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Differential regulation of type III secretion and virulence genes in *Bordetella pertussis* and *Bordetella bronchiseptica* by a secreted anti-σ factor

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Contributed by Jeff F. Miller, January 15, 2016 (sent for review November 17, 2015; reviewed by Erik L. Hewlett and John J. Mekalanos)

The BvgAS phosphorelay regulates ∼10% of the annotated genomes of *Bordetella pertussis* and *Bordetella bronchiseptica* and controls their infectious cycles. The hierarchical organization of the regulatory network allows the integration of contextual signals to control all or specific subsets of BvgAS-regulated genes. Here, we characterize a regulatory node involving a type III secretion system (T3SS)-exported protein, BtrA, and demonstrate its role in determining fundamental differences in T3SS phenotypes among *Bordetella* species. We show that BtrA binds and antagonizes BtrS, a BvgAS-regulated extracytoplasmic function (ECF) sigma factor, to couple the secretory activity of the T3SS apparatus to gene expression. In *B. bronchiseptica*, a remarkable spectrum of expression states can be resolved by manipulating *btrA*, encompassing over 80 BtrA-activated loci that include genes encoding toxins, adhesins, and other cell surface proteins, and over 200 BtrA-repressed genes that encode T3SS apparatus components, secretion substrates, the BteA effector, and numerous additional factors. In *B. pertussis*, BtrA retains activity as a BtrS antagonist and exerts tight negative control over T3SS genes. Most importantly, deletion of *btrA* in *B. pertussis* revealed T3SS-mediated, BteA-dependent cytotoxicity, which had previously eluded detection. This effect was observed in laboratory strains and in clinical isolates from a recent California pertussis epidemic. We propose that the BtrA-BtrS regulatory node determines subspecies-specific differences in T3SS expression among *Bordetella* species and that *B. pertussis* is capable of expressing a full range of T3SS-dependent phenotypes in the presence of appropriate contextual cues.

virulence gene regulation | ECF sigma factor | T3SS | *Bordetella* | host adaptation

The evolution of the human respiratory pathogen *Bordetella pertussis* from its broad host range *Bordetella bronchiseptica*-like ancestor was accompanied by genome reduction, a proliferation of pseudogenes, and host restriction (1–3). This transition also correlates with a systematic pattern of transcriptional diversity in the BvgAS virulence regulon that we hypothesized was due to functional alterations in regulatory factors that control clusters of loci in a hierarchical network (4). In *B. bronchiseptica* and *B. pertussis*, the transmembrane BvgS histidine kinase and BvgA response regulator coordinate expression of nearly all known colonization and virulence genes, an array of transcriptional regulators, and numerous genes of unknown function (3–6). BvgAS establishes a spectrum of phenotypic phases in response to environmental cues, functioning as a rheostat that controls the infectious cycle (7, 8). The Bvg^+ phase is characterized by high level BvgAS activity, expression of virulence factors [including pertussis toxin (9), adenylate cyclase toxin (10, 11), filamentous hemagglutinin (12), fimbriae (13), the *Bordetella* secretion complex (Bsc) type III secretion system ($T3SS_{Bsc}$) (14–16)], and the ability to survive and replicate on respiratory epithelia (17). In the Bvg phase, the BvgAS phosphorelay is quiescent, Bvg^+ phase genes are silent, and "virulence-repressed genes" are maximally transcribed (4, 17, 18). These genes include flagella and motility loci in *B. bronchiseptica* and genes with unknown function in *B. pertussis*. The roles of BvgAS-regulated genes that are differentially expressed between the Bvg^+ and Bvg^- extremes of the regulatory spectrum are largely unresolved (7, 19).

In vitro, $T3SS_{Bsc}$ is a particularly conspicuous virulence determinant owing to its ability to induce rapid cytotoxicity in a diverse array of eukaryotic cells, ranging from mammalian to yeast (15, 20). In vivo studies with *B. bronchiseptica* show that $T3SS_{Bsc}$ plays an immunomodulatory role that facilitates persistence in the lower respiratory tract (21, 22). Remarkably, only a single effector, BteA, has been definitively identified as a translocated substrate (15, 16). In *B. bronchiseptica*, BteA is necessary and sufficient for cytotoxicity in vitro, and mutations in *bteA* recapitulate phenotypes associated with deleting the T3SS ATPase gene, *bscN* (14–16).

As a result of their close evolutionary relationship, comparative studies exploiting the experimental tractability of *B. bronchiseptica* are relevant for understanding *B. pertussis* (3, 4, 7, 16, 23–25). *B. bronchiseptica* isolates from diverse hosts, including humans, readily display T3SS activity in vitro (20). In contrast, there are no reports of T3SS-dependent cytotoxicity by *B. pertussis*, despite the fact that T3SS loci are highly conserved, are functionally interchangeable, and bear the hallmarks of positive selection (3, 16, 25). Recent studies are beginning to shed light on this paradox. Although T3SS activity is not observed with

Significance

***Bordetella bronchiseptica* isolates from diverse hosts, including humans, display potent cytotoxicity against a broad range of mammalian cells, which is dependent on type III secretion system (T3SS) effector BteA. In contrast, neither laboratory nor clinical isolates of *Bordetella pertussis* have been observed to display T3SS-dependent cytotoxicity, despite the fact that T3SS genes are present, intact, and nearly identical to their *B. bronchiseptica* counterparts. We have characterized a regulatory node, involving a T3SS-exported anti-σ factor, BtrA, that controls virulence gene expression in *Bordetella species*. Of particular relevance to human disease, deletion of *btrA* in *B. pertussis* derepresses T3SS gene expression and confers readily detectable BteA-dependent cytotoxicity. These observations warrant a reassessment of type III secretion in the pathogenesis and prevention of pertussis.**

Author contributions: U.A. designed research; U.A., B.S., N.C., Y.C., and C.B. performed research; U.A. contributed new reagents/analytic tools; U.A., G.C., and J.F.M. analyzed data; and U.A. and J.F.M. wrote the paper.

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The authors declare no conflict of interest.

Data deposition: RNA-seq data reported in this paper have been deposited in the Sequence Read Archive, www.ncbi.nlm.nih.gov/sra (submission no. SRP064665; National Center for Biotechnology Information BioProject no. PRJNA296526).

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laboratory-adapted strains, Bsp22, a highly expressed secretion substrate that polymerizes to form a flexible filament connecting the needle to the translocon, has been detected in culture supernatants of *B. pertussis* clinical isolates (24, 26). Furthermore, mutations in the *B. pertussis bscN* allele result in elevated production of proinflammatory cytokines and accelerate clearance from the lungs of aerosol-infected mice (24). A hypothesis that could explain species-specific differences is that the regulatory module in the BvgAS regulon that includes T3SS loci has diverged with host specificity. Indeed, T3SS activity is controlled at multiple levels. *bteA* and *bsc* apparatus genes are transcriptionally activated by an extracytoplasmic function (ECF) sigma factor, BtrS, which is encoded by a BvgAS-activated gene (25). In addition, the partner-switching proteins BtrU, BtrV, and BtrW mediate a cycle of serine phosphorylation and dephosphorylation events that gate secretion through the T3SS apparatus (25, 27).

Here, we characterize a regulatory node involving a T3SS-exported anti-σ factor, BtrA (20, 28), and demonstrate its activity as a secreted BtrS antagonist that differentially controls expression of nearly 300 genes that define six distinct regulatory modules within the BvgAS virulence regulon. In *B. bronchiseptica*, the relative levels of BtrA and BtrS determine the magnitude of T3SS activity. In both laboratory and recent clinical isolates of *B. pertussis*, deletion of *btrA* derepressess T3SS gene expression and confers readily observable, BteA-dependent cytotoxicity in vitro. Our results show that *B. pertussis* is capable of expressing the full range of T3SS-dependent phenotypes and that the BtrA regulatory node is a key determinant of species-specific differences in expression. These observations provide the impetus and foundation for a comprehensive assessment of $T3SS_{Bsc}$ in the pathogenesis and prevention of human pertussis.

Results

BtrA Is a T3SS Substrate That Regulates Multiple Virulence Genes. The *bsc* locus, which includes T3SS apparatus, translocon, and chaperone genes, is flanked by a cluster of regulatory loci that encode BtrS and the BtrUWV partner-switching module (25, 27) (Fig. 1*A*). Recent studies implicate BtrA (also called BspR), which is encoded directly upstream of *btrS*, as an exported substrate of the Bsc T3SS, illustrated in Fig. 1*B* using *B. bronchiseptica* strain RB50 grown in vitro under conditions permissive for type III secretion (20, 28). BtrA and Bsp22, a known T3SS substrate (26), are exported into supernatants in a manner dependent on the BscN ATPase. Fractionation controls included pertactin (Prn), an OM autotransporter that releases an N-terminal domain after cleavage (29), and BvgA, a cytoplasmic response regulator. These results confirm that BtrA is an exported T3SS substrate in RB50.

Next, we generated an RB50 derivative with an in-frame deletion in *btrA*. A readily apparent phenotype was observed in which independently constructed mutants formed small, weakly hemolytic colonies on blood agar in comparison with RB50 (Fig. 1*C*). Relative growth rates in shake flask cultures mirrored differences in colony size because the Δ*btrA* mutant grew more slowly and reached lower levels in stationary phase than the WT parental strain (Fig. S1*A*). Because hemolytic activity, colony morphology, and growth rate are

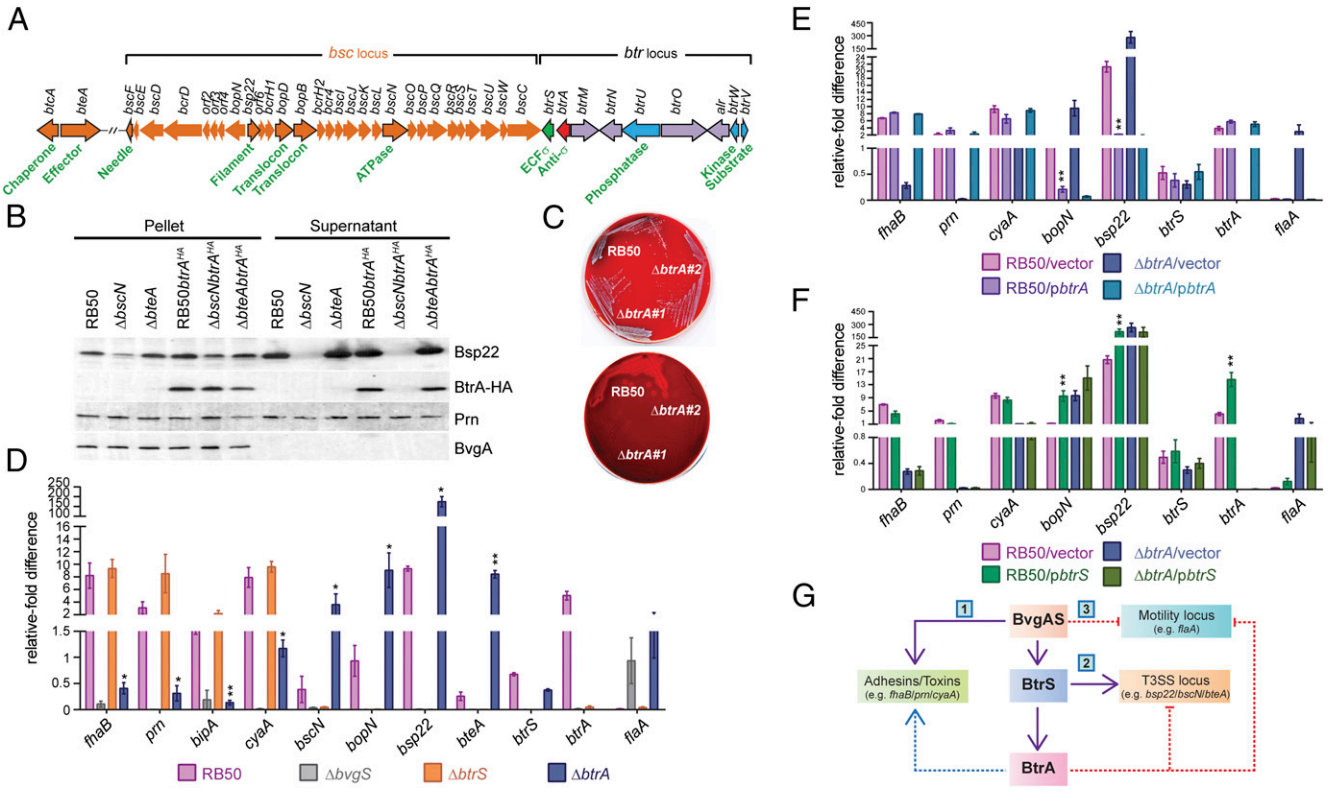

Fig. 1. BtrA differentially regulates three distinct modules of the BvgAS regulon. (*A*) The *bsc-btr* locus gene organization. Orange ORFs designate $T3SS_{Bsc}$ loci (14), blue ORFs encode partner-switching proteins (27), and purple ORFs are uncharacterized. *btrA* and *btrS* are red and green, respectively. Select ORFs are highlighted in black outline with functions shown. (*B*) BtrA secretion characterized by immunoblot analysis of culture supernatant or pellet fractions from the indicated strains. Blots were probed with antisera against BvgA, Prn, Bsp22, or the HA tag. (*C*) Colony morphology and hemolysis phenotypes of RB50 vs. independently isolated Δ*btrA* derivatives on Bordet–Gengou agar scanned with reflective mode (*Top*) or transmission mode (*Bottom*). RB50Δ*btrA*#1 was used in all subsequent studies. (*D–F*) qRT-PCR with RNA from indicated strains. Fold differences in transcript levels were calculated relative to *recA* expression. Colored bars represent average values from three independent experiments, and error bars represent ± SEM. (*G*) Summary of results showing that BtrA positively regulates adhesin and toxin genes (*i*), negatively controls *flaA* expression (*ii*), and represses a subset of T3SS genes (*iii*). Student's *t* test was used for statistical analysis, and genes showing significant differences between RB50 vs. RB50Δ*btrA* (*D*), RB50/vector vs. RB50/p*btrA* (*E*), or RB50/vector vs. RB50/p*btrS* (*F*) are labeled with an * ($P < 0.05$) or ** ($P < 0.01$). See Fig. S1 for additional supporting data.

all BvgAS-regulated phenotypes, we compared the effects of deleting *btrA* on expression of a panel of genes chosen to represent different modules of the BvgAS virulence regulon.

As shown in Fig. 1*D*, transcript levels of adhesin and toxin genes (*fhaB*, *prn*, *bipA*, and *cyaA*), T3SS loci (*bopN*, *bsp22*, and *bteA*), *btrS*, and *btrA* are highly dependent on BvgS whereas the flagellin locus (*flaA*) is Bvg-repressed (3). Expression of T3SS genes additionally requires the BtrS sigma factor, and the same is true for *btrA*. Deletion of *btrA* resulted in a marked increase in T3SS-associated transcripts, with induction ratios ranging from 10-fold (*bscN*) to 34-fold (*bteA*). In contrast, toxin and adhesin genes showed the opposite effect and were down-regulated 7-fold (*cyaA*) to 20-fold (*fhaB*) in Δ*btrA* mutants. Interestingly, *flaA* expression was increased in the Δ*btrA* strain to levels similar to the Δ*bvgS* mutant. As shown in Fig. S1*B*, these transcriptional effects are reflected at the levels of protein expression and secretion. Complementation with *btrA* reversed the effects of the Δ*btrA* deletion on transcription (Fig. 1*E*), protein expression, and secretion (Fig. S1*C*). In WT RB50, overexpression of *btrA* decreased expression of T3SS-associated genes but did not confer increased expression of toxin or adhesin loci, suggesting that BtrA levels in RB50 are sufficient for maximal expression under laboratory conditions. Together, these data confirm and extend previously published results (28) and show that BtrA differentially regulates three distinct modules of the BvgAS regulon: (*i*) Bvg^+ phase adhesin and toxin genes, which require BtrA for full level expression, (*ii*) Bvg^+ phase T3SS loci that require BtrS and are repressed by BtrA, and (*iii*) the Bvg phase flagellin locus, which is down-regulated by BtrA (Fig. 1*G*).

BtrA Is a Secreted BtrS Antagonist. The observations that BtrA and BtrS have opposing effects on T3SS gene expression and that BtrA is itself a T3SS substrate prompted the hypothesis that BtrA antagonizes BtrS. As shown in Fig. 1*F*, overexpression of *btrS* in RB50 resulted in elevated levels of *btrA*, *bsp22*, and *bopN* transcription. For the T3SS apparatus components *bsp22* and *bopN*, BtrS overexpression mimicked the effects of deleting *btrA*. In contrast, *cyaA*, *fhaB*, and *flaA* transcript levels were relatively insensitive to increased BtrS. Corroborating results were obtained by monitoring protein expression and secretion (Fig. S1*D*). These data show that high level expression of T3SS genes can be achieved by eliminating BtrA or by overexpressing BtrS, consistent with an antagonistic interaction.

Because ECF sigma factors are often controlled by antagonistic binding partners (i.e., anti-σ factors), we used a multireadout yeast two-hybrid system to determine whether BtrA and BtrS interact (Fig. 2*A* and Fig. S2*A*). BtrA fused to the Gal4 DNA binding domain strongly interacted with a BtrS-Gal4 activation domain fusion protein, and identical results were observed when bait and prey inserts were swapped. No interactions were detected with negative control constructs encoding fusions with noncytotoxic BteA (BteA644) or Lamin. To determine the relationships between BtrS binding and gene regulation, we examined the ability of a series of truncated BtrA derivatives to interact with BtrS, repress *bsp22* and *bopN* expression, and up-regulate the expression of *fhaB*. As shown in Fig. 2 *B* and *D*, removal of 33, 63, 93, or 123 residues from the BtrA C terminus eliminated the ability to repress *bsp22* and *bopN* and to activate *fhaB* in the Δ*btrA* background. The Δ93 and Δ123 truncations also eliminated BtrS binding (Fig. 2*C* and Fig. S2*A*), as did two internal deletions. All mutant BtrA proteins were expressed and secreted at levels that corresponded to their ability to repress T3SS gene expression (Fig. 2*B* and Fig. S2*B*).

As summarized in Fig. 2*E*, our data resolve three functional regions of BtrA: a C-terminal domain required for transcriptional

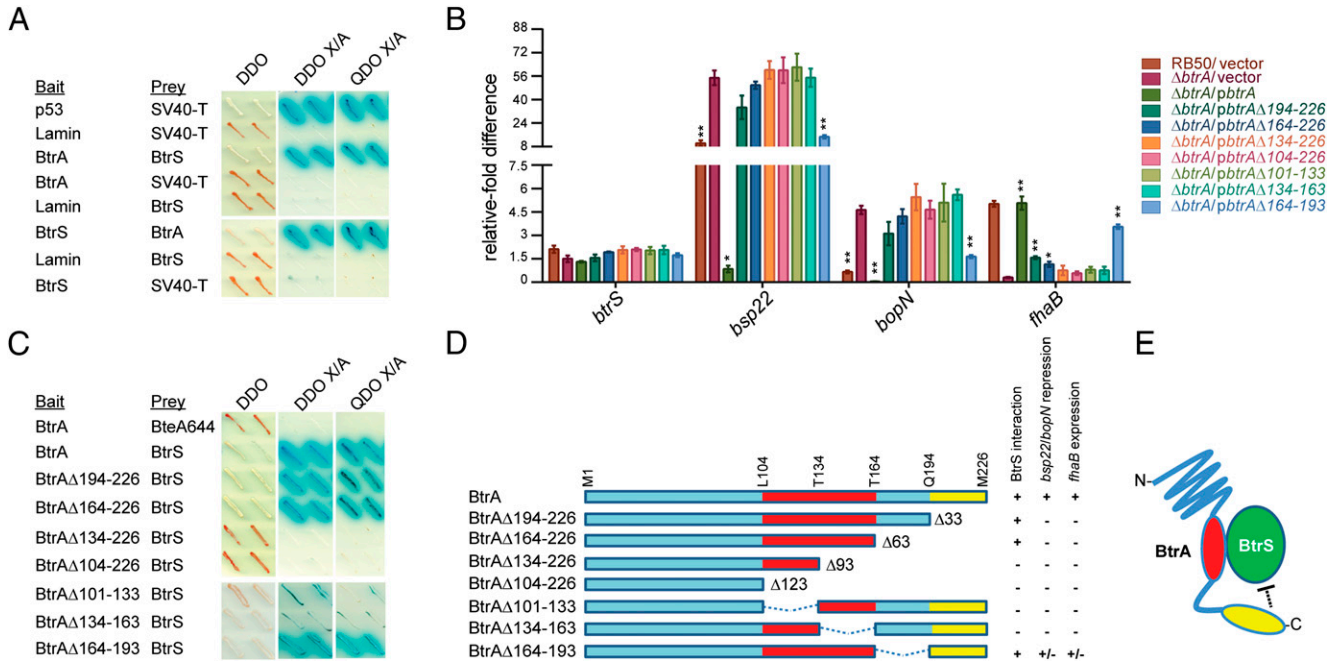

Fig. 2. BtrA binds and antagonizes BtrS. (*A*) Yeast two-hybrid analysis of BtrA–BtrS interactions. Yeast diploids were grown on double dropout (DDO) medium lacking leucine and tryptophan, DDO containing the chromogenic substrate X-α-gal and aureobasidin A (DDO X/A), and on quadruple dropout (QDO X/A) medium containing X-α-gal and aureobasidin A but lacking leucine, tryptophan, histidine, and adenine. Binding interactions result in growth on high stringency QDO X/A medium, resistance to aureobasidin A, and α-galactosidase activity indicated (blue). Interaction of SV40 large T antigen with p53 or Lamin served as positive or negative controls, respectively. Noncytotoxic BteA644 also served as a negative control. Representative results from two independent experiments are shown. All interactions were confirmed by swapping bait and prey protein fusions (Fig. S2). (*B*) qRT-PCR with RNA from indicated strains. Deletion endpoints correspond to amino acid residues in BtrA as shown in *D*. Fold differences in transcript levels were calculated relative to *recA* expression. Colored bars represent average values from three independent experiments, and error bars represent ± SEM. Student's *t* test was used for statistical analysis. For each gene, strains showing significant differences from Δ*btrA*/vector are labeled with * ($P < 0.01$) or ** ($P < 0.001$). (*C*) Yeast two-hybrid analysis of interactions between BtrS and BtrA deletion mutants. (*D*) Ability of BtrA variants to bind BtrS, repress T3SS genes, and activate *fhaB* expression. (*E*) Model of BtrA—BtrS interactions occurring in the cytoplasm, which are disrupted by BtrA secretion; see *Results* for details. Fig. S2 contains additional supporting data.

repression and activation, a central region required for BtrS binding, and N-terminal sequences that mediate recognition as a T3SS substrate. BtrS binding is likely required for transcriptional control, but it is clearly not sufficient. A simple hypothesis for the ability of BtrA to activate expression of *fhaB* and coregulated loci invokes the existence of a BtrS-activated repressor specific for this subset of genes. This model accounts for the observations that BtrS binding is required for both activation and repression by BtrA, and it is supported by RNA-seq analysis.

Effects of BtrA on Cytotoxicity and Virulence. *B. bronchiseptica* isolates have T3SS-dependent cytotoxic effects on a broad range of cell types (14, 16, 20, 30). Most respond like HeLa cells (Fig. 3*A*), which are efficiently killed by RB50 in a BscN- and BteA-dependent manner. In contrast, human pneumocyte-derived A549 cells are unusually resistant to RB50 cytotoxicity through an unknown mechanism (20) (Fig. 3*B*). Resistance was reversed, however, by deleting *btrA*, which resulted in high level BteA-dependent cytotoxicity against HeLa as well as A549 cells. The levels of cell death observed with RB50Δ*btrA* are similar to strain Bbr77, which represents a clade of hypercytotoxic *B. bronchiseptica* isolates from human respiratory infections (20).

We next used a murine intranasal challenge model to examine the effects of deleting *btrA* on respiratory colonization and virulence. Groups of C57BL/6NCr mice were intranasally infected with WT or Δ*btrA* strains using a protocol that results in infection of the entire respiratory tract by RB50, followed by gradual clearance from the lungs and trachea (31). Bacterial numbers in the trachea were indistinguishable throughout the course of the experiment (Fig. 3*C*). In lungs, the Δ*btrA* mutant showed a modest 1- to 2-log decrease in infection levels until the peak at day 7, followed by clearance with similar kinetics as the WT strain RB50. All animals seemed healthy during the course of the experiment.

Surprised by relative lack of a correlation between hypercytotoxicity in vitro (Fig. 3*B*) and the infection results shown in Fig. 3*C*, we compared RB50 and the Δ*btrA* mutant in an acute respiratory infection model in which the majority of animals become moribund within 1 wk postinoculation (20, 31). Again, the Δ*btrA* mutant was no more virulent than the WT strain (Fig. S3). Because eliminating BtrA results in increased T3SS activity and decreased adhesin and toxin expression, these opposing effects and other changes may account for the lack of an overt phenotype. An alternative possibility is that the system is normally derepressed in vivo through active secretion of BtrA, making the Δ*btrA* allele superfluous. In either case, it is important to understand the extent to which BtrA differentially regulates gene expression in *Bordetella*.

BvgAS, BtrS, and BtrA Regulatory Networks. We compared the transcriptomes of RB50 and isogenic Δ*bvgS*, Δ*btrS*, Δ*btrA*, and Δ*btrA*Δ*btrS* derivatives using RNA-seq. The transcriptional data were used to generate nonhierarchical clusters, from which four broad groups of coexpressed gene classes were derived (Fig. 4). Bvg-repressed genes (n = 297) were found in clusters 1 and 2 whereas Bvg-activated genes (n = 242) grouped in clusters 3 and 4. Genes influenced by deletion of *btrA* (n = 294) were divided between clusters 1, 3, and 4. Network models consistent with observed regulatory profiles are shown in Fig. 4*B*.

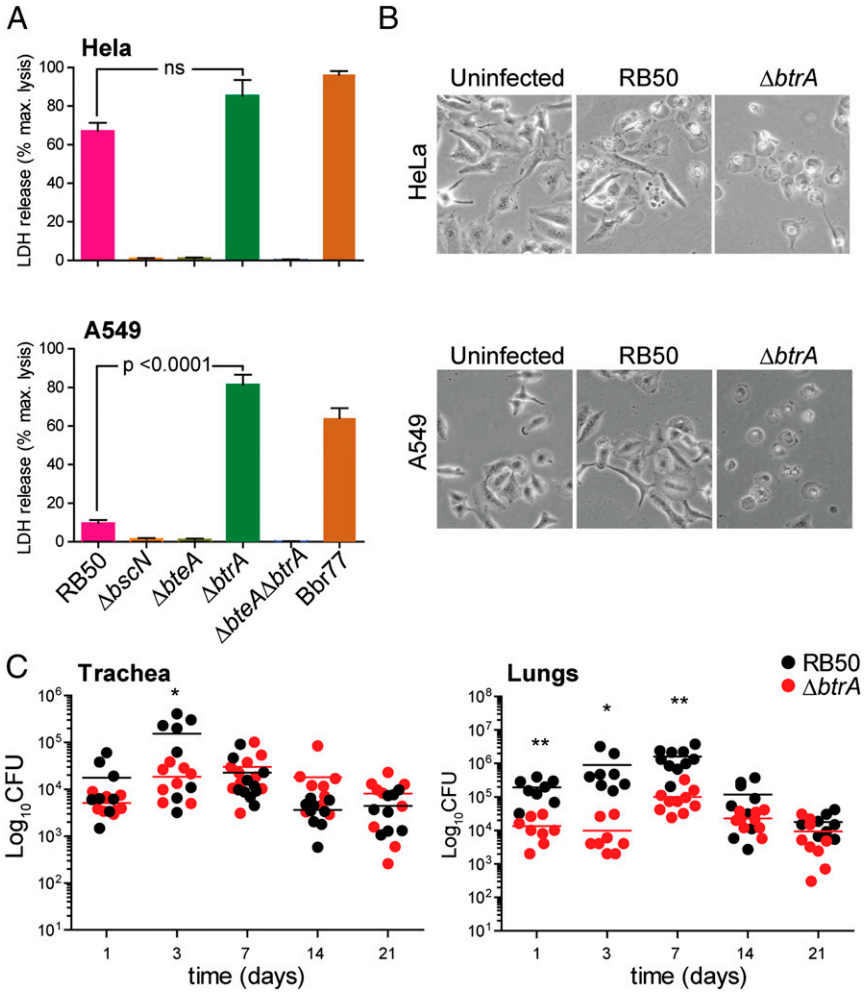

Fig. 3. Hypercytotoxicity and virulence phenotypes of RB50Δ*btrA*. (*A*) HeLa cells (*Top*) or A549 cells (*Bottom*) were infected with indicated strains at an MOI of 50:1 for 3 h, and cytotoxicity was measured by lactate dehydrogenase (LDH) release. Bars represent averages from three independent experiments, and error bars indicate ± SEM. (*B*) HeLa or A549 cells were infected with indicated strains at an MOI of 50:1 for 2 h and examined by phase-contrast microscopy at 20× magnification on a Carl Zeiss Axiovert 40 CFL microscope. Images are representative of three independent experiments. (*C*) Four-week-old female C57BL/6NCr (B6) mice were intranasally inoculated with 5×10^5 cfu of RB50 or RB50Δ*btrA* and killed on indicated days. The number of colony-forming units (CFU) per lung or trachea is shown for each animal. Statistical significance (t test) is indicated with * ($P < 0.05$) or ** ($P < 0.001$). Fig. S3 contains additional data.

Cluster 1 contains 89 genes that are coregulated with the flagellin locus *flaA*. These loci are minimally expressed in RB50, highly activated in the Bvg phase (Δ*bvgS*), and activated in the Δ*btrA* strain. Although deleting *btrS* alone has little effect, combining the Δ*btrS* and Δ*btrA* alleles reverses the Δ*btrA* phenotype. Coexpressed genes in cluster 1 encode a large subset of motility and chemotaxis proteins, along with an assortment of predicted surface and regulatory factors (Dataset S1).

Cluster 2 includes 208 Bvg-repressed genes that are maximally expressed in the Δ*bvgS* strain with no apparent involvement of BtrA or BtrS. These repressed genes include loci encoding ABC transporters, a sulfate transport system, urea uptake and metabolism (32), a host of diverse metabolic functions, and chemotaxis and motility genes distinct from those in cluster 1. The distribution of motility factors between clusters 1 and 2 accounts for the lack of motility in soft agar by the Δ*btrA* strain, in which only cluster 1 genes are derepressed, compared with active motility in the Δ*bvgS* mutant in which all chemotaxis and motility loci are actively transcribed (Fig. S4 and Dataset S1).

The 168 Bvg-activated genes in cluster 3 split into two groups. Cluster 3a loci encode many of the canonical *Bordetella* virulence determinants and their accessory factors, including FHA (*fhaB* and *-C*), adenylate cyclase toxin (*cyaA to -E*), pertactin (*prn*), and fimbria (*fimA to -D*). In addition to their dependency on BvgS, these genes are down-regulated in the Δ*btrA* strain, and this phenotype is reversed in the Δ*btrA*Δ*btrS* double mutant. In contrast, cluster 3b genes are Bvg-activated with little if any effect of BtrA or BtrS. The differential effects of BtrA/BtrS on cluster 3a vs. 3b loci are readily apparent in Fig. 4*C*. Cluster 3b includes numerous T3SS loci that are BtrS-independent. These loci encode structural components of the secretion apparatus (*bscC* to *-F*, *bscO* to *-W*), proteins involved in controlling secretion activity (*btrW*, *btrV*), and other T3SS-associated factors (Dataset S1).

Cluster 4 contains 126 genes that require BtrS for expression and are negatively regulated by BtrA. This cluster divides into two subgroups that differ in their basal levels of expression. As shown in Fig. 4*B*, cluster 4a loci are poorly transcribed in vitro by WT or Δ*bvgS* strains whereas cluster 4b loci are expressed by RB50 at relatively high levels and down-regulated in the Δ*bvgS* mutant. In both cases, transcript levels are substantially increased by deletion of *btrA*, and this effect is reversed in a Δ*btrA*Δ*btrS* double mutant (Fig. 4 *B* and *C*). Cluster 4a includes 86 genes predicted to encode several autotransporters and ABC transport systems, regulatory proteins, and various metabolic functions. Surprisingly, this cluster also includes the complete set of genes required for pertussis toxin production (*ptxA* to *-E*) and secretion (*ptlA* to *-L*), which were previously thought to be quiescent in *B. bronchiseptica*. Cluster 4b includes T3SS loci that encode extracellular components of the translocation needle (*bscI* to *-K*, *bsp22*), the membrane-penetrating translocon and other secreted

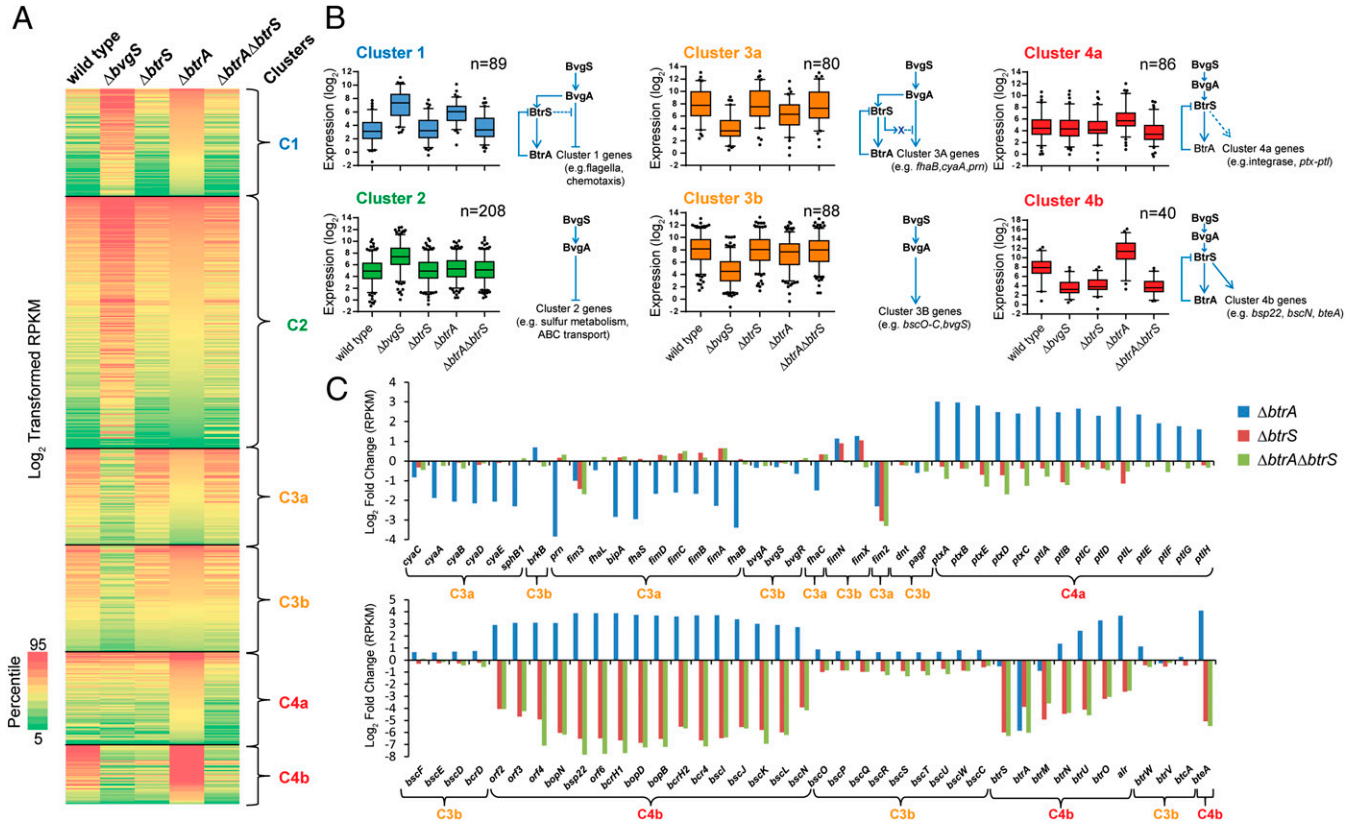

Fig. 4. BtrA, BtrS, and BvgS regulatory networks identified using RNA-seq. (*A*) Heat map of $\log_2$ transformed reads per kilobase of transcript per million mapped reads (RPKM) values showing expression levels of specific transcripts (rows) in samples from WT RB50 or Δ*bvgS*, Δ*btrS*, Δ*btrA*, or Δ*btrA*Δ*btrS* derivatives (columns). Genes with at least plus or minus threefold change in expression relative to WT were included in the analysis. Nonhierarchical clustering of RPKM values revealed four coexpressed gene clusters (C1–C4). Map colors indicate the percentile score of expression level. RNA-seq alignment statistics, datasets, and comparative analyses are listed in Table S4 and Dataset S1. (*B*) Box-and-whisker plots showing the distribution of transcripts within each gene cluster. Boxes depict data between the 25th and 75th percentiles, with horizontal lines within boxes representing the median value. Whiskers show 5th and 95th percentiles, with outliers as dots. The projected regulatory network for each cluster is drawn next to the respective plot. Arrowheads indicate transcriptional activation, and bars indicate repression. For cluster 3a, "X" designates a predicted BtrS-activated repressor that acts on a subset of BvgAS-regulated genes. (*C*) $\log_2$-fold changes in expression levels of selected genes in deletion mutants compared with WT RB50. (*Top*) Genes are ordered by relative position in the RB50 genome. (*Bottom*) T3SS genes ordered by relative position. Curly brackets designate coexpression clusters.

apparatus components (*bopB, -D,* and *-N*), the BscN ATPase, the BtrU phosphatase, and the BteA effector (Dataset S1).

As shown in Fig. S5, the effects of overexpressing *btrA* or *btrS* corroborate results obtained with deletion mutants. Taken together, transcriptional profiling experiments reveal an unexpected diversity of expression patterns among BvgAS-regulated genes revealed by manipulating BtrA. Given its pervasive effects on gene expression and the observation that the BtrA proteins of *B. bronchiseptica* and *B. pertussis* are 93% identical, we tested the possibility that BtrA regulates virulence genes in *B. pertussis*.

BtrA Controls T3SS-Mediated Cytotoxicity in *B. pertussis*. As shown in Fig. 5*A*, deletion of *btrA* in *B. pertussis* strain Bp536 derepresses *bopB*, *bopD*, *bopN*, *bsp22*, *bscN*, and *bteA* transcription, and these effects were partially reversed by complementation. Other T3SS genes, *fhaB*, *cyaA*, *prn*, *ptxA,* and *ptlA*, were unaffected by *btrA* deletion or overexpression. Protein expression and secretion assays (Fig. S6*A*) mirror results obtained by quantitative real-time polymerase chain reaction (qRT-PCR). We conclude that Bp536 has retained BtrA-mediated control of cluster 4b genes encoding T3SS substrates and components whereas BtrA regulation of virulence genes in clusters 3a and 4a has either been lost or is inapparent in vitro. Cluster 3b genes are BtrA-independent in both *B. bronchiseptica* and *B. pertussis*.

Having observed that BtrA functions in *B. pertussis*, we tested the hypothesis that the lack of discernable T3SS-mediated cytotoxicity in vitro reflects an alteration in regulation as opposed to an inability to deploy a functional secretion apparatus. Indeed, Fig. 5*B* shows a marked, dose-dependent increase in cytotoxicity conferred by deleting *btrA*, which is reversed by complementation. Reasoning that the cryptic nature of T3SS cytotoxicity is likely due to BtrS antagonism by BtrA, we overexpressed BtrS in Bp536 and, as predicted, observed a level of cell death that was nearly identical to the *ΔbtrA* mutant (Fig. 5*C*).

As previously seen with *B. bronchiseptica* (Fig. 3*A*) (16), high level cytotoxicity by *B. pertussis* required both the BteA effector and the BscN ATPase (Fig. 5*B*). We were intrigued, however, by the ability to detect toxicity by *ΔbtrAΔbteA* double mutants at high multiplicities of infection (MOIs) or longer time points, but not by *ΔbtrAΔbteAΔbscN* triple mutants (Fig. 5*B*). Although levels were low and did not reach statistical significance at 6 h, they were highly significant at 24 h, providing evidence for the presence of a BteA-independent, T3SS-dependent activity that confers cytotoxic effects after prolonged incubation.

To further assess the relevance of our results, we determined whether low passage clinical isolates representing currently circulating strains also display enhanced cytotoxicity after *btrA* deletion. *B. pertussis* strains Bp2 and Bp11 were isolated from lethal infections in infants during the 2010 pertussis epidemic in California, and, although challenging to grow and manipulate, we were able to delete *btrA* in both strains. This deletion resulted in levels of cytotoxicity similar to those seen with Bp536 (Fig. 5*D*), and a corresponding increase in Bsp22 secretion (Fig. S6*B*). Cells exposed to *B. pertussis* isolates with derepressed T3SSs displayed blebbing and other morphological changes characteristic of those seen with WT *B. bronchiseptica* (Fig. 5*E*) (30).

Our data show that BtrA is active in *B. pertussis*, that *B. pertussis* is capable of expressing a fully functional Bsc T3SS, and that the lack of cytotoxic activity in vitro is due to BtrA-mediated repression. Although BteA clearly functions as a cytotoxic effector, the observation of BteA-independent, T3SS-dependent cytotoxicity raises the possibility that additional effectors await discovery. The implications of these findings and other results are discussed below.

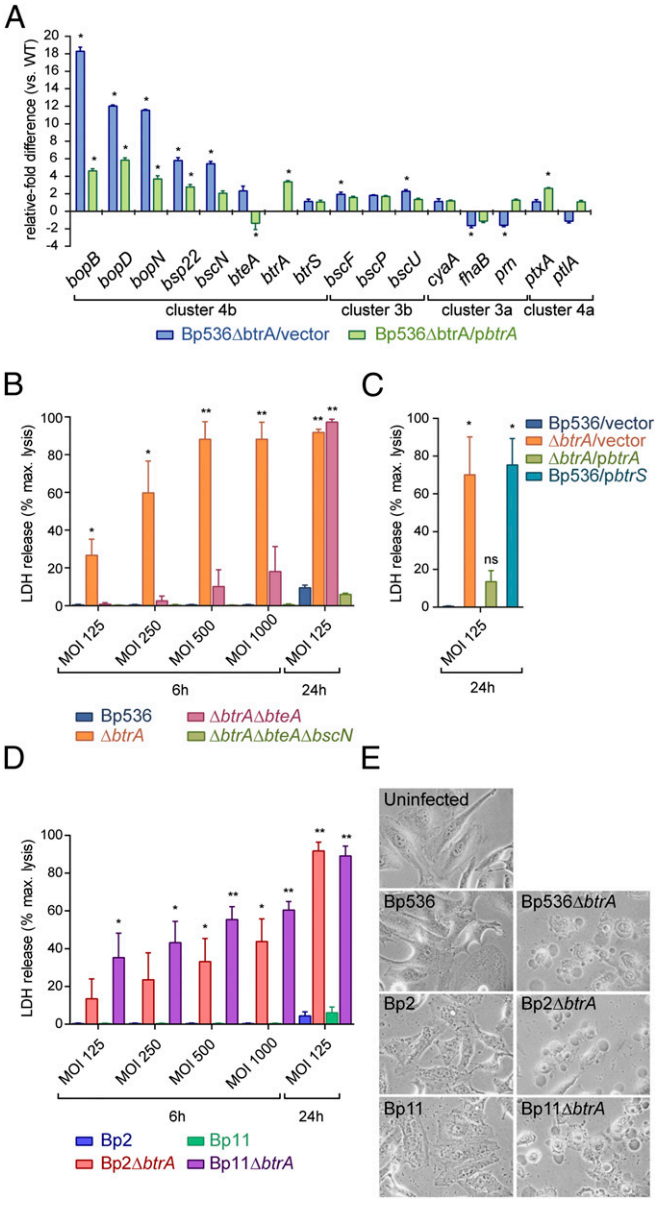

Fig. 5. BtrA-dependent regulation of BteA-mediated cytotoxicity in laboratory and clinical *B. pertussis* isolates. (*A*) qRT-PCR analysis of transcripts from indicated strains. Fold differences in transcript levels were calculated relative to Bp536/vector (WT) using the $\Delta\Delta C_t$ method. Statistical significance is indicated with * ($P < 0.05$). Colored bars represent average values obtained from three independent experiments, and error bars represent ± SEM. (*B–D*) HeLa cells were infected with bacterial strains at different MOIs for 6 or 24 h, and cytotoxicity was measured by LDH release. Error bars represent ±SEM. Statistical significance (*t* test) is indicated with * ($P < 0.05$), or ** ($P < 0.001$). (*E*) HeLa cells were infected with indicated strains at an MOI of 250:1 for 5 h and examined by phase-contrast microscopy at 40× magnification on a Carl Zeiss Axiovert 40 CFL microscope. Images are representative of three independent experiments.

Discussion

Through its function as a secreted BtrS antagonist, BtrA establishes a feedback loop that couples the activity of the T3SS apparatus with expression of T3SS genes encoding the BteA effector, other secretion substrates, and interacting factors. In *B. bronchiseptica*, BtrA is also required for high level transcription of genes encoding adenylate cyclase toxin, FHA, fimbria, Prn, SphB1, and numerous other products, establishing a reciprocal relationship between their expression and T3SS activity. Reciprocal control of adhesins and toxins vs. T3SS genes could reflect differential requirements during temporally or spatially distinct stages of infection. An alternative hypothesis is that it functions to facilitate immune evasion. It has previously been suggested that $T3SS_{Bsc}$ may target antigen presenting cells (APCs) that extravagate to the respiratory surface during infection (22, 33). If true, simultaneous down-regulation of major antigen loci and up-regulation of T3SS

genes may combine to suppress antigen processing and the generation of protective immunity. Although both regulatory functions are observed in *B. bronchiseptica*, adhesin and toxin gene expression have apparently been uncoupled from BtrA control in *B. pertussis*. This observation correlates with the long term and often asymptomatic association between *B. bronchiseptica* and natural hosts, in contrast to the acute and immunizing nature of human pertussis (3, 34).

Considerable evidence supports the conclusion that BtrA functions as a BtrS antagonist. For T3SS genes in cluster 4b, deletions in *btrA* or *btrS* have opposing transcriptional effects (Fig. 4), and *btrS* overexpression in a WT background mimics the effects of deleting *btrA* (Fig. 1 *E* and *F* and Fig. S5*D*). BtrA interacts directly with BtrS using a central domain, and regulation of gene expression requires C-terminal sequences in addition to BtrS binding (Fig. 2). The BtrA N terminus confers recognition as a secretion substrate, thereby coupling T3SS activity to transcriptional control (Fig. S2*B*), and it has also been shown to mediate nuclear localization after translocation into host cells (35). Secreted antagonists regulate flagellar genes in *Salmonella* (36, 37) and T3SS loci in *Pseudomonas aeruginosa* (38), *Yersinia* spp. (39), *Shigella* (40), and other bacteria (41). A unique feature of *btrA*, however, is the extent and diversity of expression profiles it controls.

Six coexpressed clusters of BtrA-regulated genes can be resolved by comparing WT *B. bronchiseptica* with Δ*bvgS*, Δ*btrA*, Δ*btrS*, and Δ*btrA* Δ*btrS* derivatives (Fig. 4*B*). In several cases, expression profiles predict the existence of new, unidentified control factors. For example, high level expression of cluster 3a genes (e.g., *fhaB*, *cyaA*, and *prn*) is clearly dependent on *btrA*. This requirement is relieved, however, by simultaneously eliminating *btrA* and *btrS*, indicating a negative role for BtrS. Because sigma factors are not generally associated with direct repression, we predict the existence of a BtrS-activated negative regulator (Fig. 4*B*). Based on its expression profile, this postulated control factor is likely encoded by a cluster 4 gene.

T3SS loci are distributed between cluster 3b, which contains BvgAS-activated genes that are BtrA- and BtrS-independent, and cluster 4b, which includes loci that require BvgAS and BtrS for transcription and are negatively controlled by BtrA. As shown in Fig. S7, cluster 3b encodes components of the secretion apparatus, up to and including the BscF needle. Cluster 4b encodes secreted substrates, such as the BteA effector, translocon components, and the Bsp22 filament protein, along with multiple chaperones and the BscN ATPase complex. We have previously shown that the BtrU, -W, and -V partner-switching proteins operate at a post-translational level to "gate" the secretion apparatus, controlling its function in response to unknown queues (27). Coupling transcription of genes encoding secreted factors and ancillary systems to T3SS activity via negative control by BtrA presumably ensures the availability of an adequate pool of T3SS substrates to meet demand. Although clusters 3b and 4b together include all known T3SS genes, they also include numerous coregulated loci with unexplored roles in *Bordetella*–host interactions.

We identified two surprising consequences of deleting *btrA* in *B. bronchiseptica*. First, Δ*btrA* strains display high level, BtrS-dependent expression of flagellar and chemotaxis genes in cluster 1, which were previously assumed to be exclusively expressed in the Bvg- phase (4, 42). A regulatory scheme that can account for these observations is shown in Fig. 4*B* although the advantage of activating these loci in the Bvg^+ phase is unclear given the deleterious effect of flagellar expression on respiratory infection (17). A second surprise was the observation that deletion of *btrA* activates expression of *ptxA* to -*E* and *ptlA* to -*H* genes in cluster 4a, which are homologs of the *B. pertussis* loci that encode pertussis toxin and the T4SS that exports it (3). Although all genes in cluster 4 require BvgAS and BtrS for transcription, cluster 4a genes seem to require substantially higher levels of BtrS for expression than cluster 4b genes and therefore appear quiescent in vitro.

Of the estimated 5,072 genes in RB50, 12% (n = 591) are BvgAS-regulated as determined by RNA-seq (Fig. 4). One half of all BvgAS-regulated genes are also controlled by BtrA, and these genes partition into BtrA-repressed (n = 215) and BtrA-activated (n = 80) loci. The pervasive and complex nature of BtrA-mediated transcriptional control in *B. bronchiseptica* was unexpected, particularly in light of the relatively modest effects of deleting *btrA* in murine models of infection. Increased expression of cluster 4 genes and decreased transcription of cluster 3a loci could have net effects that are nearly compensatory during short-term infection, or the Δ*btrA* allele may mimic expression levels that normally occur in vivo. Longer time points, competition formats, and other approaches may clarify subtle phenotypes in vivo. In contrast, derepression of T3SS genes by deleting BtrA conferred an increase in BteA-dependent cytotoxicity that was readily apparent, even in cells that are nearly refractory to killing by the WT strain (Fig. 3*A*). The magnitude of this effect and the extent to which BtrA controls gene expression in *B. bronchiseptica* prompted us to explore the possibility that differential regulation might explain the enigma of why T3SS-dependent cytotoxicity has never been reported in *B. pertussis* (3, 25).

B. pertussis T3SS loci are clearly responsive to BtrA, and the dichotomy between BtrA-regulated expression of secretion substrates and associated factors vs. BtrA-independent expression of apparatus genes is also conserved. Most importantly, deletion of *btrA* derepressed T3SS activity in *B. pertussis*, providing the first clear evidence, to our knowledge, for cytotoxicity dependent on the BteA effector and the BscN ATPase (Fig. 5 *B*–*E*). Overexpression of *btrS* in Bp536 mimicked the effects of deleting *btrA*, suggesting that BtrA functions by antagonizing BtrS as it does in *B. bronchiseptica*. Because Bp536 is a derivative of Tohama I (43), which was isolated in the early 1950s and has been passaged in laboratories ever since, it was essential to determine whether BtrA also controls cytotoxicity in currently circulating strains. Indeed, deletion of *btrA* in two *B. pertussis* isolates from infants that died of pertussis during the 2010 California epidemic derepressed cytotoxicity to a similar extent as observed in Bp536, thereby demonstrating the relevance of our observations to understanding human disease. In contrast to *B. bronchiseptica*, BtrA has little effect on expression of virulence genes that encode CyaA, FHA, Prn, or pertussis toxin in *B. pertussis* Bp536. As indicated in the regulatory model for genes in cluster 3a (Fig. 4*B*), BtrA control could be uncoupled in a single step by mutation of a predicted BtrS-activated negative regulator. Indeed, our previously reported comparative microarray analysis showed that numerous transcriptional regulators that are expressed and BvgAS-regulated in *B. bronchiseptica* are missing, not transcribed, or present as pseudogenes in *B. pertussis* (4).

BtrA establishes a critical regulatory loop in both *B. pertussis* and *B. bronchiseptica*. We hypothesize that differences in T3SS activity between these subspecies reflects differential control over the intracellular level of BtrA, which is itself determined by secretion activity. The BtrU, -W, and -V partner-switching proteins likely play a pivotal role (27), and experiments are underway to understand signals and signaling mechanisms that control their interactions and, consequently, the activity of the secretory apparatus. Regardless of the basis for in vitro differences from *B. bronchiseptica*, the observation that *B. pertussis* isolates are fully capable of expressing BteA-mediated cytotoxicity in vitro warrants renewed consideration of the potential role of type III secretion in the pathogenesis and prevention of human pertussis.

Materials and Methods

Bacterial cultures and mammalian cells were grown as previously described (20) and are listed in Table S1 along with plasmids used in the study. Mutants were constructed using allelic exchange as previously described (14, 44). Immunoblotting, cytotoxicity assays, microscopy, and animal experiments were performed as previously described (20). All animals were maintained in University of California, Los Angeles animal research facilities according to National Institutes of Health and University of California Institutional Animal Care and Use Committee (IACUC) guidelines. The animal experiments were approved by the UCLA Chancellor's Animal Research Committee. Yeast two-hybrid assays were performed per the manufacturer's instructions (Clontech). RNA extraction and processing, quantitative RT-PCR, RNA-seq library preparation, Illumina sequencing, and the data analysis pipeline are described in *SI Materials and Methods*. Oligonucleotides used for PCR, RT-PCR, and sequencing are listed in Tables S2

and S3. The RNA-seq alignment statistics, dataset, and comparative analyses are listed in Table S4 and Dataset S1. RNA-seq data reported in this paper have been deposited in the Sequence Read Archive, www.ncbi.nlm.nih.gov/sra (submission no. SRP064665; NCBI BioProject no. PRJNA296526).

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1. Preston A, Parkhill J, Maskell DJ (2004) The bordetellae: Lessons from genomics. *Nat Rev Microbiol* 2(5):379–390.
2. Diavatopoulos DA, et al. (2005) *Bordetella pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of *B. bronchiseptica*. *PLoS Pathog* 1(4):e45.
3. Melvin JA, Scheller EV, Miller JF, Cotter PA (2014) *Bordetella pertussis* pathogenesis: Current and future challenges. *Nat Rev Microbiol* 12(4):274–288.
4. Cummings CA, Bootsma HJ, Relman DA, Miller JF (2006) Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. *J Bacteriol* 188(5):1775–1785.
5. Aricó B, et al. (1989) Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proc Natl Acad Sci USA* 86(17):6671–6675.
6. Uhl MA, Miller JF (1996) Integration of multiple domains in a two-component sensor protein: The *Bordetella pertussis* BvgAS phosphorelay. *EMBO J* 15(5):1028–1036.
7. Cotter PA, Miller JF (1997) A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol Microbiol* 24(4):671–685.
8. Cotter PA, Jones AM (2003) Phosphorelay control of virulence gene expression in *Bordetella*. *Trends Microbiol* 11(8):367–373.
9. Carbonetti NH (2015) Contribution of pertussis toxin to the pathogenesis of pertussis disease. *Pathog Dis* 73(8):ftv073.
10. Eby JC, et al. (2013) Quantification of the adenylate cyclase toxin of *Bordetella pertussis in vitro* and during respiratory infection. *Infect Immun* 81(5):1390–1398.
11. Masin J, Osicka R, Bumba L, Sebo P (2015) *Bordetella* adenylate cyclase toxin: A unique combination of a pore-forming moiety with a cell-invading adenylate cyclase enzyme. *Pathog Dis* 73(8):ftv075.
12. Inatsuka CS, Julio SM, Cotter PA (2005) *Bordetella* filamentous hemagglutinin plays a critical role in immunomodulation, suggesting a mechanism for host specificity. *Proc Natl Acad Sci USA* 102(51):18578–18583.
13. Willems RJ, van der Heide HG, Mooi FR (1992) Characterization of a *Bordetella pertussis* fimbrial gene cluster which is located directly downstream of the filamentous haemagglutinin gene. *Mol Microbiol* 6(18):2661–2671.
14. Yuk MH, Harvill ET, Miller JF (1998) The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. *Mol Microbiol* 28(5):945–959.
15. Panina EM, et al. (2005) A genome-wide screen identifies a *Bordetella* type III secretion effector and candidate effectors in other species. *Mol Microbiol* 58(1):267–279.
16. French CT, et al. (2009) The *Bordetella* type III secretion system effector BteA contains a conserved N-terminal motif that guides bacterial virulence factors to lipid rafts. *Cell Microbiol* 11(12):1735–1749.
17. Akerley BJ, Cotter PA, Miller JF (1995) Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. *Cell* 80(4):611–620.
18. Merkel TJ, Stibitz S (1995) Identification of a locus required for the regulation of *bvg*-repressed genes in *Bordetella pertussis*. *J Bacteriol* 177(10):2727–2736.
19. Jones AM, Boucher PE, Williams CL, Stibitz S, Cotter PA (2005) Role of BvgA phosphorylation and DNA binding affinity in control of Bvg-mediated phenotypic phase transition in *Bordetella pertussis*. *Mol Microbiol* 58(3):700–713.
20. Ahuja U, et al. (2012) Phenotypic and genomic analysis of hypervirulent human-associated *Bordetella bronchiseptica*. *BMC Microbiol* 12:167.
21. Yuk MH, Harvill ET, Cotter PA, Miller JF (2000) Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the *Bordetella* type III secretion system. *Mol Microbiol* 35(5):991–1004.
22. Skinner JA, Pilione MR, Shen H, Harvill ET, Yuk MH (2005) *Bordetella* type III secretion modulates dendritic cell migration resulting in immunosuppression and bacterial persistence. *J Immunol* 175(7):4647–4652.
23. Martínez de Tejada G, Miller JF, Cotter PA (1996) Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Mol Microbiol* 22(5):895–908.
24. Fennelly NK, et al. (2008) *Bordetella pertussis* expresses a functional type III secretion system that subverts protective innate and adaptive immune responses. *Infect Immun* 76(3):1257–1266.
25. Mattoo S, Yuk MH, Huang LL, Miller JF (2004) Regulation of type III secretion in *Bordetella*. *Mol Microbiol* 52(4):1201–1214.
26. Medhekar B, Shrivastava R, Mattoo S, Gingery M, Miller JF (2009) *Bordetella* Bsp22 forms a filamentous type III secretion system tip complex and is immunoprotective *in vitro* and *in vivo*. *Mol Microbiol* 71(2):492–504.
27. Kozak NA, Mattoo S, Foreman-Wykert AK, Whitelegge JP, Miller JF (2005) Interactions between partner switcher orthologs BtrW and BtrV regulate type III secretion in *Bordetella*. *J Bacteriol* 187(16):5665–5676.
28. Kurushima J, Kuwae A, Abe A (2012) The type III secreted protein BspR regulates the virulence genes in *Bordetella bronchiseptica*. *PLoS One* 7(6):e38925.
29. Gotto JW, et al. (1993) Biochemical and immunological properties of two forms of pertactin, the 69,000-molecular-weight outer membrane protein of *Bordetella pertussis*. *Infect Immun* 61(5):2211–2215.
30. Stockbauer KE, Foreman-Wykert AK, Miller JF (2003) *Bordetella* type III secretion induces caspase 1-independent necrosis. *Cell Microbiol* 5(2):123–132.
31. Harvill ET, Cotter PA, Miller JF (1999) Pregenomic comparative analysis between *bordetella bronchiseptica* RB50 and *Bordetella pertussis* tohama I in murine models of respiratory tract infection. *Infect Immun* 67(11):6109–6118.
32. McMillan DJ, Shojaei M, Chhatwal GS, Guzmán CA, Walker MJ (1996) Molecular analysis of the *bvg*-repressed urease of *Bordetella bronchiseptica*. *Microb Pathog* 21(5):379–394.
33. Legarda D, Klein-Patel ME, Yim S, Yuk MH, Diamond G (2005) Suppression of NF-kappaB-mediated beta-defensin gene expression in the mammalian airway by the *Bordetella* type III secretion system. *Cell Microbiol* 7(4):489–497.
34. Mattoo S, Cherry JD (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin Microbiol Rev* 18(2):326–382.
35. Abe A, Nishimura R, Tanaka N, Kurushima J, Kuwae A (2015) The *Bordetella* Secreted Regulator BspR is translocated into the nucleus of host cells via Its N-terminal moiety: Evaluation of bacterial effector translocation by the *Escherichia coli* type III secretion system. *PLoS One* 10(8):e0135140.
36. Hughes KT, Gillen KL, Semon MJ, Karlinsey JE (1993) Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* 262(5137):1277–1280.
37. Kutsukake K (1994) Excretion of the anti-sigma factor through a flagellar substructure couples flagellar gene expression with flagellar assembly in *Salmonella typhimurium*. *Mol Gen Genet* 243(6):605–612.
38. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ (2005) ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 102(22):8006–8011.
39. Cheng LW, Schneewind O (2000) *Yersinia enterocolitica* TyeA, an intracellular regulator of the type III machinery, is required for specific targeting of YopE, YopH, YopM, and YopN into the cytosol of eukaryotic cells. *J Bacteriol* 182(11):3183–3190.
40. Botteaux A, Sory MP, Biskri L, Parsot C, Allaoui A (2009) MxiC is secreted by and controls the substrate specificity of the *Shigella flexneri* type III secretion apparatus. *Mol Microbiol* 71(2):449–460.
41. Kodama T, et al. (2010) Transcription of *Vibrio parahaemolyticus* T3SS1 genes is regulated by a dual regulation system consisting of the ExsACDE regulatory cascade and H-NS. *FEMS Microbiol Lett* 311(1):10–17.
42. Akerley BJ, Monack DM, Falkow S, Miller JF (1992) The bvgAS locus negatively controls motility and synthesis of flagella in *Bordetella bronchiseptica*. *J Bacteriol* 174(3):980–990.
43. Stibitz S, Yang MS (1991) Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. *J Bacteriol* 173(14):4288–4296.
44. Stibitz S, Black W, Falkow S (1986) The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. *Gene* 50(1-3):133–140.
45. Stainer DW, Scholte MJ (1970) A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J Gen Microbiol* 63(2):211–220.
46. Brennan MJ, et al. (1988) Identification of a 69-kilodalton nonfimbrial protein as an agglutinogen of *Bordetella pertussis*. *Infect Immun* 56(12):3189–3195.
47. Inatsuka CS, et al. (2010) Pertactin is required for *Bordetella* species to resist neutrophil-mediated clearance. *Infect Immun* 78(7):2901–2909.
48. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. *Nature* 340(6230):245–246.
49. Chien CT, Bartel PL, Sternglanz R, Fields S (1991) The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci USA* 88(21):9578–9582.
50. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402–408.
51. Li H, Durbin R (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 26(5):589–595.
52. Robinson MD, Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11(3):R25.
53. Quinlan AR, Hall IM (2010) BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* 26(6):841–842.
54. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 5(7):621–628.
55. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J Roy Stat Soc B Met* 57(1):289–300.