Response of the *bvg* regulon of *Bordetella pertussis* to different temperatures and short-term temperature shifts

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***Bordetella pertussis* produces a number of virulence factors whose expression is coordinately regulated by the *bvgAS* locus. Transcription of virulence genes is repressed by environmental factors such as low temperature (25 °C) and chemical stimuli. Temperature shift of bacterial cultures from 25 °C to 37 °C activates two classes of *bvg*-regulated virulence genes: the early genes, which are activated within 10 min, and late genes, which require 2–4 h for activation. During the interval between the activation of the early and late genes, the intracellular concentration of BvgA increases 50-fold. It has been proposed that this increased concentration may be required for the activation of the late genes. Here we have analysed the response of the *bvg* locus to intermediate temperatures and to repeated temperature shifts. Temperature shifts of *B. pertussis* cultures from 22 °C to 28 °C, 32 °C or 35 °C resulted in the synthesis of low, intermediate, and high amounts of BvgA. This implied that the intracellular concentration of BvgA is temperature-dependent. We have also observed that the amount of virulence factors produced correlates with the BvgA concentration. When bacteria grown at 37 °C were shifted to 22 °C, transcription from the adenylate cyclase toxin haemolysis promoter (P_{AC}) was repressed after 30 min, while transcription from the *bvg* (P_1) and filamentous haemagglutinin (P_{FHA}) promoters was repressed after 2 h. During this time, the amount of BvgA did not decrease. A subsequent temperature shift from 22 °C to 37 °C induced transcription from the P_1 and P_{FHA} promoters after 10 min and transcription from the P_{AC} promoter after 20 min. This result shows that in the presence of a high concentration of BvgA, the time lag between temperature shift and late promoter transcription is reduced from 2–4 h to 20 min. The above data support the proposal that the concentration of BvgA plays a role in activating expression of the late genes.**

Keywords: *bvg* locus, environmental regulation, thermoregulation, virulence genes, *Bordetella pertussis*

INTRODUCTION

The virulence genes of *Bordetella pertussis*, the human pathogen which causes whooping cough, are coordinately regulated by a sensory transduction system encoded by the *bvg* locus (Aricò *et al.*, 1989; Stibitz & Yang, 1991; Weiss & Hewlett, 1986). This locus codes for two proteins, BvgA and BvgS, which are members of the two-component family of bacterial signal transduction proteins (Aricò *et al.*, 1989; Coote, 1991; Miller *et al.*, 1989a; Scarlato *et al.*, 1993a; Stock *et al.*, 1990). Transcription of the pertussis toxin operon (*ptx*), the adenylate cyclase

toxin haemolysin gene (*cyaA*), the filamentous haemagglutinin gene (*fhaB*), and others, requires the *bvgAS* gene products *in trans* (Domenighini *et al.*, 1990; Gross *et al.*, 1989; Laoide & Ullmann, 1990; Miller *et al.*, 1989b; Roy *et al.*, 1989, 1990; Scarlato *et al.*, 1990).

Transcription of the *bvg* locus is controlled by three autoregulated promoters (P_1 , P_3 , P_4) and one *bvg*-independent promoter (P_2) (Scarlato *et al.*, 1990). The P_1 , P_2 and P_3 promoters direct mRNA synthesis of the *bvg* operon and the P_4 promoter directs the synthesis of an anti-sense RNA strand of unknown function which has its

5'-end region complementary to the 5' untranslated region of the *bvg* mRNAs. The *bvg*-dependent transcription is induced at 35 °C and is repressed at a low temperature (25 °C), or in the presence of MgSO₄ or nicotinic acid in the culture medium (Domenighini *et al.*, 1990; Gross & Rappuoli, 1988; Gross *et al.*, 1989; Laoide & Ullmann, 1990; Miller *et al.*, 1989b).

bvgS encodes a 135 kDa sensory protein with a hydrophobic transmembrane sequence and an N-terminal region that is localized in the periplasm, as demonstrated by the analysis of alkaline phosphatase fusion proteins (Stibitz & Yang, 1991). The N-terminal periplasmic domain of BvgS is involved in signal recognition; via phosphorylation, it transduces the signals into the transcriptional regulation network (Uhl & Miller, 1994). Evidence that BvgS is involved in signalling events is provided by the finding of a class of mutations that make the system insensitive to environmental regulation (Miller *et al.*, 1992; Manetti *et al.*, 1994; Goyard *et al.*, 1994).

bvgA encodes a 23 kDa transcriptional activator that binds upstream from the P₁ promoter of the *bvg* locus and upstream from the *fhaB* gene *in vitro* (Roy & Falkow, 1991) but does not bind upstream from the *ptx* and *cyaA* promoters, which are also regulated by BvgA. The same promoters are also activated differently *in vivo*. In fact, following a temperature shift of bacterial cultures from 25 °C to 35 °C, the P₁ and the *fhaB* promoters are activated within 10 min, while the *ptx* and *cyaA* promoters require 2–4 h for activation. Because of this behaviour, the *bvg*-activated genes have been divided into early and late, respectively.

During the interval between the activation of the early and late genes, the intracellular concentration of BvgA increases 50-fold. It has been proposed that this increased concentration may be required for the activation of the late genes. The difference between early and late genes can be observed also when the system is cloned in *Escherichia coli*: the early promoters are readily activated by the *bvg* locus *in trans*, while the transcription of the late promoters depends upon the cloning configuration of the promoter (Scarlato *et al.*, 1993b). Further evidence of the differential recognition of the two classes of promoters by BvgA has been provided by single-amino-acid mutations in BvgA which affect the recognition of early and late promoters differently (Stibitz, 1994).

In this study we used growth at different temperatures and repeated temperature shifts to study the behaviour of early and late promoters and to ask whether a high concentration of BvgA is required for the expression of the late genes. The data obtained concord with the proposal that the expression of late genes requires a high concentration of BvgA.

METHODS

Materials. Restriction enzymes, calf intestinal alkaline phosphatase, T4 polynucleotide kinase and S1 nuclease were purchased from Boehringer Mannheim or New England

Biolabs, and were used according to the manufacturer's specifications. Radiochemicals were from Amersham.

Bacterial strains and growth conditions. *B. pertussis* strain BPW28 (Bvg⁺ Sm^R) (Aricò *et al.*, 1993) was grown on BG agar plates containing 15 % (v/v) defibrinated blood (Pittman, 1984) or in SS-modified liquid medium (Stainer & Scholte, 1970). *E. coli* DH5 was used for plasmid preparations and was grown on LB agar or in LB broth (Sambrook *et al.*, 1989). Antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹; streptomycin, 400 µg ml⁻¹.

Plasmids and probes. The plasmids pFHA/EP, pVIR/EP and pAD/BS are pGem3 (Promega) derivatives and have been described by Scarlato *et al.* (1990, 1991). The probes used for S1 nuclease assays are described in the legend to Fig. 3. All DNA manipulations were carried out by standard techniques (Sambrook *et al.*, 1989).

RNA isolation. *B. pertussis* was grown overnight with shaking in a 100 ml flask containing 10 ml SS-modified medium (Stainer & Scholte, 1970) at 35 °C, then diluted to an OD₅₉₀ of 0.1 and grown to mid-exponential phase. Bacteria (25 ml) were harvested at appropriate times and stored at -20 °C. Cells were lysed in 3.7 ml 100 mM Tris/HCl, pH 7.5, 1 % (w/v) SDS, 2 mM Na₂EDTA for 5 min at 100 °C. After 5 min on ice in the presence of 75 mM KCl, cell debris was removed by spinning at 8000 r.p.m. for 10 min in a JA20 rotor. To 3.5 ml of sample, 4.56 g CsCl was added and the RNA was pelleted by sedimentation in an SW65 rotor at 35000 r.p.m. for about 20 h. The pellet was resuspended in 500 µl TE (10 mM Tris/HCl, pH 8, 1 mM EDTA), extracted with an equal volume of phenol/chloroform, ethanol precipitated, resuspended in 200 µl TE, reprecipitated with ethanol, and stored at -20 °C.

S1 nuclease mapping. S1 protection assays (Berk & Sharp, 1977) were performed by combining 6 µg RNA samples and about 20 fmol specific 5'-end-labelled DNA probe (Fig. 3). The mixture was ethanol precipitated in the presence of 1 µg *E. coli* tRNA and 1 M LiCl and processed as previously described by Scarlato *et al.* (1990, 1991). Hybridization was performed in a water bath for 16 h at appropriate temperatures (see legend to Fig. 4).

Immunoblots. Bacteria from 15 ml culture were harvested and resuspended in 25 % (w/v) saccharose, 50 mM Tris/HCl, pH 8, at an expected OD₅₉₀ of 0.3.

The suspension (20 µl) was loaded on 12.5 % (w/v) SDS-PAGE gels, subjected to electrophoresis and blotted to nitrocellulose for immunoblot analysis. Western blots were processed by standard procedures and stained by the chemiluminescence detection system (Amersham). BvgA was detected with a 1:2500 dilution of a polyclonal mouse antiserum raised against the purified protein (Scarlato *et al.*, 1990).

RESULTS

Accumulation of BvgA correlates with the growth temperature

To investigate whether at intermediate temperatures *B. pertussis* expresses intermediate amounts of BvgA and virulence factors, cells were grown to exponential phase at 22 °C for 7–10 d, and then shifted to 25 °C, 28 °C, 32 °C or 35 °C for 8 h. Aliquots of bacteria were withdrawn after 8 h and tested by Western blotting to evaluate the amount of BvgA and the outer-membrane protein pertactin.

Fig. 1. Western blot analysis of BvgA in a time-course experiment of temperature shifts. Samples were taken from cells grown at 22 °C (lane 22), or after 8 h at the indicated temperatures. The arrow points to the BvgA protein band.

Fig. 2. Western blot analysis of BvgA in a time-course experiment of temperature shifts. Numbers on top of each lane indicate the time at which cells were collected at the indicated temperatures. The sample from 37 °C was collected 1 min before the shift to 25 °C.

Fig. 1 shows immunoblots of total protein extracts stained with antibodies against BvgA. As expected, at 25 °C, when transcription of virulence genes is repressed, the intracellular amount of BvgA remains at basal level. In marked contrast, when the bacterial cultures were shifted from 22 °C to 28 °C, 32 °C or 35 °C, the amount of BvgA increased in a temperature-dependent manner. These data show that *B. pertussis* produces a different amount of regulator at different temperatures. In addition, Western blot analyses of the outer-membrane protein pertactin showed a pattern of protein accumulation qualitatively similar to the accumulation of BvgA (data not shown). Thus, the amount of virulence factors is likely to be proportional to the strength of the perceived signal.

A large amount of BvgA shortens the time response of the late-induced genes

To further study the role of BvgA concentration in late-gene activation, we designed a time-course experiment of temperature variation by shifting bacterial cultures from 37 °C to 25 °C for 2 h and then again to 37 °C for an additional 2 h. Aliquots of bacterial samples were harvested at 37 °C, after 10, 30 and 120 min at 25 °C, and after 10, 20, 60 and 120 min at 37 °C. Total protein extracts and RNA samples were extracted to evaluate the amount of BvgA and the pattern of RNA accumulation of the *bvg*-regulated promoters, respectively.

First we used Western blotting to analyse whether the amount of BvgA varied during the course of the experiment. Fig. 2 shows that the amount of BvgA did

Fig. 3. Probes and plasmids used for S1 nuclease mapping experiments. Each probe is shown as an open box; the restriction sites used to isolate each probe from its plasmid are indicated. Asterisks mark 5' ends that were radioactively labelled with T4 polynucleotide kinase and [γ -³²P]ATP. The portions of probes expected to be protected by hybridization to RNA initiating at the indicated promoters are shown as solid arrows. Arrows indicate the direction of transcription. The larger open tail box at the probe from pVIR/EP plasmid represents vector DNA. The dotted line at the P₃ transcript indicates the 5' RNA transcription which does not hybridize to the probe DNA.

not decrease in a detectable manner during the 2 h at 25 °C, and no increase was found when the cells were shifted again at 37 °C. Then we used S1 nuclease mapping to study the transcriptional regulation of the *bvg*-regulated promoters in the cells that had undergone a temperature shift while the concentration of BvgA was constant. DNA probes (Fig. 3) were 5'-end labelled, hybridized with total RNA, digested with S1 nuclease (Berk & Sharp, 1977) and the S1-resistant DNA-RNA duplexes were sized by polyacrylamide/urea gel electrophoresis.

Fig. 4 shows the electrophoretic pattern of signals generated by the P₁₋₃- (a), P_{FHA}- (b) and P_{AC}-associated (c) transcripts. As expected, the P₁, P₃, P_{FHA} and P_{AC} promoters were transcriptionally active at 37 °C (lanes 4), while transcription from the P₂ promoter was repressed (Fig. 4a, lane 4). Following a temperature transition from 37 °C to 25 °C, the pattern of RNA accumulation associated with these promoters changed in the following 2 h at 25 °C. The amounts of the P₁ (Fig. 4a) and P_{FHA} (Fig. 4b) RNAs decreased after 120 min at 25 °C (lanes 7), the amount of the P_{AC} RNA decreased after 30 min (Fig. 4c, lane 6), while the amount of the P₂ RNA was already detectable after 10 min at 25 °C (Fig. 4a, lane 5).

A quantitative analysis of the patterns of RNA accumulation associated with these promoters after a temperature shift from 37 °C to 25 °C is reported in Fig. 5. Surprisingly, the amount of the P₁, P_{FHA}- and P_{AC}-associated RNA increased after 10 min at 25 °C. The P_{FHA}-associated RNA also increased after 30 min at 25 °C, while at this time the amount of P₁ and P_{AC} RNAs decreased. From the slope of the curves shown in Fig. 5 it can be deduced that between 10 and 30 min the amount of the P_{AC} RNA decreased with a half-life of 9 min. The amounts

Fig. 4. Time course of transcription for promoters P_{1-3} , P_{FHA} and P_{AC} upon temperature shifts from 37 °C to 25 °C to 37 °C. (a) P_{1-3} . The *Ava*I–*Pvu*II DNA fragment (273 bp) from plasmid pVIR/EP was used for hybridization and S1 nuclease digestion analysis. The hybridization temperature was 48 °C. (b) P_{FHA} . The *Mlu*I–*Eco*RI fragment (356 bp) of plasmid pFHA/EP was used as probe. The hybridization temperature was 54 °C. (c) P_{AC} . The *Sal*I–*Bam*HI fragment (513 bp) of plasmid pAD/BS was used as a probe. The hybridization temperature was 55 °C. RNAs were collected at the indicated times after temperature shifts. Control samples were processed identically but contained no RNA, and were not (lanes P), or were (lanes M), digested with S1 nuclease. A denatured 1 kb ladder (Gibco-BRL) served as size markers (lanes C).

Fig. 5. RNA accumulation for P_1 (○), P_{FHA} (△) and P_{AC} (■) promoters after a temperature shift from 37 °C to 25 °C. The ordinate shows RNA accumulation relative to its maximum value (set to 100) for each promoter within 2 h growth at 25 °C.

of the P_1 and P_{FHA} RNAs decreased after 10 and 30 min at 25 °C, with estimated half-lives of 45 and 40 min, respectively.

As expected, following a temperature shift from 25 °C to 37 °C, the amount of the P_1 and the P_{FHA} RNAs increased after 10 min incubation at 37 °C (Fig. 4a, b; lanes 8), while the amount of the P_2 RNA diminished after 20 min (Fig. 4a, lane 9). Surprisingly, the transcription from P_{AC} , which is a late promoter, was activated already after 20 min of induction (Fig. 4c, lane 9). The amount of this RNA increased after 60 and 120 min at 37 °C (Fig. 4c, lanes 10–11).

These data show that the time required for activation of the late promoters can be shortened from 2–4 h to 20 min when cells have already accumulated a large amount of BvgA.

DISCUSSION

Following the discovery that the virulence genes can be divided into early- and late-induced genes, a number of studies have tried to address the question of how one transcriptional activator may differentially activate two types of promoters. Since this behaviour has never been described for other transcriptional activators, the question is very interesting, not only for *B. pertussis*, but it may provide a better understanding of the basic mechanisms of gene transactivation. So far, two major hypotheses have been proposed. One claims that transcription of the early genes is mediated directly by BvgA, while transcription of the late genes may require a second molecule specific for the late genes. To demonstrate this hypothesis, extensive studies have been performed to identify, either by expression cloning or by classical genetics, the putative factor responsible for the activation of the late genes. So far these studies have been inconclusive. The only factors that have been found to have an effect on the transcription of the late genes are mutations in the α subunit of the RNA polymerase. These mutants play a general role in transcription, but are not specific for the late genes (Carbonetti *et al.*, 1994).

The second hypothesis to explain the differential regulation of early and late genes proposed that both early and late genes were regulated directly by BvgA, without the need of additional specific factors. The hypothesis claimed that early genes could be activated by a very low concentration of BvgA, such as that present when the bacteria are grown for a long period of time at 22 °C, while activation of the late genes required large amounts of BvgA, which are present only in bacteria grown for a long period of time at 35 °C. This theory was initially supported only by the observation that bacteria grown at 22 °C have a basal level of BvgA which, following temperature shift to 37 °C, increases progressively with time, until it reaches a concentration that is 50-fold the

initial one. Transcription of the late genes initiates when the concentration of BvgA is 20-fold over the basal level and then increases proportionally with the concentration of BvgA. Today, this theory is supported by several other experimental observations. For instance, the presence of two copies of the *bvgAS* locus in *B. pertussis* was sufficient to increase the basal level of BvgA and to induce the expression of pertussis toxin at 25 °C (Scarlato *et al.*, 1991). Then it was shown that the pertussis toxin promoter can be activated in *E. coli* by the *bvgAS* locus alone, without additional factors deriving from *B. pertussis* (Scarlato *et al.*, 1993b). Finally, it was shown that mutations in the *bvgA* gene could affect only the transcription of the late genes (Stibitz, 1994). The above data support, but do not prove, the proposal that BvgA is sufficient to activate both early and late promoters. Moreover they suggest that BvgA has a high affinity for the early promoters, so that BvgA can bind them at low concentration, while BvgA has a low affinity for late promoters and can bind them only when it is present in high amounts.

In this work, we have provided additional evidence that BvgA may be the sole regulator of early and late genes, and that a high concentration of BvgA may be necessary for the activation of the late genes. In fact, we have shown that the amount of BvgA protein present in the cells is temperature-dependent. By Western blot analysis we also assayed the expression of the products coded by the late genes and found that it correlates with the concentration of BvgA (data not shown). In addition, when BvgA was kept constantly high, we have been able to show that the time required to activate the late genes can be shortened to 20 min. These data strongly support the theory that BvgA is sufficient for the activation of both early and late genes, and encourage studies to understand the molecular basis of this extremely interesting mechanism of gene transactivation. It is likely that a fine regulation of the two classes of promoters is at the level of BvgA phosphorylation.

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REFERENCES

- Aricò, B., Miller, J. F., Roy, C., Stibitz, S., Monack, D. M., Falkow, S., Gross, R. & Rappuoli, R. (1989). Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proc Natl Acad Sci USA* **86**, 6671–6675.
- Aricò, B., Nuti, S., Scarlato, V. & Rappuoli, R. (1993). Adhesion of *Bordetella pertussis* to eukaryotic cells requires a time-dependent export and maturation of filamentous hemagglutinin. *Proc Natl Acad Sci USA* **90**, 9204–9208.
- Berk, A. J. & Sharp, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**, 721–723.
- Carbonetti, N. H., Fuchs, T. M., Patamawenu, A. A., Irish, T. J., Deppisch, H. & Gross, R. (1994). Effect of mutations causing overexpression of RNA polymerase α subunit on regulation of virulence factors in *Bordetella pertussis*. *J Bacteriol* **176**, 7267–7272.
- Coote, J. G. (1991). Antigenic switching and pathogenicity: environmental effects on virulence gene expression in *Bordetella pertussis*. *J Gen Microbiol* **137**, 2493–2503.
- Domenighini, M., Relman, D., Capiou, C., Falkow, S., Prugnola, A., Scarlato, V. & Rappuoli, R. (1990). Genetic characterization of *Bordetella pertussis* filamentous hemagglutinin: a protein processed from an unusually large precursor. *Mol Microbiol* **4**, 787–800.
- Goyard, S., Bellalou, J., Mireau, H. & Ullmann, A. (1994). Mutations in the *Bordetella pertussis* *bvgS* gene that confer altered expression of the *fhaB* gene in *Escherichia coli*. *J Bacteriol* **176**, 5163–5166.
- Gross, R. & Rappuoli, R. (1988). Positive regulation of pertussis toxin expression. *Proc Natl Acad Sci USA* **85**, 3913–3917.
- Gross, R., Aricò, B. & Rappuoli, R. (1989). Families of bacterial signal transducing proteins. *Mol Microbiol* **3**, 1661–1667.
- Laoide, B. M. & Ullmann, A. (1990). Virulence dependent and independent regulation of the *Bordetella pertussis* *cya* operon. *EMBO J* **9**, 999–1005.
- Manetti, R., Aricò, B., Rappuoli, R. & Scarlato, V. (1994). Mutations in the linker region of BvgS abolish response to environmental signals for the regulation of the virulence factors in *Bordetella pertussis*. *Gene* **150**, 123–127.
- Miller, J. F., Mekalanos, J. J. & Falkow, S. (1989a). Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **243**, 916–922.
- Miller, J. F., Roy, C. R. & Falkow, S. (1989b). Analysis of *Bordetella pertussis* virulence gene regulation by use of transcriptional fusions in *Escherichia coli*. *J Bacteriol* **171**, 6345–6348.
- Miller, J. F., Johnson, S. A., Black, W. J., Beattie, D. T., Mekalanos, J. J. & Falkow, S. (1992). Constitutive sensory transduction mutations in the *Bordetella pertussis* *bvgS* gene. *J Bacteriol* **174**, 970–979.
- Pittman, M. (1984). Genus *Bordetella*. In *Bergey's Manual of Systematic Bacteriology*, pp. 388–393. Edited by N. R. Krief & J. G. Holt. Baltimore: Williams and Wilkins.
- Roy, C. R. & Falkow, S. (1991). Identification of *Bordetella pertussis* regulatory sequences required for transcriptional activation of the *bvgAS* operon. *J Bacteriol* **173**, 2385–2392.
- Roy, C. R., Miller, J. F. & Falkow, S. (1989). The *bvgA* gene of *Bordetella pertussis* encodes a transcriptional activator required for coordinate regulation of several virulence genes. *J Bacteriol* **171**, 6338–6344.
- Roy, C. R., Miller, J. F. & Falkow, S. (1990). Autogenous regulation of the *Bordetella pertussis* *bvgABC* operon. *Proc Natl Acad Sci USA* **87**, 3763–3767.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scarlato, V., Prugnola, A., Aricò, B. & Rappuoli, R. (1990). Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis*. *Proc Natl Acad Sci USA* **87**, 6753–6757.
- Scarlato, V., Aricò, B., Prugnola, A. & Rappuoli, R. (1991). Sequential activation and environmental regulation of the virulence genes in *Bordetella pertussis*. *EMBO J* **10**, 3971–3975.

Scarlato, V., Aricò, B., Domenighini, M. & Rappuoli, R. (1993a). Environmental regulation of virulence factors in *Bordetella* species. *BioEssays* **15**, 99–104.

Scarlato, V., Aricò, B. & Rappuoli, R. (1993b). DNA topology affects transcriptional regulation of the pertussis toxin gene of *Bordetella pertussis* in *Escherichia coli* and in vitro. *J Bacteriol* **175**, 4764–4771.

Stainer, D. W. & Scholte, M. J. (1970). A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J Gen Microbiol* **63**, 211–220.

Stibitz, S. (1994). Mutations in the *bvgA* gene of *Bordetella pertussis* that differentially affect regulation of virulence determinants. *J Bacteriol* **176**, 5615–5621.

Stibitz, S. & Yang, M.-S. (1991). Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. *J Bacteriol* **173**, 4288–4296.

Stock, J. F., Stock, A. M. & Mottonen, J. M. (1990). Signal transduction in bacteria. *Nature* **344**, 395–400.

Uhl, M. A. & Miller, J. F. (1994). Autophosphorylation and phosphotransfer in *Bordetella pertussis* BvgAS signal transduction cascade. *Proc Natl Acad Sci USA* **91**, 1163–1167.

Weiss, A. A. & Hewlett, E. L. (1986). Virulence factors of *Bordetella pertussis*. *Annu Rev Microbiol* **40**, 661–686.

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