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Identification of a Locus Required for the Regulation of *bvg*-Repressed Genes in *Bordetella pertussis*

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In *Bordetella pertussis*, the coordinate regulation of virulence factor expression is controlled by the products of the *bvgAS* locus. In the presence of modulating signals such as $MgSO_4$, nicotinic acid, or reduced temperature, the expression of *bvg*-activated genes is reduced while the expression of *bvg*-repressed genes is induced. One model for the regulation of *bvg*-repressed genes predicts the existence of a repressor protein encoded by a *bvg*-activated gene. Once activated, the product of this *bvg*-activated gene would bind to and repress transcription from the *bvg*-repressed genes. We isolated five genetically independent transposon insertion mutants of *B. pertussis* that have a phenotype consistent with the knockout of a putative *bvg*-regulated repressor. These mutants constitutively expressed a *vrg6-phoA* transcriptional fusion but demonstrated normal *bvgAS* function. Genomic mapping and DNA sequence analysis of the sites of transposon insertion demonstrated that these mutants define a locus downstream of *bvgAS*. Introduction of an in-frame, 12-bp insertion within this locus also conferred the mutant phenotype, confirming that the phenotype seen in the transposon mutants is the result of disruption of a distinct gene, which we have designated *bvgR*, and is not a consequence of polar effects on *bvgAS*.

Bordetella pertussis, the causative agent of whooping cough, produces a wide array of factors that are associated with its ability to cause disease (13, 22, 36, 37). These virulence factors include several toxins, such as pertussis toxin (encoded by *ptx*), adenylate cyclase toxin/hemolysin (encoded by *cya*), and dermonecrotic toxin (encoded by *dnt*), as well as factors associated with adhesion, such as filamentous hemagglutinin (encoded by *fha*), pertactin (encoded by *prn*), and fimbriae (encoded by *fim*). The production of these and other proteins is coordinately regulated in response to environmental signals, a phenomenon known as modulation (19). Although the physiologically relevant signals are unknown, the synthesis of these virulence factors by *B. pertussis* is repressed in laboratory cultures when $MgSO_4$ or nicotinic acid is present in the growth medium or when the cells are grown at lowered temperatures. It is now understood that the expression and regulation of these virulence factors are dependent upon the *vir* (now designated *bvg*) locus, which encodes two proteins, BvgA, a 23-kDa cytoplasmic protein, and BvgS, a 135-kDa transmembrane protein (1, 35). The products of the *bvg* locus have significant sequence homology with a large family of bacterial regulatory proteins commonly referred to as two-component systems (1, 30). Most of these systems consist of a "sensor" or "transmitter" protein, which spans the inner membrane and senses changes in the environment, and a "regulator" or "receiver" protein, which is cytoplasmic and is often a transcriptional activator (23). Several members of these families have been shown to communicate through the transfer of a phosphate moiety from the sensor protein to its cognate regulator, which stimulates the regulator protein to activate transcription from specific promoters (23). In keeping with these models, BvgS is proposed to respond to environmental signals and to communicate with BvgA, a transcriptional regulator which, upon modification by BvgS, binds to specific promoters and activates transcription (33).

Evidence from several laboratories indicates that the mechanism by which the products of the *bvg* locus regulate expression is probably different for different virulence loci. Although BvgA can directly activate transcription from the *fha* and *bvg* promoters, the *bvg* locus does not appear to be sufficient for the activation of transcription from the *ptx* or *cya* promoter. Evidence for this includes the observations that the regulated expression of *fha* can be reconstituted in *Escherichia coli* with the *bvg* and *fha* loci from *B. pertussis* and that the binding of BvgA to the *fha* and *bvg* promoters has been demonstrated in vitro (20, 24, 25, 34). Attempts to show the same for the *ptx* and *cya* loci were unsuccessful. In addition, BvgA binds to a sequence element that is found in both the *bvgAS* and *fha* promoters but which is not evident in the *cya* or *ptx* promoter. These differences in the regulation of known virulence loci have led to the hypothesis that accessory regulators are required for the expression of *ptx* and *cya*, as well as other virulence loci, but are not required for the expression of *bvgAS* or *fha*.

The complexity of the *bvg* regulon is even more apparent when one considers an additional class of genes that are repressed by the *bvg* locus. The expression of this class, the *bvg*-repressed genes (*vrg*s [*vir*-repressed genes]) is reduced under conditions in which the expression of the aforementioned *bvg*-activated virulence factors is maximal, and this repression has been shown to be dependent upon the presence of an intact *bvgAS* locus (17). While much is known about the virulence factors which are activated by the *bvg* locus, the nature and role(s) of the *bvg*-repressed genes are essentially unknown. It is speculated that the *vrg* gene products may be involved in the establishment or persistence of *B. pertussis* in the host, survival within a specialized niche in the host, or survival of the bacterium outside the host. A strain harboring a transposon insertion in one of the *bvg*-repressed genes, *vrg6*, was found to be dramatically reduced in its ability to persist and cause lymphocytosis in the mouse lung model, thus suggesting a role in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Source
E. coli K-12		
DH5α	High-efficiency transformation	Bethesda Research Laboratories
SM10	Tra functions of IncP plasmids integrated into chromosome	26
B. pertussis		
Tohama I	Patient isolate	16
BP947	Tohama I, Nal^r Str^r, *fhaB-lacZ*	28
TM1081	Tohama I, Nal^r Str^r, *fhaB-lacZ vrg6-phoA*	This study
TM1081-T1	TM1081, Tn*5* transposon insertion at *bvg* position 5465	This study
TM1081-T16	TM1081, Tn*5* transposon insertion at *bvg* position 5214	This study
TM1081-T17	TM1081, Tn*5* transposon insertion at *bvg* position 5214	This study
TM1081-T22	TM1081, Tn*5* transposon insertion	This study
TM1081-T25	TM1081, Tn*5* transposon insertion at *bvg* position 5214	This study
TM1126	TM1081, *Eco*RI-*Not*I-*Eco*RI linker at *bvg* position 5311	This study
TM1210	TM1081, linker UTA inserted at *bvg* position 5530	This study
SK73	Kan^r, *vrg73-phoA* translational fusion	297
SK73-T1	SK73, Tn*5* transposon insertion at *bvg* position 5465	This study
SK73-T16	SK73, Tn*5* transposon insertion at *bvg* position 5214	This study
Plasmids		
pBS KS$^+$	General cloning vector	Stratagene
pSS1311	*rpsL Sal*I fragment in pBR322	This study
pSS1577	Ap^r Kan^r *rpsL oriT cos*	32
pSS1823	*vrg6* in pSS1577	This study
pSS1894	Ap^r Kan^r *oriT cos*	This study
pSS2000	Ap^r Gen^r *rpsL oriT cos*	This study
pSS2048	pUT-Kan with *Spe*I and *Xba*I sites added	29
pSS2125	Broad-host-range cloning vector	This study
pTM023	pSS1577, *vrg6-phoA* transcriptional fusion	This study
pTM025	pSS2000, *bvg* sequences 4711–5991	This study
pTM034	pTM025, *Eco*RI-*Not*I-*Eco*RI linker at *bvg* position 5311	This study
pTM058	pTM025, translational terminator inserted at *bvg* position 5530	This study
pTM061	*bvg Bgl*II-*Bam*HI fragment in pSS2125	This study
pTM063	*bvg Sal*I-*Xho*I fragment in pSS2125	This study

virulence for at least one *bvg*-repressed gene (4). Five *bvg*-repressed genes have been identified to date, and the DNA sequence of the 5′ end of each gene has been determined (2, 3). Four of the five genes (*vrg6*, *vrg18*, *vrg24*, and *vrg53*) contain a conserved 32-bp sequence within the coding region, and 22 of the 32 consensus nucleotides are the same in at least three of the four sites. The exception is *vrg73*, which does not appear to contain this 32-bp 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 (2, 3). Replacement of the *vrg6* promoter with the non-*bvg*-regulated *asd* promoter had no effect on modulation, indicating that the sites required for regulation of the *bvg*-repressed genes lie within the coding region. These experiments suggest that for at least some of the *bvg*-repressed genes in *B. pertussis*, a regulatory protein encoded by a *bvg*-activated gene may bind to the conserved sequence element within the coding region of each gene and repress its transcription. Alternatively, BvgA itself could act as a repressor protein and bind these conserved sequence elements within the *bvg*-repressed genes.

In this study, we sought to identify accessory proteins involved in the regulation of the *bvg*-repressed genes. A genetic screen of mini-Tn*5* transposon mutants of *B. pertussis* identified five mutants that demonstrated constitutive expression of a transcriptional fusion of the *B. pertussis vrg6* gene to the gene encoding *E. coli* alkaline phosphatase (*phoA*) but showed wild-type *bvg* activity, as demonstrated by normal regulation of hemolysis and normal expression and regulation of a transcriptional fusion of the *B. pertussis fha* gene to the gene encoding *E. coli* β-galactosidase (*lacZ*). Mapping and preliminary characterization of the mutations has defined a gene closely linked to but distinct from *bvgAS* that is required for the repression of *bvg*-repressed genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are presented in Table 1. *E. coli* strains were grown on L-agar or in L-broth supplemented with antibiotics when appropriate (21). *B. pertussis* strains were grown on Bordet-Gengou agar (BG-agar) (Difco, Detroit, Mich.) containing 1% proteose peptone (Difco) and 15% defibrinated sheep blood or in Cohen-Wheeler broth (S. & S. Media, Rockville, Md.). Unless otherwise noted, the antibiotics and concentrations (micrograms per milliliter) used were gentamicin sulfate, 10; kanamycin sulfate, 10; nalidixic acid, 50; rifampin, 50; streptomycin sulfate, 100; tetracycline, 15; lividomycin, 100; and butirosin, 100. *E. coli* DH5α was obtained from Bethesda Research Laboratories (Bethesda, Md.).

Strain and plasmid construction. Strain BP947 harbors a transcriptional gene fusion of the *fhaB* gene to *lacZ* of *E. coli*. A *Bam*HI-*Sal*I fragment derived from pRS1551 (27) replaces *fhaB* sequences from the *Bam*HI site at nucleotide 2836 to the *Xho*I site at nucleotide 7279, with reference to the GenBank file BPEF HAB1. The construction of this fusion and its reintroduction into the *B. pertussis* chromosome have been described previously (28).

Strain TM1081 was constructed as follows. The *vrg6* gene was cloned into plasmid pSS1577 (32) as an *Eco*RI fragment, generating plasmid pSS1823. A synthetic *Nco*I-*Bgl*II-*Nco*I linker (5′-CATGGAGATCTC-3′) was subsequently inserted into the unique *Nco*I site within the *vrg6* sequences, creating a unique *Bgl*II site. The *phoA* gene was synthesized by PCR with oligonucleotides that annealed between positions 216 and 243 (5′-GCGGATCCGTCACGGC CGAGACTTATAGTGCGTTTG-3′) and positions 1670 and 1698 (5′-GCG GATCCTTATTTCAGCCCCAGAGCGGCTTTCATGG-3′) of the published *phoA* sequence (10). The resulting PCR product was cloned as a *Bam*HI frag-

ment into the *Bgl*II site engineered in *vrg6* to generate a *vrg6-phoA* transcriptional fusion. The resulting plasmid was designated pTM023. *E. coli* SM10 bearing plasmid pTM023 was mated with *B. pertussis* BP947 as described below, and exconjugants in which the plasmid sequences had integrated into the chromosome were isolated by selection on kanamycin, with nalidixic acid counterselection. Subsequently, isolates in which plasmid sequences were lost from the chromosome but in which the *vrg6-phoA* fusion was retained were selected by virtue of the streptomycin sensitivity encoded by the plasmid. Those isolates retaining the *vrg6-phoA* fusion were identified by screening for alkaline phosphatase activity by the colony lift method after growth in the presence of 50 mM $MgSO_4$ (see below). Approximately 50% of the streptomycin-resistant colonies demonstrated alkaline phosphatase activity in the presence of 50 mM $MgSO_4$. Eight colonies expressing alkaline phosphatase activity in the presence of 50 mM $MgSO_4$ were selected, and the *vrg6-phoA* and *fha-lacZ* activities of these isolates were determined after growth in the presence and absence of 50 mM $MgSO_4$ (data not shown). All eight isolates demonstrated the same levels of *vrg6-phoA* and *fha-lacZ* activity and the same degree of regulation of these loci. One of these isolates, designated TM1081, was selected for further study.

Plasmid pSS2000 was constructed as follows. To create pSS1872, the complementary oligonucleotides 5′-AGCTACTAGTCTAGATTTAAATTAATTAAG AATTCG-3′ and 5′-GATCCGAATTCTTAATTAATTTAAATCTAGACTA GT-3′ were added between the *Hin*dIII and *Bam*HI sites of pSS1673 (31). This resulted in the addition of *Spe*I, *Xba*I, *Swa*I, *Pac*I, and *Eco*RI sites downstream of the gentamicin resistance gene and the destruction of the *Hin*dIII site there. To create pSS1873, a recognition site for the intron-encoded restriction enzyme I-*Sce*I was added at the *Xba*I site of pSS1872 by insertion of the complementary oligonucleotides 5′-CTAGATAGGGATAACAGGGTAATT-3′ and 5′-CTA GAATTACCCTGTTATCCCTAT-3′, conserving the flanking *Xba*I sites. A *Pme*I site was introduced at the *Spe*I site of pSS1873 by the addition of the self-complementary oligonucleotide 5′-CTAGTGTTTAAACA-3′, thus conserving the flanking *Spe*I sites and creating pSS1877. A recognition site for the intron-encoded restriction enzyme I-*Ppo*I was introduced at the *Pac*I site of pSS1877 by using the complementary oligonucleotides 5′-CTCTCTTAAGG TAGCTTAAT-3′ and 5′-TAAGCTACCTTAAGAGAGAT-3′ to create pSS1881. In this case, one of the *Pac*I sites flanking the insertion was destroyed. An approximately 250-bp fragment from pSS1881 extending from the *Bgl*II site in the gentamicin resistance gene to the *Eco*RI site downstream was substituted for the homologous fragment of pSS1832 (31) in order to introduce all of these new sites downstream of the gentamicin resistance gene of that plasmid, creating plasmid pSS1894. A PCR fragment of the *rpsL* gene of *E. coli* was generated by using the oligonucleotides 5′-CGCGTCGACGACGGTAACCGCTACCTT GAAAGTC-3′ and 5′-CGCGTCGACGTTTGGCCTTACTTAACGGAGAA CC-3′, digested with *Sal*I, and cloned into the *Sal*I site of pBR322 (7) to create pSS1311. The 715-bp *Sal*I *rpsL* fragment liberated from this plasmid was cloned into the *Xho*I site of pSS1894 to create pSS2000.

Strain TM1126 was constructed as follows. The *bvg* sequence downstream of *bvgAS* (nucleotides 4711 to 5991) was subcloned as a *Sal*I-*Xho*I fragment into plasmid pSS2000, generating plasmid pTM025. A synthetic *Eco*RI-*Not*I-*Eco*RI linker (5′-AATTGCGGCCGC-3′) was inserted into the *Eco*RI site at nucleotide position 5311 in the *bvg* sequence in plasmid pTM025, generating plasmid pTM034. *E. coli* SM10 bearing plasmid pTM034 was mated with *B. pertussis* TM1081 as described below, and exconjugants in which the plasmid sequences had integrated into the chromosome were isolated by selection on kanamycin, with nalidixic acid counter-selection. An isolate in which plasmid sequences were lost from the chromosome but in which the *Eco*RI-*Not*I-*Eco*RI linker was retained was isolated by selection for streptomycin resistance and screening for alkaline phosphatase activity in the absence of modulators. Approximately 50% of the streptomycin-resistant colonies demonstrated alkaline phosphatase activity in the absence of a modulator. Eight colonies expressing alkaline phosphatase activity in the absence of modulators were selected, and the *vrg6-phoA* and *fha-lacZ* activities of these isolates were determined after growth in the presence and absence of 50 mM $MgSO_4$ (data not shown). All eight isolates demonstrated the same levels of *vrg6-phoA* and *fha-lacZ* activity and the same degree of regulation of these loci. One of these isolates, designated TM1126, was selected for further study.

Strains SK73-T1 and SK73-T16 were constructed as follows. TM1081-T1 and TM1081-T16 Hfr donor pools were generated by following the protocol described by Stibitz and Carbonetti (31). The resulting TM1081-T1 and TM1081-T16 donor pools were mated with strain SK73, and recipients that received and incorporated the region of the donor chromosome containing the T1 or T16 transposon insertion were selected as follows. Tn*phoA*, the derivative of Tn*5* used by Knapp and Mekalanos to generate the *vrg73-phoA* fusion in strain SK73 (17), encodes aminoglycoside 3′-phosphotransferase II, which confers resistance to the antibiotics kanamycin and butirosin but not lividomycin. The transposon used in the mutagenesis of strain TM1081 carries a gene encoding aminoglycoside 3′-phosphotransferase I, which confers resistance to the antibiotics kanamycin and lividomycin but not butirosin (5). Following the conjugations described above, it was possible to directly select SK73 exconjugants that had received and incorporated the region of the donor chromosome containing the T1 or T16 transposon insertion by selecting for growth in the presence of lividomycin (100 μg/ml) and butirosin (100 μg/ml).

Strain TM1210 was constructed as follows. The synthetic linker UTA was designed to allow the insertion of a universal translation terminator into *Afl*III sites (5′-CATGTTTAATTAATTAAA-3′). Linker UTA was inserted into the *Afl*III site at nucleotide position 5530 in the *bvg* sequence in plasmid pTM025, generating plasmid pTM058. *E. coli* SM10 bearing plasmid pTM058 was mated with *B. pertussis* TM1081 as described below, and exconjugants in which the plasmid sequences had integrated into the chromosome were isolated by selection on kanamycin, with nalidixic acid counter selection. Isolates in which plasmid sequences were lost from the chromosome but in which the UTA linker was retained were isolated by selection for streptomycin resistance and screening for alkaline phosphatase activity in the absence of modulators. Approximately 50% of the streptomycin-resistant colonies demonstrated alkaline phosphatase activity in the absence of a modulator. Eight colonies expressing alkaline phosphatase activity in the absence of modulators were selected, and the *vrg6-phoA* and *fha-lacZ* activities of these isolates were determined after growth in the presence and absence of 50 mM $MgSO_4$. All eight isolates demonstrated the same levels of *vrg6-phoA* and *fha-lacZ* activity and the same degree of regulation of these loci (data not shown). One of the isolates, designated TM1210, was selected for further study.

Plasmid pSS2125 was constructed as follows. The complementary oligonucleotides 5′-AATTGTTAATTAAGGATCCCTCGAGGAATTCCTTAAGTTAA TTAAC-3′ and 5′-AATTGTTAATTAACTTAAGGAATTCCTCGAGGGATC CTTAATTAAC-3′ were inserted at the *Eco*RI site of pRK290 (12). The *Eco*RI sites flanking the insertion were destroyed, and *Bam*HI, *Xho*I, *Eco*RI, and *Afl*III sites were introduced, flanked by *Pac*I sites.

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 $MgCl_2$. After 3 h of incubation at 37°C, the bacteria were swabbed onto Bordet-Gengou agar containing the appropriate antibiotics for the selection of exconjugants and incubated at 37°C in screw-top jars. Prior to mating, *B. pertussis* strains were grown for 3 days and *E. coli* strains were grown overnight.

Matings between *B. pertussis* strains were performed by resuspending fresh plate cultures of each strain in 1× phosphate-buffered saline (PBS) to an approximate density of 10^{10} cells per ml. Matings were initiated by adding 50 μl of donor strain to 500 μl of recipient strain. The strains were vortexed briefly, and 50 μl was spotted onto a Bordet-Gengou agar plate supplemented with 10 mM $MgCl_2$. After 6 to 8 h of incubation at 37°C, the bacteria were swabbed onto Bordet-Gengou agar containing the appropriate antibiotics for the selection of exconjugants and incubated at 37°C in screw-top jars.

Transposon mutagenesis. Plasmid pSS2048 was used to introduce transposon insertion mutations into *B. pertussis* strains. The minitransposon donor plasmid pUTKan (15) was used as the basis for the construction of pSS2048. Details of the construction will be reported elsewhere, but the net change to pUTKan is the replacement of the *Not*I kanamycin resistance gene fragment with a homologous *Pst*I fragment from pKanπ (6), with the addition of the following sequence between the *Pst*I and *Not*I sites at both ends of the transposon: 5′-CTGCAG GTCATCGACCCAAGTACCGCCACCTAAAAGCTACTAGTGTTTAAA CACTAGTCTAGATAGGGATAACAGGGTAATTCTAGATTTAAATTA ATCTCTCTTAAGGTAGCTTAATAACTTCGTATAGCATACATTATAC GAAGTTATCAATTGAATTGCGGCCGC-3′. The kanamycin resistance gene encoded by the *Pst*I fragment was originally derived from Tn*903* and encodes an aminoglycoside 3′-phosphotransferase I (5). For the present study, the relevant feature of the sequence inserted at the ends of the transposon is the presence of *Xba*I and *Spe*I sites, used to map insertions of the transposon in the *B. pertussis* chromosome. *E. coli* SM10 bearing plasmid pSS2048 was mated with strain TM1081 as described above, and exconjugants in which the mini-Tn*5* Km insertion sequence had inserted into the chromosome were selected by growth on Bordet-Gengou agar containing kanamycin. Colonies arising after transposon mutagenesis were screened for constitutive expression of the *vrg6-phoA* fusion as described below.

Quantitative alkaline phosphatase and β-galactosidase assays. Bacteria to be assayed were recovered by sterile swab into 3.5 ml of Tris-HCl, pH 8.0, and the A_{600} 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 (SDS) and 30 μl of chloroform followed by vortexing, and the assay was completed as described by Miller (21). For measurement of alkaline phosphatase activity, 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 (8). Units of activity in both cases were calculated as $[1{,}000 \times A_{420} - (1.75 \times A_{550})]/(T \times V \times A_{600})$, where T is the incubation time (in minutes) and V is the volume of permeabilized cells added to the assay (in milliliters).

Colony lift assay for alkaline phosphatase activity. Colonies arising after growth on BG-agar were screened for expression of alkaline phosphatase activity by allowing colonies to adhere to nitrocellulose filters and perfusing them with 1.0 M Tris-HCl (pH 8.0) and 160 μg of XP (5-bromo-4-chloro-3-indolylphosphate) per ml as described before (17).

Restriction mapping of transposon insertions. Chromosomal DNA was isolated from *B. pertussis* cells, digested with *Spe*I and *Xba*I, and subjected to pulsed-field gel electrophoresis as described elsewhere (32).

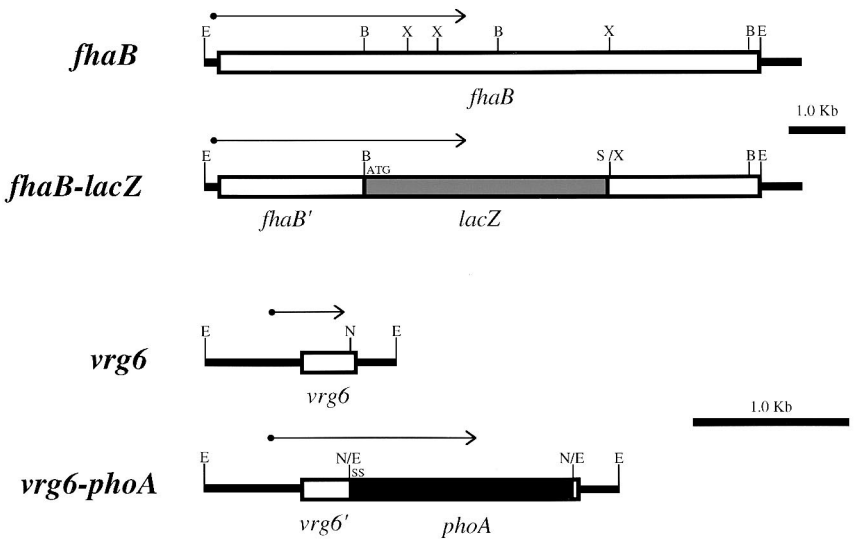

FIG. 1. *B. pertussis* TM1081. The reporter gene transcriptional fusions present in strain TM1081 are shown. The *fhaB* and *vrg6* coding sequences are both represented by open boxes. The β-galactosidase coding sequence and the alkaline phosphatase coding sequence are represented by shaded and solid boxes, respectively. The start and direction of transcription of the native genes and the transcriptional fusions are indicated by arrows. SS, *phoA* signal sequence. Restriction enzyme recognition sequences: E, *Eco*RI; B, *Bam*HI; S, *Sal*I; X, *Xho*I; N, *Nco*I.

Sequence analysis. Inserts cloned in plasmid pBS KS$^+$ (Stratagene, La Jolla, Calif.) were sequenced on an Applied BioSystems Incorporated model 370A Automated Sequencer with the Prism Ready DiDeoxy-Terminator Cycle Sequencing kit (Applied BioSystems Incorporated) and the M13-20 primer (5′-GTAAAACGACGGCCAGT-3′) and the M13 reverse primer (5′-AACAGC TATGACCATG-3′). Computer analysis of DNA and protein sequences was performed with the GCG sequence analysis software package (Genetics Computer Group Inc., Madison, Wis.) and MacVector Sequence Analysis programs (International Biotechnologies Inc., New Haven, Conn.).

RESULTS

Isolation of transposon insertion mutants. Mutants defective for the putative *bvgAS*-regulated repressor would be expected to demonstrate constitutive expression of *bvg*-repressed genes [Vrg(Con)] and normal expression of the *bvg*-activated genes. In order to screen mutagenized bacterial colonies for mutants with this phenotype, we constructed strain TM1081 as described in Materials and Methods. This strain contains transcriptional fusions of *vrg6* to *phoA* and *fhaB* to *lacZ* (Fig. 1). In this strain, the desired mutants will express alkaline phosphatase activity (Pho$^+$) in the presence and absence of modulating signals and will express β-galactosidase activity (Lac$^+$) as well as hemolytic activity (Hly$^+$) only in the absence of modulators. After transposon mutagenesis of strain TM1081 with mini-Tn*5* Km (11) and growth on BG-agar in the absence of modulators, kanamycin-resistant colonies were screened for alkaline phosphatase activity. A total of 32 independently derived isolates that constitutively expressed alkaline phosphatase activity [Pho(Con)] were identified. These candidates were analyzed by quantitative enzyme assays for alkaline phosphatase and β-galactosidase and screened for hemolysis on BG-agar plates. Twenty-seven of the 32 isolates failed to express β-galactosidase activity (Lac$^-$), were nonhemolytic on BG-agar plates (Hly$^-$), and constitutively expressed alkaline phosphatase activity. This is the expected phenotype of a *bvgAS* knockout mutation. The remaining five independently derived isolates were Pho$^+$ in the absence and presence of 50 mM $MgSO_4$ but demonstrated normal regulation of the *fha-lacZ* fusion. The results of these assays for these five mutants are shown in Table 2. In each case, the activity of the *vrg6-phoA* fusion was reduced only approximately 2-fold upon growth in the absence of modulators, compared with the reduction of

TABLE 2. Activities of mini-Tn*5* Km transposon and linker insertion mutants of TM1081[a]

Strain	Alkaline phosphatase activity (*vrg6-phoA*) (U)		β-Galactosidase activity (*fha-lacZ*) (U)		Hemolysis (*cya*)	
	+$MgSO_4$	−$MgSO_4$	+$MgSO_4$	−$MgSO_4$	+$MgSO_4$	−$MgSO_4$
TM1081	1.00 ± 0.00	0.11 ± 0.03	0.04 ± 0.01	1.00 ± 0.00	−	+
T1	1.13 ± 0.27	0.68 ± 0.26	0.02 ± 0.01	1.00 ± 0.07	−	+
T16	1.16 ± 0.33	0.64 ± 0.21	0.03 ± 0.02	1.02 ± 0.06	−	+
T17	1.14 ± 0.38	0.63 ± 0.19	0.03 ± 0.02	1.02 ± 0.09	−	+
T22	1.14 ± 0.29	0.65 ± 0.22	0.03 ± 0.02	1.02 ± 0.03	−	+
T25	1.04 ± 0.33	0.66 ± 0.25	0.03 ± 0.02	1.00 ± 0.13	−	+
TM1126	1.03 ± 0.18	0.64 ± 0.19	0.03 ± 0.01	1.13 ± 0.32	−	+
TM1210	0.089 ± 0.19	0.67 ± 0.16	0.02 ± 0.00	1.06 ± 0.12	−	+

[a] β-Galactosidase activities are reported relative to that of strain TM1081 grown in the absence of $MgSO_4$ (9,153 U). Alkaline phosphatase activities are reported relative to that of strain TM1081 grown in the presence of $MgSO_4$ (15.2 U). All values reported are the averages of at least six independent assays ± the standard deviations. Hemolysis activity was scored qualitatively by inspection of colonies after 3 to 4 days of growth on BG-agar: +, hemolytic zone visually indistinguishable from the wild-type zone; −, no discernible hemolytic zone.

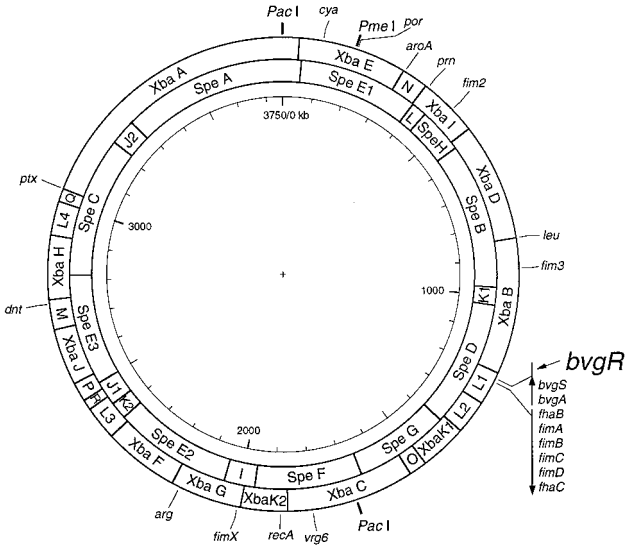

FIG. 2. Physical map of the *B. pertussis* Tohama I chromosome. Restriction map of the *B. pertussis* chromosome, showing the locations of the *Xba*I and *Spe*I fragments (32). The positions of genes for the major virulence determinants in the *bvg* regulon as well as some housekeeping genes are shown. The position of *bvgR* as determined in this study is indicated.

approximately 10-fold seen in strain TM1081 under the same conditions. All five mutants demonstrated wild-type levels of expression and regulation of the *fha-lacZ* fusion, and expression and regulation of *cya* appeared normal, as determined by scoring for the presence of zones of hemolysis on BG-agar after growth in the presence and absence of 50 mM $MgSO_4$.

Mapping of the transposon insertion sites in TM1081-T1, -T16, -T17, -T22, and -T25. Initial mapping of the sites of transposon insertion in each of the five mutants was made possible by the derivation of a restriction map of the *B. pertussis* Tohama I chromosome for the enzymes *Xba*I and *Spe*I (Fig. 2) (32) and the presence of *Xba*I and *Spe*I sites within the mini-Tn*5* Km transposon. Chromosomal DNA from the five mutants and TM1081 was digested with *Xba*I or *Spe*I and subjected to pulsed-field agarose gel electrophoresis (Fig. 3). It is clear that band SpeD is missing in the *Spe*I digests of the five mutants and that in the digests of these same mutants, two bands of approximately the same size form a doublet band between bands SpeG and SpeF which is not seen in the TM1081 digest. This result suggests that in all five mutants, the site of transposon insertion is near the center of band SpeD. Close examination of the *Xba*I digests reveals the appearance of a band of approximately 25 kb in the *Xba*I digests of the mutant chromosomes that is not present in the *Xba*I digests of the wild-type chromosome. In addition, band XbaN appears to be more intense than band XbaM in each of the *Xba*I digests of the mutant chromosomes, while in the *Xba*I digests of the wild-type chromosome, XbaN appears less intense than XbaM, suggesting that a new band of approximately 75 kb, which comigrates with band XbaN, is present in the digests of the mutant chromosomes. The appearance of two bands in the mutant digests totaling approximately 100 kb indicates that the site of transposon insertion in each of the mutants is within one of the XbaL bands. Taken together, the results from the *Spe*I and *Xba*I digests indicate that, in all five mutants, the sites of insertion are very near each other and demonstrate that the sites of transposon insertion are in the vicinity of the *bvgAS* operon (see Fig. 2).

In order to precisely determine the sites of insertion, chromosomal DNA from each of the five mutants was digested with *Eco*RI, and the resulting fragments were cloned into pBS KS^+ vector (Stratagene). The ligated products were transformed into *E. coli* DH5α, and Kan^r colonies were selected. Kanamycin-resistant transformants were isolated from the ligations of T1, T16, T17, and T25 chromosomal digests. Several attempts were made to subclone the chromosomal *Eco*RI fragment from mutant T22, containing the inserted Kan^r marker, but these attempts were unsuccessful. Plasmid DNA derived from mutants T1, T16, T17, and T25 was isolated, and the sequence across the ends of the inserted sequence was determined. From this analysis, it was possible to determine the precise site of transposon insertion in these four mutants. In mutants T16, T17, and T25, the site of transposon insertion is after nucleotide 5214 of the published *bvgAS* sequence (1). In mutant T1, the site of transposon insertion is after nucleotide 5465 (see Fig. 4).

Effect of transposon insertions on *vrg73* expression. Mapping of the positions of the four independently derived transposon insertions that confer the Vrg6(Con) phenotype to a site immediately downstream of *bvgAS* suggested that this newly defined locus may be at the top of the regulatory cascade leading to the repression of most, if not all, of the *bvgAS*-repressed genes. If this is the case, we would predict that other *bvgAS*-repressed genes are derepressed in these mutants. In order to test this prediction, we chose to examine the effect of the T1 and T16 transposon insertions on the expression of *vrg73*. Because *vrg73* is the only *bvgAS*-repressed gene that appears to be missing the putative repressor-binding site (2, 3), we reasoned that the regulation of the expression of *vrg73* is the most likely to be different from that of *vrg6*. As described in Materials and Methods, mutants T1 and T16 were converted to Hfr donor strains, and these donors were used to transfer the regions of the T1 and T16 chromosomes carrying the T1 and T16 insertions into *B. pertussis* SK73, generating strains SK73-T1 and SK73-T16, respectively. SK73 carries a translational fusion of *phoA* to the *vrg73* gene (3, 17).

The expression of the *vrg73-phoA* fusions in strains SK73-T1 and SK73-T16 was analyzed by quantitative determination of the alkaline phosphatase activity after growth in the absence and presence of 50 mM $MgSO_4$. The results of this analysis are shown in Table 3. In strains SK73-T1 and SK73-T16, the activity of the *vrg6-phoA* fusion was reduced less than 2-fold upon growth in the absence of modulators, while a reduction of approximately 30-fold was seen in strain SK73 under the same conditions. Strains SK73, SK73-T1, and SK73-T16 demonstrated normal regulation of hemolysis. The result that *vrg6* and *vrg73* are both derepressed in the T1 and T16 insertion mutants suggests that the locus defined by these insertion mutations is responsible for the repression of most, and possibly all, of the *bvgAS*-repressed genes.

Construction and characterization of an in-frame (nonpolar) insertion within the putative *bvg*-regulated repressor. Since the four independently derived transposon insertions that confer the Vrg6(Con) Bvg^+ phenotype map immediately downstream of *bvgAS*, one can infer that these insertions either disrupt a distinct gene required for the *bvg*-dependent repression of *vrg6* or, by virtue of the transcriptional activity of the inserted sequence, affect expression of the *bvgAS* operon so that *bvg* regulation of *vrg* repression is altered without affecting the ability of the *bvg* operon to activate transcription from the *bvg*-activated genes. In order to address this issue, we generated a nonpolar mutation within the region delineated by the two sites of insertion in the mutants described above. An *Eco*RI-*Not*I-*Eco*RI linker was inserted into the *Eco*RI site at

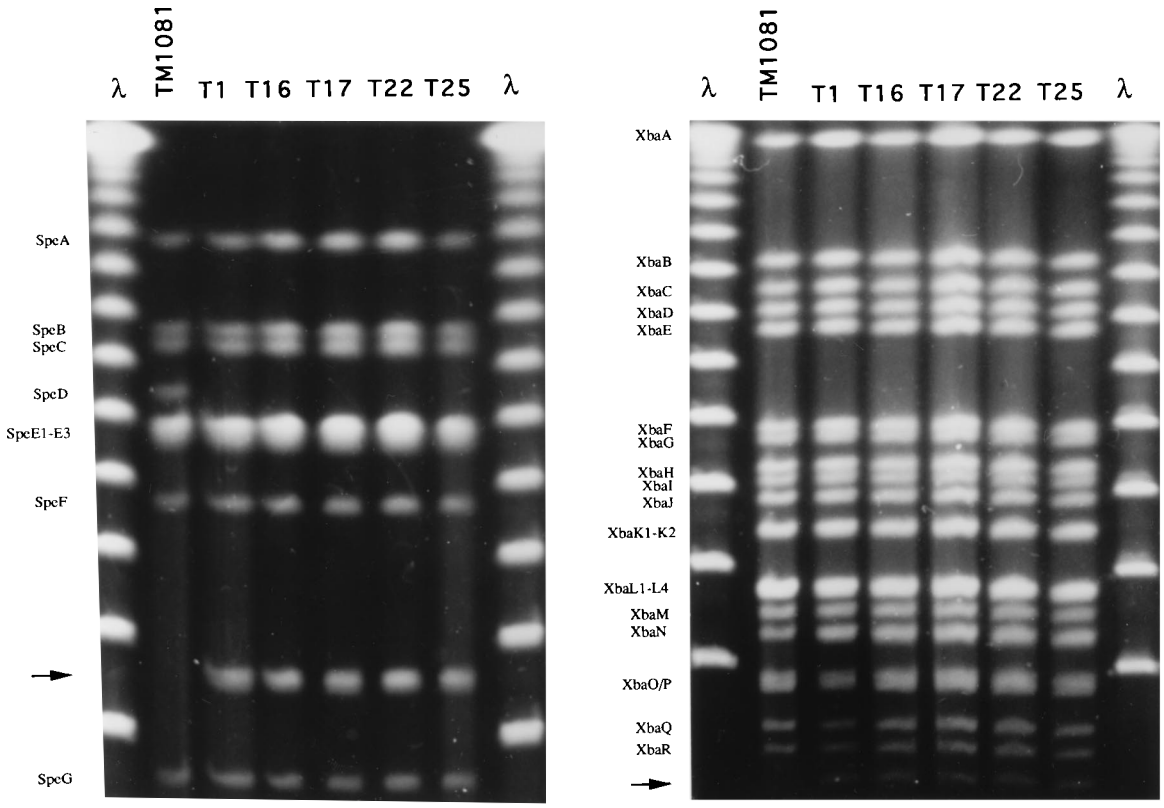

FIG. 3. Physical mapping of the sites of mini-Tn*5* Km insertions. Intact chromosomal DNA from *B. pertussis* TM1081 and from each mini-Tn*5* Km insertion mutant was restricted with *Spe*I (left panel) and with *Xba*I (right panel) and subjected to pulsed-field gel electrophoresis. The bands generated by the restriction of the wild-type Tohama I chromosome by each enzyme (SpeA to SpeG and XbaA to XbaR) are indicated to the left of each panel. Arrows indicate the positions of bands generated by the digestion of mutant chromosomal DNA that are not seen in the wild-type TM1081 digest.

nucleotide position 5311 in the *bvgAS* sequence (1). This generated a 12-bp insertion in the chromosome at a position between the two sites of mini-Tn*5* Km insertion that were shown to confer the Vrg6(Con) Bvg^+ phenotype. As this mutation creates an in-frame insertion, it would not be expected to affect the expression or function of *bvgAS*. This mutation was transferred onto the chromosome of strain TM1081, generating strain TM1126. The expression of the *vrg6-phoA* and *fha-lacZ* fusions in strain TM1126 was analyzed by quantitative determination of the alkaline phosphatase and β-galactosidase activities after growth in the absence and presence of 50 mM $MgSO_4$. The results of this analysis are shown in Table 2. In strain TM1126, the activity of the *vrg6-phoA* fusion was reduced only approximately 2-fold upon growth in the absence of modulators, while a reduction of approximately 10-fold was seen in strain TM1081 under the same conditions. Strain TM1126 demonstrated wild-type levels of expression and regulation of the *fha-lacZ* fusion and normal regulation of hemolysis.

Complementation analysis. If the locus downstream of *bvgAS* encodes a repressor protein that is responsible for the regulation of *bvgAS*-repressed genes, it should be possible to complement mutations affecting this locus with wild-type sequences introduced and maintained in *trans*. This prediction was tested by analyzing the ability of plasmids bearing sequences that include the region downstream of *bvgAS* to complement the defect in strain TM1126. The *Sal*I-*Xho*I and *Bgl*II-*Bam*HI restriction fragments from the *bvgAS* locus were cloned into plasmid pSS2125, generating plasmids pTM061 and pTM063, respectively (see Fig. 5). These plasmids were introduced into strain TM1126 by conjugation and maintained by growth in the presence of tetracycline. The expression of the *vrg6-phoA* and *fha-lacZ* fusions in strains TM1126, TM1126/pSS2125, TM1126/pTM061, and TM1126/pTM063 was analyzed by quantitative determination of the alkaline phosphatase and β-galactosidase activities after growth in the absence and presence of 50 mM $MgSO_4$. The results of this analysis are shown in Table 4. As seen previously, the activity of the *vrg6-phoA* fusion in strain TM1126 was reduced less than twofold upon growth in the absence of modulators. Strain TM1126 bearing vector sequences alone or vector with the *Sal*I-*Xho*I restriction fragment showed the same level of expression and same degree of regulation as strain TM1126 alone. In contrast, a 10-fold reduction in the expression of the *vrg6-phoA* fusion was seen in strain TM1126 bearing the vector with the *Bgl*II-*Bam*HI restriction fragment upon growth in the absence of modulator. All of the derivatives of strain TM1126 demonstrated levels of expression and regulation of the *fha-lacZ* fusion equivalent to those of the parental strain TM1126, as well as normal regulation of hemolysis. These results demonstrate that it is possible to complement mutations in the locus downstream of *bvgAS* in *trans*, although the *bvg* sequences contained on plasmid pTM063 are not sufficient to restore function.

Sequence analysis. Previous examination of the sequences downstream of *bvgAS* led to the conclusion that there was no obvious open reading frame (ORF) in this region (1). The discovery, reported here, that mutations conferring constitutive expression of *bvg*-repressed genes mapped downstream of *bvgAS* led us to reexamine this region. Our analysis revealed three ORFs, each of which was disrupted by each of the trans-

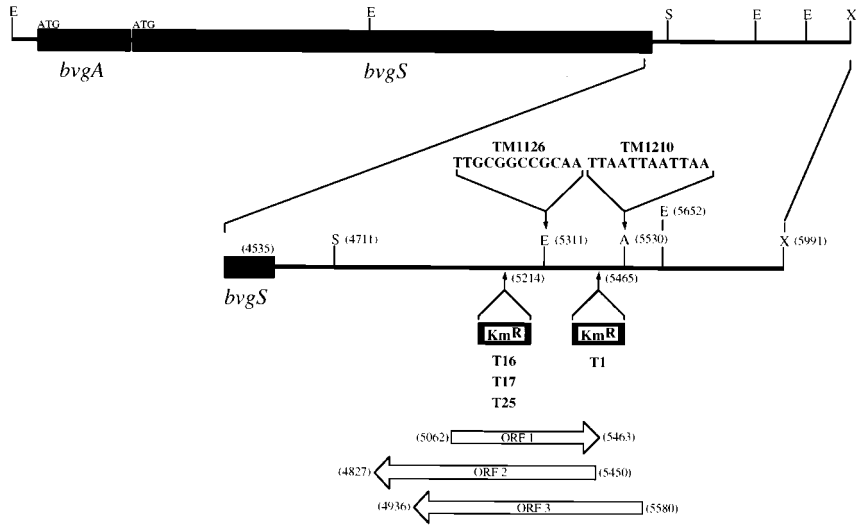

FIG. 4. Organization of the *bvg* operon. All sequences represented in the figure were published previously (1). Relevant nucleotide numbers from the published sequence are provided in parentheses. Solid boxes represent the coding sequences for the BvgA and BvgS proteins. Restriction enzyme recognition sequences: E, *Eco*RI; S, *Sal*I; X, *Xho*I; A, *Afl*II. The sites of mini-Tn*5* Km transposon insertion are indicated. The sequence of the 12-bp inserted sequence in strain TM1126 and its site of insertion are shown. The sequence of the 12-bp translational terminator in strain TM1210 and its site of insertion are shown. ORFs that are disrupted by the transposon and linker insertions are represented at the bottom of the figure by open boxes.

poson insertions as well as the linker insertion (see Fig. 4). The protein sequences predicted by each of the ORFs were determined and compared with sequences in the Swiss Protein, Protein Information Resource, and GenBank databases. The protein sequences predicted by *bvg* ORF1 and *bvg* ORF2 did not show significant homology to any sequences in the databases. A search of the databases with the BLAST program showed that *bvg* ORF3 has significant homology to four proteins which have been predicted from nucleotide sequence analysis of a virulence plasmid-encoded resolvase locus in *Salmonella typhimurium* (18) and mercuric ion resistance operons in *Serratia marcescens*, *E. coli*, and *Shigella flexneri* (9, 14). These four predicted proteins, StyResOrf, SmaMerOrf, Eco MerOrf, and SflMerOrf, respectively, have as yet unknown functions. Sequence alignment by the LFASTA program showed that a region of 79 amino acid residues was highly conserved among these proteins (Fig. 6). Within the aligned region, 75% of the residues (59 of 79) are identical between the proteins predicted by SmaMerOrf, EcoMerOrf, and SflMerOrf. Comparison of the predicted amino acid sequences of SmaMerOrf, EcoMerOrf, and SflMerOrf with StyResOrf shows that 30% of the residues (24 of 79) are invariant among all four proteins. The sequence predicted by *bvg* ORF3 is most closely related to that of StyResOrf; there are 27% identity and 70% similarity over the 79-amino-acid region. Of the 24 residues that are invariant between the SmaMerOrf, EcoMerOrf, SflMerOrf, and StyResOrf proteins, 11 (46%) are also present in Bvg ORF3, while 10 (42%) are conservative substitutions (Fig. 6).

Closing of ORF3. A linker was designed to introduce the sequence 5′-TTAATTAATTAA-3′ into the *Afl*III site at nucleotide position 5530 in the *bvgAS* sequence (1) (see Fig. 4). Introduction of this linker at this position closed ORF3 without affecting ORF1. ORF2 would only be affected by this insertion if it disrupts upstream sequences required for the expression of ORF2. This mutation was transferred onto the chromosome of strain TM1081, generating strain TM1210. The expression of the *vrg6-phoA* and *fha-lacZ* fusions in strain TM1210 was analyzed by quantitative determination of the alkaline phosphatase and β-galactosidase activities after growth in the absence and presence of 50 mM $MgSO_4$. The results of this analysis are shown in Table 2. Strain TM1210 has the same Vrg6(Con) Bvg^+ phenotype as the transposon insertion mutants and strain TM1126, further underscoring the importance of ORF3.

TABLE 3. Activities of mini-Tn*5* Km transposon mutants of SK73[a]

Strain	Alkaline phosphatase activity (*vrg73-phoA*) (U)		Hemolysis (*cya*)	
	$+MgSO_4$	$-MgSO_4$	$+MgSO_4$	$-MgSO_4$
SK73	1.00 ± 0.00	0.03 ± 0.01	−	+
SK73-T1	1.01 ± 0.26	0.66 ± 0.18	−	+
SK73-T16	1.23 ± 0.16	0.73 ± 0.16	−	+

[a] Alkaline phosphatase activities are reported relative to that of strain SK73 grown in the presence of $MgSO_4$ (248 U). All values reported are the averages of four independent assays ± the standard deviations. Hemolysis activity was scored qualitatively by inspection of colonies after 3 to 4 days of growth on BG-agar: +, hemolytic zone visually indistinguishable from the wild-type zone; −, no discernible hemolytic zone.

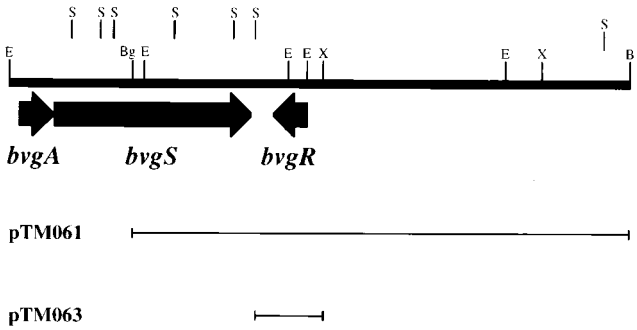

FIG. 5. *B. pertussis* sequences included in plasmids pTM061 and pTM063. E, *Eco*RI; S, *Sal*I; Bg, *Bgl*II; X, *Xho*I; B, *Bam*HI.

TABLE 4. Complementation of strain TM1126[a]

Plasmid	Alkaline phosphatase activity (*vrg6-phoA*) (U)		β-Galactosidase activity (*fha-lacZ*) (U)		Hemolysis (*cya*)	
	+$MgSO_4$	−$MgSO_4$	+$MgSO_4$	−$MgSO_4$	+$MgSO_4$	−$MgSO_4$
None	1.03 ± 0.18	0.64 ± 0.19	0.03 ± 0.01	1.13 ± 0.32	−	+
pSS2125	1.03 ± 0.21	0.61 ± 0.35	0.04 ± 0.03	0.96 ± 0.17	−	+
pTM061	1.14 ± 0.41	0.12 ± 0.07	0.04 ± 0.02	0.97 ± 0.13	−	+
pTM063	0.79 ± 0.20	0.55 ± 0.26	0.03 ± 0.01	0.89 ± 0.06	−	+

[a] β-Galactosidase activities are reported relative to that of strain TM1126 grown in the absence of $MgSO_4$ (8,766 U). Alkaline phosphatase activities are reported relative to that of strain TM1126 grown in the presence of $MgSO_4$ (13.7 U). All values reported are the averages of at least four independent assays ± the standard deviations. Hemolysis activity was scored qualitatively by inspection of colonies after 3 to 4 days of growth on BG-agar: +, hemolytic zone visually indistinguishable from the wild-type zone; −, no discernible hemolytic zone.

DISCUSSION

Although the precise mechanism by which the *bvgAS* locus activates the expression of all virulence factors has yet to be determined, its central role in the regulation of virulence genes in *B. pertussis* in response to environmental signals has been well established (36). The biphasic nature of the *bvg* regulon was revealed by the discovery of loci which are repressed by *bvgAS* under the same conditions that maximally activate the previously defined virulence factors (17). The existence of one set of genes that are induced and another set of genes that are repressed by the *bvgAS* operon during infection of the host suggests that the regulation of virulence gene expression during the course of infection is quite complex.

Five *bvg*-repressed genes have been identified in *B. pertussis*. Examination of the *cis*-acting elements involved in the regulation of these loci has revealed the existence of a conserved 32-bp element which is required for repression within the coding region of four of these five genes (2, 3). Transcriptional fusions to the *vrg6* gene and Northern (RNA blot) analysis of *vrg6* expression indicate that the regulation of *vrg* gene expression is at the level of transcription. Furthermore, Southwestern (DNA-protein blot) analysis suggested that the 32-bp consensus sequence is bound by a 34-kDa protein in *B. pertussis* extracts and that this protein is more highly expressed in the absence than in the presence of modulators (3). These observations led to a model which predicts that a regulator protein that is itself activated by *bvgAS* binds to the conserved element found in the *bvg*-repressed genes and represses transcription (3). Although it is possible that BvgA may directly repress expression from the *bvg*-repressed genes, this would require that BvgA bind a different sequence than that found in the BvgA-binding site in the *bvgAS* and *fhaB* promoters.

If a *bvgAS*-activated protein is required for the regulation of the *bvg*-repressed genes, it should be possible to isolate mutations in *B. pertussis* that eliminate the repression of the *bvg*-repressed genes without affecting the regulation of the *bvg*-activated genes. We have isolated five independently derived mini-Tn*5* Km transposon mutants with the phenotype expected of a strain defective for the putative *bvgAS*-regulated repressor. The insertion sites in four of these mutants have been shown by sequence analysis to be at two sites downstream of *bvgAS*. Two lines of evidence suggest that the phenotype seen in these mutants is a result of disruption of a distinct locus rather than an effect on the *bvgAS* genes. First, BvgAS function appears to be unimpaired in these mutants. The expression and regulation of an *fhaB-lacZ* fusion in the five mini-Tn*5* Km transposon mutants are indistinguishable from those of the wild-type parent. In addition, the regulation of hemolysis also appears to be normal in these mutants. It is unlikely that the effect of a transposon insertion is so subtle that the *bvg*-dependent repression of *vrg6* and *vrg73* is affected without having any detectable effect on the *bvg*-activated genes *fha* and *cya*. Second, a 12-bp insertion within the region defined by the sites of transposon insertion confers the same phenotype as seen in the transposon mutants. This argues against the possibility that inherent properties of the inserted sequences (such as transcriptional activity within the transposon) are somehow affecting the normal expression of *bvgAS*. The insertion of a 12-bp sequence is not likely to affect the expression of *bvgAS*, which is more than 700 bp upstream of the site of insertion. Since *bvgAS* function appears to be unaffected in these mutants, BvgAS does not appear to be directly responsible for the repression of the *vrg6* or *vrg73* gene. Instead, our results suggest that a previously unidentified gene lies immediately downstream of *bvgAS* and that this gene is responsible for the repression of *vrg6*, *vrg73*, and possibly other *bvg*-repressed genes in *B. pertussis*. We have designated this gene *bvgR*, for *Bordetella* virulence gene repression.

Examination of the sequence downstream of the *bvgAS* genes has allowed the tentative identification of the *bvgR* ORF. Of the three ORFs disrupted by the transposon and linker insertions, only Bvg ORF3 shows significant homology to other proteins predicted from nucleotide sequence analysis. The predicted Bvg ORF3 protein shows homology to the ORFs of

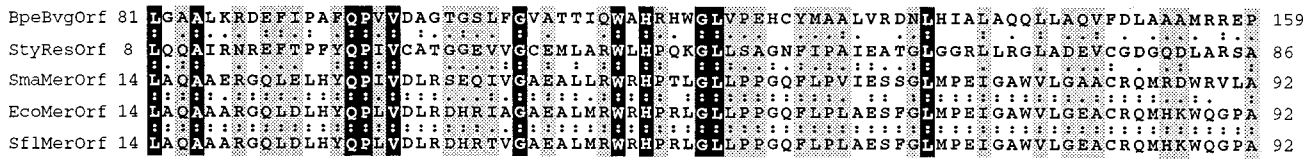

FIG. 6. Alignment of the predicted amino acid sequence of Bvg ORF with a new family of hypothetical proteins of unknown function. Bvg ORF3, unidentified ORF3 of the *B. pertussis bvg* operon; StyResOrf, ORF4 of the *S. typhimurium* resolvase operon (18); SmaMerOrf, unidentified ORF2 of *S. marcescens* mercuric ion resistance operon (pDU1358) (14); EcoMerOrf, unidentified ORF2 of *E. coli* mercuric ion resistance operon (R100) (9); SflMerOrf, unidentified ORF2 of the *S. flexneri* mercuric ion resistance operon (Tn*501*) (9). Solid boxes indicate positions conserved within all five sequences. Shaded boxes identify positions at which only conservative substitutions are found within all five sequences.

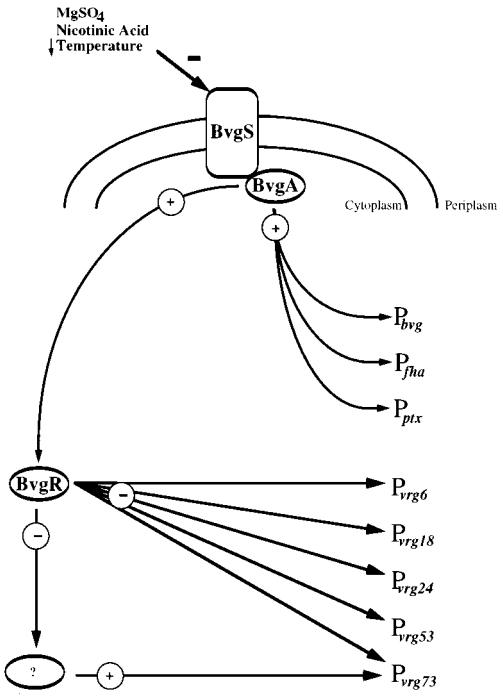

FIG. 7. Model for the regulation of *bvg*-repressed genes.

unknown function in a resolvase locus of *S. typhimurium* and mercuric ion resistance operons found in *S. marcescens*, *E. coli*, and *S. flexneri* (StyResOrf, SmaMerOrf, EcoMerOrf, and SflMerOrf, respectively). Bvg ORF3 is predicted to encode a 23-kDa basic protein (pI 10.9) and may function as a DNA-binding transcriptional regulator. The assignment of *bvgR* coding to ORF3 is supported by the result that closing ORF3 confers the *bvgR* phenotype.

Until now, the *bvg* locus has been thought to consist of only a sensory component (BvgS) and a transcriptional activator (BvgA). The discovery, reported here, that the *bvg* locus contains a third component, BvgR, which is responsible for repression, correlates very well with the observation that the *bvg* regulon includes a set of genes that are activated by the *bvg* locus as well as a set of genes that are repressed by the *bvg* locus. We propose that the apex of the *bvg* regulatory hierarchy consists of three factors: BvgS, which encodes the sensor that is sensitive to the environment; BvgA, a transcriptional activator required for the activation of the *bvg*-activated set of genes; and BvgR, which is responsible for the repression of the *bvg*-repressed set of genes (see Fig. 7). According to this model, BvgR activity is induced either by transcriptional activation of the *bvgR* gene by BvgA or, possibly, by posttranslational modification of the BvgR protein by BvgS. Beattie et al. (3) have reported the binding of a *bvg*-activated 34-kDa protein to the putative repressor-binding site of *vrg6*. This result, together with our results demonstrating that *bvgR* is required for repression of the *bvg*-repressed genes, indicates that BvgR is essential for repression but may not be the protein that directly binds the repressor-binding site. We propose that BvgR governs a regulatory circuit that leads to the repression of the *bvg*-repressed genes. It is likely that within both arms of the *bvg* regulon, multiple accessory regulators exist that are responsible for the activation or repression of specific promoters. Among the *bvg*-repressed genes, *vrg73* is clearly distinguishable from the remaining four genes in that it is missing the putative repressor-binding site. Therefore, any model of *vrg* regulation should invoke the existence of at least one additional regulator in order to account for the regulation of this gene. One possible model would invoke an activator of *vrg73* transcription which is itself repressed by BvgR.

The identification of the *bvgR* locus opens a new door in the analysis of the negatively regulated arm of the *bvg* regulon. Further characterization of this new locus is under way and includes identification of the *bvgR* gene product, the mechanism by which *bvgR* is activated by *bvgAS*, and the mechanism by which BvgR represses the *bvg*-repressed genes. Elucidation of the mechanism of BvgR regulation and function will further our understanding of the regulation of virulence in *B. pertussis.*

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