Role of the Type III Secretion System in a Hypervirulent Lineage of *Bordetella bronchiseptica* *†*

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Despite the fact that closely related bacteria can cause different levels of disease, the genetic changes that cause some isolates to be more pathogenic than others are generally not well understood. We use a combination of approaches to determine which factors contribute to the increased virulence of a *Bordetella bronchiseptica* lineage. A strain isolated from a host with *B. bronchiseptica*-induced disease, strain 1289, was 60-fold more virulent in mice than one isolated from an asymptptomatically infected host, strain RB50. Transcriptome analysis and quantitative reverse transcription-PCR showed that the type III secretion system (TTSS) genes were more highly expressed by strain 1289 than strain RB50. Compared to strain RB50, strain 1289 exhibited greater TTSS-mediated cytotoxicity of a mammalian cell line. Additionally, we show that the increase in virulence of strain 1289 compared to that of RB50 was partially attributable to the TTSS. Using multilocus sequence typing, we identified another strain from the same lineage as strain 1289. Similar to strain 1289, we implicate the TTSS in the increased virulence of this strain. Together, our data suggest that the TTSS is involved in the increased virulence of a *B. bronchiseptica* lineage which appears to be disproportionally associated with disease. These data are consistent with the view that *B. bronchiseptica* lineages can have different levels of virulence, which may contribute to this species’ ability to cause different severities of respiratory disease.

Although the disease caused by different strains of pathogenic bacteria is known to vary, the molecular basis for these differences has been difficult to disentangle from the many other genetic changes that occur as strains diverge. Recently, a growing number of studies have identified factors that contribute to increased virulence of bacterial lineages by using a combination of genome-wide analyses, phylogenetics, mutational analysis, and host infection models (15, 46, 49–51, 56, 58). Horizontal gene transfer of novel virulence factors, phage integration, phenotypic variation, gene loss, and mutation have been shown to alter the phenotype or severity of disease (15, 51, 56).

*Bordetella bronchiseptica* is a gram-negative respiratory pathogen that infects a wide range of mammals and is closely related to *Bordetella pertussis* and *Bordetella parapertussis*, the causative agents of whooping cough in humans (18, 33, 36). Colonization of hosts by *B. bronchiseptica* can lead to a range of diseases, from lethal pneumonia to asymptomatic infection (18), which is thought to be caused by differences in host immune status, polymicrobial infection, and/or bacterial strain variation (18, 33). However, in inbred and specific-pathogen-free mice, the 50% lethal dose (LD₅₀) can still differ by up to 100,000-fold between bacterial strains, suggesting that substantial differences in virulence may be due to strain variation alone (5, 19, 20).

While the population structure of these bacteria appears to be clonal, *B. pertussis* and *B. parapertussis* are more monomorphic than *B. bronchiseptica* strains, and isolates of this species can be related more distantly to each other than to either of the human-associated pathogens (11, 36, 37, 60). Previously, it was shown that differences in gene regulation between *B. bronchiseptica* strains can correlate with phylogenetic lineage (17) and strains can differ in virulence factor expression (3, 19, 31, 36, 47). Recently, we showed that phylogenetic lineages can differ in virulence factor expression and virulence, as a lineage of *B. bronchiseptica* was found to lack expression of adenylate cyclase toxin and exhibit decreased virulence (5).

*B. bronchiseptica* strains express many virulence factors, including adhesins, secretion systems, autotransporters, and toxins, that are globally regulated by the BvgAS two-component signal transduction system (7, 53). In the nonvirulent, or Bvg− phase, which occurs at 25°C or in the presence of chemical modulators such as MgSO₄ or nicotinic acid, BvgAS is unable to activate virulence-associated genes (10, 35, 38). The Bvg− phase is both necessary and sufficient for colonization of the respiratory tract (1, 8). Among the Bvg-regulated genes are those encoding a type III secretion system (TTSS) which is similar to others shown to directly translocate effector proteins through a needle-like injection apparatus directly into eukaryotic cells, disrupt host cell signaling, and induce necrotic-like cell death (27, 62). Under
Bvg<sup>+</sup> conditions, the btr regulatory locus (including btrS, btrU, btrW, and btrV) is transcribed (34). BtrS is an ECF sigma factor that is necessary and sufficient for activating the more than 20 TTSS-related genes (bsc, bop, bsp, and bte) (34). BscN is the putative ATPase that provides energy for the secretion of effector proteins and is required for the function of the TTSS apparatus (62). TTSS gene products BopB, BopC, and BteA have been shown to be secreted and required for cytotoxicity in mammalian cells (28, 29, 41). In a murine model of infection, the TTSS increases host interleukin-10 production and enhances persistence of <i>B. bronchiseptica</i> in the lower respiratory tract (44, 61, 62).

While correlations between the level of virulence or disease caused by <i>B. bronchiseptica</i> strains and specific bacterial factors have been made (5, 39, 47), limited studies have directly tested whether these factors cause some strains to be more virulent than others (4) and whether these characteristics are associated with a particular phylogenetic lineage. Here, we use genome-wide analyses, phylogenetics, allelic exchange, and a murine model of infection to determine the bacterial factors that contribute to the increased virulence of a <i>B. bronchiseptica</i> lineage. We compared the relative virulence, as measured by LD<sub>50</sub> of <i>B. bronchiseptica</i> strains from a diseased (strain 1289) or asymptotically infected (strain RB50) host (8). Strain 1289 was approximately 60-fold more virulent than strain RB50. Transcriptome analysis showed that TTSS-related genes were more highly expressed by strain 1289 than RB50. The TTSS-mediated cytotoxicity and virulence of 1289 was greater than that of strain RB50. Using multiplex sequence typing (MLST) analysis, we identified another strain from the same sequence type (ST) as strain 1289 and showed that, similar to strain 1289, the increased virulence of this strain was partially attributable to the TTSS. Together, our data indicate that the TTSS is involved in the increased virulence of a <i>B. bronchiseptica</i> lineage. This is consistent with the idea that different phylogenetic lineages can differentially regulate their virulence factors to modulate their overall level of virulence, which may contribute to the ability of <i>B. bronchiseptica</i> strains to cause different severities of respiratory disease.

**MATERIALS AND METHODS**

**Bacterial strains and growth.** <i>B. bronchiseptica</i> isolates, sources, locations, dates, anatomical sites of isolation, references, and all available health and necropsy reports (strains 1289, S308, and S314) are included herein or in Table S1 in the supplemental material or have been previously described (strain RB50) (8). In cases where there was an available health report, strains were grouped as (i.e., having an asymptomatic infection) (see Table S1 in the supplemental material). Bacterial strains and growth. All strains were maintained on Bordet-Gengou (BG) agar (Difco, Sparks, MD) at 37°C, as previously described (22, 26). Because the Bvg apparatus (62). TTSS gene products BopB, BopC, and BteA putative ATPase that provides energy for the secretion of effector proteins and is required for the function of the TTSS apparatus (62). TTSS gene products BopB, BopC, and BteA have been shown to be secreted and required for cytotoxicity in mammalian cells (28, 29, 41). In a murine model of infection, the TTSS increases host interleukin-10 production and enhances persistence of <i>B. bronchiseptica</i> in the lower respiratory tract (44, 61, 62).

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**Expression arrays and statistical analysis.** The expression array was carried out as previously described (5, 38). Briefly, bacteria were grown in SS broth, subcultured at a starting OD<sub>600</sub> of 0.02 into 50 ml of SS broth, grown at 37°C for 24 h with shaking, and harvested in log phase (OD<sub>600</sub> of 1.0). Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA), treated with RNAse free DNase I (Invitrogen, Carlsbad, CA), and purified using RNAeasy columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was isolated from two independent biological replicates of strains RB50 and 1289. A two-color hybridization format was used, and dye swap experiments were performed. For each reaction mixture, 5 μg of cDNA was fluorescently labeled. The two differentially labeled reaction mixtures to be compared were combined and hybridized to a <i>B. bronchiseptica</i> strain RB50-specific long-oligonucleotide microarray (5, 38). The slides were then scanned using a GenePix 4000B microarray scanner and analyzed with GenePix Pro software (Axon Instruments, Union City, CA). The spots were assessed visually to identify those of low quality, and the arrays were normalized so that the median of the ratio across each array was equal to 1.0. Spots of low quality were identified and were filtered out prior to analysis. Ratio data from the two biological replicates were combined and normalized based on the total Cy3 percent intensity and Cy5 percent intensity to eliminate slide-to-slide variation. Gene expression data were then normalized to the expression of 16S rRNA. The statistical significance of the gene expression changes observed was assessed by using the significance analysis of microarrays (SAM) program (59). A one-class unpaired SAM analysis using a false discovery rate of 0.30% (<0.1%) was performed. All microarray expression data are available in Table S2 in the supplemental material.

**qRTP-PCR.** Quantitative reverse transcription-PCR (qRTP-PCR) was completed as previously described (5, 38), and RNA was extracted as described for the microarray experiment. One microgram of RNA from each biological replicate was reverse transcribed using 300 ng of random oligonucleotide hexamers and SuperScript III RTase (Invitrogen, Carlsbad, CA). The resulting cDNA was diluted 1:1,000, and 1-μl amounts were used in qRTP-PCR mixtures containing 300 nM primers that were designed with Primer Express software (Applied Biosystems, Foster City, CA) and 2× SYBR green PCR master mix (Applied Biosystems, Foster City, CA). To confirm the lack of DNA contamination, reactions of mixtures without reverse transcriptase were completed. Dissociation curve analysis was performed to verify product homogeneity. Threshold fluorescence was established within the geometric phase of exponential amplification, and the cycle threshold (C<sub>T</sub>) determined for each reaction mixture. The C<sub>T</sub> from all biological replicates for each strain was compiled, and the 16S RNA amplicon was used as an internal control for data normalization. The change in transcript level was determined by using the relative C<sub>T</sub> method (ΔΔC<sub>T</sub>) (48). All primer sequences and changes in gene expression analyzed by qRTP-PCR are available (see Table S2 in the supplemental material).
Cytotoxicity assay. Cytotoxicity assays were carried out as previously described (34, 62). Briefly, J774 macrophages were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% nonessential amino acids, and 1% sodium pyruvate to 85% confluence in 5% CO2 at 37°C. Then, warmed RPMI medium lacking phenol red and with 5% fetal bovine serum, 1% l-glutamine, 1% nonessential amino acids, and 1% sodium pyruvate was used to replace the Dulbecco's modified Eagle's medium. Bacterial infections were carried out using a multiplicity of infection (MOI) of 10, and bacterial suspensions were centrifuged onto the macrophage cells at 250 x g for 5 min and incubated in 5% CO2 at 37°C for the amount of time indicated below.

The cell culture supernatants were collected, and the percent lipopolysaccharide (LPS) release was analyzed by using a CytoTox96 kit (Promega) according to the manufacturer's instructions. Statistical significance in percent cytotoxicity between strains was calculated by using a Tukey simultaneous test in Minitab (version 13.30; Minitab, Inc.) (40). A P value of ≤0.05 was taken as statistically significant.

MLST and phyllogenetic tree construction. MLST analysis was performed as previously described (5, 11). In this study, the STs of three strains (S308, S314, and 973) were determined (see Table S1 in the supplemental material). All alleles were double-strand sequenced at The Pennsylvania State University's Sequencing Center. The sequences were trimmed, and alleles and STs were designated by using the Bordetella MLST database (http://pubmlst.org/bordetella) (5, 11, 23). Using MEGA 4.0, the alleles were concatenated and aligned, and an unweighted pair group method with arithmetic mean tree with 1,000 bootstraps using the K2 model was constructed for these 3 strains and for 58 strains whose STs were previously determined (5, 11, 57) (see Table S1 in the supplemental material).

Accession numbers. All microarray expression data have been deposited in MIAExpress under the accession number E-MEXP-1736, and all CGH data have been deposited in MIAMExpress under the accession number E-MEXP-1737.

RESULTS

B. bronchiseptica strain 1289 is more virulent than strain RB50 in a mouse intranasal challenge model. Previous studies have shown that B. bronchiseptica strains can vary widely in virulence (5, 19, 20). To establish a system in which we could measure the contribution of specific factors to the difference in virulence between strains, we compared the LD50, a measure of their virulence, of B. bronchiseptica strains in inbred, specific-pathogen-free mice. B. bronchiseptica strain RB50 was isolated from the nasal cavity of an asymptptomatically infected host (8). Consistent with previous studies of this strain, inoculation with 1 x 104, 3 x 104, or 6 x 104 CFU of B. bronchiseptica strain RB50 led to 100%, 50%, and 0% survival, respectively (Fig. 1A) (5). B. bronchiseptica strain 1289 was isolated from the thoracic cavity of a host with a lethal B. bronchiseptica infection (see Table S1 in the supplemental material). When inoculated with 1 x 105, 5 x 105, or 1 x 106 CFU of strain 1289, 100%, 50%, and 0% of the mice survived the infection, respectively (Fig. 1B), which indicates that the LD50 of strain 1289 is 60-fold lower, or 2.95 million CFU fewer, than that of strain RB50. Although these two isolates did not differ in growth rate in vitro (data not shown), we examined whether the greater virulence of strain 1289 might allow it to colonize the respiratory tract to a higher level than strain RB50. Groups of 15 mice were inoculated with a sublethal dose (1 x 104 CFU) of either strain RB50 or 1289, and respiratory organs were excised to quantify bacterial loads on days 0, 3, 7, 14, and 21 postinoculation (Fig. 1C) (for bacterial strain comparisons, P < 0.002), indicating that the numbers of strain 1289 are higher than the numbers of strain RB50 early after infection (data not shown). The bacterial load did not differ significantly between strain RB50 and 1289 in the trachea or nasal cavity over the course of infection (see Fig. S4 in the supplemental material). Combined, these data indicate that strain 1289 is more virulent.
TTSS genes are upregulated in strain 1289. To identify candidate bacterial genes that correlated with the increased virulence of strain 1289, a comparison of whole-genome transcriptome analyses was performed between strains 1289 and RB50 (Fig. 2A). Of the 5,013 genes represented on the RB50-specific microarray, 646 were downregulated in strain 1289 relative to their expression levels in strain RB50. These included 49 transporter genes; 47 metabolism-related transcripts; 51 transcriptional regulator genes; 27 electron transporter genes; 11 two-component system genes; 7 transcriptional or translational genes; 1 protein biosynthesis-related transcript; 74 exported or membrane protein genes; 72 phage-related transcripts; and 202 hypothetical, predicted, or probable genes. Additionally, five genes classified as virulence factors were downregulated two- to eightfold in strain 1289 compared to their levels of expression in strain RB50; these were genes for filamentous hemagglutinin B (fhaB), filamentous hemagglutinin S (fhaS), Bordetella colonization factor (bcaA), Bordetella resistance to killing A (brkA), and one O-antigen-related gene (wbmS) (Fig. 2A) (see Table S2 in the supplemental material). The downregulated expression of brkA and fhaB in strain 1289 was confirmed by qRT-PCR (see Table S2 in the supplemental material). When CGH analysis was completed on these strains, none of the known virulence factors were identified as divergent in strain 1289 (see Table S3 in the supplemental material), suggesting that the decreased signal of these virulence factors in strain 1289 identified in our transcriptome analysis is due to down-regulation rather than sequence divergence.

We were particularly interested in determining which genes were upregulated in strain 1289, as they might contribute to the greater virulence of this strain. Six hundred seven genes were identified as upregulated in strain 1289 relative to their expression levels in strain RB50. These included 68 transporter genes; 67 metabolism-related transcripts; 23 transcriptional regulator genes; 42 electron transporter genes; 7 two-component system genes; 16 transcriptional or translational genes; 40 protein biosynthesis-related transcripts; 75 exported or membrane protein genes; 16 phage-related transcripts; and 154 hypothetical, predicted, or probable genes. Thirty-three genes associated with known virulence factors were identified as upregulated in strain 1289, as they might contribute to the greater virulence of this strain. Six hundred seven genes were identified as upregulated in strain 1289 compared to their expression levels in strain RB50. Four of these, the genes for cyclolysin-activating lysine-acyltransferase (cyaC), Bordetella resistance to killing (brkB), an O-antigen-related protein (wbmJ), and pertussis toxin subunit 4 precursor (ptxD), were upregulated by 1.8-fold or more in strain 1289 (Fig. 2A) (see Table S2 in the supplemental material). The remaining 29 virulence-associated genes are related to the TTSS and were upregulated from 1.4- to 8.5-fold in strain 1289 over their expression levels in strain RB50 (Fig. 2A and B). As expected, there was a strong correlation between expression levels analyzed by microarray and qRT-PCR results (R = 0.914) (see Table S2 in the supplemental material). Although qRT-PCR did not confirm the upregulation of cyaC, all the TTSS-related genes examined were upregulated 2.6- to 12.8-fold in strain 1289 compared to their levels of expression in strain RB50 (Fig. 2C; see also Table S2 in the supplemental material). The upregulation of these virulence factor genes in strain 1289 does not appear to be due to gene duplication, as no genes were identified as duplicated in CGH analysis (see Table S3 in the supplemental material).

The TTSS is involved in the increased cytotoxicity and virulence of strain 1289. The increased expression of such a large set of genes with a known coordinated function in virulence led us to hypothesize that strain 1289 exhibited greater TTSS-mediated effects than strain RB50. One well-described function attributed to the TTSS is cytotoxicity for a variety of mammalian cells (14, 34, 62). Since nearly all TTSS-related genes are upregulated in strain 1289, we hypothesized that this strain may cause more TTSS-mediated cytotoxicity than strain RB50. Although J774 macrophages treated with medium alone did not release cytoplasmic LDH, infection of these macrophages with strain RB50 at an MOI of 10 caused 5%, 7%, 34%, and 87% of their LDH to be released after 1, 2, 3, and 4 h of
infection, respectively, indicating that RB50 is cytotoxic toward macrophages (\(P = 0.0001\)) (Fig. 3A), as previously reported (34, 62). Upon infection with the same dose of strain 1289, the percent LDH release was higher than that caused by strain RB50 (\(P = 0.0360\)), which indicates that strain 1289 causes more rapid cytotoxicity of macrophages than strain RB50 (Fig. 3A). To determine if the cytotoxicity induced by these strains is caused by the TTSS, isogenic mutants each lacking the bscN gene (RB50\(_{bscN}\) and 1289\(_{bscN}\)) were used to compare their cytotoxicity for J774 macrophages (Fig. 3A). The percent LDH release caused by infection with RB50\(_{bscN}\) was lower than that caused by its parental strain, RB50, and was not significantly different from that in the medium control (\(P = 0.0047\) and \(P = 0.5758\), respectively), which confirms that the TTSS of strain RB50 causes cytotoxicity toward macrophages (Fig. 3A) (21, 62). Similarly, the percent LDH release caused by infection with 1289\(_{bscN}\) was lower than that caused by its wild-type counterpart and was not significantly different from that in the medium control or RB50\(_{bscN}\) (\(P = 0.9857\), respectively), the former of which indicates that strain 1289 does not have a measurable TTSS-independent mechanism of cytotoxicity (Fig. 3A). The greater TTSS-dependent cytotoxicity caused by strain 1289 at earlier time points suggests that strain 1289 causes more rapid TTSS-mediated cytotoxicity than strain RB50.

Since the TTSS causes cytotoxicity in vitro and increases bacterial numbers in vivo (21, 44), we hypothesized that the TTSS contributes more to the virulence of strain 1289 than to that of strain RB50. To test this, mice were inoculated with the bscN deletion strains of RB50 and 1289 and the LD\(_{50}\)s of these strains were determined (Fig. 3B). Groups of three or four C57BL/6 mice were inoculated intranasally with the indicated doses of strains RB50\(_{bscN}\) or 1289\(_{bscN}\). Survival curves were generated by inoculating mice with the indicated dose and determining the percent survival over a 28-day period.

FIG. 2. Whole-transcriptome and TTSS expression analysis of B. bronchiseptica strains RB50 and 1289. (A) Comparison of whole-transcriptome analyses between strains RB50 and 1289. The x axis indicates the order of genes along the B. bronchiseptica strain RB50 5.3-megabase (Mb) chromosome. The y axis indicates the change in expression level (as fold change in expression [FCE]) of each gene. Negative FCE values indicate decreased gene expression of genes in strain 1289 compared to their levels of expression in strain RB50, and positive FCE values indicate increased gene expression in strain 1289 compared to their levels in strain RB50. Genes of interest are labeled, with corresponding underscores. HP, hypothetical protein gene; \(\phi\), phage-related gene; PEP, putative exported protein gene; BB4921, putative ferredoxin gene. (B and C) Comparison of TTSS-related gene expression between strains RB50 and 1289 by microarray analysis (B) and qRT-PCR (C). The x axis indicates the genes analyzed. The y axis indicates the FCE in strain 1289 over the expression level in strain RB50. Error bars represent the plus-or-minus standard errors in panels A and B and the standard deviation in panel C.

FIG. 3. TTSS-mediated effect on cytotoxicity and virulence of B. bronchiseptica strains RB50 and 1289. (A) Cytotoxicity in J774 macrophages treated with medium or infected with RB50, RB50\(_{bscN}\), 1289, or 1289\(_{bscN}\) for 1, 2, 3, and 4 h at an MOI of 10. The error bars represent the plus-or-minus standard deviations. (B) Groups of three or four C57BL/6 mice were inoculated intranasally with the indicated doses of strains RB50\(_{bscN}\) or 1289\(_{bscN}\). Survival curves were generated by inoculating mice with the indicated dose and determining the percent survival over a 28-day period.
isogenic mutant strains were determined. When inoculated with $8.0 \times 10^6$ or $1.0 \times 10^7$ CFU of RB50ΔbscN, 100% and 0% of the mice survived the infection, respectively (Fig. 3B), indicating that the LD$_{50}$ of strain RB50ΔbscN is approximately $9.0 \times 10^6$ CFU; threefold greater than the LD$_{50}$ of strain RB50 (Fig. 3B and 1A). When mice were inoculated with $1.0 \times 10^5$, $1.0 \times 10^6$, or $2.0 \times 10^6$ CFU of 1289ΔbscN, 100%, 66%, and 0% of the mice survived the infection, respectively (Fig. 3B), indicating that the LD$_{50}$ of strain 1289ΔbscN is approximately $1.2 \times 10^6$ CFU, 24-fold greater than that of strain 1289 (Fig. 3B and 1B). Therefore, the TTSS appears to contribute more to the virulence of strain 1289 than to that of strain RB50 (Fig. 3A, 1A, and 3B). Since the LD$_{50}$ of 1289ΔbscN is lower than that of RB50ΔbscN, it suggests that another factor besides the TTSS also contributes to the increased virulence of strain 1289 (Fig. 3B). Together, these data indicate that while the TTSS is not the sole factor, it is partially responsible for the increased virulence of strain 1289 compared to the virulence of strain RB50.

The TTSS is implicated in the increased virulence of ST32 strains. To examine whether isolates associated with B. bronchiseptica-related disease were of the same phylogenetic lineage, we completed MLST and phylogenetic analyses using two B. bronchiseptica strains from diseased hosts to determine if they fell into the same ST as strain 1289. In addition to these three disease-associated isolates, our analysis also included 58 additional Bordetella isolates, of which 55 were B. bronchiseptica strains (none known to be associated with diseased hosts and all from a broad range of locations, dates, and hosts), 2 B. pertussis strains, and 1 B. parapertussis strain (Fig. 4; see also Table S1 in the supplemental material) (5, 11). These 58 isolates served to demonstrate the genetic relatedness of strains RB50 and 1289 and to confirm the evolutionary history of the classical bordetellae. Consistent with other phylogenetic analyses, both B. pertussis and B. parapertussis appear to have evolved independently from B. bronchiseptica-like progenitors (Fig. 4) (11, 42, 60). As previously described, strains RB50 and 1289 were identified as ST12 and ST32 isolates, respectively (Fig. 4) (5, 11). Importantly, strains belonging to the same STs as strains RB50 (11) and 1289 (Fig. 4; see also Table S1 in the supplemental material) have been isolated from several continents, suggesting that these two STs exist worldwide. Two isolates, strains S308 and S314, were selected for MLST analysis because they were collected from hosts with B. bronchiseptica-induced disease (see Table S1 in the supplemental material). While one of these strains, S314, belongs to ST7, the other strain, S308, belongs to the same ST as strain 1289 (Fig. 4). These data suggest that not all strains causing B. bronchiseptica-induced disease are of the same phylogenetic lineage. However, within the ST32 lineage, both the ST32 disease and the complex is labeled next to each set of STs (complex I, which includes B. bronchiseptica strains, complex II, which includes B. pertussis strains, complex III, which includes B. parapertussis strains, and complex IV, which includes B. bronchiseptica strains that appear to be most closely related to B. pertussis) as previously described (5, 11). The numbers on the tree branches indicate branch strength. All branch strengths below 50 were removed. The scale indicates the relative genetic distances along the branches.

![FIG. 4. MLST analysis of 61 Bordetella strains. Unweighted pair group method with arithmetic mean tree with 1,000 bootstraps based on concatenated MLST gene sequences of 61 Bordetella isolates (58 B. bronchiseptica, 2 B. pertussis, and 1 B. parapertussis isolates). The identification number of each strain is listed. The asterisks indicate strains that have undergone or are undergoing full-genome sequencing (42). The ST is labeled next to each strain (see also Table S1 in the supplemental material). The identification number of each strain is listed. The numbers on the tree branches indicate branch strength. All branch strengths below 50 were removed. The scale indicates the relative genetic distances along the branches.)](https://journals.asm.org/doi/abs/10.1128/infimmun.00368-08?journalCode=infimm)
to that of strain 1289 (5 × 10⁶ CFU) and approximately 40-fold lower than that of strain RB50 (3 × 10⁶ CFU) (Fig. 1A, 1B, and 5). To determine if the TTSS contributed to the increased virulence of strain S308, we deleted the bscN gene from this strain and determined the LD₅₀. When inoculated with 3 × 10⁶ or 1 × 10⁶ CFU of S308ΔbscN, 0% and 100% of the mice, respectively, survived the infection (Fig. 5). Therefore, the LD₅₀ of this strain is approximately 2 × 10⁶ CFU, which is 27-fold higher than that of its parental wild-type strain (Fig. 5). These data indicate that the TTSS contributes more to the virulence of strain S308 than that of strain RB50 (Fig. 5 and 3B). Similar to strain 1289ΔbscN, the LD₅₀ of S308ΔbscN is lower than that of RB50ΔbscN, which suggests that another factor besides the TTSS also contributes to the increased virulence of strain S308. Together, these data suggest that the increased virulence of ST32 strains is partially dependent on the TTSS.

MLST analysis has been completed for approximately 260 Bordetella strains, the vast majority of which are B. bronchiseptica (S.E. Hester, K. E. Creppage, M. C. Dunagin, K. Register, and E. T. Harvill, unpublished data) (5, 11). Of these, only three ST32 strains have been identified (Fig. 4), which suggests that while these strains exist worldwide (see Table S1 in the supplemental material), this ST may not contain as many strains as other STs. Therefore, we wanted to determine if other strains closely related to ST32 are more virulent than strain RB50. We analyzed strain 448 from ST23, as it was the strain most closely related to ST32 and having Bvg⁺ morphology that was available at the time of this study (Fig. 4). The LD₅₀ of strain 448 was approximately 1 × 10⁶ CFU, threefold lower than that of strain RB50 (data not shown), and its bacterial loads in the lung were higher than those of strain RB50 (see Fig. S4 in the supplemental material) over the course of the infection. Therefore, these data suggest that strains closely related to ST32 are also more virulent than strain RB50 and may represent lineages of increased virulence.

**DISCUSSION**

The severity of a B. bronchiseptica infection can range from long-term asymptomatic carriage in the upper respiratory tract to fatal pneumonia (18). While previous studies have correlated differences in virulence or severity of disease to particular bacterial factors (5, 39, 47), few studies have shown that these factors actually contribute to the virulence of particular lineages (4). Here, we identify a bacterial factor that contributes to the increased virulence of a B. bronchiseptica lineage by combining comparative genomic analyses, bacterial mutagenesis, phylogenetics, and a host infection model. B. bronchiseptica strain RB50, which was isolated from an asymptptomatically infected host, was less virulent than strain 1289, which was isolated from a diseased host (Fig. 1; see also Table S1 in the supplemental material) (8). Transcriptome analysis revealed that TTSS-related genes were more highly expressed in strain 1289 than in strain RB50 (Fig. 2). Using allelic exchange, we determined that the TTSS causes more-rapid cytotoxic effects in macrophages, that there was negligible cytotoxicity in its absence, and that it contributes more to the virulence of strain 1289 than to that of strain RB50 (Fig. 3). When assessing another strain that belonged to the same ST as strain 1289 and was also associated with B. bronchiseptica-induced disease, we found that the increased virulence of this strain was also partially attributable to the TTSS (Fig. 4 and 5). Combined, these data suggest that the TTSS is involved in the increased virulence of a B. bronchiseptica lineage.

The amount of genomic content shared between strains of a single microbial species can vary substantially (30). The “core genome” represents all genes shared between strains of the same species, while the “flexible genome” includes those genes that are variably present. The flexible genome is thought to confer differences in phenotypes, such as virulence, host range, and/or environmental niches, of different strains. Recently, Cummings et al. proposed an analogous distinction to describe those genes similarly expressed (the core regulon) or differentially expressed (the flexible regulon) between strains, as not all genes are similarly regulated by BvgAS among Bordetella strains (10). The TTSS of B. pertussis, which mediates cellular attachment rather than cytotoxicity, is expressed by some strains but not others (14). Therefore, the TTSS of B. pertussis appears to be part of the flexible regulon, as some B. pertussis strains express the TTSS while others do not (14, 62). The work described herein provides evidence that the TTSS is also part of the flexible regulon of B. bronchiseptica, as strains of this species express TTSS-related genes differentially. Together, these data suggest that the TTSS can have different functions and/or levels of these functions in different strains or species of Bordetella.

Since nearly all known TTSS-related genes were upregulated in strain 1289 compared to their levels of expression in strain RB50, we speculate that the underlying mechanism behind the increased TTSS-mediated virulence of strain 1289 is that increased TTSS gene expression leads to enhanced protein expression and secretion, which in turn increases TTSS-mediated cytotoxicity and virulence. While TTSS-related genes were upregulated in strain 1289, the genes encoding the master regulator of the TTSS, bvgAS, were not differentially expressed. Since the TTSS is controlled by a complex, multilayered, trans-regulatory gene network (34, 62), we propose that the increased expression of a yet-unidentified, Bvg-activated activator or decreased expression of a Bvg-activated repressor may contribute to the differential expression of the TTSS between strains. This regulator may be one of the 23 upregulated
or 51 downregulated transcriptional regulators identified in strain 1289.

Since 1289ΔbscN and S308ΔbscN are more virulent than RB50ΔbscN, we conclude that the TTSS is not the only factor that contributes to the increased virulence of ST32 strains. Novel genes acquired via phage or horizontal gene transfer, loss or downregulation of hypovirulence genes, or mutations in strain 1289 may also contribute to this strain’s increased virulence, although they do not appear to be sufficient for cytotoxicity in vitro (15, 16). While many phage-related genes present in strain RB50 were identified as absent in strain 1289, another study of ours showed that a B. bronchiseptica strain lacking these prophage genes is less virulent than strain RB50, making it unlikely that the lack of these genes contributes to the increased virulence of strain 1289 (5). A few known virulence-related genes and many genes with unknown function were identified as differentially expressed or divergent between these strains. These genes may also contribute to the increase in virulence of strain 1289 compared to that of strain RB50. Differences in gene expression over the course of infection, promoter mutations, or a gain of novel genes in strain 1289 would not be detected in our analyses and may also contribute to the increased virulence of strain 1289. We are currently sequencing the genome of strain 1289 and will then be able to assess promoter mutations and novel genes that may play a role in the increased virulence of this strain (A. M. Buboltz, X. Zhang, S. C. Schüster, E. T. Harvill et al., unpublished data).

The most widely accepted view of virulence evolution assumes that there is a cost-benefit trade-off to virulence, which is defined as any reduction in host fitness following infection (2, 12, 45). Under this framework, evolutionary processes that lead to the maintenance of harmful effects are thought to be characterized by the presence of other beneficial qualities (12). Thus, a fitness-decreasing change in one trait in the pathogen is accompanied by a fitness-increasing change in a different trait (12). The ST32 strains described herein appear to be quite successful, as they have been isolated from three separate continents (South America, Europe, and the United States) (see Table S1 in the supplemental material) (60). Here, we show that ST32 strains appear to be associated with respiratory disease and exhibit increased TTSS-mediated virulence, a potential cost for the bacteria because pathogen success is dependent upon host survival before transmission. Since the TTSS increases colonization and persistence of B. bronchiseptica in the lungs of mice (44, 62), this effect may benefit the bacteria in maximizing transmission, allowing for the selection and maintenance of these highly virulent ST32 strains. Thus, the fitness enhancement caused by increased TTSS-mediated effects may be accompanied by the unavoidable side effect of increased virulence (45).

A high degree of clonal diversity appears to exist among B. bronchiseptica strains (3, 17, 19, 31, 36, 47). Recently, we reported that strains belonging to ST27 and ST40, which were collected from a wide geographical area, are hypovirulent and have lost the genes encoding adenylate cyclase toxin, which was previously believed to be among the few core factors required for the success of the classical bordetellae (5). Here, we report that the TTSS contributes to the increased virulence of strains belonging to ST32, which appear to exist worldwide. Combined, these studies support the conclusion that phylogenetic lineages of B. bronchiseptica differentially regulate and utilize distinct sets of virulence factors which can affect the overall virulence of these STs. This versatility may contribute to the wide variety in severity of respiratory disease observed upon B. bronchiseptica infection.

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REFERENCES