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Characterization of a Highly Conserved Island in the Otherwise Divergent *Bordetella holmesii* and *Bordetella pertussis* Genomes▽§

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The recently discovered pathogen *Bordetella holmesii* has been isolated from the airways and blood of diseased humans. Genetic events contributing to the emergence of *B. holmesii* are not understood, and its phylogenetic position among the bordetellae remains unclear. To address these questions, *B. holmesii* strains were analyzed by comparative genomic hybridization (CGH) to a *Bordetella pertussis* microarray and by multilocus sequence typing. Both methods indicated substantial sequence divergence between *B. pertussis* and *B. holmesii*. However, CGH identified a putative pathogenicity island of 66 kb that is highly conserved between these species and contains several IS*481* elements that may have been laterally transferred from *B. pertussis* to *B. holmesii*. This island contains, among other genes, a functional, iron-regulated locus encoding the biosynthesis, export, and uptake of the siderophore alcaligin. The acquisition of this genomic island by *B. holmesii* may have significantly contributed to its emergence as a human pathogen. Horizontal gene transfer between *B. pertussis* and *B. holmesii* may also explain the unusually high sequence identity of their 16S rRNA genes.

Bordetella holmesii is a recently described human pathogen first isolated in 1983 from the blood of a septicemic patient (48). Although most known systemic *B. holmesii* infections occur in immunocompromised individuals (41), serious systemic infection of a healthy adolescent has also been reported (36). Furthermore, although infrequently isolated, *B. holmesii* has been recovered from the nasopharynges of immunocompetent patients with pertussis-like symptoms (50), suggesting that *B. holmesii* may also cause respiratory disease. This bacterium is readily cultured on a variety of standard clinical microbiology media, so its discovery just a few decades ago suggests its recent emergence as a human pathogen (48).

In addition to *B. holmesii*, a number of other pathogenic species are found in the genus *Bordetella*. Best studied are the respiratory pathogens known as the classical or mammalian bordetellae: *B. pertussis*, human *B. parapertussis*, ovine *B. parapertussis*, and *B. bronchiseptica*. *B. pertussis* and human *B. parapertussis* are the causative agents of whooping cough, or pertussis, a disease which has reemerged despite widespread vaccination (8, 42). Ovine *B. parapertussis* infects the respiratory tracts of sheep, and *B. bronchiseptica* infects a wide range of mammalian species. The nonclassical *Bordetella* species include *B. avium*, *B. hinzii*, *B. petrii*, "*B. ansorpii*" (proposed name), and *B. trematum*, and these form a genetically diverse group that is clearly distinct from the mammalian bordetellae (16, 21).

Comparative analysis of their 16S rRNA sequences suggested that *B. holmesii* is very closely related to *B. pertussis* (48), a hypothesis supported by the discovery of the *B. pertussis*-specific insertion sequence element IS*481* in *B. holmesii* (34). However, subsequent sequencing of housekeeping genes, analysis of cellular fatty acid composition, and characterization of the *bvgAS* locus suggested that *B. holmesii* may not be as closely related to *B. pertussis* as was first assumed (15, 48).

To identify genetic factors that may have contributed to the emergence of *B. holmesii* as a human pathogen and to clarify its relation to other members of the *Bordetella* genus, we used microarray-based comparative genome hybridization (CGH) and sequencing of housekeeping genes. Although gene sequencing indicated that *B. holmesii* is a uniform group that is more closely related to the nonclassical *B. hinzii* and *B. avium* than to the mammalian bordetellae, CGH detected a genomic region of approximately 66 kb that is highly conserved between *B. pertussis* and *B. holmesii*. This genomic island, encoding genes that could promote pathogenesis, may have been transferred from *B. pertussis* to *B. holmesii*, possibly contributing to the emergence of *B. holmesii* as a human pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Characteristics of the strains used in this study are listed in Table 1. For iron depletion tests, a previously described chemically defined medium (9) was adapted by adding 330 μM L-cysteine, 114 μM ascorbic acid, 33 μM niacin, and 325 μM reduced glutathione. Chelex-100 resin (Bio-Rad, Hercules, CA) was used for iron depletion, as described previ-

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TABLE 1. *B. holmesii* strain characteristics

Strain	Original name	Source[a]	Isolation site	Country	Region	Isolation yr
B0436	104394	Institut Pasteur	—[b]	—	—	—
B0437	104395	Institut Pasteur	—	—	—	—
B1850	C690	Pam Cassiday, CDC	Nasopharynx	United States	Massachusetts	1994
B1851	C691	Pam Cassiday, CDC	Blood	United States	Massachusetts	1995
B1852	C692	Pam Cassiday, CDC	Blood	United States	Ohio	—
B1853	C693	Pam Cassiday, CDC	Blood	United States	Colorado	—
B1854	C694	Pam Cassiday, CDC	Blood	United States	North Carolina	—
B1855	USA 8/8/00	Cathy Canthaboo, NIBSC	—	—	—	—
B2738	BP244-01	Norman Fry, University of Glasgow	Blood	United Kingdom	Isle of Wight	2001
B2739	BP246-01	Norman Fry, University of Glasgow	Blood	United Kingdom	London	2001
B2767	RR 0000 0020	Norman Fry, University of Glasgow	Blood	United Kingdom	—	2003
B2768	HO 4290 0199	Norman Fry, University of Glasgow	Blood	United Kingdom	Oxford	2004
Bho29	G8350[c]	Robbin Weyant, CDC	Blood	Switzerland	—	—

[a] CDC, Centers for Disease Control and Prevention, United States; NIBSC, National Institute for Biological Standards and Control, United Kingdom.
[b] —, unknown.
[c] Isolation reported in reference 48.

ously (9). Iron-replete medium was made by adding 66 μM ferrous sulfate to iron-depleted medium.

Comparative genomic hybridization. Experiments utilized a comprehensive classical *Bordetella* genome-wide DNA microarray, based on the microarray described in references 10 and 11. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI) by following the protocol for gram-negative bacteria. *B. holmesii* genomic DNA was labeled with Cy5 and hybridized to arrays with Cy3-labeled reference DNA (equimolar amounts of *B. pertussis* Tohama I, *B. bronchiseptica* RB50, and *B. parapertussis* 12822 genomic DNA) as described in reference 11. Arrays were scanned on a two-color GenePix 4000B scanner and analyzed with GenePix Pro 6.0 (Axon Instruments, Union City, CA).

For each spot on the array, the fluorescence intensity of each of the two dyes was calculated by subtracting the mean intensity of the background pixels from the median intensity of the spot pixels. The logarithm of the ratio of background-subtracted Cy5 intensity over background-subtracted Cy3 intensity [$\log_2$(Cy5/Cy3)] is near 0 when both samples hybridize equally well to the probe and significantly below 0 when the *B. holmesii* DNA hybridizes poorly compared to the reference DNA. Data normalization was achieved by calculating the mean $\log_2$(Cy5/Cy3) for 16 probes within the iron uptake island (IUI) that were verified by sequencing to be at least 99% identical to the *B. holmesii* genome (see Table S1 in the supplemental material) and then subtracting this value from the $\log_2$(Cy5/Cy3) for each probe on the array. Data from replicate spots for the same probe were averaged. A probe was considered to have a positive *B. holmesii* hybridization signal when its hybridization ratio exceeded the fifth percentile of ratios from a control hybridization of *B. pertussis* Tohama I. Further details are in Methods in the supplemental material.

Organization of the IUI. The IUI was analyzed by amplifying overlapping 5- to 10-kb DNA fragments across the region. PCRs were analyzed by agarose gel electrophoresis to compare the fragment sizes from *B. holmesii* and *B. pertussis* Tohama I (see Table S2 in the supplemental material).

A total of 21 genes in the IUI were partially sequenced from three *B. holmesii* isolates. The nucleotide sequence of the 4.8-kb *B. holmesii* insert, absent from the classical bordetellae, was determined by genome walking inwards from the adjacent genes. In order to assess the organization of the BB3888 homolog, one of the open reading frames (ORFs) contained in the IUI, in *B. pertussis* and *B. holmesii*, we designed PCR primers in BB3888 that span the IS*481* insertion and rearrangement point from *B. pertussis* Tohama I. Nucleotide sequence data adjacent to the left breakpoint of the IUI was obtained by PCR, using one primer inside the IUI, in BB0794, and one primer outside the IUI, in BB0795, which was not detected in *B. holmesii* by CGH. The sequence adjacent to the right breakpoint of the IUI was obtained using the TOPO walker kit (Invitrogen, Carlsbad, CA). Further details are in Methods in the supplemental material, and primers are listed in Table S3 in the supplemental material.

Phylogenetic analysis and broad-range PCR. PCR primers for phylogenetic analysis were designed to hybridize to conserved regions of the *atpD*, *rpoB*, *tuf*, and *rnpB* genes (see Table S3 in the supplemental material). PCR products were directly sequenced. Concatenated sequences of these four genes were aligned for the construction of neighbor-joining trees using PHYLIP software (J. Felenstein. 2005. Phylogeny Inference Package, version 3.6 [distributed by the author]. Department of Genomic Sciences, University of Washington, Seattle). Details are in Methods in the supplemental material.

B. holmesii 16S rRNA genes were amplified by broad-range PCR using the 8F and 806R primers (35), and 23S rRNA genes were amplified using the MS37 and MS38 primers (22). PCR products were cloned using the Topo TA cloning kit (Invitrogen). Twelve clones were picked for each strain, and plasmids were purified using the Wizard miniprep kit (Promega) and sequenced using M13 forward and reverse primers.

Southern blot hybridization. The genomic DNA of *B. holmesii*, *B. pertussis*, and *B. avium* isolates was digested with the restriction enzymes ClaI and NcoI. Southern blotting and hybridization with biotin-labeled oligonucleotide probes were performed essentially as described by Schouls et al. (37). The same blot was used for both 16S rRNA gene probe hybridizations, with a stripping step between experiments. Table S3 in the supplemental material lists the probes that were hybridized to the genomes of the *Bordetella* isolates.

Transcription of alcaligin genes. *B. holmesii* strains were grown to mid-logarithmic phase in iron-depleted or iron-replete medium. Total RNA was isolated using the QIAGEN RNeasy kit (QIAGEN Inc, Valencia, CA). Real-time PCR experiments were performed with a LightCycler (Roche Diagnostics, Indianapolis, IN) using the LightCycler DNA master hybridization probe kit (Roche Diagnostics). *alcA* and *fauA* transcript levels were compared to that of *rpoB*, which served as the internal reference. Further details are in Methods in the supplemental material.

MS. Culture supernatant samples were analyzed by nanoscale reversed-phase liquid chromatography (LC) on a model HP1100 LC system (Hewlett Packard GmbH, Waldbronn, Germany) coupled to an electrospray mass spectrometer (MS) (liquid chromatography quadrupole classic ion trap), essentially as described by Meiring et al. (25). Technical details are in Methods in the supplemental material.

Microarray data accession number. Raw microarray data have been deposited at ArrayExpress under accession number E-TABM-55.

Nucleotide sequence accession numbers. GenBank accession numbers DQ402382 to DQ402420 and DQ420056 to DQ420164 are published for the first time.

RESULTS

Comparative genome hybridization. The genomes of 12 *B. holmesii* strains independently isolated in diverse locations during different years (Table 1) were each hybridized to a microarray representing the ORFs of *B. pertussis* Tohama I, *B. parapertussis* 12822, and *B. bronchiseptica* RB50 (11). Although most of the 5,515 microarray probes failed to hybridize significantly to *B. holmesii* genomic DNA, an average of 157 probes (ranging from 59 to 311) hybridized to the *B. holmesii* genomes as strongly as they did to *B. pertussis* genomic DNA (Fig. 1A; see normalized microarray data available as Table S4 in the

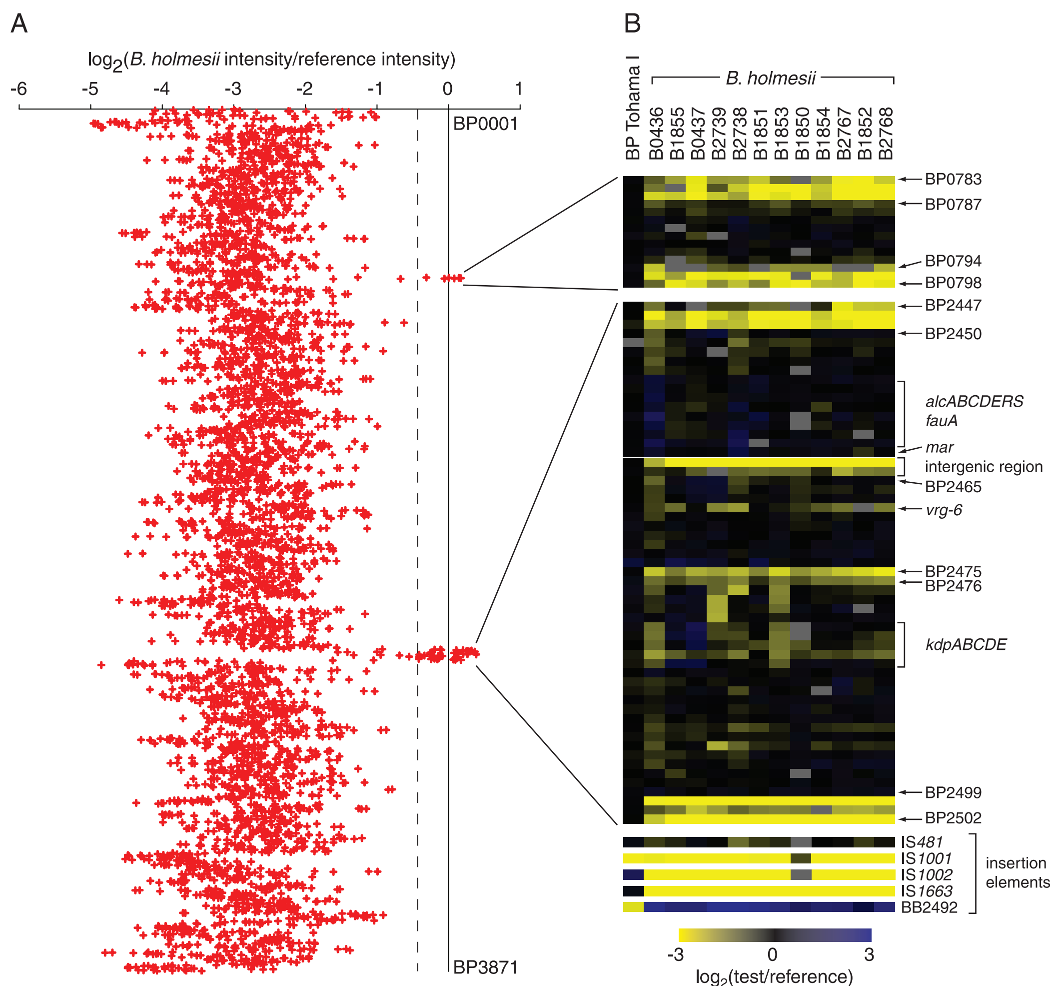

FIG. 1. Comparative genome hybridization of *B. holmesii* strains to a classical *Bordetella* DNA microarray. (A) CGH of 12 *B. holmesii* isolates to a microarray comprising the genomes of *B. pertussis* Tohama I, *B. parapertussis* 12822, and *B. bronchiseptica* RB50. The moving average (with a sliding window of three adjacent values) of the mean $\log_2$(Cy5/Cy3) of 12 *B. holmesii* genomes is plotted on the *x* axis. Microarray probes that are represented in the *B. pertussis* Tohama I genome are arranged on the *y* axis in *B. pertussis* Tohama I genome order. The dashed line indicates the fifth percentile of *B. pertussis* Tohama I hybridization ratios; ratios below the fifth percentile are considered to indicate the lack of *B. holmesii* hybridization. (B) Probes that hybridized to the *B. holmesii* genome with a strength comparable to that of the reference and adjacent nonhybridizing probes are shown in detail for individual *B. holmesii* strains and for *B. pertussis* Tohama I. ORF and gene designations for selected loci are provided as landmarks. Probes representing insertion sequence elements are also shown. BB2492 is a putative IS*3* family transposase found in *B. bronchiseptica* RB50 but not in *B. pertussis*. Strain numbers are indicated above the columns. Each row represents one probe in *B. pertussis* Tohama I gene order. The logarithm of the hybridization ratio [$\log_2$(Cy5/Cy3)] is indicated by the yellow-black-blue color scale. Missing data are represented in gray. The data represented in this figure are available as Table S4 in the supplemental material.

supplemental material). On average, 41 of these positive probes mapped to two contiguous regions in the Tohama I genome: BP0787-BP0794 and BP2450-BP2499 (Fig. 1B). The remaining probes were scattered across the genome and usually represented genes with relatively high sequence conservation across the *Bordetella* genus (e.g., genes encoding ribosomal proteins [data not shown]). The hybridization of *B. holmesii* genomic DNA to a probe for IS*481* was consistent with previous work that indicated the presence of eight IS*481* copies in the *B. holmesii* genome (34).

The two *B. pertussis* genomic regions shared by the classical bordetellae and *B. holmesii* contained, among a number of other genes, a locus comprising *alcABCDERS*, encoding the alcaligin biosynthetic pathway, the exporter for alcaligin (AlcS), and a transcriptional activator of the locus (AlcR), and *fauA*, encoding the alcaligin uptake receptor (3, 6, 7, 19, 31). Alcaligin is a siderophore produced by *B. pertussis*, *B. bronchiseptica*, and *Alcaligenes denitrificans* (27) that scavenges free iron from the extracellular milieu. Accordingly, we designated this genomic fragment the *B. holmesii* IUI.

Molecular characterization of the *B. holmesii* IUI. Partial nucleotide sequences of 21 genes in the IUI, indicated to be present by CGH, were obtained from *B. holmesii* isolates B0436, B1850, and B1852 after PCR amplification using *B. pertussis* primer pairs (GenBank accession numbers DQ402382 to DQ402418). All of these sequences were at least 99.4% identical to the *B. pertussis* sequence, with the exception of BP0794 (94.1%), located at the putative right end of the IUI (see below; see also Table S1 in the supplemental material).

The GC content of the IUI (66.0%) is substantially higher

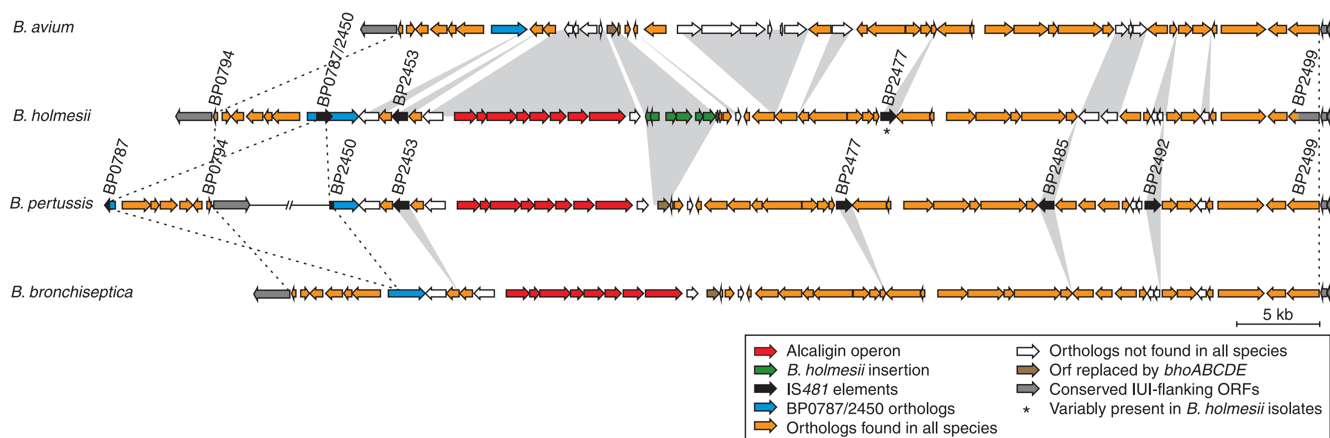

FIG. 2. Comparison of IUI genomic organizations in *B. holmesii*, *B. pertussis*, *B. bronchiseptica*, and *B. avium*. Deletions and insertions between species are indicated by gray surfaces. The dashed lines connect the ORFs at the borders of the orthologous sequences. The ORFs are color coded as described in the key. The ORF composition of *B. holmesii* was deduced from PCR and CGH data, while the representations of *B. avium* 197N, *B. pertussis* Tohama I, and *B. bronchiseptica* RB50 ORFs were based on published genome sequences.

than the average GC content of *B. holmesii* genes (61.5 to 62.3%) (48; see below) but comparable to that of the *B. pertussis* genome (67.7%) (30).

In *B. bronchiseptica* and *B. parapertussis*, but not in *B. pertussis*, the two genomic regions identified by CGH constitute a single continuous chromosomal fragment. The presence of IS*481* sequences at the putative rearrangement breakpoint in BP0787 and BP2450, which are the two halves of a single ORF in *B. bronchiseptica* and *B. parapertussis*, suggests that this region has been rearranged in the *B. pertussis* lineage by recombination between transposon copies (Fig. 2). PCR was performed using a primer pair that spans the breakpoint (BB3888-480f and BB3888-720r; see also Table S3 in the supplemental material). This reaction yielded a 240-bp fragment from *B. bronchiseptica* RB50 but failed to amplify a product from *B. pertussis* Tohama I due to the chromosomal rearrangement between these primers.

PCR amplification of DNA from all *B. holmesii* strains using this primer pair yielded a product of approximately 1.3 kb, indicating the chromosomal proximity of the primer-binding sites but suggesting the presence of a 1-kb insertion, which is the approximate size of an IS*481* element. Nucleotide sequencing confirmed that the IUI is present as a single, contiguous locus in *B. holmesii* but interrupted by an IS*481* element at the same position as the Tohama I breakpoint (Fig. 2). PCR screening of 45 *B. pertussis* strains identified one strain, 18323, that carried the same contiguous but IS*481*-disrupted allele of BP0787-BP2450 as *B. holmesii* (GenBank accession numbers DQ420073 to DQ420074).

Fourteen PCR primer pairs that amplify overlapping fragments from the *B. pertussis* Tohama I IUI sequence were used to map the IUIs in 12 *B. holmesii* strains by PCR amplification (see Table S2 in the supplemental material). As shown by agarose gel electrophoresis, most *B. holmesii* PCR amplicons were identical in size to those from *B. pertussis*, suggesting conserved genomic organization, but small insertions or deletions or point mutations that could result in pseudogene formation would not have been detected by electrophoresis of PCR amplicons or CGH. Two primer pairs yielded PCR products from *B. holmesii* that were approximately 1 kb shorter than the corresponding Tohama I products; sequencing of these products indicated that the IS*481* elements BP2485 and BP2492 were missing in *B. holmesii*. Like *B. holmesii*, *B. pertussis* 18323 lacked these two IS*481* elements. A third IS*481* element, BP2477, was variably present among the *B. holmesii* strains.

PCR amplification of one genomic region in the IUI, downstream of the *alc* operon, revealed a 4.8-kb insertion in *B. holmesii* which is not present in the classical bordetellae (Fig. 2). Nucleotide sequencing identified the left terminus of this insert 237 nucleotides downstream of the BP2464 stop codon and its right terminus in BP2465, which has been partially deleted (GenBank accession number DQ402419).

BLASTX comparison of the 4.8-kb *B. holmesii*-specific sequence against GenBank identified five putative genes, designated *bhoABCDE*, with highly significant hits (E value $< 1 \times 10^{-17}$) to known proteins (Table 2). *bhoA* is predicted to encode an IS*3* family transposase with two out-of-frame overlapping ORFs (*orfA* and *orfB*), as previously described for IS*3* transposases (39). However, a frameshift mutation detected in the 3′ ORF may have inactivated the transposase. Southern blot hybridization with a *bhoA*-specific probe identified 40 to 50 copies in the genome of *B. holmesii* but no copies in the genome of *B. pertussis* (data not shown). *bhoB* and *bhoC* are homologous to adjacent genes from the *Ralstonia metallidurans* CH34 genome. These encode a probable extracytoplasmic solute receptor and a D-isomer-specific 2-hydroxyacid dehydrogenase. *bhoD* encodes a putative extracytoplasmic function sigma factor, and *bhoE* encodes a homolog of the FecR proteins (Table 2). The GC content of the insert is 62%, similar to the average GC content of *B. holmesii* genes (61.5 to 62.3%) (48) but lower than the shared component of the IUI (66.0%).

The CGH data suggested a sharp chromosomal boundary between the IUI and the flanking *B. holmesii* genomic sequence, prompting a search for the discrete breakpoints that define the ends of the IUI. The left breakpoint in *B. holmesii* was mapped by PCR and sequencing (GenBank accession numbers DQ420096 to DQ420098) to approximately codon 31

TABLE 2. Annotation of the *B. holmesii*-specific insertion in IUI

Gene	Predicted product	BLASTX top hit			Pfam significant hit(s)		
		Entrez locus	Species	E value	Accession no.	Domain	E value
bhoA	IS*3* family transposase (pseudogene)	YP_107572	*Burkholderia pseudomallei*	2E−74	PF00665	Integrase core domain	9E−16
bhoB	Extracytoplasmic solute receptor	EAN49994	*Ralstonia metallidurans*	7E−79	PF03401	*Bordetella* uptake gene (bug) product	4E−55
bhoC	D-Isomer-specific 2-hydroxyacid dehydrogenase	EAN49993	*R. metallidurans*	3E−76	PF00389	D-Isomer-specific 2-hydroxyacid dehydrogenase, catalytic domain	0.27
bhoD	ECF sigma factor	CAD84465	*Nitrosomonas europaea*	2E−21	PF04542 PF04545	Sigma-70, region 2 Sigma-70, region 4	2E−10 5E−08
bhoE	FecR protein	YP_349526	*Pseudomonas fluorescens*	6E−18	PF04773	FecR protein	4E−33

of BP0794. The right breakpoint was mapped by chromosomal walking and sequencing (GenBank accession number DQ402420) to approximately codon 276 of BP2499. In both cases, the breakpoints were approximated as the point at which sequence identity, reading out from the IUI, dropped from greater than 99% to below 90%. The ORFs at both breakpoints are intact in *B. holmesii*. No evidence was found at either end of the IUI for the presence of phage, plasmid, or IS sequences.

The IUI sequence was also compared to the *B. avium* 197N genome sequence (38). Using the BLAST programs, a syntenic region with homology extending beyond the ends of the IUI was identified (Fig. 2). The average nucleotide sequence identity of the *B. avium* region to the *B. holmesii* IUI was 73%, indicating that these sequences are not highly conserved across all members of the genus, but sequences near the ends of the IUI were more highly conserved (at least 88%) than sequences in the middle. Although gene order is otherwise largely conserved, the *B. avium* genome shows no detectable homology to the *alcABCDERS*, the *fauA*, the *bhoABCDE*, and the IS*481* loci (Fig. 2; see also Table S1 in the supplemental material).

Alcaligin expression and detection. To determine whether the IUI alcaligin biosynthesis, export, and uptake locus is expressed in *B. holmesii*, the transcript levels of *alcA* and *fauA* were measured by quantitative reverse transcription-PCR of B0437 grown under iron-depleted and iron-replete conditions. The transcript abundances of *alcA* and *fauA* were 3.3- and 4.8-fold higher, respectively, in the iron-depleted sample than in the nondepleted sample, indicating that the locus is expressed and iron regulated in vitro.

LC-MS of culture supernatant from iron-starved *B. holmesii* detected a compound in the iron-depleted supernatant but not in the iron-replete supernatant with m/z 405 $(M + H)^+$ (Fig. 3), corresponding to the mass of desferri-alcaligin (27). MS fragmentation analysis detected two fragment ions with m/z 283 and m/z 387, corresponding to previously described alcaligin fragment ions (27).

Evolutionary relationship of *B. holmesii* to other bordetellae. Contrary to 16S rRNA-based phylogenies, our CGH data suggested substantial sequence divergence between *B. pertussis* and *B. holmesii*. To determine the phylogenetic position of *B. holmesii* within the *Bordetella* genus, conserved regions of the *atpD*, *rpoB*, *tuf*, and *rnpB* genes, which have been used for phylogenetic inference in other groups of closely related bacterial species (18, 26, 29), were sequenced from seven *B. holmesii* strains and from representative isolates of *B. hinzii*, *B. trematum*, *B. petrii*, and the closely related β-proteobacterial species *Achromobacter xylosoxidans* (GenBank accession numbers DQ420062 to DQ420072 and DQ420099 to DQ420164). Orthologous sequences from the classical *Bordetella* species (GenBank accession numbers NC_002927, NC_002928, and NC_002929), *B. avium*, and another β-proteobacterial species, *Burkholderia pseudomallei* (GenBank accession numbers NC_006350 and NC_006351), were obtained from available genome sequence data. A neighbor-joining tree, based on 3,559 fully informative characters in the alignment of concatenated nucleotide sequences of all these genes, indicated that *B. holmesii* is more closely related to *B. avium* and *B. hinzii* than to the mammalian bordetellae (Fig. 4). The exclusion of *B. holmesii* from the mammalian *Bordetella* clade was supported by all individual gene trees. Comparison of sequences from seven independent *B. holmesii* strains revealed only two single-nucleotide polymorphisms (one synonymous and one nonsynonymous) among 3,666 aligned bases, both of which were variant only in Bho29. Because of the near identity of these seven strains, no further *B. holmesii* isolates were sequenced.

Molecular characterization of *B. holmesii* rRNA loci. In light of the phylogeny determined above, the near identity of the *B. pertussis* and *B. holmesii* 16S rRNA genes (99.7%) is anomalous. One possible explanation for this discrepancy is lateral transfer of the 16S rRNA gene from *B. pertussis* to *B. holmesii*. To determine the number of *B. pertussis*-like 16S rRNA gene copies and search for possibly divergent 16S rRNA genes, Southern blot hybridization was performed. A *B. pertussis*-specific 16S rDNA probe that did not hybridize to the *B. avium* genome detected three copies in the *B. holmesii* genome (Fig. 5). A broad-range 16S rRNA gene probe (37) that hybridized equally well to *B. pertussis* and *B. avium* DNA did not detect any additional bands in the *B. holmesii* genome, suggesting that the only 16S rRNA loci are the three *B. pertussis*-like copies (Fig. 5). 16S rRNA broad-range PCR was also employed to identify possible variant 16S rRNA sequences. Twelve cloned PCR products from each of three *B. holmesii* strains were sequenced. All were at least 99.5% identical to the *B. pertussis*

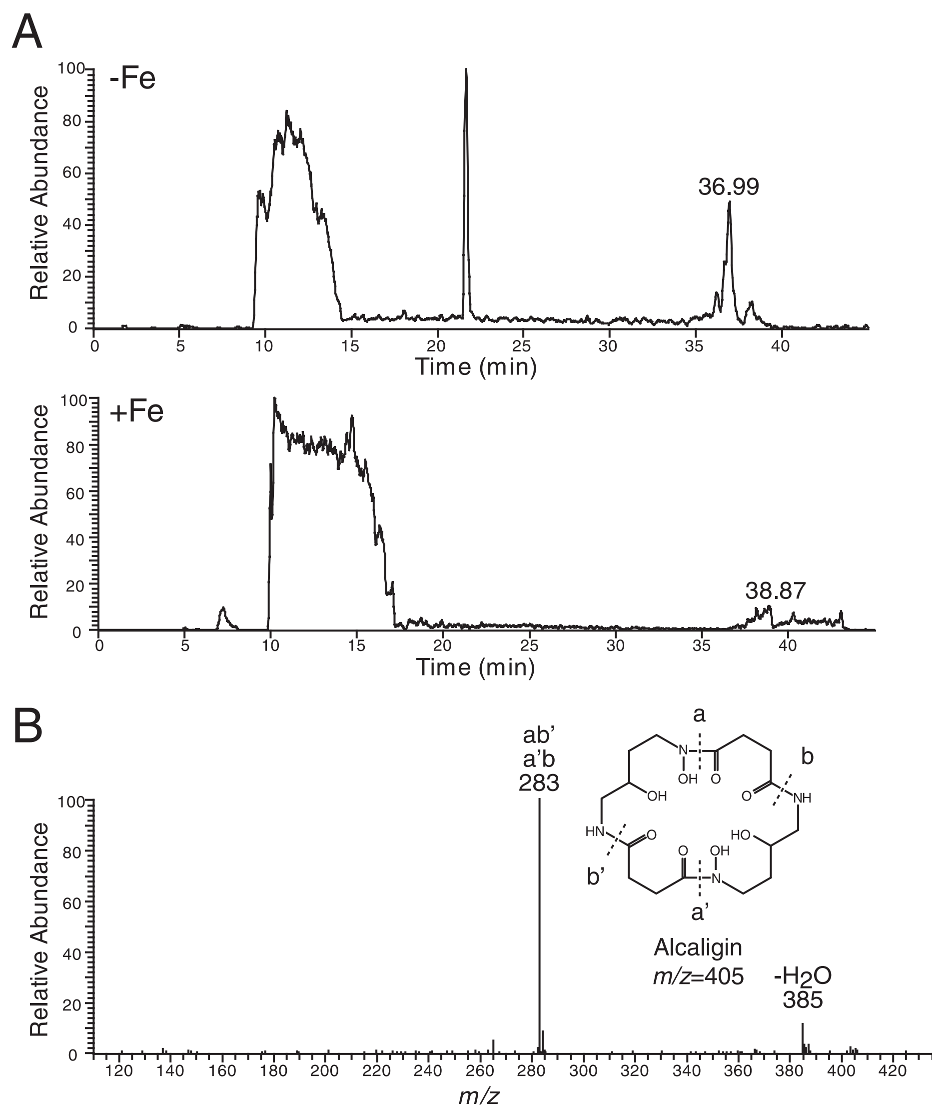

FIG. 3. LC-MS spectra of *B. holmesii* supernatants from iron-depleted and iron-replete cultures. (A) LC-MS spectra of iron-depleted (−Fe) and iron-replete (+Fe) *B. holmesii* supernatants, depicting ions with *m/z* 405 (corresponding to alcaligin) and with retention times between 0 and 45 min. (B) Fragment ions detected after collisional activation (35% energy) of the peak from (A), with a retention time of 36.99 min. After measurement of the reference standard, the calibration deviated by approximately 1.5 mass units, explaining the difference in *m/z* values compared to those of the fragment ions as reported in reference 22. The nomenclature of fragment ions is according to that in reference 27.

16S rRNA, further suggesting that all copies of the 16S rRNA in *B. holmesii* are essentially identical to the *B. pertussis* gene (GenBank accession numbers DQ420056 to DQ420058).

No copies of the 16S rRNA gene were identified within the boundaries of the IUI as defined above. To test further whether a 16S rRNA gene was located near either end of the IUI in *B. holmesii*, PCR amplification was attempted using primers in the 16S rRNA gene and in the putative 5′ and 3′ termini of the IUI. PCR amplification was unsuccessful, suggesting that no 16S rRNA genes are in close proximity to the IUI.

Among the sequenced *Bordetella* genomes, the intergenic transcribed spacer region (ITS) between the 16S and 23S rRNA genes is more varied than the sequences of the structural rRNA genes (Table 3). However, ITS sequences from seven *B. holmesii* strains, all of which were identical to each other, were 99.6% identical to that from *B. pertussis* (GenBank accession numbers DQ420075 to DQ420095) and more similar than even the ITS sequence from *B. bronchiseptica*. A partial sequence of the 23S rRNA gene (*B. pertussis* bases 1043 to 1919) was also determined following broad-range PCR of three *B. holmesii* strains. Nine sequences (three from each strain; GenBank accession numbers DQ420059 to DQ420061) were identical to each other but only 97.2% identical to *B. pertussis* and more divergent than the *B. avium* sequence (Table 3). For comparison, *B. pertussis* and *B. bronchiseptica* vary at only a single nucleotide in this region. Therefore, the region of unexpectedly high identity between the *B. pertussis* and *B. holmesii* rRNA gene operons includes the 16S rRNA gene and the ITS but does not include the 3′ end of the 23S rRNA gene. The boundary between nearly identical (>99.5%) and divergent sequences

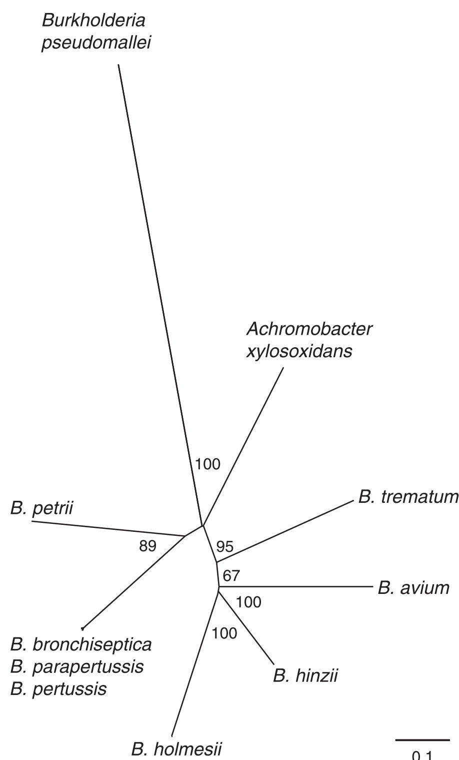

FIG. 4. Neighbor-joining phylogeny of *Bordetella* spp. and related β-proteobacteria based on four genes outside the rRNA operon. The unrooted tree is based on 3,559 fully informative nucleotides from an alignment of concatenated sequences of *atpD*, *rpoB*, *tuf*, and *rnpB* from seven *B. holmesii* strains, *B. pertussis* Tohama I, *B. parapertussis* 12822, *B. bronchiseptica* RB50, *B. avium* 197N, *B. hinzii* BC304, *B. trematum* DSM11334, and *B. petrii* SE1111R, and from *A. xylosoxidans* ATCC 15173 and *B. pseudomallei* K96243. Bootstrapping values greater than 50% (in 1,000 resamplings) are indicated at branches.

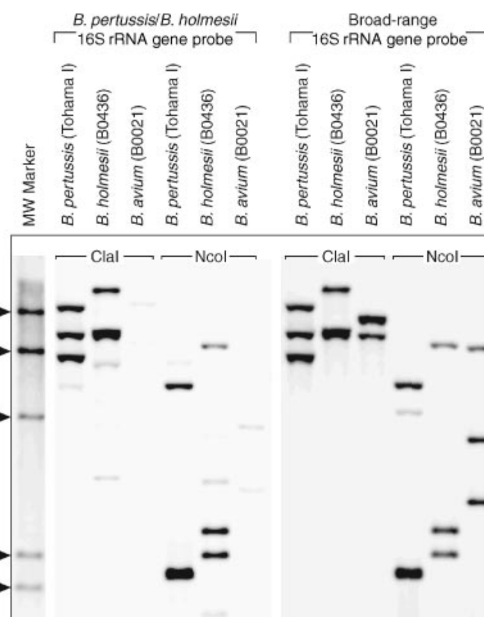

FIG. 5. Southern blot hybridizations of *B. pertussis*, *B. holmesii*, and *B. avium* with 16S rRNA gene probes. Genomic DNA from each of the three strains was restriction digested with ClaI or NcoI as indicated and hybridized to a *B. pertussis*-specific probe (left panel) and a broad-range 16S rRNA gene probe (B-16S8F [right panel]) (15). Biotinylated DNA markers are in the first lane, and numbers at the left are fragment sizes (in base pairs).

lies in the first kilobase of the 23S gene, which was not sequenced in this study.

DISCUSSION

Although 16S rRNA phylogeny and clinical criteria had placed *B. holmesii* close to *B. pertussis*, other evidence, including gene sequencing and cellular fatty acid analysis, had in fact suggested a more distant evolutionary relationship (15, 16, 48). The multilocus sequence analysis presented here provides further evidence that *B. holmesii* is not, in fact, a member of the classical *Bordetella* lineage. Rather, phylogenetic analysis based on the nucleotide sequences of four different housekeeping genes strongly suggested that *B. holmesii* is a member of the phylogenetically diverse nonclassical *Bordetella* group (Fig. 4). Furthermore, at these loci, *B. holmesii* and *B. avium* were similarly distant from the classical bordetellae (89.1% and 90.0%, respectively). By demonstrating that the tree based on the 16S rRNA gene differs significantly from the tree based on four independent housekeeping genes, these results underscore the importance of using multiple loci when reconstructing evolutionary histories. An explanation for the anomalously high sequence identity of the *B. holmesii* and *B. pertussis* 16S rRNA genes is proposed below.

Only 2 of 3,666 nucleotide positions in the housekeeping genes were polymorphic among seven geographically distinct *B. holmesii* strains. This level of sequence conservation is comparable to the three polymorphisms of 3,666 nucleotides observed between *B. bronchiseptica* and *B. pertussis*, which diverged and expanded clonally from the *B. bronchiseptica* lineage 2 to 5 million years ago (12, 30). Likewise, *B. holmesii* may have diverged and expanded within a similar time frame.

Comparative genomic hybridization identified a 66-kb DNA region, designated the IUI, which, unlike most of the *B. holmesii* genome, was highly conserved in *B. holmesii* and the classical bordetellae. No conserved chromosomal regions were identified when *B. avium* genomic DNA was hybridized to the classical *Bordetella* microarray (data not shown). Detailed molecular characterization verified that the shared sequences are more than 99% identical, much higher even than the expectation based on strongly conserved phylogenetic marker genes (under the assumption that the nucleotide substitution rates are similar across the genome). Cloning of the IUI ends also demonstrated sharp boundaries between the conserved IUI sequence and the weakly conserved flanking sequences. These

TABLE 3. Percentages of nucleotide identity in the rRNA operon between *B. holmesii* and selected *Bordetella* species

Species	% Nucleotide identity		
	16S rRNA	ITS	23S rRNA
B. pertussis	99.7	99.6	97.2
B. bronchiseptica	99.4	96.4	96.6
B. avium	97.2	72.5	98.1

data are consistent with the recent transfer of the IUI from a classical *Bordetella* genome to the *B. holmesii* genome. Transfer from a *B. bronchiseptica* or *B. parapertussis* genome cannot be ruled out, but the presence in the IUI of IS*481* elements, which have been found in all *B. pertussis* and *B. holmesii* strains analyzed but in only two *B. bronchiseptica* strains (12, 45), argues strongly that a *B. pertussis* strain was the donor of this DNA. Although the IUI region is rearranged in *B. pertussis* Tohama I relative to this region in *B. holmesii* and the other mammalian bordetellae, another *B. pertussis* strain, 18323, had a genomic architecture and an IS*481* element distribution very similar to those observed in the *B. holmesii* IUI, suggesting that the *B. holmesii* IUI may have been transferred from an 18323-like *B. pertussis* strain. *B. pertussis* 18323, isolated from a pertussis patient in 1947, is an atypical *B. pertussis* strain by many criteria, including pulsed-field gel electrophoresis (17), multilocus sequence typing (12), multilocus enzyme electrophoresis (45), and CGH (11) results, but a genetically related strain, CZ, was isolated from a pertussis case in 1993 (4, 17), suggesting that this is a rare, but circulating, variant.

The data presented here do not rule out the possibility that the IUI was transferred in the other direction, from *B. holmesii* to the classical bordetellae. However, our interpretation is supported by two lines of evidence. First, the GC content of the IUI is similar to that of the classical *Bordetella* chromosomes but significantly higher than that of *B. holmesii* protein-coding genes outside this region. Because atypical nucleotide composition is a hallmark of recently acquired DNA (reviewed in reference 13), this suggests that the IUI was recently transferred into *B. holmesii*. Second, the presence of multiple IS*481* elements in the *B. holmesii* and *B. pertussis* IUIs, but not in the other classical *Bordetella* genomes, is more easily explained by our model, which does not require transfer of an IS*481*-containing DNA fragment to the ancestor of the classical *Bordetella* lineage, followed by precise excision of those transposons in two of three derived species. *B. pertussis* and *B. holmesii* have both been isolated from the respiratory tracts of humans, making the human airway a likely environment in which the transfer of the IUI could have occurred. At the resolution of CGH and PCR analysis, IUIs were identical in all examined *B. holmesii* isolates, with the exception of a variably present IS*481* element, suggesting that the IUI was acquired recently or is under selective pressure to maintain its genomic organization.

A region of synteny between *B. avium*, *B. holmesii*, and the classical bordetellae extended through the IUI and into the flanking sequences on either side, indicating that the gene order of this chromosomal region is conserved across distantly related *Bordetella* species (Fig. 2). This finding, together with the failure to identify signatures of mobile elements (e.g., phage or conjugative transposon genes) at the ends of the IUI, suggests that the most likely mechanism for the proposed integration of the IUI into the *B. holmesii* genome is homologous recombination between the laterally transferred IUI and the ancestral *B. holmesii* genome, which may have diverged from the *B. pertussis* sequence to an extent similar to the extent of the divergence of the *B. avium* sequence. The degree of nucleotide sequence identity between the *B. holmesii* IUI and the orthologous sequences in *B. avium* is highest in the vicinities of the left and right insertion breakpoints, indicating that these genes may be more highly conserved across the *Bordetella* genus than the average gene. These sequences are proposed to have served as the substrates for the putative double-recombination event that replaced the *B. holmesii* sequence with a transferred *B. pertussis* fragment.

Given the evidence presented above for lateral transfer of the IUI from *B. pertussis* to *B. holmesii*, lateral transfer is also a plausible explanation for the unexpectedly high sequence similarity of their 16S rRNA genes. The failure to identify a 16S rRNA gene within, or in close proximity to, the IUI suggests that it was independently transferred from *B. pertussis* or that a subsequent rearrangement separated the 16S rRNA gene from the IUI. The proposed exchange includes the 16S rRNA gene and the ITS and may include a 5′ fragment of the 23S rRNA gene.

Immediately following the proposed 16S rRNA gene transfer event, the recipient *B. holmesii* chromosome would have carried a single copy of the *B. pertussis* 16S rRNA gene and, unless there was only a single rRNA locus that was replaced by homologous recombination with the foreign DNA, one or more copies of its ancestral 16S rRNA gene. Other microbial genomes have been found to harbor one or more foreign copies of the small-subunit rRNA locus in addition to their presumed native copies (1, 37, 49). In order to detect putative ancestral *B. holmesii* 16S rRNA loci, independent hybridization- and PCR-based methods were used to search for copies of the 16S rRNA gene that diverged from the *B. pertussis*-like sequence. However, only *B. pertussis*-like 16S rRNA genes were found, suggesting that all copies of the ancestral recipient strain's 16S rRNA gene have been either lost or converted by recombination since acquisition of the *B. pertussis*-like 16S rRNA locus. The putative replacement of the ancestral *B. holmesii* 16S rRNA, which, by analogy to *B. avium*, might have been more than 98% identical to the *B. pertussis* sequence, may not have had serious functional consequences for the organism, because heterologous rRNA genes have been shown to function in some cases. For example, the *Salmonella enterica* serovar Typhimurium rRNA operon, although only 97% identical to that of *Escherichia coli*, can functionally replace the native *E. coli* loci (2).

The complement of genes carried by the IUI is of particular interest because it may include virulence factors or host specificity determinants that have contributed to the emergence of *B. holmesii* as a human pathogen. Of the 57 putative and confirmed genes (excluding transposons) carried on the IUI, the alcaligin locus, consisting of 8 genes, is the most recognizable candidate for a role in virulence. Alcaligin is an important iron-scavenging siderophore in *B. pertussis* and *B. bronchiseptica* (3, 6, 7, 19, 31) and is required for the maximal virulence of *B. bronchiseptica* in a piglet infection model (33). The demonstration of iron-regulated *alcA* and *fauA* transcription and the detection of alcaligin in the supernatants of iron-limited *B. holmesii* cultures indicated that the alcaligin biosynthesis and export locus was functional in *B. holmesii*. Although *B. avium* is capable of acquiring iron through other mechanisms (e.g., heme uptake [28]), alcaligin production has not been detected in *B. avium*, and its genome does not appear to encode an alcaligin biosynthesis locus, indicating that not all *Bordetella* species possess the ability to produce this siderophore. Therefore, prior to the IUI acquisition, the hypothetical progenitor of *B. holmesii* may not have been competent to produce alca-

ligin. Wholesale acquisition of this function by lateral transfer from *B. pertussis* could have provided *B. holmesii* with a new, highly efficient iron uptake system, potentially leading to an enhancement of its ability to colonize the human host, in which free iron is sequestered. Additionally, BhoD and BhoE, encoded by ORFs in the *B. holmesii*-specific IUI fragment, are homologous to *E. coli* FecI and FecR, which regulate ferric citrate transport (5, 14, 46, 47), and to related *Bordetella* heme uptake regulators (20, 32, 43, 44), raising the possibility that these may regulate iron uptake systems in *B. holmesii*.

Other genes in the IUI may also contribute to *B. holmesii* host specificity or virulence. For example, *mar*, adjacent to the alcaligin locus, encodes a member of the MarC family of multiple antibiotic resistance efflux pumps. Multidrug resistance transporters have been shown to confer resistance to specific host antimicrobial peptides in diverse bacterial pathogens (e.g., *Neisseria meningitidis* [40] and *Staphylococcus aureus* [23]). Perhaps the *mar* gene product enhances colonization of the host airway by *B. holmesii* by increasing resistance to host defensins and other endogenous antimicrobial peptides. Some ORFs in the IUI encode putative cell surface or exported proteins, including a putative lipoprotein (BP0793) and a putative exported protein (BP2486), that could influence pathogenesis by modifying interactions with the host epithelium and immune system. The presence of the *B. pertussis* Bvg-repressed gene *vrg6* in the IUI is interesting, but this gene is not required for virulence in *B. bronchiseptica* (24), making its role in *B. holmesii* virulence unlikely. The numerous IUI genes encoding so-called housekeeping functions (e.g., metabolic enzymes, cytochromes, potassium transporters, cell division proteins, and molecular chaperones) might contribute to pathogenicity by enhancing fitness and so cannot be ruled out as virulence factors without further functional characterization. The potential roles of the hypothetical and conserved hypothetical ORFs, which account for about 20% of the genes in IUIs, remain to be explored.

Only recently has *B. holmesii* been identified as a human pathogen and recognized to cause pertussis-like disease. However, in spite of its similarities in host range and clinical course to those of *B. pertussis*, *B. holmesii* is not as closely related to *B. pertussis* as are the other mammalian bordetellae. The results presented here suggest that lateral transfer of a genomic island from *B. pertussis* may have contributed to the emergence of *B. holmesii* and its adaptation to the human host. These results represent a significant advance in the characterization of *B. holmesii* and in our understanding of the evolution of virulence in the important group of pathogens comprising the genus *Bordetella*.

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