JOURNAL OF BACTERIOLOGY, May 1998, p. 2484–2492 Vol. 180, No. 9
0021-9193/98/$04.00+0

Mutations Affecting the α Subunit of *Bordetella pertussis* RNA Polymerase Suppress Growth Inhibition Conferred by Short C-Terminal Deletions of the Response Regulator BvgA

SCOTT STIBITZ*

Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

Received 19 August 1997/Accepted 19 February 1998

The effects of short deletions of the C terminus of the BvgA response regulator protein of the BvgAS two-component system were examined in *Bordetella pertussis*. When present as a single copy in the chromosome, deletions removing as few as two amino acids conferred a completely Bvg$^-$ phenotype. When provided in *trans*, on the broad-host-range plasmid pRK290, under the control of the native *bvgAS* promoter, deletions of two or three amino acids conferred a profound growth inhibition which was dependent on the integrity and activity of the wild-type chromosomal *bvgAS* locus. It is proposed that this phenotype was the result of an inappropriate interaction of the mutant BvgA protein with the RNA polymerase enzyme, specifically the α subunit. Mutant strains in which this growth inhibition was relieved were isolated and characterized. Although most of the suppressor mutations affected either the mutant plasmid copy or the wild-type chromosomal *bvg* locus, three mutations which affected the α subunit of *B. pertussis* RNA polymerase were also isolated. Two of these resulted in increased levels of the α subunit, and one caused a substitution of glycine for the aspartic acid residue at position 171, in the N-terminal domain. All three mutations also resulted in a differential phenotype in that expression of *fha* was essentially normal, but expression of *ptx* was greatly reduced.

Environmentally responsive regulation of virulence gene expression in the human pathogen *Bordetella pertussis* is mediated by the *bvgAS* locus, which encodes a member of the two-component family of bacterial signal transduction systems (33). BvgS is an unorthodox sensor/transmitter protein which contains, in addition to the typical periplasmic, transmembrane, and histidine kinase domains, a response regulator domain and an additional domain termed the output domain. Through a phosphorelay mechanism, this protein is capable of transferring phosphate to the response regulator BvgA, resulting in increased affinity of BvgA for its binding sites in virulence gene promoters (1, 4, 5, 16, 37). In addition to regulating its own expression, the *bvgAS* locus governs the expression of all known protein virulence factors of this organism, including those encoding filamentous hemagglutinin (*fha*), pertussis toxin (*ptx*), and adenylate cyclase toxin/hemolysin (*cya*). Under standard culture conditions, BvgS activates BvgA to stimulate expression of these virulence genes. However, when grown in media containing $MgSO_4$ or nicotinic acid, or at reduced temperatures, *bvg*-regulated expression of virulence factors is repressed—a phenomenon known as antigenic, or phenotypic, modulation (19).

In vitro transcription experiments involving the *ptx* and *fha* promoters have demonstrated a dependence on phosphorylated BvgA (6, 7, 28). At both promoters, binding of BvgA-phosphate to consensus inverted heptameric repeats upstream of the −35 promoter motif has been seen, with additional sequences between these repeats and the −35 motif also being protected in DNase I footprinting experiments (5, 6, 42). However, the repeats in the *ptx* promoter region are further upstream than in the *fha* promoter, and the inverted heptamers are separated by 10 bp (33). These differences in promoter architecture may be responsible for functional differences in the two promoters observed in vivo, in that higher concentrations of BvgA-phosphate are required to activate *ptx* transcription than are needed to activate *fha* transcription. This phenomenon has been observed in *Escherichia coli* as well as in *B. pertussis* (25, 36).

Further demonstration of functional differences between these two promoters is provided by missense mutations affecting the extreme C terminus of BvgA which abolish *ptx* transcription but have little or no effect on *fha* transcription (30). The region of the BvgA protein affected by these mutations may be involved in interaction with RNA polymerase at regulated promoters. This is suggested by studies of other members of the subfamily of response regulators to which BvgA is related by sequence similarity in its C-terminal domain. In both the LuxR protein of *Vibrio fischeri* and the UhpA protein of *E. coli*, short C-terminal deletions affected the ability of these proteins to stimulate transcription at regulated promoters (10, 38).

We report here an analysis of the phenotype conferred by short C-terminal deletions of BvgA. Unexpectedly, when provided in *trans* in *B. pertussis*, these deletions conferred a profound growth inhibition which was dependent on the presence of a functional chromosomal *bvg* locus. Mutations which relieved this growth inhibition included mutations which affected the level of expression or the primary structure of the *B. pertussis* RNA polymerase α subunit. These observations provide additional genetic evidence for an interaction between the C-terminal portion of the BvgA protein and RNA polymerase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* DH5α, which was used as a transformation recipient for all cloning steps, was obtained from Bethesda Research Laboratories. *E. coli* strains were grown on L agar or in L broth (21) supplemented with antibiotics as appropriate. *B. pertussis* strains were grown on Bordet-

* Mailing address: Division of Bacterial Products, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892. Phone: (301) 496-1785. Fax: (301) 402-2776. E-mail: stibitz@helix.nih.gov.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Source or reference
E. coli K-12		
DH5α	High-efficiency transformation	BRL
SM10	Conjugation-proficient donor	26
SS1840	LE392[pSS1840]	32
B. pertussis		
Tohama I	Patient isolate	17
BP338	Tohama I, Nal^r	39
BP536	Tohama I, Nal^r Sm^r	29
BP947	Tohama I, Nal^r Sm^r *fha-lacZ*	This study
BP953	Tohama I, Nal^r Sm^r *fha-lacZ ptx-phoA*	This study
RPV5	Rif^r derivative of a spontaneous Ptx^- Cya^- Fha^+ mutant (↑ RpoA)	32
BP1079	Tohama I, Sm^r Nal^r Rif^r *fha-lacZ ptx-phoA*	This study
BP1187	BP953, *rpoA1187*	This study
BP1196	BP953 mutant (↑ RpoA)	This study
BP1242	BP953 with *rpoA* gene replaced by using pSS2316 (↑ RpoA)	This study
BP1246	BP1079 mutant (↑ RpoA)	This study
BP1247	BP1079, *bvg-1247*	This study
BP1248	BP1079, *bvg-1248* (*bvg*::IS*1002*)	This study
BP1250	BP1079, *bvg-1250* (*bvg*::IS*481*)	This study
BP1252	BP1079, *bvg-1251* (*bvg*::IS*481*)	This study
BP1253	BP1079, *bvg-1252*) (*bvg*::IS*481*)	This study
BP1254	BP1079, *bvg-1253* (*bvg*::IS*1002*)	This study
Plasmids		
pRK290	Broad-host-range cloning vector, Tet^r, RP4 *oriV*, RP4 *oriT*	13
pSS1129	Allelic exchange vector, *bla gen rpsL oriVColE1 oriT* λ *cos*	31
pSS1581	pSS1129 containing *fhaB-lacZ* with flanking sequences	This study
pSS1615	pSS1129 containing *ptx-phoA* with flanking sequences	This study
pSS1840	Amp^r, RP4 *oriT*, RP4 *tra*, λ *cos*	32
pSS1853	Amp^r Gen^r, RP4 *oriT*, RP4 *tra*, λ *cos*	32
pSS1894	Amp^r Gen^r, RP4 *oriT*	20
pSS2000	Amp^r Gen^r, RP4 *oriT*, *rpsL*	20
pSS2141	*Sal*I deletion derivative of pSS2000	This study
pSS2197	*bvgA* allelic retrieval vector	34
pSS2306	pSS2141 containing 7-kb *B. pertussis rpoA Bam*HI fragment	This study
pSS2647	pSS1129 containing *bvgAΔC1* with flanking sequences	This study
pSS2650	pSS1129 containing *bvgAΔC2* with flanking sequences	This study
pSS2189	pSS1129 containing *bvgAΔC3* with flanking sequences	This study
pSS2190	pSS1129 containing *bvgAΔC6* with flanking sequences	This study
pSS2191	pSS1129 containing *bvgAΔC9* with flanking sequences	This study
pSS2192	pSS1129 containing *bvgAΔC12* with flanking sequences	This study
pSS2193	pSS1129 containing *bvgAΔC15* with flanking sequences	This study
pSS2194	pSS1129 containing *bvgAΔC18* with flanking sequences	This study
pSS2068	pRK290 containing *Eco*RI-*Sph*I *bvgA* fragment	This study
pSS2342	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC1* fragment	This study
pSS2344	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC2* fragment	This study
pSS2244	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC3* fragment	This study
pSS2219	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC6* fragment	This study
pSS2246	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC9* fragment	This study
pSS2248	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC12* fragment	This study
pSS2250	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC15* fragment	This study
pSS2252	pRK290 containing *Eco*RI-*Sph*I *bvgAΔC18* fragment	This study
pSS2320	pSS2244, *bvgA1263*	This study
pSS2321	pSS2244, *bvgA1264*	This study
pSS2322	pSS2244, *bvgA1265*	This study
pSS2324	pSS2244, *bvgA1267*	This study
pSS2325	pSS2244, *bvgA1268*	This study
pSS2326	pSS2244, *bvgA1269*	This study
pSS2316	pSS2306 containing *rpoA* allele from RPV5	This study
pSS2318	pSS2306 containing *rpoA1187* from BP1187	This study

Gengou agar (Difco) containing 1% proteose peptone (Difco) and 15% defibrinated sheep blood. Concentrations of antibiotics were 10 μg/ml for tetracycline, kanamycin, and gentamicin; 50 μg/ml for nalidixic acid; and 100 μg/ml for streptomycin. For growth of *B. pertussis* strains under modulating conditions, 50 mM $MgSO_4$ was added.

Plasmid construction. Deletions of the *bvgA* gene were constructed by PCR. A fragment containing the entire *bvgA* gene and its promoter region was amplified by using an upstream primer including the *Eco*RI site at position 1 and a downstream primer including the *Sph*I site at position 817, 6 bp downstream of the *bvgA* stop codon (coordinates as in GenBank accession no. M25401). Dele-

tions removing codons immediately upstream of the stop codon were introduced by specifying the appropriate sequence in the downstream primer. In this way, genes coding for BvgA which lacked 1, 2, 3, 6, 9, 12, 15, or 18 C-terminal amino acids (*bvgAΔC1-bvgAΔC18*) were synthesized and subsequently cloned between the *Eco*RI and *Sph*I sites of pSS1894. To permit introduction of these *bvgA* deletions into the *B. pertussis* chromosome, flanking sequences were restored and the resulting constructs were recloned into the allelic exchange vector pSS1129. In this way, plasmids pSS2189, pSS2190, pSS2191, pSS2192, pSS2193, pSS2194, pSS2647, and pSS2650 were created. To permit introduction of the mutant *bvgA* genes in *trans*, an *Eco*RI site was added at the *Sph*I site by cleavage and ligation with the self-complementary oligonucleotide 5′-CATGCGAATTCG-3′. The resulting *Eco*RI fragments were recloned into pRK290 to create pSS2342, pSS2344, pSS2244, pSS2219, pSS2246, pSS2248, pSS2250, and pSS2252. In a similar way, pSS2068, containing the wild-type *bvgA* gene was created.

To create pSS2306, a *Bam*HI fragment containing the *rpoA* gene of *B. pertussis* (9) was cloned into the allelic exchange vector pSS2141 (20).

Strain construction. *B. pertussis* BP953 contains *fhaB-lacZ* and *ptx-phoA* transcriptional gene fusions to allow monitoring of virulence factor gene expression regulated by the *bvgAS* locus. The *fhaB-lacZ* fusion was created as follows. A *Bam*HI-*Sal*I fragment containing *lacZ* and derived from pRS1551 (27) was added between the most upstream *Bam*HI site and the most downstream *Xho*I site in the 10.0-kb *Eco*RI fragment containing *fhaB*. The resulting *Eco*RI fragment containing the fusion was recloned into pSS1129 to create pSS1581. This plasmid was used to replace the chromosomal *fhaB* gene to create BP947, using previously described methodology (31). The *ptx-phoA* fusion was created as follows. Plasmid ptxA4-50-6, kindly provided by Cynthia Lee, consists of a 4.7-kb *Eco*RI fragment containing the pertussis toxin operon cloned into pBR325. The oligonucleotide linker 5′-pGATCTCGCGGCCGCGA-3′ was added at the unique *Bgl*II site in this plasmid, resulting in the addition of a *Not*I site. A fragment containing the *phoA* gene of *E. coli* was created by PCR. The upstream primer sequence was 5′-ATTCCCGGGCGAGTACATTGCGAAAATAAAGT GAAACAAAGCACTA-3′. This primer includes the first 16 bp of the *phoA* gene, including the native GTG start codon, 9 bp upstream of the start codon, and an *Xma*I site at the 5′ end. The downstream primer sequence was 5′-CGC AAGCTTGCGGCCGCATTTCAGCCCCAGAGCGGCTTTCATG-3′, which contains the last 25 bp of the *phoA* gene prior to the termination codon and a *Not*I site near the 5′ end. This PCR fragment was cloned by using its *Xma*I and *Not*I sites into the *ptx* operon between the naturally occurring *Xma*I site and the *Not*I site introduced at the *Bgl*II site. The resulting *phoA* open reading frame is predicted to code for 13 additional amino acids at its C terminus relative to wild-type *phoA* as a result of the lack of the native termination codon. This fusion was cloned as a *Bst*BI fragment into the *Cla*I site of pSS1129 to create pSS1615, which was then used to replace the *ptx* locus of BP947 to create BP953 as previously described (31).

BP1079 is a spontaneous rifampin-resistant mutant of BP953 which was isolated on Bordet-Gengou agar containing 100 μg of rifampin per ml.

B. pertussis strains containing the replication-proficient derivatives of pRK290 were the result of conjugation with *E. coli* donor strains. Plasmids were transformed into *E. coli* SM10 for transfer or were transferred from DH5α in a triparental mating using SS1840.

Selection of mutants surviving growth inhibition conferred by *bvgAΔC3*. Independent cultures of BP953[pS2244] or BP1079[pS2244] were grown on Bordet-Gengou agar containing tetracycline and $MgSO_4$. After growth for 3 to 4 days, bacteria were resuspended in phosphate-buffered saline, the bacterial density was estimated by measurement of A_{600}, and dilutions were plated on Bordet-Gengou agar containing tetracycline but lacking $MgSO_4$. After incubation for 3 to 4 days at 37°C, colonies surviving selection were examined for expression of alkaline phosphatase and β-galactosidase to differentiate Bvg phenotypes. Mutants were restreaked onto Bordet-Gengou medium lacking tetracycline, and colonies were screened for tetracycline sensitivity. In this way, the mutant strains were cured of plasmid pSS2244 prior to further analysis.

Visualization of alkaline phosphatase and β-galactosidase expression by *B. pertussis* colonies. Colonies were allowed to adhere to nitrocellulose filters, which were then perfused by placing on 3MM filter paper (Whatman) saturated with Tris-HCl (pH 8.0) containing 200 μg of BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) per ml and 500 μg of Magenta-Gal (Biosynth) per ml to visualize expression of alkaline phosphatase and β-galactosidase, respectively.

Enzyme assays. For measurement of β-galactosidase, bacteria to be assayed were recovered by sterile swab from Bordet-Gengou agar and suspended in Z buffer (21). The A_{600} was measured, and 0.05 ml was diluted with 1 ml of Z buffer. Cells were permeabilized by the addition of 30 μl of 0.1% sodium dodecyl sulfate and 30 μl of chloroform followed by vortexing, and the assay was completed as described by Miller (21). For measurement of alkaline phosphatase, bacteria were suspended in 1.0 M Tris-HCl (pH 8.0). The A_{600} was measured, and 0.5 ml of cell suspension was diluted with 0.5 ml of 1.0 M Tris-HCl (pH 8.0). The cells were permeabilized as described above, and the assay was completed as described by Brickman and Beckwith (8). Units in both cases were defined by the following equation: $\text{units} = [1{,}000 \times A_{420} - (1.75 \times A_{550})]/(T \times V \times A_{600})$, where T is the incubation time in minutes and V is the volume of permeabilized cells in milliliters.

Mapping of mutations in *B. pertussis*. Donors used for Hfr mapping in this study contained selectable markers in the form of insertions of Tn*2048*, a derivative of the mini-Tn*5* transposon delivered by pUT-Kan (11, 20). From a set of 12 such insertions spaced evenly around the *B. pertussis* Tohama I chromosome, three markers near the *bvg* locus, and three markers near the *rpoA* locus were used to localize mutations to these regions. The genetic background of the donor strains was Tohama I, *str rif ptx-phoA*. Chromosomal sequences were mobilized by a library of random *B. pertussis* chromosomal fragments cloned into pSS1853 by using generalized conjugation as described previously (32) into the mutant strains, with selection on Bordet-Gengou agar containing kanamycin and nalidixic acid. Linkage of a mutation to the different Tn*2048* insertions was determined by visualization of the expression of *ptx-phoA* and *fhaB-lacZ* by exconjugant colonies as described above.

Mutations which behaved in these crosses in a manner consistent with a location in the *bvg* locus were further mapped by using suicide plasmids containing portions of the *bvg* locus as previously described (30) to derive an approximate location within the *bvg* locus. Mutations for which Hfr mapping suggested a location near *rpoA* were tested for rescue of their phenotype by using allelic exchange directed by pSS2306, which contains a 7-kb *Bam*HI fragment including *rpoA* of *B. pertussis* (9).

Recovery of mutations from *B. pertussis*. Plasmids pSS2320, pSS2321, pSS2322, pSS2324, pSS2325, and pSS2326 were recovered from *B. pertussis* mutant strains by purification of DNA by the alkaline lysis method (3) and transformation into *E. coli* DH5α. Plasmids containing the chromosomal *bvgA* gene from *B. pertussis* mutant strains were isolated by using the allelic recovery plasmid pSS2197 previously described for this purpose (34). Plasmids containing the *rpoA* gene from *B. pertussis* mutant strains RPV5 and BP1187 were isolated in a similar manner. Briefly, pSS2306 was transferred to the mutant strains with selection for gentamicin resistance and counterselection for nalidixic acid resistance. Strains containing pSS2306 thus integrated at the *rpoA* locus by a single crossover in the 7-kb *Bam*HI fragment containing the *rpoA* gene were subsequently mated with SS1840 to recover the plasmid liberated by a second crossover. Plasmids so isolated were screened for the ability to confer the differential phenotype following reintroduction into BP953. In this way, pSS2316 and pSS2318 were isolated.

Western analysis. Western blot analyses were performed as previously described (29). Samples were prepared from suspensions of *B. pertussis* strains grown on Bordet-Gengou agar which were adjusted to have the same optical density. The monoclonal antibody 4RA2, recognizing epitopes on the α subunit conserved between *E. coli* and *B. pertussis*, was kindly provided by Nancy Thompson and Richard Burgess (35). To detect BvgS, the murine monoclonal antibody 23/5 isolated in this laboratory was used (40).

DNA sequence analysis. DNA sequence analysis was performed by the dideoxy-chain termination method, using a Sequenase kit with deazaGTP to reduce artifacts due to G:C compression (U.S. Biochemical). Single-stranded templates for sequencing were isolated following cloning into mp18 and mp19 (41).

RESULTS

Short C-terminal deletions of BvgA confer a *bvg*-dependent growth inhibition in *trans*. To assess the phenotype conferred by deletion mutations affecting the C terminus of BvgA, deletions removing 1, 2, 3, 6, 9, 12, 15, and 18 amino acids were constructed and are illustrated in Fig. 1. These deleted *bvgA* genes were used to replace the wild-type *bvgA* gene of *B. pertussis* BP953. This strain contains transcriptional gene fusions of the *fhaB* gene, encoding filamentous hemagglutinin, to the *lacZ* gene of *E. coli*, encoding β-galactosidase, and of the *ptx* operon, encoding pertussis toxin, to the *phoA* gene of *E. coli*, which encodes alkaline phosphatase. Deletions removing more than one C-terminal amino acid conferred a Bvg^- phenotype in that colonies were Lac^- and Pho^- as assessed by colony lifts and perfusion with colorimetric substrates BCIP and Magenta-Gal (data not shown). In addition, these colonies were nonhemolytic, indicating a lack of expression of the *cya* operon, encoding adenylate cyclase toxin/hemolysin (data not shown). The *bvgAΔC1* mutation conferred a mild regulatory phenotype in that *ptx-phoA* expression was reduced approximately twofold, while *fha-lacZ* expression remained at wild-type levels (data not shown). To assess the effects of these mutations in *trans*, the deleted *bvgA* genes were cloned into pRK290, which is capable of replication in *B. pertussis* with a copy number of 5 to 7 (14). These plasmids were transferred by conjugation to BP953. As indicated in Fig. 1, deletion of one amino acid had no effect, but deletion of two or three amino acids of the *bvgA* gene in *trans* resulted in a complete inhibition

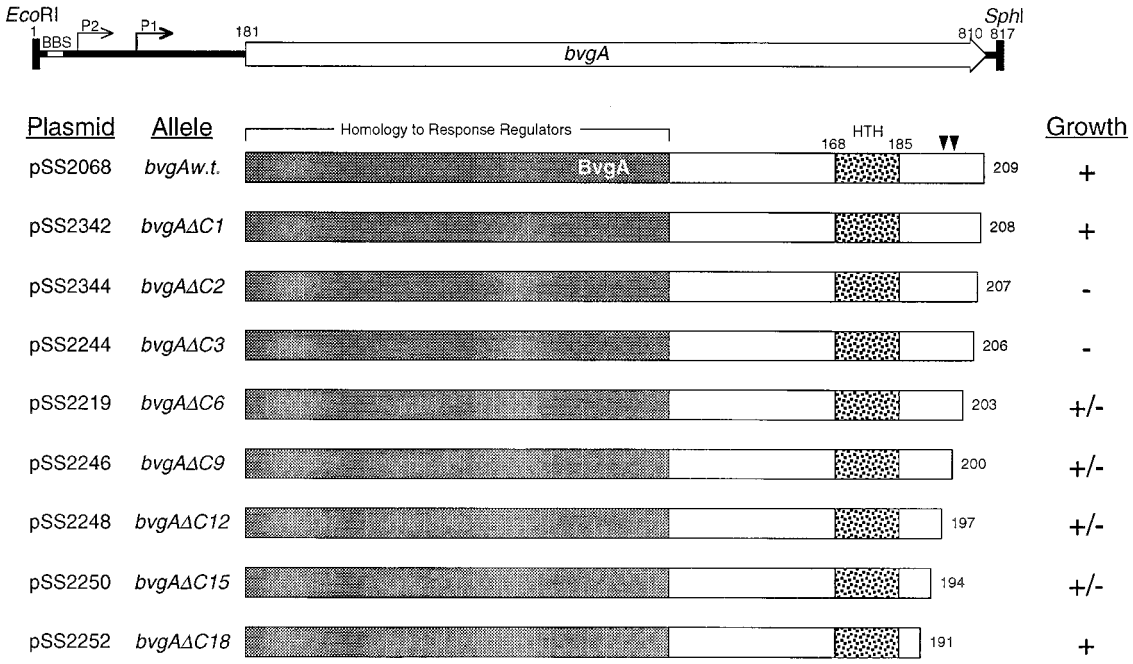

FIG. 1. BvgA proteins expressed by plasmid-borne *bvgA* alleles in *trans*. The top line depicts DNA sequence features of the *Eco*RI-*Sph*I fragment containing *bvgA*. P1 and P2 are Bvg-inducible and constitutive promoters, respectively (23, 24). BBS is a BvgA binding site (23). The coding sequence of wild-type *bvgA* (*bvgAw.t.*) is depicted by an open arrow. Nucleotide coordinates for the *Eco*RI and *Sph*I sites refer to the first nucleotide of those sites with reference to GenBank accession no. M25401. The BvgA proteins are depicted as open boxes below the DNA map, with the number of residues in each protein to the right. The shaded areas correspond to the region of amino acid sequence similarity with other two-component response regulator proteins, and the stippled areas correspond to the putative helix-turn-helix (HTH) DNA binding motif. The positions of the *bvgA1056* and *bvgA1060* mutations which affect *ptx* transcription but not *fha* transcription (30) are depicted by arrowheads on the BvgA wild-type protein. Plasmid names and their encoded *bvgA* alleles are shown at the left; the growth phenotype conferred upon BP953 is shown at the right.

of detectable growth on Bordet-Gengou agar. Deletions removing 6, 9, 12, or 15 amino acids had an intermediate effect, as revealed by a reduction in colony size but not number, and deletion of 18 residues relieved the inhibition. Expression of the growth-inhibitory phenotype conferred by the *bvgAΔC3* gene of plasmid pSS2244 was dependent on the activity of the wild-type chromosomal *bvgAS* locus, as no inhibition was observed with a strain containing a chromosomal *bvgA* null mutation, or of BP953[pSS2244] grown in the presence of 50 mM $MgSO_4$, which represses synthesis of *bvgAS*-activated genes, including the *bvgAS* locus itself (data not shown).

Selection for mutations which relieve growth inhibition conferred by BvgAΔC3. Cultures of BP953[pSS2244] or BP1079 [pSS2244] grown on Bordet-Gengou agar containing $MgSO_4$ (permissive conditions) were plated on Bordet-Gengou agar lacking $MgSO_4$ (nonpermissive conditions). This permitted the isolation of mutants which relieved the growth-inhibitory effect of the BvgAΔC3 protein. As shown in Fig. 2, when colonies surviving this selection were examined for expression of *fhaB-lacZ* and *ptx-phoA*, a variety of colonial phenotypes were observed. Dark blue colonies expressed both fusions normally and were Cya^+ by hemolysis (data not shown), a Bvg^+ phenotype. Colorless colonies lacked expression of either gene fusion and were nonhemolytic, a Bvg^- phenotype. Pink colonies had reduced hemolysis and expression of *ptx-phoA* but normal or nearly normal expression of *fha-lacZ*, a differential Bvg phenotype. Each of these classes were further characterized as follows.

Four independently isolated mutant strains displaying a Bvg^+ phenotype were analyzed. Plasmid pSS2244, directing expression of the BvgAΔC3 protein, was extracted from these strains and reintroduced into BP953. Upon reintroduction, these plasmids failed to confer a growth-inhibitory phenotype and did not affect the Bvg^+ phenotype of BP953 (data not shown). From these observations, it was concluded that mutations in this class of survivors probably represent knockouts of the *bvgAΔC3* gene.

A total of 12 independent mutants displaying a Bvg^- phenotype were analyzed. All retained their Bvg^- phenotype when cured of pSS2244. The approximate chromosomal locations of these mutations were determined by Hfr mapping as described in Materials and Methods. All behaved in Hfr crosses in a manner indistinguishable from that of mutations in the *bvg* locus (data not shown). Three of these mutations were further mapped by using marker rescue by suicide plasmids, and their positions were found to be distributed in the *bvgA* and *bvgS* genes (data not shown). These mutations appear to be knockouts of the *bvgAS* locus which relieve growth inhibition by simply reducing expression of the *bvgAΔC3*, itself a *bvg*-regulated gene.

A total of 26 independent mutants displaying a differential Bvg phenotype were analyzed. In eight of these, the mutant genotype allowing survival and conferring a differential Bvg phenotype was demonstrated to be associated with plasmid pSS2244 by recovery of the plasmids from the mutant strains and reintroduction into BP953 by conjugation. In all eight cases, reintroduction of the plasmid resulted in the mutant phenotype (data not shown). The remaining 18 mutants were subjected to Hfr mapping in order to determine an approximate chromosomal location. Fifteen of these behaved in these crosses in a manner consistent with a location in the *bvg* locus, and the other three showed linkage to markers near the *rpoA*

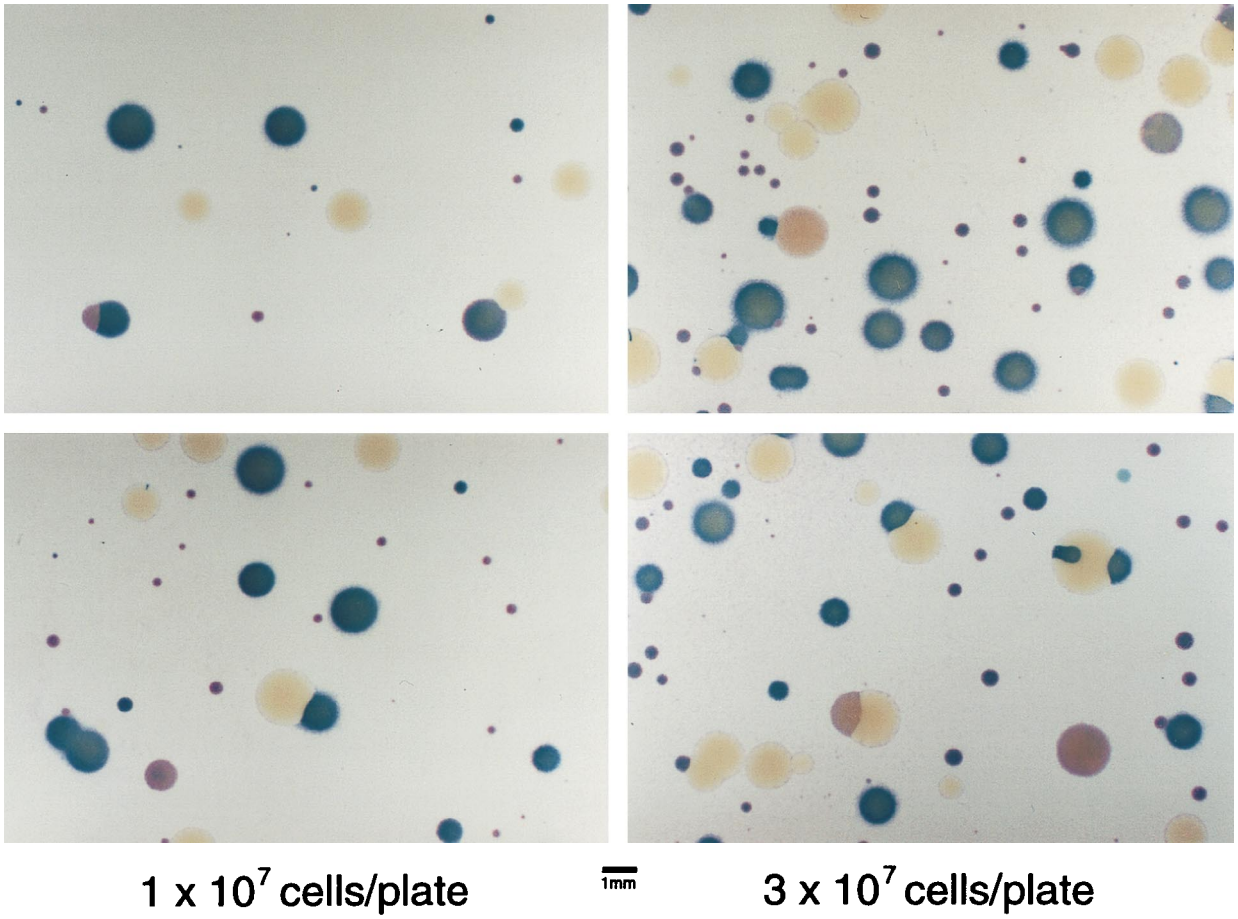

FIG. 2. Selection for mutations which suppress the growth-inhibitory phenotype conferred by pSS2244. BP953[pSS2244] was grown under permissive conditions and plated under nonpermissive conditions at an approximate density of 1×10^7 or 3×10^7 per plate (8.5-cm diameter). Colonies were allowed to adhere to nitrocellulose filters and developed with BCIP and Magenta-Gal as described in Materials and Methods. Dark blue colonies express *ptx-phoA* and *fha-lacZ*, pink colonies express primarily *fha-lacZ*, and white colonies express neither fusion.

locus of *B. pertussis* (data not shown). Further characterization of these three mutant classes displaying a differential Bvg phenotype are described below.

Plasmid-borne *bvgA* mutations conferring a differential Bvg phenotype. The differential phenotypes conferred by six of the plasmid-borne *bvgA* alleles were analyzed by β-galactosidase and alkaline phosphatase assays in order to quantitate the differential expression of *ptx-phoA* and *fhaB-lacZ*. These data are presented in Table 2 (plasmid-borne *bvgA* mutations) and demonstrate, in comparison to BP953 containing the pRK290 vector alone, that *ptx-phoA* expression is repressed upon introduction of these alleles in *trans.*

These six pSS2244 plasmid derivatives were subjected to DNA sequence analysis to define the nature of their mutations. As depicted in Fig. 3, one nonsense mutation, two IS*481* insertions, and three +2 frameshift mutations were observed. The effect of all six mutations is to truncate the mutant BvgAΔC3 protein in the C-terminal domain upstream of the helix-turn-helix motif. This presumably relieves the growth inhibition associated with the novel C terminus of this protein by removing this apparently toxic portion of the BvgA molecule. We hypothesize that the inhibitory effect on Bvg-regulated expression of *ptx* is due to the interaction of truncated BvgA with wild-type BvgA or by its competition with wild-type BvgA for phosphorylation by BvgS. In either case, the overall level of BvgA activity is reduced and a differential phenotype results. That an intermediate level of BvgAS activity can lead to a differential phenotype is suggested by temporal patterns of gene expression following induction of *bvgAS* by temperature shift (25). This can also be demonstrated through partial modulation of the activity of BvgAS. As shown in Fig. 4, the expression of *ptx-phoA* in BP953 is more sensitive to the addition of $MgSO_4$ than is *fha-lacZ*, being repressed at lower $MgSO_4$ concentrations. Thus, intermediate concentrations of a modulator can result in partial BvgAS activity and reduced expression of *ptx-phoA* relative to *fha-lacZ.*

Chromosomal *bvg*-linked mutations conferring a differential Bvg phenotype. In all but 1 of the 15 strains harboring chromosomal *bvg*-linked mutations, the location of the mutation conferring the differential phenotype could be unequivocally localized to the 816-bp *Eco*RI-*Sph*I fragment containing the *bvgA* gene and upstream region. This was accomplished by using the allelic recovery plasmid pSS2197 to recover this fragment from the mutant strains and reintroduce it into the BP953 genetic background according to procedures described elsewhere (34). Except in the case of *bvg-1247*, which appeared to map to the region encoding the periplasmic domain of BvgS, the mutation was associated with the *Eco*RI-*Sph*I *bvgA* fragment in all cases. Six mutants of this class were selected for further characterization. The results of enzyme assays per-

TABLE 2. β-Galactosidase and alkaline phosphatase assays of mutant strains displaying a differential phenotype

Strain	β-Galactosidase (*fha* expression)[a]	Alkaline phosphatase (*ptx* expression)[a]
Plasmid-borne *bvgA* mutations		
BP953[pRK290] (vector)	81 (9)	91 (25)
BP953[pSS2320] (*bvgA1263*)	82 (9)	13 (2)
BP953[pSS2321] (*bvgA1264*)	96 (13)	14 (7)
BP953[pSS2322] (*bvgA1265*)	93 (11)	31 (5)
BP953[pSS2324] (*bvgA1267*)	90 (9)	46 (1)
BP953[pSS2325] (*bvgA1268*)	85 (3)	20 (4)
BP953[pSS2326] (*bvgA1269*)	69 (4)	9 (1)
Chromosomal *bvgAS* mutations		
BP1247 (*bvg-1247*)	40 (21)	3 (1)
BP1248 (*bvg-1248*)	49 (1)	3 (1)
BP1250 (*bvg-1250*)	66 (3)	5 (1)
BP1252 (*bvg-1252*)	79 (3)	4 (0)
BP1253 (*bvg-1253*)	90 (4)	5 (1)
BP1254 (*bvg-1254*)	74 (7)	6 (1)
rpoA-linked mutations		
BP1187 (*rpoA1187*)	98 (11)	11 (2)
BP1196 (↑ RpoA)	75 (5)	11 (4)
BP1246 (↑ RpoA)	72 (10)	9 (4)
BP1242 (RPV5 *rpoA*) (↑ RpoA)	94 (4)	35 (9)

[a] Values are expressed as percentages of those obtained for BP953. Standard deviations calculated from the results of three separate assays performed on different cultures on different days are given in parentheses.

formed to quantitate the differential expression of *ptx-phoA* and *fhaB-lacZ* in these strains are presented in Table 2 (chromosomal *bvgAS* mutations). The expression of the *ptx* locus in all six strains is reduced to very low levels, while expression of the *fha* locus is reduced at most twofold. In all cases, the expression of *fha-lacZ* was repressed by addition of $MgSO_4$ to the growth media, demonstrating that these mutations did not affect modulation mediated by the *bvgAS* locus (data not shown).

The *Eco*RI-*Sph*I *bvgA* fragments from five of these six mutant strains (the exception being BP1247) were subjected to DNA sequence analysis. In all five cases, the mutation was found to be the result of an insertion sequence. Three examples of IS*481* and two examples of IS*1002* were found. In all five cases, insertion occurred at exactly the same spot between the *bvgA* and *bvgS* genes such that neither open reading frame was disrupted. Restriction analysis of the remaining 8 mutants in this class suggested that a similar insertion mutation had occurred in 14 of the 15 mutants (data not shown). These mutations would be expected to affect the level of BvgA expression and/or activity by reducing expression of BvgS, possibly allowing its expression, at a lower level, from a promoter contained in IS*481* or its relative IS*1002*. The ability of IS*481* to direct transcription of a neighboring gene from a promoter within IS*481* has been observed previously (12). Western blot analysis in fact demonstrated that BvgS levels were markedly reduced in these mutant strains (data not shown). Therefore, it appears that these mutations relieve inhibition of growth by reducing expression of the BvgAΔC3 protein, and the differential Bvg phenotype is a result of lower levels of BvgA activity, in a manner similar to that suggested above and illustrated in Fig. 4.

Chromosomal *rpoA*-linked mutations conferring a differential Bvg phenotype. Mutations affecting RpoA have been shown previously to cause a differential Bvg phenotype (9). One of these previously described *rpoA* alleles was examined and was also found to relieve the growth-inhibitory phenotype conferred by pSS2244 but apparently not to the same degree,

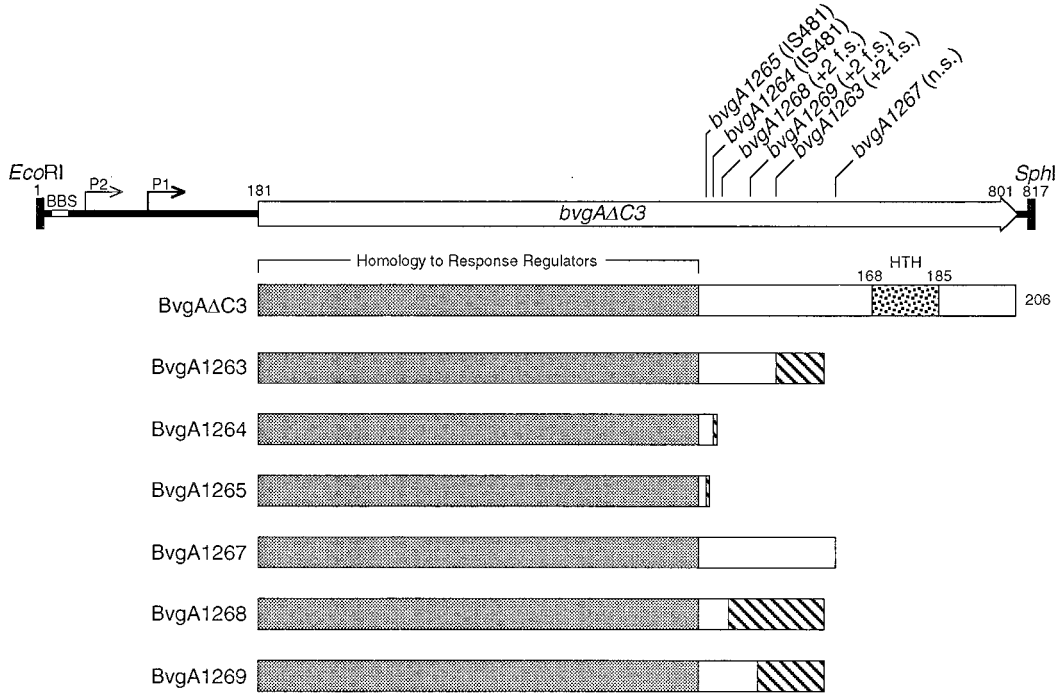

FIG. 3. Plasmid-borne mutations in the *bvgAΔC3* gene which relieve growth inhibition of pSS2244 and confer a differential phenotype. The positions and nature of the mutations are shown above the map of the *Eco*RI-*Sph*I *bvgAΔC3*-containing fragment. Other features are as described for Fig. 1, with the exception of the hatched boxes, which represent predicted nonsense (n.s.) peptide introduced into the *bvgA* open reading frame as a result of frameshift (f.s.) mutation or IS insertion.

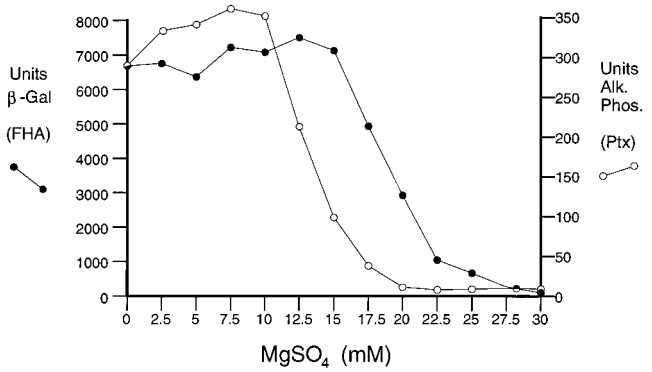

FIG. 4. $MgSO_4$ modulation of the *bvg*-regulated gene fusions *fha-lacZ* and *ptx-phoA*. BP953 was grown on Bordet-Gengou agar containing different concentrations of $MgSO_4$. Cells were recovered and assayed as described in Materials and Methods. Each point represents the mean of three separate assays. β -Gal, β-galactosidase; Alk. PhoS., alkaline phosphatase.

as colony size was reduced. This *rpoA* allele was recovered from its parental strain RPV5 and transferred by allelic exchange to the BP953 chromosome to create BP1242. A quantitative comparison of the regulatory phenotypes caused by *rpoA*-linked mutations isolated in this study with that from RPV5 is shown in Table 2 (*rpoA*-linked mutations). It can be seen from these data that the effects of these mutations were similar, although BP1187, BP1196, and BP1246 showed a somewhat more pronounced differential effect.

The *rpoA* allele from RPV5 directs increased expression of the protein encoded by this gene, the α subunit of RNA polymerase (9). To determine whether the same was true of the *rpoA*-linked mutations isolated here, Western analysis was performed. As shown in Fig. 5, both BP1196 and BP1246 show increased expression of the α subunit, while BP1187 displays wild-type levels. The magnitude of this increased expression appears comparable to that previously reported for RPV5, i.e., approximately twofold (9). Thus, although the exact locations of the mutations in these strains have not been determined, their effect appears to be due to an increase in the amount of the α subunit.

Plasmid pSS2306, which contains a 7-kb *Bam*HI fragment bearing the wild-type *rpoA* gene and flanking sequences, was used to test these three strains for restoration of the wild-type phenotype by allelic exchange. By this method, only *rpoA1187* was demonstrated to be within this 7-kb *Bam*HI fragment. DNA sequence analysis of the *rpoA* gene from BP1187 revealed the presence of a missense mutation in the coding region of this gene whereby aspartic acid residue 171 is changed to a glycine. This mutation is shown in Fig. 6, where the *B. pertussis* RpoA sequence is aligned with that of *E. coli*. It can be seen that the residue affected is near the middle of the α subunit sequence, in the N-terminal domain, a location not typically seen for mutations in the α subunit that affect its interaction with positive regulatory factors.

DISCUSSION

An unexpected phenotype results from the expression, in *trans*, of deletions of two or three amino acid residues from the C terminus of the response regulator protein BvgA of *B. pertussis*. This phenotype, a profound inhibition of growth, is proposed to be the result of an inappropriate interaction of the mutant BvgA protein with the RNA polymerase enzyme, specifically the α subunit. Experimental support for this hypothesis comes from the observation that mutations which alter the amino acid sequence of the α subunit or which increase levels of the α subunit can relieve this inhibition of growth.

The portion of the BvgA molecule associated with this growth inhibition, and by inference the interaction with the α subunit, is the C terminus, downstream of the putative helix-turn-helix DNA binding motif. Experimental support for this conclusion comes from the observation that deletion of this region in BvgAΔC18 relieves the growth inhibition, as do larger truncations isolated as spontaneous mutations of the plasmid-borne *bvgAΔC3* gene. It is suggested that this portion of the BvgA molecule normally interacts with the α subunit but in the BvgΔC2 and BvgΔC3 deletions does so in an uncontrolled or otherwise inappropriate way. Results from studies on other response regulators, with sequence similarity to BvgA in the C-terminal domain, are consistent with a role for the C-terminal sequence in interaction with RNA polymerase. For example, short C-terminal truncations result in an inability of LuxR to activate transcription, although it appears to be able to bind DNA normally (10). Similarly, UhpA C-terminal deletions are inactive in transcriptional activation of the *uhpT* promoter (38). The Bvg^- phenotypes observed here with chromosomally encoded BvgA C-terminal deletions are consistent with these observations in that removal of as few as two amino acids abolishes BvgA activity. Interestingly, this region of the BvgA molecule includes the sites of amino acid substitutions in two mutants which no longer activate *ptx* transcription but which activate *fha* transcription normally (30), consistent with the hypothesis that the differential effect of those mutations involves a change in the interaction of BvgA with RNA polymerase at the *ptx* promoter.

The growth-inhibitory phenotype observed with *bvgAΔC3* in *trans* can also be overcome by mutations which affect the wild-type chromosomal *bvg* locus, either by eliminating expression (*bvg* knockouts) or by reducing expression and/or activity, through the insertion of IS*481* or IS*1002* just upstream of the *bvgS* gene. In the latter case, the differential phenotype observed in terms of *ptx* and fha transcription is likely to be due to differences in the amount of BvgA-phosphate required for maximal expression at these two loci. In a similar fashion, the truncated BvgA molecules presented in Fig. 3 may result in intermediate levels of activity, thus explaining the differential phenotype observed under these conditions. One explanation for this finding would involve the formation of mixed dimers of BvgA. Several different experimental approaches have suggested that BvgA forms dimers (2, 4, 24). Alternatively, these truncated derivatives may compete with wild-type BvgA for phosphorylation by BvgS. Genetic analysis of a similar trans-

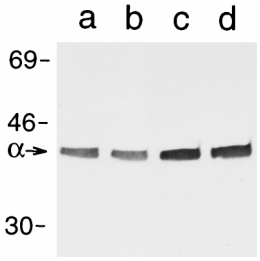

FIG. 5. Western analysis of RNA polymerase α subunit expression. Samples were whole-cell extracts of bacterial suspensions normalized by optical density to contain the same numbers of cells. The blot was probed with monoclonal antibody 4RA2, which recognizes an epitope on the α subunit conserved between *E. coli* and *B. pertussis*, kindly provided by Nancy Thompson and Richard Burgess. Sizes are indicated in kilodaltons. Lanes: a, BP953; b, BP1187; c, BP1196; d, BP1246.

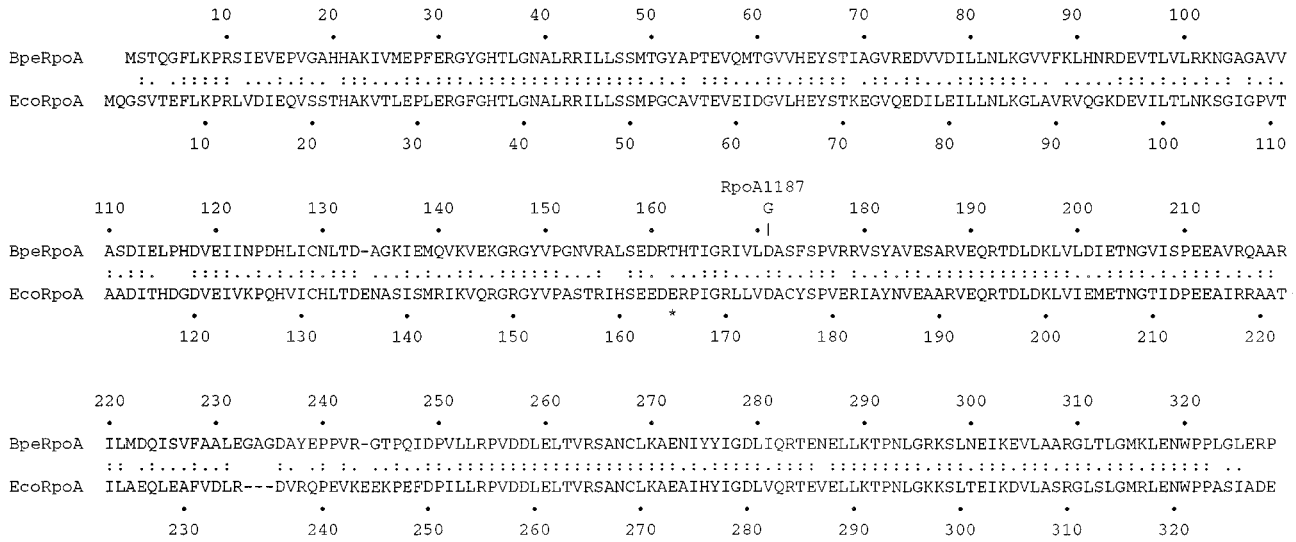

FIG. 6. Amino acid sequence alignment of the α subunits of *B. pertussis* and *E. coli*. Alignment was performed at the National Center for Biotechnology Information by using the LFASTA network service. The substitution in BP1187 is indicated above the *B. pertussis* RpoA (BpeRpoA) sequence, and the position of a mutation in the *E. coli* α subunit affecting activation of class II promoters (22) is marked by an asterisk below the *E. coli* RpoA (EcoRpoA) sequence.

dominant phenotype conferred by truncated UhpA molecules would suggest that the latter possibility is most likely correct (38). The fact that several of the mutant strains harboring chromosomal *bvg* mutations have reduced transcriptional activity of *fha-lacZ* is consistent with their very low levels of *ptx-phoA* expression and the observation that the ranges of responsiveness of these two fusions to modulating signals overlap somewhat (Fig. 4). This finding suggests that these strains have even lower levels of Bvg activity than the plasmid-bearing strains shown above them in Table 2.

The dependence of the growth-inhibitory phenotype on expression of an intact *bvg* locus can be explained in several ways. BvgAS may simply be required for the expression of *bvgAΔC3*, itself a *bvg*-regulated gene. Alternatively, wild-type BvgA protein may play a direct role in the growth inhibition, perhaps through interaction with BvgAΔC3 to form a heteromultimer, which would then be the toxic form of the protein. To date, our efforts to distinguish between these two possibilities by expressing the *bvgAΔC3* gene from a non-*bvg*-regulated promoter have been hampered by the unavailability of a promoter that is inducible in *B. pertussis* and that is of sufficient strength to achieve the growth-inhibitory phenotype, even in the presence of a wild-type chromosomal copy of *bvgAS* (data not shown). A third possibility is that manifestation of toxicity requires phosphorylation of BvgAΔC3 by BvgS. This hypothesis is currently being addressed by assessing the toxicity of BvgAΔC3 into which a D54N mutation has been incorporated, thus rendering it incapable of phosphorylation.

It has been previously reported that mutations affecting the level of the α subunit in *B. pertussis* affect *bvg*-regulated gene expression (9). In this case, the effect seen was a differential phenotype (Fha^+ Ptx^- Cya^-). This has been interpreted to possibly indicate (i) an interaction between the α subunit and BvgA and (ii) titration of active BvgA-phosphate by excess α subunit. Biochemical data provide strong evidence of such an interaction as well, in that the C-terminal portion of the α subunit is required for *bvg*-activated transcription of the *fha* gene (6). The results reported here provide additional genetic data supporting such an interaction in that mutations which increase α subunit levels suppress the phenotype associated with a mutant BvgA protein.

Interestingly, a mutation affecting the primary structure of the α subunit was also found to overcome the growth inhibition conferred by BvgAΔC3. Typically, mutations in *rpoA* which suppress mutations in positive regulatory proteins have been found in the C-terminal third of this protein, a region believed to make up a separate conformational domain which is attached to the N-terminal portion of α by a flexible peptide linker (15). Two possible explanations for the effect of this mutation are proposed. One is that this mutation defines a site on the N-terminal domain of α which contacts the BvgA molecule in the process of transcriptional activation of the *ptx* promoter. However, this would not be the only contact required, because in vitro, BvgA-activated transcription of the ptx promoter is dependent on the C-terminal domain of the α subunit as well (7). A precedent for interaction of the N-terminal portion of α in transcriptional activation comes from recent reports of catabolite gene activator protein-activated transcription of the synthetic class II promoter CC(−41.5) in *E. coli*. In this case, a mutation affecting the glutamic acid residue at position 165 of the *E. coli* α subunit had a significant negative effect (22). Interestingly, as shown in Fig. 5, the aspartic acid residue that was identified in the *B. pertussis* α subunit, at position 171, is nine amino acids away from the *B. pertussis* α residue corresponding to the *E. coli* α glutamic acid 165 and may represent an analogous mutation. Another possibility is that the glycine 171 mutation affects the assembly of α into the RNA polymerase core enzyme, either in terms of the efficiency of assembly or in terms of the configuration adopted by α in the assembled enzyme. If efficiency of assembly was low, unincorporated α subunit could accumulate with a differential effect on transcription of the *ptx* and *fha* genes as previously suggested (9). However, inefficient assembly either would be expected to result in a reduced growth rate due to reduced levels of RNA polymerase or would require higher levels of α to maintain the same level of RNA polymerase. However, neither of these conditions was observed. Fortuitously, the contribution of the corresponding residue in the *E.*

coli α subunit on assembly of RNA polymerase in vitro has been previously examined. It was found that a change of this aspartic acid at position 174 to an alanine negatively affected dimerization of the α subunit, the initial step in assembly, but subsequent assembly of $\alpha_2\beta$ and $\alpha_2\beta\beta'$ was normal (18). Inferring from this study and the observation of normal growth of the mutant *B. pertussis* strain, we may still ask whether the configuration and presentation of the mutant α subunit in the assembled RNA polymerase might not be altered and thus affect interaction with the BvgA protein at the *ptx* promoter. Clearly more study is required to distinguish between these models and to explain the behavior of this interesting mutant.

ACKNOWLEDGMENTS

I gratefully acknowledge Mei-Shin Yang for invaluable technical assistance, Nancy Thompson and Richard Burgess for providing monoclonal antibody directed against the α subunit, Richard Ebright, Phil Boucher, and Nicholas Carbonetti for useful discussions, and Joseph Devito, Michael Schmitt, and Judy Kassis for critical reading of the manuscript.

REFERENCES

1. **Beier, D., H. Deppisch, and R. Gross.** 1996. Conserved sequence motifs in the unorthodox BvgS two-component sensor protein of *Bordetella pertussis*. Mol. Gen. Genet. **252:**169–176.
2. **Beier, D., B. Schwarz, T. M. Fuchs, and R. Gross.** 1995. In vivo characterization of the unorthodox BvgS two-component sensor protein of *Bordetella pertussis*. J. Mol. Biol. **248:**596–610.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1520.
4. **Boucher, P. E., F. D. Menozzi, and C. Locht.** 1994. The modular architecture of bacterial response regulators. Insights into the activation mechanism of the BvgA transactivator of *Bordetella pertussis*. J. Mol. Biol. **241:**363–377.
5. **Boucher, P. E., and S. Stibitz.** 1995. Synergistic binding of RNA polymerase and BvgA phosphate to the pertussis toxin promoter of *Bordetella pertussis*. J. Bacteriol. **177:**6486–6491.
6. **Boucher, P. E., K. Murakami, A. Ishihama, and S. Stibitz.** 1997. Nature of DNA binding and RNA polymerase interaction of the *Bordetella pertussis* BvgA transcriptional activator at the *fha* promoter. J. Bacteriol. **179:**1755–1763.
7. **Boucher, P.** Unpublished data.
8. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96:**307–316.
9. **Carbonetti, N. H., T. M. Fuchs, A. A. Patamawenu, T. J. Irish, H. Deppisch, and R. Gross.** 1994. Effect of mutations causing overexpression of RNA polymerase α subunit on regulation of virulence factors in *Bordetella pertussis*. J. Bacteriol. **176:**7267–7273.
10. **Choi, S. H., and E. P. Greenberg.** 1991. The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. Proc. Natl. Acad. Sci. USA **88:**11115–11119.
11. **DeLorenzo, V., M. Herrero, J. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
12. **DeShazer, D., G. E. Wood, and R. L. Friedman.** 1994. Molecular characterization of catalase from *Bordetella pertussis*: identification of the *katA* promoter in an upstream insertion sequence. Mol. Microbiol. **14:**123–130.
13. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
14. **Figurski, D., R. Meyer, and D. R. Helinski.** 1979. Suppression of ColEI replication properties by the incP-1 plasmid RK2 in hybrid plasmids constructed in vitro. J. Mol. Biol. **133:**295–318.
15. **Ishihama, A.** 1993. Protein-protein communication within the transcription apparatus. J. Bacteriol. **175:**2483–2489.
16. **Karimova, G., J. Bellalou, and A. Ullmann.** 1996. Phosphorylation-dependent binding of BvgA to the upstream region of the *cyaA* gene of *Bordetella pertussis*. Mol. Microbiol. **20:**489–496.
17. **Kasuga, T., Y. Nakase, K. Ukishima, and K. Takatsu.** 1954. Studies on *Haemophilus pertussis*. V. Relation between the phase of bacilli and the progress of the whooping-cough. Kitasato Arch. Exp. Med. **27:**57–62.
18. **Kimura, M., and A. Ishihama.** 1995. Functional map of the α subunit of *Escherichia coli* RNA polymerase: amino acid substitution within the amino-terminal assembly domain. J. Mol. Biol. **254:**342–349.
19. **Lacey, B. W.** 1960. Antigenic modulation of *Bordetella pertussis*. J. Hyg. **31:**423–434.
20. **Merkel, T. J., and S. Stibitz.** 1995. Identification of a locus required for the regulation of *bvg*-repressed genes in *Bordetella pertussis*. J. Bacteriol. **177:**2727–2736.
21. **Miller, J.** Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Niu, W., Y. Kim, G. Tau, T. Heyduk, and R. H. Ebright.** 1996. Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. Cell **87:**1123–1134.
23. **Roy, C., J. F. Miller, and S. Falkow.** 1990. Autogenous regulation of the *bvgABC* operon of the bacterial pathogen *Bordetella pertussis*. Proc. Natl. Acad. Sci. USA **87:**3763–3767.
24. **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.
25. **Scarlato, V., B. Arico, A. Prugnola, and R. Rappuoli.** 1991. Sequential activation and environmental regulation of virulence genes in *Bordetella pertussis*. EMBO J. **10:**3971–3975.
26. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–789.
27. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
28. **Steffen, P., S. Goyard, and A. Ullmann.** 1996. Phosphorylated BvgA is sufficient for transcriptional activation of virulence-regulated genes in *Bordetella pertussis*. EMBO J. **15:**102–109.
29. **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.
30. **Stibitz, S.** 1994. Mutations in the *bvgA* gene of *Bordetella pertussis* that differentially affect regulation of virulence determinants. J. Bacteriol. **176:**5615–5621.
31. **Stibitz, S.** 1994. Use of conditionally counterselectable suicide vectors for allelic exchange. Methods Enzymol. **235:**458–465.
32. **Stibitz, S., and N. H. Carbonetti.** 1994. Hfr mapping of mutations in *Bordetella pertussis* that define a genetic locus involved in virulence gene regulation. J. Bacteriol. **176:**7260–7266.
33. **Stibitz, S., and J. F. Miller.** 1994. Coordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*) locus. *In* V. L. Miller, J. B. Kaper, D. A. Portnoy, and R. I. Isberg (ed.), Molecular genetics of bacterial pathogenesis. American Society for Microbiology, Washington, D.C.
34. **Stibitz, S.** 1998. Allelic retrieval: a scheme to facilitate the repeated isolation of a specific segment of the *Bordetella pertussis* chromosome. Gene **208:**183–189.
35. **Thompson, N. E.** Unpublished data.
36. **Uhl, M. A., J. F. Miller.** 1995. BvgAS is sufficient for activation of the *Bordetella pertussis ptx* locus in *Escherichia coli*. J. Bacteriol. **177:**6477–6485.
37. **Uhl, M. A., and J. F. Miller.** 1996. Central role of the BvgS receiver as a phosphorylated intermediate in a complex two-component phosphorelay. J. Biol. Chem. **271:**33176–33180.
38. **Webber, C. A., and R. J. Kadner.** 1995. Action of receiver and activator modules of UhpA in transcriptional control of the *Escherichia coli* sugar phosphate transport system. Mol. Microbiol. **15:**883–893.
39. **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.
40. **Yang, M.-S., and S. Stibitz.** Unpublished data.
41. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
42. **Zu, T., R. Manetti, R. Rappuoli, and V. Scarlato.** 1996. Differential binding of BvgA to two classes of virulence genes of *Bordetella pertussis* directs promoter selectivity by RNA polymerase. Mol. Microbiol. **21:**557–565.