an active RisS reduced RisA phosphorylation levels, lowered expression of the classical *vrg*s, capsular polysaccharide synthesis genes, and BRP, and increased expression of flagellar and chemotaxis genes (Chen *et al.*, unpublished data). Thus, it appears that the inactivation of *risS* in the *B. pertussis* lineage provided a selectable advantage due to the expression of *vrg*s in the Bvg^- mode. One such an advantage may have been an enhanced ability of the evolving *B. pertussis* to transmit to new hosts. This would have been particularly important as its host range was narrowing from multiple mammalian reservoirs to only humans.

Conclusion

Study of the BvgASR regulon, which includes both virulence genes and the Bvg-repressed genes, has revealed several novel regulatory mechanisms with clear significance for regulatory systems in other bacteria. It has also provided a framework in which to consider the evolution of the acutely virulent obligate human pathogen *B. pertussis* from the chronically colonizing multihost animal pathogen *B. bronchiseptica*. In the Bvg^+ mode, a relatively small number of mutations in the otherwise silent promoter of the pertussis toxin operon have turned on the expression of this hallmark *B. pertussis* virulence factor within the human host. In addition, through a single basepair change in the *risS* gene, an entire group of Bvg-repressed genes have been activated in the Bvg^- mode in *B. pertussis*. Long seen as having no purpose, these genes are now believed to contribute to efficient aerosol transmission, another hallmark of *B. pertussis* pathogenesis. These findings make clear the value of studying the regulation of pathogenesis. To put it another way, it is not just knowing the tools that a bacterial pathogen has, that is its virulence factors, but also an understanding of how they are implemented, that is needed for a complete description of its pathogenic lifestyle.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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