Review Article

Antigenic switching and pathogenicity: environmental effects on virulence gene expression in *Bordetella pertussis*

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Introduction

The virulence of bacteria, their capacity to cause disease, is generally dependent on the expression of a variety of properties which create a complex series of interactions between the invading pathogen and a susceptible host. Different factors may be required at different stages of infection: initially, the bacteria will usually need to adhere to tissues in sufficient numbers to establish infection; then they will need to obtain nutrients and to multiply while at the same time evading the host defence mechanisms. In some instances, they may need to invade host cells for survival or further dissemination within the host (Finlay & Falkow, 1989). The recognition that bacterial pathogenicity is multifactorial has been followed by the more recent finding that many pathogens regulate the expression of sets of virulence factors in a coordinated manner as a response to changes in their environment (J. F. Miller *et al.*, 1989*a*). These changes would be seen when the pathogen first enters the host from an outside environment, when it transfers to different sites within the host or as a response to changing conditions within the host as a result of inflammation or an increased immune response. Coordination is achieved by a regulatory locus whose expression is itself responsive to environmental signals and which then in turn alters the expression of virulence-associated genes. This review will focus on the expression of the virulence factors of *Bordetella pertussis*, a Gram-negative bacterium that causes the human respiratory disease whooping cough. The techniques of molecular genetics found early application with *B. pertussis* during attempts to define important virulence factors and to ascertain

their role in immunogenicity and disease as a necessary prelude to the design of a new acellular vaccine (Weiss & Falkow, 1983). This groundwork has subsequently led to the development of this organism as a model system for the genetic analysis of virulence.

The virulence factors of *B. pertussis*

Although the events which occur during the normal course of infection are still far from clear, progress has been made in identifying several factors that may function as virulence determinants. These have been reviewed extensively (Weiss & Hewlett, 1986; Friedman, 1988; Wardlaw & Parton, 1988; Parton, 1989) and will be described here only briefly. It is not known precisely how *B. pertussis* adheres to the cells of the respiratory tract, but several factors have been identified as putative adhesins: sero-specific agglutinogens (AGGs) 2 and 3 which are fimbrial antigens and elicit formation of agglutinating antibodies; a 69 kDa outer-membrane protein, pertactin, which is a non-fimbrial agglutinin; and filamentous haemagglutinin (FHA), a high-*M_r* rod-like surface protein. The latter two components contain arginine-glycine-aspartic acid (RGD) sequences similar to those found in eukaryotic proteins such as fibronectin and which are involved in binding to integrin receptors on mammalian cells. Pertussis toxin (PTX), which is considered a major virulence factor of *B. pertussis*, appears also to act as an adhesin. Once established, growth of the organism is undoubtedly facilitated by the production of several toxins which interfere with the clearance mechanisms of the respiratory epithelium, damage host cells to provide nutrients and suppress the immune response. In addition, *B. pertussis* produces hydroxamate siderophores to obtain essential iron and also has the capacity to internalize iron directly from

Abbreviations: ACT, adenylate cyclase toxin; AGG, agglutinin; FHA, filamentous haemagglutinin; HLT, heat-labile toxin; HLY, haemolysin; PTX, pertussis toxin; TCT, tracheal cytotoxin.

transferrin. PTX has a wide range of activities and may be responsible for the paroxysmal cough characteristic of pertussis. It is a protective immunogen and together with FHA is included in current acellular vaccines (Parton, 1991; Rappuoli *et al.*, 1991). Adenylate cyclase toxin (ACT or cyclolysin) is a bifunctional protein with adenylate cyclase and haemolysin (HLY) activities. It is able to penetrate and intoxicate mammalian cells by elevation of internal cyclic AMP levels and is thought to act primarily as an anti-phagocytic factor. Heat-labile or dermonecrotic toxin (HLT) and tracheal cytotoxin (TCT) may contribute to the disease by inducing ciliary paralysis and localized necrosis and tissue damage. Finally, the ability of *B. pertussis* to invade and survive within various mammalian cells has recently been established and is considered in more detail below.

Antigenic variation in *B. pertussis*

The notable capacity of *B. pertussis* to alternate between virulent and avirulent forms by both phenotypic (antigenic modulation) and genotypic (phase variation) means has been reviewed elsewhere (Robinson *et al.*, 1986; Coote & Brownlie, 1988). Antigenic modulation is the term applied to a freely reversible, phenotypic change, first noted by Lacey (1960), whereby all the cells in a population can alter the expression of virulence determinants in a coordinate manner in response to environmental changes. Growth with NaCl produces virulent organisms, expressing all the virulence factors mentioned above, whereas the presence of modulators such as MgSO₄ or nicotinic acid promotes simultaneous loss of these factors (except TCT), and the expression of other antigens instead. Phase variation is a genotypic change also characterized by simultaneous loss of expression of virulence factors. Avirulent phase variants arise in a population at a frequency of 10⁻³ to 10⁻⁶ and in some instances this has been shown to be reversible, although at a lower frequency (Weiss & Falkow, 1984; Stibitz *et al.*, 1989). These two processes form the main subjects of this review. The expression of AGGs 2 and 3 may be regulated by either of these processes but they can also be lost and regained independently of each other at frequencies of 10⁻³ to 10⁻⁴ in a process known as serotype variation (Stanbridge & Preston, 1974). Recent data indicate that both phase and serotype variation may occur by a similar mechanism.

Identification of a virulence regulon

The concept of a central regulatory locus which dictates the expression of a number of virulence-associated genes in *B. pertussis* was first brought to light by the pioneering

work of Weiss *et al.* (1983, 1984). They examined the virulence properties of a series of Tn5 insertion mutants defective in the expression of putative virulence determinants. Thus, for example, mutants defective in either PTX or ACT production were essentially avirulent in an infant mouse model of infection. Aside from establishing the importance of certain individual virulence factors in pathogenicity, this work identified a pleiotropically negative avirulent phenotype, designated Vir⁻, where a single Tn5 insertion prevented the expression of multiple virulence factors, including PTX, ACT, FHA and HLT. Weiss *et al.* (1983) suggested that a *trans*-acting factor(s) was encoded by the locus inactivated by Tn5 insertion and that this factor acted as a positive effector for the expression of virulence-associated genes. Weiss & Falkow (1984) proposed that modulating conditions influenced the expression of this locus (designated *vir* and since renamed *bvg*, *Bordetella* virulence gene), which in turn altered the expression of the virulence determinants. Molecular cloning techniques have since confirmed that the *bvg* locus can act in *trans* to restore virulence expression to both avirulent phase variants and strains carrying Tn5-induced *bvg* mutations (Brownlie *et al.*, 1988; Knapp & Mekalanos, 1988; Stibitz *et al.*, 1988; McGillivray *et al.*, 1989).

It was soon realized that if environmental signals affect the expression of virulence factors in a coordinate manner, then this affords a means for identification of unknown genes controlled by modulating signals. Knapp & Mekalanos (1988) further reasoned that virulence factors would for the most part be expressed at the cell surface. They used Tn5*phoA*, a reporter for loci which encode secreted proteins, to characterize mutations in genes whose promoters were subject to modulation. Two sets of genes were identified: *vir*-activated genes, *vag*, from which expression was maximal in the absence of modulators (in the same manner as the major virulence factors described above) and *vir*-repressed genes, *virg*, from which expression was derepressed in the presence of modulators. Two of the *virg-phoA* fusions were cloned and returned to *B. pertussis* on a low copy number vector (Knapp & Mekalanos, 1988; Beattie *et al.*, 1990). In the wild-type background the loci were not expressed in the absence of modulators, but were fully derepressed in a strain lacking *bvg* activity. This confirmed that their expression was subject to repression by *bvg* products. Weiss *et al.* (1989) used Tn5*lac*, which has a promoterless β -galactosidase gene, to identify mutations in genes subject to modulation. Although, as with the study of Knapp & Mekalanos (1988), several mutations were identified in previously undefined loci, all the genes identified by Weiss *et al.* (1989) behaved as *vag* loci and no *virg* loci were detected. This was explained by the fact that *virg* loci are residually active under Vir⁺ conditions

Fig. 1. Representation of the pivotal role played by the *bvg* locus in regulating expression of genes as a response to environmental signals. *vag* loci, *vir*-activated genes; *vrg* loci, *vir*-repressed genes.

and are simply derepressed in the absence of *bvg* activity. This is in contrast to the *vag* loci, which have an absolute requirement for *bvg* activity and so produce a more readily distinguishable phenotype under modulating conditions. Fig. 1 summarizes the role of the *bvg* locus in directing the expression of the *vag* and *vrg* loci.

The *bvg* regulatory locus

The locus has two genes, *bvgA* and *bvgS*, which constitute an operon and encode proteins with predicted sizes of 23 kDa and 135 kDa respectively (Arico *et al.*, 1989; Scarlato *et al.*, 1990; Stibitz & Yang, 1991; Fig. 2). The predicted amino acid sequences of BvgA and BvgS have extensive homology to a family of regulatory proteins that transmit sensory signals using conserved sequences in a two-component sensor/regulator system (Ronson *et al.*, 1987; Gross *et al.*, 1989). These systems generally consist of a sensory transmembrane protein which has kinase activity and a cytoplasmic DNA-binding protein that when phosphorylated can activate gene expression. Proteins in the sensor class usually have a conserved C-terminal region which interacts with the regulator protein and a variable N-terminal portion that senses stimulatory signals. The regulator proteins display a conserved N-terminal receiver domain and a variable C-terminal region that interacts with gene sequences. These homologies suggested a model (Arico *et al.*, 1989; Stibitz & Yang, 1991) whereby BvgS acts as a transmembrane protein with a periplasmic N-terminal receiver domain which senses external signals and transmits them to the second component, BvgA, via phosphorylation which allows BvgA to regulate specific loci. Available data fully substantiate this model, but suggest that a hierarchy of regulation exists with different requirements for the control of expression of various *bvg*-dependent loci.

The *bvg* locus is closely linked to three genes involved

in FHA production (Stibitz *et al.*, 1988; Fig. 2). A cosmid clone containing *bvg* and *fha* loci expressed FHA in *E. coli* and this expression was sensitive to modulation (Stibitz *et al.*, 1988). A subclone containing only the *fha* loci did not express FHA and all other attempts to express virulence-associated loci from their own promoters in *E. coli* have been unsuccessful. This fact can be exploited to determine if the presence of the *bvg* locus alone is sufficient to restore expression in the *E. coli* background. This was confirmed for the *fha* locus by the construction of a single copy *lacZYA* fusion to the promoter region of *fhaB* (the structural gene for FHA) integrated into the *E. coli* chromosome. Such a construct was activated 400-fold by the *bvg* locus supplied in *trans* and activity was severely reduced by modulation (J. F. Miller *et al.*, 1989b). In this instance, the *bvg* locus was sufficient for sensory transduction and transcriptional activation in *E. coli*. This was not true, however, for similar *lacZYA* fusions to *ptxA* and *cyaA*, the promoter-proximal genes of the operons for PTX and ACT production (Nicosia *et al.*, 1986; Loch & Keith, 1986; Brownlie *et al.*, 1988; Glaser *et al.*, 1988), or for *phoA* fusions to two *vrg* genes. In these instances, the presence of the *bvg* locus in *trans* had no effect on the transcription of these genes (J. F. Miller *et al.*, 1989b; Beattie *et al.*, 1990; Goyard & Ullmann, 1991). These data indicated that the influence of *bvg* on these loci was indirect and that additional factors were perhaps required for their transcription.

Other work with the *fhaB::lacZYA* fusion in the *E. coli* chromosome showed that BvgA alone could act as a transcriptional activator (Roy *et al.*, 1989). Overproduction of this protein from a strong heterologous promoter activated the *fhaB* fusion 600-fold. There was no such activation of a similar chromosomal fusion to the *ptxA* promoter. The activation of the *fhaB* fusion was not subject to modulation, indicating that the BvgS protein was required for sensing environmental signals. The observation that overproduction of a regulatory protein creates constitutive activity in the absence of its cognate sensor protein has been noted in other systems (see Roy *et al.*, 1989 for references), including that of ToxR in *Vibrio cholerae*. ToxR is a regulatory protein analogous to BvgA that is responsible for the coordinate expression of several virulence factors in *V. cholerae* (V. L. Miller *et al.*, 1987). When expressed from a strong constitutive promoter in *E. coli*, ToxR activates expression from the virulence loci, but if the native *toxR* promoter is retained then full activation requires another protein, ToxS, the product of a gene immediately downstream of *toxR* in an operon similar to the *bvg* operon (V. L. Miller *et al.*, 1989). There is evidence that low level activation of regulatory proteins can occur via sensor proteins from other similar two-component systems present in the *E.*

Fig. 2. Organization of the *bvg* and *fha* loci. Large open arrows indicate the direction of translation of the relevant open reading frames (ORFs) and small arrows the direction of transcription from the various promoters. Heptameric sequences forming inverted repeats and direct repeats upstream of *fhaB* and *bvgA* respectively are shown as solid arrows. Transmitter and receiver bars in *bvgA* or *bvgS* indicate the locations of sequences homologous to other members of sensory transduction systems that contain similar domains. Numbers represent base pairs from the start of the *bvgA* gene, but the *fhaB*/*bvgA* intergenic region is not drawn to the same scale as the ORFs, which consist of the following numbers of amino acids: *bvgA* (209), *bvgS* (1211), *fhaB* (3591). Data from Arico *et al.* (1989), Delisse-Gathoye *et al.* (1990), Domenighini *et al.* (1990), Roy *et al.* (1990), Scarlato *et al.* (1990) and Roy & Falkow (1991).

coli cell. This process, termed cross-talk, has been shown to occur *in vitro* (Ninfa *et al.*, 1988), but is inefficient and requires larger amounts of regulatory protein to be effective.

Construction of an in-frame deletion in the chromosomal *bvgA* gene in *B. pertussis* prevented transcription from the *fhaB* and *ptxA* promoters (Roy *et al.*, 1989). Thus, although BvgA cannot activate the *ptx* promoter in *E. coli*, it is clearly required for activation of the *ptx* operon in *B. pertussis*. This suggests that a regulatory hierarchy exists in *B. pertussis* for the expression of *vag* and *vrg* genes. Roy *et al.* (1989) proposed that BvgA would activate transcription of the *fha* locus and, in addition, one or more genes whose products are required for the positive expression of other *vag* loci or the repression of *vrg* loci. A cascade effect such as this might mediate temporal expression of virulence determinants as a result of environmental change.

The genes for auxiliary regulatory factors should be identifiable by mutations that affect a subset of virulence determinants. This possibility is supported by the observations of Goldman *et al.* (1984), who examined phase variants of *B. pertussis* which arose at a frequency of 10^{-6} to 10^{-7} . They screened over 100 variants for HLY, PTX and FHA production and four phenotypic classes were observed: Hly⁺Ptx⁺Fha⁺ (9%), Hly⁻Ptx⁺Fha⁺ (17%), Hly⁻Ptx⁻Fha⁺ (8%) and Hly⁻Ptx⁻Fha⁻ (65%). The latter group probably represented the full avirulent Vir⁻ phenotype. The isolation of only four phenotypes from a possible eight indicated that the loss of virulence factors had occurred in a non-random way, with FHA production being the last to be lost in accordance with the hierarchy of expression outlined above. A search for a cosmid clone from a gene library of *B. pertussis* able to promote *bvg* activation of *ptxA::lacZYA* transcription in *E. coli* proved unsuccessful (J. F. Miller *et al.*, 1990). However, gel-retardation studies have recently identified a 23 kDa protein, found in *bvg*⁺ but not *bvg*⁻ strains, that binds to the promoter regions of the *ptx* and *cya* loci (Huh & Weiss, 1991). This is a protein distinct from BvgA which has been shown not to bind to the *ptx* promoter (Roy & Falkow, 1991;

Huh & Weiss, 1991). It is likely that this protein represents an auxiliary factor required for the expression of the *ptx* and *cya* genes. A locus, *toxT*, has recently been described in *V. cholerae* whose product(s) activates the promoters of certain virulence-associated genes dependent on ToxR (DiRita *et al.*, 1991). These promoters, unlike, for example, that of *ctxAB*, the cholera toxin operon, are not directly activated by ToxR, but rather the *toxT* locus is activated by ToxR and then produces the auxiliary regulatory factor(s). This is obviously similar to the pattern of regulation emerging in *B. pertussis*.

Transcription of *bvg*-locus-controlled genes

The *bvg* and *fha* loci

The *bvg* locus itself is subject to positive autoregulation. A single copy *lacZYA* fusion to the *bvg* promoter region integrated into the *E. coli* chromosome was activated 10-fold by the *bvg* locus *in trans* on a multicopy plasmid (Roy *et al.*, 1990). Activity was reduced to a basal level under modulating conditions. Similar results were obtained with *bvg* gene fusions created by allelic exchange in the chromosome of *B. pertussis* which confirmed a previous report in which Northern blot analysis had shown that modulation eliminated transcription of *bvg*-regulated genes, such as the *ptx* and *cya* loci, and reduced transcription from the *bvg* locus itself (Melton & Weiss, 1989).

The close proximity of the *bvg* and *fha* loci and their capacity to be transcribed in *E. coli* has allowed the regulatory sequences controlling these genes to be thoroughly investigated (Fig. 2). In all, five promoters have been identified in the 432 bp intergenic region between *fhaB* and *bvgA* (J. F. Miller *et al.*, 1990; Scarlato *et al.*, 1990; Roy *et al.*, 1990). Primer extension analysis identified two transcriptional start sites 90 bp (P1) and 141 bp (P2) upstream of the *bvgA* gene both in *B. pertussis* and from the cloned genes in *E. coli* (Roy *et al.*, 1990). S1 nuclease protection studies in *B. pertussis* located a third start site 270 bp (P3) from the translational start codon of *bvgA* and a fourth promoter (P4) on the DNA strand complementary to the *bvg* coding

strand, starting 20 bp from *bvgA* and driving transcription of a 155 bp anti-sense RNA complementary to the untranslated regions of the three RNAs transcribed from promoters P1, P2 and P3 (Scarlato *et al.*, 1990). Finally, a start site for transcription of the *fhaB* gene was located on the same DNA strand as P4, but beginning 70 bp from the start of the *fhaB* gene in both *B. pertussis* and *E. coli* (J. F. Miller *et al.*, 1990; Scarlato *et al.*, 1990). The *bvg* and *fha* loci are therefore regulated by divergent promoters which are subject to activation by the products of the *bvg* locus.

Inactivation of either the *bvgA* or the *bvgS* gene, or the imposition of modulating conditions, shut off transcription from the *bvg* P1, P3 and P4 promoters and the *fhaB* promoter both in *B. pertussis* and from the cloned genes in *E. coli* (Roy *et al.*, 1990; Scarlato *et al.*, 1990). This confirmed that these promoters were positively regulated by the products of the *bvg* locus and that both a functional BvgA and a BvgS protein were required for their activation. Transcription from the *bvg* P2 promoter was maintained under these conditions and this constitutive activity is assumed to direct the synthesis of a low level of BvgA and S proteins.

Scarlato *et al.* (1990) raised antibody against the BvgA protein and showed by Western blotting that BvgA was located in the *B. pertussis* cytoplasm, in keeping with its role as a DNA-binding protein. A constitutive low level of the protein was produced in BvgS-defective cells or in the wild-type under modulating conditions, presumably produced via activity at the *bvg* P2 promoter. In the absence of modulation, BvgA concentration increased 50-fold, yet was lost only slowly upon re-addition of a modulator. Gross & Rappuoli (1989) had reported the immediate cessation of transcription from the *bvg*-controlled *ptxA* promoter upon addition of the modulator MgSO₄. Within 10 min of addition of the modulator no mRNA directed by this promoter was found by Northern blotting. This observation indicated that modulation changed the activity but not the amount of BvgA. Addition of a cross-linking reagent to cell lysates established the presence only in non-modulated cells of a 46 kDa protein which cross-reacted with anti-BvgA serum (Scarlato *et al.*, 1990). These authors therefore suggested that the 46 kDa protein may have corresponded to a complex of BvgA with another protein or to a dimer of BvgA itself. As a working hypothesis, they proposed that under non-inducing conditions only *bvg* P2 is active to produce a low level of the BvgAS proteins. BvgA is in the monomeric form, but after a signal received by BvgS from the periplasm, BvgA is activated (possibly by phosphorylation) and adopts the dimeric form. This form *trans*-activates *bvg* P1, P3, P4 and the *fhaB* promoters which results in increased synthesis of BvgA in sufficient amounts to activate the other *bvg*-

dependent genes in the regulon. The active form of BvgA depends on continued signal transmission by BvgS and if this ceases, BvgA is rapidly inactivated. At the present time, several features of this model remain to be elucidated. It is not clear how the active form of BvgA is inactivated in such a way as to bring about an almost immediate cessation of transcription of *bvg*-regulated loci on modulation. In addition, it has to be established if BvgA requires an auxiliary factor to function at loci such as *ptx* and *cya* or whether it is simply required to activate genes for synthesis of secondary effectors which function in place of BvgA. The cross-linked 46 kDa protein described by Scarlato *et al.* (1990) could be BvgA combined with the 23 kDa protein described by Huh & Weiss (1991), which associated with the promoter region of the *ptx* locus. This possibility might suggest that only one auxiliary protein was required to co-activate several loci as only the one cross-linked polypeptide was found besides BvgA alone. Alternatively, one or more auxiliary proteins of a similar *M_r* might associate with BvgA. The role of the putative anti-sense RNA produced from *bvg* P4 has not been established. It might be expected to interfere with translation of the complementary mRNA by binding to the ribosomal-binding site, but the data of Scarlato *et al.* (1990) show that the anti-sense transcript starts at least 5 bp beyond the putative ribosomal-binding site located 8 to 15 nucleotides upstream of the translational start site of the *bvgA* gene. In this case it may not interfere with the initiation of translation of the *bvgA* mRNA and the authors suggested that it may instead have a positive regulatory function favouring interaction of the mRNA with the ribosomes.

Stibitz & Yang (1991) raised antibody against PhoA fusions to both BvgA and BvgS. They confirmed that the amount of BvgA protein detectable in *B. pertussis* by immunoblotting decreased under modulating conditions, but interestingly, the amount of BvgS protein showed little reduction. This indicated that the synthesis of BvgS was not regulated in the same way as BvgA. The way in which that is achieved has yet to be elucidated, but the mechanism presumably maintains a constant level of the sensor protein for continual interaction with the environment.

As the *bvg* and *fha* loci were transcribed in a similar way both in *B. pertussis* and in *E. coli*, deletion analysis was used to establish that a minimum of 85 bp of sequence upstream of the *fhaB* transcriptional start site was required for activation by the *bvg* locus in *E. coli* (Roy & Falkow, 1991). DNAase protection studies showed that BvgA protected regions between -101 and -78 and -71 to -67 of the *fhaB* promoter. Within the -96 to -83 region was a 14 bp inverted repeat and deletions which eliminated this repeat domain were no longer activated by *bvg* in *trans*. Moreover, the oligo-

nucleotide sequence corresponding to the -99 to -80 region, when present on a multicopy plasmid, completely inhibited activation of the *fhaB* promoter by the *bvg* locus (J. F. Miller *et al.*, 1990). This effect was assumed to be due to sequence-specific titration of the BvgA protein. DNAase protection studies also showed that BvgA protected a 32 bp region just upstream of *bvg* P2. This region contained the heptameric sequences TTTCTTA and TTTGGTA as direct repeats. The former sequence made up one half of the inverted repeat in the *fhaB* promoter; the other half was TTTCTTA. The *bvg* promoter region containing the direct repeats was also able to reduce *trans* activation of the *fhaB* promoter by BvgAS, presumably again due to titration of BvgA (J. F. Miller *et al.*, 1990). A similar construct carrying the *ptxA* promoter region had no effect in the titration assay. This is in keeping with the absence of the heptameric sequences in the *ptx* promoter and the lack of BvgA activation of this locus. Differences noted in the capacity of BvgAS to *trans*-activate *lacZYA* fusions to the *fha* and *bvg* loci, 300-fold and less than 10-fold respectively, were ascribed to a proposed higher affinity of BvgA for the inverted repeat of the *fha* promoter rather than the direct repeat present in the *bvg* promoter (Roy & Falkow, 1991). The heptameric sequences present in the *bvg* and *fha* promoter regions are similar to the TTTTGAT element tandemly repeated in the promoter region of the *ctxAB* operon of *V. cholerae* where the ToxR regulatory protein has been shown to bind (V. L. Miller *et al.*, 1987).

Other *bvg*-regulated loci

Deletion analysis of the *ptx* promoter region showed that at least 170 bp upstream of the transcriptional start site was necessary for *bvg*-dependent activation in *B. pertussis* (Gross & Rappuoli 1988, 1989). Deletions between -170 and -120 bp progressively reduced activity in a *bvg*-proficient background. This region contained two 21 bp direct repeats between bases -157 and -117 where it was proposed that products associated with BvgAS activity were bound to activate transcription. In keeping with this, the binding of the 23 kDa protein, identified by Huh & Weiss (1991), to the *ptx* promoter was prevented by an oligonucleotide corresponding to either repeat region. Huh & Weiss (1991) identified two similar 21 bp tandemly repeated sequences between bases -157 and -114 in the *cya* promoter region. Again, oligonucleotides corresponding to either of these repeat sequences bound strongly to the 23 kDa protein, but not to BvgA.

The promoter regions of several other virulence-associated genes have now been sequenced (Livey *et al.*, 1987; Charles *et al.*, 1989; Willems *et al.*, 1990; Beattie *et al.*, 1990). The repeat sequences characterized in the

promoter regions of the *fha*, *bvg*, *ptx* or *cya* operons have not been identified in these determinants, although with the *fim-3* gene for AGG3 synthesis, transcription was shown to require functional *bvg* activity (Willems *et al.*, 1990; MacGregor *et al.*, 1991). Similarly, in a *bvg*⁻ background, the gene encoding the 69 kDa adhesin pertactin was not expressed (Charles *et al.*, 1989) and two *vrg* genes were fully derepressed (Beattie *et al.*, 1990). However, the 21 bp sequence present in the *ptx* promoter recurs at nucleotide 60 within the first gene of the *ptx* operon encoding the S1 subunit (Gross & Rappuoli, 1989). Two related inverted repeat sequences of 12 and 15 bp have been described in the first 60 bp of the coding region of the *fhaB* gene (Relman *et al.*, 1989). The significance, if any, of these repeat units within the coding regions of these genes remains to be examined. It is clear, however, that modulation of transcription of two *vrg* genes occurs downstream of the promoter region (Beattie *et al.*, 1990). The expression of *vrg-6* and *vrg-18* was repressed in the presence of an active *bvg* locus. Progressive deletion of the promoter region in both cases caused a drop in transcription efficiency due to loss of the promoter, but modulation of transcription was nevertheless maintained even when only 24 bp of *vrg-18* upstream of the translational start codon remained. A 21 bp region of dyad symmetry forming a 10 bp inverted repeat was identified in the N-terminal coding region of each *vrg* gene and linker insertion in this region, identified as a hydrophobic signal sequence, abolished repression by the *bvg* locus. It is possible to visualize how *bvg* regulation might occur within the translated regions of the *vrg* genes. An effector molecule might bind to the mRNA and prevent translation which, coupled to increased mRNA degradation, would result in loss of function.

A 29 bp region containing an 11 bp inverted repeat was found in the *fim-2* promoter region between 167 and 140 bp upstream of the translational start codon (Livey *et al.*, 1987). Interestingly, primer extension analysis placed the transcriptional start site at -146 bp which overlaps this repeat region (Walker *et al.*, 1991), although the size of the *fim-3* mRNA detected by Northern blotting conflicted with this data and indicated a shorter distance between the transcriptional and translational start sites (Willems *et al.*, 1990). A similarly positioned inverted repeat was found in the *fim-3* gene promoter region, but was not identified in a determinant designated *fim-X* which has close homology to the *fim-2* and *fim-3* genes, yet does not express an identifiable fimbrial protein (Pedroni *et al.*, 1988). It remains to be determined if transcriptional regulators interact with these repeat regions.

Taken together these data indicate that, although the virulence-associated determinants of *B. pertussis* constitute a regulon controlled by the *bvg* locus, the mechanism

regulating expression may vary with different determinants. This variation almost certainly involves promoter-specific factors in the case of the *vag* genes, but control of *vrg* gene expression may be at the level of mRNA stability.

A model for antigenic modulation and phase variation

The human respiratory tract is the only known habitat for *B. pertussis* and the nature of the signals that might influence the activity of the *bvg* regulon *in vivo* are unknown. Nor is it clear how these signals influence the properties of BvgS, the proposed sensory component of the system. A number of environmental conditions besides the presence of MgSO_4 and nicotinic acid, including other salts, fatty acids and growth at 25 °C create modulating conditions (Lacey, 1960; Brownlie *et al.*, 1985). With regard to salts it is clear that the sulphate anion is important as MgCl_2 has no modulating effect, but other cations such as Na^+ , K^+ or NH_4^+ can replace Mg^{2+} (Brownlie *et al.*, 1985; Melton & Weiss, 1989). Modulation can be induced by analogues of nicotinic acid where the pyridine ring is substituted with a carboxyl group, but not by nicotinamide (Wardlaw *et al.*, 1976; Schneider & Parker, 1982). Modification of the carboxyl group by methylation or acetylation abolishes the modulating activity of nicotinic acid. These studies suggest that sulphate ions and nicotinic acid induce modulation through specific intermolecular reactions, but the nature of these and their relevance to events *in vivo* is not known. It has been suggested that excess sulphate, an ion with a similar structure to that of phosphate, may interfere with the phosphorylation mechanism whereby BvgS transmits external signals to BvgA (Melton & Weiss, 1989). In other systems, such as the *toxRS* regulon in *V. cholerae*, osmolarity and temperature have been shown to play an important role in the expression of virulence determinants (V. L. Miller & Mekalanos, 1988; Parsot & Mekalanos, 1990).

There is indirect evidence to indicate that BvgS may act as a dimer to activate BvgA. Stibitz & Yang (1990) created several in-frame mutations in the *bvgS* gene by linker insertion and returned these constructs to *B. pertussis* on a multicopy plasmid. Four of five mutations were *trans*-dominant to the wild-type. One interpretation of this is that BvgS acts as a multimer and that excess mutant protein expressed from the plasmid created non-functional oligomers having at least one defective protein constituent. In keeping with this, in the reverse situation, when the wild-type protein was overproduced in the mutant strains, a wild-type phenotype resulted. Moreover, intracistronic complementation occurred

Fig. 3. A model for the sensory activation of the *bvg* regulon. Appropriate signals sensed by the N-terminal domain of BvgS in the periplasm promote the protein to adopt either a monomeric or dimeric form in the inner membrane. In the dimeric form BvgS is active as a kinase and promotes the formation of active BvgA in the form of a dimer in the cytoplasm. Active BvgA is able to interact with the promoter regions of appropriate loci within the *bvg* regulon. N and C represent N- and C-terminal domains of the proteins, C₁ of BvgS has homology to C-terminal transmitter domains of other sensory proteins, but C₂ is unusual and has homology to receiver domains of regulatory proteins. The N-terminus of BvgA has homology to the receiver domains of other regulatory proteins. Data from Arico *et al.* (1989), Gross *et al.* (1989) and Stibitz & Yang (1990).

between two mutations at either end of the *bvgS* gene. The two mutant proteins were non-functional individually, but functional when expressed together in the same cell. Stibitz & Yang (1990) proposed that activation of BvgA (which itself may act as a dimer) is dependent on the dimeric state of BvgS which is sensitive to modulating conditions. A model for signal transmission can be envisaged (Fig. 3) which relies on dimerization of BvgS starting in the periplasm and which when completed serves to activate BvgA in the cytoplasm. BvgA in turn may be induced to take up an active dimeric form or more readily be able to associate with another effector molecule. In *V. cholerae*, dimerization of ToxR catalysed by ToxS has been proposed as a critical event for the subsequent activity of ToxR as a DNA-binding protein (DiRita & Mekalanos, 1991). Although the ToxR and S proteins appear not to have the transmitter/receiver relationship characteristic of other two-component systems, ToxR is nevertheless homologous at its N-terminal end to the DNA-binding domains of several regulator proteins (Gross *et al.*, 1989). One unusual feature of BvgS is that it has at its C-terminal end (C₂ in Fig. 3) a region homologous to receiver domains normally present in regulatory molecules, including BvgA (Arico *et al.*, 1989). The protein thus has a 'receiver' module (C₂, residues 947 to 1064) fused to the

typical transmitter module (C₁, residues 698 to 930) normally associated with sensor proteins. The role of this extra 'receiver' module is at present unknown, but it is certainly required for *bvg* regulation as a Tn5 insertion in this region, or an in-frame linker insertion a short distance downstream of the region, gave a Bvg⁻ phenotype (Arico *et al.*, 1989). However, Lee *et al.* (1990) examined a *B. pertussis* derivative with a plasmid insertion in the *bvgS* gene which effectively removed 85 amino acids from the C-terminal end of the protein. This strain had an intermediate phenotype with regard to virulence factor expression; it was Hly⁻Ptx⁻, but produced small amounts of FHA and pertactin and was able to invade HeLa cells (see below). These features were not exhibited by the Bvg⁻ strains mentioned above, nor by a Bvg⁻ strain carrying a frame-shift mutation at residue 1075 in a repetitive cytosine sequence (Stibitz *et al.*, 1989). This latter mutation resulted in the loss of an additional 49 residues from BvgS starting just beyond the C₂ 'receiver' module. The additional 'receiver' domain is thus important for *bvg* activity, but the extreme C-terminal end of the protein may not be required for expression from promoters directly activated by BvgA, such as that for FHA, and possibly those for pertactin synthesis and cell invasion, but may have a role in the activation of other *bvg*-dependent loci.

Knapp & Mekalanos (1988) described a series of spontaneous mutants that expressed *vag* functions such as HLY, PTX and FHA under modulating conditions and which showed no derepression of *vrg* functions. The constitutive phenotype was apparent in the presence of either MgSO₄, nicotinic acid or low temperature, confirming that the different modulators affected gene expression by a common component associated with the constitutive mutation, designated *mod*. It might have been expected that *mod* either represented a mutation in the *bvg* locus which locked BvgS or BvgA in the active form, making either unable to respond to modulating signals, or it identified another gene whose product interacted directly with modulating signals. The mutation was *trans*-dominant to the wild-type and was closely linked to the *bvg* locus. Knapp & Mekalanos (1988) suggested that the *mod* gene may encode, by analogy with ToxS of *V. cholerae*, a protein which interacts with BvgS to modulate its activity. The additional 'receiver' domain on BvgS could be the site of such an interaction. A complicating factor for this interpretation is that in *E. coli* only the *bvg* locus carried on a plasmid was apparently required to *trans*-activate the chromosomal *fhaB::lacZYA* fusion (J. F. Miller *et al.*, 1989b). The 5.1 kb insert in the plasmid corresponded to the 5'- and 3'-boundaries of the *bvgAS* operon and further deletions at either end eliminated activity. In no case was a deletion that affected modulation, but not activation

detected. This might suggest that BvgA and S function in *E. coli* without the *mod* gene product, but J. F. Miller *et al.* (1989b) reported similar constitutive mutants which retained their activity in *E. coli* and *trans*-activated *fhaB::lacZYA* expression even under modulating conditions. The precise nature of these constitutive *bvg*-associated mutations has thus still to be defined.

For one strain of *B. pertussis* that has been studied in detail, the genotypic change to the avirulent form (phase variation) has been shown to be a reversible process caused by insertion or deletion of a cytosine residue in a run of six such residues within the *bvgS* gene (Stibitz *et al.*, 1989). In other instances, stable phase variants have been shown to possess small deletions or additions to the *bvg* locus (McGillivray *et al.*, 1989; Monack *et al.*, 1989). Expression of AGGs 2 and 3 is controlled by the *bvg* locus (Walker *et al.*, 1990; Willems *et al.*, 1990; MacGregor *et al.*, 1991), but they can be lost and regained independently during serotype variation. This change is dependent on *cis*-acting elements associated with the *fim* genes rather than the production of *trans*-acting molecules (Willems *et al.*, 1990; MacGregor *et al.*, 1991). Willems *et al.* (1990) produced evidence which suggested that serotype variation may be due to insertion/deletion events within a cytosine-rich region located some 70 bp upstream of the translational start site of the *fim* genes. This region contained 15 and 13 cytosine residues respectively in the *fim-2* and *fim-3* genes sequenced from strains producing high levels of these AGGs, to between 12 and 9 residues from strains producing low levels and down to 7 residues for a gene *fim-X* assumed not to produce a fimbrial protein. A variant of a serotype 2⁺,3⁻ strain was selected by *in vivo* passage after infection of a rabbit. After 40 d, serotype 2⁺,3⁺ strains were isolated and the promoter region of one of these strains, when sequenced, had an extra cytosine inserted in the cytosine-rich region. The regions where these alterations occurred lies outside of the coding region of the *fim* genes and Willems *et al.* (1990) suggested that the number of cytosine residues may influence the ability of the transcriptional apparatus to express the *fim* genes. Insertions or deletions in the run of cytosine residues may, for example, alter the alignment of regulatory factors with respect to the RNA polymerase. Reiterated base sequences are known to cause duplications or deletions during DNA replication, and antigenic variation by insertions or deletions has been documented for several pathogens (Stern & Meyer, 1987; Rosquist *et al.*, 1988; Weiser *et al.*, 1989). However, in all these instances, and including that of BvgS in *B. pertussis*, translation is affected due to frame-shift alterations. The significance of the alterations outside of the coding regions of the *fim* genes remains to be established.

Antigenic modulation and phase variation *in vivo*

The role of the BvgAS sensory and regulatory system in pathogenesis is unknown. However, it is unlikely that such a system would exist if it was not important for the organism to adapt to a changing environment by altering the expression of important antigens. The human respiratory tract is the only known habitat for *B. pertussis* and no other reservoir or asymptomatic carrier state has been identified. Two early reports indicated that both phase variation and antigenic modulation occur *in vivo* and are not simply laboratory phenomena. Kasuga *et al.* (1954) isolated avirulent phase variants from pertussis patients only in the later stages of the disease. It is unknown at present if the reversible frame-shift mutations in the extended cytosine residues of BvgS (Stibitz *et al.*, 1989) represent a programmed genotypic change which is of use to the bacterium and has consequently been maintained against evolutionary elimination in what is a highly adapted organism. Lacey (1960) observed that, of sera from 50 convalescent patients, 42 possessed antibodies specific to the virulent phase and three to the avirulent phase. Only two of the sera with virulent phase antibodies were from adults whereas all three sera with avirulent phase antibodies were from patients over the age of nineteen. These latter patients had only a mild cough, but were culture positive for 2 to 5 months. Thus the avirulent phase may represent a pathogenically dormant state associated with the later stages of infection, when the host immune response has developed, and may be a strategy for immune evasion and persistence within the host. Lacey (1960) noted that avirulent phase antigens expressed in the presence of modulators were less immunogenic than those from the virulent phase and that these were the antigens characteristic of the long-term carriers of *B. pertussis*. The avirulent phase antigens described by Lacey (1960) may thus be the products of the *vrg* genes which may contribute to the persistence of the organism. This is of concern if new acellular vaccines are to consist of antigens produced only during the virulent phase.

Robinson *et al.* (1986) suggested that, as modulated organisms adhere less readily to mammalian cells than those expressing virulent phase antigens, modulation may aid expulsion of bacteria and thus enhance transmission of the disease. As disease progresses local tissue damage may give rise to signals that favour modulation and, as temperatures below 28 °C induce modulation, this condition would be maintained outside the host. It is not known at present if *B. pertussis* can maintain an environmental reservoir outside the human host and it has not been shown experimentally that modulated cells are capable of switching to the virulent phase and initiating infection in animal models.

The colonization site of the bacterium is assumed to be the surface of the ciliated respiratory epithelium, but recent reports have shown that *B. pertussis* can be taken up by and survive within mammalian epithelial cells and macrophages (Ewanowich *et al.*, 1989; Lee *et al.*, 1990; Saukkonen *et al.*, 1991). In non-professional phagocytes the process is mediated by microfilament-dependent endocytosis and requires products controlled by the *bvg* locus, but these have not been identified. In macrophages, modulated cells and cells deficient in BvgAS activity were as efficiently internalized as virulent organisms (Lee *et al.*, 1990) by a host-specified mechanism (Saukkonen *et al.*, 1991). Uptake was followed by bacterial survival and slow multiplication over a 4 h period. Recent data have shown that bacteria taken up by leucocytes induced high levels of the respiratory burst normally associated with bacterial killing by phagocytes, but prevented phagosome-lysosome fusion, and this feature was suggested as the means of survival (Steed *et al.*, 1991). When rabbits were infected with *B. pertussis*, approximately half of the total bacterial population recovered from tracheal lavage fluid 24 h after infection appeared to reside within macrophages (Saukkonen *et al.*, 1991). Pulmonary oedema was attributed to the extracellular cilia-associated bacterial population, however, which indicated either that the intracellular location of bacteria did not permit release of toxins or that the bacteria were induced to modulate to the avirulent phenotype. This latter possibility is supported by the observation that a mutant strain of *B. pertussis* constitutively expressing ACT, independently of *bvg* activity, continued to produce enzymic activity after entry into alveolar macrophages *in vitro* whereas the parent strain showed a steady decline in activity over a 2 h period (Masure & Barrett, 1991). Cessation of ACT production by the wild-type bacteria was interpreted to mean that antigenic modulation had occurred within the macrophages.

The significance of a possible intracellular population of *B. pertussis in vivo* is open to speculation. Bacteria would be protected from antibody and complement and, in a quiescent state, would represent an asymptomatic carrier condition. The factors that might influence their re-emergence from host cells have not been examined. The full implications of regulatory systems like *bvgAS* in *B. pertussis* and *toxRS* in *V. cholerae* have yet to be realized, but they both clearly offer a means of antigen switching as a response to environmental change. Moreover, there appears to be a hierarchy of expression of antigens, at least in the virulent phase. In the case of *B. pertussis*, BvgA serves to activate genes for its own production and for FHA and possibly also for pertactin and proteins for host cell invasion, together presumably with at least one other unidentified gene whose product

almost certainly acts as an effector for the expression of other virulence factors such as PTX and ACT. It is possible that this latter gene expression may be subject to different environmental signals relayed through the *bvg* locus. From the viewpoint of the bacterium, efficient attachment to respiratory tissue would be followed in a controlled sequence by production of factors that promote establishment of the organism and subsequent evasion of host defences. In the later stages of infection the organism may adopt the avirulent, immunogenically inert condition to allow survival or onward transmission to a new host.

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