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Characterization of the *bvgR* Locus of *Bordetella pertussis*

TOD J. MERKEL,[1]* CASSIA BARROS,[1] AND SCOTT STIBITZ[2]

National Institute of Dental Research, National Institutes of Health,[1] and Center for Biologics Evaluation and Research, U.S. Food and Drug Administration,[2] Bethesda, Maryland 20892

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***Bordetella pertussis*, the causative agent of whooping cough, produces a wide array of factors that are associated with its ability to cause disease. The expression and regulation of these virulence factors is dependent upon the *bvg* locus (originally designated the *vir* locus), which encodes two proteins: BvgA, a 23-kDa cytoplasmic protein, and BvgS, a 135-kDa transmembrane protein. It is proposed that BvgS responds to environmental signals and interacts with BvgA, a transcriptional regulator which upon modification by BvgS binds to specific promoters and activates transcription. An additional class of genes is repressed by the *bvg* locus. Expression of this class, the *bvg*-repressed genes (vrgs [for *vir*-repressed genes]), is reduced under conditions in which expression of the aforementioned *bvg*-activated virulence factors is maximal; this repression is dependent upon the presence of an intact *bvgAS* locus. We have previously identified a locus required for regulation of all of the known *bvg*-repressed genes in *B. pertussis*. This locus, designated *bvgR*, maps to a location immediately downstream of *bvgAS*. We have undertaken deletion and complementation studies, as well as sequence analysis, in order to identify the *bvgR* open reading frame and identify the *cis*-acting sequences required for regulated expression of *bvgR*. Studies utilizing transcriptional fusions of *bvgR* to the gene encoding alkaline phosphatase have demonstrated that *bvgR* is activated at the level of transcription and that this activation is dependent upon an intact *bvgAS* locus.**

Whooping cough is an acute respiratory disease caused by the small gram-negative bacterium *Bordetella pertussis. B. pertussis* expresses several factors that contribute to its ability to cause disease (15, 16, 33, 43, 45). Several of these factors, including filamentous hemagglutinin, pertactin, fimbriae, and the recently described tracheal colonization factor, contribute to the interaction between the bacterium and host cells. Other virulence factors are exotoxins which impair the function of immune cells and/or are capable of causing damage to host tissues. These include pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, and tracheal cytotoxin. Expression of these virulence factors, with the exception of tracheal cytotoxin, is activated at the level of transcription by a single locus, referred to as the *bvg* locus (originally designated the *vir* locus) (3, 38, 39, 42, 44). The *bvg* locus encodes a two-component regulatory system consisting of a sensor protein, BvgS, and a transcriptional activator, BvgA. Although the relevant signals for regulation of the *bvg* locus in vivo are unknown, activity of the *bvg* locus is repressed when cells are grown in the presence of $MgSO_4$ or nicotinic acid or when they are grown at a reduced temperature in vitro (26). This *bvg*-mediated change in the patterns of transcription in response to environmental signals is referred to as phenotypic modulation. Under nonmodulating conditions, autophosphorylation of BvgS on a conserved histidine residue is followed by two intramolecular phosphotransfer reactions and the transfer of the phosphate moiety to a conserved aspartate residue on BvgA (41). Upon phosphorylation by BvgS, BvgA binds to *cis*-acting sequences in the promoter regions of the *bvg*-activated genes and activates transcription (7, 8, 22).

In addition to the virulence factors which are activated by the *bvg* locus, a second class of genes, which is repressed by the *bvg* locus, has been described (24). The function(s) of the *bvg*-repressed genes is unknown. The fact that these genes are repressed by the locus responsible for regulation of known virulence genes suggests that they play a role in the pathogenesis of the bacterium, perhaps by contributing to late stages in the infectious cycle. Alternatively, their inappropriate expression may interfere with the bacterium's ability to cause disease. Five *bvg*-repressed genes have been identified to date (*vrg6*, *vrg18*, *vrg24*, *vrg53*, and *vrg73*), and the DNA sequences of the 5′ ends of these genes have been determined (4, 5). Examination of the upstream regions of these genes revealed the presence of a conserved sequence element in four of the five genes (*vrg6*, *vrg18*, *vrg24*, and *vrg53*). The exception, *vrg73*, does not appear to contain this element. A 6-bp linker inserted into the conserved sequence element, as well as a single base pair change within the element in one of these genes, *vrg6*, eliminated responsiveness to modulation and resulted in constitutive expression (4, 5). A construct in which the sequences upstream of the initiating codon in *vrg6* were replaced with a constitutive *B. pertussis* promoter was still regulated normally, demonstrating that the *cis*-acting sequences required for *bvg*-dependent repression of this locus are located downstream of the translation start site (5). The conserved sequence element in *vrg6* was shown by Southwestern analysis to be bound by a 34-kDa protein which is present in nonmodulated cells but absent in modulated cells (5). These results, taken together, have led to a proposed model of *bvg* repression in which the *bvg*-repressed genes are regulated by a repressor protein, the expression or activity of which is activated by the *bvg* locus. A locus required for expression of repressor activity has been identified and shown to be located immediately downstream of the *bvgS* gene (30). This locus has been designated *bvgR*.

In this report, we describe the identification and characterization of the BvgR open reading frame. The presence of several errors in the published sequence of the region downstream of *bvgS* resulted in a failure to recognize previously the presence of this large open reading frame in the *bvg* locus (3). Analysis of the corrected sequence, presented herein, and the

* Corresponding author. Mailing address: OIIB/NIDR/NIH, Building 30, Rm. 303, 30 Convent Dr. MSC 4350, Bethesda, MD 20892-4350. Phone: (301) 496-6060. Fax: (301) 402-0396. E-mail: merkel@yoda.nidr.nih.gov.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Reference or source
E. coli K-12 strains		
DH5α	High-efficiency transformation	BRL[a]
SM10	Tra functions of IncP plasmids integrated into chromosome	35
S17	Tra functions of IncP plasmids integrated into chromosome	35
B. pertussis strains		
Tohama I	Patient isolate	23
BP907	Tohama I; Nal^r Str^r Δ*bvgA*	40
BP947	Tohama I; Nal^r Str^r *fhaB-lacZ*	37
TM1081	Tohama I; Nal^r Str^r *fhaB-lacZ vrg6-phoA*	30
TM1126	TM1081-*bvgR* (in-frame insertional mutant)	30
TM1311	Δ*bvgA orf2-phoA*	This study
TM1312	Δ*bvgA orf1-phoA* (= *bvgR-phoA*)	This study
TM1315	*fhaB-lacZ orf2-phoA*	This study
TM1316	*fhaB-lacZ orf1-phoA* (= *bvgR-phoA*)	This study
Plasmids		
pBBRKAN	Replication in *Bordetella*; mobilizable by IncP Tra functions	2
pTM193	pBBRKAN with multiple cloning site inserted	This study
pSS2000	Ap^r Gm^r *rpsL oriT cos*	30

[a] BRL, Bethesda Research Laboratories.

results of complementation analyses demonstrate that the *bvgR* gene is located immediately downstream of *bvgS* and that it is transcribed convergently to *bvgAS*. Activation of expression of the *bvgR* gene by BvgA was demonstrated to be at the level of transcription.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and media. The bacterial strains and plasmids used in this study are presented in Table 1. *Escherichia coli* strains were grown on L agar or in L broth supplemented with antibiotics when appropriate (31). *B. pertussis* strains were grown on Bordet-Gengou agar (Difco) containing 1% proteose peptone (Difco) and 15% defibrinated sheep blood. Concentrations of antibiotics, unless stated otherwise, were as follows: gentamicin sulfate, 10 μg/ml; kanamycin sulfate, 10 μg/ml; nalidixic acid, 50 μg/ml; rifampin, 50 μg/ml; streptomycin sulfate, 100 μg/ml. Plasmids were transformed into *E. coli* DH5α (Bethesda Research Laboratories, Bethesda, Md.).

Strain and plasmid construction. pTM193 derivatives bearing *B. pertussis* chromosomal fragments derived from the region downstream of the *bvgA* and *bvgS* genes were constructed as follows. Oligonucleotides MCS-1 (5′-CGCCG CGGATCCATATGAGCTCTAGATCTAGTACTAGTCGACCATGGTACC AATTGAATTCGCTAGCATGC-3′) and MCS-2 (5′-CGGCATGCTAGCGAA TTCAATTGGTACCATGGTCGACTAGTACTAGATCTAGAGCTCATAT GGATCCGCGG-3′) were annealed to generate a linker bearing the restriction enzyme sites *Nar*I, *Sac*II, *Bam*HI, *Nde*I, *Sac*I, *Xba*I, *Bgl*II, *Sca*I, *Spe*I, *Sal*I, *Hinc*II, *Nco*I, *Kpn*I, *Mun*I, *Eco*RI, *Nhe*I, *Sph*I, and *Nar*I. This linker was cloned into the *Nar*I site of plasmid pBBRKAN to generate plasmid pTM193. The 9.0-kb *Bgl*II-*Bam*HI, 6.0-kb *Sal*I, 4.0-kb *Xho*I, 3.5-kb *Bgl*II-*Xho*I, or 1.3-kb *Sal*I-*Xho*I restriction fragment derived from the region downstream of the *bvg* locus was cloned into the multiple cloning site of plasmid pTM193 to generate plasmids pTM193: BgB, pTM193:S, pTM193:X, pTM193:BgX, and pTM193:SX, respectively. Restriction fragments bearing *Bgl*II-proximal deletions of the 3.5-kb *Bgl*II-*Xho*I fragment were generated by PCR with primers X1 (5′-CTGACTCGAGAATG GCCTGCCGGTCCGCCACATCGAGCAG-3′), and either Bg2 (5′-GTCAAG ATCTGGCTACGAATTGGCGCGCCGCATACGCGCC-3′), Bg3 (5′-GTCA AGATCTGGCCGAAGGTCTCGGACATGGCGCACAGGC-3′), Bg4 (5′-GT CAAGATCTTGGTACGTACGCTTGCGGCGCAGTCCGCCG-3′), Bg5 (5′-GTCAAGATCTCCTTACGGATAATTGGGCGGCATCTCGCGG-3′), or Bg6 (5′-GTCAAGATCTCATGGGCGGGGCCACATGGTCGCCCAGCAG-3′). Restriction fragments bearing *Xho*I-proximal deletions of the 3.5-kb *Bgl*II-*Xho*I fragment were generated by PCR with primers Bg1 (5′-GACTAGATCT CGAGAATGGCCTGCCGGTCCGCCACATC-3′) and either X2 (5′-GTCAC TCGAGCGCTGGTGGTTTCGATGCGGCCGCTGAAGG-3′), X3 (5′-GTCA CTCGAGGCGACCCCGCATTGATCGGTATCGCCGACC-3′), X4 (5′-GTC ACTCGAGCGGCCACATGCTGGTCACCGATCTGCTCAAC-3′), or X5 (5′-GTCACTCGAGCATGATCCACTGGACCAACAGCGCTCGCAG-3′). The products of these PCRs were double-stranded DNA fragments which contained terminal *Bgl*II and *Xho*I restriction enzyme sites. After digestion with *Bgl*II and *Xho*I, these fragments were inserted into plasmid pTM193, which had been digested with *Bgl*II and *Sal*I. Although the entire sequences of the PCR products were not determined, at least four independent PCRs were performed to generate those products and the PCR products from each reaction gave the same result, indicating that differences in activity between the truncated DNA fragments generated by PCR and the full-length wild-type fragment are consequences of the deletion of required sequences at the termini rather than results of misincorporation of nucleotides during the extension reactions.

Strains with transcriptional fusions of the *E. coli* gene for alkaline phosphatase (*phoA*) to open reading frames downstream of *bvgAS* were constructed as follows. Oligonucleotide XApX (5′-TCGAGGGCCC-3′) was self-annealed to generate a linker which permits the insertion of an *Apa*I site into an *Xho*I site. This linker was inserted into the *Xho*I site in plasmid pBS KS$^+$, generating plasmid pBS:XApX. The *Bgl*II-*Bam*HI fragment from the *B. pertussis bvg* locus was inserted into plasmid pSS2000 to generate plasmid pTM030. Plasmid pTM030 was digested with *Bgl*II, *Xho*I, and mung bean nuclease and religated to generate plasmid pTM057. A double-stranded DNA fragment bearing the *E. coli phoA* gene was synthesized by PCR with primers phoA1 (5′-GCGGATCCGTATTT GTACATGGAGAAAATAAAATGAAACAAAGCAC-3′) and phoA2 (5′-GC GGATCCTTATTTCAGCCCCAGAGCGGCTTTCATGG-3′). This PCR resulted in the production of an altered form of the *phoA* gene in which the native GTG initiating codon was replaced by an ATG initiating codon. Oligonucleotides phoA1 and phoA2 both contain a *Bam*HI restriction enzyme site, allowing insertion of the *phoA*-bearing PCR product into the *Bam*HI site of plasmid pBS:XApX. This fragment can be inserted in either of two possible orientations, generating plasmids pTM073 and pTM074. The *phoA* gene was transferred from plasmid pTM073 as an *Apa*I fragment into the *Apa*I site of plasmid pTM057. Clones containing the *phoA* fragment were identified and designated either pTM092 or pTM093, depending on the orientation of the inserted DNA fragment. *E. coli* SM10 bearing pTM092 or pTM093 was mated with *B. pertussis* BP907 and BP947, as described below, and exconjugates in which the plasmid sequences had integrated into the chromosome were isolated by selection with gentamicin. Isolates in which plasmid sequences were lost from the chromosome but in which the *orf1-phoA* or *orf2-phoA* transcriptional fusion was retained were isolated by selection for streptomycin resistance on Bordet-Gengou agar plates and by screening for alkaline phosphatase activity in the absence of modulators. PhoA$^+$ Str^r exconjugates of BP907 bearing the *orf1-phoA* and *orf2-phoA* fusions, as well as PhoA$^+$ Str^r exconjugates of BP947 bearing the *orf1-phoA* and *orf2-phoA* fusions, were isolated and have been designated TM1312, TM1311, TM1316, and TM1315, respectively (Fig. 1).

Bacterial conjugations. Matings between *E. coli* and *B. pertussis* strains were performed by swabbing bacteria from fresh plate cultures of each strain onto a Bordet-Gengou agar plate supplemented with 10 mM $MgCl_2$ but without any antibiotics. After 3 h of incubation at 37°C, bacteria were swabbed onto Bordet-Gengou agar containing the appropriate antibiotics for selection of exconjugates, and incubation was continued at 37°C. Prior to mating, *B. pertussis* strains were grown for 3 days and *E. coli* strains were grown overnight at 37°C.

Quantitative alkaline phosphatase and β-galactosidase assays. Bacteria to be assayed were recovered by sterile swabs into 3.5 ml of Tris-HCl, pH 8.0, and the absorbance at 600 nm was measured. For measurement of β-galactosidase activity, 0.05 ml of cell suspension was added to 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

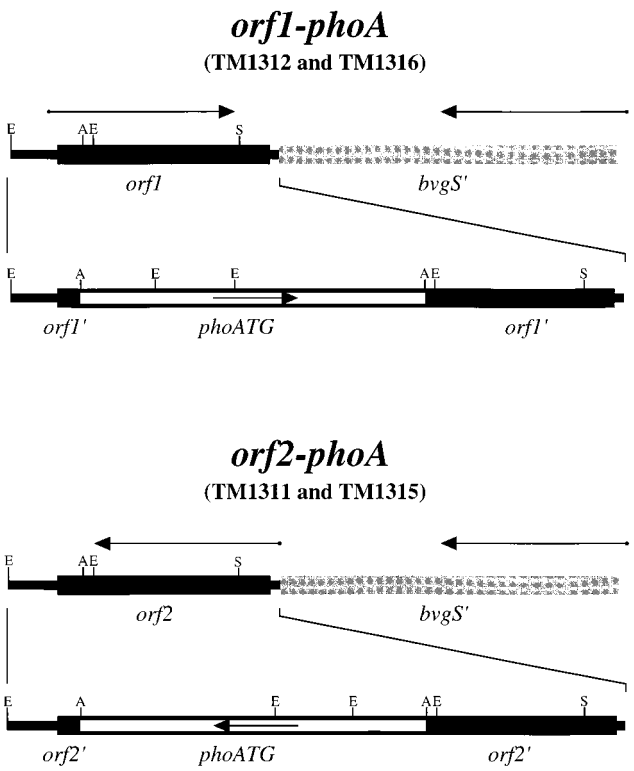


FIG. 1. *B. pertussis* TM1311, TM1312, TM1315, and TM1316. The *orf1-phoA* transcriptional fusion present in strains TM1312 and TM1316 and the *orf2-phoA* transcriptional fusion present in strains TM1311 and TM1315 are shown. The putative BvgR (ORF1 and ORF2) coding sequences are represented by black boxes. The 3′ terminus of the *bvgS* coding sequence is represented by gray boxes. The alkaline phosphatase coding sequence is represented by open boxes. The putative directions of transcription of the native genes and transcriptional fusions are indicated by arrows. Restriction enzyme recognition sequences are indicated as follows: E, *Eco*RI; B, *Bam*HI; S, *Sca*I; X, *Xho*I; N, *Nco*I; A, *Apa*I.

by Miller (31). For measurement of alkaline phosphatase, 0.5 ml of cell suspension was added to 0.5 ml of Tris-HCl (pH 8.0), the cells were permeabilized as above, and the assay was completed as described by Brickman and Beckwith (9). Units in both cases were defined by the following equation: 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 added to the assay mixture, in milliliters.

Sequence analysis. DNA fragments cloned in plasmid pBS KS$^+$ were sequenced by the dideoxy sequencing method with the Sequenase 7-deaza dGTP DNA sequencing kit (United States Biochemical, Cleveland, Ohio). Computer analysis of DNA and protein sequences was performed with the GCG sequence analysis software package (Genetics Computer Group Inc., Madison, Wis.) and with the MacVector sequence analysis programs (International Biotechnologies Inc., New Haven, Conn.).

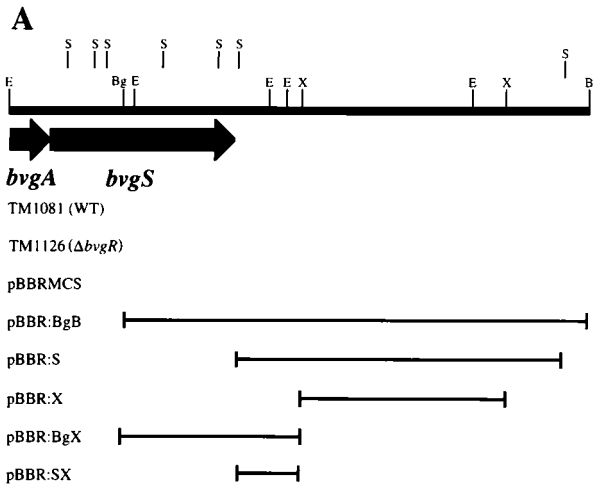
RESULTS

Complementation studies of the *bvgR* locus. We identified previously a locus in *B. pertussis* that is required for regulation of the *bvg*-repressed genes (30). This locus, designated *bvgR*, was mapped to a position immediately downstream of *bvgAS*. In order to functionally define the limits of the *bvgR* locus, we utilized the fact that *bvgR*-dependent regulation of a *vrg6-phoA* fusion can be restored in the *bvgR*-deficient strain TM1126 when the intact *bvgR* locus is provided in *trans* (30). A collection of restriction fragments containing different segments of DNA from the region immediately downstream of *bvgAS* were cloned into plasmid pTM193 (Fig. 2). These constructs were transferred by conjugation into strain TM1126, which contains a 12-bp in-frame insertion within the *bvgR* locus, a transcriptional fusion of the *E. coli lacZ* gene to the *bvg*-activated gene *fhaB*, and a transcriptional fusion of the *E. coli phoA* gene to the *bvg*-repressed gene *vrg6*. Quantitative enzyme assays for alkaline phosphatase and β-galactosidase activities after growth in either the presence or absence of 50 mM $MgSO_4$ were performed in order to evaluate the ability of the exconjugates to complement the *bvgR* deficiency in strain TM1126. In all of the strains assayed, expression of the *fha-lacZ* fusion was induced between 13- and 38-fold, indicating that the functions of BvgA and BvgS were not altered in these constructs relative to the wild-type strain. As seen previously, the activity of the *vrg6-phoA* fusion in the wild-type strain shows an approximately 10-fold reduction in activity when the cells are grown in the absence of $MgSO_4$, while the activity of the *vrg6-phoA* fusion in strain TM1126 is reduced less than twofold under the same conditions (Fig. 2A). Strain TM1126 bearing plasmid pTM193 alone or pTM193:S, pTM193:X, or pTM193:SX failed to restore wild-type regulation of the *vrg6-phoA* fusion in strain TM1126 (Fig. 2A). Introduction of plasmid pTM193:BgB or pTM193:BgX restored wild-type regulation of the reporter fusion in strain TM1126. These results indicated that the sequences required for regulated expression of BvgR were located between the *Bgl*II site in *bvgS* and the *Xho*I site 1.5 kb downstream of *bvgS*.

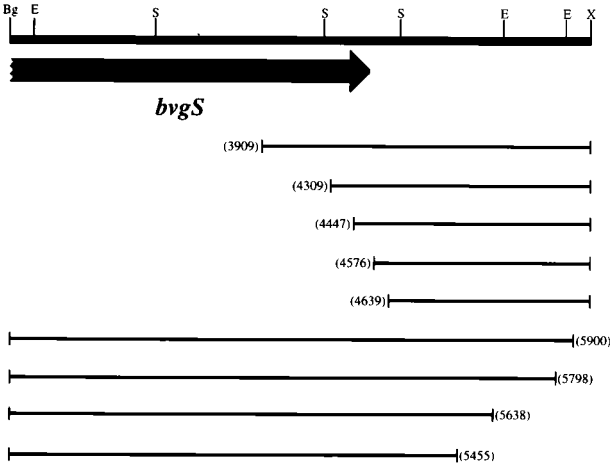
In order to more precisely define the limits of the *bvgR* locus, a nested set of deletions of the *Bgl*II-*Xho*I fragment were generated by PCR and DNA fragments containing these deletions were inserted into plasmid pTM193. These constructs were transferred by conjugation into strain TM1126, and their ability to complement the *bvgR* deficiency in strain TM1126 was determined. Deletion of the sequences between the *Bgl*II site and position 4447 in the published sequence of *bvgAS* did not effect the ability of the plasmid to complement the *bvgR* deficiency in strain TM1126 (Fig. 2B). However, a deletion extending to position 4576 eliminated the ability of the plasmid to provide *bvgR* function in *trans*. Deletion of the sequences between the *Xho*I site and position 5638 in the published sequence of *bvgAS* did not affect the ability of the plasmid to complement the *bvgR* deficiency in strain TM1126; however, a deletion of sequences up to position 5455 eliminated the ability of the plasmid to provide *bvgR* function in *trans*.

None of the *bvgR*-bearing pTM193 derivatives conferred constitutive repression of the *vrg6-phoA* fusion in strain TM1126. This result indicates that there is not sufficient transcriptional activity through the multiple cloning site of the plasmid to confer expression of enough BvgR to repress transcription at the *vrg6* locus. This conclusion is supported by the fact that insertion of all DNA fragments yielded the same result regardless of the orientation of the insert within the plasmid vector (data not shown). These results indicate that expression of the *bvgR* locus carried on the pTM193 derivatives is being driven by the native *bvgR* promoter rather than by promoters provided by the vector. These results indicate that the *cis*-acting sequences required for the regulated expression of *bvgR* are located between positions 4447 and 5638 of the published *bvgAS* sequence.

Sequencing of the *bvgR* locus. A previous examination of the published sequence of the region immediately downstream of the *bvgAS* genes revealed three open reading frames which were disrupted by all of the mutations known to eliminate repressor activity in *B. pertussis* (30). Although the work of Beattie et al. (5) suggested that the *bvg*-dependent repressor was a protein of approximately 34 kDa, none of the open reading frames downstream of *bvgS* were predicted to encode a protein of that size. In addition, the boundaries of the *bvgR* locus, as determined by deletion and complementation analy-



	vrg6-phoA Activity		
	+ $MgSO_4$	- $MgSO_4$	Induction
TM1081 (WT)	1.00±0.00	0.11±0.03	9.1
TM1126 (Δ*bvgR*)	1.03±0.18	0.64±0.19	1.6
pBBRMCS	1.00±0.00	0.38±0.08	2.6
pBBR:BgB	0.90±0.08	0.08±0.01	11.3
pBBR:S	1.14±0.27	0.34±0.06	3.4
pBBR:X	1.16±0.19	0.35±0.08	3.3
pBBR:BgX	1.20±0.33	0.07±0.02	17
pBBR:SX	1.12±0.12	0.27±0.07	4.1



	vrg6-phoA Activity		
	+ $MgSO_4$	- $MgSO_4$	Induction
(3909)	1.13±0.26	0.10±0.05	11.3
(4309)	1.35±0.20	0.11±0.07	12.3
(4447)	1.19±0.23	0.10±0.03	11.9
(4576)	1.17±0.40	0.33±0.07	3.5
(4639)	1.37±0.16	0.43±0.11	3.2
(5900)	1.17±0.23	0.09±0.05	13.0
(5798)	1.08±0.29	0.09±0.05	12.0
(5638)	1.00±0.20	0.09±0.03	11.1
(5455)	1.01±0.35	0.39±0.08	2.6

FIG. 2. Deletion and complementation analysis of the *bvgR* locus. The transcriptional activities of the *vrg6-phoA* transcriptional fusions in wild-type (WT) strain TM1081, strain TM1126, and strain TM1126 bearing the indicated pTM193 derivatives are shown. The *bvg* sequences inserted into the multiple cloning site of each pTM193 derivative are indicated by solid lines. (A) Effects of pTM193 alone and pTM193 bearing large restriction fragments derived from the *bvg* locus on expression of the *vrg6* locus. (B) Effects of pTM193 bearing truncated versions of the *bvg Bgl*II-*Xho*I restriction fragment on expression of the *vrg6* locus. The endpoints of deletions are indicated in parentheses. Nucleotide numbers correspond to those in the published sequence of *bvgAS* (3). Alkaline phosphatase activities are reported relative to strain TM1081 grown in the presence of $MgSO_4$ (36.1 units). All reported values are averages of at least six independent assays. Restriction enzyme recognition sequences are indicated as follows: E, *Eco*RI; B, *Bam*HI; S, *Sal*I; X, *Xho*I; Bg, *Bgl*II.

sis, were mapped to positions nearly 200 bp downstream from the ends of any of the three predicted open reading frames. In order to examine the possibility that the region defined by the deletion and complementation analysis contained an open reading frame large enough to encode a protein of approximately 34 kDa, we determined the DNA sequence between the *Sal*I site at position 4272 in the published sequence and the *Xho*I site at position 5991. We identified 8 nucleotide residues between the 3′ end of the *bvgS* gene and the *Xho*I site downstream of *bvgS* which were in error in the published sequence (see Fig. 3). Sequencing was performed on both strands of DNA for the entire region specified above. Nucleotides which were not in agreement with the published sequence were sequenced at least two times on each strand. Thus, a total of at least four independent sequencing reactions were performed, and the readings for each corrected nucleotide were in agreement in all of the reactions. Examination of the corrected sequence revealed the presence of two open reading frames predicted to encode proteins of approximately 30 kDa (Fig. 1). Open reading frame 1 (ORF1) extends from position 547 to position 1422 in the corrected sequence (Fig. 3) and is predicted to encode a 32-kDa protein. Open reading frame 2 (ORF2) extends from position 1347 to position 520 in the corrected sequence and is predicted to encode a 29-kDa protein. Transcription of ORF1 and *bvgAS* is predicted to occur convergently. Sequences which are good matches to the *E. coli* ribosomal binding site and the *E. coli* −10 promoter element are found upstream of ORF1 at positions 474 to 479 and positions 510 to 516, respectively. ORF2 and *bvgAS* are predicted to be transcribed in the same direction. Sequences

FIG. 3. Nucleotide sequence of *bvgR*. The nucleotide sequence of *bvgR* and the 3′ end of *bvgS* and the corresponding amino acids are shown. A potential promoter element (−10) and ribosomal binding site (RBS) are indicated by boxed-in sequences. Corrections of the published sequence are indicated as follows: nucleotides that are deleted in the published sequence are indicated by asterisks, and at those positions where a nucleotide was inserted or substituted in the published sequence, the nucleotide present in the published sequence is shown above the corrected sequence.

which are good matches to the *E. coli* ribosomal binding site and −10 promoter element are also found upstream of ORF2, at positions 1379 to 1373 and 1417 to 1412, respectively.

Activation of *bvgR*. In an effort to determine which of the two open reading frames encodes the *bvg*-dependent repressor, an *Apa*I fragment containing a promoterless *E. coli phoA* gene was inserted in both orientations into the *Apa*I site located at position 5853 of the published *bvgAS* sequence (Fig. 1). This allowed construction of transcriptional fusions of the *phoA* gene to ORF1 and ORF2. These constructs were transferred by conjugation into strain BP947, which contains a transcriptional fusion of the *E. coli lacZ* gene to *fha*, and strain BP907, which contains an in-frame deletion of the *bvgA* locus. The orientation of the *phoA* gene with respect to the *bvg* locus was determined by restriction enzyme digestion of plasmid DNA prior to introduction into strains BP947 and BP907 and also by PCR analysis of chromosomal DNA derived from strains TM1311, TM1312, TM1315, and TM1316 (data not shown). The activities of the *bvg-phoA* and *fha-lacZ* fusions in each strain were determined by analysis of the exconjugates with quantitative enzyme assays for alkaline phosphatase and β-galactosidase after growth at 37°C. The results of this analysis are shown in Fig. 4. The *fha-lacZ* fusions in strains TM1315 and TM1316 demonstrated high levels of expression when the cells were grown in the absence of modulators. This activity was reduced approximately 25-fold upon growth of the cells in the presence of 50 mM $MgSO_4$ or 20 mM nicotinic acid and approximately 5-fold upon growth at 25°C (data not shown). Low-level constitutive alkaline phosphatase activity was detected under all of the conditions examined for the transcriptional fusion of the *phoA* gene to ORF2 (*orf2-phoA*) (strains TM1311 and TM1315). The transcriptional fusion of the *phoA* gene to ORF1 demonstrated high levels of expression in the

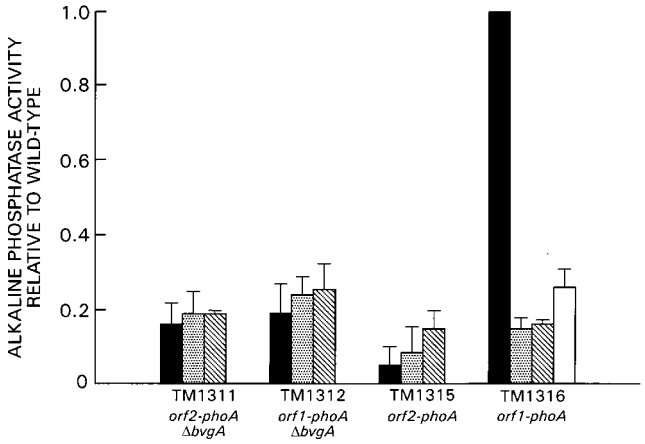


FIG. 4. Transcriptional activation of *bvgR*. Alkaline phosphatase activities of transcriptional fusions of the *E. coli* gene encoding alkaline phosphatase to ORF1 and ORF2 are shown after growth in the presence and absence of modulators. Black bars, no modulator; dotted bars, 50 mM $MgSO_4$; diagonally striped bars, 20 mM nicotinic acid; white bar, growth at 25°C. Activities are reported relative to strain TM1316 grown in the absence of $MgSO_4$ (153.26 units). All reported values are averages of at least four independent assays.

wild-type background (strain TM1316) in the absence of modulators. This expression was reduced approximately 6.5-fold when the bacteria were grown in the presence of 50 mM $MgSO_4$. The *orf1-phoATG* fusion demonstrated low-level constitutive activity in strain TM1312, which bears an in-frame deletion of the *bvgA* locus. We investigated the regulation of expression of the *orf1-phoA* fusion further by determining the level of alkaline phosphatase activity expressed in strain TM1316 after growth in the presence of 20 mM nicotinic acid or after growth at 25°C, two conditions that have also been shown to modulate the activity of *bvg*-activated genes. The high level of alkaline phosphatase activity seen in strain TM1316 upon growth in the absence of modulators was reduced approximately 6.5-fold when the cells were grown in the presence of 20 mM nicotinic acid and approximately 4-fold when the cells were grown at 25°C.

These results demonstrate that expression of ORF1 is activated at the level of transcription and that this expression is dependent upon the presence of an intact *bvgA* locus. Moreover, this activity is reduced upon growth under conditions known to down-modulate the expression of *bvgA*-activated genes. Our observations indicate that ORF1 encodes a *bvgA*-activated protein. This, along with the result that only a low constitutive level of transcriptional activity was detected through ORF2 under conditions known to promote expression of *bvg*-activated genes, has led us to assign a *bvgR* coding function to ORF1.

Analysis of the *bvgR* sequence. The predicted protein sequence of BvgR was determined and compared to sequences in the Swiss Protein, Protein Information Resource, and GenBank translated sequence databases. A search of the databases with the BLAST program showed that BvgR has significant homology to 18 proteins or predicted proteins found in a variety of eubacterial organisms, although the functions of these proteins are unknown (Fig. 5). The *rtn* locus of *E. coli* was identified by a transposon insertion that conferred an increased rate of adaptive mutation (19). The mechanism by which the *rtn* locus confers this phenotype, or if disruption of the *rtn* open reading frame is even responsible for the phenotype, has yet to be determined. The 22-kDa antigen of *Borrelia burgdorferi* has been identified, but its function is unknown (28). The functions of two of these predicted proteins have only been inferred, based upon their sequence homologies to characterized proteins in other organisms. These are the NifL homolog of *Synechocystis* sp. and the FixL homolog of *E. coli* (21) (D90789). It should be noted that the homology between these predicted proteins and BvgR does not extend to the characterized examples of these proteins (NifL of *Klebsiella pneumoniae* and FixL of *Rhizobium meliloti*). The functions of the remaining 14 of these predicted proteins are unknown, although their open reading frames are within or are tightly linked to operons with defined functions. Sequence alignment by the LFASTA program initially showed that a region of 55 amino acid residues was highly conserved among 16 of the 19 proteins (Fig. 5A). Domain I of BvgR is most closely related to domain I of YAHA_ECOLI, with 31% identity and 47% similarity over the 55-amino-acid region. Alignment of the 15 protein sequences identified as having homology to BvgR within domain I revealed the presence of a second region of high sequence homology (Fig. 5B). Although this family of proteins was originally defined by homology to domain I of BvgR, BvgR itself shows very poor conservation of domain II. Within this family, we have observed that in addition to the high degree of sequence conservation within the two domains, the spacing between the two domains and the distance between domain II and the carboxy-terminal ends of the predicted proteins are also conserved. (Fig. 5C). It should be noted that we have included in this analysis only those proteins and predicted proteins for which some information is available. An additional 12 open reading frames in the *Synechocystis* genome, 9 open reading frames in the *E. coli* genome, and 2 open reading frames in the *Mycobacterium tuberculosis* genome that contain these two conserved sequence elements were identified, bringing the total number of potential members of this protein family to 42.

Examination of the predicted BvgR sequence with the Motifs program did not reveal the presence of any of the sequence motifs defined in the PROSITE dictionary of protein sites and patterns. No sequences with significant homologies were identified when the predicted protein sequence encoded by ORF2 was compared to sequences in the Swiss Protein, Protein Information Resource, and GenBank translated sequence databases with the BLAST program.

DISCUSSION

The locus required for regulation of the *bvg*-repressed genes in *B. pertussis* is located immediately downstream of *bvgAS* (30). This locus was designated *bvgR*, for *Bordetella* virulence gene repression. Examination of the published sequence of the *bvgAS* locus revealed the presence of three open reading frames in the region immediately downstream of *bvgAS* that are predicted to be affected by all of the mutations that have been demonstrated to abolish repressor activity (30). The largest of these open reading frames is predicted to encode a protein of approximately 25 kDa. Previous results published by Beattie et al. have suggested that the repressor protein that binds to the conserved sequence element in the *vrg6* gene is a protein of approximately 34 kDa (5). It is possible that rather than repressing the *bvg*-repressed genes directly, the product of the *bvgR* locus may be required for the expression or activity of the repressor protein. If, however, *bvgR* does encode the repressor protein, then either the repressor is smaller than 34 kDa in size or the *bvgR* open reading frame is larger than those open reading frames present in the published sequence. In order to begin to address these possibilities, we sought to functionally define the upstream and downstream limits of the *bvgR* locus. Various plasmid derivatives of plasmid pTM193 bearing sequences derived from the *bvgR* locus were introduced into strain TM1126, which bears an insertional mutation in the *bvgR* locus which abrogates *bvgR* function. The ability of these derivatives to provide *bvgR* function in *trans* was determined. This analysis allowed localization of the *cis*-acting sequences required for regulated expression of *bvgR* to between positions 4447 and 5639 in the published *bvgAS* sequence. It should be noted, however, that although this system provides an effective method for determining the boundaries of the *bvgR* locus, it provides only an indirect measure of the level of *bvgR* expression. It is possible, for example, that promoter elements contributing to *bvgR* expression reside upstream of position 5639, since a requirement for these sequences would not be detected by the assay utilized in this study if less than 100% of *bvgR* expression was sufficient to fully activate expression at the *vrg6* locus. Future studies will focus on the identification and characterization of all *cis*-acting sequences that contribute to *bvgR* expression.

Having determined the functional boundaries of the *bvgR* locus, we next undertook the sequencing of the region between the end of the *bvgS* open reading frame and the *Xho*I site 1.5 kb downstream of this site. This analysis resulted in the identification of 8 nucleotides which were in error in the published sequence (Fig. 3). Examination of the corrected sequence re-

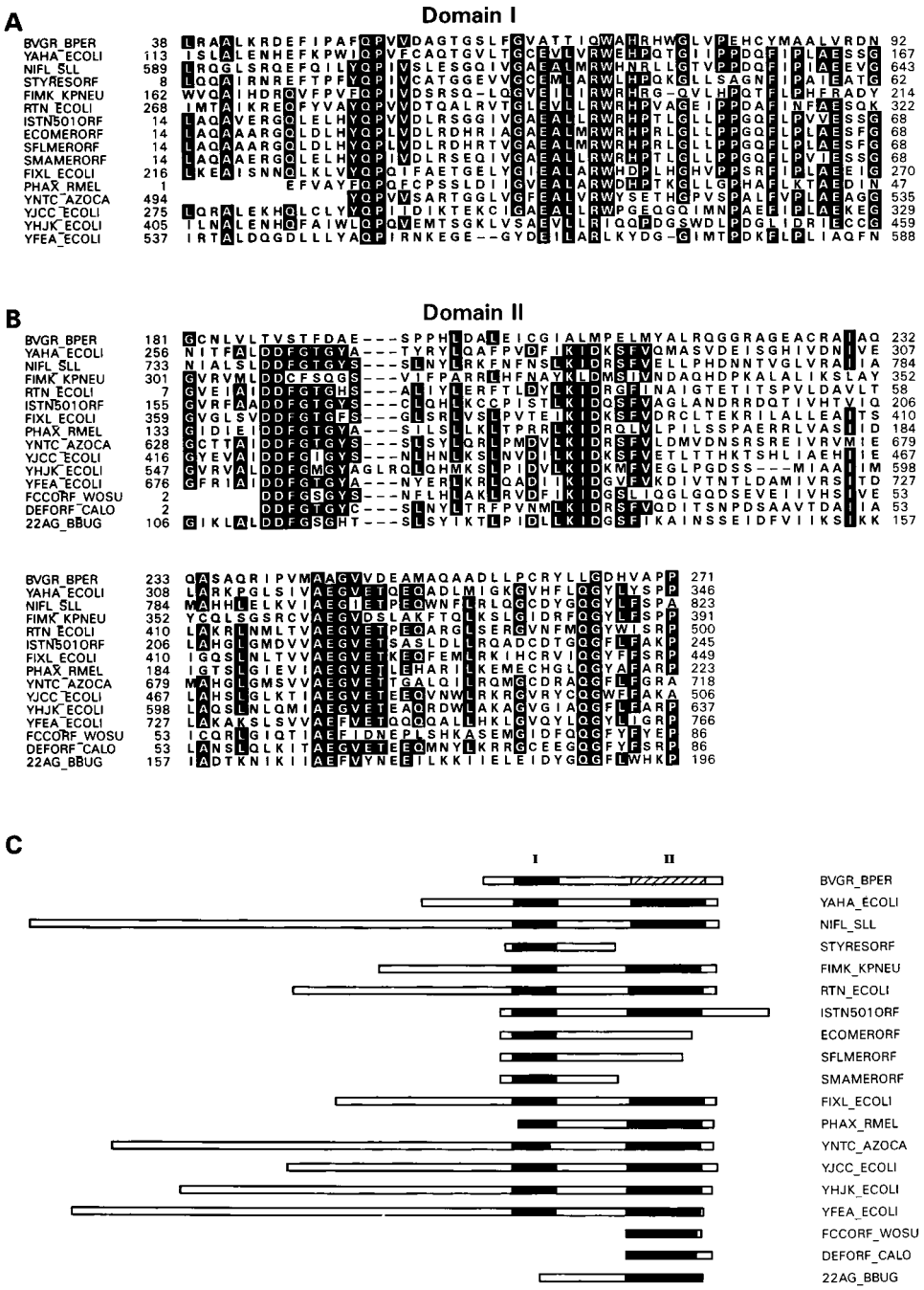


FIG. 5. Alignment of the predicted amino acid sequence of BvgR with a new family of proteins. BVGR_BPER, predicted protein BvgR of *B. pertussis*; YAHA_ECOLI, hypothetical 40.7-kDa protein encoded by the *betT* 3′ region of *E. coli* (27); NIFL_SLL, NifL homolog of *Synechocystis* sp. (21); STYRESORF, open reading frame 4 of the *Salmonella typhimurium* resolvase operon (25); FIMK_KPNEU, FimK protein of *K. pneumoniae* (17); RTN_ECOLI, Rtn protein of *E. coli* (19, 20); ISTN501ORF, unidentified reading frame 2 of the ISTN501-encoded mercuric ion resistance operon of *Pseudomonas aeruginosa* (10–12, 14, 32); ECOMERORF, unidentified reading frame 2 of the *E. coli* mercuric ion resistance operon (R100) (1, 11); SFLMERORF, unidentified reading frame 2 of the *Shigella flexneri* mercuric ion resistance operon (Tn*501*) (11); SMAMERORF, unidentified reading frame 2 of the *Serratia marcesens* mercuric ion resistance operon (pDU1358) (18); FIXL_ECOLI, FixL homolog of *E. coli* (D90789); PHAX_RMEL, unidentified reading frame X of the *pha* operon of *R. meliloti* (X93358); YNTC_AZOCA, hypothetical 80.5-kDa protein encoded by the *ntrC* 5′ region of *Azorhizobium caulinodans* (34); YJCC_ECOLI, hypothetical 60.8-kDa protein encoded by the *ssb-soxS* intergenic region (6); YHJK_ECOLI, hypothetical 73.1-kDa protein encoded by the *dctA-dppF* intergenic region (36); YFEA_ECOLI, hypothetical protein encoded by the *gltX* 5′ region of *E. coli* (13, 46). FCCORF_WOSU, unidentified open reading frame in the *fcc* 5′ region of *Wolinella succinogenes* (Y10581); DEFORF_CALO, unidentified open reading frame in the *def* 3′ region of *Calothrix* sp. (29); 22AG_BBUG, 22-kDa antigen of *B. burgdorferi* (28). Black boxes indicate positions conserved in at least 51% of the aligned sequences.

vealed the presence of two large open reading frames downstream of *bvgS* within the boundaries determined by deletion and complementation analysis to be sufficient to provide *bvgR* activity in *trans* (Fig. 3). One open reading frame (ORF2) was located almost in its entirety within the other open reading frame (ORF1), although the two open reading frames are encoded on opposite strands of the DNA. Both open reading frames would be expected to be disrupted by all of the muta-

tions known to abolish repressor activity in *B. pertussis*, and both open reading frames are large enough to encode the protein identified as the repressor protein in the studies of Beattie et al. (5). The presence of two large open reading frames immediately downstream of *bvgAS*, in opposite orientation to each other, suggests two models for the regulated transcription of *bvgR*. If *bvgR* is encoded by ORF2, it lies immediately downstream of *bvgS* oriented in the same direction as *bvgA* and *bvgS*, which would allow all three proteins to be expressed from a single transcript under the regulation of the *bvgAS* promoter. If, however, *bvgR* is encoded by ORF1, its transcription is presumably driven from its own *bvgA*-activated promoter. Analysis of the transcription in the region downstream of *bvgS* revealed that there is only a low constitutive level of transcription through ORF2. In contrast, there was a high level of transcriptional activity through ORF1 that was reduced upon growth under conditions known to modulate the expression of *bvg*-activated genes; this transcription was dependent upon an intact *bvgA* locus. These results demonstrate that ORF1, but not ORF2, encodes a *bvg*-activated gene and support the conclusion that *bvgR* is encoded by ORF1. Although it remains to be demonstrated that *bvgR* encodes the actual repressor of the *bvg*-repressed genes, the fact that the product of the *bvgR* locus is predicted to be a protein of 32 kDa is consistent with that conclusion. It is interesting that the *bvg*-activated transcription of *bvgA* and *bvgS* does not extend through the *bvgR* locus. This is perhaps a little surprising, given that the end of *bvgR* lies only 43 nucleotides downstream from *bvgS*. Examination of the sequence between *bvgS* and *bvgR* reveals the presence of several large potential stem-loop structures. The presence of one or more of these stem-loop structures may lead to transcriptional termination in this region. Perhaps this is required in order to ensure that overlapping transcription of *bvgAS* and *bvgR* does not interfere with expression of the products of these genes.

The predicted BvgR protein encoded by ORF1 contains a domain that is strongly conserved among a large number of predicted proteins from a variety of eubacterial species (Fig. 5). This family of predicted proteins has been identified based on its homology to domain I of BvgR. Most of the members of this family are characterized by two domains of strong sequence homology which are located at the carboxy-terminal ends of the proteins and which reside approximately 130 residues apart. Several members of this family of proteins have either domain I or domain II but not both. The assignment of BvgR to this family of proteins is based on its high degree of conservation within domain I. In this region, BvgR is most closely related to YAHA_ECOLI, with 30% identity and 46% similarity over the 55 residues. BvgR shows weak but discernible conservation in domain II, suggesting that BvgR either has lost the requirement for the function of Domain II or has adapted this domain for an alternative function. The functional role(s), if any, of domain I and domain II is only speculative at this time, since no known function has been demonstrated for any of the members of this protein family. It is interesting to note that members of this family of proteins are encoded by open reading frames which either lie within or are very closely linked to functional operons. The identification and characterization of BvgR allows, for the first time, the assignment of an activity to one of the members of this family of proteins. It will be interesting to evaluate whether the members of this family are involved in regulation of expression of the operons to which they are so closely linked.

In this study, we have identified the *bvgR* open reading frame. The close correlation between the predicted size of the *bvgR* product and the protein shown by Beattie et al. (5) to bind the putative repressor binding site in the *vrg6* gene suggests that BvgR is the actual repressor protein that binds to the conserved sequence element found within the coding sequences of the *bvg*-repressed genes. Our results demonstrate that the expression of *bvgR* is activated at the level of transcription by the products of the *bvgAS* genes. We propose that this activation is the result of binding of phosphorylated BvgA to the *bvgR* promoter. Ongoing and future work is and will be focused on characterizing in detail the activation of *bvgR* expression and determining the mechanism by which BvgR represses the expression of its target genes.

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