

Bordetella holmesii: Comparison of Two Isolates from Blood and a Respiratory Sample

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ABSTRACT

Interest in *Bordetella holmesii* is increasing, but very little is known about this bacterium, which can be isolated from both blood and respiratory samples. In this study, we compared a *B. holmesii* isolate from the blood sample of an adult with bacteremia with another isolate from a nasopharyngeal swab from an adult with whooping cough syndrome. Genetic analysis was carried out, targeting relevant genes, and virulence properties were studied in cellular and animal models. Our genomic analysis provided no evidence of traits specific to either blood or respiratory isolates of *B. holmesii*. Neither isolate was cytotoxic to human tracheal epithelial cells. Both isolates were only weakly invasive and they did not persist within epithelial cells for less than 48 h.

Keywords: *Bordetella holmesii*; Bacteremia; Respiratory Isolate

1. Introduction

Bordetella holmesii is a Gram-negative bacterium, first described in 1995, following its isolation from the blood of a patient with septicemia [1]. Phylogeny analysis based on 16S rRNA genes initially placed *B. holmesii* close to *B. pertussis*. However, multilocus sequence typing showed that *B. holmesii* did not belong to the classical *Bordetella* group (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*), being instead more closely related to *B. avium* [2]. Very few putative virulence factors have been identified in this species: a two-component *bvg* operon (*bvgA* and *bvgS*) [3,4], a filamentous hemagglutinin-like protein FHA-bho [5] and an alcaligin operon [2]. By contrast, no toxin or adhesion similar to those involved in pathogenesis in classical *Bordetella* species has been identified. There has recently been an increase in the number of cases linking *B. holmesii* to whooping cough syndromes. The *B. holmesii* genome harbors insertion sequences, such as IS481, which is also present in the genome of *B. pertussis*, but at a lower copy number [6]. This poses a potential problem, because the diagnosis of *B. pertussis* infection is based on the PCR detection of IS481 sequences. The development of specific PCR diagnosis for *B. holmesii* infection, targeting the *recA* gene [7-9], or the transposase IS1001*bho* [10], the amplifica-

tion of specific target as bhoE [11] or the sequencing of 16sRNA or OmpA [12] has made it possible for a number of retrospective studies to demonstrate an increase in the number of reported cases of respiratory infections due to *B. holmesii* over the last few years [10,11,13-21]. We wondered whether this increase in detection reflected a real increase in the number of infections or was simply a consequence of the increasing use of RT-PCR targeting IS481 for the diagnosis of *B. pertussis* since 2005. Indeed, Njamkepo *et al.* [16] reported a prevalence of 6.8% for *B. holmesii* in respiratory samples from patients with a principal diagnosis of pertussis. Prevalence was highest (20.3%) in adolescents and adults, and no cases were found in children under the age of nine years. By contrast, 1) during an outbreak in Chile, Miranda *et al.* [22] reported a prevalence of 11.1% for *B. holmesii*, mostly in adolescents and young adults, but they also identified three cases (3%) in patients under the age of one year; 2) in Argentina in 2010, Bottero *et al.*, [21] reported 9 cases due to *B. holmesii*, among 1475 pertussis suspected cases patients, 7 under 6 months of age, one 7 months old and one 13 years old. Rodgers *et al.* [10] recently reported that, during an outbreak of pertussis in Ohio (USA), 30% of the patients were infected with *B. holmesii* and not with *B. pertussis*. These findings raise questions concerning the nature of *B. holmesii*: is it an op-

opportunistic bacterium or an invasive or respiratory pathogen? They also raise questions about the possible differences between invasive and respiratory isolates. The aim of our study was to compare two *B. holmesii* isolates, one from blood [23] and the other from a respiratory sample [6], in terms of their genetics, targeting relevant genes and considering their virulence in cellular and animal tests.

2. Materials and Methods

2.1. Bacterial Growth and DNA Extraction

All *B. pertussis* and *B. holmesii* isolates used were obtained from the Collection of the Pasteur Institute or the French National Reference Center. They were grown at 36°C for 72 hours on Bordet-Gengou agar (BGA) supplemented with 15% defibrinated sheep blood, and replated on the same medium and incubated for 24 hours before use. A list of the isolates used is presented in **Table 1**. For pyrosequencing, genomic DNA was prepared on Genomic-tip 500/G anion-exchange columns (Qiagen), according to the manufacturer's recommendations. For PCR validation, DNA extractions were performed with the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions.

2.2. Pulsed-Field Gel Electrophoresis

DNA fingerprinting was performed by pulsed-field gel electrophoresis (PFGE), as previously described [24].

2.3. Western-Blot Analysis

Western blots were carried out as described by Weber *et al.* [25].

2.4. Pyrosequencing and In-House Sequence Verifications

Pyrosequencing was carried out with a 454GS-FLX Next-Gen sequencing platform (Roche Diagnostics GmbH, Beckman Coulter Genomics). Three mate-pair libraries were constructed for both isolates. The two samples were then simultaneously sequenced in one GS-FLX run, with a 70 × 75 mm Pico-Titer plate device (Roche Diagnostics GmbH) and the GS LR-70 sequencing kit (Roche Diagnostics GmbH), as previously described [26]. A standard sequencing assembly was generated with Newbler assembler version 2.3 from 454/Roche, generating 11 scaffolds for Bho1 and 33 for FR4020. However, as no reference annotated genome is available for *B. holmesii*, we initially focused on the few genes of interest already identified for this species: *bvgA/S* [3], *fhaB* [5], the alcaligin operon and *B. holmesii*-specific genes [2]. Moreover, as pyrosequencing technology is known to generate errors, particularly in homopolymeric nucleotide tracts, we checked all the sequence differences ob-

served in these genes by PCR and classical Sanger sequencing for both the isolates studied and for some additional isolates (**Table 1**). The primers used are listed in **Table 2**. Concatenated sequences of the four sequences of interest (located in *bvgA/bvgS/fhaB* and *fimC*) were aligned for the construction of a neighbor-joining tree, with PHYLIP software [27] from the Pasteur Mobyle Portal (**Figure 1**).

We then used the NCBI blastn and blastp programs to identify other genes potentially important for the putative pathogenicity of *B. holmesii*.

2.5. Cell Culture

The murine monocyte/macrophage-like cell line J774.A1 and human tracheal epithelial cells (HTE) were cultured as previously described [26].

2.6. Cytotoxicity Assays

Bacterial cytotoxicity to J774.A1 and HTE cells was assessed as previously described [26]. Briefly, bacteria were added to cells at a ratio of 100 bacteria per cell and gently centrifuged. Infected cells were incubated at 37°C, in the presence of 5% CO₂, for 8 hours. Cytotoxicity was determined every 2 hours, with the Cytotox 96@ Non-Radioactive Cytotoxicity Assay (Promega), which measures the activity of the lactate dehydrogenase released into the culture supernatant. In each experiment with J774.A1, we used Tohama, the *B. pertussis* reference strain known to be cytotoxic to these cells, as a positive control [28]. In each experiment with HTE cells, we used RB50, the *B. bronchiseptica* reference strain known to be cytotoxic to these cells, as a positive control [29].

2.7. HTE Cell Invasion and Persistence Assays

Invasion and persistence assays were conducted with HTE cells, as previously described [26]. Briefly, bacteria were added to cells at a ratio of 100 bacteria per cell and gently centrifuged. Infected cells were incubated at 37°C, in the presence of 5% CO₂. After 5 hours of incubation, cells were thoroughly washed and incubated for a further two hours with gentamicin (Sigma) at a final concentration of 100 µg/ml, to kill any remaining extracellular bacteria. For persistence assays, the concentration of gentamicin in the cell culture medium was then decreased to 10 µg/ml over the rest of the incubation period. The number of intracellular *Bordetella* was determined by cell lysis and determinations of the number of CFU after 5 hours for invasion assays and after 48 hours of incubation for persistence assays.

The results presented are the means and standard deviations of at least three experiments. The symbol (*) in **Figure 2** indicates $p < 0.05$ in comparisons with the re-

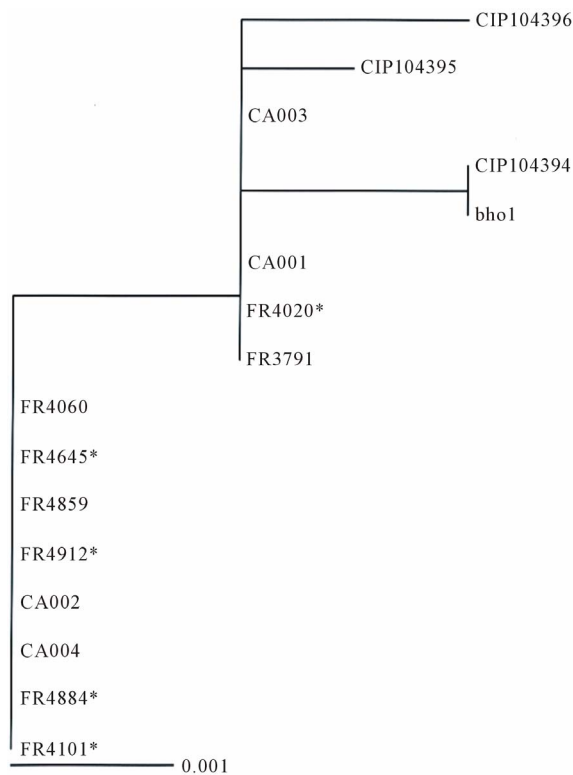
Table 1. List of clinical isolates used in the study.

Name of isolate	Species	Year of collection	Origin	Age of the patient	Disease/pathological detection	Reference
CIP104394 (ATCC51541-F5101)	Bh*	1983	USA	37	Mild leukocytosis, cardiomegaly	[1]
CIP104395 (G7702)	Bh	1992	USA	36	Dog bite	[1]
CIP104396	Bh	Nk [§]	Saudi Arabia	32	Endocarditis	[1]
Bho1	Bh	1996	France	20	Hemoculture/asplenic	[23]
FR3713	Bp**	2007	France	0.17	Respiratory	[6]
FR3791	Bh	2007	France	20	Hemoculture/asplenic	[6]
FR4020	Bh	2008	France	44	Respiratory	[6]
CA001	Bh	before 2008	Canada	13	Hemoculture/asplenic	[20]
CA002	Bh	before 2008	Canada	17	Hemoculture/asplenic	[20]
CA003	Bh	before 2008	Canada	13	Hemoculture/asplenic	[20]
CA004	Bh	before 2008	Canada	15	Hemoculture/asplenic	[20]
FR4060	Bh	2009	France	10	Hemoculture	[6]
FR4101	Bh	2009	France	15	Respiratory	[6]
FR4645	Bh	2009	France	15	Respiratory	[6]
FR4859	Bh	2010	France	67	Hemoculture	[6]
FR4884	Bh	2011	France	68	Respiratory	[6]
FR4912	Bh	2011	France	61	Respiratory	[6]

Nk[§]: Not known; Bh*: *Bordetella holmesii*; Bp**: *Bordetella pertussis*.

Table 2. List of primers used in the study.

Target gene	Primer name	Primer sequence (5'→3')	PCR product size (bp)
<i>bvgA</i>	<i>bvgA-bho-F</i>	AAAGAGGGTTTCGAGGTCGT	543
	<i>bvgA-bho-R</i>	TCGTTTGGCCATGTCTATCA	
<i>bvgS</i>	<i>bvgS1-bho-F</i>	AGTCACAACCTGGGCTTGAT	760
	<i>bvgS1-bho-R</i>	CGATCTGGAAATGCAATCCT	
	<i>bvgS2-bho-F</i>	CGCAACAATTGGCATAACATC	611
	<i>bvgS2-bho-R</i>	CATATCGCCCTTGCTTTGAC	
<i>fhaB-bho</i>	<i>fhaB1-bho-F</i>	GGTTTGACAGACCTTCGACAT	587
	<i>fhaB1-bho-R</i>	ATGTCACGCGACAGGATGAT	
	<i>fhaB2-bho-F</i>	GGCACGCAGGTCTTGATG	808
	<i>fhaB2-bho-R</i>	CCGTGCTGCTGAAGTTGG	
	<i>fhaB3-bho-F</i>	GACCATCAAGACCGAGCAGT	656
	<i>fhaB3-bho-R</i>	GTACGAAGTTTGCCTGTGGA	
<i>fhaB-bho</i>	<i>fhaB4-bho-F</i>	CCGGTATCCGAACTCAAGAC	301
	<i>fhaB4-bho-R</i>	CGTTCTGCTTGCTGTTGTTG	
	<i>fhaB5-bho-F</i>	GCAAGATCCAGGCTGACAAC	316
	<i>fhaB5-bho-R</i>	GCGATCTGGCTGTAGTTGGT	
Upstream	<i>fhaB6-bho-F</i>	TTCCAGAACTGGGCTATCAA	273
	<i>fhaB6-bho-R</i>	GTTTATCCGCGTCCAGACAT	
<i>fhaB</i>	<i>pfhaBbho-F</i>	GGCCTGTTGCATGAGTCTCT	368
	<i>pfhaBbho-R</i>	GGCGTTCACGGTGTATCTCT	
<i>fimC</i>	<i>fimCbho-F</i>	TTCTACGGGACGAGCTATCG	307
	<i>fimCbho-R</i>	AAACGCCTACCCCTTTGACT	



Four concatenated sequences of interest (located in *bvgA/bvgS/fhaB* and *fimC*) were aligned for the construction of a neighbor-joining tree. The symbol (*) is used to identify isolates of respiratory origin.

Figure 1. Neighbor-joining tree based on partial sequences of *bvgA/bvgS/fhaB/fimC*.

cently isolated *B. pertussis* FR3713.

2.8. Animal Models

All procedures involving animals were conducted in accordance with the Institut Pasteur animal care and use committee guidelines. LD₅₀ was determined as previously described [26].

3. Results

3.1. Description of the Isolates

Table 1 presents the isolates used in this study. We compared one isolate from a man with sickle cell anemia and *B. holmesii* bacteremia (Bho1, [23]) with another isolate from the respiratory tract of a woman with whooping cough syndrome (FR4020, [6]). These two isolates had PFGE profiles (data not shown) identical to those of the other isolates tested (**Table 1**) [6,20,23]. None of the toxins and adhesins involved in the pathogenicity of classical *Bordetella* species, such as pertussis toxin (PT), adenylate cyclase-hemolysin toxin (ACHly), *Bordetella* type 3 secretion system effector A (BteA), filamentous hemagglutinin (FHA), pertactin (PRN) and fimbrial proteins (Fim 2 and Fim 3), were detected in suspensions of

the isolates with specific antibodies raised against purified *B. pertussis* virulence factors.

3.2. Pyrosequencing and In-House Sequence Verification

We used pyrosequencing data and carried out in-house PCR sequence verifications, focusing on the small number of genes already identified as of potential interest for studies of the physiopathology of this species.

3.2.1. The *bvgAS* Regulatory System

We compared the sequences obtained for *bvgA* and *bvgS* between Bho1 and FR4020. They were found to display 99.9% and 99.8% nucleotide identity, respectively. We also compared these sequences with the sequences deposited in GenBank for the *B. holmesii* G7702 isolate (AJ748854.1) [3] and with the corresponding sequences obtained from additional isolates.

Differences in the *bvgS* sequence were found in positions 1973 (G//T), 2036 (A//G), 2191(G//T), 2252 (A//G), 4894 (G//A) and 4889 (T//G) (**Table 3**). All but one of these SNPs resulted in an amino-acid substitution, the remaining SNP, that in position 2252, being silent. Three SNPs (in positions 2252, 4894 and 4889) were found in all the isolates tested, regardless of the sequencing technique used. These SNPs were also found in CIP104395 (derived from the G7702 isolate used for the *bvgS* GenBank sequence determination), suggesting a possible error in the sequence present in GenBank at these positions. CIP104396 was the only isolate with a G in position 2036 and Bho1 and CIP101394 were the only isolates with a T rather than a G in positions 1973 and 2191. In conclusion, with the exception of CIP104396, Bho1 and CIP104394, the respiratory and bacteremia-related isolates tested had identical *bvgS* sequences.

Differences were also observed in *bvgA*, at positions 1085 (additional A), 1220 (G//A) and 1403 (A//G) (**Table 3**). All isolates except CIP104395 (derived from G7702) displayed a silent SNP (A//G) at position 1403. CIP104396 was the only isolate with an A in position 1220, whereas both the Bho1 and CIP104394 isolates had an additional A residue in position 1085, leading to premature termination of the activator *bvgA*.

3.2.2. The FHA-Related Protein

We observed a sequence with some similarities to that of *fhaB*, as reported by Link *et al.* [5], in the genomes of Bho1 and FR4020. This sequence displayed 99.98% nucleotide sequence identity between these two isolates. We also compared these sequences with the sequences for *B. holmesii* obtained with the G7702 isolate and deposited in GenBank (AM491633.1) [5] and with the corresponding sequences obtained from additional isolates. Differences were observed in positions 151 (C//T), 3094

Table 3. Sequence differences observed in *B. holmesii* isolates for *bvgS*, *bvgA*, *fhaB-bho* and *fimC*.

Gene	Isolate	Position
<i>bvgS</i>		1973 2036 2191 2252 4894 4889
	AJ48854.1	G A G A G T
	Bho1-CIP104394	T A T G A G
	CIP104396	G G G G A G
	(CIP104395=G7702)-CA001-CA002-CA003-CA004-FR3791-FR4020-FR4060-FR4101-FR4645-FR4859-FR4884-FR4912	G A G G A G
<i>bvgA</i>		1085 1220 1403
	AJ748854.1 and CIP104395=G7702	- G G
	Bho1-CIP104394	A G A
	CIP104396	- A A
	CA001-CA002-CA003-CA004-FR3791-FR4020-FR4060-FR4101-FR4645-FR4859-FR4884-FR4912	- G A
<i>fhaB-bho</i>		151 3094 3095 3098 4697 5589
	AM491633.1	C T A A C G
	Bho1-CIP104394-(CIP104395=G7702)-CIP104396	C A G G A G
	CA001-CA003-FR3791-FR4020	C A G G A A
	CA002-CA004-FR4060-FR4101-FR4645-FR4859-FR4884-FR4912	T A G G A A
<i>fimC</i>		1555
	Bho1-CIP104394-(CIP104395=G7702)- CIP104396- CA001-CA003-FR3791-FR4020	G
	CA002-CA004-FR4060-FR4101-FR4645-FR4859-FR4884-FR4912	A

(T//A), 3095 (A//G), 3098 (A//G), 4697 (C//A) and 5589 (G//A) (**Table 3**). Four of these SNPs (position 3094, 3095, 3098 and 4697) were found in all the isolates tested, including CIP104395 (derived from G7702), regardless of the sequencing technique used, suggesting a possible error in the GenBank sequence at these positions. Bho1, CIP104394, CIP104395 and CIP104396 displayed only these four differences. An additional silent mutation was found at position 5589 in all the other isolates and a SNP at position 151 was found only in the most recent isolates, whether obtained from blood or respiratory system samples. This SNP results in a Y₅₁ residue, rather than the L₅₁ present in the signal domain of the protein.

3.2.3. Other Putative Virulence Related CDS

CDS analogous to *fimABCD-fhaC* were detected, with a % GC of up to 62% (GenBank accession KC203605). The sequences for each CDS were identical in the two isolates tested, except for *fimC* which displayed one difference: Bho1 and FR4020 had a G in position 1555 of *fimC*, whereas the other isolates had an A. This difference results in a difference in the amino acid sequence of the corresponding protein, with an S residue replacing the original residue present at position 372 in *fimC*.

A CDS analogous to *ampG* involved in TCT [30] release has also been identified in both isolates.

3.2.4. Neighbor-Joining Tree

Concatenated sequences of the four sequences of in-

terest (located in *bvgA/bvgS/fhaB* and *fimC*) were aligned for the construction of a neighbor-joining tree (**Figure 1**). This tree showed that the *B. holmesii* isolates tested belonged to two main groups. The first consisted of FR4020, FR3791, CA001 and CA003 and three nodes corresponding to Bho1 and CIP104394 together, CIP104395 and CIP104396. The second group contained all the other isolates tested, corresponding to the most recent isolate, regardless of their blood or respiratory origin.

3.2.5. Iron Uptake Island, Specific *bho* CDS and the Alcaligin Operon

We confirmed the presence of the *B. holmesii*-specific *bhoABCDE* genes in all the isolates tested and found that the sequences of these genes were identical to those submitted to GenBank (DQ402419) [2]. The genes of the alcaligin operon, *alcABCDERS-fauA*, were conserved in both isolates, which displayed similar differences from those of *B. pertussis* (BP2456 to BP2463, see **Table 4**). PCR with previously described primers [2] targeting this region was carried out to check that the organization of this alcaligin operon was conserved.

3.2.6. Diagnosis Targets

Bho1 and FR4020 displayed no differences in *recA*, *bhoE* or *ompA* sequences.

3.2.7. Putative Acquired CDS

We identified two loci as potentially acquired from other

Table 4. Sequence differences observed with *B. pertussis* sequences (NC_002929) for the alcaligin operon of Bho1 and FR4020.

Gene	Ref. <i>B. pertussis</i> NC_002929	Position				<i>B. holmesii</i> isolates			
<i>alcA</i>	BP2456	732(T)	788(T)	1088(C)		Bho1-FR4020			
		C	C	T					
<i>alcB</i>	BP2457	45(G)	Bho1-FR4020						
		A							
<i>alcC</i>	BP2458	145(T)	583(T)	645(G)	858(C)	891(C)	1282(G)	Bho1-FR4020	
		C	C	A	T	T	A		
<i>alcD</i>	BP2459	222(T)	474(C)	571(G)		Bho1-FR4020			
		C	T	A					
<i>alcR</i>	BP2461	6(A)	295(A)	Bho1-FR4020					
		G	G						
<i>alcS</i>	BP2463	938(C)	972(G)	1204(G)		Bho1-FR4020			
		T	A	A					
<i>FauA</i>	BP2463	75(C)	1101(C)	1392(C)	1603(A)	1850(C)	Bho1-FR4020		
		T	T	A	G	T			

species (GenBank accession KC203606-KC203607): 1) the first had a mean % GC of 57% and contained genes displaying sequence similarity to genes encoding a benzene monooxygenase, *bmoA*, and a toluene O-xylene monooxygenase subunit from *Pseudomonas mendocina*, 2) the second had a mean % GC of 61% and contained genes displaying sequence similarity to hypothetical protein-encoding genes from *Azoarcus aromaticum*.

3.3. LD50

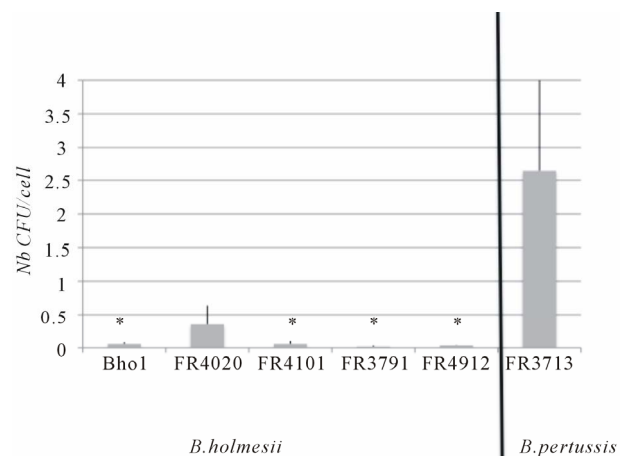
Whatever their origin, Bho1 and FR4020 were not lethal in a murine model of respiratory infection, even after inoculation with a dose of 2×10^8 CFU/mouse.

3.4. Interaction with Cells

Contrary to *B. pertussis*, both *B. holmesii* isolates tested were not cytotoxic for murine macrophages cell line J774.A1, correlating with the absence of AC-Hly activity and the animal model experiment. Both *B. holmesii* isolates were not cytotoxic for HTE cell lines, even after eight hours of contact. We also assessed their invasive properties and persistence within HTE cells. FR4020 was more invasive than the other *B. holmesii* isolates tested (**Figure 2**), but all these isolates were significantly less invasive than a recently collected *B. pertussis* isolate ($p < 0.05$). Moreover, they did not persist or multiply in epithelial cells over a period of 48 hours.

4. Discussion

We compared an isolate from a man with sickle cell anemia and *B. holmesii* bacteremia (Bho1, [23]) with another isolated from the respiratory tract of a woman with



Each isolate was added to an individual well of a 24-well tissue culture plate containing 5×10^5 cells/ml, to achieve a ratio of 100 bacteria per cell. The invasion of HTE cells was assayed as described by Bouchez 2009 [26]. The results shown are the means and standard deviations of at least three experiments. The symbol (*) indicates $p < 0.05$ in comparison with the *B. pertussis* strain FR3713.

Figure 2. Invasive properties of *B. holmesii* isolates.

whooping cough syndrome (FR4020, [6]).

These two isolates had PFGE profiles identical to each other and to those of isolates from the Institut Pasteur and National Reference Center collections [6,20,23], regardless of the origin or year of collection of these strains. None of the toxins and adhesins involved in the pathogenicity of classical *Bordetella* species, were detected in suspensions of the isolates with specific antibodies raised against purified *B. pertussis* virulence factors, confirming our previous findings [20,23]. Thus, these proteins are either not produced by *B. holmesii* isolates or the antibodies raised against the classical *Bordetella* virulence

factors do not cross-react with the corresponding proteins in *B. holmesii*. Transcriptomic analyses were not performed since most of genes encoding virulence factors are not harbored in the genome of the *B. holmesii* isolates.

The *bvgAS* two-component regulatory system controls the expression of most virulence factors in *Bordetella* species. Regulatory sensors and activators have already been described in *B. holmesii*, with the identification of proteins with sequences 59.9% identical to that of BvgS from *B. pertussis* and 72.2% identical to that of BvgA from *B. pertussis* [4]. Gerlach and coworkers [4] demonstrated that the cytoplasmic transduction domain of the BvgS histidine kinases of *B. pertussis* and *B. holmesii* were functionally interchangeable, whereas the extracellular sensor domains of these two proteins did not seem to respond to the same signals. We found that two of the isolates tested (Bho1 and CIP104394) had a truncated, and therefore nonfunction BvgA protein, because of the insertion of an A residue into *bvgA*, as previously described [3]. However, all the other *B. holmesii* isolates (including FR4020) had identical *bvgS* and *bvgA* sequences, whatever their origin (blood or respiratory), suggesting that regulation via the *bvg* two-component system should be similar in isolates from blood and respiratory samples.

Link and coworkers [5] described an FHA-related protein in *B. holmesii*. We confirmed the presence of such an *fhaB*-*bho* gene in Bho1 and FR4020. All the isolates collected after 2009 presented the same sequence, with a single non-silent SNP in the signal domain of the protein. Upstream from *fhaB*-*bho*, Link *et al.* [5] also demonstrated the presence of four heptanucleotide inverted-repeat sequences. This motif is very similar to the BvgA binding site in the *B. pertussis* *fhaB* promoter region, highlighting a possible role for *B. holmesii* BvgA in regulating the transcription of *fhaB*-*bho*. We found no genetic variation in this particular region for the *B. holmesii* isolates tested. Despite the presence of the gene, FhaB-*bho* was not detected by western blotting on suspensions of the isolates tested with specific antibodies raised against purified *B. pertussis* FHA. This is not surprising, because the amino-acid sequence of FhaB-*bho* is only 39% identical to that of *B. pertussis* FHA. Furthermore, the amino-acid sequence of FhaB-*bho* is 55% identical to that of a similar protein present in *B. avium* [5,31], and we were unable to detect this protein by probing *B. avium* extracts with the same antibodies raised against purified *B. pertussis* FHA.

A locus analogous to *fimABCD-fhaC* was detected in the genomes of both Bho1 and FR4020. Such a locus has also been documented in classical *Bordetella* and in *B. avium*, in which it has been shown to be functional, regulated by temperature and involved in adhesion to the host

respiratory tract tissues [32]. However, we detected no fimbrial proteins in the *B. holmesii* suspensions tested, with antibodies specific for the *B. pertussis* fimbrial proteins Fim 2 and Fim 3.

A CDS analogous to *ampG* was observed in both isolates. AmpG is an integral cytoplasmic membrane protein involved in peptidoglycan fragment recycling [33]. In *B. pertussis*, *ampG* gene has an insertion sequence IS481 that may affect its transcription, leading to the release of great quantities of Tracheal CytoToxin (TCT) a disaccharide-tetrapeptide monomer of peptidoglycan in culture supernatants [30,34]. No such insertion sequence is found around *ampG* sequence in *B. holmesii* suggesting that the level of TCT released should not be as high as for *B. pertussis* but rather lower as observed for other *Bordetella* species.

Diavatopoulos *et al.* [2] characterized a putative pathogenic island in the genome of *B. holmesii* isolates containing a functional iron-regulated alcaligin operon (known as the iron uptake island, or IUI) and other genes. This pathogenic island may have been acquired from *B. pertussis*, in which it is involved in the biosynthesis, export and uptake of the alcaligin siderophore in the absence of iron, leading to iron starvation. In microarray studies, Mooi *et al.* [11] reported the presence of this alcaligin operon in the genome of *B. holmesii* isolated from Dutch patients with pertussis-like illness. This operon (*alcABCDERS-fauA*) is conserved in the genome of Bho1 and FR4020, and its sequence displays only minor differences with respect to the sequences reported for *B. pertussis* BP2456 to BP2463. The IUI [2] also contains five genes (*bhoABCDE*) thought to be specific to *B. holmesii* species. We confirm their presence in Bho1 and FR4020. Furthermore, the sequences of these genes were found to be identical to the sequences submitted to GenBank (DQ402419) in both isolates, regardless of their origin (blood and a respiratory sample).

We identified two loci involved in aromatic compound metabolism. One of these loci contains genes encoding a benzene monooxygenase (*bmoA*) and a toluene O-xylene monooxygenase subunit similar to that of *Pseudomonas mendocina*, a Gram-negative environmental bacterium that can cause opportunistic nosocomial infections, such as infective endocarditis and spondylodiscitis [35]. The other locus contains genes displaying sequence identity to hypothetical protein-encoding genes from *Azoarcus aromaticum*, a proteobacterium capable of breaking down organic pollutants. The putative acquisition of proteins involved in aromatic compound metabolism has been reported for *B. petrii*, the only *Bordetella* species for which an environmental stage has been demonstrated [36,37].

Our genomic analysis did not distinguish between isolates responsible for bacteremia and those responsible

for respiratory disease. These findings were supported by the phylogenetic analysis based on partial sequences from *bvgA/bvgS/fhaB* and *fimC*. Isolates segregated according to the time at which they were collected rather than the type of source (blood or respiratory samples).

We already know that the genome of *B. holmesii* harbors IS481 sequences, but with fewer copies than are present in the genome of *B. pertussis*. This is why *B. holmesii* infection is diagnosed on the basis of a real-time PCR targeting a segment of the housekeeping gene *recA* (GenBank accession AF399664), which is polymorphic between *B. holmesii*, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. hinzii*, and *B. avium* [7-9]. This sequence was found to be fully conserved in both isolates, Bho1 and FR4020. Mooi *et al.* [11] recently used *bhoE* to confirm the identification of *B. holmesii* isolates based on 16S rRNA sequencing. Bho1 and FR4020 present a *bhoE* sequence identical to that in GenBank (DQ402419). *OmpA*, encoding a porin-like protein, is sometimes preferred for identification purposes, because it displays a higher degree of mutational variation than 16S rRNA [12]. The sequences of *ompA* in Bho1 and FR4020 were 100% identical to that in GenBank for other *B. holmesii* isolates (Accession no.: AM748266, AM748567 and JQ650246). Due to the high degree of similarity between the targeted sequences and the low level of divergence between *B. holmesii* isolates, current diagnostic tools cannot differentiate between isolates recovered from blood and those recovered from respiratory samples.

We found that *B. holmesii* isolates were not cytotoxic to the cells tested and that none of these isolates was lethal in a murine model of infection. These results are not surprising because no orthologs of either AC-Hly, which is responsible for macrophage cell death through apoptosis [28], or BteA, which is responsible for epithelial cell death through necrosis [38], have been found. No other cytotoxic agent seems to be produced by *B. holmesii*. Furthermore, although FR4020 was slightly more invasive than the other isolates of *B. holmesii* tested, none of these isolates was particularly invasive. We observed no difference in invasive properties for the Bho1 isolate, which has a nonfunctional BvgA protein, suggesting that Bvg system is unlikely to control the proteins involved in cell invasion processes.

In classical *Bordetella* species, adhesins and toxins are key factors in the interactions between bacteria and cells [39]. No ortholog of pertactin has been identified in *B. holmesii*, but the *fhaB*-*bho* gene of this species has some similarities with that of the *B. pertussis* *fhaB* gene encoding the adhesin FHA. FHA promotes the invasion of human respiratory epithelial cells through the interaction of its RGD motif with the host cell 5-1 integrin [40]. *B. holmesii* produces an FHA-related protein, but this protein more closely resembles that of *B. avium* and contains

a KGD motif rather than an RGD motif [5]. The role of this KGD motif in interactions with cells has yet to be established. Finally, a fimbrial locus has been reported for *B. holmesii* isolates, as for *B. avium*, in which it encodes fimbrial proteins involved in adhesion to the host respiratory epithelium [5]. The slightly greater invasive capacity of FR4020 cannot be linked to sequence differences in either *fhaB*-*bho* or *fimC*, because FR3791, an isolate harboring the same sequences, is as poorly invasive as the other *B. holmesii* isolates tested. Other proteins may thus be involved. None of the isolates tested persisted or multiplied in HTE cells over a period of 48 hours, regardless of their origin.

5. Conclusions

We were unable to identify any particular traits specific to *B. holmesii* isolates collected from blood or to isolates causing pertussis-like symptoms. We nevertheless report that Bho1 is unusual in having an incomplete BvgA protein. The more recent isolates tested (*i.e.* collected after 2009) had identical *bvgS*, *bvgA* and *fhaB* sequences, regardless of their origin. We identified a fimbrial locus and genes involved in aromatic compound metabolism in both Bho1 and FR4020. Our genomic comparisons were restricted to particular genes, due to the absence of a reference genome for *B. holmesii*. A whole-genome annotation for a recently collected *B. holmesii* isolate would facilitate future genomic studies. Using *in vitro* cellular and *in vivo* animal models, we were unable to differentiate between isolates from blood and isolates from respiratory samples: none of these isolates was cytotoxic to J774.A1 murine macrophage cells or HTE cells. In addition, all of the isolates tested were only weakly invasive and none multiplied or persisted in HTE cells over a 48 hour period. Many questions about *B. holmesii* remain unanswered:

- Is *B. holmesii* a human respiratory pathogen or an opportunistic bacterium carried mostly by adolescents and adults (and, in rare cases, by newborns) but only detected during an episode of coughing due to another *Bordetella* species (as suggested by Rodgers *et al.*, [10]) or another microorganism, such as a virus? Systematic searches for other respiratory pathogens must be undertaken. For example, in most studies, *B. holmesii* was isolated from respiratory samples from patients with pertussis-like symptoms, but tests for *B. pertussis* or *B. parapertussis* infections or viral infections were not systematically carried out.
- Can *B. holmesii* be carried asymptotically? It is recovered from the blood of immunocompromised patients, such as asplenic patients and patients on chemotherapy [19]. It is probably important to obtain a nasopharyngeal swab from all patients with *B. holmesii* bacteremia.

- Is *B. holmesii* adapting to humans? Multilocus sequence typing analysis revealed that *B. holmesii* is not a classical *Bordetella* species, instead lying at a similar distance from this group to *B. avium* [2]. Weyant *et al.* [1], in their first description of this species, reported that *B. holmesii* isolates had a cellular fatty-acid profile closer to that of *B. avium* than to that of classical *Bordetella* species. Our genomic comparisons, based on only a few genes (as *bvg fhaB*, *fim*), also tended to suggest that the *B. holmesii* genome was closer to that of *B. avium*, which is thought to be a strictly avian pathogen. Nevertheless, human cases of respiratory disease associated with *B. avium* have already been reported in patients with cystic fibrosis [41], and *B. avium* isolates have also been collected from patients with pneumonia, thereby demonstrating that *B. avium* can also act as an opportunistic pathogen in humans, despite having an avian reservoir [42]. However, *B. holmesii* has acquired some genes from *B. pertussis*. It is thus possible that these two species coexist and exchange genetic material. Yih [17] and Rodgers [10] reported the cocirculation of *B. pertussis* and *B. holmesii* during outbreaks of pertussis. Is there a link between *B. avium* and *B. holmesii*? Does *B. holmesii* have an extrahuman reservoir that has yet to be identified? The sequencing and annotation of a complete *B. holmesii* genome are required, together with detailed genomic comparisons, to address these questions.
- Can *B. holmesii* be transmitted between humans? During an outbreak in Japan, Kamiya [13] observed epidemiological links between patients, suggesting that *B. holmesii* might have been transmitted from person to person. However, as previously stated, no other diagnostic tests were undertaken to check that *B. holmesii* was really the source of the whooping cough symptoms. This highlights the need to collect as much information about patients as possible during studies. It also highlights the possible transmission and carriage of *B. holmesii* between humans.
- Do vaccines against pertussis protect against *B. holmesii*? Zhang *et al.* [9] have already reported that neither whole-cell (wP) nor acellular (aP) *B. pertussis* vaccines confer protection against *B. holmesii* isolates in mice. Rodgers *et al.* [10] showed that aP booster vaccination was more effective against *B. pertussis* than against *B. holmesii*.

Clearly, further characterization of this bacterium is required. Much remains to be done and attentive monitoring should continue.

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