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The *bvgA* Gene of *Bordetella pertussis* Encodes a Transcriptional Activator Required for Coordinate Regulation of Several Virulence Genes

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The *bvg* region of the respiratory pathogen *Bordetella pertussis* coordinately regulates the expression of several unlinked virulence determinants in response to environmental signals. The DNA sequence of the *bvg* region contains three genes (*bvgA*, *bvgB*, and *bvgC*). Transcription of a single-copy fusion consisting of the upstream region of a *bvg*-activated *B. pertussis* gene (*fhaB*) attached to the promoterless *lac* operon in *Escherichia coli* requires the entire *bvgABC* region in *trans*. Activation of the *fhaB*::*lacZYA* fusion is sensitive to the same environmental stimuli in *E. coli* that modulate the expression of *bvg*-activated genes in *B. pertussis*. Our data show that overexpression of the *bvgA* gene from a strong heterologous promoter results in transcriptional activation of the *fhaB*::*lacZYA* fusion even in the absence of the *bvgB* and *bvgC* products. Activation of *fhaB* transcription by *bvgA* overexpression in *E. coli* is no longer repressed by environmental conditions. The *bvgA* product has been identified by maxicell analysis as a 23-kilodalton protein. A *B. pertussis* mutant containing an in-frame deletion in *bvgA* was constructed. This mutant was nonhemolytic and no longer produced filamentous hemagglutinin and pertussis toxin. The mutation in this strain was complemented by returning the *bvgA* gene in *trans*. Transcriptional chloramphenicol acetyltransferase fusions to the *fhaB* and *ptx* promoter regions were returned to both the *B. pertussis bvgA* deletion mutant and its parental wild-type strain. Analysis of these strains indicated that the deletion mutant was defective in transcription of both *ptx* and *fhaB*. We conclude from these data that *bvgA*, *bvgB*, and *bvgC* comprise an operon encoding the components essential for coordinate regulation and sensory transduction. The BvgA protein is a transcriptional regulatory factor. The *bvgB* and *bvgC* products may be important in regulating the activity of BvgA in response to the changing environmental stimuli that *B. pertussis* encounters during the disease whooping cough.

The *bvg* (*vir*) region of *Bordetella pertussis*, the causative agent of whooping cough, is a central regulatory locus required for the expression of several unlinked genes (26). A number of *B. pertussis* virulence factors, including pertussis toxin (Ptx), filamentous hemagglutinin (Fha), and hemolysin, are encoded by *bvg*-activated genes (27, 28). Bacteria containing mutations that eliminate expression of *bvg*-activated genes are reduced in their ability to cause disease and therefore have been phenotypically designated Vir$^-$ (28). These mutations can be complemented by returning the wild-type *bvg* region on a plasmid capable of replicating in *B. pertussis*, demonstrating that the *bvg* genes are *trans* acting (24). Both chemical and physical stimuli, including $MgSO_4$, nicotinic acid, and low temperature (30°C), have been shown to reduce expression of *bvg*-activated genes (7, 12). This coordinate regulation of *bvg*-activated genes in response to different environmental conditions is known as phenotypic modulation (for a review, see reference 13).

We have shown recently that the *bvg* functions can be reconstructed in *Escherichia coli* by using a transcriptional *lacZYA* fusion to the structural gene for Fha (*fhaB*) from *B. pertussis* (13a).Transcription of the *fhaB*::*lacZYA* fusion in *E. coli* requires the *bvg* region, which agrees with the observation by Stibitz et al. that detection of an Fha-immunoreactive product in *E. coli* correlates with the presence of an intact *bvg* locus (24). Activation of *fhaB* transcription by the *bvg* region is reduced in *E. coli* by the addition of $MgSO_4$ or nicotinic acid as well as by growing the bacteria at 30°C. This suggests that the *bvg* region is sufficient for both positive regulation and phenotypic modulation and that positive regulation occurs at the level of transcription.

The nucleotide sequence of the *bvg* region contains three tandem open reading frames designated *bvgA*, *bvgB*, and *bvgC* (1). Translation of the nucleic acid sequence reveals that the predicted BvgA and BvgC proteins are similar to a family of proteins involved in several bacterial two-component signal transduction systems (18). These systems characteristically consist of a sensor protein and its cognate regulator protein. The BvgC protein is homologous to the protein kinases CheA and NtrB (sensors), which process and transmit sensory information (15, 18, 25). The BvgA protein is homologous to the effector proteins CheY and NtrC (regulators), which are activated by CheA and NtrB, respectively (5, 8). Once activated, CheY and NtrC have different effector functions. CheY changes the rotational direction of the flagellar motor (9), whereas NtrC activates transcription of the *glnAp2* promoter (6).

In this paper we analyze the *bvg* components required for transcriptional activation and modulation of *fhaB* by using JFMC3, an *E. coli* strain which contains a transcriptional *fhaB*::*lacZYA* fusion chromosomally integrated on a recombinant lambda phage. We demonstrate that transcriptional activation of the *fhaB*::*lacZYA* fusion is mediated by the product of the *bvgA* gene but that phenotypic modulation requires the intact *bvg* region. Finally, we show that BvgA is required in *B. pertussis* for the transcription of *fhaB* and the pertussis toxin operon (*ptx*).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
E. coli		
DH5α	F⁻ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA relA1*	Bethesda Research Laboratories
HB101	*hsdS20* (r_B^- m_B^+) *supE44 ara-14* λ⁻ *galK2 lacY1 proA2 rspL20 xyl-5 mtl-1 recA13*	11
SM10	Contains RP4 transfer genes on chromosome	21
MC4101	F⁻ *araD139* Δ(*lacZYA-argF*)U169 *rspL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA1*	W. J. Black
JFMC3	MC4101 λ*fhaB*::*lacZYA*	13a
JFME3	MC4101 λ*ptx*::*lacZYA*	13a
B. pertussis		
BP369	Vir⁻ Rif^r	26
BP370	Vir⁺ Rif^r	24
BP359	*bvgA1*::Tn*5* Vir⁻	26
BP3703	BP370 Δ*bvgA* Vir⁻	This study
Plasmids		
pBR322	Ap^r Tc^r cloning vector	1a
pKS-	High-copy-number cloning vector with *lac* promoter downstream from polylinker	Stratagene
pSK-	pKS- with polylinker in opposite orientation	Stratagene
pMMB67HE	RSF1010 derivative containing pTAC promoter and *lacI*^{q}	2
pRTP1	Ap^r, Sm^s gene replacement vector	23
pCR401	pKS- with 2.5-kilobase (kb) *Eco*RI fragment	This study
pCR405	pSK- with 1.9-kb *Eco*RI-*Sal*I fragment	This study
pCR407	pSK- with 1.3-kb *Eco*RI-*Sal*I fragment	This study
pCR414	pKS- with 1.1-kb *Pst*I fragment	This study
pCR416	pKS- with 746-bp *Eco*RI-*Pst*I fragment	This study
pCR422	pMMB67HE with 2.5-kb *Eco*RI fragment downstream of pTAC promoter	This study
pCR435	pKS- with 1.1-kb *Eco*RI-*Stu*I fragment	This study
pCR436	pMMB67HE with 1.1-kb *Eco*RI-*Stu*I fragment downstream of pTAC promoter	This study
pCR440	pCR416 with *Ppu*MI-*Sty*I deletion creating an in-frame *bvgA* deletion	This study
pCR445	pCR401 with a *Mlu*I deletion	This study
pCR446	Δ*bvgA* gene in pRTP1	This study
pJM26	pBR322 containing the entire *bvgABC* operon	13a
pTOX35	pRTP1 containing CAT fusion to *ptx* promoter region	W. J. Black
pFHACAT	pRTP1 containing CAT fusion to *fhaB* promoter region	D. Monack

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown in L broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) or on L-agar plates. Antibiotics were supplemented for *E. coli* strains at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 40 μg/ml. *B. pertussis* strains were grown on Bordet-Gengou (BG; Difco Laboratories, Detroit, Mich.) agar plates containing 15% sheep blood. Antibiotics were supplemented at the following concentrations (if necessary): ampicillin, 50 μg/ml; streptomycin, 400 μg/ml; and rifampin, 20 μg/ml.

Recombinant DNA methods. Standard methods were used for the isolation and transformation of plasmid DNA, restriction enzyme digestions, agarose gel electrophoresis, and DNA ligations (11). Enzymes were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.

β-Galactosidase assay. *E. coli* strains containing *lacZYA* fusions were grown overnight in L broth at 37°C. The next day the bacteria were diluted 1/100 in L broth and grown to an optical density at 600 nm of approximately 0.5. $MgSO_4$ (40 mM, final concentration) or nicotinic acid (5 mM, final concentration) were supplemented as indicated. Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Chemical Co., St. Louis, Mo.) was added (5 mM, final concentration) to induce transcription from the pTAC promoter when necessary. β-Galactosidase (β-gal) activity was assayed by the Miller method (14). *E. coli* strains were screened for β-gal production by growing them on L-agar plates containing 40 μg of X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; Sigma) per ml.

Analysis of plasmid-encoded proteins. The maxicell method was used to analyze plasmid-encoded proteins as described elsewhere (20).

Construction of a *bvgA* in-frame deletion in *B. pertussis*. An in-frame deletion in *bvgA* was constructed in vitro. The plasmid pCR416 was cleaved with the restriction enzymes *Ppu*MI and *Sty*I. The vector was then isolated away from the resulting 287-base-pair (bp) fragment containing internal *bvgA* sequences by fractionation on low-melting-point agarose. Blunt ends were then created by treating the purified vector with mung bean nuclease. The plasmid was recircularized by the addition of DNA ligase and transformed into DH5α. Several transformants contained plasmids that had the intended in-frame *bvgA* deletion as determined by DNA sequence analysis. A restriction fragment containing the in-frame deletion was then subcloned into pRTP1 and returned to the chromosome of BP370 by allelic exchange as described previously (23). BP370 Δ*bvgA* mutants were identified by Southern blot analysis.

Conjugative transfer of plasmid DNA from *E. coli* to *B. pertussis*. SM10 was used as the *E. coli* donor strain for all conjugations. Matings between *E. coli* and *B. pertussis* were carried out as described previously (28).

Polyacrylamide gel electrophoresis and immunoblotting.

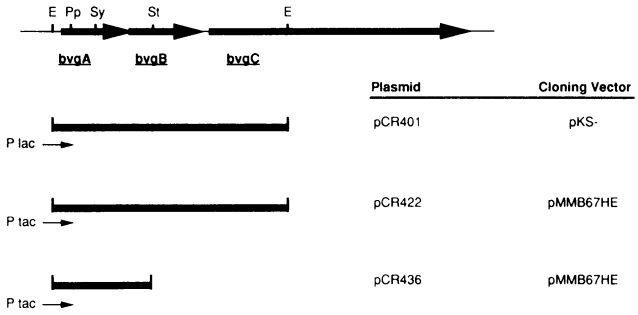

FIG. 1. Schematic representation of the *bvg* inserts contained in three plasmids used in these studies. The directions of transcription by exogenous promoters present in the cloning vectors are indicated for each construct. Restriction sites: E, *Eco*RI; Pp, *Ppu*MI; Sy, *Sty*I; St, *Stu*I.

Proteins were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis from *B. pertussis* by suspending cells taken from 2-day-old (for Fha) or 3-day-old (for Ptx) BG-agar plates in Laemmli buffer (10) and boiling for 5 min. Proteins were electrophoresed on 12% polyacrylamide slab gels. The proteins were transferred to nitrocellulose filters and probed by standard methods (4). Briefly, the filters were probed with either a Fha monoclonal antibody mixture or with a monoclonal antibody to the S_1 subunit of pertussis toxin. After the filters were washed, they were probed with goat anti-mouse antibody conjugated to alkaline phosphatase and developed by using bromochloroindoyl phosphate-Nitro Blue Tetrazolium as the colorometric substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Construction of *B. pertussis* CAT fusion strains. Suicide plasmids containing chloramphenicol acetyltransferase (CAT) fusions to the upstream regions of *ptx* (pTOX35) and *fhaB* (pFHACAT) were conjugated into *B. pertussis* strains as described above. Exconjugants carrying plasmids that had undergone a single crossover event mediated by homologous recombination with the chromosome were selected by their resistance to ampicillin. The locations of the fusion plasmid cointegrates were verified by Southern analysis.

CAT activity assay. Sonicated extracts of *B. pertussis* taken from 2-day-old BG-agar plates were prepared as described by Nicosia and Rappuoli (16). The extracts were incubated with [^{14}C]chloramphenicol (Amersham Corp., Arlington Heights, Ill.) to determine CAT activity as described elsewhere (3).

RESULTS

Transcriptional activation of *fhaB* in the presence of *bvg* sequences. We have previously shown that an intact *bvg* region containing *bvgABC* is required for transcriptional activation of *E. coli* JFMC3, which contains a chromosomal fusion consisting of the *fhaB* gene from *B. pertussis* attached to the promoterless *lacZYA* operon (13a). Plasmids encoding the entire *bvg* region were able to activate transcription of the *fhaB*::*lacZYA* fusion in *E. coli*. Plasmids containing small deletions in either the 5′ end of *bvgA* or the 3′ end of *bvgC* did not activate the fusion, showing that the *bvg* region is required for transcription of *fhaB* in *E. coli*.

When the plasmid pCR401 (Fig. 1) was transformed into JFMC3, extremely high levels of β-gal were produced. This was surprising, considering that pCR401 contained *bvgA* and *bvgB* but did not encode all of *bvgC*. If the orientation of the *Eco*RI fragment in pCR401 is reversed, the resulting plasmid no longer activates the fusion in JFMC3. This result suggested that the *lac* promoter present in the cloning vector pKS− was enhancing transcription of *bvgA* and *bvgB*, permitting the system to overcome its requirement for BvgC. To examine this possibility further, the *Eco*RI fragment from pCR401 was placed downstream of the IPTG-inducible pTAC promoter in the expression vector pMMB67HE. The pTAC promoter is normally repressed by the product of the *lacI*^{q} gene, which is also present on pMMB67HE. The resulting plasmid (pCR422) was then assayed for *fhaB*::*lacZYA* activation in JFMC3 with and without the addition of IPTG (Table 2). Over a 600-fold increase in β-gal units was observed in JFMC3(pCR422) upon IPTG induction. JFMC3(pMMB67HE) produced only basal levels of β-gal in both the presence and absence of IPTG. When the orientation of the *bvg* insert in pCR422 was reversed, the resulting plasmid was no longer able to activate the fusion. This indicates that transcriptional activation of *fhaB* in the absence of *bvgC* can be achieved by overexpressing *bvgA* and/or *bvgB* from a strong heterologous promoter.

TABLE 2. Levels of β-gal produced by *E. coli* JFMC3 (*fhaB*::*lacZYA*) containing various plasmids[a]

Plasmid	Growth conditions[b]	β-gal units[c]
pMMB67HE	37°C, IPTG	5.9
pCR422	37°C, no IPTG	6.0
pCR422	37°C, IPTG	3,900
pCR436	37°C, no IPTG	5.6
pCR436	37°C, IPTG	5,800
pCR436	37°C, IPTG, $MgSO_4$	6,800
pCR436	37°C, IPTG, nicotinic acid	5,900
pCR436	30°C, IPTG	1,800
pJM26	37°C	7,500[d]
pJM26	37°C, $MgSO_4$	51[d]
pJM26	37°C, nicotinic acid	69[d]
pJM26	30°C	140[d]

[a] Bacteria were diluted 1/100 from an overnight culture into L broth and incubated with aeration until reaching an optical density of approximately 0.5. β-gal levels were determined by the Miller method (14).
[b] Cultures were supplemented with IPTG (5 mM), $MgSO_4$ (40 mM), and nicotinic acid (5 mM) as indicated.
[c] β-gal units are expressed as the average of three independent assays.
[d] Taken from reference 13a.

***bvgA* encodes a transcriptional regulatory factor.** Restriction fragments from the *bvg* region were subcloned into pKS− or pSK− so that transcription of the *bvg* genes was enhanced by the *lac* promoter present in the cloning vectors. The resulting plasmids were transformed into JFMC3 to determine the minimal components required for transcriptional activation of *fhaB*. Strains containing plasmids capable of activating the *fhaB*::*lacZYA* fusion appeared as dark blue colonies on L-agar plates supplemented with X-gal. Only the strains with an intact copy of *bvgA* in *trans* to the fusion produced detectable levels of β-gal (Fig. 2). The plasmid pCR435, containing all of *bvgA* and only a portion of *bvgB*, was the smallest subclone capable of activating transcription of the *fhaB*::*lacZYA* fusion. Plasmid pCR416 has a 68-bp deletion in the 3′ end of *bvgA*, and pCR445 has 227-bp 5′ deletion in *bvgA*; neither of these plasmids activated transcription of the *fhaB*::*lacZYA* fusion. From these data it appears that *bvgA* encodes a *trans*-acting regulatory factor capable of activating transcription of *fhaB*.

When either pCR401 or pCR435 was transformed into JFME3 (which contains a chromosomal *lacZYA* fusion to the *ptx* promoter region), there was no detectable increase in β-gal production, suggesting that BvgA is not sufficient for

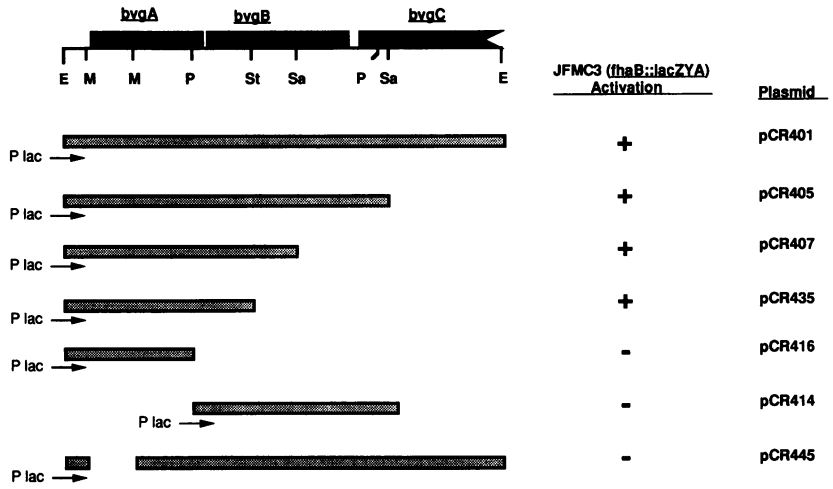

FIG. 2. Schematic representation of *bvg* subclones used to delineate the sequences sufficient for transcriptional activation of the *fhaB*::*lacZYA* fusion in *E. coli* JFMC3. β-gal production was determined by plating the bacteria on L-agar medium containing 40 mg of X-gal per ml. Positive clones produced blue colonies, and negative clones were white. Restriction sites: E, *Eco*RI; M, *Mlu*I; P, *Pst*I; St, *Stu*I; Sa, *Sal*I.

activation of *ptx* transcription in *E. coli*. This finding agrees with our earlier data that the entire *bvg* region does not activate transcription of *ptx* in *E. coli* (13a), although it has been shown that the *bvg* region is necessary for transcription of *ptx* in *B. pertussis* (16).

Identification of the *bvgA* product. To identify a *bvgA*-encoded gene product we examined the *bvg* subclones described above in a maxicell system that preferentially detects plasmid-encoded proteins. When cell lysates from [^{35}S]methionine-labeled HB101 maxicells containing *bvg* subclones were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a unique 23-kilodalton product was observed only in strains containing a complete copy of *bvgA* (Fig. 3, lanes C and D). The size of this product correlates with the predicted molecular weight of the *bvgA* protein as determined by translation of the DNA sequence. We were unable to detect a truncated *bvgA* product in strains containing pCR416. This may be due to the instability of incomplete *bvgA* products in *E. coli*.

Effect of environmental stimuli on transcriptional activation of *fhaB* by overproduction of *bvgA*. In *E. coli*, transcriptional activation of *fhaB* requires either the entire *bvg* region or overexpression of *bvgA* by a strong heterologous promoter. We have previously shown that activation of *fhaB* by the entire *bvg* region in *E. coli* is sensitive to the same environmental stimuli that modulate the expression of *bvg*-activated genes in *B. pertussis* (13a). Therefore, we investigated whether transcriptional activation of *fhaB* by the overexpression of *bvgA* was also inhibited by environmental conditions by using pCR436 (Fig. 1), a pMMB67HE derivative containing the *bvgA* gene under control of the inducible pTAC promoter.

As expected, upon induction with IPTG high levels of β-gal were produced by JFMC3(pCR436) under normal growth conditions (Table 2). Activation of the *fhaB*::*lacZYA* fusion by *bvgA* overexpression, however, was not inhibited by the addition of $MgSO_4$ or nicotinic acid or growth at 30°C (Table 2). These stimuli did inhibit activation of the fusion in JFMC3 when the entire *bvg* region is present on the plasmid pJM26. These data suggest that the activity of the *bvgA* product is not directly affected by environmental conditions.

Phenotypic analysis and genetic complementation of an in-frame *bvgA* deletion in the *B. pertussis* chromosome. Our analysis of the function of BvgA had been based upon the activation of *fhaB* transcription in *E. coli*. To determine whether other *B. pertussis* genes require BvgA for expression, we analyzed *B. pertussis bvgA* mutants. The Tn*5* mutant BP359 is the only strain of *B. pertussis* that contains a genetically defined *bvgA* mutation (26). Phenotypically, this mutant is Vir$^-$ and therefore does not express Fha, Ptx, and other *bvg*-activated gene products. Although the mutation is in *bvgA*, it was not complemented by plasmids encoding *bvgA* (pCR436) or by plasmids containing both *bvgA* and *bvgB* (pCR422; data not shown). This evidence suggested that the Tn*5* mutation in BP359 has a polar effect on the expression of *bvgB* and *bvgC* and that the *bvg* genes comprise an operon as proposed by Arico et al. (1).

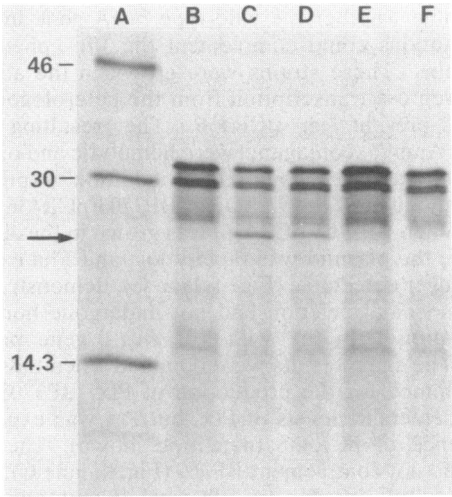

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled HB101 maxicells containing *bvg* subclones. The arrow indicates the location of a unique 23-kilodalton product found only in strains containing plasmids with the intact *bvgA* gene. Lanes: A, ^{14}C-labeled molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.); B, HB101(pKS−); C, HB101(pCR407); D, HB101(pCR435); E, HB101(pCR416); F, HB101(pCR445).

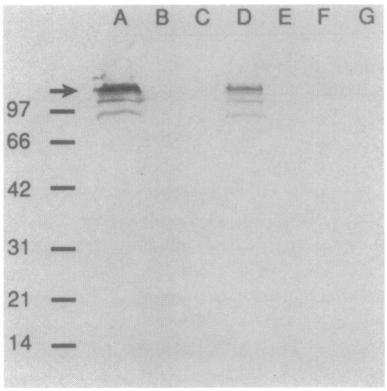

FIG. 4. Immunoblots of *B. pertussis* strains probed with a monoclonal antibody mixture against Fha. The arrow indicates the location of the full-length Fha product of approximately 220 kilodaltons. Lanes: A, BP370(pMMB67HE); B, BP3703(pMMB67HE); C, BP369(pCR436); D, BP3703(pCR436); E, BP370(pMMB67HE) plus $MgSO_4$; F, BP3703(pCR436) plus $MgSO_4$; G, BP3703(pCR436) without ampicillin selection.

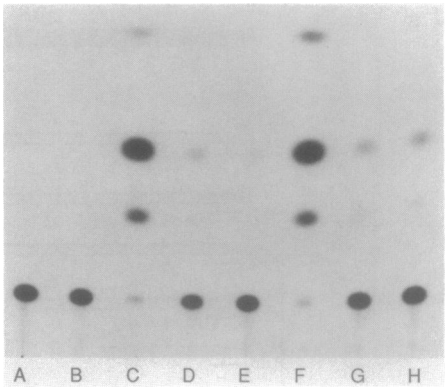

FIG. 5. [^{14}C]CAT assays on sonicated extracts of *B. pertussis* strains. Lanes: A, no extract; B, BP370; C, BP370::FHACAT; D, BP369::FHACAT; E, BP3703::FHACAT; F, BP370::TOX35; G, BP369::TOX35; H, BP3703::TOX35.

To investigate the function of BvgA in *B. pertussis*, we decided to construct a *bvgA* in-frame deletion and return this mutation to the chromosome of *B. pertussis*. An in-frame deletion in *bvgA* should eliminate BvgA function without disrupting the expression of *bvgB* and *bvgC*. The *bvgA* deletion was made by removing the 287-bp *Ppu*MI-*Sty*I fragment from pCR416 as described in Materials and Methods. The deletion was then returned to the chromosome of BP370 (Vir^+) by using the vector pRTP1. BP370 (Δ*bvgA*) mutants were confirmed by Southern hybridization analysis.

All of the BP370 (Δ*bvgA*) mutants we identified produced nonhemolytic colonies on BG-agar plates. In addition to being nonhemolytic, the mutants did not produce Fha as determined by immunoblot analysis (Fig. 4, lane B). One of these mutants, BP3703, was chosen for further characterization. The plasmid pCR436 was conjugatively transferred into BP3703 and BP369 (an isogenic Vir^- phase variant of BP370) to determine whether the wild-type *bvgA* gene in *trans* to these mutations could complement the Vir^- phenotype in these strains. These strains were grown in the absence of IPTG to repress transcription from the heterologous pTAC promoter present on pCR436. The resulting BP3703 (pCR436) Amp^r exconjugants were hemolytic and once again expressed Fha (Fig. 4, lane D). In addition, expression of Fha was modulated by $MgSO_4$ in BP3703(pCR436) (Fig. 4, lane F). When BP3703(pCR436) was grown in the absence of ampicillin, the plasmid was rapidly lost and Fha expression was no longer detectable (Fig. 4, lane G), demonstrating that the chromosomal deletion had not undergone homologous recombination with the wild-type *bvgA* gene present in pCR436. The same results were obtained when these strains were examined for the production of Ptx. BP3703 did not produce detectable levels of Ptx, but Ptx was expressed in the presence of pCR436 (data not shown). The plasmid pCR436 did not complement BP369 (Fig. 4, lane C), which is phenotypically Vir^- due to a frameshift mutation in *bvgC* (22), providing evidence that BvgC is necessary for complementation of the Vir^- phenotype by pCR436. Therefore, the ability of pCR436 to complement the in-frame *bvgA* deletion in BP3703 suggests that this deletion does not have a polar effect on *bvgC* expression. From these data we conclude that *bvgA* is required not only for the regulation of Fha but also for the expression of Ptx and hemolysin.

To examine whether the Δ*bvgA* mutation in BP3703 affected transcription of *fhaB* or the *ptx* operon, we used CAT fusions to the promoter region of *fhaB* and *ptx*. The CAT fusions were located at the same restriction sites as the *lacZYA* fusions in JFMC3 and JFME3. The CAT fusions were returned to the chromosomes of BP369 (Vir^-), BP370 (Vir^+), and BP3703 (Δ*bvgA*) by homologous recombination. The resulting strains were then assayed for CAT production. Transcription of both *fhaB* and *ptx* in BP3703 was reduced significantly when compared with that the parental strain, BP370, and appeared identical to that of the Vir^- control, BP369 (Fig. 5). These data were in agreement with the findings in *E. coli* that BvgA is a transcriptional regulatory factor. Although BvgA does not directly activate transcription of *ptx* in *E. coli*, it is necessary for transcriptional activation of the pertussis toxin operon in *B. pertussis*.

DISCUSSION

In an effort to determine the molecular mechanism of coordinate regulation and sensory transduction involved in the expression of several *B. pertussis* virulence determinants, we have demonstrated that *bvgA* encodes a transcriptional regulatory factor.

The entire *bvgABC* region of *B. pertussis* is normally required for transcription of *fhaB* in a *bvg*-regulated system reconstructed in *E. coli* (13a). Our data, however, indicate that overexpression of *bvgA* alone is sufficient for transcriptional activation of *fhaB* in *E. coli*, providing evidence that BvgA is the regulatory component that mediates this process. This evidence was supported by the construction of a *B. pertussis* mutant containing an in-frame deletion in *bvgA*, which did not have a polar effect on the expression of *bvgB* and *bvgC*. This mutant appeared to be phenotypically Vir^- and showed a significant decrease in *fhaB* and *ptx* transcription. This analysis confirmed that BvgA is required for the expression of several *bvg*-activated genes in *B. pertussis*.

The sequence homology between the *bvg* products and the proteins of several two-component regulatory systems suggests that BvgC is a sensor protein that activates its cognate regulator protein, BvgA, when the proper environmental signals are present. Once activated, BvgA would then carry out its appropriate effector function, which according to our data is to act as a positive regulator of *bvg*-activated genes. Results obtained from the genetic analysis of *bvg* activation of the *fhaB*::*lacZYA* fusion in *E. coli* and complementation

of the Vir$^-$ phenotype in a *B. pertussis* (Δ*bvgA*) mutant show that BvgA normally requires BvgC to function as a transcriptional activator. These results support the proposed two-component sensor-regulator relationship between BvgC and BvgA; however, proof of this association awaits further biochemical and genetic analysis.

The phenomenon of overproduction of a regulator protein resulting in constitutive activity, even in the absence of its cognate sensor protein, has been reported in other systems but is not completely understood (9, 19, 29). Weston and Kadner have demonstrated that transcription of the *E. coli* gene encoding the sugar-phosphate (Uhp) transport system protein UhpT requires the products of the *uhpABC* genes (29). The UhpA protein is homologous to BvgA and other regulator-class proteins and can activate transcription of *uhpT* in the absence of UhpB and UhpC if overproduced. One possible explanation for this phenomenon is that overproduction of a regulator-class protein allows its activation by sensor proteins from heterologous two-component systems. This process, termed cross-talk, has been demonstrated in vitro with purified proteins from the *E. coli* nitrogen utilization system (Ntr) and the *Salmonella typhimurium* chemotaxis system (Che) (17). The chemotaxis sensor protein CheA can substitute for NtrB in an in vitro transcription assay and phosphorylate NtrC, which triggers NtrC to function as a transcriptional activator of the *glnAp2* promoter. CheA phosphorylation of NtrC, however, is inefficient, which would explain why overproduction of the regulator is necessary for this process to occur in vivo. It is also conceivable that in our *E. coli* experiments *bvgBC*-independent activation of the *fhaB*::*lacZYA* fusion is achieved solely by increasing the intracellular concentration of BvgA and does not require cross-talk with heterologous sensor proteins.

BvgA is required for the transcription of *B. pertussis ptx* genes, but BvgA is not sufficient for transcriptional activation of *ptx* in *E. coli*. These data suggest a regulatory mechanism for *ptx* expression which differs from that of *fhaB*. There are several possible explanations for this finding: (i) BvgA directly activates transcription of *ptx* in *B. pertussis* but not in *E. coli*; (ii) other factors are required for transcriptional activation of *ptx* in addition to BvgA; and (iii) a regulatory hierarchy exists in *B. pertussis* whereby the *bvg* region directly regulates the expression of several genes, and one of the *bvg*-regulated gene products is a transcription factor which activates *ptx*. So far, results from our studies of the *bvg* region with *lac* fusions in *E. coli* have been consistent with results obtained in *B. pertussis*, which leads us to believe that the first hypothesis is unlikely. We propose that the *bvg* region is at the top level of a regulatory hierarchy that controls the expression of numerous *B. pertussis* virulence determinants. In this model BvgA would activate transcription of *fhaB* and one or more genes required for the expression of pertussis toxin and perhaps other *bvg*-activated virulence factors. A cascade effect such as this could mediate temporal expression of individual virulence determinants during the disease process and still retain the ability to coordinately modulate the expression of these factors given the appropriate stimuli.

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