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Differential Response of the *bvg* Virulence Regulon of *Bordetella pertussis* to $MgSO_4$ Modulation

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Magnesium sulfate is known to repress the expression of the virulence factors of *Bordetella pertussis* that are coordinately regulated by the *bvg* locus. We have tested the time required by $MgSO_4$ to repress the synthesis of several *bvg*-regulated mRNA species and found that the promoters of the virulence genes (pertussis toxin, adenylate cyclase, and filamentous hemagglutinin) are repressed in 6 min, while the autogenously regulated promoters of the *bvg* locus (P_1, P_3, and P_4) are repressed only several hours later. These data show a differential behavior between regulated and autoregulated genes of the *bvg* regulon.

Pathogenic bacteria have evolved a regulatory system which responds to environmental changes in a coordinate manner. In *Bordetella pertussis*, the coordinate regulation of the virulence-associated determinants in response to environmental stimuli is controlled by the *bvg* locus (17, 26, 27). This locus codes for two proteins, BvgA and BvgS, originally called BvgABC (1, 4, 12, 13, 21, 25, 26), which by sequence homology fall into the two-component family of bacterial signal transduction proteins (1, 4, 16, 19, 25). Transcription at the *bvg* locus of *B. pertussis* is controlled by three autoregulated promoters (P_1, P_3, and P_4) and one *bvg*-independent promoter (P_2) (23). The P_1, P_2, and P_3 promoters direct mRNA synthesis for the *bvg* operon, and the P_4 promoter synthesizes an RNA complementary to the 5′ untranslated region of the *bvg* mRNAs. With the exception of the P_2 promoter, all of these promoters and the promoters of the virulence factors such as pertussis toxin (P_{TOX}) (5, 15), adenylate cyclase (P_{Ad}) (9), and filamentous hemagglutinin (P_{FHA}) (3, 18, 23) are regulated by environmental stimuli; that is, they are induced at 37°C and are repressed at a low temperature (25°C) or in the presence of 50 mM $MgSO_4$ or 10 mM nicotinic acid (5, 7, 9, 11, 13, 22, 23). It has been demonstrated that addition of $MgSO_4$ to the culture medium represses synthesis of pertussis toxin protein in 10 min (6).

In this study, we have investigated the modulative response of the expression *B. pertussis* virulence factors by adding 50 mM $MgSO_4$ to the culture medium and studying in vivo RNA accumulation driven by six *bvg*-regulated promoters in the subsequent 8 h. We have found that the virulence-associated genes are repressed within 6 min after treatment, while the *bvg* mRNAs accumulate for several hours after treatment.

$MgSO_4$ blocks transcription of the virulence genes in 6 min. To study the repression of the *bvg*-regulated promoters, *B. pertussis* W28 (23) was grown in 500 ml of SS modified medium (24) at 37°C in a 1-liter flask to an optical density of 0.7. Twenty-five milliliters of growing bacteria was harvested and chilled in a 100-ml flask containing 20 ml of frozen medium. Bacteria were then centrifuged and stored at −20°C. $MgSO_4$ was added to the growing bacteria to a final concentration of 50 mM, and 25-ml aliquots were harvested after 3, 6, 10, 15, 30, 60, 120, and 480 min and processed as stated before. During this time, bacteria maintained exponential growth.

Total RNA was extracted from each sample (23) and used to investigate the patterns of transcription of the *bvg*-regulated promoters by assaying the accumulation of RNA in S1 nuclease mapping experiments. Specific DNA probes (Fig. 1), 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, were hybridized with total RNA under conditions which favor the formation of DNA-RNA hybrids and then digested with S1 nuclease (2, 23). The S1 nuclease-resistant RNA-DNA duplexes were denatured, and the labeled DNA was visualized following fractionation on a denaturing acrylamide gel.

Figure 2 shows the electrophoretic pattern of signals generated by the P_{FHA} (panel A), P_{TOX} (panel B), and P_{Ad} (panel C) transcripts. Before addition of $MgSO_4$ to the culture medium, all promoters were transcriptionally active (lane a). Following addition of $MgSO_4$ to the medium, the amount of RNA dependent on the P_{FHA}, P_{TOX}, and P_{Ad} promoters had decreased already by 3, 6, and 10 min (lanes b to d). The accumulation of RNA mapping to these promoters was drastically diminishing by 15 to 30 min after treatment (lanes e and f).

To correlate better the patterns of RNA accumulation associated with these promoters during $MgSO_4$ treatment, the autoradiograms shown in Fig. 2, and other autoradiograms, were quantified by laser scanning. Figure 2D shows that, in the first 6 min after addition of $MgSO_4$, there is little change in the amount of accumulated RNA; then the P_{FHA}, P_{TOX}, and P_{Ad} mRNAs decay with half-lives of 4, 2, and 2.5 min, respectively. These data demonstrate that the external signal, transduced into the cell, requires only 6 min to block RNA transcription of the virulence-associated genes.

The autoregulation of the *bvg* locus is slowly repressed. It has been shown previously that transcription of the *bvg* locus is controlled by four promoters, P_1, P_2, P_3, and P_4, and that P_1, P_3, and P_4 are repressed by the addition of $MgSO_4$ to the medium (22, 23).

To analyze the response of the *bvg*-autoregulated locus to

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FIG. 2. Time course of transcription for promoters P_{FHA}, P_{TOX}, and P_{Ad} upon addition of $MgSO_4$ to the culture medium. The *Mlu*I-*Eco*RI (M-E, panel A), *Sal*I-*Kpn*I (S-K, panel B), and *Sal*I-*Bam*HI (S-B, panel C) DNA fragments from plasmids pFHA/EP, pT110.2, and pAD/BS (Fig. 1) were used for hybridization and S1 nuclease digestion analysis (2) of RNA collected 3 (lane b), 6 (lane c), 10 (lane d), 15 (lane e), 30 (lane f), 60 (lane g), 120 (lane h), or 480 (lane i) min after the addition of 50 mM $MgSO_4$ to the culture medium. Lanes a, RNA collected 1 min before $MgSO_4$ treatment. Control samples were processed identically but contained no RNA and either were (lane C) or were not (lane P) digested with S1 nuclease. S1 protection assays were performed by combining 2 μg of RNA and ~20 fmol of probe. A denatured 1-kb ladder (Bethesda Research Laboratories-GIBCO) served as size markers (lane M). Promoters are indicated at the right of each panel. Hybridization was performed for 15 h at the following temperatures: 54°C (A), 58°C (B), and 55°C (C). (D) Decay of accumulated RNA after addition of $MgSO_4$ to the culture medium. The ordinate scale is logarithmic. The amounts of RNA species, obtained by laser scanning (LKB densitometer), were not normalized and are not comparable. The curve for each mRNA species is indicated by the promoter name, and each half-life (see text) was determined from the slope of the curve.

$MgSO_4$ addition, we hybridized the RNAs used for the time course experiment of Fig. 2 to the *bvg* probes shown in Fig. 1. The results of the S1 nuclease protection assays of the P_1-, P_2-, P_3-, and P_4-associated mRNAs are shown in Fig. 3. All RNAs were present in the growing cell before addition of $MgSO_4$ (lane a), and their accumulation persisted for at least 2 h after treatment began (lanes b to h). P_1- and P_3-associated mRNAs became undetectable only 8 h after $MgSO_4$ addition (lane i, panel A), while the P_4 mRNA was still present, although in a lesser amount (lane i, panel B). Since it has been shown (23) that the P_4 promoter is repressed in cells cultured in the presence of $MgSO_4$, it is likely that, to be repressed, this promoter requires treatment for a longer time. To establish whether the presence of the long-lasting RNAs was due to longer half-lives or to continuous synthesis, we performed an S1 nuclease experiment on RNA extracted from cells treated with 100 μg of rifampin per ml, which blocks RNA synthesis (8). In this case, all *bvg* RNAs disappeared in 10 min (data not shown). This shows that the P_1 to P_4 promoters were active for several hours after $MgSO_4$ addition.

To analyze the behavior of P_1 and P_2 RNAs, the autoradiogram of Fig. 3A with a shorter exposure time, and other autoradiograms, were quantified. Figure 3C shows that during the first 30 min there is a rapid decrease of the P_1 mRNA and a corresponding increase of the P_2 mRNA. Although the P_2 promoter is not regulated by environmental stimuli, Roy and Falkow (20) demonstrated that the BvgA protein protects against DNase I digestion the nucleotide sequence from position −52 to position −84 of the P_1 initiation transcription site which overlaps the P_2 promoter sequence. As a result, the BvgA protein is likely to compete with RNA polymerase in interacting with the same DNA region (20, 22) so that high activity of the P_1 promoter results in low activity of the P_2 promoter and vice versa. This mechanism is likely to guarantee an initial efficient transcription of the *bvg* locus when external stimuli repress the transcription of the other *bvg*-regulated promoters.

In conclusion, we have found a differential behavior of the *bvg*-regulated promoters: the promoters transcribing the virulence genes are repressed immediately after addition of $MgSO_4$ to the culture medium, while those transcribing the *bvg* locus require a longer time to reach repression. Whether the differential response of regulated and autoregulated promoters plays an important role during *B. pertussis* infection is an intriguing question which requires further studies.

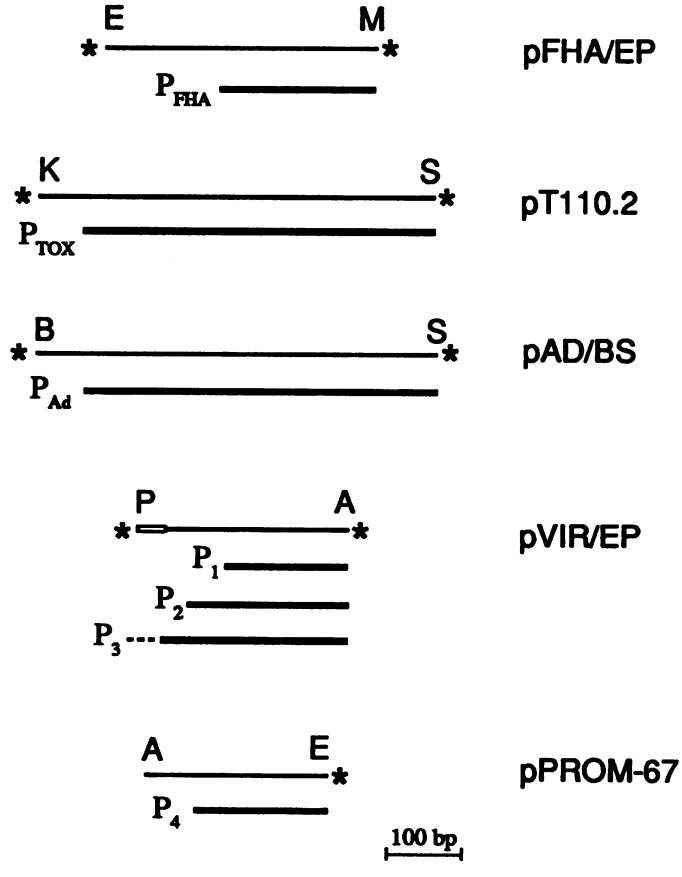
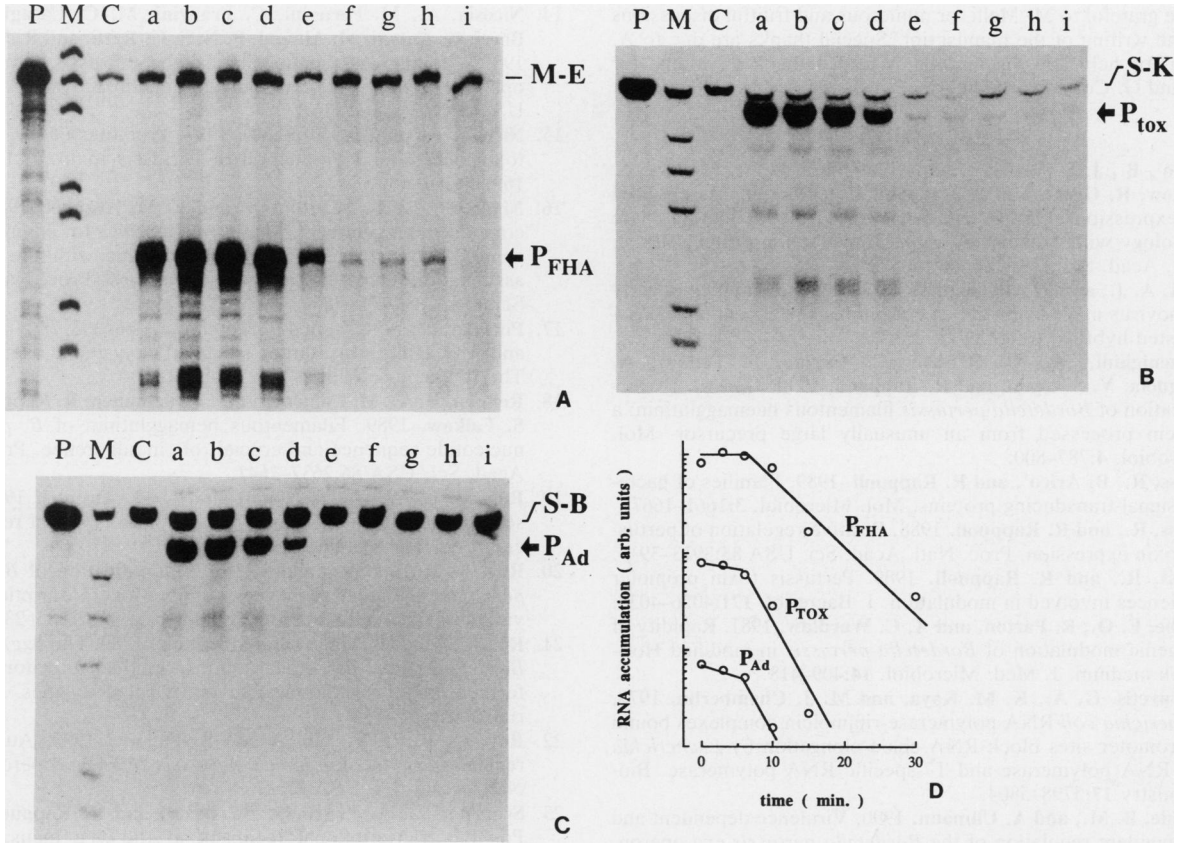


FIG. 1. Probes and plasmids used for S1 nuclease protection assays (2). Each probe is shown as a thin line. The restriction sites used to isolate each probe from its plasmid are indicated: A, *Ava*II; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; M, *Mlu*I; P, *Pvu*II; S, *Sal*I. Asterisks mark 5′ ends that were radioactively labeled. The portion of probes expected to be protected by hybridization to RNA initiating at the indicated promoters are shown as solid bars. The open tail box at the probe from plasmid pVIR/EP represents 35 bp of vector DNA. The dotted line at the P_3 transcript indicates the 5′ RNA transcription which does not hybridize to the probe DNA. Host cells were *Escherichia coli* DH5 and JM101. Plasmids pVIR/EP, pFHA/EP, and pPROM-67 have been described before (23), as has pT110.2 (14). Plasmid pAD/BS was obtained by a polymerase chain reaction (Perkin-Elmer, Cetus) amplification procedure on the *B. pertussis* chromosome and contains a 536-bp DNA fragment of the adenylate cyclase gene spanning from nucleotides 789 to 1323 (GenBank accession number Y00545). All enzymatic manipulations were carried out by standard procedures (10).



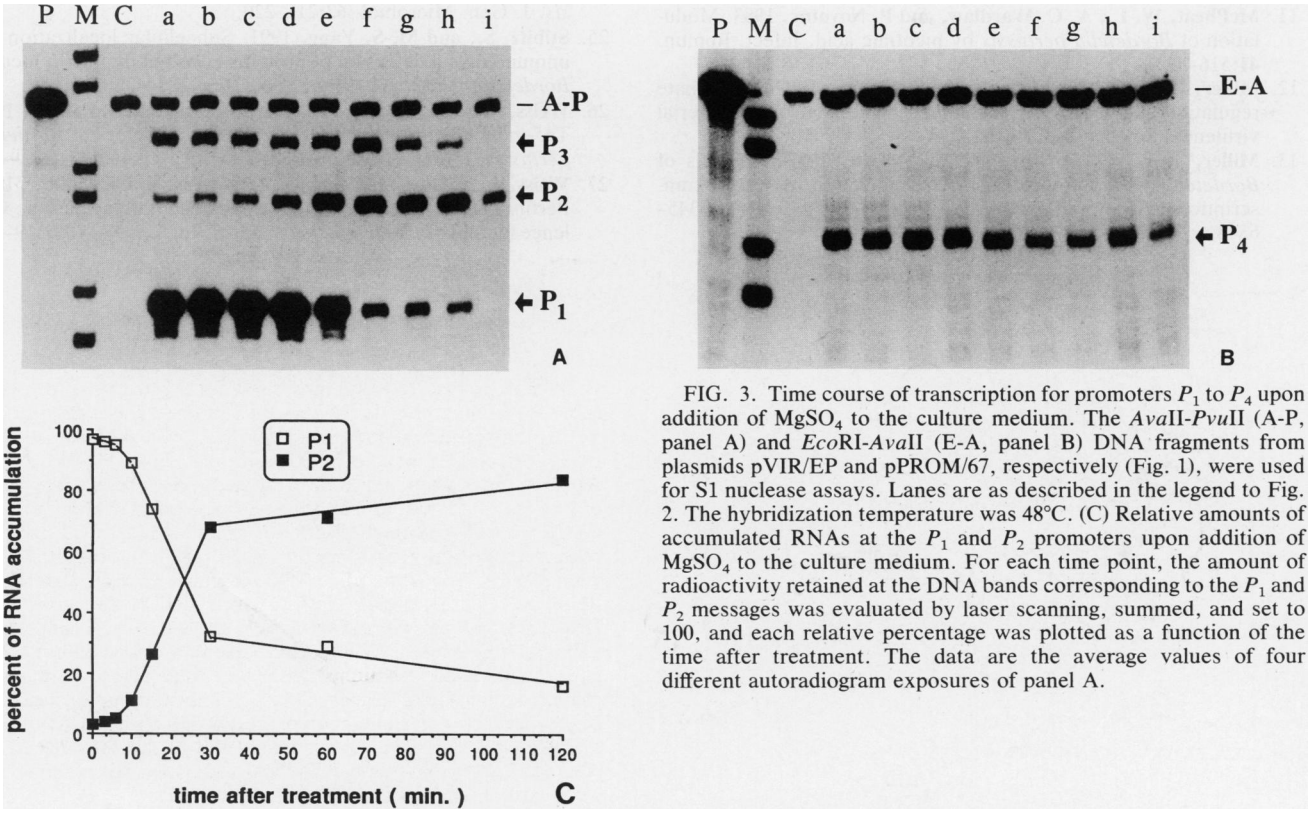


FIG. 3. Time course of transcription for promoters P_1 to P_4 upon addition of $MgSO_4$ to the culture medium. The *Ava*II-*Pvu*II (A-P, panel A) and *Eco*RI-*Ava*II (E-A, panel B) DNA fragments from plasmids pVIR/EP and pPROM/67, respectively (Fig. 1), were used for S1 nuclease assays. Lanes are as described in the legend to Fig. 2. The hybridization temperature was 48°C. (C) Relative amounts of accumulated RNAs at the P_1 and P_2 promoters upon addition of $MgSO_4$ to the culture medium. For each time point, the amount of radioactivity retained at the DNA bands corresponding to the P_1 and P_2 messages was evaluated by laser scanning, summed, and set to 100, and each relative percentage was plotted as a function of the time after treatment. The data are the average values of four different autoradiogram exposures of panel A.

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REFERENCES

1. **Arico', B., J. F. Miller, C. Roy, S. Stibitz, D. M. Monack, S. Falkow, R. Gross, and R. Rappuoli.** 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.
2. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–723.
3. **Domenighini, M., D. Relman, C. Capiau, S. Falkow, A. Prugnola, V. Scarlato, and R. Rappuoli.** 1990. Genetic characterization of *Bordetella pertussis* filamentous haemagglutinin: a protein processed from an unusually large precursor. Mol. Microbiol. **4**:787–800.
4. **Gross, R., B. Arico', and R. Rappuoli.** 1989. Families of bacterial signal-transducing proteins. Mol. Microbiol. **3**:1661–1667.
5. **Gross, R., and R. Rappuoli.** 1988. Positive regulation of pertussis toxin expression. Proc. Natl. Acad. Sci. USA **85**:3913–3917.
6. **Gross, R., and R. Rappuoli.** 1989. Pertussis toxin promoter sequences involved in modulation. J. Bacteriol. **171**:4026–4030.
7. **Idigbe, E. O., R. Parton, and A. C. Wardlaw.** 1981. Rapidity of antigenic modulation of *Bordetella pertussis* in modified Horibrook medium. J. Med. Microbiol. **14**:409–418.
8. **Kassavetis, G. A., K. M. Kaya, and M. J. Chamberlin.** 1978. *Escherichia coli* RNA polymerase-rifampicin complexes bound at promoter sites block RNA chain elongation by *Escherichia coli* RNA polymerase and T7-specific RNA polymerase. Biochemistry **17**:5798–5804.
9. **Laoide, B. M., and A. Ullmann.** 1990. Virulence dependent and independent regulation of the *Bordetella pertussis* cya operon. EMBO J. **9**:999–1005.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **McPheat, W. L., A. C. Wardlaw, and P. Novotny.** 1983. Modulation of *Bordetella pertussis* by nicotinic acid. Infect. Immun. **41**:516–522.
12. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243**:916–922.
13. **Miller, J. F., C. R. Roy, and S. Falkow.** 1989. Analysis of *Bordetella pertussis* virulence gene regulation by use of transcriptional fusions in *Escherichia coli*. J. Bacteriol. **171**:6345–6348.
14. **Nicosia, A., M. Perugini, C. Franzini, M. C. Casagli, M. G. Borri, G. Antoni, M. Almoni, P. Neri, G. Ratti, and R. Rappuoli.** 1986. Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. Proc. Natl. Acad. Sci. USA **83**:4631–4635.
15. **Nicosia, A., and R. Rappuoli.** 1987. Promoter of the pertussis toxin operon and production of pertussis toxin. J. Bacteriol. **169**:2843–2846.
16. **Nixon, B. T., C. W. Ronson, and F. M. Ausubel.** 1986. Two-component regulatory system responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. Proc. Natl. Acad. Sci. USA **83**:7850–7854.
17. **Pittman, M.** 1984. Genus *Bordetella*, p. 338–393. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systemic bacteriology, The Williams & Wilkins Co., Baltimore.
18. **Relman, D. A., M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *B. pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86**:2637–2641.
19. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579–581.
20. **Roy, C. R., and S. Falkow.** 1991. Identification of *Bordetella pertussis* regulatory sequences required for transcriptional activation of the *bvgAS* operon. J. Bacteriol. **173**:2385–2392.
21. **Roy, C. R., J. F. Miller, and S. Falkow.** 1989. The *bvgA* gene of *Bordetella pertussis* encodes a transcriptional activator required for coordinate regulation of several virulence genes. J. Bacteriol. **171**:6338–6344.
22. **Roy, C. R., J. F. Miller, and S. Falkow.** 1990. Autogenous regulation of the *Bordetella pertussis bvgABC* operon. Proc. Natl. Acad. Sci. USA **87**:3763–3767.
23. **Scarlato, V., A. Prugnola, B. Arico', and R. Rappuoli.** 1990. Positive transcriptional feedback at the *bvg* locus controls expression of virulence factors in *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **87**:6753–6757.
24. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63**:211–220.
25. **Stibitz, S., and M.-S. Yang.** 1991. Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. J. Bacteriol. **173**:4288–4296.
26. **Weiss, A. A., E. L. Hewlett, G. A. Meyers, and S. Falkow.** 1983. Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. **42**:33–41.
27. **Weiss, A. A., E. L. Hewlett, G. A. Meyers, and S. Falkow.** 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. J. Infect. Dis. **150**:219–222.