Activation of the *vrg6* Promoter of *Bordetella pertussis* by RisA

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The BvgAS two-component system positively regulates the expression of the virulence genes of *Bordetella pertussis* and negatively regulates a second set of genes whose function is unknown. The BvgAS-mediated regulation of the *bvg*-repressed genes is accomplished through the activation of expression of the negative regulator, BvgR. A second two-component regulatory system, RisAS, is required for expression of the *bvg*-repressed surface antigens VraA and VraB. We examined the roles of BvgR and RisA in the regulation of four *bvg*-repressed genes in *B. pertussis*. Our analyses demonstrated that all four genes are repressed by the product of the *bvgR* locus and are activated by the product of the *risA* locus. Deletion analysis of the *vrg6* promoter identified the upstream and downstream boundaries of the promoter and, in contrast to previously published results, demonstrated that sequences downstream of the start of transcription are not required for the regulation of expression of *vrg6*. Gel mobility-shift experiments demonstrated sequence-specific binding of RisA to the *vrg6* and *vrg18* promoters, and led to the identification of two putative RisA binding sites. Finally, transcriptional analysis and Western blot analysis demonstrated that BvgR regulates neither the expression nor the stability of RisA.

Bordetella pertussis is the causative agent of the respiratory infection of humans known as whooping cough (13). A wide variety of virulence factors have been identified which contribute to the bacterium's ability to colonize the host and cause disease (10–12, 14, 18, 23, 24, 26–28, 40). These factors include adhesins such as filamentous hemagglutinin, pertactin, and fimbriae, as well as several virulence determinants that cause damage to host tissues, including pertussis toxin, adenylate cyclase, and dermonecrotic toxin. The expression of most of these virulence factors is regulated in response to environmental signals by a two-component regulatory system encoded by the *bvg* locus (1, 29, 33, 34, 37). BvgS is a membrane-spanning protein that presumably acts as a sensor of the external environment, and BvgA is a soluble transcriptional activator. When *B. pertussis* is grown under normal laboratory conditions in rich media at 37°C, the BvgS protein is autophosphorylated and mediates the phosphorylation of BvgA through a series of phospho-transfer reactions (36, 38, 39). Upon phosphorylation, BvgA binds to the promoters of the virulence genes, inducing transcription of those genes (5–7, 9, 16, 29). Although the signals sensed by BvgS *in vivo* are unknown, it has been shown that when *B. pertussis* is grown at low temperatures, or in the presence of MgSO₄ or nicotinic acid, the BvgS-mediated phospho-transfer reactions are inhibited, and the expression of the *bvg*-activated genes is down regulated (a condition referred to as modulation) (36, 38, 39).

In addition to the set of genes that is activated by the *bvg* locus, a second set of genes have been identified which are repressed by the *bvg* locus (17). Initial studies identified five

bvg-repressed genes: *vrg6*, *vrg18*, *vrg24*, *vrg53*, and *vrg73* (17). A conserved 21-bp sequence, located within the coding region, was identified in each of these genes, and mutations in the conserved region in the *vrg6* gene were reported to cause a loss of repression resulting in constitutive expression of the gene (4). Replacement of the *vrg6* promoter sequence with that of the nonregulated *asd* gene was reported to have no effect on the *bvg*-mediated repression of the gene (2, 3). Southwestern analysis demonstrated the binding of a *bvg*-activated, 34-kDa protein to the consensus sequence of the *vrg6* gene (3). Taken together, these results suggested that the expression of the *bvg*-repressed genes was repressed by the binding of a *bvg*-activated repressor to the conserved element found at the 5' end of each of the *bvg*-repressed genes.

Transposon mutagenesis studies have identified *bvgR*, a *bvg*-activated gene located immediately downstream of *bvgS*, as the repressor of the *bvg*-repressed genes (19, 21). In-frame deletions of *bvgR* result in constitutive expression of the *bvg*-repressed genes without affecting the regulated expression of the *bvg*-activated genes (19, 21). A more recent study demonstrated that expression of BvgR is activated by the binding of phosphorylated BvgA to the *bvgR* promoter (20). Taken together, these studies indicate that BvgA represses the expression of the *bvg*-repressed genes through the activation of the repressor, BvgR.

In addition to the *bvgAS* regulatory system that represses expression of the *bvg*-repressed genes, a second two-component regulatory system that is required for the expression of the *bvg*-repressed genes was identified. This locus was designated as the *risAS* locus due to its association with reduced intracellular survival by *B. bronchiseptica* (15). Two groups independently identified the *risAS* locus: Jungnitz et al. identified the *risAS* locus as a region that is required for intracellular survival of *B. bronchiseptica*, while Stenson et al. identified the same locus as a region that is required for the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Source
Strains		
<i>E. coli</i> K-12		
TOP10	High-efficiency transformation	Invitrogen, Carlsbad, Calif.
S17	Tra functions of IncP plasmids integrated into chromosome	30
BL21(DE3)pLysS	<i>lacI</i> , <i>P_{lacUV5}</i> -T7 RNA polymerase, $\Delta ompT$, Δlon	Novagen, San Diego, Calif.
<i>B. pertussis</i>		
BP536	Tohama I, <i>Nal^r</i> <i>Str^r</i>	40
TM1627	Tohama I, <i>Nal^r</i> <i>Str^r</i> $\Delta risA$	This study
TM1793	Tohama I, <i>Nal^r</i> , <i>Str^r</i> , $\Delta bvgR$	This study
Bp106	Clinical isolate	CBER <i>Bordetella</i> Collection ^a
Bp188	Clinical isolate	CBER <i>Bordetella</i> Collection
Bp509	Clinical isolate	CBER <i>Bordetella</i> Collection
Bp10536	Clinical isolate	CBER <i>Bordetella</i> Collection
Plasmids		
pSS1577	<i>Ap^r</i> <i>Kan^r</i> <i>rpsL</i> <i>oriT</i> <i>cos</i>	35
pSS2809	<i>Ap^r</i> <i>Gm^r</i> <i>oriT</i> ; <i>B. pertussis</i> DNA, <i>E. coli</i> <i>lac</i> operon	8
pCR2.1-TOPO	TA cloning vector	Invitrogen
pET22b	LacI-repressed T7 promoter, 6His tag	Novagen
pTM119	<i>Ap^r</i> <i>Km^r</i> <i>rpsL</i> <i>oriT</i> <i>cos</i> <i>bvgR</i>	This study
pTM120	<i>Ap^r</i> <i>Km^r</i> <i>rpsL</i> <i>oriT</i> <i>cos</i> $\Delta bvgR$	This study
pTM255	pCR2.1-TOPO- <i>risA</i>	This study
pTM266	pCR2.1-TOPO- $\Delta risA$	This study
pTM268	<i>Ap^r</i> <i>Gm^r</i> <i>rpsL</i> <i>oriT</i> <i>cos</i> $\Delta risA$	This study
pTM275	pCR2.1-TOPO- <i>risA</i>	This study
pTM276	pET22b- <i>risA</i>	This study

^a CBER, Center for Biologics Evaluation and Research.

expression of two *bvg*-repressed surface antigens, *VraA* and *VraB*, in *B. pertussis* (15, 31, 32).

In this study, we investigated the regulation of the *bvg*-repressed gene, *vrg6*, by the *bvg4SR* and *ris4S* regulatory systems. We found that *risA* is essential for expression of the *bvg*-repressed genes and that the *RisA* protein binds to the promoter region of both the *vrg6* and *vrg18* genes. Although our data clearly demonstrate that *BvgR* is required for the repression of the *bvg*-repressed genes, we show that the putative repressor-binding site, conserved in each of the five *bvg*-repressed genes, is not required for the *Bvg*- or *Ris*-mediated regulation of the *bvg*-repressed genes in *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, 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. *B. pertussis* strains were grown on Bordet-Gengou (BG) agar (Difco, Detroit, Mich.) containing 1% proteose peptone (Difco) and 15% defibrinated sheep blood. Unless otherwise noted, the concentrations of antibiotics included in the medium were 10- μ g/ml gentamicin sulfate, 10- μ g/ml kanamycin sulfate, 100- μ g/ml streptomycin sulfate, and 10- μ g/ml nalidixic acid.

Bacterial conjugations. Prior to mating, *B. pertussis* strains were grown for 3 days and *E. coli* strains were grown overnight at 37°C. Matings between *E. coli* and *B. pertussis* strains were performed by swabbing bacteria from fresh plate cultures of each strain onto a BG agar plate supplemented with 10 mM MgCl₂. After 3 h of incubation at 37°C, bacteria were swabbed onto BG agar plates containing the appropriate antibiotics for the selection of exconjugants, and the plates were incubated at 37°C.

Construction of isogenic *bvgR* and *risA* knockout mutants. Strain TM1627, which bears an internal, in-frame deletion in *risA*, was constructed as follows: Oligonucleotide *risA*-F (5'-GCAGCGGGAAGACGAAGTTTCGA-3') was used in combination with oligonucleotide *risA*-R (5'-CCGTATGCGAATAGACAGGGCCGT-3') in a PCR using Tohama I chromosomal DNA as template.

The PCR product generated by the reaction was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.), generating pTM255. Plasmid pTM255 was digested with *Sac*II and was religated, generating pTM266, which has a deletion of a 204-bp fragment from the *risA* gene. Plasmid pTM266 was digested with *Eco*RI, and the fragment bearing the truncated *risA* gene was inserted into the *Eco*RI site of pSS1577 (35), generating pTM268. *E. coli* strain S17 bearing pTM268 was mated with *B. pertussis* strain BP536, and exconjugates in which the plasmid sequences had integrated into the chromosome were isolated by selection with gentamicin. An isolate in which plasmid sequences were lost from the chromosome, but in which the in-frame truncation of *risA* was retained, was isolated by selection for streptomycin resistance on BG plates and by screening with PCR. This strain was designated TM1627.

Strain TM1793, which bears an internal in-frame deletion in *bvgR*, was constructed as follows: The *Sa*II restriction fragment that contains the 3' end of the *bvgR* gene, and extends 304 bp downstream of *bvgR*, was excised from pBBR:BgB (19) and inserted into the *Sa*II site in pTM025 (21) to generate pTM119. Plasmid pTM119 was digested with *Apa*I, followed by treatment with mung bean nuclease to generate blunt ends, digestion with *Stu*I, and finally religation to circularize the plasmid. These manipulations resulted in the loss of an internal 606-bp fragment from *bvgR*. The plasmid bearing the in-frame internal truncation of *bvgR* was designated pTM120. *E. coli* strain S17 bearing pTM120 was mated with *B. pertussis* strain BP536, and exconjugates in which the plasmid sequences had integrated into the chromosome were isolated by selection with gentamicin. An isolate in which plasmid sequences were lost from the chromosome, but in which the in-frame truncation in *bvgR* was retained, was isolated by selection for streptomycin resistance on BG plates and by screening with PCR. This strain was designated TM1793.

Construction of *vrg* promoter fusions. *B. pertussis* strains bearing fusions of the *vrg6*, *vrg18*, *vrg24*, and *vrg73* promoters to *lacZ* were constructed as follows. Oligonucleotide pairs *vrg6*-F1/*vrg6*-B1, *vrg18*-F1/*vrg18*-B1, *vrg24*-F1/*vrg24*-B1, and *vrg73*-F1/*vrg73*-B1 were used in PCRs using Tohama I chromosomal DNA as a template (Table 2). The PCR products generated by the aforementioned PCRs were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.). The full-length *vrg6*, *vrg18*, *vrg24*, and *vrg73* promoter fragments were excised from pCR2.1-TOPO using the restriction enzymes, *Xba*I and *Sa*II, and the excised fragments were cloned into the previously described reporter plasmid, pSS2809 (8). The pSS2809 derivatives were subsequently transferred by conjugation from *E. coli*

TABLE 2. Sequences of oligonucleotides used to generate promoter deletion mutants

Primer	Sequence
<i>vrg6</i> -F1.....	5'-TCTAGATACTGCCACACATGACACCCG G-3'
<i>vrg6</i> -F2.....	5'-TCTAGATACGGCAAGCCAGGCGACCTGA TC-3'
<i>vrg6</i> -F3.....	5'-TCTAGAGATGAATTCTTGCCGAAACCCC G-3'
<i>vrg6</i> -F4.....	5'-TCTAGATATGGGTGCTGGAATTCGTCTG CTGAACC-3'
<i>vrg6</i> -F5.....	5'-TCTAGA-CCGCGTTTCAACCCATTTCAAAG CCGCGCC-3'
<i>vrg6</i> -F6.....	5'-TCTAGACGCGCGCTCCCGGACTGCGGGC GGTTGCGG-3'
<i>vrg6</i> -F7.....	5'-TCTAGACACAAATTTACGCGCAAAACAG CCGAGAAT-3'
<i>vrg6</i> -F8.....	5'-TCTAGAGCGCAGCAAGGCAGGTGAAAA TGAAAAAG-3'
<i>vrg6</i> -B1.....	5'-GTCGACGCATAACGGCTGGTGGAAGG-3'
<i>vrg6</i> -B2.....	5'-GTCGACAGTCCGGCAGCGCCGATGCCGG CAGCAAC-3'
<i>vrg6</i> -B3.....	5'-GTCGACGAACCACTTTTTCATTTTTCACCT GCCTTGC-3'
<i>vrg6</i> -B4.....	5'-GTCGACCGTGCAGCCAAGTTGACTAATC GACGGGCC-3'
<i>vrg6</i> -B5.....	5'-GTCGACCCGCAACCGCCCGCAGTCCGGG ACGCCGGC-3'
<i>vrg6</i> -B6.....	5'-GTCGACGGCGCGGCTTTGAAATGGGTTG AAACGCGG-3'
<i>vrg18</i> -F1.....	5'-TCTAGAGCCCTGTGAGTGATCGTGG-3'
<i>vrg18</i> -B1.....	5'-GTCGACTCTAGAGGATCCACCACGGC-3'
<i>vrg24</i> -F1.....	5'-TCTAGACCACGGCGCGTTGACTTGTG-3'
<i>vrg24</i> -B1.....	5'-GTCGACCATGGACAGCATGGGCATCA-3'
<i>vrg73</i> -F1.....	5'-TCTAGACTACCGCAGCGACTACATCA-3'
<i>vrg73</i> -B1.....	5'-GTCGACGTTTCGGAAACCAGCCTGAG-3'

strain S17 into *B. pertussis* strains BP536, TM1627, and TM1793. Selection for exconjugates was performed by plating onto BG plates containing gentamicin and nalidixic acid. The identities of the resulting strains were confirmed by PCR.

Construction of promoter deletion fusions. A nested set of 5' deletions of the *vrg6* promoter fused to the *lacZ* gene were constructed as follows. Oligonucleotide *vrg6*-B1 (Table 2) was used in combination with oligonucleotides *vrg6*-F1, -F2, -F3, -F4, -F5, -F6, -F7, and -F8 in PCRs using Tohamia I chromosomal DNA as a template. A nested set of 3' deletions of the *vrg6* promoter fused to the *lacZ* gene were constructed as follows. Oligonucleotide *vrg6*-F1 (Table 2) was used in combination with oligonucleotides *vrg6*-B2, -B3, -B4, -B5, and -B6 in PCRs using Tohamia I chromosomal DNA as a template. The PCR products generated by the aforementioned PCRs were cloned into pCR2.1-TOPO. The full-length promoter fragment and each of the 5' and 3' deletions were excised by digesting with XbaI and SalI, and the resulting fragments were cloned into the previously described reporter plasmid, pSS2809 (8). These plasmids were then transferred by conjugation from *E. coli* strain S17 into *B. pertussis* strains BP536, TM1627, and TM1793. Selection for exconjugates was performed by plating onto BG plates containing gentamicin and nalidixic acid. The identities of the resulting strains were confirmed by PCR.

Quantitative β -galactosidase assays. β -Galactosidase assays were performed as described by Miller (22) with minor modifications. Bacteria were recovered from the plates with a sterile swab and were resuspended in 3.5 ml of 1 M Tris-HCl, pH 8.0. The A_{600} was measured. For measurement of β -galactosidase activity, 50 μ l of cell suspension was added to 1 ml of Z-buffer (0.1 M sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 50 mM mercaptoethanol). Cells were permeabilized by adding 30 μ l of 0.1% sodium dodecyl sulfate and 30 μ l of chloroform, followed by vortexing. The remainder of the assay was performed as described by Miller (22). For quantification of β -galactosidase activity, units 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 (in milliliters) of permeabilized cells added to the assay.

Sequence analysis of the *risA* locus. Oligonucleotide *risA*-F (5'-GCCGGC GCGTGCCAGCAATTCCTG-3') was used in combination with oligonucleo-

tide *risA*-B (5'-GGCCTCAAGCCCTAAATTCTACGCT-3') in PCRs using chromosomal DNA from four randomly selected *B. pertussis* clinical isolates (Bp106, Bp188, Bp509, and Bp10536) as the template. The resulting PCR products containing the *risA* locus were sequenced with a BigDye Terminator v1.1 sequencing kit (Applied Biosystems), and the reactions were analyzed on ABI PRISM 3730xl DNA analyzers using Applied Biosystems sequence analysis software. The sequences were edited with Sequencher version 4.1.2, and the data obtained were assembled into contiguous sequences. The sequence of both strands of each of the four amplified DNA fragments was determined and compared to published sequences (15, 25).

Preparation of recombinant protein. An *E. coli* strain expressing recombinant RisA protein was constructed as follows. Oligonucleotide *risA*-F1 (5'-CTCGA GATGAACACGCAAAACACCACTCT-3') was used in combination with oligonucleotide *risA*-B1 (5'-CTCGAGACTGCCGCATCCGGAACGAAAC-3') in a PCR using Tohamia I chromosomal DNA as a template. The resulting PCR product containing the *risA* open reading frame was cloned into pCR2.1-TOPO, generating pTM275. The *risA* open reading frame was excised from pTM275 as an XhoI fragment and was inserted into the XhoI site in the expression vector pET22b (Novagen/EMD Biosciences, Inc., San Diego, Calif.), generating pTM276. Plasmid pTM276 bears a C-terminal fusion of a sequence encoding six histidine residues to the *risA* open reading frame under the transcriptional regulation of a recombinant T7 promoter engineered to be regulated by the *E. coli lac* repressor (LacI). Plasmid pTM276 was transformed into *E. coli* strain BL21(DE3)pLysS (Novagen/EMD Biosciences, Inc., San Diego, Calif.). *E. coli* strain BL21(DE3)pLysS, bearing pTM276, was grown in Luria broth at 37°C to an optical density of 0.6, and subsequently the expression of the *risA* gene was induced by the addition of IPTG (isopropyl- β -D-thiogalactopyranoside) to a final concentration of 1 mM. Four hours after induction, cells were harvested by centrifugation for 10 min at $5,000 \times g$ and were lysed with 8 M urea. Denatured protein was purified with the QIAGEN Ni-nitrilotriacetic acid protein purification kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. Refolding of denatured protein was carried out by slow gradient dialysis against dialysis buffer (10 mM HEPES-NaOH, 1 mM EDTA, 0.1 mM dithiothreitol [DTT], pH 7.4). After removal of insoluble protein by centrifugation, the supernatant was concentrated in a Centricon-10 centrifugal concentrator (Millipore, Billerica, Mass.). Glycerol was added to a final concentration of 40%, and the samples were stored at -20°C.

Gel shift assays. The *vrg6* promoter fragment generated by PCR using oligonucleotides F4 and B1 as described above, was excised from pCR2.1-TOPO by digestion with XbaI and SalI. Following gel purification, the fragments were end labeled with ^{32}P by T4-polynucleotide kinase reaction (Lofstrand, Gaithersburg, Md.). Gel-shift reaction mixtures contained 10 ng of probe (2×10^4 dpm/ng), 100 ng of poly(dI:dC), and 10 μ g of purified protein in binding buffer (10 mM Tris-HCl [pH 7.8], 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM DTT). Reactions were incubated for 20 min at 30°C. Prior to loading samples, the 6% polyacrylamide-Tris-borate-EDTA (TBE) gels (Invitrogen, Carlsbad, Calif.) were pre-electrophoresed for 30 min. Electrophoresis was performed at 15 V/cm, after which gels were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, Calif.), and the images were visualized with Imagequant software (Molecular Dynamics).

Generating mouse antiserum to recombinant RisA. Recombinant RisA was mixed 1:1 (vol/vol) with a 0.65% solution of Alhydrogel (aluminum hydroxide; Superfos a/s, Vedbaek, Denmark) to give a final protein concentration of 500 μ g/ml. Each of five female BALB/c mice was injected intraperitoneally with 0.1 ml of the protein-adjuvant solution (50 μ g of protein). Booster doses were given 2 and 4 weeks after the initial injection. After the second booster dose, blood was collected from the periorbital artery of each mouse. The serum was collected by centrifugation and was stored at -20°C.

Immunoblotting. After growth on BG plates in the presence or absence of 50 mM $MgSO_4$, bacteria were resuspended in Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 2.35% SDS, 100 mM DTT, 10% glycerol, 1 mM EDTA, 0.001% bromophenol blue), and were lysed by boiling. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide), and proteins were transferred to nitrocellulose membranes using a wet tank immunoblotter (Bio-Rad, Hercules, Calif.). Nonspecific binding sites on the membranes were blocked using 5% (wt/vol) dehydrated milk (Marvel) in phosphate-buffered saline (PBS; blocking solution), and were probed with anti-RisA mouse polyclonal antiserum diluted 1:1,000 in blocking solution. Membranes were washed in PBS, and antigen-antibody complexes were detected with rabbit anti-mouse immunoglobulin G (IgG) antibodies conjugated to horseradish peroxidase diluted 1:500 in blocking solution. Cross-reacting proteins were visualized with the TMB membrane peroxidase substrate system (KPL, Gaithersburg, Md.).

RESULTS

Regulation of *bvg*-repressed genes by the *risAS* and *bvgR* loci. Stenson et al. demonstrated that the expression of two distinct *bvg*-repressed surface antigens was dependent upon an intact *risAS* locus (31, 32). Based on this finding, we sought to determine if other *bvg*-repressed genes in *B. pertussis* were dependent upon the *risAS* locus for full expression. We cloned the promoter regions of four *bvg*-repressed genes (*vrg6*, *vrg18*, *vrg24*, and *vrg73*) into the promoter assay vector, pSS2809 (8), as described in Materials and Methods. This vector has a multiple cloning site for cloning promoter fragments upstream of a promoterless *lac* operon. It also has multiple tandem transcription terminators upstream of the cloning sites in order to minimize transcriptional read-through in this region. A 2-kb fragment of *B. pertussis* genomic DNA in the plasmid enables insertion of the plasmid via homologous recombination into the *B. pertussis* chromosome at a site distant from the *vrg6*, *vrg18*, *vrg24*, or *vrg73* loci. The activity of each promoter fusion was assayed after growth of the bacteria in the absence or presence of MgSO_4 in the wild-type, $\Delta bvgR$, and $\Delta risA$ genetic backgrounds (Fig. 1). As expected, all four *bvg*-repressed genes were expressed in the wild-type strain when bacteria were grown on BG plates in the presence of 50 mM MgSO_4 but were not expressed when grown on BG plates in the absence of MgSO_4 (Fig. 1). The expression of the four *bvg*-repressed genes was increased between 6- and 20-fold when the bacteria were grown in the presence of 50 mM MgSO_4 . When these same promoter fusions were tested in a strain bearing an in-frame deletion of the locus containing *bvgR* ($\Delta bvgR$), all four loci demonstrated the same high level of expression in the presence of 50 mM MgSO_4 (Fig. 1). Although all four loci demonstrated reduced levels of expression upon growth in the absence of 50 mM MgSO_4 , the level of expression of each of these genes was significantly higher than that observed in the wild-type background in the absence of 50 mM MgSO_4 . This result indicates that the expression of these four genes is derepressed in the absence of BvgR. When the transcriptional activity of the four *bvg*-repressed promoters was tested in a strain bearing an in-frame deletion of the *risA* gene ($\Delta risA$), the activity of each promoter was reduced to basal levels under all conditions tested (Fig. 1). These results confirm that expression of the four *bvg*-repressed genes, examined herein, is repressed by the product of the *bvgR* locus. These data also demonstrate that expression of these four genes is dependent on an intact *risA* locus.

RisS sequence analysis. The *B. pertussis* genome sequence was determined by Parkhill and colleagues at The Wellcome Trust Sanger Institute (25). According to the Sanger Institute sequence, the *B. pertussis* *risS* coding sequence has a frameshift mutation within a tract of three cytosine residues following codon 233. This frameshift was not present in the *B. pertussis* *risS* sequence reported by Jungnitz and colleagues (15). In order to determine if *risS* is a pseudogene in *B. pertussis*, we amplified and sequenced the DNA region containing the *risAS* locus from four independent clinical isolates of *B. pertussis*. The four isolates were selected randomly from the Center for Biologics Evaluation and Research *B. pertussis* strain collection. Isolates Bp106 and Bp108 were isolated in the 1930s. Isolate Bp509 was isolated in 1982. The isolation date of

FIG. 1. Expression of *bvg*-repressed genes in *bvgR* and *risA* knock-out strains. Fusions of the *vrg6*, *vrg18*, *vrg24*, and *vrg73* promoters to the *E. coli* gene encoding β -galactosidase were constructed and crossed onto the chromosome of wild-type strain BP536, strain BP536: $\Delta bvgR$, and strain BP536: $\Delta risA$ as described in Materials and Methods. The β -galactosidase activity expressed by each of the resulting reporter strains was determined after growth on BG plates in the presence (black bars) or absence (gray bars) of 50 mM MgSO_4 . The statistical significance for selected comparisons was determined by Student's *t* test analysis. The mean activity from each promoter in the $\Delta bvgR$ background when grown in the presence or absence of MgSO_4 was compared to those levels measured in wild-type bacteria under the same environmental conditions. Each result reported is the mean of at least four independent assays. Error bars represent the standard deviation from the mean. Statistically significant differences ($P < 0.01$) are indicated with two asterisks.

Bp10536 is unknown. The sequence of the *risAS* locus in all four clinical isolates was the same as that reported for the *B. pertussis* *risAS* locus by Parkhill et al.

Deletion analysis of the *vrg6* promoter. We generated deletion mutants in an effort to define the upstream and downstream boundaries of the *vrg6* promoter. A nested set of 5' and 3' deletions of the *vrg6* promoter was constructed by PCR as described in Materials and Methods (Fig. 2A). In order to determine the activities of these promoter derivatives in vivo,

FIG. 2. Deletion analysis of the *vrg6* promoter. 5' and 3' deletions of the *vrg6* promoter, fused to the *E. coli* gene encoding β -galactosidase, were constructed and crossed onto the chromosome of wild-type strain BP536, strain BP536: $\Delta bvgR$, and strain BP536: $\Delta risA$ as described in Materials and Methods. (A) Schematic diagram of the *vrg6* promoter deletions. The endpoints of each deletion, relative to the transcription start site, are shown in parentheses. The *vrg6* coding region is indicated by a black box, and the putative repressor-binding site is indicated by a gray box. (B) The

the deletion mutants were inserted into promoter assay vector pSS2809. The promoter deletion constructs were crossed into the wild-type, $\Delta bvgR$, and $\Delta risA$ genetic backgrounds, and the β -galactosidase activity of each reporter strain was determined after growth of the bacteria in the presence or absence of 50 mM $MgSO_4$. In the $\Delta risA$ background, all of the deletion fragments exhibited only a very low level of activity (Fig. 2B). This observation was in agreement with the previous finding that an intact *risA* locus is required for expression of the *vrp6* promoter (Fig. 1). In the wild-type background, promoter deletions $\Delta 1$ to $\Delta 4$ showed normal expression when the cells were grown in the presence of 50 mM $MgSO_4$, and approximately sixfold repression when the cells were grown in the absence of $MgSO_4$ (Fig. 2B; constructs $\Delta 1$ to $\Delta 4$). These levels are similar to that seen with the full-length promoter. In the $\Delta bvgR$ background, promoter deletions $\Delta 1$ to $\Delta 4$ had the same high level of expression as the full-length *vrp6* promoter when the bacteria were grown in the presence of 50 mM $MgSO_4$. In the absence of 50 mM $MgSO_4$, the level of expression observed in the $\Delta bvgR$ background was lower than that observed in the presence of $MgSO_4$ but was significantly higher than the expression observed in the wild-type background in the absence of $MgSO_4$. This analysis demonstrated that deletion of all of the sequences upstream of position -271 , relative to the transcription start site, does not affect the regulated expression from the *vrp6* promoter. Deletion of the sequences up to position -156 resulted in a complete loss of *vrp6* promoter activity under all conditions tested, indicating that sequences between -271 and -156 are essential for *vrp6* promoter activity (Fig. 2B; constructs $\Delta 5$ to $\Delta 8$).

Examination of the 3' promoter deletions revealed that deletion of all of the sequences downstream of position $+24$, relative to the transcription start site, does not affect the activation or repression of the *vrp6* promoter (Fig. 2B; constructs ΔC and ΔE). In the wild-type background, promoter deletions ΔC and ΔE showed normal levels of expression when the cells were grown in the presence of 50 mM $MgSO_4$ and approximately sixfold repression when the cells were grown in the absence of $MgSO_4$. In the $\Delta bvgR$ background, promoter deletions ΔC and ΔE demonstrated levels of expression similar to that of the full-length *vrp6* promoter when cells were grown in the presence of 50 mM $MgSO_4$. In the absence of 50 mM $MgSO_4$, the level of expression observed in the $\Delta bvgR$ background was lower than that observed in the presence of $MgSO_4$ but was significantly higher than the expression observed in the wild-type background in the absence of $MgSO_4$. In both the wild-type and $\Delta bvgR$ backgrounds, promoter deletion ΔD , which has a 3' endpoint between deletions ΔC and ΔE , demonstrated a higher level of expression than the full-length promoter and promoter deletions ΔC and ΔE . The expression of

promoter deletion ΔD was repressed upon growth in the absence of $MgSO_4$ in the wild-type background, and that degree of repression was significantly reduced in the $\Delta bvgR$ background. Deletion of sequences from the 3' end up to position -63 and beyond resulted in a complete loss of *vrp6* promoter activity under all conditions tested (Fig. 2B; constructs ΔA and ΔB). Taken together, these results indicate that no sequences upstream of position -271 or downstream of position $+24$ are required for the RisA-mediated activation or the BvgR-mediated repression of the *vrp6* gene. The results also indicate that the sequences downstream of $+1$, previously identified as the BvgR-binding site, are not required for repression of *vrp6* expression.

In vitro binding of RisA to the *vrp6* promoter. The 5' and 3' deletion analyses identified the upstream and downstream boundaries of the *vrp6* promoter, defining a 295-bp region (-271 to $+24$) that was required for the regulated activity of the *vrp6* promoter. Since both RisA and BvgR have been shown to affect expression of *vrp6*, we hypothesized that binding sites for both proteins may be found within this 295-bp region. In order to directly evaluate the interaction between RisA and BvgR with the *bvg*-repressed promoters, we conducted gel-shift assays. The coding sequences for both BvgR and RisA were cloned into expression vector pET22b, and the expressed proteins were purified as described in the Materials and Methods. Both the BvgR and RisA proteins were expressed in large quantities upon induction and formed inclusion bodies in *E. coli*. RisA refolded in soluble form upon serial dialysis. However, BvgR remained an insoluble aggregate even after dialysis. Therefore, we conducted the gel-shift assays using only RisA. The addition of purified RisA protein to a reaction mixture containing ^{32}P -labeled *vrp6* promoter fragment resulted in a mobility shift of the labeled promoter fragment (Fig. 3A). Increasing the amount of protein added to a constant amount of ^{32}P -labeled promoter fragment resulted in an increase in the amount of probe that was shifted upward (Fig. 3A). The binding interaction was specific as demonstrated by competition for binding of RisA to the ^{32}P -labeled fragment by unlabeled *vrp6* promoter fragments (Fig. 3B). The RisA protein bound to the ^{32}P -labeled *vrp6* promoter fragment in the absence of unlabeled DNA competitor. This binding was completely blocked by the addition of unlabeled *vrp6* and *vrp18* promoter DNA but was not blocked by the addition of unlabeled *asd* or *sodB* promoter DNA (Fig. 3B).

We utilized 45-bp, double-stranded oligonucleotides bearing sequences derived from the *vrp6* promoter region, to identify RisA binding regions on the *vrp6* promoter. We evaluated the ability of the double-stranded oligonucleotides to compete with the ^{32}P -labeled *vrp6* probe for binding of RisA. A set of 15 double-stranded oligonucleotides that spanned the *vrp6* pro-

β -galactosidase activity expressed by each of the reporter strains was determined after growth on BG plates in the presence (black bars) or absence (gray bars) of 50 mM $MgSO_4$. The statistical significance for selected comparisons was determined by Student's *t* test analysis. The mean of the activity of each promoter deletion in the presence of $MgSO_4$, in the wild-type and $\Delta bvgR$ backgrounds, was compared to the mean of the activity of the full-length promoter in the presence of $MgSO_4$, in the wild-type background. *, $P < 0.05$; **, $P < 0.01$. For each promoter, no statistically significant difference was observed between the mean of the activity in the presence of $MgSO_4$, in the $\Delta bvgR$ background, and the mean of the activity of the same construct in the presence of $MgSO_4$, in the wild-type background. For each promoter, the mean of the activity in the absence of $MgSO_4$, in the $\Delta bvgR$ background, was compared to the mean of the activity of the same construct in the absence of $MgSO_4$, in the wild-type background. †, $P < 0.01$. Each result reported is the mean of at least four independent assays. Error bars represent the standard deviation from the mean.

FIG. 3. Binding of RisA to the *vrg6* promoter. Gel shifts were performed with 32 P-labeled *vrg6* promoter DNA and purified recombinant RisA protein as described in Materials and Methods. (A) 32 P-labeled *vrg6* promoter DNA was added to the gel-shift reaction mixture containing either no RisA, undiluted RisA, or serial fivefold dilutions of RisA. (B) 32 P-labeled *vrg6* promoter DNA was added to the gel-shift reaction mixture containing either no RisA protein, RisA protein with no DNA competitor, or RisA protein with excess amounts of double-stranded competitor DNA bearing either the *vrg6*, *vrg18*, *asd*, or *sodB* promoter sequences.

motor was constructed (Table 3). The first eight oligonucleotides (no. 1 to 8) spanned the 295-bp region known to be required for the RisA-mediated activation of the promoter. The next seven oligonucleotides (no. 12, 23, 34, 45, 56, 67, and 78) were constructed to overlap the first eight oligonucleotides in order to account for any binding sites that may be disrupted by linker design. The ability of these linkers to compete with the 32 P-labeled *vrg6* promoter DNA for RisA-binding was eval-

TABLE 3. Sequences of the double-stranded DNA linkers used for competition of RisA binding to the *vrg6* promoter

Linker 1	5'-GAACCAGAACGGGCATTGATCCTCGTGGC GGACGGGCCACGCAAG-3' 3'-CTTGCGTGGCCCGTCCGCCACGAGGATCA ATGCCCGTTCTGGTTC-5'
Linker 2	5'-GTGTGGAAATGTAACCCACCTGGGGCCC GCCTTGGCTCCCCCG-3' 3'-CACACCTTTACATTGGGGTGGACCCCGGG CGGAACCGAGGGGGGC-5'
Linker 3	5'-CGTTTCAACCATTTCAAAGCCGCGCCCGC CCCAGCGGGACAAAT-3' 3'-GCAAAGTTGGGTAAAGTTTCGGCGCGGGC GGGGTCGCCCTGTTTA-5'
Linker 4	5'-TTGCCGCAACGCAGCAAACGCCGGCGTCC CGGACTGCGGGCGGT-3' 3'-AACCGCCGCGAGTCCGGGACGCCGGCGTT TGCTGCGTTGCGGCA-5'
Linker 5	5'-GCGGATTGTCGCCATTGCGGGGGCTGTTT TCACAAATTACGCGC-3' 3'-CGCCTAACAGCGGTAACGCCCCGACAAA AGTGTTTAAATGCGCG-5'
Linker 6	5'-AAAACAGCCGAGAATAGGCCCGTCGATTA GTCAACTTGGCTGCAC-3' 3'-TTTTGTGCGGCTCTTATCCGGGCAGCTAATC AGTTGAACCGACGTG-5'
Linker 7	5'-GCGCAGCAAGGCAGGTGAAAAATGAAAA AGTGGTTCGTTGCTGCC-3' 3'-CGCGTCGTTCCGTCCACTTTTACTTTTCA CCAAGCAACGACGG-5'
Linker 8	5'-GGCATCGGCGCTGCCGGACTCATGCTCTC CAGCGCCGCCCTGGCC-3' 3'-CCGTAGCCGCGACGGCCTGAGTACGAGAG GTCGCGGCGGGACCGG-5'
Linker 12	5'-TCGTGGCGGACGGGCCACGCAAGGTGTG GAAATGTAACCCACCT-3' 3'-AGCACCGCCTGCCGGTGCCTTCCACACCT TTACATTGGGGTGA-5'
Linker 23	5'-GGGGCCCGCCTTGGCTCCCCCGCGTTTCA ACCCATTTCAAAGCC-3' 3'-CCCCGGGCGGAACCGAGGGGGCGCAAA GTTGGGTAAAGTTTCGG-5'
Linker 34	5'-GCGCCCGCCCCAGCGGGACAAATTTGCCG CAACGCAGCAAACGCC-3' 3'-CGCGGGCGGGTGCCTGTTTAAACGGC GTTGCGTCGTTTTCGG-5'
Linker 45	5'-GGCGTCCCGGACTGCGGGCGGTTGCGGAT TGTCGCCATTGCGGGG-3' 3'-CCGCAGGGCCTGACGCCGCCAACGCCTA ACAGCGGTAACGCCCC-5'
Linker 56	5'-GCTGTTTTACAAATTACGCGCAAAACAG CCGAGAATAGGCCCG-3' 3'-CGACAAAAGTGTAAATGCGCGTTTGTG GGCTCTTATCCGGGC-5'
Linker 67	5'-TCGATTAGTCAACTTGGCTGCACGCGCAG CAAGGCAGGTGAAAAA-3' 3'-AGCTAATCAGTTGAACCGACGTGCGCGTC GTTCCGTCCACTTTT-5'
Linker 78	5'-TGAAAAAGTGGTTCGTTGCTGCCGGCATC GGCGTGCCTGGACTCA-3' 3'-ACTTTTTCACCAAGCAACGACGGCCGTAG CCGCGACGGCCTGAGT-5'

uated with gel-shift assays (Fig. 4). Four linkers (no. 2, 5, 12, and 56) were identified that competed with the 32 P-labeled *vrg6* probe (Fig. 4). Linkers 2 and 12 overlap each other, as do linkers 5 and 56. However, linkers 2 and 12 do not overlap with linkers 5 and 56 (Fig. 5B). Our results, therefore, define two

FIG. 4. Identification of RisA binding regions. Gel-shift assays were performed with 32 P-labeled *vrg6* promoter DNA and purified recombinant RisA protein as described in Materials and Methods. 32 P-labeled *vrg6* promoter DNA was added to gel-shift reaction mixture containing either no RisA protein or undiluted RisA protein and excess double-stranded linker DNA derived from the *vrg6* promoter sequence. The sequence of each of the linkers is provided in Table 3. The position of each linker sequence within the *vrg6* promoter is shown schematically in Fig. 5. (A) Competition of RisA binding to 32 P-labeled *vrg6* promoter DNA by linkers 1 to 8. (B) Competition of RisA binding to 32 P-labeled *vrg6* promoter DNA by linkers 12 to 78.

distinct regions of approximately 25 bp each, which contain a RisA binding site.

Sequence analysis of putative RisA binding site. An examination of the sequences of the two 25-bp regions that were shown to bind RisA revealed a conserved 7-bp sequence (5'-AAATG/TTA-3'; Fig. 5B). A search of the 500 bp upstream of the start of translation of the *vrg6*, *vrg18*, *vrg24*, and *vrg73* genes identified three matches to this sequence in the *vrg6* promoter region, two matches to this conserved sequence in the *vrg18* promoter region, five matches in the *vrg24* promoter region, and a single match in the *vrg73* promoter region (Fig. 5C).

Effect of *bvgR* and modulation on RisA expression and stability. The observation that the presumed repressor-binding site in *vrg6* does not contribute to repression of gene expression compelled us to consider the alternative mechanisms by which BvgR may repress its target genes. We examined the possible role of BvgR in the expression and stability of RisA. The *risA* and *bvgR* promoters were inserted into the promoter assay vector pSS2809. The promoter constructs were crossed into the wild-type, Δ *bvgR*, and Δ *risA* backgrounds, and the β -galactosidase activity of each reporter strain was determined after growth in the presence or absence of 50 mM MgSO_4 (Fig. 6A and B). The expression and regulation of the *bvgR* promoter were the same in the wild-type, Δ *bvgR*, and Δ *risA* backgrounds, indicating that *bvgR* does not regulate its own expression, nor is its expression regulated by *risA* (Fig. 6A). The expression of the *risA* promoter was the same in the wild-type and Δ *risA* backgrounds but higher in the Δ *bvgR* background, indicating that *risA* does not regulate its own expression (Fig. 6B).

Polyclonal antiserum to recombinant RisA was raised in mice and was used to probe immunoblots containing cell lysates prepared from wild-type, Δ *bvgR*, and Δ *risA* strains after growth in the presence or absence of 50 mM MgSO_4 (Fig. 6C). As expected, no RisA could be detected in the Δ *risA* mutant strain. The amounts of RisA protein present in cell lysates were the same in both the wild-type and Δ *bvgR* backgrounds regardless of the growth conditions. These results indicate that the level of expression and the stability of RisA are not affected by BvgR- or *bvg*-mediated regulation.

DISCUSSION

The mechanism by which the expression of the *bvg*-repressed genes is regulated is unknown. We demonstrated previously that, in *B. pertussis*, the expression of the *vrg6* and *vrg73* genes is repressed by the *bvgAS*-activated protein BvgR (21). Jungnitz et al. identified a locus in *B. bronchiseptica*, which they designated as the *risAS* locus, that was required for survival of *B. bronchiseptica* in eukaryotic cells and contributed to persistence of the bacterium in a mouse infection model (15). Stenson et al. independently identified the *risAS* locus in *B. pertussis* and demonstrated that it was required for the expression of two distinct *bvgR*-repressed surface antigens (31, 32). In this study, we examined the roles of BvgR and RisA in the regulation of four *bvg*-repressed genes in *B. pertussis* (*vrg6*, *vrg18*, *vrg24*, and *vrg73*). Our results demonstrate that the repression of expression of all four of these genes is dependent upon BvgR and the activation of expression of these genes is dependent upon RisA (Fig. 1). The observation that the expression of all of the *bvg*-repressed genes examined to date (*vrg6*, *vrg18*, *vrg24*, *vrg73*, and the *bvg*-repressed surface antigens described by Stenson et al.) is dependent upon RisA is striking. It suggests that there is a large, if not complete, overlap of the BvgR and RisA regulons. This might be expected if, for example, BvgR represses expression of the *bvg*-repressed genes by controlling the activity of RisA. Microarray and proteomic approaches should be undertaken to define the BvgR and RisA regulons, in order to evaluate the extent to which they overlap.

Parkhill and colleagues first determined that in *B. pertussis* the *risS* gene is disrupted by a frameshift mutation (25). We have confirmed the presence of the same frameshift in the *risS* gene in four randomly selected clinical isolates. The isolation of these four strains was separated both geographically and by time (separated by nearly 50 years). It is clear from our results that the *risA* locus is required for expression of the *bvgR*-repressed genes; yet it is equally clear that the *risS* gene does not encode a functional RisS protein. Given these facts, we conclude that either RisA is able to activate transcription at its target promoters in the unphosphorylated state or RisA is phosphorylated by a sensor kinase other than RisS.

FIG. 5. Putative consensus RisA binding site. (A) Schematic diagram of the *vrg6* promoter sequence. The black box marks the *vrg6* coding region, and the gray box marks the putative repressor-binding site. The arrow marks the transcription start site (+1). (B) Schematic diagram showing the positions of the double-stranded linkers used as competitors in the gel-shift assays. The linkers that inhibited binding of RisA protein to the ³²P-labeled *vrg6* promoter DNA are circled, and the sequences of the regions of overlap between the two sets of linkers that inhibited binding of RisA protein to the *vrg6* promoter DNA are shown. A conserved sequence found in both overlapping regions is shaded. (C) Alignment of sequences from the *vrg6*, *vrg18*, *vrg24*, and *vrg73* promoter regions that were found to contain the conserved element (putative RisA binding site). The conserved element in each sequence is shaded. Because the transcription start site for four of the five genes is unknown, the upstream and downstream boundaries of each sequence relative to the translation start site (ATG) of each gene are shown in parentheses.

Once we demonstrated that RisA activates the expression of the *bvg*-repressed genes, we focused on identifying the *cis*-acting sequences that are required for RisA activation of the *bvg*-repressed genes. We noted that Beattie et al. had performed a deletion analysis of the *vrg6* promoter (2) and demonstrated that as progressively larger fragments of the promoter were deleted from the upstream side between positions -428 and -221, relative to the transcription start site, almost a fourfold drop in *vrg6* promoter activity was observed. Beattie et al. also observed a fivefold drop in promoter activity when the deletions were extended further to position -186, and activity was further diminished as the sequence was deleted even further toward the transcription start site. The activity of all of the constructs generated by Beattie et al. appeared to be

FIG. 6. Regulation of *bvgR* and *risA* expression. Fusions of the *bvgR* and *risA* promoters to the *E. coli* gene encoding β -galactosidase were constructed and crossed onto the chromosome of wild-type strain BP536, strain BP536: Δ *bvgR*, and strain BP536: Δ *risA* as described in Materials and Methods. The β -galactosidase activity expressed by each of the *bvgR-lacZ* reporter strains (A) and each of the *risA-lacZ* reporter strains (B) was determined after growth on BG plates in the presence (black bars) or absence (gray bars) of 50 mM MgSO₄. (C) Immunoblot analysis of samples prepared with protein extracts obtained from wild-type strain BP536, strain BP536: Δ *bvgR*, and strain BP536: Δ *risA* after growth on BG plates in the presence (+) or absence (-) of 50 mM MgSO₄. The blots were probed with anti-RisA polyclonal antibody. The statistical significance for selected comparisons was determined by Student's *t* test analysis. The mean activity from either the *bvgR* or *risA* promoter, in the presence or absence of MgSO₄ in each mutant background, was compared to that of the same promoter in wild-type bacteria grown under the same environmental conditions. Statistically significant differences are indicated with an asterisk ($P < 0.05$) or a double asterisk ($P = 0.01$). Each result reported is the mean of at least four independent assays. Error bars represent the standard deviation from the mean.

repressed upon growth under modulating conditions. These data suggested that an important promoter element lay between positions -221 and -186 . However, it also appeared that sequences upstream of position -221 contributed to promoter activity. We noted that Beattie and colleagues performed their analysis with promoter fusions carried on a plasmid present at a concentration of five to seven copies per cell. We hoped a deletion analysis performed with fusions maintained in single copy would yield a clearer picture of the upstream boundary of the *vrg6* promoter. We also sought to take advantage of the ability to examine the activity of each fusion in both a $\Delta bvgR$ background and a $\Delta risA$ background. Our deletion analysis of the *vrg6* promoter demonstrated that all of the sequences required for regulated expression of *vrg6* were downstream of position -271 relative to the transcription start site (Fig. 2). All of the deletion fragments with upstream boundaries either at or further upstream than position -271 retained 100% of promoter activity. This activity was dependent upon RisA and was repressed by BvgR. All of the upstream deletions that extended into the promoter to position -159 or beyond lost all RisA-dependent promoter activity, and the basal level of expression observed in those fusions was not repressed by BvgR (Fig. 2). Our results are in general agreement with those of Beattie et al. Our results indicate that the upstream boundary of an important promoter element lies between positions -271 and -159 relative to the transcription start site. However, our results clearly demonstrate that no sequences upstream of position -271 contribute to promoter activity. Finally, we did not see any evidence of BvgR-mediated repression of promoter fusions that had 5' deletions that extended beyond position -159 .

We extended the promoter deletion analysis by generating deletions from the 3' end (Fig. 2). Our results demonstrate that promoter fragments with deletions from the 3' end that extended as far as position $+24$, relative to the transcription start site, retained 100% of promoter activity. The activity of all three of these promoter fusions was dependent upon RisA, and, most significantly, despite the lack of the putative repressor-binding site in two of the constructs, BvgR repressed the activities of all three of these fusions. As expected, deletions from the 3' end that extended as far as positions -63 and -130 , relative to the transcription start site, lost all RisA-dependent promoter activity and BvgR did not repress the basal level of expression observed in those fusions (Fig. 2). Therefore, we have concluded that sequences downstream of the start of transcription are not required for repression of the *vrg6* gene and probably are not required for repression of any of the *bvg*-repressed genes. In retrospect, reexamination of the data that led to the identification of the repressor-binding site within the coding region of *vrg6* suggests that what was interpreted previously as a loss of repression of the *vrg6* promoter constructs (2, 3) was in fact a loss of *vrg6* promoter induction.

Our promoter deletion analysis demonstrated that all of the sequences required for the regulated expression of the *vrg6* gene lay between position -271 and $+24$, relative to the transcription start site. Since all of the promoter fragments that demonstrated activity were repressed by BvgR, we concluded that the sequences required for activation of the promoter by RisA, and repression of the promoter by BvgR, lie within that 295-bp region. We attempted to overexpress and purify both

BvgR and RisA for use in an in vitro DNA-binding assay. Although the purification of RisA was relatively straightforward, multiple strategies to purify soluble BvgR were unsuccessful. A gel-shift analysis clearly demonstrated that purified RisA binds to the *vrg6* promoter (Fig. 3). Binding of RisA to a 32 P-labeled *vrg6* promoter fragment was blocked by excess unlabeled DNA fragments containing the *vrg6* or *vrg18* promoters but not by unlabeled fragments containing the *asd* or *sodB* promoters. This result indicated that binding of RisA to the *vrg6* promoter was sequence specific and also demonstrated that RisA binds to the *vrg18* promoter. By evaluating the ability of a collection of 15 overlapping 45-bp double-stranded oligonucleotides bearing sequences from the *vrg6* promoter, to block binding of RisA to the 32 P-labeled *vrg6* promoter fragment, two distinct RisA-binding regions were identified (Fig. 4). Although none of the linkers completely eliminated the gel mobility shift, four linkers clearly inhibited RisA binding to the *vrg6* probe, resulting in a reduced shift in mobility. Interestingly, these four linkers consisted of two pairs of overlapping linkers. This observation suggested that the RisA binding regions are located on the two 22-bp regions defined by the overlap between linkers 2 and 12 and linkers 5 and 56. A comparison of these two sequences revealed a conserved 7-bp sequence that is present in both regions (5'-AAA TT/GTA-3') (Fig. 5B). Although close approximations of this sequence are found in the *vrg18*, *vrg24*, and *vrg73* promoter sequences (Fig. 5C), it should be noted that a nearly perfect match to this sequence (5'-AAATTTG-3') is found at a third position in the *vrg6* promoter, and is incorporated into linker 34, which did not compete with the *vrg6* promoter probe for binding of RisA. Although it is tempting to focus on this sequence element, definition of the specific sequences that contribute to the binding of RisA will require a more rigorous analysis of RisA binding to the *risA*-activated promoters.

To date, attempts at identifying specific *cis*-acting sequences involved in repression of expression of the *vrg6* gene have not been successful. This has led us to consider alternative models for BvgR-mediated repression of *vrg6* promoter activity (i.e., models that do not require direct binding of BvgR to the *bvg*-repressed promoters). One possibility is that BvgR represses the expression of RisA or reduces the stability of RisA once it is synthesized. To address this question, we examined the expression of *risA-lacZ* fusions in the wild-type, $\Delta bvgR$, and $\Delta risA$ backgrounds (Fig. 6B). Our results indicated that BvgR does not repress *risA* transcription. It also indicated that *risA* transcription is not autoregulated. This conclusion was confirmed by immunoblot analysis which demonstrated the presence of steady-state levels of RisA in wild-type, $\Delta bvgR$, and $\Delta risA$ strains (Fig. 6C). The observation that the amount of RisA present in cells is constant in the wild-type and $\Delta bvgR$ genetic backgrounds, after growth in the presence or absence of $MgSO_4$, demonstrates that BvgR neither regulates the transcription or translation of RisA, nor does it mediate its stability once the protein is expressed. It seems unlikely that BvgR acts independently of RisA at the *vrg6* promoter since BvgR does not repress the residual RisA-independent activity of the *vrg6* promoter. We speculate that if BvgR acts by binding to the *vrg6* promoter, its binding may interfere with the binding of RisA rather than interfere with the binding of the polymerase. Alternatively, BvgR may not directly repress expression of the

bvg-repressed genes, but rather, it may exert its effect indirectly through *RisA* either by binding *RisA* and preventing its interaction with the promoter or by modifying *RisA* so that the protein is no longer active. Ongoing work in our laboratory is directed toward distinguishing between these alternative models.

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