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

Trevor H. Stenson,[1]* Andrew G. Allen,[2]† Jehan A. al-Meer,[2]‡ Duncan Maskell,[2]§ and Mark S. Peppler[1]

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

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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*, in this process 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 β-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 phosphorelay 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 B*v*g-*a*ctivated *g*enes, or *vag*s (52). An additional level of regulatory complexity is evident in that the *vag*s 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 *vag*s 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 B*v*g-*r*epressed *g*enes, or *vrg*s (33).

The *vag*s 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 *vag*-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 *vrg*s encode factors whose expression is more

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, University of Alberta, 1-69 Medical Sciences Bldg., Edmonton, Alberta T6G 2H7, Canada. Phone: (780) 492-0934. Fax: (780) 492-7521. E-mail: trevor.stenson@ualberta.ca.

† Present address: Oxford Gene Technology IP Ltd., Begbroke Business & Science Park, Sandy Lane, Yarnton, Oxford OX5 1PF, United Kingdom.

‡ Present address: University of Qatar, Faculty of Science, Chemistry and Biochemistry Department, P.O. Box 2713, Doha, Qatar.

§ Present address: The Centre for Veterinary Science, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, United Kingdom.

divergent 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 *vrg*s and antigens (Vras) with unknown functions (3, 29, 33, 57). The repression of the *vrg*s appears to be mediated principally through the *vag*-encoded transcriptional repressor BvgR, which is believed to bind at *cis*-repressive sites of the *vrg* genes (7, 41, 42). The expression of the *vrg*s is maximal late in the bacterial growth phase (55), and individual *vrg*s are differentially modulated by individual in vitro modulators (29). In addition to the *vag*s and the *vrg*s, 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 *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Shigella flexneri* (22) that reciprocally regulate the expression of porins OmpC and OmpF in response to osmolarity. 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 tobacco 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 a homolog, of OmpR/EnvZ, as it does not respond to osmolarity 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 *vrg*s but not the *vag*s, whereas *risS* does not affect *vrg* or *vag* expression probably because it is a pseudogene in *B. pertussis*. *risA* mutants adhere just as well as wild-type bacteria but are less efficient at invasion of HeLa cells, indicating a role in the host-pathogen 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 (Dalynn, 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 $MgSO_4$ (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 insertionally disrupted *risA* in BP536 (59) was performed using the allelic exchange vector pRTP1 (58). Briefly, PCR analysis of a transfected *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 *Sau*3A and transfected into *E. coli* XL1-Blue (Stratagene, La Jolla, CA). PCR was performed with primers bpORF (5′-CTGCTGGTTCTCGACCTG-3′) and bpORR (5′-GGGTTGAAGGGCTT GGAC-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 pBluescript II KS(+) (Stratagene) to create pBP3. A kanamycin resistance (*neo*) cassette was inserted at the EcoRI site of the *risA* fragment in pBP3 to create pBP15. Insertionally disrupted *risA* DNA was released as a 3.5-kb PvuII fragment and cloned into pRTP1 to create pBP16. Triparental matings were used to mobilize pBP16 from donor *E. coli* CC118λ*pir* (15) into recipient *B. pertussis* BP536 using *E. coli* plasmid-mobilizing helper strain S17-1(pNJ5000) (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 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′-AAGCTTAGCGG GAAGACGAAGTTTCGAAGGCAA-3′) and risAfor (5′-CTCGAGGGGGCA 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* (Δ*risA*) by restriction digestion with SstII followed by religation to create pTS210. The Δ*risA* construct was cloned as an EcoRI fragment from pTS210 into pSS2141 to generate pTS219. Triparental 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(pRK2013) (20). Integration of the plasmid into the chromosomal copy 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 Δ*risA* mutant by PCR using the risAfor and risArev primer pair and template prepared from bacterial colonies (13). The in-frame Δ*risA* mutant was confirmed in one clone, BPΔOR, 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 risASfor (5′-AAGCTTCACCGCCTCATGCGACAC-3′) and risASrev (5′-AA GCTTGCCAGCACCGACGAAATGTC-3′), which generated flanking HindIII sites (underlined). The *risAS* PCR product was cloned into vector pCR2.1 to generate pTS264. The *risAS* 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* (Δ*risS*) by restriction digestion with SstI followed by religation to create pTS270. The Δ*risS*-containing fragment was amplified by PCR from pTS270 using primers risASfor and risASrev and was cloned into vector pCR2.1 to generate pTS272. The Δ*risS*-containing fragment was then cloned into pSS2141 from pTS272 as a BamHI fragment to generate pTS273. Allelic replacement of *risS* with Δ*risS* in *B. pertussis* strain BP536 was performed using pTS273 and the mating and selection procedures described for the generation of the Δ*risA* mutant. Clones were screened for the Δ*risS* mutant by PCR using the risASfor and risASrev primer pair. The in-frame Δ*risS* mutant was confirmed in one clone, BPΔRS, which was selected for further analysis.

A mutant with constitutive expression of the *vag*s and insensitivity to modu-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source and/or reference
Strains		
B. pertussis		
CN2992	*B. pertussis* wild type	Wellcome collection, Kent, United Kingdom
BP338	Tohama I background; Nal^r	64
BP536	Tohama I background; Nal^r Str^r	59
BPOR	BP536, *risA*::*neo*; Nal^r Str^r Km^r	This study
BPΔOR	BP536, Δ*risA*; Nal^r Str^r	This study
BPΔRS	BP536, Δ*risS*; Nal^r Str^r	This study
BPSC	BP536, *bvgS*-C3; Nal^r Str^r	This study
SCOR	BP536, *bvgS*-C3, Δ*risA*; Nal^r Str^r	This study
BP-72, -75, -76, -79, -82, and -83	*B. pertussis* clinical isolates	Pertussis Reference Laboratory, Manchester, United Kingdom
B. parapertussis		
CN2951	*B. parapertussis* human clinical isolate	Wellcome collection
BPP-C, -H1, and -K2	*B. parapertussis* clinical isolates	Moredun Institute, Midlothian, Scotland
B. bronchiseptica		
CN7635E	*B. bronchiseptica* wild type	Wellcome collection
Bbr-4 to Bbr-9	*B. bronchiseptica* clinical isolates	N. Guiso (26)
E. coli K-12		
XL1-Blue	High-efficiency transformation	Stratagene
One Shot	High-efficiency transformation	Invitrogen
DH5α	High-efficiency transformation	Invitrogen
MM294(pRK2013)	Conjugation helper strain; carries Km^r mobilizing plasmid; IncP1 Tra^+ *oriE1*	20
S17-1(pNJ5000)	Conjugation helper strain; carries Tc^r mobilizing plasmid; RP4 Res^-, Tra^+, *pri*, $PstIC^-$	12
SM10	Conjugation helper strain; RP4-2Tc::Mu, Km^r	56
CC118λpir	Lysogenized with λpir phage; conjugation-proficient donor	15
Plasmids		
pHC79	Cosmid vector; Tc^r Ap^r	Boehringer
pUC9	Cloning vector; Ap^r	44
pBluescript II KS(+)	Cloning vector; Ap^r	Stratagene
pRTP1	Allelic exchange vector for *Bordetella pertussis*; Ap^r	S. Stibitz (58)
pSS2141	Allelic exchange vector for *Bordetella pertussis*; Ap^r Gm^r	S. Stibitz (42)
pCR2.1	TA cloning vector; Ap^r Km^r	Invitrogen
pFUS2	Integrational vector for generating β-Gal fusions; RP4 *oriT*, Gm^r	F. Jacob-Dubuisson (3)
pBP2	*B. pertussis risA* fragment in cosmid pHC79	This study
pBP3	1.7-kb SalI-ClaI fragment from cosmid clone inserted in pBluescript II KS(+); Ap^r	This study
pBP15	pBP3 religated with EcoRI-digested 1.3-kb *neo* fragment; Ap^r Km^r	This study
pBP16	3.5-kb PvuII fragment from pBP15 ligated with blunt-ended pRTP1 vector	This study
pTS198	pCR2.1 containing *risA* fragment	This study
pTS210	pTS198 with SstII deletion of *risA*	This study
pTS219	pSS2141 with EcoRI Δ*risA* fragment from pTS210	This study
pTS264	pCR2.1 containing *risAS* fragment	This study
pTS269	pUC9 containing HindIII *risAS* fragment from pTS264	This study
pTS270	pTS269 with SstI deletion of *risS*	This study
pTS272	pCR2.1 containing *risAS* fragment amplified from pTS269	This study
pTS273	pSS2141 with BamHI Δ*risS*-containing fragment from pTS272	This study
pJM503	Allelic exchange vector containing *bvgS* fragment with *bvgS-C3* mutation	J. Miller (45)
pTS202	pCR2.1 containing *kpsM* fragment	This study
pTS203	pCR2.1 containing *vrg-6* fragment	This study
pTS206	pFUS2 derivative with *bvgR-lacZ* fusion	This study
pTS208	pFUS2 derivative with *kpsM-lacZ* fusion	This study
pTS209	pFUS2 derivative with *vrg-6-lacZ* fusion	This study
pRK310	Broad-host-range, low-copy-number plasmid; Tc^r	S. Stibitz (17)
pTS263	pCR2.1 containing *risA*	This study
pRisA	pRK310 with HindIII *risA* from pTS263	This study

[a] Nal^r, naladixic acid resistance; Str^r, streptomycin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance.

lation was generated as a control strain in the analysis of *bvg*-regulated genes and phenotypic analysis of the Δ*risA* mutant BPΔOR. This was achieved by allelic exchange of the constitutive *bvgS* mutant *bvgS-C3* into the chromosome of parental 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 $MgSO_4$. One strain, BPSC, was chosen for further analysis. To assess the affect of a mutation in *risA* in a strain with insensitivity to modulation, we constructed *B. pertussis* strain SCOR by allelic exchange of Δ*risA* as described using BPSC as the parental strain.

Comparisons of the *B. pertussis risAS* locus with the *risAS* locus of other bordetellae was done with genome sequences (49) made available from the Sanger Centre (these sequence data were produced by the *B. bronchiseptica* and *B. parapertussis* Sequencing Groups at the Sanger Centre and were obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bp/, from our own unpublished sequence data, and by BLAST analysis of published sequences available in the public databases [GenBank nonredundant nucleotide and protein databases, last accessed January 2005]).

Complementation of the Δ*risA* mutant. To confirm that the phenotype described in Δ*risA* bacteria is a result of the disruption of *risA* only, we constructed a plasmid containing *risA* to use for complementation using the low-copy-number vector pRK310 (17). PCR was performed using primers risASfor (5′-AAGCTTCACCGCCTCATGCGACAC-3′) and risACrev (5′-AAGCTTAGGCTGACCAGCATCAGTGC-3′) to generate a 1,314-bp piece of DNA with flanking HindIII sites (underlined). This DNA contains *risA* starting 438 bp upstream of DNA of the *risA* open reading frame and ending 138 bp into the *risS* open reading frame. The PCR product was cloned into vector pCR2.1 to generate plasmid pTS263, which contains the *risA* gene flanked by BamHI sites. The *risA* gene was cloned into pRK310 as a HindIII fragment to create pRisA. Plasmids pRK310 and pRisA were introduced into *B. pertussis* strains from *E. coli* DH5α by triparental matings using *E. coli* MM294(pRK2013) and selecting for tetracycline-resistant *B. pertussis*.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as previously described (13). Proteins were resolved using 15% (37.5:1) acrylamide/bis-acrylamide separating gels. The S1 subunit of pertussis toxin, pertactin, Vra-a, or Vra-a and Vra-b were detected with monoclonal antibodies X2X5 (31), BBO5 (11), and 1G7 or monoclonal antibody 7H1 (57), respectively. Peroxidase-conjugated goat anti-mouse immunoglobulin G (whole-molecule) secondary antibody was purchased from Pierce (Rockford, IL). Antibody binding was visualized by chemiluminescence using enhanced chemiluminescence Western blotting detecting reagents (Amersham, Piscataway, NJ). Apparent molecular weights were determined by comparison with the BenchMark prestained molecular weight ladder (Invitrogen).

Construction of transcriptional reporters. β-Galactosidase (β-Gal) transcriptional reporters were generated using the suicide vector pFUS2 (3) and PCR-amplified *B. pertussis* DNA. The following primers were designed to amplify an internal fragment of *bvgR*, *kpsM*, and *vrg-6*, with flanking HindIII and KpnI restriction sites (underlined): 5′-AAGCTTTGTTCATCCAGCCAGAGCCC-3′ (bvgRF) and 5′-GGTACCCATGGTCGCCCAGCAGATACC-3′ (bvgRR), 5′-AAGCTTCGTTCTTCCTGTTCAGCCACAC-3′ (kpsMF) and 5′-GGTACCAATAAAGGTGCTCCCACGACTTG-3′ (kpsMR), and 5′-AAGCTTGCCGGACTCATGCTCTCCAGCG-3′ (vrg-6F) and 5′-GGTACCGCGGTCATAGTGCCGATGGC-3′ (vrg-6R). The sizes of the cloned fragments were 441 bp, 309 bp, and 234 bp for *bvgR*, *kpsM*, and *vrg-6*, respectively. PCR was performed using *B. pertussis* strain BP338 DNA as the template, and the products were cloned into pCR2.1. The resulting plasmids were digested with HindIII-KpnI and cloned in-frame with the *groES* gene of pFUS2, resulting in a transcriptional fusion with the *lacZ* gene. These fusion constructs were introduced into the chromosomes of strains BPOR, BPΔOR, BPSC, and BPΔRS and their isogenic parent, BP536, from *E. coli* DH5α in matings using *E. coli* MM294(pRK2013). Gentamicin selects for insertional duplication mutations in the chromosomal copy of the genes and creates a β-Gal transcriptional reporter under the control of the chromosomal promoters. Strains containing reporters were maintained under gentamicin selection to select against potential excision of the plasmids. To complement the Δ*risA* mutant, pRisA (or control pRK310) was mated into BPΔOR reporter strains from DH5α in matings using *E. coli* MM294(pRK2013). Unique clones were generated for each transcription assay.

β-Galactosidase assay. Transcriptional analysis was done using bacteria grown on solid media as described previously (3). Transcription was monitored using β-Gal as a reporter using a standard colorimetric assay (40). Data were collected from four independent assays. Student's *t* test was used to analyze the data.

HeLa cell adherence assay. The HeLa cell adherence assay was performed as a modification of the *B. pertussis* HeLa cell invasion assay (see below). Bacteria were incubated with HeLa cell monolayers for 90 min in Eagle minimal essential medium (MEM; Invitrogen) supplemented with 3% fetal bovine serum (FBS; Flow Laboratories, McLean, VA) at 37°C in 5% CO_2. HeLa monolayers were then washed five times for 5 min each with rotation at 120 rpm at room temperature using 500 μl MEM–3% FBS. Monolayers were then rinsed with trypsin buffer and treated with 0.25% trypsin solution (Invitrogen) to lift the monolayers and dislodge adherent bacteria. Cells were diluted in MEM–3% FBS and plated onto BGA in duplicate for determination of total CFU. Data were collected from three independent assays. Student's *t* test was used to analyze the data.

HeLa cell invasion assay. The gentamicin invasion assay was performed as previously described (19). Separate control experiments demonstrated that the *risA* mutant did not result in an appreciably greater sensitivity to gentamicin than in parental strains containing wild-type *risA* (data not shown). Colony counts were collected from at least three independent assays. Student's *t* test was used to analyze the data.

RESULTS

Sequence analysis of the *risAS* locus of *B. pertussis*. The *ompR*/*envZ* orthologs *risA* and *risS* were compared between *Bordetella* spp. using the published genome sequences of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*; published *risAS* 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* 35086. 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 nematophilus* is only 342 amino acids long but can complement *E. coli envZ*-null mutants in an osmodependent manner; however it is truncated in its N-terminal perplasmic 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* (CN2591 and three ovine strains, BPP-HI, BPP-C, and BPP-k2) and *B. bronchiseptica* (CN 7635E and six rabbit isolates, BBr-4 through BBr-9) did not contain the

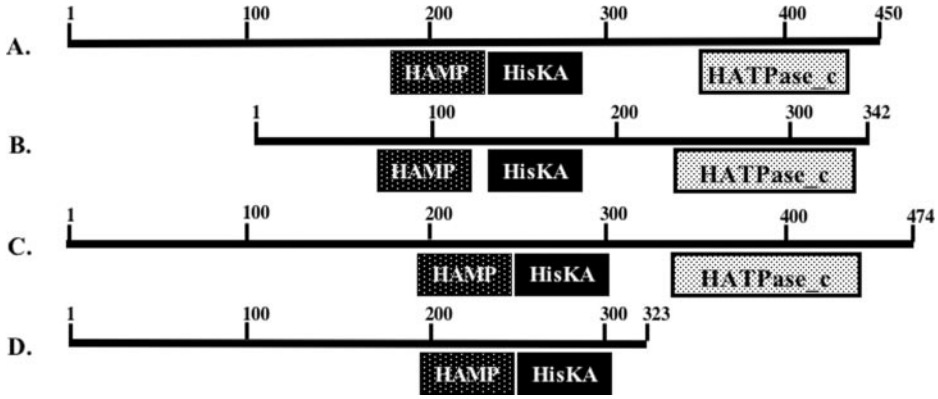

FIG. 1. Comparison of domain structures of EnvZ and RisS proteins. (A) *E. coli* EnvZ; (B) *Xenorhabdus nematophilus* AN6/1 EnvZ; (C) *B. bronchiseptica* RB50 RisS; (D) *B. pertussis* Tohama I RisS. HAMP, receptor histidine kinase domain; HisKA, dimerization/phosphoacceptor, HATPase_c; histidine kinase-like ATPases.

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* (30). 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 deduced *B. pertussis* RisA. Residue D60 of RisA lies in a domain between V58 and R76 that is completely conserved between *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. 2 and Fig. 3, lower-molecular-mass bands) and Vra-b under both nonmodulating and modulating conditions. *B. pertussis* strain BPOR, which has *risA* insertionally disrupted by a *neo* cassette, demonstrated decreased expression of the Vras even when the bacteria were grown under modulating conditions but did not appear to have altered expression of the *vag* products pertactin (Prn) or pertussis toxin subunit S1 (data not shown). To confirm that the decrease in expression of the Vras was due specifically to the mutation in *risA* and not polar effects due to the *neo* insertion, we constructed mutants with single in-frame deletions in *risA* (BPΔOR) or *risS* (BPΔRS). Levels of Vra-a and Vra-b were greatly diminished in the Δ*risA* 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 Δ*risS* 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 Δ*risA* 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. Immunoblots 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 Δ*risA* mutant regardless of the presence of pRK310 or pRisA, and their expression levels did not differ appreciably between the Δ*risA* stains and their wild-type isogenic parent (Fig. 3A and B). In addition, standard zones of hemolysis were observed when *risA* mutants

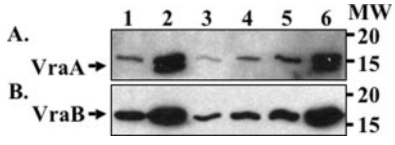

FIG. 2. Expression of Bvg-repressed antigens in *risA* and *risS* mutants. Lanes: 1, parental *Bordetella pertussis* strain BP536 grown on BGA without modulators; 2, *B. pertussis* strain BP536 grown on BGA with the modulators 5 mM nicotinic acid and 40 mM $MgSO_4$; 3, Δ*risA* mutant (BPΔOR) grown on BGA; 4, Δ*risA* mutant grown on BGA with modulators; 5, Δ*risS* mutant (BPΔRS) grown on BGA; 6, Δ*risS* 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.

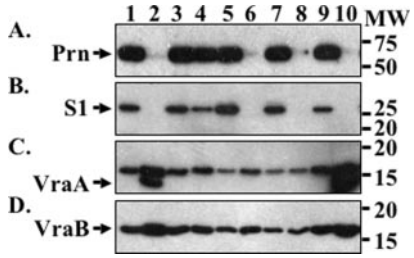

FIG. 3. Expression of Bvg-regulated proteins. Lanes: 1, parental *Bordetella pertussis* strain BP536 grown on BGA without modulators; 2, *B. pertussis* strain BP536 grown on BGA with the modulators 5 mM nicotinic acid and 40 mM $MgSO_4$; 3, *bvgS-C* (BPSC) mutant grown on BGA; 4, *bvgS-C* mutant grown on BGA with modulators; 5, Δ*risA* mutant (BPΔOR) grown on BGA; 6, Δ*risA* mutant grown on BGA with modulators; 7, Δ*risA* mutant with low-copy-number vector [BPΔOR (pRK310)] grown on BGA; 8, BPΔOR(pRK310) grown on BGA with modulators; 9, Δ*risA* 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 Δ*risA* mutant compared to the wild type even in the presence of modulators, with levels of the Vras in the modulated Δ*risA* 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 either nonmodulating or modulating conditions (Fig. 3). However, introduction of pRisA complemented the Δ*risA* mutant, restoring strong expression of the Vras to a level severalfold higher than that produced by the wild-type strain in the presence of modulators (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 products. 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 Δ*risA* 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 *vrg*s was greatly reduced in the Δ*risA* mutant BPΔOR, even under modulating conditions. However, introduction of pRisA restored transcription of the *vrg*s 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 Δ*risA* mutant BPΔOR were compared under modulating conditions, transcription of the *vrg*s *kspM* 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 *vag*s. Analysis of transcription data for pRisA-complemented Δ*risA* 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, *kspM* 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 *vrg*s regardless of the affect of Bvg regulation. In summary, these data indicate that mutation of *risA* greatly diminishes the ex-

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

Gene	β-Galactosidase units[a]							
	BP536 (wild type)		BPSC (*bvgS-C*)		BPΔOR (Δ*risA*)		BPΔOR(pRisA)	
	NM[b]	SO_4-Nic[c]	NM	SO_4-Nic	NM	SO_4-Nic	NM	SO_4-Nic
bvgR	1,801 ± 93	128 ± 42	1,700 ± 152	2,527 ± 165	1,479 ± 54	110 ± 10	1,545 ± 39	24 ± 5
kpsM	77 ± 5	350 ± 52	80 ± 13	102 ± 33	82 ± 18	104 ± 26	0 ± 2	807 ± 62
vrg-6	337 ± 24	2,312 ± 66	245 ± 52	607 ± 85	90 ± 14	124 ± 32	911 ± 51	4,354 ± 308

[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 $MgSO_4$.

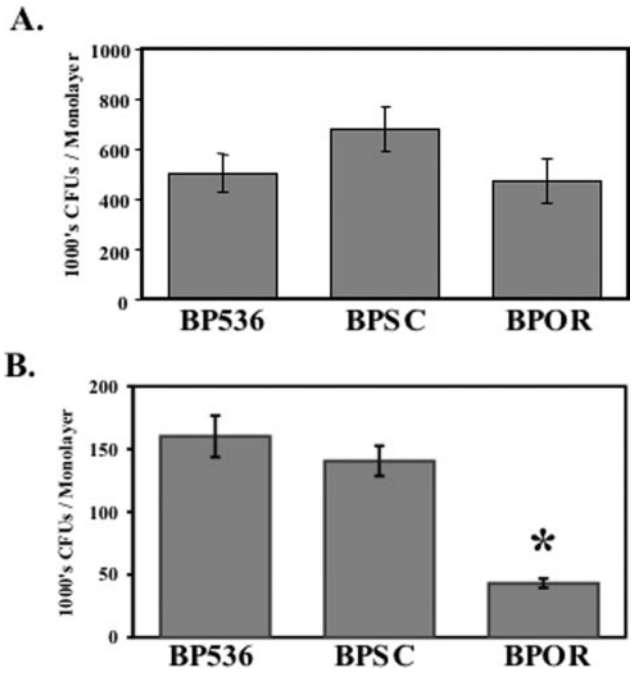

FIG. 4. HeLa cell adherence or invasion by a *risA* mutant. (A) adherence assay; (B) invasion assay. BP536, parental *B. pertussis* strain; BPSC, *bvgS-C* mutant; BPOR, *risA* mutant. Values represent 10^3 CFU recovered from gentamicin-treated (panel B only) and washed monolayers. Error bars represent standard errors of the means for three replicates. *, $P < 0.01$, compared to BP536.

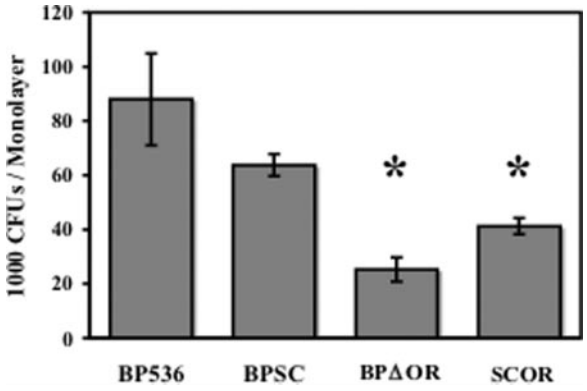

FIG. 5. HeLa cell invasion by Bvg-constitutive strains. BP536, parental *B. pertussis* strain; BPSC, *bvgS-C* mutant; BPΔOR, Δ*risA* mutant; SCOR; *bvgS-C*/Δ*risA* double mutant. Values represent 10^3 CFU recovered from gentamicin-treated and washed monolayers. Error bars represent standard errors of the means for four replicates. *, $P < 0.01$, compared to BP536.

pression of Bvg-repressed products at the level of transcription, while not apparently affecting the transcription of the *vag*s.

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-Δ*risA* 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 Δ*risA* mutant BPΔOR and the BvgS-constitutive/Δ*risA* 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. pertussis*. This suggests that an increased level of RisA transactivator increases expression of the Vras.

Transcriptional analysis using gene fusions demonstrated a dependence on *risA* for transcription of the *vrg*s. 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* expression and Vra production at the level of transcription. Furthermore, 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 transactivator induces higher levels of expression of the *vrg*s. It will be interesting to determine whether RisA acts directly to bind the *cis*-activating sites of the *vrg*s or indirectly by altering the expression of other regulatory molecules.

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

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 autophosphorylation. This mutation was not found in *risS* of *B. bronchiseptica* or *B. parapertussis*. When *B. pertussis risS* was mutated 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 dependent on functional *risA* but is independent of *risS*. It is interesting 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 phosphotransfer has been demonstrated between the purified components 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 between the AcrB sensor of anaerobiosis and the OmpR regulator of porin expression in *E. coli*, suggesting interplay between phosphorelay systems (37). Therefore, it is possible that another regulatory system, other than BvgAS or RisAS, may regulate the expression of the *vrg*s in *B. pertussis* via RisA activation.

B. bronchiseptica risAS expression is maximal at 37°C in the absence of magnesium cations, when the bacteria are intracellular (30). It will be important to determine which signals regulate RisA expression and activation in *B. pertussis*. This is of particular interest given that *risS* 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 determine if shuttling a wild-type *B. bronchiseptica risS* gene to *B. pertussis* alters the expression patterns of Bvg-repressed molecules 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. bronchiseptica* 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 requirement for RisA in *B. pertussis* for maximal expression of the *vrg*s contrasts with the pleiotropic nature of the Ris-regulated 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 indicate that the genes regulated by *risAS* and the mechanisms of RisA-mediated gene regulation have diverged during evolution of the bordetellae. It is possible that differences in *risA* gene regulation and differences in *vrg* expression in the bordetellae have resulted from the same selective pressure that resulted in the evolution of *Bordetella* subspecies with different host ranges, physiology, and disease presentations.

B. bronchiseptica ris mutants have reduced survival in macrophage invasion models (30, 65). *B. pertussis* can invade epithelial-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* independent. Since *vrg* expression is greatly diminished in *risA* mutants, it could be hypothesized that the *vrg*s are required for this intracellular survival or invasion. However, we found that Bvg-activated phase-locked bacteria, which constitutively repress the *vrg*s, 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 bacterium. 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*s 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 *B. pertussis* to survive stressful factors in its environment.

In *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 *B. pertussis*, a relevant *vrg*-encoded biological phenotype is uncertain. Although a Bvg-repressed capsule has not been described for *B. pertussis*, *kpsM*, a capsule biosynthesis gene, has been described as a *vrg* in *B. pertussis* (3, 29). This is intriguing, as polysaccharide capsules are known to be important virulence factors for a number of bacteria (47). We have confirmed that *kpsM* is a *B. pertussis vrg*; however, it is unknown whether the KpsM protein is expressed in *B. pertussis*. Furthermore, in *B. pertussis*, several other genes of the capsule biosynthesis locus have been inactivated or have been shuttled to alternate positions in the chromosome (49). It is possible that the altered subset of capsule synthesis genes could have a biological function in *B. pertussis*.

The smaller number of *vrg*s expressed in *B. pertussis* compared with *B. bronchiseptica* may indicate that the *B. pertussis vrg*s are decaying relative to *B. bronchiseptica*. However, many *vrg*s are still expressed in *B. pertussis*, and moreover, the expression patterns of the *vrg*s 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 *vrg*s in the life cycle of the human pathogen *B. pertussis*.

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