Bordetella pertussis risA, but Not risS, Is Required for Maximal Expression of Bvg-Repressed Genes

Trevor H. Stenson, Andrew G. Allen, Jehan A. al-Meer, Duncan Maskell, and Mark S. Peppler

Department of Medical Microbiology and Immunology, University of Alberta, 1-69 Medical Sciences Bldg., Edmonton, Alberta T6G 2H7, Canada, and Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, United Kingdom

Expression of virulence determinants by Bordetella pertussis, the primary etiological agent of whooping cough, is regulated by the BvgAS two-component regulatory system. The role of a second two-component regulatory system, encoded by risAS, is not defined. Here, we show that mutation of B. pertussis risA does not affect Bvg-activated genes or proteins. However, mutation of risA resulted in greatly diminished expression of Bvg-repressed antigens and decreased transcription of Bvg-repressed genes. In contrast, mutation of risS had no effect on the expression of Bvg-regulated molecules. Mutation of risA also resulted in decreased bacterial invasion in a HeLa cell model. However, decreased invasion could not be attributed to the decreased expression of Bvg-repressed products, suggesting that mutation of risA may affect the expression of a variety of genes. Unlike the risAS operons in B. parapertussis and B. bronchiseptica, B. pertussis risS is a pseudogene that encodes a truncated RisS sensor. Deletion of the intact part of the B. pertussis risS gene does not affect the expression of risA-dependent, Bvg-repressed genes. These observations suggest that RisA activation occurs through cross-regulation by a heterologous system.

Bordetellae are gram-negative coccobacilli that cause respiratory diseases in several host species. These include whooping cough in humans, caused by Bordetella pertussis and human strains of B. parapertussis, B. pertussis, B. parapertussis, and B. bronchiseptica are closely related and are considered to be subspecies within the B. bronchiseptica cluster (4, 24, 49, 63). In particular, B. pertussis and B. parapertussis appear to have evolved independently from a progenitor bacterium most similar to B. bronchiseptica through selective gene loss and inactivation (14, 49). These subspecies share many similarities but possess interesting differences, which are likely to account for their different host specificities and abilities to cause disease (27, 28). One clear similarity is the presence in all of these three bordetellae of the BvgAS two-component regulatory system, encoded by the bvg locus (for Bordetella virulence gene), which controls the expression of virulence determinants in all three subspecies (36, 53).

BvgAS is a member of the large family of two-component response regulators. These are comprised of a sensor protein (that is generally transmembrane) and a cytoplasmic transcriptional regulator protein. These systems respond to environmental signals and regulate target gene transcription (46). Specifically, the BvgS sensor can respond to signals such as temperature and high concentrations of b-vitamin derivatives (e.g., nicotinic acid) and sulfate anions (34, 40). Under permissive conditions, such as growth at 37°C in low sulfate and nicotinic acid concentrations, BvgS initiates a complex phosphorylation cascade that ultimately results in phosphorylation of BvgA (62). Phosphorylated BvgA then acts as a transcriptional activator at cis-acting sites for a number of genes known as Bvg-activated genes, or vags (52). An additional level of regulatory complexity is evident in that the vags can be separated into early- and late-activated genes whose temporal expression differs when the bacteria are switched between conditions (32, 54). When Bordetella spp. are grown in vitro at low temperatures or in the presence of high sulfate or nicotinic acid concentrations, the BvgAS system is inactive: there are low levels of phosphorylated BvgA transactivator, resulting in low, basal, or virtually absent transcription of the individual vags and low expression of their encoded virulence factors. This process has been called antigenic or phenotypic modulation, and the chemicals that induce it are called modulators (34, 40). Modulation also results in the up-regulation of the expression of another set of genes, known as Bvg-repressed genes, or vrgs (33).

The vags include the bvgAS locus itself (51), bvgR encoding a transcriptional repressor (41), and a broad set of other genes (9, 25, 35, 38, 50). The vrg-encoded products include the majority of known virulence factors of these pathogens and are shared mostly between B. pertussis, B. parapertussis, and B. bronchiseptica. Notable exceptions include pertussis toxin, which is believed to be expressed only in B. pertussis (5); PagP, which is apparently not expressed in B. pertussis (50); and the Bcr type III secretion system, which, despite being transcriptionally active in B. pertussis, is nonfunctional (29, 38).

In contrast, the vrgs encode factors whose expression is more...
dissimilar between the broad-host-range pathogen *B. bronchiseptica* and the obligate human pathogen *B. pertussis* (14, 49). Their products include *B. bronchiseptica*-specific flagella (2) and urease (39) and *B. pertussis*-specific vrs and antigens (Vras) with unknown functions (3, 29, 33, 57). The repression of the vgs appears to be mediated principally through the vag-encoded transcriptional repressor BvgR, which is believed to bind at cis-repressive sites of the vgs genes (7, 41, 42). The expression of the vgs is maximal late in the bacterial growth phase (55), and individual vgs are differentially modulated by individual in vitro modulators (29). In addition to the vgs and the vrgs, there is a third gene class, Bvg-intermediate, exemplified by the *bipA* gene (60) whose transcription is maximal at semimodulating conditions (16).

A second two-component regulatory system, RisAS, has been described and analyzed in *B. bronchiseptica* (30, 65). This system is related to the EnvZ-OmpR systems of other gram-negative bacteria including *Encherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Shigella flexneri* (22) that have cis-regulately induce the expression of porins OmpC and OmpF in response to osmolality. Interestingly, they are also implicated in bacterial virulence. For example, *Xenorhabdus nematophilus envZ* mutants have decreased the production of a number of outer membrane proteins and are less pathogenic in host to-bacco worms (23), *S. enterica serovar Typhimurium* *ompR* mutants are less virulent in mice (18), *ompR* is required for *Yersinia enterocolitica* survival in macrophages (10), and both *ompR* and *envZ* mutants of *S. flexneri* are less able to invade HeLa cells (8).

The RisAS system is considered to be an ortholog, not an homolog, of OmpR/EnvZ. Its expression is not regulated as an environmental signal and apparently does not regulate the expression of porins in *B. bronchiseptica* (30). Rather, RisAS coordinately regulates a variety of factors, acting independently of Bvg-mediated regulation. Furthermore, Ris expression is induced when *B. bronchiseptica* is intracellular and is required for intracellular survival, resistance to oxidative stress, and persistence in a mouse infection model (30, 65). This implicates Ris as a second two-component regulatory system important in the pathogenesis of the bordetellae.

In this study, we describe a comprehensive analysis of the RisAS system in *B. pertussis* and show that *risA* affects the transcription and expression of vgs but not the vrgs, whereas *risS* does not affect vrg or vag expression probably because it is a pseudogene in *B. pertussis*. *risA* mutants adhere just as well and wild-type bacteria but are less efficient at invasion of HeLa cells, indicating a role in the pathogen-host relationship for this system. RisA can function independent of RisS, suggesting the possibility of cross talk between regulatory systems and the integration of multiple regulatory signals in the expression of Bvg-regulated molecules in *B. pertussis*.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. *Bordetella pertussis* strains were grown on Bordet-Gengou agar (BGA; Difco, Detroit, MI) containing 15% sheep's blood (Dalyn, Calgary, Alberta, Canada) for 3 days at 37°C in a humidified atmosphere prior to experimental assays, unless otherwise noted. To induce modulation, *B. pertussis* strains were grown on BGA containing 5 mM nicotinic acid and 40 mM MgSO₄ (7, 25). *E. coli* strains were grown in L broth or on L agar. When necessary, the following antibiotics at the indicated concentrations were added to the media: nalidixic acid, 30 μg/ml; gentamicin, 15 μg/ml (for maintenance of *B. pertussis* and *E. coli* strains) or 30 μg/ml (for selection of transconjugants); ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; streptomycin, 200 μg/ml; and tetracycline, 10 μg/ml. PCR, DNA isolation, cloning, and sequencing were performed by standard methods. Clones were confirmed by sequence analysis, restriction analysis, or Southern hybridization as appropriate.

**Mutagenesis of the risA and risS genes in *B. pertussis***. The Bordetella pertussis *risAS* locus was identified and cloned by our own unpublished analysis and confirmed through analysis of published sequences (30) and the genomic sequence of *B. pertussis* (49). The genomic sequence data were produced by the *B. pertussis* Sequencing Group at the Sanger Centre and were obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bp/. Allelic replacement of an insertional disrupted *risA* in BP536 (59) was performed using the allelic exchange vector pRPT1 (58). Briefly, PCR analysis of a transduced *B. pertussis* DNA cosmid library identified one colony whose DNA acted as a template for the amplification of *risA*. The cosmid library was constructed in the vector pHC79 (Boehringer Ingelheim, Ridgefield, CT) from *B. pertussis* CN2992 (Wellcome collection) chromosomal DNA partially digested with Sau3A and transfected into *E. coli* XL1-Blue (Stratagene, La Jolla, CA). PCR was performed with primers bpORF (5′-CTGCTGGTTCATCCGCTC-3′) and bpORR (5′-GTTGAAGGCGGTTGGAC-3′), which generated a 200-bp *risA* fragment. The *risA*-containing cosmid, pBP2, was digested with SalI-ClaI to release a 1.7-kb DNA fragment which was cloned into plBluescript II KS (+) (Stratagene) to create pBP2. A kanamycin resistance (neo) cassette was inserted at the EcoRI site of the *risA* fragment in pBP2 to create pBP15. Insertionally disrupted *risA* DNA was released as a 3.5-kb PvuII fragment and cloned into pRPT1 to create pBP16. Trparental matings were used to mobilize pBP16 from donor *E. coli* CC118 (15) into recipient *B. pertussis* BP536 using *E. coli* plasmid-mobilizing helper strain SM10 (12). Double-crossover events were selected on media containing streptomycin and kanamycin. Southern hybridization using genomic DNA from resultant colonies confirmed recombination and mutagenesis. One mutant, BPOR, was chosen for further analysis.

To address the possibility that the insertion of the neo gene into *risA* created downstream effects on other genes, we created an in-frame deletion of *risA* by homologous recombination in *B. pertussis*. Allelic replacement of the deletion mutant into BP536 was performed using the allelic exchange vector pSS2141 (42). Briefly, PCR was performed with primers risArev (5′-AAGGTCATACGGGGAAGACCAAGTTTTTCGAAAC-3′) and risAfor (5′-CTCGAGGGGGGCA CGAGACGGCGCTCCTG-3′), which generated a 948-bp *risA*-containing fragment. PCR was performed using *B. pertussis* strain BP338 (64) DNA as the template. The PCR product was cloned into vector pCR2.1 using the TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA) to generate pTS198. A 204-bp in-frame deletion was generated in *risA* by restriction digestion with SstI followed by religation with SstIII followed by religation to create pTS210. The *ΔrisA* construct was cloned as an EcoRI fragment from pTS210 into pSS2141 to create pTS192. Trparental matings were used to mobilize pTS219 from donor *E. coli* DH5α (Invitrogen) into recipient *B. pertussis* BP536 using *E. coli* plasmid-mobilizing helper strain MM294 (20). Integration of the plasmid into the chromosomal map of *risA* was selected for by plating on media containing gentamicin. A second homologous recombination resulting in the excision of pSS2141 DNA was selected for by plating transconjugates on media containing streptomycin. Isolates were screened for the *ΔrisA* mutant by PCR using the risAfor and risArev primer pair and template prepared from bacterial colonies (13). The in-frame *ΔrisA* mutant was confirmed in one clone, BPOR1, which was selected for further analysis.

An in-frame deletion of the *risS* gene was performed by first amplifying a 2,140-bp fragment of DNA containing the *risAS* locus by PCR using the primers risAfor (5′-AAAGGTTCACCGCCACATCGGCAC-3′) and risSrev (5′-AA GCTTCGACCACGGCGGAAATGC-3′), which generated flanking HindIII sites (underlined). The *risS*-containing *B. pertussis* PCR product was cloned into vector pCR2.1 to generate pTS264. The *risS* fragment was then cloned from pTS264 into pUC9 (44) as a HindIII fragment to generate pTS269. A 432-bp in-frame deletion was generated in *risS* (ΔrisS) by restriction digestion with SstI followed by religation to create pTS270. The *ΔrisS*-containing fragment was amplified by PCR from pTS270 using primers risAfor and risSrev and cloned into vector pCR2.1 to generate pTS272. The *ΔrisS*-containing fragment was then cloned into pSS2141 from pTS272 as a BamHI fragment to generate pTS273. Allelic replacement of *risA* with *ΔrisS* in *B. pertussis* strain BP536 was performed using pRPT1. The mating and selection procedures described for the generation of the *ΔrisA* mutant. Clones were screened for the *ΔrisS* mutant by PCR using the risAfor and risSrev primer pair. The in-frame *ΔrisS* mutant was confirmed in one clone, BPAR2, which was selected for further analysis.

A mutant with constitutive expression of the vrgs and insensitivity to modulation...
### TABLE 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. pertussis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN2992</td>
<td>B. pertussis wild type</td>
<td>Wellcome collection, Kent, United Kingdom</td>
</tr>
<tr>
<td>BP338</td>
<td>Tohama I background; NaI</td>
<td>64</td>
</tr>
<tr>
<td>BP356</td>
<td>Tohama I background; NaI'</td>
<td>59</td>
</tr>
<tr>
<td>BPOR</td>
<td>BP356, risA::neo; NaI' Str' Km'</td>
<td>This study</td>
</tr>
<tr>
<td>BPaOR</td>
<td>BP356, risA; NaI' Str'</td>
<td>This study</td>
</tr>
<tr>
<td>BPARS</td>
<td>BP356, risS; NaI' Str'</td>
<td>This study</td>
</tr>
<tr>
<td>BPSC</td>
<td>BP356, bgS-C3; NaI' Str'</td>
<td>This study</td>
</tr>
<tr>
<td>SCOR</td>
<td>BP356, bgS-C3, risA; NaI'</td>
<td>This study</td>
</tr>
<tr>
<td>BP-72, -75, -76, -79, -82, and -83</td>
<td>B. pertussis clinical isolates</td>
<td>Pertussis Reference Laboratory, Manchester, United Kingdom</td>
</tr>
<tr>
<td><strong>B. parapertussis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN2951</td>
<td>B. parapertussis human clinical isolate</td>
<td>Wellcome collection</td>
</tr>
<tr>
<td>BPP-C, -H1, and -K2</td>
<td>B. parapertussis clinical isolates</td>
<td>Moredun Institute, Midlothian, Scotland</td>
</tr>
<tr>
<td><strong>B. bronchiseptica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN7635E</td>
<td>B. bronchiseptica wild type</td>
<td>Wellcome collection</td>
</tr>
<tr>
<td>Bbr-4 to Bbr-9</td>
<td>B. bronchiseptica clinical isolates</td>
<td>N. Guiso (26)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-12</td>
<td>High-efficiency transformation</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>High-efficiency transformation</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>One Shot</td>
<td>High-efficiency transformation</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DH5α</td>
<td>Conjugation helper strain; carries Km' mobilizing plasmid; IncP1 Tra' oriE1</td>
<td>20</td>
</tr>
<tr>
<td>MM294(pRK2013)</td>
<td>Conjugation helper strain; carries Te' mobilizing plasmid; RP4 Res', Tra', pri, PstIC</td>
<td>12</td>
</tr>
<tr>
<td>S17-1(pN5000)</td>
<td>Conjugation helper strain; RP4-2Te::Mu, Km'</td>
<td>56</td>
</tr>
<tr>
<td>SM10</td>
<td>Lysogenized with Δpir phage; conjugation-proficient donor</td>
<td>15</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHC79</td>
<td>Cosmid vector; Te' Ap'</td>
<td>Boehringer</td>
</tr>
<tr>
<td>pUC9</td>
<td>Cloning vector; Ap'</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBlue script II KS(+)</td>
<td>Cloning vector; Ap'</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRTP1</td>
<td>Allelic exchange vector for Bonetella pertussis; Ap'</td>
<td>S. Stibitz (58)</td>
</tr>
<tr>
<td>pSS2141</td>
<td>Allelic exchange vector for Bonetella pertussis; Ap' Gm'</td>
<td>S. Stibitz (42)</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>TA cloning vector; Ap' Km'</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pFU/S2</td>
<td>Integration vector for generating β-Gal fusions; RP4 oriT, Gm'</td>
<td>F. Jacob-Dubuisson (3)</td>
</tr>
<tr>
<td>pBP2</td>
<td>B. pertussis risA fragment in cosmid pHC79</td>
<td>This study</td>
</tr>
<tr>
<td>pBP3</td>
<td>1.7-kb SalI-ClaI fragment from cosmid clone inserted in pBlue script II KS(+) Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pBP15</td>
<td>pBP3 ligated with EcoRI-digested 1.3-kb neo fragment; Ap' Km'</td>
<td>This study</td>
</tr>
<tr>
<td>pBP16</td>
<td>3.5-kb PvuII fragment from pBP15 ligated with blunt-ended pRTPI vector</td>
<td>This study</td>
</tr>
<tr>
<td>pTS198</td>
<td>pCR2.1 containing risA fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pTS210</td>
<td>pTS198 with SstI deletion of risA</td>
<td>This study</td>
</tr>
<tr>
<td>pTS219</td>
<td>pSS2141 with EcoRI ΔrisA fragment from pTS210</td>
<td>This study</td>
</tr>
<tr>
<td>pTS264</td>
<td>pCR2.1 containing risA fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pTS269</td>
<td>pUC9 containing HindIII risA fragment from pTS264</td>
<td>This study</td>
</tr>
<tr>
<td>pTS270</td>
<td>pTS269 with SstI deletion of risS</td>
<td>This study</td>
</tr>
<tr>
<td>pTS272</td>
<td>pCR2.1 containing risA fragment amplified from pTS269</td>
<td>This study</td>
</tr>
<tr>
<td>pTS273</td>
<td>pSS2141 with BamHI ΔrisS-containing fragment from pTS272</td>
<td>This study</td>
</tr>
<tr>
<td>pJM503</td>
<td>Allelic exchange vector containing bgS fragment with bgS-C3 mutation</td>
<td>J. Miller (45)</td>
</tr>
<tr>
<td>pTS202</td>
<td>pCR2.1 containing kpsM fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pTS203</td>
<td>pCR2.1 containing vrg-6 fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pTS206</td>
<td>pFU/S2 derivative with bgVR-lacZ fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pTS208</td>
<td>pFU/S2 derivative with kpsM-lacZ fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pTS209</td>
<td>pFU/S2 derivative with vrg-6-lacZ fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pRK310</td>
<td>Broad-host-range, low-copy-number plasmid; Te'</td>
<td>S. Stibitz (17)</td>
</tr>
<tr>
<td>pTS263</td>
<td>pCR2.1 containing risA</td>
<td>This study</td>
</tr>
<tr>
<td>pRisA</td>
<td>pRK310 with HindIII risA from pTS263</td>
<td>This study</td>
</tr>
</tbody>
</table>

*NaI', naladixic acid resistance; Str', streptomycin resistance; Gm', gentamicin resistance; Km', kanamycin resistance; Te', tetracycline resistance; Ap', ampicillin resistance.
lation was generated as a control strain in the analysis of bvg-regulated genes and phenotypic analysis of the ΔrisA mutant BP40OR. This was achieved by allelic exchange of the constitutive bvgS open reading frame into the chromosomal parent strain BP536 using E. coli SM10(pJM503) (45) as previously described. Screening for hemolysis identified constitutive bvgS strains that remained hemolytic on BGA containing the modulators 5 mM nicotinic acid and 40 mM MgSO4. One strain, BPSC, was chosen for further analysis. To assess the effect of a mutation in risA in a strain with insensitivity to modulation, we constructed B. pertussis strain SCOR by allelic exchange of risA from four independent assays. Student’s t test was used to analyze the data.

RESULTS

Sequence analysis of the risAS locus of B. pertussis. The ompRinvZ orthologs risA and risS were compared between Bordetella spp. using the published genome sequences of B. pertussis, B. parapertussis, and B. bronchiseptica; published ri-

SAS gene sequences; and our own sequence analysis. The DNA sequences of risAS are nearly identical, with a few, mostly conservative, nucleotide changes between alleles (30). The strains studied were B. bronchiseptica BB7866, B. pertussis Tohama I, B. parapertussis 15311 and ML/180, and B. avium 3086. The genomic sequences of B. pertussis Tohama I, B. parapertussis 12822, and B. bronchiseptica RB50 also reveal fundamentally identical risAS alleles, with the notable exception of B. pertussis Tohama I (49). The Tohama I risAS sequence differs by only a few bases but most notably contains an additional C at position 1848. This is within the coding region of risS and introduces a frameshift in the risS gene. This results in a downstream opal stop codon and a deduced RisS protein that is truncated at residue 323 with a predicted molecular mass of 36 kDa instead of the 52 kDa predicted for the RisS protein of the other bordetellae. EnvZ of Xenorhabdus nematophila is only 342 amino acids long but can complement E. coli envZ-null mutants in an osmoregulated manner; however it is truncated in its N-terminal periplasmic region and still contains all of the domains important for signal transduction (61). In contrast, B. pertussis RisS is predicted to lack an EnvZ-like transmitter domain, which includes the autophosphorylation site as well as other invariant amino acids and conserved domains. It is thus highly unlikely that the EnvZ ortholog RisS is functional as an environmental sensor in B. pertussis. The domain structures of EnvZ and RisS variants are illustrated in Fig. 1.

It is possible that this single base change is peculiar to the strains examined. We therefore used PCR to amplify risS from other Bordetella isolates and performed sequence analysis to compare risS alleles. The strains tested were CN2992 and six clinical isolates from the United Kingdom Pertussis Reference Laboratory which had been passaged in vitro a maximum of two times: BP-K72, BP-K75, BP-K76, BP-K79, BP-K82, and BP-K83. All of these strains contained the extra cytosine resulting in the frameshift mutation in risS. Control strains of B. parapertussis (CN2991 and three ovine strains, BP-BH, BPP-C, and BP-P2) and B. bronchiseptica (CN 76356 and five rabbit isolates, BBR-4 through BBR-9) did not contain the...
frameshift mutation and had a full-length risS gene. Hence, this mutation appears to be common in B. pertussis strains but rare or absent in B. parapertussis and B. bronchiseptica.

The risA gene is upstream of risS, and the two genes are separated by only 4 bp, as found in ompR-envZ systems. They are cotranscribed in B. bronchiseptica. The deduced B. pertussis RisA protein comprises 244 amino acid residues with a molecular mass of 27.8 kDa and shares a 65.7% identity with the E. coli OmpR protein. This response regulator family of proteins is defined by a conserved domain of approximately 100 amino acids that extends over the N terminus of each of these proteins. In particular, the residues that correspond to D12, D55, and K105 in the E. coli OmpR protein tend to be conserved among the response regulator protein family. These are found at amino acid positions D17, D60, and K110 in the B. pertussis RisA and E. coli OmpR and is likely to be the site of RisA phosphorylation. In addition, the region between P83 and I121 that accommodates the invariant K110 is almost completely conserved between the two proteins (30).

Expression of Bvg-regulated proteins. We have previously noted that two B. pertussis Vras are not expressed in a risA transposon mutant (our unpublished observations). Furthermore, mutation of the risAS locus is known to have pleiotropic effects on B. bronchiseptica gene expression (30). We thus analyzed protein expression in risAS mutants to investigate their patterns of Bvg-regulated protein expression. Blots were probed for expression of the Bvg-activated proteins pertussis toxin and pertactin and the Bvg-repressed antigens Vra-a (Fig. 3A and B). In addition, the levels did not differ appreciably between the wild-type strain and ΔrisA mutant compared to the wild type under both nonmodulating and modulating conditions, while the overall expression of the Vras was still increased under modulating versus nonmodulating conditions (Fig. 2, lanes 1 through 4). In contrast, the ΔrisS mutant did not show any alteration in the expression of the Vras compared to the wild type (Fig. 2, lanes 1 and 2 and 5 and 6).

To ensure that mutation of risA was specifically responsible for the decreased expression of the Vras, we introduced either low-copy-number control vector pRK310 (17) or this vector containing the wild-type risA gene (pRisA) into the ΔrisA mutant BPΔOR and then analyzed the plasmid-containing strains in complementation experiments. In addition, we constructed and analyzed an isogenic mutant containing the bvgS-C3 mutant (BPSC) (45), which has a Bvg-constitutive phenotype, to act as a Bvg-activated phenotypic control. Immunooblots were probed for pertussis toxin (subunit S1) and pertactin and the Bvg-repressed antigens Vra-a and Vra-b (Fig. 3). Pertussis toxin and pertactin were expressed only in the absence of modulators in the wild-type strain and ΔrisA mutant regardless of the presence of pRK310 or pRisA, and their expression levels did not differ appreciably between the ΔrisA strains and their wild-type isogenic parent (Fig. 3A and B). In addition, standard zones of hemolysis were observed when risA mutants

FIG. 1. Comparison of domain structures of EnvZ and RisS proteins. (A) E. coli EnvZ; (B) Xenorhabdus nematophilus AN6/1 EnvZ; (C) B. bronchiseptica RB80 RisS; (D) B. pertussis Tohama I RisS. HAMP, receptor histidine kinase domain; HisKA, dimerization/phosphoacceptor, HATPase c, histidine kinase-like ATPases.

Levels of Vra-a and Vra-b were greatly diminished in the ΔrisA mutant compared to the wild type under both nonmodulating and modulating conditions, while the overall expression of the Vras was still increased under modulating versus nonmodulating conditions (Fig. 2, lanes 1 through 4). In contrast, the ΔrisS mutant did not show any alteration in the expression of the Vras compared to the wild type (Fig. 2, lanes 1 and 2 and 5 and 6).

To ensure that mutation of risA was specifically responsible for the decreased expression of the Vras, we introduced either low-copy-number control vector pRK310 (17) or this vector containing the wild-type risA gene (pRisA) into the ΔrisA mutant BPΔOR and then analyzed the plasmid-containing strains in complementation experiments. In addition, we constructed and analyzed an isogenic mutant containing the bvgS-C3 mutant (BPSC) (45), which has a Bvg-constitutive phenotype, to act as a Bvg-activated phenotypic control. Immunooblots were probed for pertussis toxin (subunit S1) and pertactin and the Bvg-repressed antigens Vra-a and Vra-b (Fig. 3). Pertussis toxin and pertactin were expressed only in the absence of modulators in the wild-type strain and ΔrisA mutant regardless of the presence of pRK310 or pRisA, and their expression levels did not differ appreciably between the ΔrisA strains and their wild-type isogenic parent (Fig. 3A and B). In addition, standard zones of hemolysis were observed when risA mutants

FIG. 2. Expression of Bvg-repressed antigens in risA and risS mutants. Lanes: 1, parental Bordetella pertussis strain BP536 grown on BGA without modulators; 2, B. pertussis strain BP536 grown on BGA with the modulators 5 mM nicotinic acid and 40 mM MgSO4; 3, ΔrisA mutant (BPΔOR) grown on BGA; 4, ΔrisA mutant grown on BGA with modulators; 5, ΔrisS mutant (BPΔRS) grown on BGA; 6, ΔrisS mutant grown on BGA with modulators. Relative protein levels were assessed by Western blotting as described in Materials and Methods. (A) Vra-a (VraA); (B) Vra-b (VraB). Migrations of molecular weight (MW) markers are indicated on the right.
Values are reported as means unless otherwise indicated. 

were grown on blood agar (data not shown), indicating normal 

**FIG. 3.** Expression of Bvg-regulated proteins. Lanes: 1, parental Bordetella pertussis strain BP536 grown on BGA without modulators; 2, B. pertussis strain BP536 grown on BGA with the modulators 5 mM nicotinic acid and 40 mM MgSO₄; 3, bvgA-C (BPSC) mutant grown on BGA; 4, bvgS-C mutant grown on BGA with modulators; 5, ΔrisA mutant (BPΔOR) grown on BGA; 6, ΔrisA mutant grown on BGA with modulators; 7, ΔrisA mutant with low-copy-number vector [BPΔOR (pRK310)] grown on BGA; 8, BPΔOR(pRK310) grown on BGA with modulators; 9, ΔrisA mutant with complementing risA plasmid [BPΔOR(pRisA)] grown on BGA; 10, BPΔOR(pRisA) grown on BGA with modulators. Relative protein levels were assessed by Western blotting as described in Materials and Methods. (A) pertactin (Prn); (B) the S1 subunit of pertussis toxin (S1); (C) Vra-a (VraA); (D) Vra-b (VraB). Migrations of molecular weight (MW) markers are indicated on the right.

were grown on blood agar (data not shown), indicating normal levels of the vag-encoded bifunctional adenylate cyclase-hemolysin. As expected, the Bvg-constitutive mutant BPSC constitutively expressed pertussis toxin, pertactin, and the hemolytic phenotype regardless of the presence of modulators. Conversely, Vra-a and Vra-b were highly induced under modulating conditions in wild-type bacteria but were constitutively repressed in BPSC under the conditions tested (Fig. 3C and D).

Expression of these Bvg-repressed products was greatly diminished in the ΔrisA mutant compared to the wild type even in the presence of modulators, with levels of the Vras in the modulated ΔrisA mutant comparable to nonmodulated levels in the wild-type strain. Introduction of vector control pRK310 did not restore wild-type expression levels of the Vras under the conditions tested (Fig. 3C, lane 10). These data indicate that risA appears to be required for the expression of Bvg-repressed products, perhaps in a specific manner that does not affect the expression of Bvg-activated proteins. In addition, the increased copy number of risA when supplied even on a low-copy-number plasmid appears to augment expression of the Vras, suggesting that increasing levels of the RisA activator may increase expression of the Bvg-repressed antigens.

**Transcriptional analysis of Bvg-regulated genes.** To determine if the reduction of expression of Bvg-repressed products in a ΔrisA background was due to reduced transcription, we analyzed insertional lacZ fusion reporters (Table 2). As expected, transcription of the Bvg-repressed genes kpsM (which is predicted to encode a capsule transport protein) (3, 29) and vrg-6 (which is predicted to encode a small protein of unknown function) (7) were greatly increased under modulating conditions in the wild-type strain, while their transcription was constitutively repressed in BPSC. Transcription of these vrgs was greatly reduced in the ΔrisA mutant BPΔOR, even under modulating conditions. However, introduction of pRisA restored transcription of the vrgs to a level that was about double that of the wild type. In contrast, the Bvg-activated gene bvgR was transcribed at a much higher level under nonmodulating conditions than under modulating conditions in wild-type bacteria, while it had constitutively high transcription in the control strain BPSC. As in the wild type, transcription of bvgR was still high under nonmodulating conditions in the risA mutant and again was greatly reduced in the presence of modulators. Introduction of pRisA did not affect the expression of bvgR in any of the strains tested.

To test the statistical significance of the transcriptional fusion data, Student’s t test was used. When wild-type BP536 and the ΔrisA mutant BPΔOR were compared under modulating conditions, transcription of the vrgs kpsM and vrg-6 was significantly decreased in BPΔOR (P < 0.01), demonstrating that vrg transcription is significantly lower when risA is mutated. Transcription of the vag bvgR was not significantly different between the wild-type bacteria and the risA mutant, confirming that risA has no apparent role in the transcription of the vrgs. Analysis of transcription data for pRisA-complemented ΔrisA mutants demonstrated significantly increased transcription of vrg-6 under both modulating and nonmodulating conditions (P < 0.01). Significantly increased transcription of kpsM after pRisA complementation was seen only under modulating conditions; however, kpsM transcription was very low under nonmodulating conditions and near the limit of detection for the assay used. These data strongly suggest that the presence of multiple copies of risA significantly increases transcription of the vrgs regardless of the affect of Bvg regulation. In summary, these data indicate that mutation of risA greatly diminishes the expression of the Bvg-repressed proteins in the absence of RisA.

**TABLE 2. β-Galactosidase activities of B. pertussis pFus2 integrants**

<table>
<thead>
<tr>
<th>Gene</th>
<th>BP536 (wild type)</th>
<th>BPSC (bvgS-C)</th>
<th>BPΔOR (ΔrisA)</th>
<th>BPΔOR(pRisA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NM</td>
<td>SO₄-Nic</td>
<td>NM</td>
<td>SO₄-Nic</td>
</tr>
<tr>
<td>bvgR</td>
<td>1,801 ± 93</td>
<td>128 ± 42</td>
<td>1,700 ± 152</td>
<td>2,527 ± 165</td>
</tr>
<tr>
<td>kpsM</td>
<td>77 ± 5</td>
<td>350 ± 52</td>
<td>80 ± 13</td>
<td>102 ± 33</td>
</tr>
<tr>
<td>vrg-6</td>
<td>337 ± 24</td>
<td>2,312 ± 66</td>
<td>245 ± 52</td>
<td>607 ± 85</td>
</tr>
</tbody>
</table>

a Relative transcription levels were determined by measuring β-galactosidase activities from lacZ transcriptional fusions as described in Materials and Methods. Values are reported as means ± the standard errors of the means from four independent assays.
b Strains grown on BGA with no modulators (NM).
c Strains grown on BGA with the modulators 5 mM nicotinic acid and 40 mM MgSO₄.

Downloaded from iai.asm.org on December 28, 2009
pression of Bvg-repressed products at the level of transcription, while not apparently affecting the transcription of the vags.

**Analysis of risA mutants for adherence and invasion in HeLa cell models.** Transcription of risA occurs when *B. bronchiseptica* is inside eukaryotic cells, and risA mutants have reduced ability to survive within these cells (30, 65). We tested *B. pertussis* risA mutant BPOR, the constitutive mutant BPSC, and their isogenic wild-type parent for adherence to, and survival in, HeLa cells (Fig. 4).

A reduction in survival in the HeLa cell invasion assay could be due to reduced adherence, invasion, or survival. To distinguish between reduced adherence and reduced invasion or intracellular survival, we performed an adherence assay using HeLa cells. After 90-min incubation and washing to remove nonadherent bacteria, there was no difference in adherence to HeLa cell monolayers between BPOR and BPSC (*P > 0.1*) relative to the wild-type control (Fig. 4A).

The invasion of wild-type bacteria and BPSC was not significantly different (*P > 0.1*). However, there was approximately fivefold less survival of BPOR relative to the wild type (*P < 0.01*) (Fig. 4B). The total numbers of invasive wild-type *B. pertussis* were in close agreement with those previously published (19), and the strains tested did not have any appreciable differences in sensitivity to gentamicin (data not shown).

**Analysis of Bvg-constitutive mutants in the HeLa cell invasion model.** The fact that BPSC and wild-type bacteria survived equally well suggests that the reduction in survival of the risA mutant is not due to the diminished expression of Bvg-repressed molecules. We therefore constructed a BvgS-constitutive/ΔrisA double mutant and tested it for survival in the HeLa cell invasion assay to determine if risA mutation affected survival in the Bvg-constitutive background. The ΔrisA mutant BPΔOR and the BvgS-constitutive/ΔrisA double mutant SCOR both survived less well than the wild type in the HeLa cell invasion assay (Fig. 5) (*P < 0.01*), even though the Bvg-constitutive strain BPSC did not differ in its survival capability in the assay (*P > 0.1*). Interestingly, SCOR survived better than BPΔOR (*P < 0.01*), suggesting that an element of the decreased survival in risA mutants may be due to alteration in the expression of factors not regulated by Bvg.

**DISCUSSION**

The BvgAS two-component regulatory system is at the apex in the hierarchical control of virulence factor expression required for the pathogenesis of the bordetellae. However, in other pathogens, multiple regulatory networks contribute to virulence and this is likely to be the case here, too. The OmpR/EnvZ ortholog RisAS is the second two-component regulatory system to be described in the bordetellae through experimentation with *B. bronchiseptica* strains (30, 65). In particular, ris-dependent products have been implicated in resistance within macrophages to acid and oxidative stresses by *B. bronchiseptica* (30, 65) and ris is required for bacterial persistence in a mouse model of infection (65). Expression of ris is bvg independent, and mutation of ris produces pleiotropic effects on protein expression. We investigated potential roles for the RisAS system of *B. pertussis* in the regulation of virulence, using risAS mutants.

Our results suggest that RisA is required for the expression of Bvg-repressed products in *B. pertussis*. Vra-a and Vra-b are surface-exposed proteins that are tightly regulated by the BvgAS system in *B. pertussis* (43, 57). Mutation of risA results in greatly decreased expression of Vra-a and Vra-b with levels produced by modulated risA mutants only about as high as those produced by unmodulated wild-type *B. pertussis*. However, Vra-a and Vra-b were still induced by modulation, sug-
gesting that their expression is still repressed by the BvgASR
regulatory cascade but that a functional risA gene is required
for high-level expression of the Vras. Complementation of the
risA mutant with risA on a low-copy-number plasmid restored
the high-level, BvgAS-regulated expression of the Vras to a
level a severalfold higher than that found in wild-type B. per-
tussis. This suggests that an increased level of RisA transacti-
vator increases expression of the Vras.

Transcriptional analysis using gene fusions demonstrated a
dependence on risA for transcription of the vrgs. Transcription of
vrg-6 and kpsM is virtually absent when risA is disrupted. When
risA is supplied in trans, the levels of vrg expression in
modulated bacteria are about twice those of modulated wild-
type bacteria. These results suggest that risA affects vrg ex-
pression and Vra production at the level of transcription. Further-
more, the increased expression of these Bvg-repressed
molecules as a result of there being multiple copies of risA
suggests that increasing concentrations of the RisA transacti-
vator induces higher levels of expression of the vrgs. It will be
interesting to determine whether RisA acts directly to bind the
cis-activating sites of the vrgs or indirectly by altering the ex-
pression of other regulatory molecules.

The Bvg-activated products appear not to be dependent on
RisA for expression, as evidenced by equivalent levels of he-
molysis on blood agar (a result of adenylate cyclase produc-
tion) and comparable levels of pertussis toxin and pertactin
expression between the wild type and risA mutants under non-
modulating conditions. Similarly, ectopic or unregulated pro-
duction of Bvg-regulated products was not observed in risA
mutants. For instance, bvgR has a normal bvg-activated tran-
scription pattern when risA is mutated and pertussis toxin and
pertactin are also not detected under nonmodulating condi-
tions. Thus, it would seem that RisA could play a specific
function in gene regulation as an activator of expression of the
vrgs. However, it will be important to look at an expanded set
of genes and proteins, though genomic and proteomic tech-
niques, to address the likelihood that RisA affects genes other
than just the vrgs.

It is perhaps surprising that a phenotype was detected in B.
pertussis risA mutants, considering that the gene encoding the
 cognate sensor RisS is a pseudogene. All the B. pertussis strains
tested have the frameshift mutation in risS, which would result
in a prematurely truncated RisS sensor lacking an EnvZ-like
transmitter domain, and the putative site of RisS autophos-
phorylation. This mutation was not found in risS of B. bron-
chiseptica or B. parapertussis. When B. pertussis risS was mu-
tated to contain a large deletion upstream of the frameshift, it
resulted in no obvious phenotypic effect on gene expression.
Thus, maximal expression of Bvg-repressed products is depend-
ent on functional risA but is independent of risS. It is inter-
esting to speculate that RisA of B. pertussis may be activated by
phosphorylation by a kinase other than RisS via cross talk
between regulatory systems. Interestingly, heterologous phos-
photransfer has been demonstrated between the purified com-
ponents of two-component regulatory systems in vitro (48). In
addition, phosphorylation of OmpR has been observed in
envZ-null mutants of E. coli, suggesting that OmpR can be
phosphorylated by a kinase that is functionally homologous to
EnvZ (21). Cross-regulation has been observed in vivo be-
tween the AcrB sensor of anaerobiosis and the OmpR regula-
tor of porin expression in E. coli, suggesting interplay between
phosphorelay systems (37). Therefore, it is possible that an-
other regulatory system, other than BvgAS or RisAS, may
regulate the expression of the vrgs in B. pertussis via RisA
activation.

B. bronchiseptica risA expression is maximal at 37°C in the
absence of magnesium cations, when the bacteria are intracel-
lar (30). It will be important to determine which signals
regulate RisA expression and activation in B. pertussis. This is
of particular interest given that risA is a pseudogene in B.
pertussis. It would not be surprising if RisA were to be activated
by different regulatory sensors in B. pertussis and thus also
different environmental signals. It will be interesting to deter-
mine if shutting a wild-type B. bronchiseptica risS gene to B.
pertussis alters the expression patterns of Bvg-repressed mole-
cules in response to the presence of particular environmental
signals.

It is apparent that the Bvg-repressed arm of the Bvg regulon
has diverged during evolution of the subspecies of the B. bron-
chiseptica cluster (55). B. pertussis in particular expresses a
more limited, but somewhat distinct, set of Bvg-repressed
products compared to B. bronchiseptica. In addition, the re-
quirement for RisA in B. pertussis for maximal expression of
the vrgs contrasts with the pleiotropic nature of the Ris-regu-
lated products in B. bronchiseptica wherein ris is required for
expression of the B. bronchiseptica-specific acid phosphatase,
but not bvg-repressed motility or urease (30). Our results in-
dicate that the genes regulated by risAS and the mechanisms
of RisA-mediated gene regulation have diverged during evolu-
tion of the bordetellae. It is possible that differences in risA
gen regulation and differences in vrg expression in the borde-
tellae have resulted from the same selective pressure that re-
sulted in the evolution of Bordetella subspecies with different
host ranges, physiology, and disease presentations.

B. bronchiseptica ris mutants have reduced survival in mac-
rophage invasion models (30, 65). B. pertussis can invade epi-
thelial-like cells, and the expression of the Bvg-activated phase
is required for adhesion and invasion in these models (6, 19).
Our results in the HeLa cell model suggest that B. pertussis
invasion is risA dependent, while adherence is risA indepen-
dent. Since vrg expression is greatly diminished in risA mutants,
it could be hypothesized that the vrgs are required for this
intracellular survival or invasion. However, we found that Bvg-
activated phase-locked bacteria, which constitutively repress
the vrgs, are as able as wild-type bacteria to survive in HeLa
cells. This suggests that the observed phenotype is probably
due to effects on other genes that are dependent on risA for
expression but that are not Bvg repressed and argues against
modulation of the bacteria to affect increased survival in this
model system. Although risA mutants of B. pertussis enter or
survive less well in HeLa cells, the relevance of epithelial cell
invasion by B. pertussis to the disease state is uncertain, as an
intracellular niche has not been demonstrated for this bacte-
rion. It is possible that invasion occurs in low numbers in
humans in a location in which the bacteria are hard to detect.
Alternatively, the readout in the invasion models could reflect
another biologically relevant aspect of B. pertussis ecology,
even if the bacterium does not invade eukaryotic cells in vivo,
and it is conceivable that RisA has a biologically important
function in B. pertussis in vivo through an influence on the
expression of vrg or other genes. Further work will be required to determine if the HeLa cell invasion data reflect a biologically relevant interaction of the bacterium with host cells or if they reflect a more general phenotype related to the ability of \textit{B. pertussis} to survive stressful factors in its environment.

In \textit{B. bronchiseptica}, biologically relevant phenotypes have been attributed to vrg-encoded products such as motility for flagella (1, 2) and urea degradation for urease (39). In \textit{B. pertussis}, a relevant vrg-encoded biological phenotype is uncertain. Although a Bvg-repressed capsule has not been described, vrgs are known to be important virulence factors for a number of bacteria (47). We have confirmed that kpsM is a \textit{B. pertussis} vrg; however, it is unknown whether the KpsM protein is expressed in \textit{B. pertussis}. Furthermore, in \textit{B. pertussis}, several other genes of the capsule biosynthesis locus have been inactivated or have been shuffled to alternate positions in the chromosome (49). It is possible that the altered subset of capsule synthesis genes could have a biological function in \textit{B. pertussis}.

The smaller number of vrgs expressed in \textit{B. pertussis} compared with \textit{B. bronchiseptica} may indicate that the \textit{B. pertussis} vrgs are decaying relative to \textit{B. bronchiseptica}. However, many vrgs are still expressed in \textit{B. pertussis}, and moreover, the expression patterns of the vrgs differ between the two subspecies. It is hoped that further investigation of Ris- and Bvg-mediated regulation will elucidate a role (either current or ancestral) for both ris and the vrgs in the life cycle of the human pathogen \textit{B. pertussis}.

ACKNOWLEDGMENTS

We thank F. Jacob-Dubuisson for providing plasmid pFUS2, J. Miller for providing \textit{E. coli} strain SM10(pJM503), S. Stibitz for providing plasmids pSS2141 and pRK310, N. Guiso for providing \textit{B. bronchiseptica} clinical isolates, and A. Weiss for providing monoclonal antibodies 2X2S and BBO5. We also gratefully acknowledge the Sanger Centre for allowing public access to \textit{Bordetella} genome data prior to publication. This work was supported by a Canadian Bacterial Diseases Network grant (through N.C.E. Canada) to M.S.P. and a Wellcome Trust Programme grant to D.M.

REFERENCES


