

Identification of *Bordetella pertussis* Regulatory Sequences Required for Transcriptional Activation of the *fhaB* Gene and Autoregulation of the *bvgAS* Operon

CRAIG R. ROY* AND STANLEY FALKOW

Department of Microbiology and Immunology, Sherman Fairchild Science Building,
Stanford University, Stanford, California 94301

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Transcription of numerous virulence genes in *Bordetella pertussis* is positively regulated by the products of the *bvgAS* genes. In this study a series of *lacZYA* fusions containing deletions in either the *fhaB* or *bvgA* promoter regions was used to identify *cis*-acting regulatory regions required for *bvg* activation of these two genes. Gel retardation and DNase I protection analyses have shown that specific protein-DNA interactions occur at these regulatory regions and that these interactions require the transcriptional activator protein BvgA. The regulatory regions found upstream of *fhaB* and *bvgA* which are involved in protein binding both contain the sequence TTTCCTA. This sequence is part of an inverted repeat upstream of *fhaB* and a direct repeat upstream of *bvgA*. Homologous repeats are not apparent upstream of other *bvg*-activated genes, such as *ptx* and *cyaA*. These data suggest that the mechanism for transcriptional regulation of the other *bvg*-activated genes is complex and may require regulatory factors in addition to the *bvgAS* gene products.

In the respiratory pathogen *Bordetella pertussis*, transcription of several unlinked genes is regulated in response to changing environmental stimuli (6, 7, 10). Included in this set of environmentally regulated loci are the genes encoding pertussis toxin (*ptx*), hemolysin-adenylate cyclase toxin (*cyaA*), and filamentous hemagglutinin (*fhaB*) (5, 8, 14). Transcription of these genes is inhibited by growth of the bacteria in medium containing MgSO₄ or nicotinic acid as well as by growing the bacteria at 30°C rather than 37°C. The *bvgAS* operon (*vir*) encodes the proteins which coordinately regulate this environmental response (24, 26). The *bvgAS* gene products are members of the two-component family of bacterial sensory transduction proteins (1, 13, 22). The *bvgA* gene product is a transcriptional activator (15). The *bvgS* gene product (initially reported as two genes, *bvgB* and *bvgC*) is predicted to be an environmental sensor which alters the function of BvgA in response to changing stimuli (21). The *ptx*, *cyaA*, and *fhaB* genes require the *bvgAS* genes for expression and therefore are referred to as *bvg*-activated loci.

A *bvg* regulatory system was constructed in *Escherichia coli* by creating a single-copy transcriptional fusion to the *fhaB* gene and then introducing the *bvgAS* genes in *trans* to the fusion on a multicopy plasmid (12). By using this system, it was shown that transcription of an *fhaB::lacZYA* fusion in *E. coli* was *bvg* dependent. In addition, transcriptional activation of *fhaB* by *bvgAS* was inhibited when the bacteria were grown in the presence of MgSO₄ or nicotinic acid or at 30°C, demonstrating that the fidelity of the *bvg*-mediated environmental response was maintained in the *E. coli* system. In contrast, a *ptx::lacZYA* fusion was not activated in *E. coli* with the *bvgAS* genes in *trans*, suggesting that the mechanism for *bvg* activation of the *fhaB* gene differs from *bvg* activation of *ptx*.

It has been shown that expression of the *bvgAS* genes is subject to positive autoregulation (16). Transcriptional fu-

sions in both *B. pertussis* and *E. coli* have been used to demonstrate that expression of the *bvgAS* genes is greater in bacteria containing copies of an intact *bvgAS* operon in *trans*. Like other *bvg*-activated loci, this *bvg*-dependent increase in expression can be inhibited by environmental stimuli such as growth in MgSO₄. Two differentially regulated promoters have been identified upstream of the *bvgAS* operon (16, 18). The *bvgP*₁ promoter is positively autoregulated and therefore requires the *bvgAS* gene products for activity. Transcriptional activation of this promoter is sensitive to the same environmental stimuli as the other *bvg*-activated promoters. The *bvgP*₂ promoter is located upstream of *bvgP*₁ and appears to be repressed when *bvgP*₁ is activated (see Fig. 2). When the bacteria are grown in MgSO₄, the *bvgP*₁ promoter is inactive and the *bvgP*₂ promoter mediates low-level transcription of the operon.

The *bvgP*₁ promoter and the *fhaB* promoter are both activated by the *bvgAS* gene products in *E. coli*, suggesting that the mechanism for *bvg* activation of these promoters is similar. In this study, we determined the *cis*-acting sequences required for transcriptional activation of the *bvgP*₁ and *fhaB* promoters and investigated BvgA-mediated protein-DNA interactions which occur within these promoter regions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1 and in the figures. The deletion fragments shown in Fig. 2 to 4 are contained on plasmids in the vectors pKS- and pRS551 and have been recombined onto the phage λRS45. Several of the *fhaB* deletions shown in Fig. 3 and 4 are also cloned in the vector pRTP1. *E. coli* strains were grown in L broth (1% tryptone, 0.5% yeast extract, 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,

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Description	Source or reference
Strains		
<i>E. coli</i>		
SM10	RP4-2 Tc::Mu	19
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> λ ⁻ <i>thi-1 gyrA relA1</i>	Bethesda Research Laboratories
XL1-Blue	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac [F' proAB lacI^a ZΔM15 Tn10]</i>	Stratagene
MC4101	MC4100 <i>recA1</i>	12
CR430	MC4101 λ CR430 <i>bvgA::lacZYA</i>	16
<i>B. pertussis</i> BP536	Wild type, Nal ^r Str ^r	14
Plasmids		
pRS551	<i>lacZYA</i> fusion vector	20
pCR428	<i>bvgA EcoRI-StyI</i> fragment in pKS-	16
pCR435	<i>bvgA</i> in pKS-	15
pCR304	<i>fhaB EcoRI-AccI</i> fragment in pKS-	This study
pCR0.1BEX	<i>fhaB-bvgA</i> fragment in pKS-	This study
pT7.7	Vector containing T7 ϕ 10 promoter and ribosome binding site	S. Tabor
pT7.7BvgA	<i>bvgA</i> in pT7.7	This study
pJM26	<i>bvgAS</i> in pBR322	12
pRTP1	Gene replacement vector for <i>B. pertussis</i>	23
Phage		
λ RS45	Phage used to recombine <i>lacZYA</i> fusions from pRS551 onto λ	20
mGP1-2	M13 phage encoding T7 RNA polymerase	S. Tabor

Mich.) agar plates containing 15% sheep blood. Antibiotics were supplemented at the following concentrations if necessary: ampicillin, 50 μ g/ml; nalidixic acid, 50 μ g/ml.

Recombinant DNA methods. DNA ligations, restriction endonuclease digestions, BAL 31 digestions, and gel electrophoresis were performed as described previously (9). Bacterial electroporation was used to introduce plasmid DNA into *E. coli* (2). A Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) was used to determine the DNA sequence of double-stranded plasmid templates as described by the manufacturer. Enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., and Bethesda Research Laboratories, Inc., Gaithersburg, Md.

Construction of *bvg* and *fhaB* deletions. To generate sequential deletions of DNA upstream of the *bvg* promoters, the plasmid pCR428 was linearized with the restriction enzyme *EcoRI* and then treated for various lengths of time with the exonuclease BAL 31 (Fig. 1). After BAL 31 digestion, the vector was religated in the presence of *EcoRI* linkers and transformed into DH5 α . Plasmid DNA was isolated from single colonies of bacteria, and the extent of the upstream deletion was determined by sequence analysis for several clones. Plasmids containing deletions of an appropriate size were cleaved with *EcoRI* and *BamHI*.

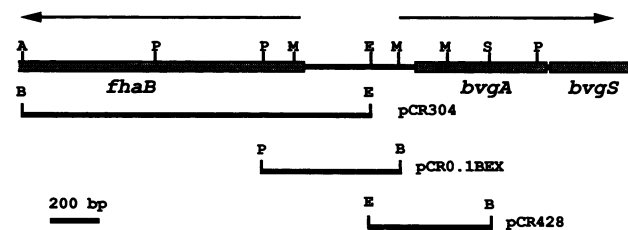


FIG. 1. Schematic representation of plasmids used in this study. The genomic restriction map of the *fhaB-bvgA* intergenic region is displayed at the top. The restriction fragments contained on the plasmids pCR304, pCR428, and pCR0.1BEX are aligned below the map. All of the restriction fragments are in the Bluescript vector pKS-. The plasmid pCR304 has the *AccI* site in *fhaB* replaced with a *BamHI* linker. A *BamHI* linker has also replaced the *MluI* site upstream of *bvgA* in pCR0.1BEX and the *StyI* site within *bvgA* in pCR428. Restriction sites: A, *AccI*; E, *EcoRI*; M, *MluI*; P, *PstI*; S, *StyI*.

Restriction fragments encoding the *bvg* promoters were isolated from low-melting-point agarose gels. These fragments were ligated into the *lacZYA* fusion vector pRS551, and the fusions were then recombined onto the λ phage RS45 as described previously (12). Single-copy *bvg::lacZYA* fusions resulted from infecting *E. coli* MC4101 with the recombinant phage and selecting for lysogens by plating the bacteria on L agar containing kanamycin.

To generate sequential deletions of DNA downstream of *bvgP*₂, the vector pCR0.1BEX was linearized with the restriction enzyme *BamHI* and treated with BAL 31 (Fig. 1). The vector was religated in the presence of *BamHI* linkers and electroporated into DH5 α . Individual clones were sequenced and restriction fragments containing the *bvg* promoter region were isolated after cleavage of the vector with *BamHI* and *EcoRV*. Fusions were constructed from these fragments in the vector pRS551, and lysogens containing single-copy fusions were isolated as described above.

Deletions in the *fhaB* upstream region were generated by BAL 31 digestion of *EcoRI*-digested pCR304 (Fig. 1). After BAL 31 digestion, the vector was religated in the presence of *EcoRI* linkers and transformed into DH5 α . After sequence analysis, several deletions were isolated as *EcoRI-BamHI* fragments and cloned into pRS551. *E. coli* lysogens containing single-copy fusions were isolated as described above. A double-stranded oligonucleotide homologous to the *fhaB* -99 to -80 region was generated by annealing the two complementary single-stranded oligonucleotides with the sequences 5'-GATCGACTAAGAAATTTCTTACAA-3' and 5'-AATTTTGTAGGAAATTTCTTAGTC-3'. The resulting double-stranded oligonucleotide contained 5' single-stranded ends with the sequences 5'-GATC-3' and 5'-AATT-3'. This oligonucleotide was then ligated upstream of the deletions 2-1FE, 3-12FE, and 4-2FE after the plasmids containing these deletions had been digested with *EcoRI*. The 5' termini of the oligonucleotides were not phosphorylated; therefore, only one oligonucleotide could ligate upstream of each deletion. After the ligation reactions, the DNA was cut with *BamHI*, and the deletion fragments containing the oligonucleotide upstream were isolated from low-melting-point agarose gels. These fragments were then ligated into *BamHI*-digested pRS551, and the resulting plasmids were used to make single-copy *lacZYA* fusions on λ phage in the *E. coli* chromosome.

β -Galactosidase assay. *E. coli* strains containing *lacZYA*

fusions were grown in L broth to mid-log phase as previously described (12). β -Galactosidase (β -gal) activity was assayed by the method of Miller (11).

Analysis of Fha expression in *B. pertussis*. The *fhaB* upstream deletions were isolated as *EcoRI*-*Bam*HI fragments and ligated into the plasmid pRTP1. The resulting plasmids were transformed into *E. coli* SM10 and then transferred by conjugation into *B. pertussis* BP536 as previously described (27). Plasmid cointegrates formed by homologous recombination between vector insert and chromosomally encoded *fhaB* sequences were selected on BG agar containing ampicillin and nalidixic acid. Whole-cell lysates of the bacteria were electrophoresed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and probed with Fha monoclonal antibodies as previously described (15).

Overexpression of BvgA. The *bvgA* gene was amplified from the plasmid pCR435 by the polymerase chain reaction by using a primer homologous to the beginning of the structural gene (5'-CCGGATCCATATGTACAACAAAGT CCTC-3') and the T7 sequencing primer. The polymerase chain reaction product and the vector pT7.7 (gift from S. Tabor, Harvard Medical School) were digested with *Nde*I and *Bam*HI and purified on low-melting-point agarose. The polymerase chain reaction-generated restriction fragment containing *bvgA* was ligated into pT7.7, and the ligations were electroporated into *E. coli* XL1. The resulting plasmid, pT7BvgA, contains the *bvgA* gene downstream of the T7 phage Φ 10 promoter and ribosome binding site. To overproduce BvgA, the strain XL1(pT7BvgA) was grown to an optical density at 600 nm of 1.5 in L broth at 37°C. The culture was then infected with the recombinant M13 phage mGP1-2 at a multiplicity of infection of 10, and isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 1 mM. The phage mGP1-2 contains the gene encoding T7 RNA polymerase which activates the Φ 10 promoter in pT7BvgA, thereby inducing high-level expression of BvgA (25). An hour after induction, the bacteria were pelleted at $5,000 \times g$ for 15 min at 4°C, washed in an equal volume of ice-cold 10 mM Tris HCl (pH 7.5), pelleted, and resuspended in 1/20 volume of ice-cold binding buffer (10 mM Tris HCl [pH 7.5], 1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 20% glycerol). The bacterial cells were then disrupted by sonication at 4°C. The sonicated extracts were spun at $10,000 \times g$ for 15 min at 4°C to remove intact cells and debris. The extracts were stored at -70°C. Extracts were also prepared from XL1(pT7.7) by this same procedure to serve as negative controls in the in vitro DNA binding studies.

Gel retardation and DNase I protection assays. Gel retardation and DNase I footprinting assays were performed as previously described (3, 4). Binding reactions were carried out in $1 \times$ binding buffer in a total volume of 20 μ l. Each reaction mixture contained 2 to 40 μ g of protein extract, 4 μ g of calf thymus DNA, and 1 to 10 ng of probe DNA. The reaction mixtures were incubated at 37°C for 20 min. For gel retardation assays, 4 μ l of loading buffer (33% glycerol, 0.03% bromophenol blue) was added gently to the reaction mixtures after the 20-min incubation period. The reaction mixtures were then loaded onto a 5% polyacrylamide gel containing $0.5 \times$ TBE and run at room temperature in $0.5 \times$ TBE. The gels were dried and exposed to X-ray film for 4 to 16 h. For DNase I protection assays, 2 μ l of 100 mM MgCl₂ was added to the binding-reaction mixtures after incubation at 37°C. DNase I was then added, and the reaction mixtures were incubated for 1 min at room temperature. To terminate

the reactions, 30 μ l of stop buffer (1% SDS, 20 mM EDTA) was added. The reaction mixtures were then extracted with phenol-chloroform (50:50), and the DNA was ethanol precipitated. The DNA was resuspended in sequencing loading buffer, denatured at 95°C for 5 min, and run on an 8% polyacrylamide sequencing gel.

RESULTS

Identification of cis-acting regulatory sequences required for differential transcription of the *bvgAS* operon. The *bvgAS* operon is transcribed by two differentially regulated promoters designated *bvgP*₁ and *bvgP*₂. The previously characterized strain CR430 contains the intact *bvg* upstream region attached to the promoterless *lacZYA* genes on a λ prophage in the *E. coli* chromosome (16). This strain produces 85 U of β -gal because of transcription from the *bvgP*₂ promoter. β -Gal levels increase to 665 U when the *bvgAS* operon, encoded on the plasmid pJM26, is placed in *trans* to the fusion in CR430. This increase in β -gal production is presumably due to activation of the *bvgP*₁ promoter. To determine the DNA sequences required for expression and regulation of the *bvg* promoters, deletions upstream of *bvgA* were generated in vitro by using BAL 31 exonuclease as described above. Restriction fragments containing these deletions were then used to construct single-copy *lacZYA* transcriptional fusions on λ prophage in the *E. coli* chromosome. *E. coli* *bvg::lacZYA* fusion strains containing deletions in the *bvgA* upstream region were then assayed for β -gal production both with and without pJM26 in *trans*. All of the *bvg::lacZYA* fusions constructed with the deletion fragments were defective in β -gal expression when compared with the parental fusion in CR430, producing less than 30 U of β -gal with or without pJM26 in *trans* (Fig. 2). The most conservative deletion examined was 428-17, which has 21 bp missing from its 5' end when compared with the parental fusion in CR430. These results suggest that the 21 bp which were deleted encode *cis* regulatory signals required for transcription of *bvgP*₂ and activation of *bvgP*₁.

To determine whether the high levels of β -gal detected in CR430(pJM26) were due to activation of the *bvgP*₁ promoter, a series of deletions coming from within the *bvgA* structural gene towards the promoters was generated by BAL 31 digestion. These truncations of downstream sequences are referred to as *bvg* 3' deletions. Selected 3' deletions were used to construct single-copy *lacZYA* fusions in *E. coli*, and β -gal activity was assayed for each *bvg* 3' deletion fusion both with and without the *bvg* operon in *trans*. β -Gal activity was detected for all of the 3' deletion fusions in the absence of the *bvg* operon because of the presence of an intact *bvgP*₂ promoter (Fig. 2). All of the 3' deletion fusions which retained the +1 site of the *bvgP*₁ promoter produced higher levels of β -gal with the *bvg* operon in *trans* (Fig. 2; 60-5BEX, 30-5BEX, 15-2BEX, 15-3BEX, and 0.1BEX). The 3' deletions which removed the *bvgP*₁ transcriptional start site did not show an increase in β -gal activity in the presence of the *bvgAS* genes (Fig. 2; 60-2BEX, 30-3BEX, and 30-6BEX). These data demonstrate that the *bvgP*₁ promoter is required for high-level autoregulated expression of the *bvg* operon.

Identification of cis-acting sequences required for transcriptional activation of *fhaB*. To determine the *cis*-acting sequences required for transcriptional activation of the *fhaB* promoter, a series of deletions in the *fhaB* upstream region was generated by using BAL 31. *E. coli* lysogens were made with λ phage containing the *fhaB* deletions attached to the

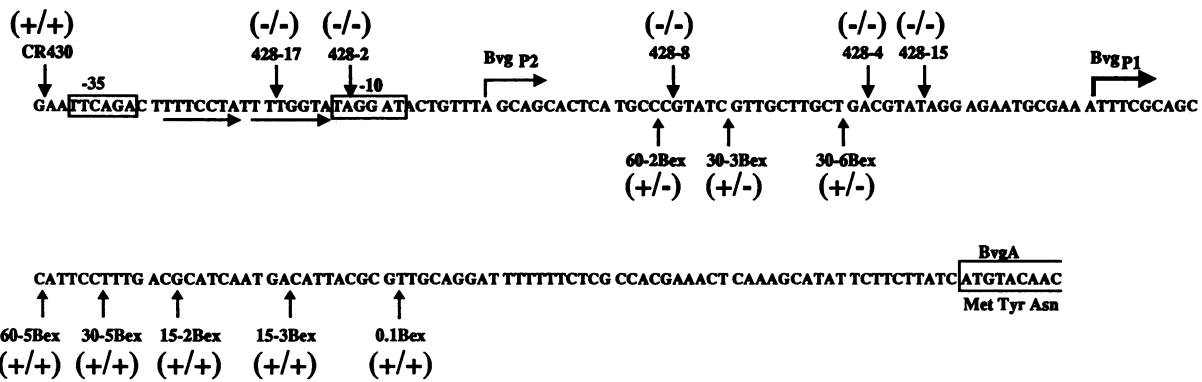


FIG. 2. Location of deletions within the *bvg* promoter region. The coding sequence upstream of the *bvgA* gene is displayed at the top. Arrows above the sequence indicate the 5' termini of deletions in the upstream region of *bvgA* derived from BAL 31 digestion of *EcoRI*-cleaved pCR428. Arrows below the sequence indicate the 3' termini of deletions coming towards the *bvg* promoters derived from BAL 31 digestion of *BamHI*-cleaved pCR0.1BEX. The deletions were used to construct *lacZYA* fusions in *E. coli* MC4101, and β -gal activity was determined for each of the resulting strains both with and without the *bvgAS* operon in *trans* to the fusions. Fusions which demonstrated *bvg*-independent levels of β -gal expression produced at least 85 U of β -gal without *bvgAS* in *trans*. Fusions which were *bvg* activated produced greater than 600 U of β -gal with the *bvgAS* genes in *trans*. Symbols: (+/+), fusions which demonstrated both *bvg*-independent and *bvg*-activated levels of β -gal expression; (+/-), fusions which demonstrated *bvg*-independent but not *bvg*-activated levels of β -gal expression; (-/-), fusions which demonstrated neither *bvg*-independent or *bvg*-activated levels of β -gal expression.

promoterless *lacZYA* genes. The plasmid pJM26 was placed in *trans* to the *fhaB* deletion fusions, and transcriptional activation was determined by assaying β -gal production. The results show that a minimum of 85 bp of sequence upstream of the *fhaB* +1 is required for transcriptional activation of the *fhaB* promoter by the *bvg* operon (Fig. 3). The 2-1FE deletion contained 60 bp of sequence upstream of the *fhaB* +1; however, *lacZYA* fusions constructed with this deletion fragment were not activated by *bvgAS*. These data suggest that sequences located within the -85 to -60 region of the *fhaB* promoter are important for transcriptional activation by *bvgAS*.

We have previously shown that an oligonucleotide homologous to the -99 to -80 region of the *fhaB* promoter completely inhibits *bvgAS* activation of an *fhaB* fusion when placed in *trans* on a high-copy-number plasmid in *E. coli* (17). This inhibition is likely caused by sequence-specific titration of a *trans*-acting factor. To investigate whether the -99 to -80 region of the *fhaB* promoter contains *cis* sequences sufficient for transcriptional activation, an oligonucleotide homologous to this region was ligated upstream

of deletions 2-1FE, 3-12FE, and 4-2FE. After ligation of the oligonucleotide upstream of the deletions, the fragments were used to construct *lacZYA* fusions in *E. coli*. The resulting strains were then assayed for transcriptional activation by placing pJM26 in *trans* to the fusions and measuring β -gal production. The addition of the oligonucleotide resulted in a 100-fold increase in β -gal production for the 2-1FE deletion and a 4-fold increase for the 3-12 deletion and had no effect on the 4-2FE deletion in which the *fhaB* promoter has been completely deleted (Fig. 4). The addition of 40 mM $MgSO_4$ reduced β -gal production in CR2-1FE-Pal(pJM26) and CR3-12FE-Pal(pJM26) to basal levels, demonstrating that transcriptional activation of these fusions by *bvgAS* is sensitive to environmental stimuli. These data show that sequences located within the -99 to -80 region are important for transcriptional activation of the *fhaB* promoter by the *bvgAS* genes.

To determine whether the *fhaB cis* sequences defined in *E. coli* were also important for expression of Fha in *B. pertussis*, the *fhaB* deletions were cloned into the plasmid pRTP1 and recombined onto the *B. pertussis* chromosome. A single

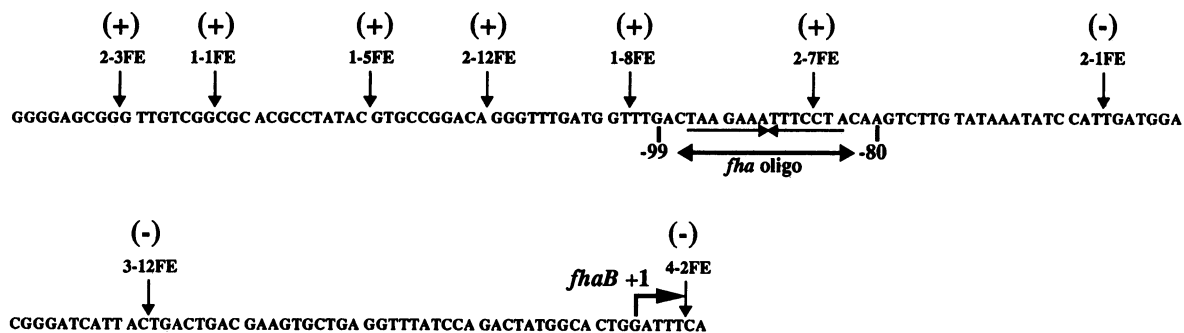


FIG. 3. Location of deletions within the *fhaB* promoter region. The coding sequence upstream of the *fhaB* gene is displayed at the top. Arrows above the sequence indicate the 5' termini of deletions in the upstream region of *fhaB* derived from BAL 31 digestion of *EcoRI*-cleaved pCR304. The deletions were used to construct *lacZYA* fusions in *E. coli* MC4101, and β -gal activity was determined for each of the resulting strains with the *bvgAS* operon in *trans* to the fusions. Fusions which were *bvg* activated (+) produced at least 8,000 U of β -gal with *bvgAS* in *trans*. Fusions which were not *bvg* activated (-) produced less than 30 U of β -gal with the *bvgAS* genes in *trans*.

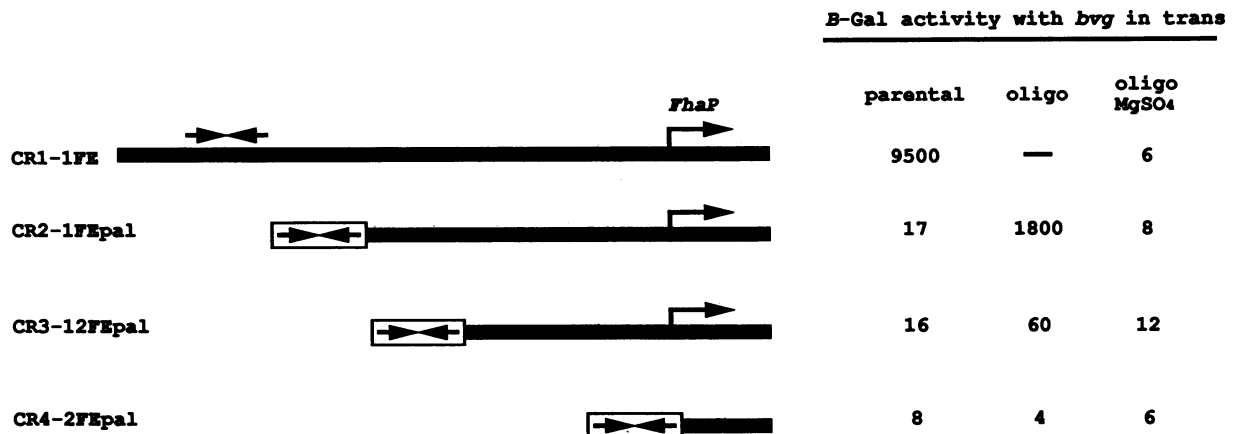


FIG. 4. Transcriptional regulation of defective *phaB* fusions after the addition of an oligonucleotide homologous to the *phaB* -99 to -80 region upstream. The effect of the oligonucleotide on *bvg* activation was determined for several *phaB::lacZYA* fusion strains by determining β -gal activity before (parental) and after (oligo) the addition of the oligonucleotide. The *bvgAS* operon was present in *trans* to the fusions on the plasmid pJM26. The fusion strains were also grown in the presence of 40 mM MgSO₄ to determine the effect of environmental stimuli on regulation. The β -gal units given are the average of three independent assays which did not differ by more than 20%.

crossover event between the plasmid and the *B. pertussis* chromosome resulted in the placement of the deletions upstream of the intact *phaB* gene. Whole-cell lysates from *B. pertussis* containing the integrated plasmids were electrophoresed on an SDS-10% polyacrylamide gel, and Fha expression was determined by Western blot (immunoblot) analysis (Fig. 5). The results show that Fha expression was eliminated after recombination of the 2-1FE deletion upstream of the *phaB* gene and that introduction of the -99 to -80 oligonucleotide upstream of the 2-1FE deletion restores Fha expression. These results confirm the data obtained in *E. coli* and show that the *cis* sequences defined in *E. coli* as being important in transcriptional activation of the *phaB* promoter are also required for Fha expression in *B. pertussis*.

Polyacrylamide gel retardation of *phaB* restriction frag-

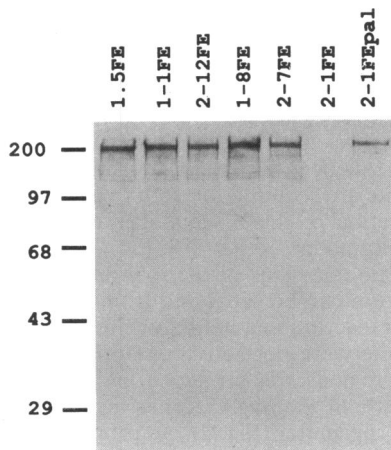


FIG. 5. Expression of Fha in *B. pertussis* strains containing deletions upstream of the *phaB* gene. The deletions upstream of *phaB* illustrated in Fig. 3 were returned to the *B. pertussis* chromosome. Whole-cell lysates of *B. pertussis* containing the indicated deletion were fractionated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Fha production was assayed by probing the membranes with anti-Fha monoclonal antibodies. Molecular weights in thousands are indicated on the left.

ments. The product of the *bvgA* gene has been shown to be sufficient for transcriptional activation of an *phaB::lacZYA* fusion in *E. coli* when overexpressed from a strong heterologous promoter (15). To examine BvgA-mediated protein-DNA interactions in vitro, the *bvgA* gene was cloned into the expression vector pT7.7 and high levels of BvgA protein were produced in *E. coli* upon induction of the T7 promoter. Sonicated extracts of *E. coli* induced for BvgA expression and control extracts of *E. coli* containing the expression vector alone were tested for DNA binding activity. The extracts were incubated in the presence of two ³²P-labeled restriction fragments, a 352-bp *EcoRI-MluI* fragment containing the *phaB* promoter and a downstream 150-bp *MluI-PstI* fragment containing only the *phaB* coding sequence. After incubation at 37°C for 20 min, the binding reaction mixtures were fractionated on a 5% polyacrylamide gel. The results show that the mobility of the 352-bp fragment containing the *phaB* promoter was specifically retarded when incubated with extracts containing BvgA, whereas the mobility of the 150-bp fragment was not affected by BvgA (Fig. 6A). Identical *E. coli* extracts lacking BvgA did not affect the mobility of either fragment, suggesting that the altered mobility observed for the restriction fragment containing the *phaB* promoter is mediated by BvgA.

The *phaB* deletion fragments used to construct the *lacZYA* fusions (Fig. 3) were then tested for alterations in gel mobility after incubation with extracts containing BvgA. The ³²P-labeled deletion fragments were incubated with extracts containing BvgA or with binding buffer alone and then fractionated on a 5% polyacrylamide gel. The results show that only fragments containing an intact -99 to -80 region of the *phaB* promoter were retarded by BvgA-containing extracts (Fig. 6B). The mobility of a double-stranded oligonucleotide homologous to the -99 to -80 region was also retarded in the presence of BvgA, suggesting that this is the site of protein-DNA interaction (Fig. 6C).

DNase I protection of the *phaB* and *bvgA* promoter regions by extracts containing BvgA. To identify the region of protein-DNA interaction mediated by BvgA, a restriction fragment encompassing the *phaB* promoter region was isolated from pCR1-1FE after digestion of the plasmid with *EcoRI* and *PstI*. The fragment was labeled with ³²P at one end and

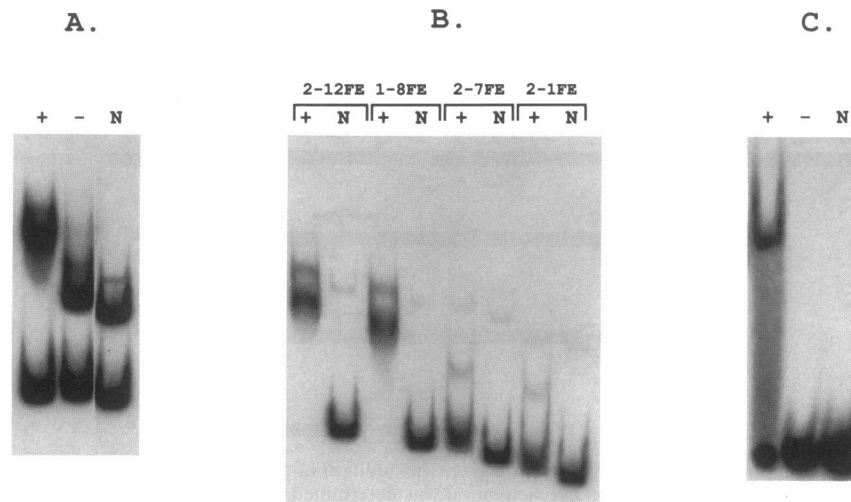


FIG. 6. Polyacrylamide gel retardation analysis. DNA fragments were end labeled with ^{32}P and incubated with 20 μg of protein extract from either *E. coli* overproducing BvgA (+), *E. coli* containing the vector pT7.7 alone (-), or no extract (N). The reaction mixtures were fractionated on a 5% polyacrylamide gel in $0.5\times$ TBE. (A) Gel retardation analysis of a 352-bp fragment containing the *fhaB* promoter region and a 150-bp fragment containing only *fhaB* coding sequence is shown. (B) Gel retardation analysis of *fhaB* deletion fragments is shown. The fragments were purified after digestion of the plasmids encoding the deletions with *EcoRI* and *MluI*. (C) Gel retardation analysis of a double-stranded oligonucleotide homologous to the -99 to -80 region upstream of *fhaB* is shown.

incubated either with extracts containing BvgA or with control *E. coli* extracts lacking BvgA. The binding reaction mixtures were partially digested with DNase I and fractionated on an 8% polyacrylamide sequencing gel (Fig. 7A). The restriction fragment was also chemically cleaved by the Maxam and Gilbert sequencing reactions and run in parallel lanes (data not shown). The results show an area of DNase I protection spanning the -101 to -78 region of the *fhaB* promoter (Fig. 8). A second area of protection was also observed in the -71 to -67 region of the *fhaB* promoter. No DNase I protection was observed when the restriction fragments were incubated with *E. coli* extracts lacking BvgA.

The *bvgA* and *fhaB* genes are transcribed divergently, and the *bvgP*₁ and *fhaB* promoters are separated by only 268 bp. A *Bam*HI-*Mlu*I restriction fragment containing both the *fhaB* and *bvgA* promoter regions was isolated from pCR0.1BEX, ^{32}P labeled, and incubated with various concentrations of protein extract. After limited digestion with DNase I, the reaction mixtures were run on an 8% polyacrylamide sequencing gel to identify the areas of protein-DNA interaction occurring within the *bvgA-fhaB* intergenic region. The same region of DNase I protection identified above was observed upstream of *fhaB* at all protein concentrations tested (Fig. 7B). As the protein concentration was increased, an area of protection appeared upstream of the *bvg* promoters. This latter area of protection spans 33 bp and encompasses the -34 to -1 region of the *bvgP*₂ promoter (Fig. 8). On the basis of the deletion analysis of the *bvgA* and *fhaB* upstream regions, the areas of DNase I protection identified above correlate with the sequences required for *bvgAS* activation of these genes.

DISCUSSION

In this study we have investigated the molecular mechanisms of *bvgAS* autoregulation and activation of the *fhaB* gene from *B. pertussis*. By using a *lacZYA* fusion system and two techniques for examining protein-DNA interactions in

vitro, we have identified *cis*-acting elements located upstream of *bvgA* and *fhaB* which are required for *bvg* activation of these genes.

We have examined deletions upstream of the *bvgA* gene and found that essential *cis* elements for *bvgP*₁ and *bvgP*₂ promoter activity are tightly linked. An *E. coli* strain containing a *bvg::lacZYA* fusion constructed with the 428-17 deletion fragment was deficient in both *bvg*-dependent and *bvg*-independent expression of β -gal (Fig. 2). The elimination of *bvg*-independent β -gal expression from the 428-17 fusion is likely due to the deletion of σ^{70} consensus sequences from the *bvgP*₂ promoter, but the lack of high-level β -gal expression from the 428-17 fusion with the *bvgAS* operon in *trans* suggests that sequences required for transcriptional activation of the autoregulated *bvgP*₁ promoter must also be missing. The CR430 *bvg::lacZYA* fusion, which expresses both *bvg*-dependent and *bvg*-independent levels of β -gal, contains 89 bp of sequence upstream of the *bvgP*₁ promoter (Fig. 2). The 428-17 deletion contains only 69 bp of sequence upstream of *bvgP*₁. These data demonstrate that *cis*-acting sequences located 69 to 89 bp downstream of the *bvgP*₁ +1 site are required for both *bvgP*₁ and *bvgP*₂ activity since a deletion of these sequences eliminates expression from both promoters.

A 3' deletion analysis of the *bvg* promoters showed that *bvgP*₂ function can be separated from *bvgP*₁ by eliminating the *bvgP*₁ transcriptional initiation site. Fusions missing the *bvgP*₁ +1 site were not activated by *bvgAS* in *trans*; however, these fusions still produced low levels of β -gal (both with and without the *bvgAS* genes in *trans*) because of the intact *bvgP*₂ promoter (Fig. 2). As proposed previously (16), these data demonstrate that the *bvgP*₂ promoter is responsible for low-level *bvg*-independent expression of the operon, while the *bvgP*₁ promoter is required for high-level autoregulated expression of the *bvgAS* genes.

A deletion analysis of the *fhaB* upstream region showed that a minimum of 85 bp of sequence upstream of the transcriptional initiation site is required for *bvgAS* activation of the *fhaB* promoter. Within the -83 to -96 region of the

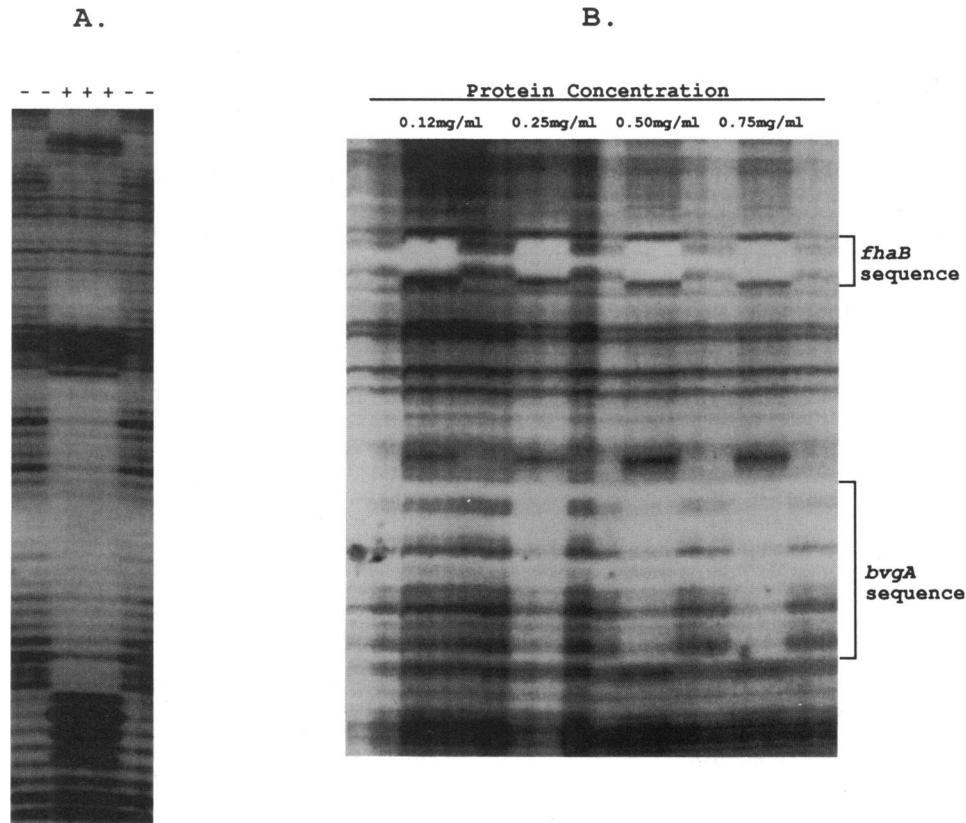


FIG. 7. DNase I protection analysis. (A) Binding reaction mixtures containing a restriction fragment encompassing the *fhaB* promoter region and 40 μ g of protein extract from either *E. coli* overproducing BvgA (+) or *E. coli* containing pT7.7 alone (-) were partially digested with DNase I and fractionated on an 8% polyacrylamide sequencing gel. (B) Binding reaction mixtures containing a restriction fragment encompassing the *fhaB*-*bvgA* intergenic region and various concentrations of protein extract were partially digested with DNase I and fractionated on an 8% polyacrylamide sequencing gel.

fhaB promoter is a 14-bp inverted repeat (Fig. 3). We found that deletions in the *fhaB* upstream region which eliminated this inverted repeat were no longer activated by *bvgAS* in *trans*. The fusion constructed with the 2-7FE deletion had most of the inverted repeat deleted but was still activated by *bvgAS*. We believe that the *EcoRI* linker (5'-CGGAAT TCCG-3') upstream of the 2-7FE deletion replaces sequences similar enough to those of the inverted repeat that this region is still recognized by the *trans*-acting factors resulting in transcriptional activation of the fusion. When an oligonucleotide encoding the inverted repeat was ligated upstream of the defective 2-1FE and 3-12FE deletions, *bvgAS* activation of the *fhaB* promoter was restored. The *fhaB* deletions were also returned to the *B. pertussis* chromosome, and expression of Fha was observed only in strains containing deletions which were activated by *bvgAS* in the *E. coli* system. These data suggest that the 14-bp inverted repeat is a recognition site for a *trans*-acting factor required for transcriptional activation of the *fhaB* promoter.

An analysis of protein-DNA interactions in vitro has revealed that the upstream regulatory sequences required for *bvgAS* activation of the *bvgP*₁ and *fhaB* promoters are bound by a *trans*-acting factor only in extracts containing BvgA. DNase I protection experiments showed that protein extracts enriched for BvgA recognized sequences located 52 to 84 bp upstream of *bvgP*₁ and sequences 67 to 101 bp upstream of the *fhaB* promoter (Fig. 8). These two protected

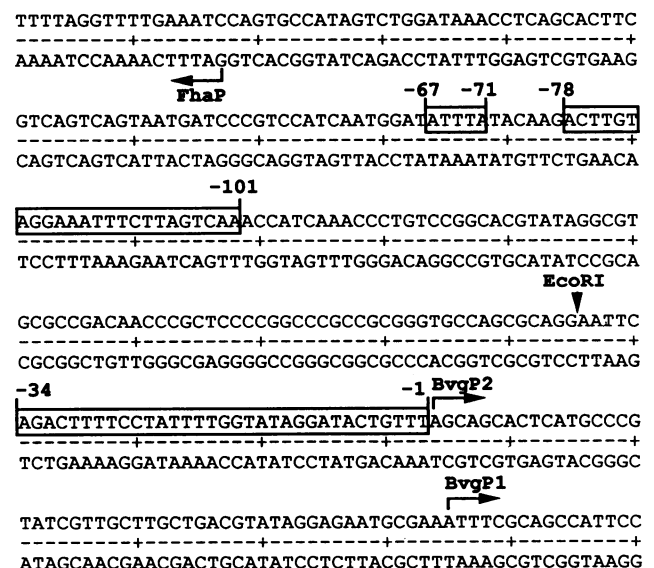


FIG. 8. Nucleotides protected from DNase I digestion in the presence of BvgA. The nucleotide sequence of the *fhaB*-*bvgA* intergenic region is shown. The locations of sequences protected from DNase I digestion by extracts containing BvgA are indicated with boxes.

regions both contain the sequence TTTCTA. This sequence is found in one half of the 14-bp inverted repeat upstream of *phaB*, with the other half containing TTTCTTA. The sequences TTTCTA and TTTGGTA are both found within the protected region upstream of *bvgA*. Unlike the *phaB* binding site, these two heptameric sequences are found as direct repeats within the *bvgA* upstream region. It appeared in vitro that the *phaB* binding site has a higher affinity for the transcription factor than the *bvg* site. This may be due to the opposite orientation of the binding sites upstream of these two genes, with the inverted repeat being a higher-affinity site than the direct repeat. On the basis of the deletion analysis and DNase I protection studies, we conclude that these repeats serve as binding sites for a transcription factor in vivo, resulting in activation of the *phaB* and *bvgP*₁ promoters. This factor is most likely the BvgA protein, since BvgA is required for transcriptional activation of these promoters in vivo (15, 16) and for DNase I protection of the *cis*-acting regulatory regions in vitro.

We have previously reported that *bvgP*₁ and *bvgP*₂ activity appears to be mutually exclusive (16). According to the data presented in this report, binding of a *trans*-acting factor within the -10 to -35 region of the *bvgP*₂ promoter is required for activation of the *bvgP*₁ promoter. We propose that BvgA binding to this region in vivo activates transcription from the *bvgP*₁ promoter; however, occupation of these sequences by BvgA also represses transcriptional initiation from *bvgP*₂ by making essential *cis* elements of the *bvgP*₂ promoter inaccessible to RNA polymerase.

The mechanism by which the *bvgAS* genes regulate the expression of other virulence determinants in *B. pertussis* is still unclear. The BvgA extracts used to examine protein-DNA interactions within the *bvg-phaB* intergenic region did not specifically retard the mobility of a DNA fragment containing the *ptx* promoter region when run on a polyacrylamide gel and did not protect any region of the *ptx* fragment from digestion in the presence of DNase I (data not shown). In addition, there do not appear to be sequences homologous to either the *phaB* inverted repeat or the *bvgA* direct repeat upstream of the *ptx* promoter (5) or upstream of the *cyaA* promoter (8). It is possible that transcription of the *ptx* and *cyaA* genes requires factors in addition to the *bvgAS* products; however, further genetic and molecular studies are necessary to elucidate the mechanism of transcriptional activation of these genes.

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