CHARACTERIZATION AND EVOLUTION OF INTRACELLULAR SURVIVAL IN BORDETELLA

by

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(Under the Direction of Eric T. Harvill)

ABSTRACT

The classical bordetellae possess several partially characterized virulence mechanisms that are studied in the context of a complete extracellular life cycle in their mammalian host. Yet, classical bordetellae have repeatedly been reported within dendritic cells and alveolar macrophages in clinical samples, and *in vitro* experiments convincingly demonstrate that the bacteria can survive intracellularly within mammalian phagocytic cells, an ability that appears to have descended from ancestral progenitor species that lived in the environment and acquired the mechanisms to resist unicellular phagocytic predator. Many pathogens, including *Mycobacterium tuberculosis*, *Salmonella enterica*, *Francisella tularensis*, and *Legionella pneumophila* are known to parasitize and multiply inside eukaryotic host cells. This strategy provides protection, nutrients, and the ability to disseminate systemically. While some work has been dedicated at characterizing *B. pertussis* intracellular survival phenotype, there is very little understanding of how this strategy has evolved within the genus *Bordetella* and contributes to bacterial pathogenicity, evasion of host immunity and systemic dissemination. Here, we explore the mechanisms that control the changes accompanying intracellular survival and how these have been

acquired and conserved throughout the divergent evolutionary histories of *Bordetella* species.

INDEX WORDS: *Bordetella*, evolution, intracellular survival, macrophages, transcriptomics, stress response and adaptation.

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B.S, University of Puerto Rico, 2011

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2020

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August 2020

DEDICATION

This work is dedicated to my kids Xionelys and Gabriel. You are my inspiration. You have taught me the beauty in simplicity, the fun in the unexpected and to embrace the unknown with hope and excitement. Your innocent wisdom has been my muse, your curiosity, my guidance, and the simple though of you, my solace in times of tribulation. Let curiosity guide your way, always be resolved in your ideals, and let compassion accompany you during this wonderful journey.

ACKNOWLEDGEMENTS

This work wouldn’t have been possible without the influence, help and guidance of great scientists and my dear friends, Bodo Linz, Kalyan Dewan, and Hamidou Illiassou. Their lessons have shaped my academic and personal development. I want to also thank Dr. Eric T. Harvill for the opportunity to join and be part of an amazing team. As a kid, I idealized the character of will, determination and pragmatism of scientists. Today, I can assert that your standards go well beyond the qualities I could have ever foreseen.

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CHAPTER 1

INTRODUCTION

The genus bordetellae are gram negative cocobacili of the class betaproteobacteria known to cause disease in a wide range of animals including small mammals and humans. The classical *Bordetella* possess several partially characterized virulence mechanisms that are studied in the context of a complete extracellular life cycle in their mammalian hosts. Yet, classical bordetellae have repeatedly been reported within dendritic cells and alveolar macrophages in clinical samples, and *in vitro* experiments convincingly demonstrate that the bacteria can survive intracellularly within mammalian phagocytic cells. Moreover, *B. bronchiseptica (Bb)*, the species closest to the ancestral lineage of the classical bordetellae, is known to have an alternate life cycle, persisting, replicating and disseminating with amoeba. These observations suggest ability to persist inside phagocytes may have descended from ancestral progenitor species that lived in the environment and acquired the mechanisms to resist unicellular phagocytic predators.

Many pathogens, including *Mycobacterium tuberculosis*, *Salmonella enterica*, *Francisella tularensis*, and *Legionella pneumophila* are known to parasitize and multiply inside eukaryotic host cells. This strategy provides protection, nutrients, and the ability to disseminate systemically. While some work has been dedicated at characterizing *B. pertussis* intracellular survival phenotype, there is very little understanding of how this strategy has evolved within the genus *Bordetella* and the contributions of this ability in bacterial pathogenicity, evasion of host immunity and systemic dissemination. Here,

using *B. bronchiseptica*, we aim to explore the transcriptional changes accompanying intracellular survival and how these have been acquired and conserved throughout the divergent evolutionary history of bordetellae. To study *Bordetella* intracellular survival, we propose the following aims:

Specific Aim 1. Evaluate *B. bronchiseptica* ability to persist inside macrophages. We had earlier observed using antibiotic protection assays, that *B. bronchiseptica* strain RB50 (*Bb*) can enter and survive within murine macrophage-like cell line RAW 264.7 *in vitro*. To determine the number and proportion of bacteria entering these macrophages, we will perform an assay of macrophages infected with *Bb* at multiplicities of infection (MOI) of 100, 10 and 1 for 1h and assess the ability to persist inside macrophages after 2, 4, 8, 24 and 48 hours post infection.

Specific Aim 2. Identify *B. bronchiseptica* transcriptional changes during growth inside murine-derived macrophages. We hypothesized that to survive within professional phagocytic cells, *Bb* would require distinct groups of genes to be transcriptionally modulated once the bacteria reached the intracellular niche. To examine this transcriptional response, we will analyze the mRNA profile of intracellular *Bb* at 2 h post inoculation and compared it to that of bacteria grown in vitro.

Specific Aim 3. Compare the genes involved in intracellular survival within the *Bordetella* genus. *In vitro* experiments convincingly demonstrated that the classical bordetellae can survive intracellularly within mammalian phagocytic cells, an ability that appears to have descended from ancestral progenitor species that lived in the environment and acquired the ability to resist phagocytic killing by amoebae that are ubiquitous

environmental predators. While many non-classical bordetellae are also human and animal pathogens, their ability to persist inside phagocytic cells has not been evaluated. Therefore, using the genes identified in Aim 2, we will evaluate the conservation of genes upregulated during intracellular survival among the non-classical *Bordetella* species.

<u>Specific Aim 4.</u> Assess the survival and persistence of knockout-mutants inside macrophages. We predict that genes highly expressed by intracellular *B. bronchiseptica* play a key role in bacterial persistence. To test this hypothesis, we will generate knockout mutants of genes identified in Aim 1 using *Bb* RB50 and assess bacterial fitness inside macrophages and the impact of these genes during early and long-term survival inside RAW 264.7 macrophages.

The proposed research represents an important step towards understanding the impact of intracellular survival and its contribution to bacterial persistence inside host phagocytes. The planned experiments will provide valuable insight on the bacterial factors required for survival inside macrophages and help elucidate the evolution of this strategy throughout the *Bordetella* genus. Understanding these principles could promote and guide the development of novel vaccines and strategies to interfere with the spread of human and animal pathogenic *Bordetella*.

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CHAPTER 2

LITERATURE REVIEW

Genus *Bordetella*

The genus *Bordetellae* comprise gram negative cocobacili of the class betaproteobacteria known to cause disease in a wide range of animals including small mammals and humans (1-7). Despite widespread vaccination, *Bordetella pertussis* –the etiological agent of whooping cough or Pertussis, affects 24.1 million with 160,700 deaths in children younger than 5 years (Yeung KHT, et.al, 2017; (8-33). The symptomatic stage of Pertussis is characterized by severe paroxysmal fits of coughing often followed by an inspirational "whooping" sound and can be accompanied with post-tussive vomiting owing to the severity of the coughing fits. Left untreated, the disease can last for months and cause severe complications and mortality in infant and individuals with underlying health conditions (20, 29, 34-41).

Along with *Bordetella pertussis*, two other very closely related species, *Bordetella parapertussis* and *Bordetella bronchiseptica* , constitute a three-member group known as the "*Classical bordetella*", sharing 99% sequence similarity among themselves (42-66). *B. parapertussis* is known to infect humans causing "Pertussis"-like pathology while another strain of the species has also been isolated from sheep (44, 67-72). The third species, *B. bronchiseptica* (*Bb*) is considered the most closely related to ancestral lineage of the "classical *Bordetella*". In contrast to the other members of the group, *B. bronchiseptica* has been isolated from the respiratory tract of a wide range of

mammals including cats, dogs, rabbits, mice, rats, sheep, pig, and cattle. In addition, *B. bronchiseptica* has also been identified in human infections, but these events are rare and considered opportunistic involving immunocompromised individuals. Despite differences among the classical *Bordetella* (e.g.: host specificity, genome size, etc.), they are considered sub-species and share striking similarities in pathogenicity, including the ability to infect and cause disease pathology of the lungs and long term persistence in the upper respiratory track.

In addition to the closely related classical *Bordetella,* several other species are being identified; collectively referred to as "non-classical" and representing a broader genetic diversity (73) (Fig 2.1). Many of these species have been isolated directly from the environment and have no known animal hosts such as *Bordetella petrii* originally isolated from an anaerobic bioreactor culture enriched from river sediment (74) and subsequently isolated from many soil samples (75, 76). However, several of the "non-classical species have been known to cause respiratory infections. These include species such as *Bordetella avium* and *Bordetella hinzii* found infecting poultry and wild birds (77-79); *B. pseudohinzii* identified as a pathobiont in several mouse breeding colonies (80, 81) and recently shown to cause chronic ear infection in mice (82); *B. trematum* an opportunistic human pathogen that can cause severe skin disease and chronic otitis media (83); Although these pathogenic non-classical *Bordetella* species display a broad genetic diversity (including acquisition and loss of multiple virulence-associated genes), these species still share many recognizable characteristics that makes them successful animal pathogens (73, 84).

Environmental origin of *Bordetella*

Bordetella species include species that are host-restricted pathogens (*B. pertussis, B. parapertussis , B. avium, B. pseudohinzii*) and others that have broader and variable host specificity (*B. bronchiseptica*) recent work has revealed that the pathogenic species of the bordetellae likely arose from an environmental ancestor. In 2011, *Bordetella petrii* –the first environmental bordetellae discovered, was isolated from a mixed anaerobic, dechlorinating culture enriched with sediment (85-88), while in 2015, another set of environmental species, named *B. muralis*, *B. tumbae* and *B. tumiloca* were isolated from a 1300-year-old mural painting inside the stone chamber of Takamatsuzuka Tomb in Japan (89). Alongside these recent findings, in 2017, Hamidou *et.al.*, reported the identification of environmental *Bordetella* species by analyzing 16S ribosomal DNA sequences collected from the NCBI database (75). In this work, sequences originating from an environmental setting (soil and water) displayed a significantly higher genetic diversity in comparison to animal-adapted species. In addition, phylogenetic assessment revealed that environmental samples were located closer to the root, indicating that these environmental species postdate adaptation into animal hosts (Fig. 2.2). Upon examination, the authors demonstrated that *Bordetella bronchiseptica* and *Bordetella hinzii* have retained the ability to grow and proliferate in soil extracts. Another piece of evidence that further supports the "environmental origin" hypothesis can be found in a study published in 2016 by Dawn *et.al*. In this work, the authors demonstrated that *B. bronchiseptica* can infect, persist and disseminated using the lifecycle of the environmental amoeba *Dictiostelium discoideum* as a vector (Fig. 2.3) (90). Taken together, these observations suggest that soil and/or water, as well as ancient interactions

with amoeba-like protists may have constituted an environmental niche and potentially provided the training grounds for the later adaptation into higher animals.

Adaptation to animal hosts

Ability to colonize, replicate and disseminate can provide critical advantages in competitive soil/water environments. Many bacteria are known to form an endosymbiotic relationship with environmental reservoirs such as amoeba, an interaction believed to provide protection against external danger, while enhancing bacterial dissemination along with the host. *B. bronchiseptica* can persist inside amoeba trophozoites and utilize its complex lifecycle to disseminate into new environments. Importantly, *B. bronchiseptica* is highly versatile, and while the bacteria can colonize and persist in a wide range of animal hosts including swine, rats, rabbits, sheep, cattle, dogs and cats, it still retains the ability to establish a successful symbiotic relationship with amoeba. Considering the similarities between amoeba trophozoites and mammalian phagocytes, the ability of *B. bronchiseptica* to maintain a stable association with amoeba could certainly have contributed to its adaptation to infect its animal hosts.

However, despite *B. bronchiseptica* ability to infect a wide range of mammals, the rest of the "classical" *Bordetella* species have specialized at causing disease and spreading within a single mammalian host (Fig. 2.4)(82). The robustly studied *B. pertussis* successfully colonizes and persist in the respiratory tract of humans for extended periods. *B. pertussis* speciation in humans has resulted in the loss of many genes, rendering it unable to efficiently infect other mammals (e.g.: mice). Substantial genomic rearrangements and gene loss in the species (75, 91) have been identified, with about 20% reduction in its genome size compared with *B. bronchiseptica*, most of which

are the result of allelic exchange of insertion element IS481 and loss of virulence factors including Pilus D, and LPS surface molecule O-antigen.

Bordetella parapertussis ovine strains Bpp5, have successfully adapted to infect the respiratory tract of sheep but do not colonize mice. *Bordetella parapertussis* ovine have experienced gene loss and accumulation of mutations in genes involved in O-antigen production (44, 67-69, 92). Interestingly, human-restricted *B. parapertussis* 12822 still possess the genes encoding for the O-antigen, which have been shown to promote protection against complement activation. This observation suggests that despite providing protection, loss of O-antigen might confer a fitness advantage during infection (93-98).

Several of the animal pathogenic non-classical *Bordetella* species have also evolved to infect a specific host. *Bordetella holmesii* –originally isolated from human patients displaying pertussis like symptoms, mainly affects adolescents from the ages 11-18 years, an observation that contrast with historical figures for *B. pertussis* which commonly affects age groups ranging from 0-10 years of age (99-105). Reports have shown an incidence rate of 5.8% during low and high Pertussis prevalence periods, which suggests that the bacteria has adapted to co-circulate alongside *B. pertussis* (106, 107).

Host specific adaptation have also been described in many non-classical *Bordetella* species. For example, *Bordetella avium* and *Bordetella hinzii* have been isolated from poultry and turkey chicks, while *Bordetella pseudohinzii* is known to infect the respiratory tract and ear canal of laboratory bred mice (81, 108-112). Despite several examples of adaptation to a specific host, several species have also been documented to cause infection in patient with underlying health conditions. These includes *B. hinzii*, *B*

trematum, and *B. ansorpii* which have been identified in wound, and ear infections in immunocompromised individuals (113-119).

Host specialization has led to significant genomic changes including reduction and rearrangements (Fig 2.5). Comparative genome analysis shows substantial gene loss for highly specialized *Bordetella* species. For example, human-restricted *Bordetella pertussis* is among the species with the smallest genome size. This pattern is also observed for *B. avium*, *B. pseudohinzii*, and *B. holmesii*. In contrast, environmental species such as *B. petrii* or species that are still able to persist in the environmental such as *B. bronchiseptica*, have the largest genome size among all the *Bordetella* species. Possibly, the presence of many ancestral genes allows for environmental persistence by providing the genomic plasticity necessary to tackle multiple and diverse environmental threats. This suggests that highly specialized bacteria have undergone significant and specific changes while adapting to a more stable environment, rendering unnecessary the maintenance of genes that do not provide any significant advantage under this highly specialized scenario (73).

Virulence of *Bordetella*

Bordetella species, which collectively thrive over a broad range of environments have evolved mechanisms to modulate gene expression to cope with the demands of varied environmental conditions, both within and outside of a host. Among the many genetic tools the bvgA/S regulon stands out, and has been robustly described in bordetellae literature (120-131). The two-component system BvgAS, is a master regulator that controls the activation of virulence in *Bordetella*. The system consists of a sensor protein, BvgS, a transcriptional activator, BvgA, and a transcriptional repressor, BvgR

(Fig 2.6). In response to changes in temperature and/or the presence of chemical components such as nicotinic acids or magnesium sulfate, *Bordetella* can switch between a virulence-activated and a virulence-repressed state, a phenomenon known as "phenotypic modulation". In its active state or Bvg+ phase, BvgS phosphorylates BvgA which in turn binds to the promoter regions of the Bvg-activated genes, and initiates transcription.

Nearly all virulence factors in human restricted *B. pertussis* are expressed upon phosphorylation of BvgA and subsequent activation of the system. A study in 2017, revealed that the BvgAS regulon induces the expression of more than 550 genes (121). Furthermore, a study using *B. pertussis* mutants demonstrated that modulation of Bvg+ is necessary and sufficient for establishing respiratory infections in mice (132), and that switching to a Bvg- phase (virulence-repressed) aversively impacts this. Similar observations have been reported for *B. bronchiseptica* using a swine model (133). Here, using an isogenic mutant of a virulent *B. bronchiseptica* strain, the authors compared the mutant's ability to colonize and cause disease, and demonstrated that activation of the Bvg + phase is required for bacterial colonization and transmission in swine.

The conditions within the mammalian host (such as: 37ºC) induce the activation of Bvg+ phase which results in transcription of virulence genes some of which includes the filamentous hemagglutinin (*fhaB* and *fhaC*), adenylate cyclase toxin (*cyaABCDE*), pertussis toxin (*ptxABDEC*), type 4 secretion system proteins (*ptlABCDIEFGH*), fimbriae (*fimABCD*) and pertactin (*prn*). These genes encode for proteins known to promote bacterial colonization and persistence. For example, Pertactin a 69 kDa autotransporter, mediates adherence of *B. pertussis* to mammalian cells and have been shown to promote

resistance against neutrophils in *B. bronchiseptica* (134-138). Filamentous hemagglutinin, a major attachment molecule, binds to human neutrophil receptors and inhibits Interleukin 12 (IL-12) secretion while enhancing the generation of Regulatory T cells (T-reg) that in turn suppresses the T helper type 1 (Th1) response. Another important virulence factor, Adenylate Cyclase Toxin (ACT) binds to macrophages and neutrophils and leads to a disruption of proper cellular signaling by uncontrolled conversion of ATP to cAMP (139, 140). Secretion of ACT has also been shown to inhibit antigen presentation, IL-12 production, and the generation of a Th1 response, resulting in suboptimal immunity (141-143). Other proteins strongly secreted upon activation includes, the Type Three and Type Six secretion Systems (T3SS and T6SS), adhesion proteins Fimbria 2 and 3 (Fim2 and Fim3), and Pertussis Toxin (PTX). These proteins constitute only a small set of virulence factors known to promote pathogenesis in *Bordetella*.

Conservation of the virulence-repressed Bvg$^-$ phase in *Bordetella*

Under laboratory conditions, classical *Bordetella* species such as *B. bronchiseptica*, can respond to different environmental stimuli by switching between two distinct virulence states (120, 123). When cultured at 37°C, which mimics mammalian host temperatures, expression of genes associated with virulence and colonization in mammalian hosts is upregulated. However, *B. bronchiseptica* adopts a second when cultured at 25°C or lower, during which expression of virulence-associated genes is down-regulated, while expression of a large, alternative set of genes –suspected to enhance survival in environmental settings, is up-regulated (73). For example, *B. petrii*, *B. bronchiseptica*, and *B. hinzii*, have been shown to grow efficiently in soil at

25°C (75). In addition, during intracellular survival in environmental amoeba *Dictyostelium discoideum* (incubated at 25°C), *B. bronchiseptica* Bvg- phase lock mutants displayed increase persistence in comparison to wildtype and Bvg+ phase lock mutant strains (90).

Despite substantial research on the *bvg*A/S regulon, the biological function of the Bvg- phase (virulence-repressed phase) is still not clearly understood. The lack of a clear function for the Bvg- phase have put into question its role during infection and has led to speculation that activation of the virulence-repressed genes might contribute to *Bordetella* environmental persistence. Interestingly, despite possessing the ability to phenotypically modulate between the two Bvg states, no evidence of an environmental niche outside humans has been found for the human-restricted *Bordetella pertussis*. This has led to the hypothesis that the Bvg- minus phase is likely the vestigial remnants of an important phenotype in ancient *Bordetella*. However, contrary to expectations of this hypothesis, homologs for the genes *bvgA* and *bvgS* have been identified in the genomes of animal associated as well as environmental *Bordetella* species (Table 2.1) (84, 144, 145). Moreover, the ability to switch between lifestyles seem to be conserved throughout the genus bordetellae. These observations mark a sharp contrast to the "vestigial" hypothesis, and so, it's likely that the virulence-repressed state have been under evolutionary selection and indeed still plays an active role in the biology of *Bordetella*.

Evidence of the potential role for Bvg- phase can be found in Karataev *et.al.* where the authors found an accumulation of Bvg- mutants among *B. pertussis* population in the nasopharynx of monkeys, suggesting an active role in facilitating bacterial persistence (146). In addition, in 2017, Moon et.al., identified the expression of genes

involved in metabolism including, fatty acid and lipid metabolism, sugar and amino acid transporter, pyruvate dehydrogenase, phenylacetic acid and the glycolate/glyoxilate pathway active during the Bvg- minus state (121). These findings led the authors to suspect that metabolic changes in response to rapid temperature drops during transmission and thus switch from Bvg+ to Bvg- phase might be an important phenotype during transmission of *B. pertussis*. Taken together, these observations strongly suggest that the Bvg- phase constitute a conserved strategy that may have contributed to the survival of ancient *Bordetella*, and even though many *Bordetella* species have evolved to infect mammalian hosts, the ability to respond and adapt to environmental changes remains active.

Intracellular survival in *Bordetella*

The classical bordetellae possess several partially characterized virulence mechanisms that are studied in the context of a complete extracellular life cycle in their mammalian hosts (147-162). However, despite not being commonly considered intracellular pathogens, *Bordetella pertussis* has repeatedly been observed in dendritic cells and alveolar macrophages of clinical samples (163-165). Furthermore, *in vitro* experiments have convincingly demonstrated that the classical *Bordetella* can survive intracellularly within mammalian phagocytic cells (163, 166-183); an ability that appears to have descended from an ancestral progenitor species that lived in the environment and which had acquired the mechanisms to resist unicellular phagocytic predators (90). It is broadly considered that this strategy provides protection, nutrients, and the ability to disseminate with the host. Experimental evidence shows that *B. pertussis* enter human alveolar epithelium cell line A549 and can evade phagolysosomal fusion, remaining

viable in nonacidic compartments. Once inside, the number of viable bacteria increase for at least 24 hours (Fig. 2.7) using a mechanism dependent on microtubule assembly, lipid raft integrity, and the activation of a tyrosine-kinase-mediated signaling (184). Similarly, other studies have shown *B. pertussis* enter within mononuclear cells isolated from venous blood treated with EDTA and persisting for over 3 days in the absence of serum or specific antibodies.

B. pertussis modulate human macrophages by secreting a wide range of proteins upon entry and reside within host cells. Functional protein analysis at 3 and 48 hours revealed an enrichment in the abundance of proteins involved in stress response, iron uptake, metabolism, regulation and virulence (185). Among these, secretion of metabolism related proteins including, glutamate synthase, succinate dehydrogenase, dihydrolipoamide acetyltransferase and ATP synthase, suggests that *B. pertussis* re-directs its carbon flux from glutamate and then channels it into the TCA cycle. Activation of genes involved in virulence and in iron metabolism were also observed for intracellular *B. pertussis*. For example, elevated levels of BrkA and iron uptake protein BfrD and BfrE were identified in intracellular bacteria soon after infection (186-192). In addition, proteins associated with bacterial stress response including chaperones GroES, ClpB and DnaK were identified (193). Interestingly, secretion of these proteins, known to promote the establishment of intracellular infections, have also been identified in other species including *Escherichia coli*, *Staphylococcus aureus*, and *Piscirikettsia salmonis*. Taken together, these observations serve as evidence of *B. pertussis* possess the facultative ability to adapt and persist inside host cells.

Ability to reside inside macrophages is not unique to *B. pertussis*. Recovery from macrophages have been documented for all classical *Bordetella*, including *B. parapertussis* and *B. bronchiseptica* (168, 170). In 1994, Guzman *et.al.*, showed that *Bordetella bronchiseptica*, can attached to mouse dendritic cells and persist intracellularly for at least 72 hours. Spontaneous mutants lacking the *bvgS* gene –sensor component of the BvgAS regulon, bound less efficiently despite long term persistence, suggesting that uptake and intracellular survival is Bvg independent or involves genes induced by the virulence-repressed phase (178, 194). Although it remains unclear whether the Bvg- phase plays a significant role during infection, previous work reported the suppression of the Bvg+ phase during infection of animal-phagocytes. Research on *B. bronchiseptica* demonstrated that expression of Bvg- phase genes promotes bacterial grow and persistence in amoeba trophozoites and mouse-derived macrophages (90, 170). In addition, work by Jungnitz *et.al.*, identified a two-component regulatory system named RisAS, required for bacterial persistence inside spleen dendritic cell line CB1 and macrophage-like cell line J774A.1 (195). Phenotypic exploration of RisA-deficient mutants revealed that the regulon promotes resistance against oxidative stress, production of acid phosphatase and *in vivo* persistence. Notably, activation of RisA is regulated independently of the Bvg regulon and is optimally expressed at 37ºC in the absence of Mg+2, and when the bacteria are within eukaryotic phagocytes. The recently identify role of RisA, alongside the expression of genes active during the Bvg- phase, denotes the complexity of the regulatory network required for adaptation to the host intracellular environment. These observations are highly suggestive of how extant *Bordetella*e have employed an ancestral environmental trait acquired during its interaction with ancient

unicellular predators; a period during its evolution that served as “training” to become animal pathogens (73, 90).

Host immunity against *Bordetella*

Host response to *Bordetella* infections have been largely studied using the murine model, and other mammals (ferrets, cats, dogs, pigs and baboons). Upon infection *Bordetella pertussis* secrets a wide array of virulence factors that among other functions, mediates adherence to the host epithelia and phagocytic cells such as macrophages and neutrophils, and promotes bacterial persistence in the host respiratory tract. Studies in murine models have shown that innate immune cells, and antimicrobial peptides help control the infection, while complete bacterial clearance requires activation of cellular immunity, largely mediated by T-helper type 1 (Th1) and Th17 cells. During the early stages of infection, local and innate immune cells, including macrophages, dendritic cells (DC), neutrophils, and natural killer (NK) cells, are recruited and largely accountable for the control and reduction of *B. pertussis* (Fig 2.8) (196). Among the effectors that promote bacterial clearance, the secretion of interferon-gamma (INF-γ) by NK cells, dendritic cell (DC) and T-helper type 1 (Th1) cells, play a crucial role in enhancing macrophage-mediated killing. Mice lacking INF-γ develop lethal infections after challenge with *B. pertussis*. In addition to INF-γ, secretion of interleukin 12 (IL-12) by mature dendritic cells promotes the recruitment of Th1 cells and enhances cell-mediated immunity. Early infection with *B. pertussis* also promotes the secretion of antimicrobial peptides such as ß-defensins, and activation of the complement component. However, the efficacy of these defense mechanisms remains uncertain as previous research have resulted in mixed results(196). Work on *Bordetella bronchiseptica* has shown that

bacterial O-antigen provides resistance against serum complement activation, however, despite not possessing O-antigen, *B. pertussis* also seem unaffected by the complement component (95, 96, 98, 197).

Activation of the adaptive immune response plays a crucial role in clearance of *B. pertussis* infection. Significant research has shown that activation and recruitment of a Th1 and Th17 adaptive immune response mediates clearance from the lower and upper respiratory tract of mice. Modulation of a cellular Th1 immune response primarily results in the secretion of IL-12 and INF-γ which in turn promotes neutrophil recruitment and enhances macrophage phagocytic activity. It is largely recognized that infection with *Bordetella pertussis* naturally primes Th1 cells, and this response constitutes the optimal immunity to combat primary and secondary infections.

Analogous to naturally occurring *B. pertussis* infection, immunization with whole cell pertussis vaccine (wP)—based on killed *B. pertussis* bacteria, also promotes the secretion of Th1 derived cytokines, and enhances macrophage phagocytic activity. The rapid decline in the number of *Pertussis* cases following it introduction provides compelling evidence of its efficacy (Figure 2.9). However, although wP vaccines were highly effective at clearing the infection and providing long-term protection, several cases of vaccine reactogenicity were reported, which led to it widespread replacement in favor of the acellular Pertussis (aP) vaccine. In contrast, vaccination with acellular pertussis (aP) vaccine –composed of up to 5 proteins, induces a Th2-mediated humoral immunity and mainly promotes the secretion of Immunoglobulins IgG and IgA. In contrast to wP, aP has been shown that, while effective in clearing Pertussis infection from the lungs and protecting from disease pathology, vaccination with aP does not

provide protection against bacterial colonization of the upper respiratory tract of mice and fails to prevent transmission in non-human primates (198).

Suboptimal Th2 immunity against intracellular bacteria contributes to *Bordetella* persistence

Despite wide vaccination coverage, the incidence of pertussis cases is increasing, which prompted the National Institute of Allergy and Infectious Diseases (NIAID) to add *B. pertussis* to the list of priority Emerging Infectious Diseases/Pathogens in 2015. Following the widespread introduction of aP vaccines, the number of Pertussis have been on the rise for the past two decades. Suboptimal immunity, including inability to prevent bacterial colonization and transmission have been proposed as a some of the reasons for the resurgence (199, 200).

Protective immunity generated by wP vaccine mediates a Th1 adaptive response, whereas vaccination with aP has been shown to promote a less efficacious Th2 response. Given the evidence for intracellular *B. pertussis*, the humoral response elicited following aP vaccination, could fail to efficiently target intracellular bacteria and result in suboptimal immune protection. It has been demonstrated that infection with *B. bronchiseptica* and *B. pertussis* naturally induces the activation of a Th1-type T-lymphocyte cytokine response, characterized by high levels of IL-2, IFN-γ, and TNF-α. Intriguingly, optimal immunity against intracellular pathogens frequently involves the activation of such a cellular Th1 response. It's possible that inability to clear intracellular bacteria following aP vaccination, could allow *B. pertussis* to evade host immunity by residing inside host phagocytes, while protecting the bacteria from antibodies, complement activation, and bactericidal agents. With all these considerations, there is

currently the hypothesis that an inadequate immune response to intracellular *B. pertussis* by currently used aP vaccines may be contributing to the reemergence of "whooping cough". Despite this possible scenario, little work has been dedicated at identifying the factors that promote to *Bordetella* intracellular survival.

Bordetella bronchiseptica as a model system for the study of intracellular survival

Data on *B. bronchiseptica* and *B. pertussis* shows that shortly after phagocytosis most ingested bacteria are killed, but a proportion of the bacteria can evade destruction and replicate in macrophages, resulting in long term bacterial persistence. Survival inside macrophages is characterized by significant changes in the bacterial transcriptome, followed by adaptation to the intracellular environment. Collectively, recent observations suggest that the ability to persist inside host phagocytic cells may constitute an important, previously underestimated feature in *Bordetella* infection. However, unlike *B. pertussis* which is human-restricted, *B. bronchiseptica* robustly infects mice providing a natural infection model with a robustly characterized host immune system thereby allowing molecular studies of both pathogen and host to be undertaken.

The mouse model of *B. bronchiseptica* infection is highly efficient. An inoculum containing five CFU administered to the nares is sufficient for colonization and lifelong persistence in the mouse nasal cavity. In addition, the high degree of genetic similarity that *B. bronchiseptica* shares with the human-pathogen *B. pertussis*, serves as an ideal system to explore and characterize the poorly understood features of intracellular survival. In combination with *B. bronchiseptica* relativeness to the most common ancestor of "classical" *Bordetella*, allows for the evolutionary exploration of this strategy throughout the genus bordetellae.

Conclusion

Pathogenic species of the genus bordetellae have adapted to colonize, replicate and transmit in animal hosts. The environmental origin of the species suggests that the ability to survive and persist within environmental phagocytes could have protected the bacteria from external dangers while ensuring transmission to novel environments and in many ways the current pathogenic species of the bordetellae likely still acquires the same advantages from the host immune response.

Studies in murine models have demonstrated that innate immune cells, and antimicrobial peptides help control the infection, while complete bacterial clearance requires activation of cellular immunity mediated by T-helper type 1 (Th1) and Th17 cells. Similar to convalescent immunity, vaccination with whole cell pertussis wP vaccine promotes the secretion of Th1 derived cytokines which result in complete bacterial clearance from the respiratory track and long-term protection. In contrast, while vaccination with acellular pertussis effectively clears bacteria from the lungs and protects from disease pathology, it does not prevent persistence in the upper respiratory tract or transmission. Its plausible that a Th2 humoral response to the aP vaccine could fail to efficiently target intracellular *B. pertussis*, resulting in suboptimal vaccine protection. Therefore, evaluation of the *Bordetella* ability to reside inside host cells and the impact of this strategy during infection can provide valuable insight in the fight against the reemergence of *Bordetella pertussis*.

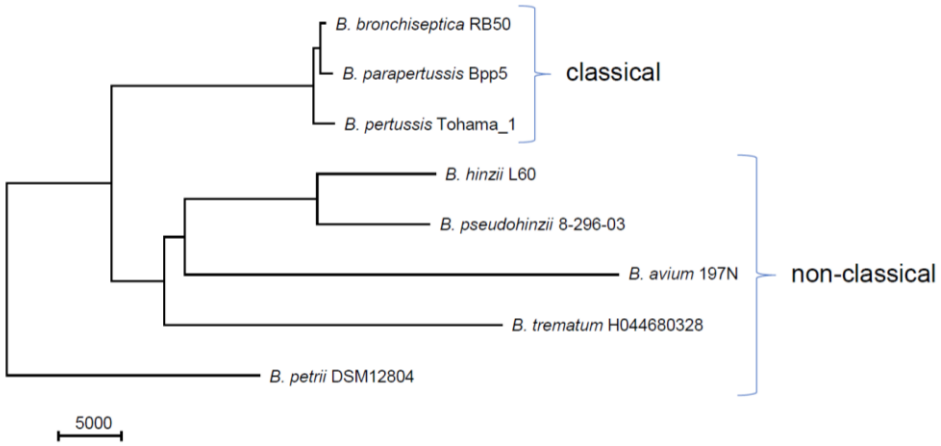

Figure 2.1. Genome-wide SNP-based phylogeny of *Bordetella* species. The genomes of the eight analyzed species formed three phylogenetic clades. The phylogeny was based on 373,499 base pairs shared between all eight genomes. Genes with more than one copy per genome such as 16S rRNA were not included. Scale bar: number of differences. Extracted from Rivera, et al., 2019, Supplementary Figure 1.

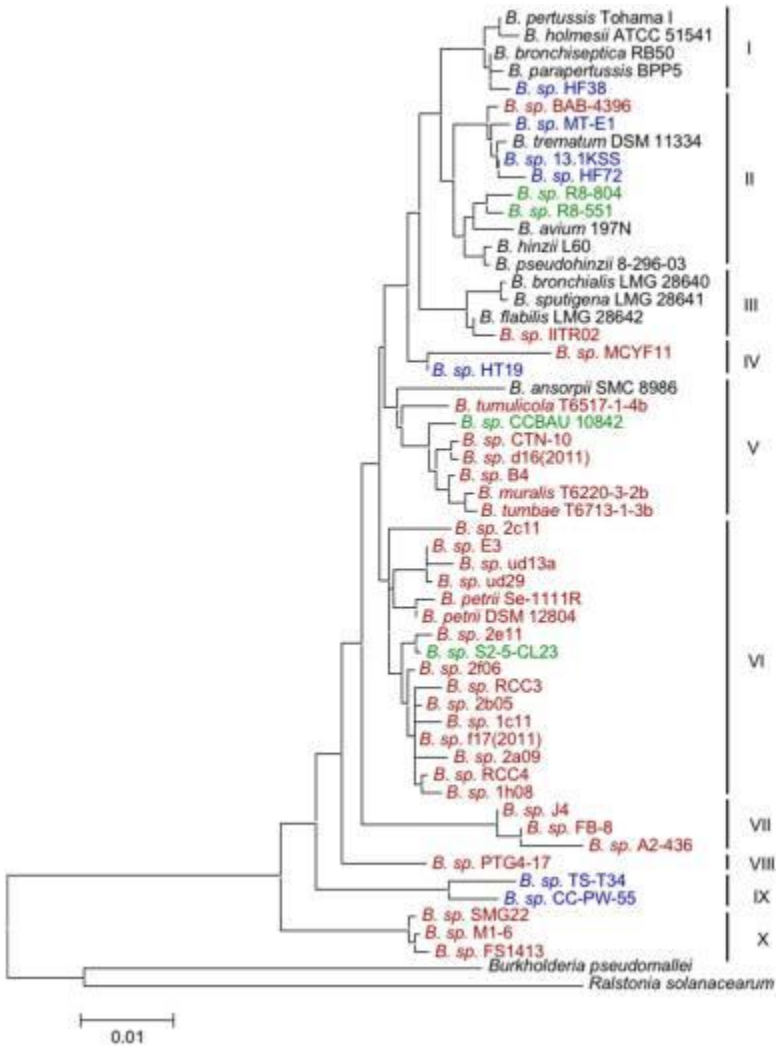

Figure 2.2. Neighbor-Joining tree based on 16S rRNA gene sequences of animal-associated and environmental strains of *Bordetella*. The 52 near full-length sequences (1376 bp) formed 10 clades (I–X) of phylogenetically closely related *Bordetella* isolates/species recovered from soil (brown), water (blue), plants (green) and animals (black). The 16S rRNA gene sequences of the beta-proteobacteria *Burkholderia pseudomallei* and *Ralstonia* solanacearum were used as outgroups. Extracted from Hamidou et al., 2017, Figure 1.

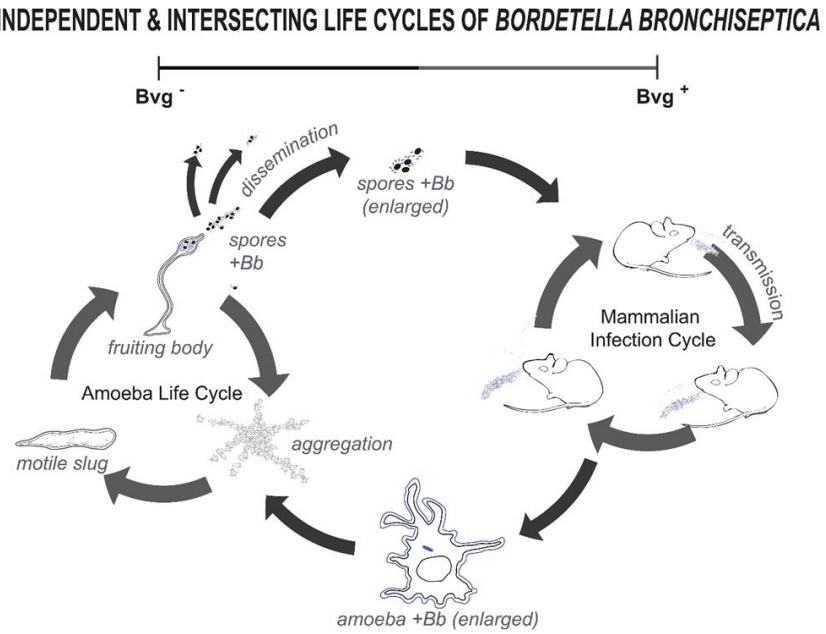

Figure 2.3. Model illustrating how BvgAS may regulate two independent but interconnected life cycles of *B. bronchiseptica*. The model illustrates the survival and transmission of *B. bronchiseptica* (blue) both in the mammalian host (in Bvg+ phase) and along with the amoeba (in Bvg- phase) and the connections between these cycles. Infected mice shed *B. bronchiseptica*, which can both transmit to colonize other mammalian hosts and spread in the environment. The Bvg+ phase genes are known to be necessary for *B. bronchiseptica* colonization and transmission between mammalian hosts. Outside the mammalian host, in the Bvg- phase, *B. bronchiseptica* can form a stable association with the amoebae, like *D. discoideum*, such that it is incorporated into the fruiting body sori and transmitted from sorus to sorus. This association may constitute an alternative life cycle for bordetellae, involving the many Bvg- specific genes that are highly conserved yet not apparently expressed during the mammalian infection cycle. Importantly, *B. bronchiseptica* recovered from amoeba sori can efficiently infect mice, indicating that these two independent life cycles are interlinked. *Bb*, *B. bronchiseptica*. Extracted from Taylor-Mulneix et al., 2017, Figure 12.

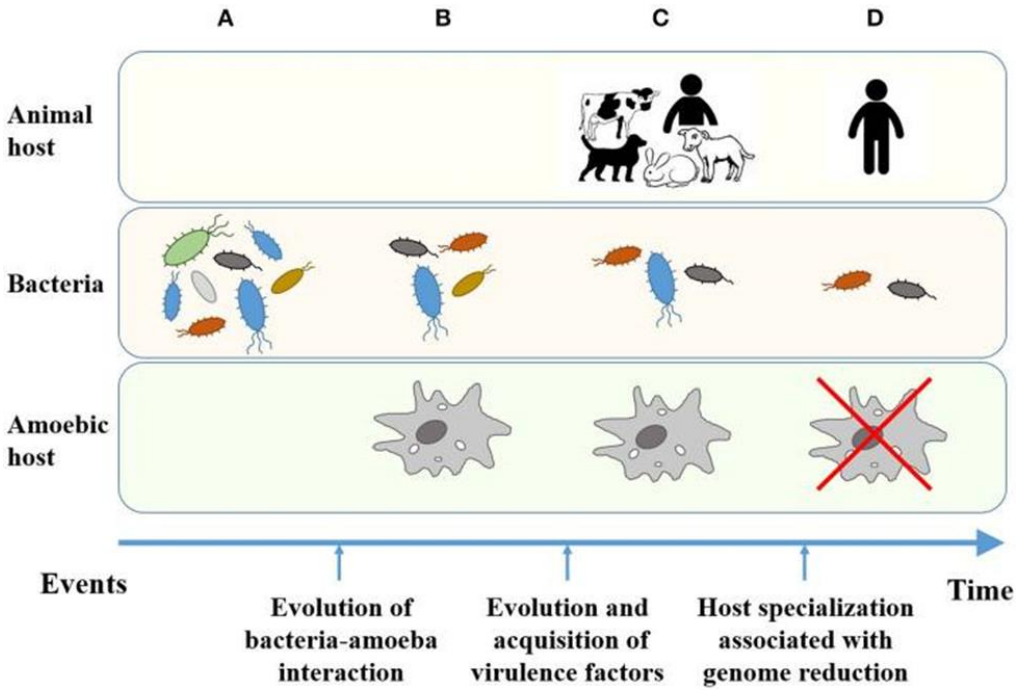

Figure 2.4. Evolution of bacteria from environmental microbes to human-restricted pathogens. (A) Environmental bacteria as a food source for amoebae. (B) Bacteria developed resistance to digestion and the ability to interact with the new eukaryotic host. (C) Bacteria able to interact with and utilize amoebae evolved to animal pathogens. (D) Host-specialized bacterial pathogens lost the ability to resist predation and interact with lower eukaryotes. Extracted from Taylor-Mulneix et al., 2017, Figure 2.

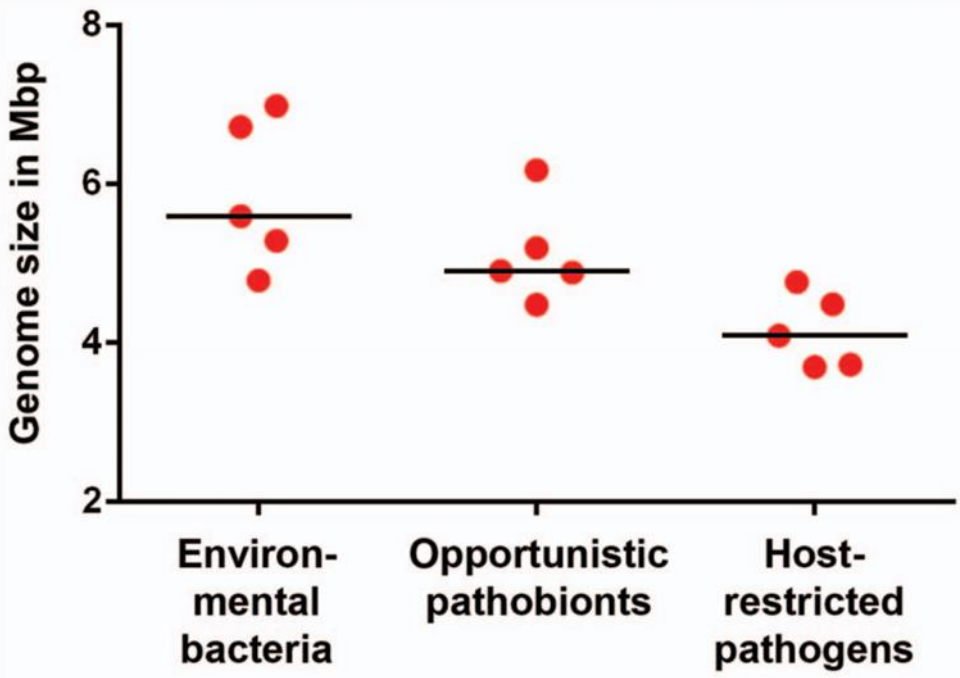

Figure 2.5. Genome size of environmental bacteria, pathobionts and host-restricted pathogens in the genus *Bordetella*. *Bordetella* sp. SCN 68-11 (accession: MEFS00000000), *B. sp.* SCN 67-23 (MEDQ00000000), *B. sp.* BFMG2 (PKCD00000000), *B. petrii* (NC_010170) and *B. sp.* N (NZ_CP013111), isolated from environmental sources, possess the largest genomes in the genus *Bordetella*. In contrast, the genomes of the obligate host-restricted pathogens *B. holmesii* (NZ_CP007494), *B. avium* (NC_010645.1), *B. pertussis* (NC_002929), *B. parapertussis* $_{hu}$ (NC_002928) and *B. pseudohinzii* (NZ_CP016440) featured substantial reduction. Pathobionts *B. trematum* (NZ_LT546645), *B. hinzii* (NZ_CP012076), *B. ansorpii* (NZ_FKIF00000000) and *B. bronchiseptica* (NC_002927) have been isolated from multiple animal and human sources. Extracted from Linz, et al., 2019, Figure 2.

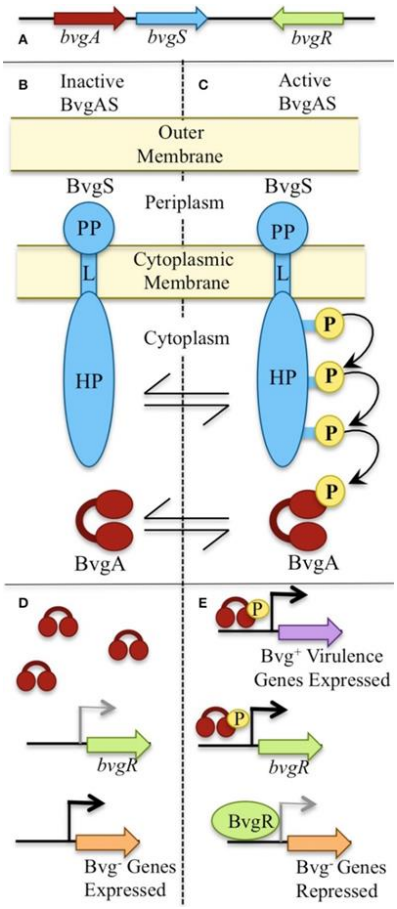

Figure 2.6. The BvgAS phosphorelay. (A) The master regulatory system of bordetellae, *Bordetella* Virulence Genes (BVG), is expressed by bvgS and bvgA. (B,C) BvgS is a transmembrane sensor protein consisting of a periplasmid domain (PP) connected to the histidine phosphotransfer domains (HP) in the cytosol through a linker domain (L). (B) BvgS is inactive and un-phosphorylated when bacteria grow at temperatures below 25°C. (D) Bvg− phase genes are transcribed when the BvgAS system is inactive. (C) Upon receiving inducing signals such as 37°C, BvgS autophosphorylates and initiates a phosphor-relay that leads to phosphorylation and activation of BvgA. (E) When the BvgAS system is active, Bvg+ phase-associated genes are transcribed, including bvgR. BvgR represses expression of Bvg− phase associated genes. Extracted from Taylor-Mulneix, et al., 2017, Figure 1.

Table 2.1. Presence and absence of specific virulence-associated key factors in the genomes of 9 *Bordetella* species. Extracted from Linz, et al., 2016, Table 3.

Key factor\Species	*B. bronchiseptica*	*B. parapertussis*	*B. pertussis*	*B. holmesii*	*B. hinzii*	*B. avium*	*B. trematum*	*B. petrii*	*B. ansorpii*
BvgA/BvgS/FHA	+	+	+	+	+	+	+	+	-
DNT	45/58	+	+	-	-	+	-	-	-
T1SS-ACT	55/58	+	+	-	-	-	-	-	-
T2SSa	-	-	-	-	+	+	-	2/3	+
T2SSb	-	-	-	-	-	-	-	-	+
T2SSc	-	-	-	-	-	-	-	-	1/2
Type IV Pilus A	+	+	d	d	+	d	-	+	+
Type IV Pilus B	+	+	d	d	+	+	+	+	+
Type IV Pilus C	+	+	d	d	+	+	+	+	+
Type IV Pilus D	+	1/2	-	-	-	-	-	-	-
Type IV Pilus E	-	-	-	-	-	-	-	-	+
Type IV Pilus F	-	-	-	-	-	-	-	-	+
T3SS	+	+	+	-	-	-	-	-	+
T4SS-Pertussis Toxin	42/58	d	+	-	-	-	-	-	-
T5SS-Pertactin	+	+	+	-	-	-	-	-	-
T6SSa	51/58	+	-	-	-	-	-	+	+
T6SSb	-	-	-	-	5/6	+	-	-	-
T6SSc	-	-	-	-	-	-	-	1/3	-
O-antigenA (*wbm* locus)[a]	51/58	1/2	-	-	-	-	-	-	-
O-antigenB (BAV0081-89)	-	-	-	-	-	+	-	-	-
Capsule A	+	+	+	+	+	-	-	-	-
Capsule B	-	-	-	-	+	+	+	-	-
Capsule C	-	-	-	-	-	-	-	-	1/2
Cellulose synthesis	-	-	-	-	+	+	+	-	+
Flagella	+	1/2	+	-	+	+	+	+	+
Alcaligin receptor	+	+	+	+	-	-	-	-	-
Heme receptor	+	+	+	+	+	+	+	-	d
Enterobactin receptor	+	d	+	+	+	+	+	+	-

d degenerate, likely not functional

[a]- *B. trematum* and *B. ansorpii* may potentially contain other, additional O-antigen synthesis loci

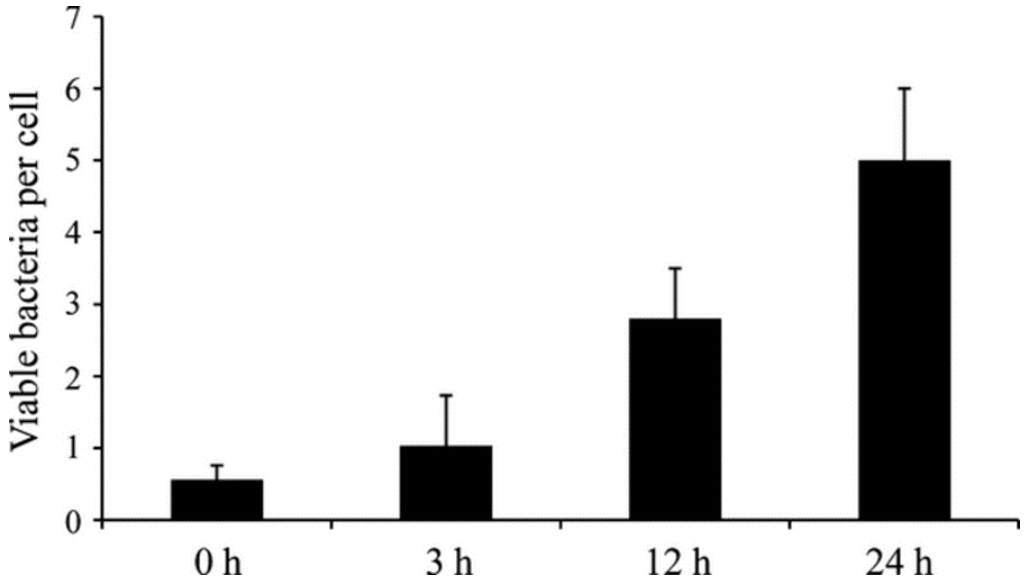

Figure 2.7. Kinetics of entry of *Bordetella pertussis* into epithelial cells. A549 cells were infected with *B. pertussis* (MOI 150) during 2 h, washed to remove nonadherent bacteria, and further incubated for other 0, 3, 12, or 24 h. The number of viable intracellular *B. pertussis* at the various time points was determined by the polymyxin B protection assay. The data represent the mean ± SD of two independent experiments performed in triplicate. Extracted from Lamberti, et al., 2013, Figure 1.

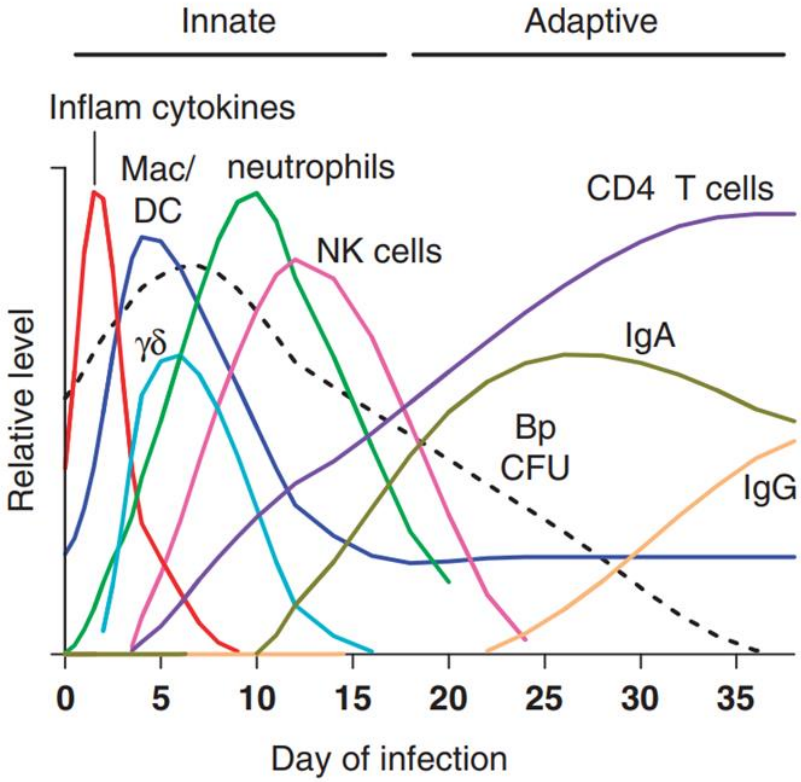

Figure 2.8. Kinetics of cell recruitment to the lungs and immune responses following infection with *Bordetella pertussis*. Hypothetical curves representing relative numbers of immune cells recruited to the lungs of mice following primary infection with *B. pertussis*. CD4T cells: recruitment of CD4+ T cells to the lungs and response of the lung T cells to *B. pertussis* antigens. Immunoglobulin (Ig) G: anti-*B. pertussis* IgG in serum. IgA: anti-*B. pertussis* IgA in lungs. Bp CFU: *B. pertussis* bacterial burden in the lungs. DC, dendritic cells; Mac, macrophage; NK, natural killer. Extracted from Higgs, et al., 2012, Figure 1.

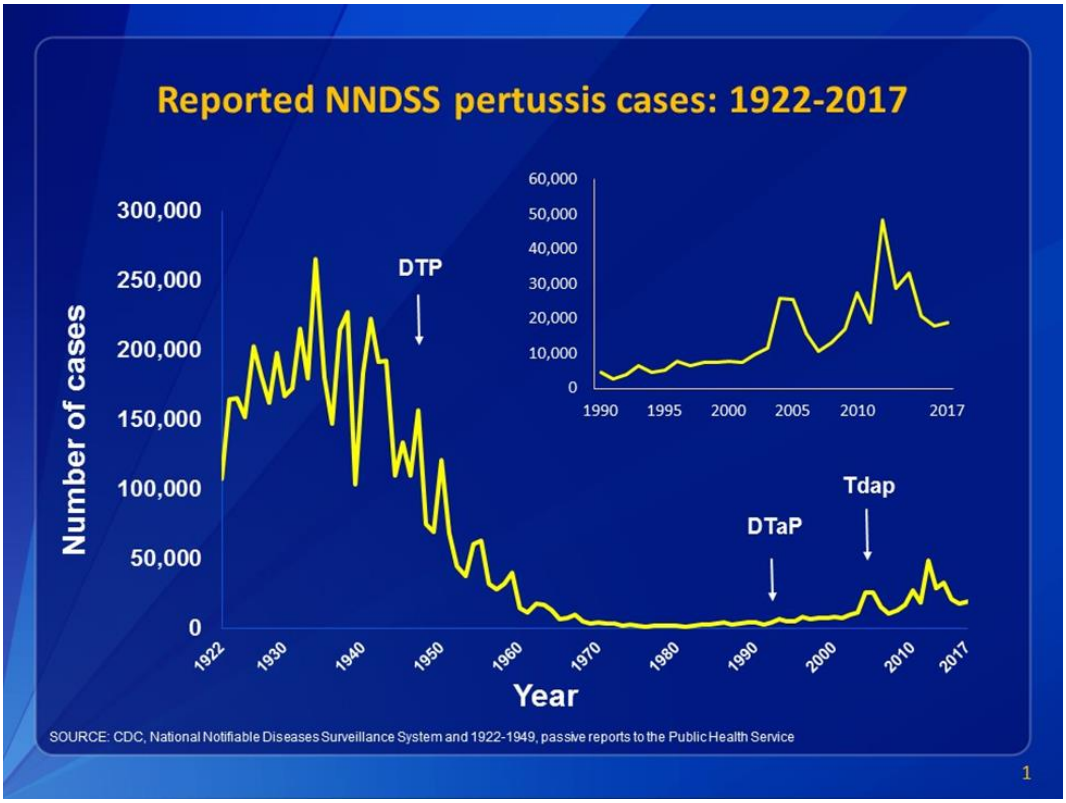

Figure 2.9. Reported number of cases of pertussis from 1922 to 2017. CDC.

Extracted from National Notifiable Disease Surveillance System and 1922-1949, passive reports on the Public Health Service.

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CHAPTER 3

CONSERVATION OF ANCIENT GENETIC PATHWAYS FOR INTRACELLULAR PERSISTENCE AMONG ANIMAL PATHOGENIC BORDETELLAE

Rivera I., Linz B, Dewan KK, Ma L, Rice CA, Kyle DE, Harvill. 2019. *Front Microbiol.* 2019;10:2839. Accepted by Frontiers in microbiology. Reprinted here with permission of the publisher.

Abstract

Animal and human pathogens of the genus *Bordetella* are not commonly considered to be intracellular pathogens, although members of the closely related classical bordetellae are known to enter and persist within macrophages in vitro and have anecdotally been reported to be intracellular in clinical samples. *B. bronchiseptica*, the species closest to the ancestral lineage of the classical bordetellae, infects a wide range of mammals but is known to have an alternate life cycle, persisting, replicating and disseminating with amoeba. These observations give rise to the hypothesis that the ability for intracellular survival has an ancestral origin and is common among animal-pathogenic and environmental *Bordetella* species. Here we analyzed the survival of *B. bronchiseptica* and defined its transcriptional response to internalization by murine macrophage-like cell line RAW 264.7. Although the majority of the bacteria were killed and digested by the macrophages, a consistent fraction survived and persisted inside the phagocytes. Internalization prompted the activation of a prominent stress response characterized by upregulation of genes involved in DNA repair, oxidative stress response, pH homeostasis, chaperone functions, and activation of specific metabolic pathways. Cross species genome comparisons revealed that most of these upregulated genes are highly conserved among both the classical and non-classical *Bordetella* species. The diverse *Bordetella* species also shared the ability to survive inside RAW 264.7 cells, with the single exception being the bird pathogen *B. avium*, which has lost several of those genes. Knock-out mutations in genes expressed intracellularly resulted in decreased persistence inside the phagocytic cells, emphasizing the importance of these genes in this environment. These data show that the ability to persist inside macrophage-like RAW

264.7 cells is shared among nearly all *Bordetella* species, suggesting that resisting phagocytes may be an ancient mechanism that precedes speciation in the genus and may have facilitated the adaptation of *Bordetella* species from environmental bacteria to mammalian respiratory pathogens.

Introduction

Three closely related species of the gram-negative bacterial genus *Bordetella* make up the group of respiratory pathogens known as the classical bordetellae. These include the notorious human pathogen *Bordetella pertussis*, which is the etiological agent of pertussis or whooping cough (Mattoo and Cherry, 2005) and the closely related *B. parapertussis*, a species which consists of two distinct lineages that cause pertussis-like disease in humans and pneumonia in sheep, respectively (Porter et al., 1994). The third species, *B. bronchiseptica*, infects a wide range of mammals including many domesticated animals (Goodnow, 1980), causing a variety of pathologies ranging from chronic asymptomatic infection to acute bronchopneumonia. Multi locus sequence typing and genome comparisons revealed that *B. pertussis* and *B. parapertussis* independently arose from a *B. bronchiseptica*-like ancestor (Parkhill et al., 2003; Diavatopoulos et al., 2005). The classical bordetellae possess several partially characterized virulence mechanisms (Skarlupka et al., 2019) that are studied in the context of what is viewed as a completely extracellular life cycle in their mammalian hosts (Melvin et al., 2014). Yet, in vitro experiments convincingly demonstrated that the classical bordetellae can survive intracellularly within mammalian phagocytic cells (Banemann and Gross, 1997; Lamberti et al., 2010; Gorgojo et al., 2012), an ability that appears to have descended from ancestral progenitor species that lived in the environment (Hamidou Soumana et al., 2017) and acquired the ability to resist phagocytic killing by amoebae that are ubiquitous environmental predators (Taylor-Mulneix et al., 2017b). In fact, *B. bronchiseptica*, the species that most closely resembles the environmental ancestor of the classical bordetellae, can survive within amoeba and also disperse along with amoebic spores,

highlighting a novel strategy for an environmental life cycle (Taylor-Mulneix et al., 2017a). These observations strongly suggest that intracellular survival may be an ancestral trait that might have affected the adaptation of *Bordetella* spp. from environmental bacteria to mammalian respiratory pathogens (Taylor-Mulneix et al., 2017b; Linz et al., 2019).

Despite not being commonly considered an intracellular pathogen, *B. pertussis* has repeatedly been recovered from dendritic cells and alveolar macrophages (Hellwig et al., 1999; Carbonetti et al., 2007; Paddock et al., 2008). These studies showed that *B. pertussis* is able to modulate human macrophages by secreting a wide range of proteins upon entry, which allows them to reside within the host cells. Interestingly, the ability to reside inside macrophages is not unique to *B. pertussis*, as recovery from macrophages have been confirmed for all classical bordetellae, including *B. parapertussis* and *B. bronchiseptica* (Gorgojo et al., 2012; Bendor et al., 2015).

In addition to the closely related classical bordetellae, which share about 99% sequence identity throughout their genomes, several other *Bordetella* species have been identified, collectively referred to as non-classical, that display much broader genetic diversity (Supplementary Figure S3.1). Of these, *B. avium* and *B. hinzii* cause respiratory infections in poultry and wild birds (Kersters et al., 1984; Vandamme et al., 1995). *B. pseudohinzii* was identified as a pathobiont in several mouse breeding colonies (Ivanov et al., 2015, 2016) and was recently shown to cause chronic ear infection in mice (Dewan et al., 2019). *B. trematum* is an opportunistic human pathogen that can cause severe skin disease and chronic otitis media (Vandamme et al., 1996). *B. petrii* was originally isolated from an anaerobic bioreactor culture enriched from river sediment

(von Wintzingerode et al., 2001) and was subsequently isolated from many soil samples (Hamidou Soumana et al., 2017; Garrido-Sanz et al., 2018). Although several genomic features have changed throughout their independent evolution, including acquisition and loss of multiple virulence-associated genes (Linz et al., 2016, 2019), these *Bordetella* species share many characteristics that make them successful animal pathogens.

Since many of the non-classical bordetellae are animal pathogens too, we hypothesized that intracellular survival, the ability to resist digestion by phagocytic cells, may constitute an ancient environmental defense mechanism that facilitated the adaptation of *Bordetella* species to animals. If this were the case, then the ability to survive intracellularly would be expected to be widespread among both classical and non-classical bordetellae with shared, conserved genetic pathways. To test this hypothesis, we analyzed the transcriptome of *B. bronchiseptica* following internalization by macrophages and identified the induced key genes and pathways. Cross species genome comparisons revealed that most of the upregulated genes are highly conserved among the *Bordetella* genus. In agreement, both the classical and non-classical *Bordetella* species have retained the ability to survive inside murine macrophages. The only exception, *B. avium* – a species that has been found only among birds – has lost several of those genes and has lost the ability to survive within macrophages. Deletion of these genes in *B. bronchiseptica* substantially decreased its intracellular survival. These data indicate that the ability to resist phagocytic killing by host macrophages is widespread among the animal pathogenic *Bordetella* species and may have been an important step enabling the evolution of *Bordetella* species as animal pathogens.

Methods

Bacterial Strains and Growth

Bordetella bronchiseptica strain RB50, *B. pseudohinzii* 8-296-03, *B. hinzii* L60, *B. petrii* DSM12804, *B. avium* 197N and *B. trematum* H044680328 were grown and maintained on BG agar (Difco) supplemented with 10% defibrinated sheep's blood (Hema Resources). Liquid cultures were grown overnight at 37°C to mid-log phase (OD ~0.6) in Stainer Scholte (SS) liquid broth (Stainer and Scholte, 1970). *Klebsiella aerogenes* was grown and maintained on Luria-Bertani (LB) agar (Difco) and liquid cultures were grown at 37°C to mid-log phase in LB broth (Difco).

Intracellular Bacterial Assays

RAW 264.7 macrophages cells were grown to 80% confluency (~1 × 105 CFU/well) in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS, glucose and glutamine in 48-well tissue-culture plates at 37°C. Bacteria were added in 10 µl PBS containing 107 CFU (MOI of 100), 106 CFU (MOI of 10) or 105 CFU (MOI of 1) as indicated. Plates were centrifuged at 250 g for 5 min at room temperature and incubated at 37°C for 1 h, after which gentamicin solution (Sigma-Aldrich) was added to a final concentration of 300 µg/ml. Plates were incubated at 37°C for an additional 1, 3, 7, or 23 h and subsequently washed with PBS. 0.1% Triton-X solution was administered, followed by 5 min incubation and vigorous pipetting to lyse the macrophages. The samples were serially diluted and plated on BG agar plates to quantify total bacteria numbers.

Electron Microscopy

RAW 264.7 macrophages were seeded in 6-well tissue-culture plates at a density of 1.5 × 105 cells/ml, inoculated with *B. bronchiseptica* RB50 at a MOI of 10:1 and centrifuged for 5 min at 250 g. Following 1 h incubation at 37°C, the macrophages were washed with PBS, and DMEM media containing 300 μg/ml gentamicin was added. After 1 h, the macrophages were washed with PBS and suspended in a final volume of 300 μl of PBS. The macrophages were then collected by centrifugation and fixed with fresh 2% glutaraldehyde for Transmission Electron Microscopy at the University of Georgia Electron Microscopy Core Facility.

Confocal Fluorescent Microscopy

Green fluorescent protein (GFP)-expressing *B. bronchiseptica* strain RB50 (Taylor-Mulneix et al., 2017a) was exposed to RAW 264.7 macrophages at a MOI of 100:1 for 1 h, followed by gentamycin treatment for 1 h. Live cell fluorescence microscopy was performed using a Zeiss Axio Obsever.Z1/7 microscope. Imaging was performed at 488 nm for GFP (green), and transmitted light for DIC II (white) at a magnification of 40x using an LD Plan-Neoflaur 40x/0.4 Korr M27 objective.

Z-Stack Imaging

RAW 264.7 cells were seeded in 6-well tissue-culture-treated plates with coverslips in the bottom at a density of 1.5 × 105 cells/ml in 3 ml and inoculated with *B. bronchiseptica* RB50 at a MOI of 10 (2 × 106 CFU) 12 h later. To synchronize the bacterial exposure to macrophages the plates were centrifuged at 300 g for 10 min. After 45 min incubation at 37°C, bacteria in the supernatant were removed by washing the macrophages three times with 1X PBS. The plates were incubated with DMEM medium

containing 300 μg/ml gentamicin for 2 h to kill the remaining extracellular bacteria, and then washed 3 times with 3 ml of 1X PBS. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then washed three times with 1X PBS and subsequently permeabilized with 0.1% Triton X-100 in 1X PBS for 20 min at room temperature. Primary antibodies derived from sera of *B. bronchiseptica*-infected mice were added after dilution in 1X PBS containing 2% BSA. After incubation at room temperature for 1 h, the cells were washed 3 times in 1X PBS. Then, the preparation was incubated with secondary donkey anti-mouse antibodies conjugated to FITC and with phalloidin for actin staining for 1 h. After 3 washes in 1X PBS, the coverslips were removed from the plates and fixed on glass slides with mounting medium containing DAPI. The images were taken with a Zeiss LSM 710 Confocal Laser Microscope for Z-stack imaging at 0.5 μm intervals.

Intracellular Bacterial Assay for Transcriptional Analysis

RAW 264.7 macrophages were seeded in 6-well tissue-culture plates and inoculated with *B. bronchiseptica* RB50 at a MOI of 100:1. A subset of the bacteria was cultured in DMEM medium without macrophages as the negative control. Following 1 h incubation at 37°C, the remaining bacteria in the supernatant were removed by washing the macrophages with PBS and followed by addition of DMEM medium containing 300 μg/ml gentamicin. After 1 h the DMEM was removed, and the macrophages were washed with PBS. The samples were suspended in 1 ml of TRIzol for RNA extraction.

RNA Isolation and Sequencing

RNA was extracted from RB50 lysates using TRIzol (Ambion) and the Bacterial RNA isolation Kit (Max Bacterial Enhancement Reagent, Ambion) with implemented

PureLink DNase treatment (Invitrogen) following the manufacturer's instructions. RNA quality was assessed using the NanoDrop 2000 (Thermo Scientific) and BioAnalyzer (Agilent). Samples were submitted for Illumina sequencing at the Molecular Research Laboratory in Shallowater, TX, United States. Ribosomal RNA was depleted from each biological replicate (n = 3) during preparation of the Illumina sequencing library.

Bioinformatic Analyses

Quality control of raw reads was performed using FASTQC and TRIMMOMATIC for filtering of low-quality reads and trimming of Illumina library adapters. Filtered reads were mapped to *Bordetella bronchiseptica* RB50 genome assembly NC_002927.3 using "Bowtie2." The resulting output files were used to evaluate differential gene expression between three biological replicates of intracellular *B. bronchiseptica* (n = 3) and controls (n = 3) using the "EdgeR" package for the statistical environment R distributed within the Bioconductor project.

Protein Similarity Analysis

Total protein sequences were extracted from the NCBI archive for: *B. bronchiseptica* RB50 (RefSeq assembly accession: GCF_000195675.1), *B. parapertussis* 12822 (GCF_000195695.1), *B. pertussis* Tohama I (CF_000195715.1), *B. hinzii* L60 (GCF_000657715.1), *B. pseudohinzii* 8-296-03 (GCF_000657795.2), *B. avium* 197N (GCF_000070465.1), *B. petrii* DSM12804 (GCF_000067205.1), and *B. trematum* H044680328 (GCF_900078695.1). Similarities between *B. bronchiseptica* proteins and proteins of the non-classical species were calculated in mGenomeSubtractor (Shao et al., 2010) as the H value for each protein, defined as H = i x (lm/lq). H is the highest BLASTp identity score (i), multiplied by the ratio of the matching sequence

length (lm) and the query length (lq). Based on our previous work (Linz et al., 2018), proteins with an H value < 0.5 were considered absent. Pairwise tBLASTx genome comparisons in the Artemis Comparison Tool (Carver et al., 2008) validated proteins with values of H > 0.5 as true orthologs.

Quantitative Real-Time PCR

Real-time PCR analyses were performed on a QuantStudio (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Complementary DNA (cDNA) transcript libraries were prepared from biological triplicates of the control and of bacteria incubated with macrophages in DMEM + 10% FBS. Samples were processed for RNA extraction using TRIzol Reagent (Ambion by Life Technologies) and treated with PureLink DNase (Invitrogen). Primers were manually designed and purchased from IDT (Supplementary Table S3.1). The cycling parameters were as follows: 5-min preincubation at 95°C followed by 40 cycles of a 2-step PCR at 95°C and 60°C. Gene expression was calculated using the ΔΔCt method with expression of the 16S rRNA used as reference. Data were analyzed using DataAssist version 3.0 (Applied Biosystems).

Deletion Mutants

The allelic exchange vector pSS4245 (Inatsuka et al., 2010) was used for the generation of deletion mutants. Briefly, ~1 kb of DNA flanking each end of the target gene was PCR amplified using primers provided in Supplementary Table S3.2, joined and inserted into the vector by PIPE cloning (Klock and Lesley, 2009). The construct was verified by sequencing, transformed into E. coli SM10λpir, and transferred into the parental *B. bronchiseptica* strain RB50 by mating. Colonies containing the integrated plasmid were selected and incubated on BG agar to stimulate allelic exchange by

homologous recombination. Emerging colonies were screened by PCR for replacement of the wildtype by the mutant allele and confirmed by Sanger sequencing. In vitro growth curves showed that none of the deletion mutants had growth defects compared to the wildtype strain RB50 (data not shown). For complementation, the target gene was cloned into plasmid pBBR1 (Antoine and Locht, 1992).

Statistical Analysis

The mean ± standard error (error bars in figures) was determined for all appropriate data. Two-tailed, unpaired student's t-tests were used to determine the statistical significance between two normally distributed populations. GraphPad Prism version 6.04 was used to conduct these statistical tests and to generate figures.

Results

B. bronchiseptica Entry and Persistence in Murine Macrophages

We had earlier observed using gentamicin protection assays that the prototype *B. bronchiseptica* strain RB50 (*Bb*) can enter and survive within murine macrophage-like cell line RAW 264.7 in vitro (Bendor et al., 2015). To determine the number and proportion of bacteria entering these macrophages, we performed an assay of macrophages infected with *Bb* at multiplicities of infection (MOI) of 100, 10 and 1 for 1 h. The percentage of recovery ranged from 0.7 to 1% of the original inoculum at all three MOIs, indicating that a relatively constant fraction of bacteria entered and resisted digestion by macrophages (Figure 3.1A and Supplementary Figure S3.2). The observation that the ratio of bacteria to macrophage did not affect survival rate suggested that this is not simply macrophages being overwhelmed or overcome by bacterial numbers. Electron microscopy (Figure 3.1C), confocal microscopy (Supplementary

Figure S3.3A) and z-stack images (Figure 3.1B and Supplementary Figure S3.3B) taken after 2 h incubation confirmed the presence of bacteria within phagocytic vacuoles. Once inside the RAW 264.7 cells, bacterial numbers remained relatively stable and decreased only slowly over time. Bacterial CFUs recovered at 4 and 8 h showed no significant change in numbers for any of the MOIs used, and even at 24 and 48 h intracellular *Bb* were recovered in substantial numbers (Figure 3.1A). In contrast to *Bb*, *Klebsiella aerogenes* (Figure 3.1A) failed to persist in RAW 264.7 cells and was recovered at numbers over two orders of magnitude lower.

B. bronchiseptica Transcriptional Response to Internalization by Macrophages

We hypothesized that to survive within professional phagocytic cells, *Bb* would require distinct groups of genes to be transcriptionally modulated once the bacteria reached the intracellular niche. To examine this transcriptional response, we analyzed the RNA profile of intracellular *Bb* at 2 h post inoculation and compared it to that of bacteria grown in vitro. Total RNA was isolated from samples collected after antibiotic treatment and sequenced on an Illumina MiSeq (RNA-Seq). On average, 8.8 × 105 reads of intracellular *Bb* (n = 3) and 5.6 × 106 reads of the planktonic bacterial control (n = 3) mapped to non-rRNA regions of the *Bb* reference genome (NC_002927.3). Those reads were used to evaluate the differential gene expression of *Bb* inside macrophages in comparison to that of bacteria grown in vitro.

A Principal Component Analysis (PCA) of the normalized read distribution revealed a clear difference in the global gene expression between intracellular and in vitro grown *Bb* (Supplementary Figure S3.4). The PCA plot showed clustering of the replicates and separation of the two groups along the first principal component (PC1),

indicating a distinct transcriptional response to the intracellular environment. Differentially expressed genes that displayed a log2-fold change of either ≥ 1.5 (upregulated genes) or ≤−1.5 (downregulated genes) with a p-value < 0.05 were selected for further analysis, which resulted in a list of 318 upregulated and 243 downregulated genes. To validate our RNA-seq dataset we performed a quantitative real-time (qRT) PCR to assess the transcriptional changes in five highly upregulated and four strongly downregulated genes (Supplementary Figure S3.5), which confirmed the RNA-Seq data.

Upregulated Genes

Functional analysis of the transcriptionally upregulated genes showed major changes at the functional levels of metabolic process (102 genes), of cellular process (116 genes), regulation (24 genes) and response to stimuli (8 genes). Gene ontology evaluation revealed enrichment for genes whose products are involved in cellular processes (113 genes) including: DNA repair, protein folding and repair, oxidative stress response, and pH homeostasis, as well as enrichment for metabolic processes (102 genes) such as nutrient assimilation (24 genes) (Table 3.1). A list of all 318 upregulated genes can be found in Supplementary File 3.1.

Expression of recA, dnaB, dps, and dksA, all implicated in the activation of the SOS response and DNA repair (Bearson et al., 1997; Simmons et al., 2008; Lund et al., 2014), was upregulated upon internalization by macrophages. Likewise, genes for protein folding and recycling such as molecular chaperones groES, groEL, and htpG, and protease genes hslV and hslU, were highly upregulated, as was expression of several other osmotic and heat shock response genes, including clpB, grpE, dnaK, and dnaJ.

Congruent to previous studies in other bacterial species (Buchmeier et al., 1997; Zimna et al., 2001; Clements et al., 2002; Gilberthorpe et al., 2007; Chiang and Schellhorn, 2012; Fang et al., 2016), expression of genes that promote resistance against oxidative stress and low pH was upregulated intracellularly, including transcription factor iscR and adjacent genes iscS, hscB, and fdx, and the transcription regulators slyA, risA, and fur. Additionally, RNA polymerase sigma factor genes rpoH, rpoN, and rpoE were highly upregulated (Laskos et al., 2004; Delory et al., 2006; Hanawa et al., 2013), as was expression of the RNA chaperone gene hfq, which is known to increase resistance against killing by macrophages (Bibova et al., 2013).

Increased transcription of glyoxylate cycle genes such as mdh, sdhC, glcB, glcC, and acnB, as well as of numerous ribosomal protein genes implies extensive metabolic activity in the bacterial cell in response to internalization by macrophages. Several genes of fatty acid synthesis pathways such as 3-oxoacyl-ACP reductase BB4150, long chain fatty acid Co-A ligase BB0233, outer membrane protein ompA, and ABC transport protein encoded by BB1556, were found to be strongly induced, suggesting increased membrane biosynthesis. Also, we observed an increase in expression of genes involved in amino acid biosynthesis and transport, including BB4592, carA, argC, and argG, and of de novo nucleotide biosynthesis (ndk, pyrH, cmk, and nrdA).

The fimbria encoding genes fim2, fimA and BB3424 were the only genes encoding virulence-associated factors among the 318 transcriptionally upregulated genes.

Downregulated Genes

The cya genes encoding the adenylate cyclase toxin were among the most downregulated genes in our dataset with a log2fold change of about -3 (Table 3.2 and

Supplementary File 3.1), and expression of the dermonecrotic toxin gene dnt was also downregulated. In agreement with previous studies (Hausman et al., 1996; Antoine et al., 2000; Hausman and Burns, 2000), expression of the pertussis toxin operon and the associated type IV secretion system (T4SS) was barely detectable under either condition. Similarly, expression of the type III secretion system (T3SS) encoded by the bsc locus was strongly suppressed, resulting in decreased expression of both the apparatus-related and the secretion-related components (Table 3.2 and Supplementary File 3.1). In addition to toxins and secretion systems, expression of the O-Antigen–encoding wbm locus (wbmO – bplJ), was significantly downregulated.

Notably, several genes with important functions in cell structure biogenesis and proliferation were also downregulated inside macrophages, including cell division genes ftsZ, ftsA, and ftsQ and cell wall synthesis genes murC, murG, ftsW, and murD. Similarly, expression of a large gene locus (BB3827 to BB3836) encoding the oxidative respiratory chain was significantly downregulated, including NADH dehydrogenase genes nuoN, nuoM, nuoL, and nuoH.

Taken together, *B. bronchiseptica* responded to internalization by macrophages by rapid changes in its transcriptional profile, that were marked by suppression of growth and virulence, and strong activation of the bacterial stress response, including DNA and protein repair and pH homeostasis, and suppression of cell division, putative virulence factors and oxidative respiration.

The Non-classical *Bordetella*e and Intracellular Persistence

Since the human-restricted pathogens *B. pertussis* and *B. parapertussis* arose from *B. bronchiseptica*-like ancestors and can persist inside human macrophages, we

evaluated whether the 318 upregulated genes were conserved among the three classical *Bordetella* species. Two hundred and seventy two intact genes (86%) were identified in the genome of *B. pertussis* strain Tohama I, the other genes were missing or truncated by frameshifts or premature stop codons. Similarly, 301 of the 318 genes (95%) were present in the genome of *B. parapertussis* strain 12822, showing conservation of most genes (Figures 3.2A,C).

While many non-classical bordetellae are also human and animal pathogens, their ability to persist inside phagocytic cells has not been evaluated. Therefore, we tested the presence or absence of the upregulated genes among the non-classical *Bordetella* species, including the bird pathogens *B. hinzii* (Vandamme et al., 1995) and *B. avium* (Kersters et al., 1984), the mouse pathogen *B. pseudohinzii* (Ivanov et al., 2016), the human opportunistic pathogen *B. trematum* (Vandamme et al., 1996), and the environmental species *B. petrii* (von Wintzingerode et al., 2001). We calculated the protein similarity (H value) of the 318 genes upregulated in *B. bronchiseptica* inside macrophages and their corresponding homologs in the non-classical bordetellae, with a gene considered to be present with a protein similarity value of $H \geq 0.5$. An average of 77–81% of the 318 upregulated genes were present in the non-classical species (Figures 3.2A,C) with 95 (30%) of the genes displaying similarity values of $H \geq 0.9$ (Figure 3.2E). In contrast, only 46–55% of the total of 4,981 evaluated *B. bronchiseptica* genes were identified in the genomes of the non-classical species ($P < 0.0001$), where only 448 (9%) of the genes reached protein similarity scores of $H \geq 0.9$ (Figures 3.2B,D,E).

This high evolutionary conservation of genes that are upregulated in *B. bronchiseptica* during intracellular survival in phagocytic cells suggests that the non-

classical bordetellae may be able to persist inside macrophages. To test this hypothesis, the non-classical species were assessed for intracellular survival in RAW 264.7 macrophages for 2 and 4 h. All examined *Bordetella* species were recovered, with the exception of *B. avium* (Figure 3.3). The inoculated *B. pseudohinzii*, *B. hinzii*, *B. trematum* and *B. petrii* bacteria survived internalization by macrophages at similar rates to *B. bronchiseptica*. The genomes of these species share 222 out of the 318 (70%) transcriptionally upregulated genes, which implies a critical function for intracellular persistence in mammalian phagocytic cells.

In contrast to the other analyzed *Bordetella* species, *B. avium* was severely impaired in its ability to persist inside macrophages. Only 0.001% of the inoculum was recovered after 2 h and no viable bacteria were detected after 4 h. Therefore, we performed a comparative genome analysis to identify transcriptionally upregulated genes that were only missing in *B. avium*, which resulted in the identification of six genes (Table 3.3). Deletion of two of these genes (BB0096 and BB1908) resulted in a significant reduction in intracellular survival (Figure 3.4 and Supplementary Figure S3.6). Complementation of these knock-out mutants with plasmid-borne gene copies restored the wildtype phenotype in both mutants (Figure 3.4), confirming that loss of malate synthase transcriptional regulator glcC (BB0096) or the tripartite tricarboxylate transporter BB1908 negatively impacts intracellular persistence in macrophages. In addition, previous studies showed important roles of transcriptionally upregulated (Table 3.1) risA and hfq genes in intracellular persistence of *Bb* and *B. pertussis* (Zimna et al., 2001; Bibova et al., 2013). We also assessed *Bb* knock-out mutants of several other transcriptionally upregulated genes (Supplementary Table S3.3), however, intracellular

survival of none of the tested mutants was significantly different from the RB50 wildtype strain.

Discussion

Most bacterial pathogens have specialized to either an intracellular or extracellular lifestyle, which determines the focus in studies on bacterial pathogenesis. The classical species of the genus *Bordetella* are broadly known as extracellular pathogens. However, an increasing number of publications have reported recovery of viable bacteria from phagocytic host cells in vitro, providing evidence for at least transient intracellular survival or persistence. There are also anecdotal reports of clinical samples harboring intracellular *B. pertussis* leading to speculation on the significance of this intracellular bacterial population in pathogenicity (Higgs et al., 2012).

Here we showed that intracellular survival and persistence is not restricted to the three classical bordetellae, but that the non-classical species *B. hinzii*, *B. pseudohinzii*, *B. trematum*, and *B. petrii* survived at equally high proportions, establishing the ability to survive internalization by phagocytic host cells as a common feature among the animal pathogenic bordetellae. Considering that the environmental species *B. petrii* did not differ from the animal pathogenic species, this common genotypic and phenotypic trait suggests the ability to survive predation by eukaryotic phagocytic cell precedes speciation in the genus.

A common ability suggests an ancestral origin and a common set of genes that are required for intracellular survival. Indeed, of the 318 *B. bronchiseptica* genes found to be transcriptionally upregulated during intracellular exposure, about 80% were present in the genomes of non-classical *Bordetella*, with 222 genes (70%) shared between all species

(Figure 3.2A). The only exception was the bird pathogen *B. avium*, which was missing six out of these 222 genes and failed to persist inside macrophages (Table 3.3). Interestingly, *B. avium* has one of the smallest genomes in the *Bordetella* genus with only 3.73 Mb in size (Sebaihia et al., 2006) suggesting that it may have undergone genome reduction during its evolution and adaptation to a specific host (Parkhill et al., 2003; Linz et al., 2016; Taylor-Mulneix et al., 2017b).

The apparent localization of the intracellular *B. bronchiseptica* within phagosomes (Figure 3.1C) suggests that the bacteria are rapidly exposed to a variety of damaging conditions, including low pH, bactericidal factors and resource starvation. To survive such harsh conditions many pathogenic bacteria have evolved mechanisms to maintain cell homeostasis and prevent DNA damage and cell death (Simmons et al., 2008). We observed the transcriptional hallmarks of a general SOS response (Bearson et al., 1997; Lund et al., 2014), characterized by suppression of cell division via downregulation of the fts locus (Table 3.2) and by upregulation of DNA repair genes, of protein chaperone genes, and of *B. bronchiseptica* homologs (rpoH, fur, risA) of the E. coli acid tolerance genes rpoS, fur and phoP (Table 3.1 and Figure 3.5) (Simmons et al., 2008).

Intracellular persistence was also accompanied by metabolic changes (Figure 3.5 and Tables 3.1, 3.2). As expected under micro-aerophilic/hypoxic conditions inside macrophages, expression of the nuoF – nuoN genes that encode the oxidative respiratory chain was strongly suppressed. In contrast, genes of the glyoxylate/TCA cycle showed elevated expression levels, including malate synthase G gene glcB and its transcriptional activator glcC, malate dehydrogenase mdh, citrate synthase gltA, and aconitase acnB.

The glyoxylate cycle is important in the utilization of acetate or fatty acids as the main carbon source and may be essential to provide hexoses for nucleotide and amino acid biosynthesis under intracellular conditions (Pellicer et al., 1999; Munoz-Elias and McKinney, 2006). *B. bronchiseptica* genes involved in biosynthesis of nucleotides, amino acids and fatty acids were indeed upregulated, consistent with limited access to these molecules. The absence of the malate transcriptional activator glcC and tricarboxylic transporter BB1908 may explain the failure of *B. avium* to persist inside macrophages.

Many intracellular pathogens such as Burkholderia pseudomallei employ protein secretion systems to facilitate replication and spread inside their hosts (Stevens et al., 2002). Interestingly, our assays were conducted at 37°C, a temperature known to induce phosphorylation of bvgA, which in turn induces expression of virulence factors (Prugnola et al., 1995). Yet under these intracellular conditions *B. bronchiseptica* displayed strong suppression of other known virulence factors, including the operons encoding both T3SS and the adenylate cyclase toxin (ACT) expression, modification and secretion. While stress conditions such as low pH have been reported to induce the expression of virulence factors in many pathogenic bacteria (Rathman et al., 1996; Bearson et al., 1997), the suppression of virulence in *B. bronchiseptica* occurred despite their intracellular vacuolar location where similar low pH environments are expected. We had earlier reported that the avirulent stage is required for survival, persistence and replication of *B. bronchiseptica* within amoeba (Taylor-Mulneix et al., 2017a), which strongly suggests that repression of virulence within the intracellular environment is part of an ancient conserved stress response in the genus.

Taken together, our results show that upon internalization by macrophages a certain proportion of bordetellae are killed, but thousands of bacteria can adapt and modulate gene expression to cope with this new environment. Rapid transcriptional adaptation was marked by what can be considered a general stress response against professional phagocytes that included increased expression of genes involved in DNA and protein repair, acid tolerance and metabolism (Figure 3.5). Conservation of these genes throughout the genus and the demonstrated ability of non-classical species, including the environmental *B. petrii,* to persist inside macrophages suggests that this response to phagocytes is not confined to the commonly studied classical bordetellae. It appears to represent an ancient pathway that preceded speciation in the genus and thus likely arose from a common ancestor. The two independent but interconnected transmission cycles of *B. bronchiseptica* in environmental amebae and in mammalian hosts (Figure 3.5) lead us to speculate that early interaction with these environmental phagocytes may have played a role in the origin of this response, which subsequently facilitated the adaptation to higher animals and thus the evolution of *Bordetella* from environmental microbes to animal and human pathogens.

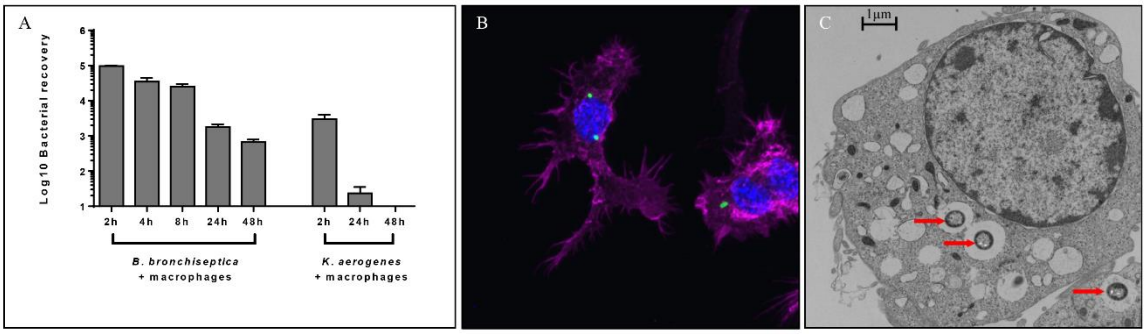


Figure 3.1. Intracellular survival of *B. bronchiseptica* in murine-derived macrophages. (A) Recovery of viable *B. bronchiseptica* RB50 and K. aerogenes post internalization by RAW 264.7 macrophages. **(B)** Z-stack fluorescence microscopy localizing *B. bronchiseptica* inside RAW 246.7 cells at 2 h post internalization (p.i.). Purple - F-actin; blue - nucleus; green - *B. bronchiseptica*. (C) Transmission electron microscopy of a RAW 264.7 macrophage containing *B. bronchiseptica* RB50 at 2 h p.i. Red arrows depict bacteria in the cell phagosomes. Scale bar: 1 μm.

Table 3.1. Upregulated genes in intracellular *B. bronchiseptica*.

Locus_tag	Gene	logFC	P-value	Description
DNA repair				
BB0180	*dksA*	3.8	2.4E-12	RNA polymerase-binding transcription factor
BB1919	*dnaB*	1.4	5.4E-08	Replicative DNA helicase
BB2076	*recA*	1.8	2.1E-08	Recombinase RecA
BB2935	*dps*	2.0	2.0E-07	Putative DNA-binding protein
Oxidative stress and pH homeostasis				
BB0020	*risA*	2.1	4.3E-06	Transcriptional regulator RisA
BB1837	*rpoE*	3.2	1.7E-12	RNA polymerase sigma factor RpoE
BB2275	*iscR*	4.2	1.7E-12	Transcriptional regulator IscR
BB2276	*iscS*	2.5	3.3E-10	Cysteine desulfurase
BB2279	*hscB*	1.6	1.5E-07	HscB chaperone
BB2281	*fdx*	1.6	5.5E-04	Ferredoxin, 2Fe-2S
BB3080	*slyA*	3.0	7.2E-06	Transcriptional regulator SlyA
BB3766	*msrP*	1.6	8.7E-08	Protein-methionine-sulfoxide reductase
BB3800	*msrB*	2.1	1.9E-07	Peptide methionine sulfoxide reductase
BB4506	*rpoN*	3.4	9.5E-12	Sigma(54) modulation protein RpoN
BB3942	*fur*	2.5	3.3E-08	Ferric uptake regulator
BB4835	*rpoH*	4.5	2.4E-12	RNA polymerase sigma factor RpoH
Protein folding				
BB0178	*hslU*	4.1	2.3E-12	ATP-dependent protease
BB0179	*hslV*	6.0	1.9E-12	ATP-dependent protease
BB0295	*secB*	1.6	1.3E-07	Protein-export protein
BB0501	*htpG*	4.2	2.8E-10	Chaperone protein
BB0962	*groEL*	4.4	8.4E-11	60 kDa chaperonin
BB0963	*groES*	7.8	6.7E-14	10 kDa chaperonin
BB2256		2.4	8.4E-11	ATP-dependent protease
BB3170	*hfq*	2.4	3.0E-07	RNA-binding protein
BB3293	*clpB*	2.6	5.5E-10	Chaperone protein
BB3933	*dnaJ*	1.7	1.1E-07	Chaperone protein
BB3934	*dnaK*	3.9	9.7E-11	Chaperone protein
BB3936	*grpE*	3.9	2.6E-10	Chaperone protein
Metabolism				
BB0095	*glcB*	1.9	4.2E-06	Malate synthase G
BB0096	*glcC*	1.9	1.5E-06	Malate synthase G transcriptional regulator
BB3682	*sdhC*	2.3	1.0E-09	Succinate dehydrogenase cytochrome B
BB3684	*mdh*	2.8	8.9E-10	Malate dehydrogenase
BB1850	*acnB*	1.4	1.04.E-7	Aconitate hydratase B

BB4150		2.4	1.5E-07	Putative short-chain dehydrogenase
BB3474	*ompA*	3.1	3.9E-11	Outer membrane protein A
BB3759	*plsX*	1.6	1.9E-09	Phosphate acyltransferase
BB3771	*pagL*	2.4	1.3E-08	Lipid A deacylase
BB0233		2.9	2.3E-09	Putative AMP-binding enzyme
BB1556		4.1	2.4E-12	ABC transporter, ATP-binding protein
BB4592		4.1	1.7E-12	Putative binding-protein-dependent transport
BB0097		3.9	6.4E-10	Putative dehydrogenase
BB1446	*carA*	1.5	6.8E-09	Carbamoyl-phosphate synthase
BB0235		5.1	9.5E-12	Probable transporter
BB4355	*argC*	2.6	5.4E-11	N-acetyl-gamma-glutamyl-phosphate reductase
BB1986	*argG*	1.5	2.4E-08	Argininosuccinate synthase
BB2000		2.5	1.3E-09	Putative aldolase
BB3179	*ndk*	2.3	1.9E-09	Nucleoside diphosphate kinase
BB2607	*pyrH*	1.8	2.0E-09	Uridylate kinase
BB3468	*cmk*	1.5	2.8E-08	Cytidylate kinase
BB4376	*nrdA*	2.3	1.6E-09	Ribonucleoside-diphosphate reductase
Virulence				
BB2992	*fimA*	2.6	9.7E-11	Fimbrial protein
BB2994	*bvgA*	2.6	2.6E-09	Transcriptional regulator of virulence
BB3424		1.7	3.7E-07	Fimbrial protein
BB3674	*fim2*	6.7	1.7E-12	Serotype 2 fimbrial subunit

logFC – log2 fold change

Table 3.2. Downregulated genes in intracellular *B. bronchiseptica*.

Locus_tag	Gene	LogFC	P-value	Description
O-Antigen				
BB0130	*wbmO*	-1.8	4.6E-06	O-Antigen biosynthesis protein
BB0138	*wbmG*	-2.2	1.1E-05	Nucleotide sugar epimerase/dehydratase
BB0139	*wbmF*	-1.8	9.4E-06	Nucleotide sugar epimerase/dehydratase
BB0145	*bplL*	-1.8	3.2E-06	Lipopolysaccharide biosynthesis protein
BB0146	*bplJ*	-1.6	1.2E-05	Membrane protein
Adenylate cyclase toxin				
BB0325	*cyaB*	-2.9	3.3E-09	Cyclolysin secretion ATP-binding protein
BB0326	*cyaD*	-3.4	1.0E-08	Membrane fusion protein (MFP) family protein
BB0327	*cyaE*	-3.3	5.8E-10	Protein CyaE
BB0328	*cyaX*	-3.9	1.1E-08	Adenylate cyclase transcriptional regulator
Type 3 secretion system				
BB1609	*bscF*	-2.1	6.5E-06	Putative type III secretion protein
BB1623	*bcr4*	-2.6	6.9E-08	Uncharacterized protein
BB1624	*bscI*	-2.0	1.3E-04	Putative type III secretion protein
BB1625	*bscJ*	-2.3	4.4E-07	Lipoprotein
BB1627	*bscL*	-2.5	1.1E-04	Type III secretion protein
BB1628	*bscN*	-1.9	5.3E-06	Type III secretion ATP synthase
BB1630	*bscP*	-2.8	4.8E-07	Type III secretion protein
BB1631	*bscQ*	-3.0	4.3E-08	Type III secretion protein
BB1632	*bscR*	-2.1	6.5E-05	Type III secretion protein
BB1634	*bscT*	-3.0	3.0E-06	Type III secretion protein
BB1635	*bscU*	-2.8	7.9E-07	Type III secretion protein
BB1636	*bscW*	-2.8	9.9E-05	Type III secretion protein
BB1637	*bscC*	-1.9	3.9E-07	Type III secretion protein
Electron transport				
BB3827		-4.6	6.0E-10	Putative membrane protein
BB3828	*nuoN*	-3.0	6.9E-10	NADH-quinone oxidoreductase subunit N
BB3829	*nuoM*	-2.3	2.5E-08	NADH-ubiquinone oxidoreductase, chain M
BB3830	*nuoL*	-2.4	6.9E-10	NADH-ubiquinone oxidoreductase, chain L
BB3834	*nuoH*	-1.7	1.3E-07	NADH-quinone oxidoreductase subunit H
BB3835	*nuoG*	-1.9	3.7E-08	NADH-quinone oxidoreductase
BB3836	*nuoF*	-2.0	5.7E-09	NADH-quinone oxidoreductase subunit F
Cell division				
BB4188		-2.2	2.4E-08	Uncharacterized protein
BB4193	*ftsZ*	-2.5	4.3E-10	Cell division protein FtsZ
BB4194	*ftsA*	-3.4	3.3E-09	Cell division protein FtsA
BB4195	*ftsQ*	-3.4	6.0E-09	Cell division protein FtsQ
BB4196	*ddl*	-3.5	2.1E-07	D-alanine--D-alanine ligase

BB4197	*murC*	-3.0	1.1E-08	UDP-N-acetylmuramate--L-alanine ligase
BB4198	*murG*	-3.6	8.1E-10	Undecaprenyl-PP-MurNAc-pentapeptide-UDPGlcNAc GlcNAc transferase
BB4199	*ftsW*	-3.6	3.6E-08	Cell division protein FtsW
BB4200	*murD*	-3.0	5.2E-09	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase
BB4201	*mraY*	-2.8	1.3E-08	Phospho-N-acetylmuramoyl-pentapeptide-transferase
BB4202	*murE*	-2.1	1.0E-09	Multifunctional fusion protein

logFC – log2 fold change

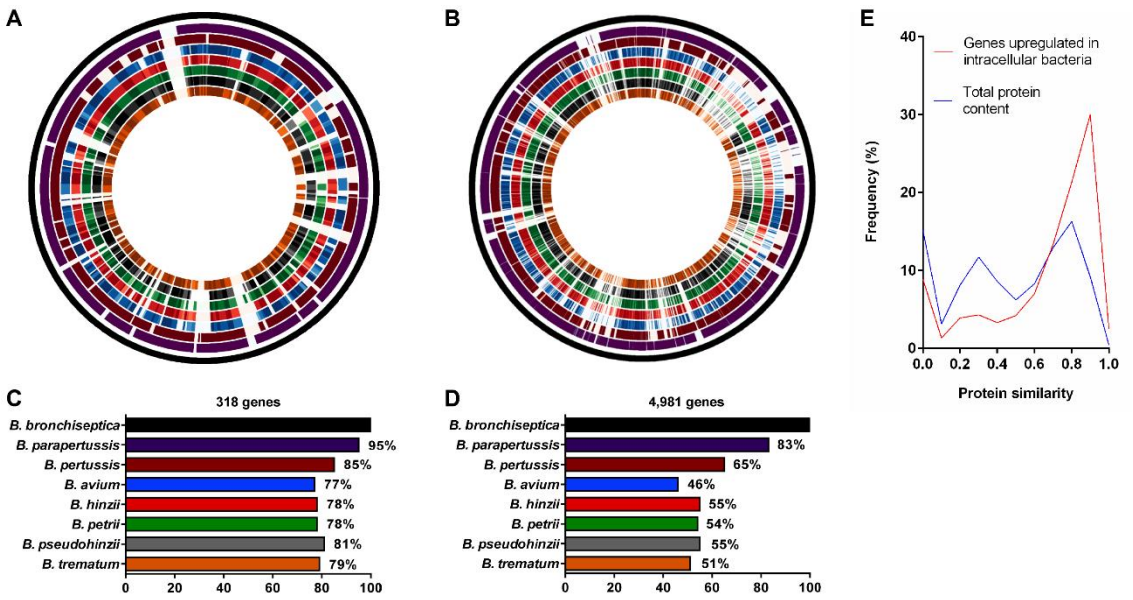


Figure 3.2. Comparative analysis of genes upregulated during intracellular survival and their presence/absence in non-classical *Bordetella* species. Analysis of protein similarity of (A) 318 *B. bronchiseptica* genes upregulated in macrophages and (B) 4,981 genes in the entire genome of *B. bronchiseptica* strain RB50 in comparison to the non-classical *Bordetella* species. From outside to inside: Circle 1: Virtual genome of *B. bronchiseptica* strain RB50. Circles 2–8: Visual representation of protein similarity between *B. bronchiseptica* RB50 and classical (circles 2–3) and the non-classical species (circles 4–8) represented as color shades with darker shades indicating higher protein similarity. (C) 77–81% of the genes upregulated in intracellular *B. bronchiseptica* were conserved among the non-classical species, (D) in contrast to only 46–55% of the 4,981 *B. bronchiseptica* genes in the entire genome. (E) Line plot showing the frequency of protein similarities.

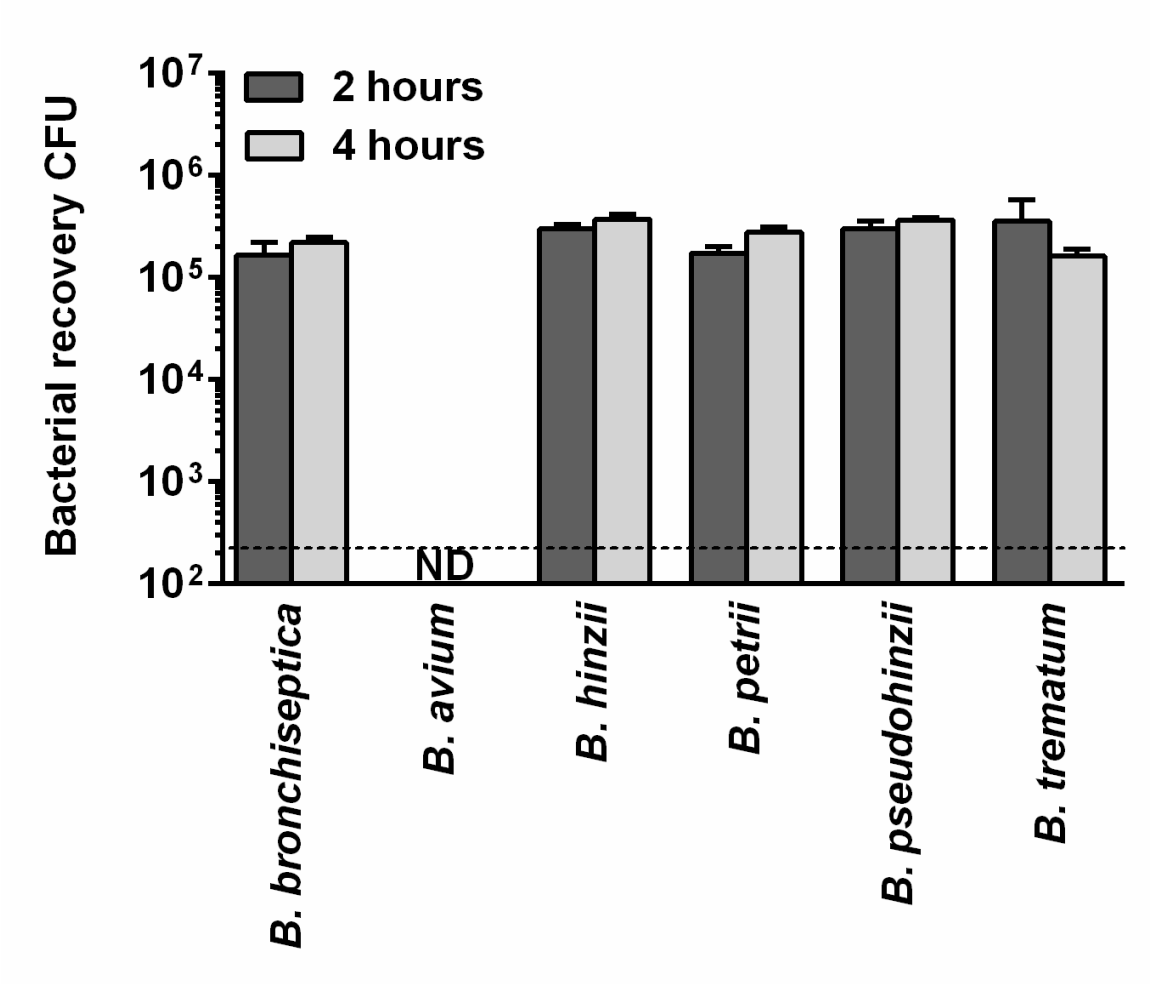


Figure 3.3. Intracellular survival of non-classical bordetellae within macrophages. The non-classical bordetellae were recovered from macrophages in numbers similar to *B. bronchiseptica*. The only exception, *B. avium*, failed to survive internalization by macrophages.

Table 3.3. Genes upregulated in intracellular *B. bronchiseptica* and absent in *B. avium.*

Locus_tag/gene	logFC	Protein
BB0096/*glcC*	1.9	Malate synthase transcriptional regulator
BB1908	1.6	Tripartite tricarboxylate transporter receptor
BB1948	1.9	Glutamate transport periplasmic receptor
BB1999	2.5	Tripartite tricarboxylate transporter receptor
BB2944	1.6	LysR-family transcriptional regulator
BB4150	2.4	3-ketoacyl-(acyl-carrier-protein) reductase

logFC – log2 fold change

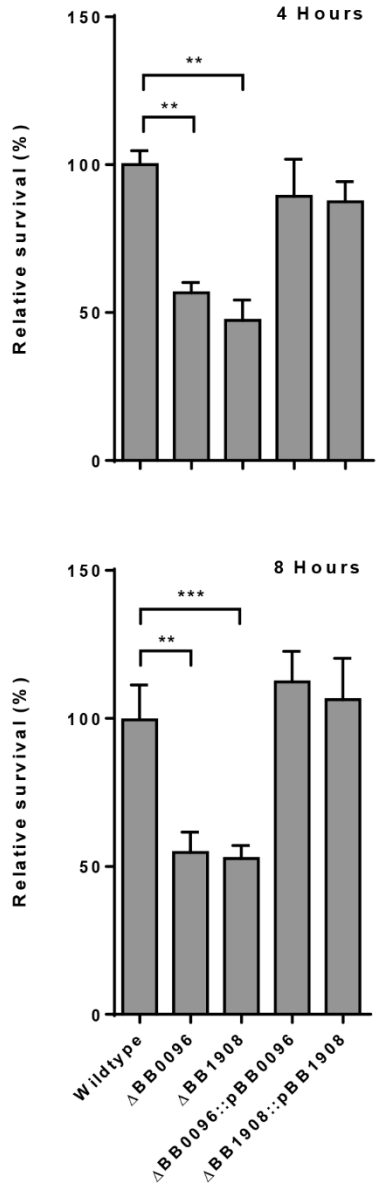


Figure 3.4. Assessment of *B. bronchiseptica* deletion mutants for intracellular survival. Deletion of malate synthase regulator gene BB0096 or tripartite tricarboxylate transporter gene BB1908 resulted in significantly reduced bacterial recovery. Plasmid-borne complementation of the gene deletion restored the wildtype phenotype. $**p < 0.01$; $***p < 0.001$.

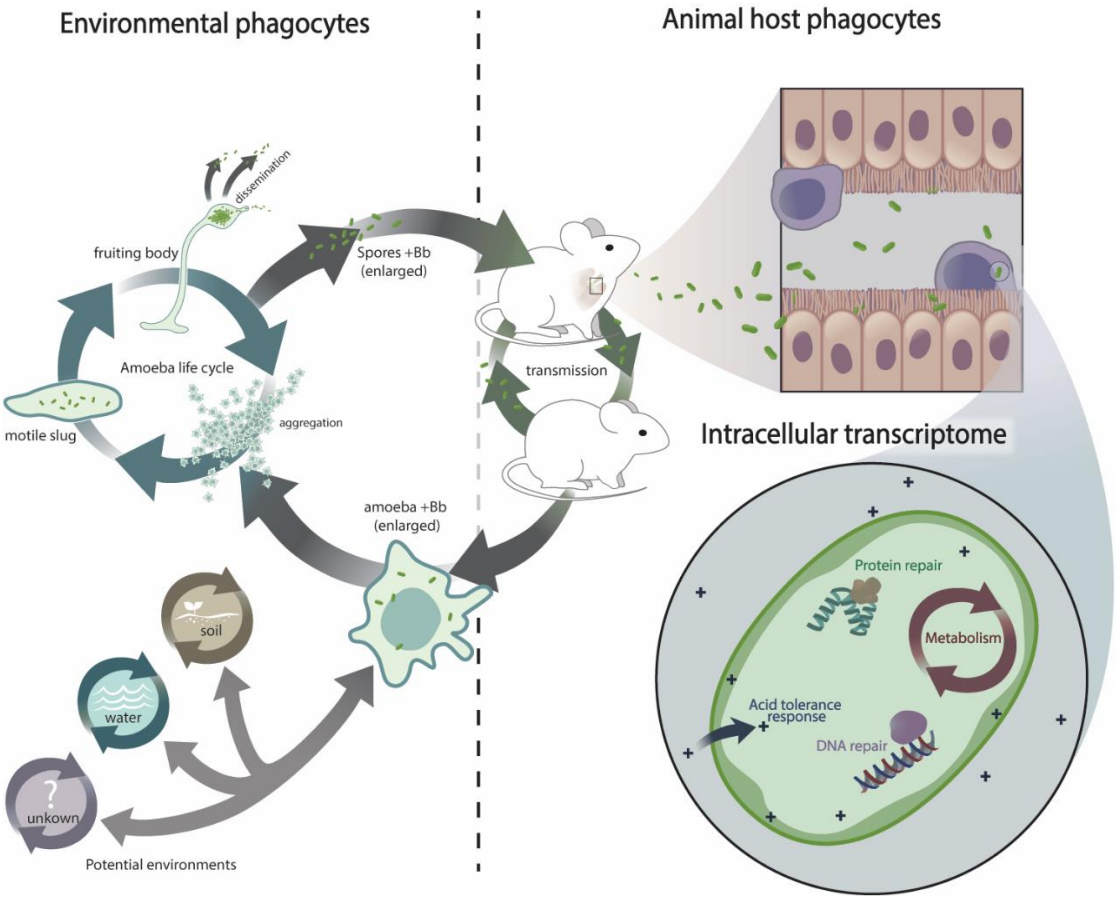


Figure 3.5. Schematic representation of *Bordetella* exposure to eukaryotic phagocytes and its transcriptional response. Hypothetical scenario depicting *Bordetella* exposure to interconnected lifecycles and adaptation from environmental phagocytes to animal phagocytes.

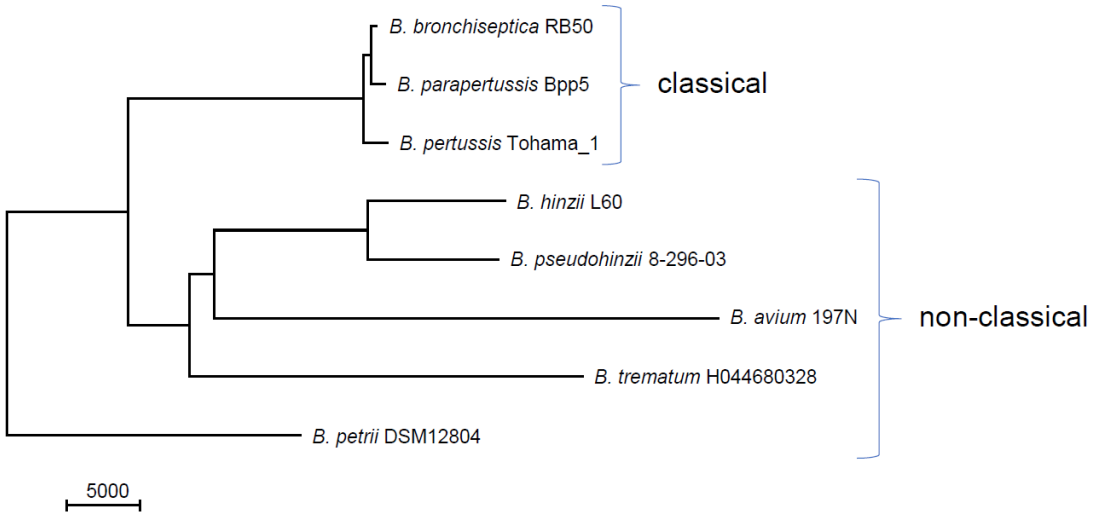
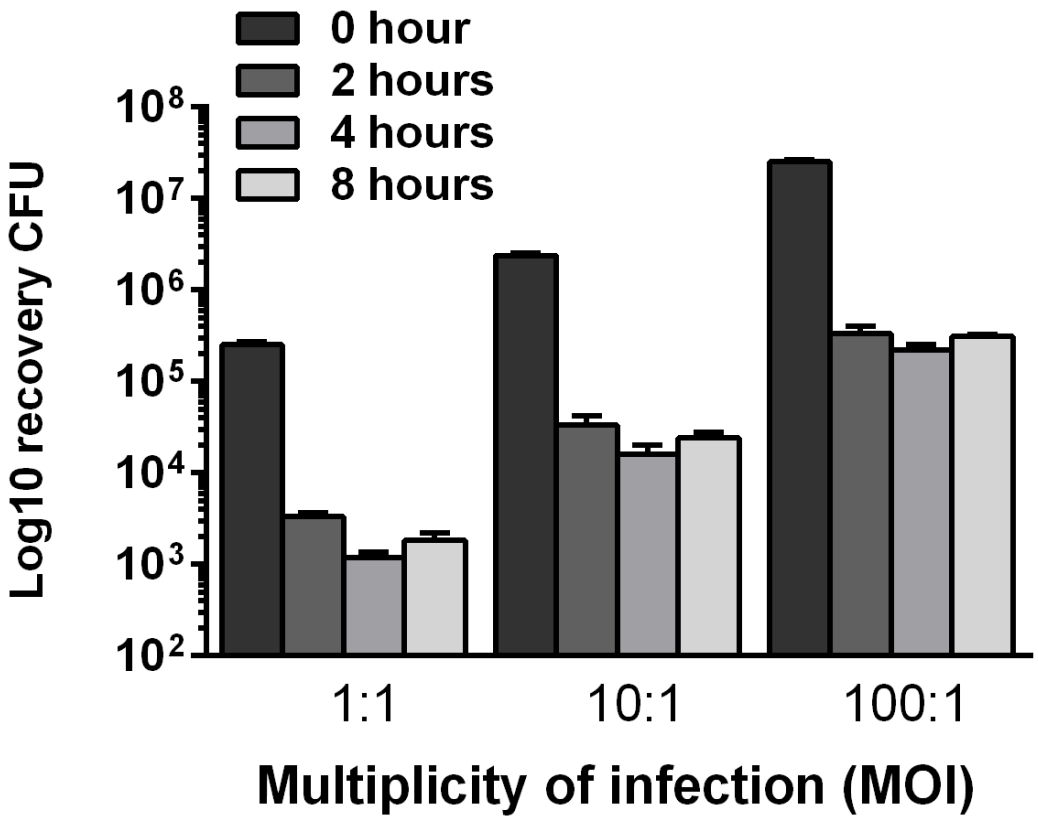


Figure S3.1. Genome-wide SNP-based phylogeny of *Bordetella* species.

The genomes of the eight analyzed species formed three phylogenetic clades, similar toLinz et al., 2016. The phylogeny was based on 373,499 base pairs shared between all eight genomes. Genes with more than one copy per genome such as 16S rRNA were not included. Scale bar: number of differences.



FigureS3.2. Intracellular survival of *B. bronchiseptica*strain RB50 in macrophages At several time points post inoculation with a multiplicity of infection (MOI) of 1:1, 10:1or100:1. Bacteria were consistently recovered at 0.7% to 1.0% of the inoculum at each of the used MOIs.

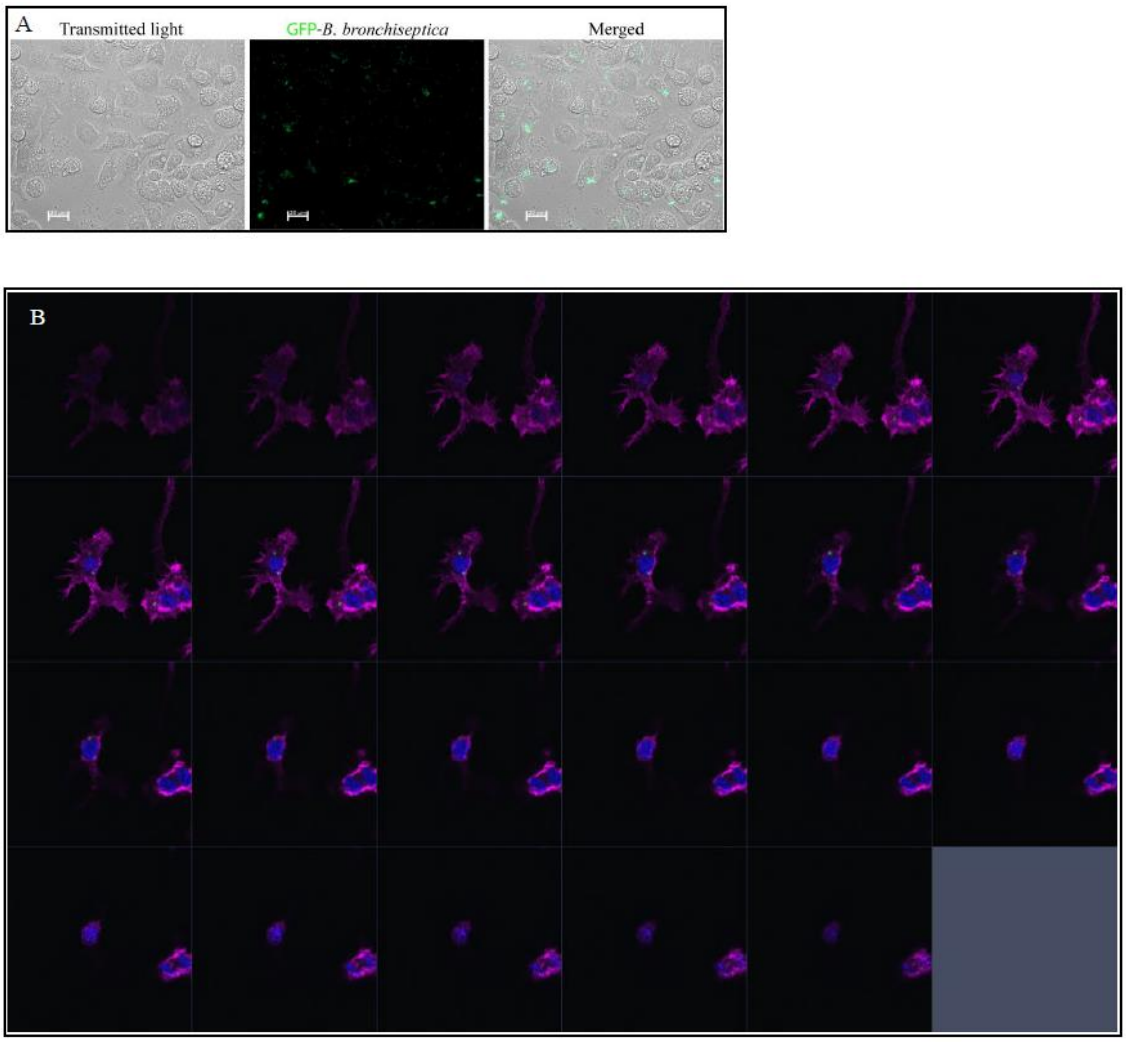


Figure S3.3. Confocal microscopy and Z-stack gallery images. A) Confocal fluorescent microscopy of RAW 264.7 macrophages with GFP-tagged B.*bronchiseptica* RB50 2 hours p.i. Scalebar: 20μm. **B)** Complete gallery of z-stack images confirming intracellular localization of *B. bronchiseptica* RB50. Z-stack images were taken at 0.5μm intervals. purple – F-actin; blue – nucleus; green–*B. bronchiseptica*.

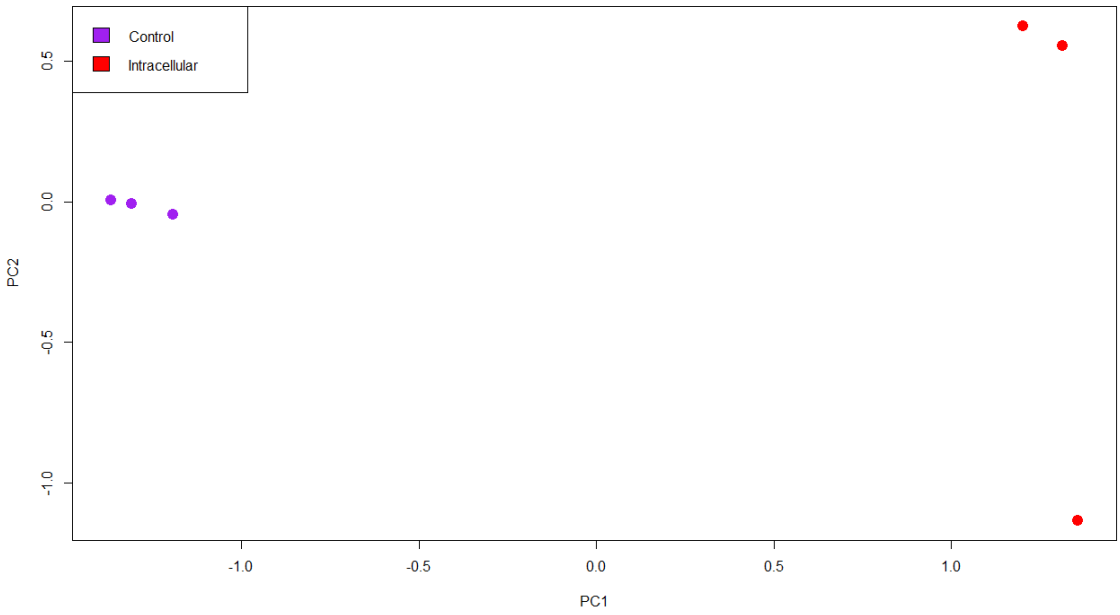


Figure S3.4. Principal component analysis. Principal-component (PC) analysis of normalized transcript abundance in intracellular *B. bronchiseptica* RB50 incubated with macrophages for 2 hours compared to RB50 cultured in DMEM + 10% FBS medium as the control.

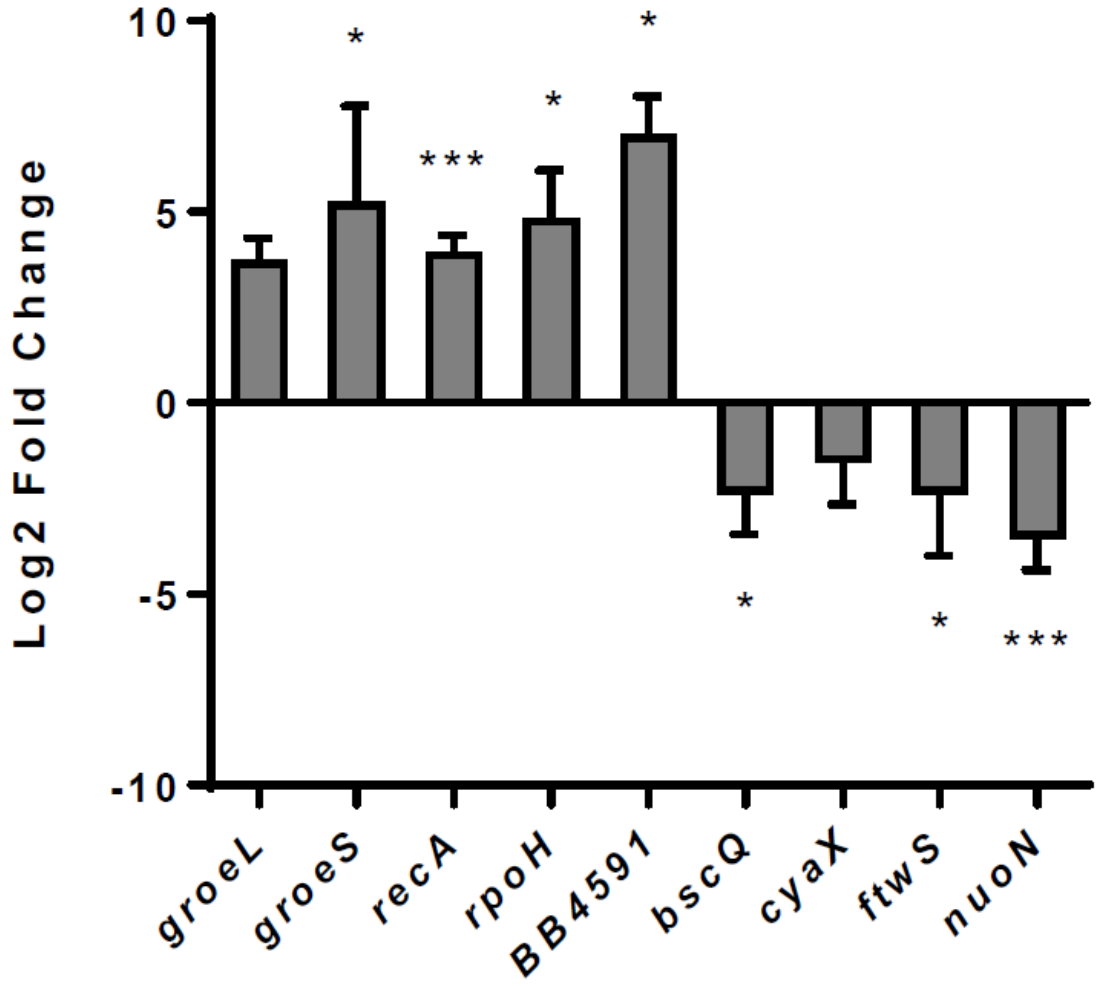


Figure S3.5. Verification of *B. bronchiseptica* gene expression inside RAW 264.7 Macrophages by qRTPCR. Expression of 9 genes identified in the transcription data displayed as the mean 2-fold change in gene expression levels (with standard deviations) of *B. bronchiseptica* RB50 isolated from macrophages at 2hours p.i. relative to RB50 cultured in DMEM + 10% FBS medium. Values are based on 3 independent experiments. Asterisks indicate significantly different expression levels; *$p \leq 0.05$;***$p < 0.001$.

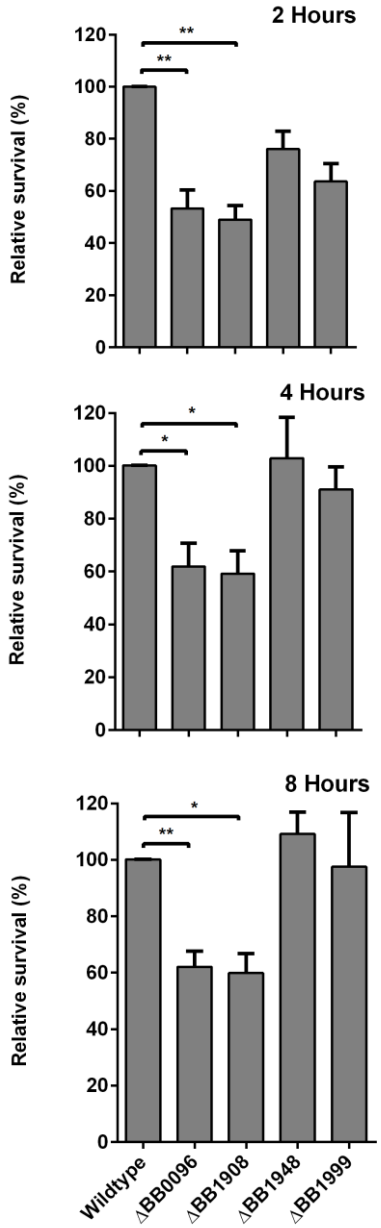


Figure S3.6. Assessment of *B. bronchiseptica* deletion mutants for intracellular survival. Deletion of malate synthase regulator gene BB0096 or tripartite tricarboxylate transporter gene BB1908 resulted in significantly reduced bacterial recovery at 2, 4, and 8 hours post inoculation. In contrast, the knock-out mutants of genes BB1948 and BB1999 did not display significant differences in comparison to wild type bacteria.

Table S3.1. Primers for quantitative real-time PCR.

Gene	Direction	Sequence (5' → 3')	Bp	Tm
groeL	Forward	GACGATCCGTATGTCCTGATCTA	23	63.0°C
	Reverse	CGGATGTTGTTCACGACCAG	20	63.0°C
groeS	Forward	GAAGACCGAAGACGGCAAGAT	21	64.0°C
	Reverse	CTGGATCACGGCGAGGATTT	20	64.0°C
recA	Forward	CAAGCTGACGGCCACCAT	18	58.5°C
	Reverse	GCACCGAGGAATAGAACTTGAG	22	55.3°C
rpoH	Forward	CGCAAGCTGTTCTTCAACCT	20	63.0°C
	Reverse	TCATCGTCGTCCTGGCTTTC	20	64.0°C
BB4591	Forward	ACCTGACCCTTAGCCACAAC	20	64.0°C
	Reverse	TTCGTCGATGAAGGCGAACA	20	64.0°C
bscQ	Forward	TACATCGGCCTGACGGTTC	19	64.0°C
	Reverse	CATGCCTCGACAGACATCCT	20	64.0°C
cyaX	Forward	GCTCGATGCGCAGAGTTAT	19	62.0°C
	Reverse	CGCATACGACACATAGGGATAG	22	62.0°C
ftwS	Forward	GGCATCAACGGCAAGTATTTC	21	62.0°C
	Reverse	AATGCGACAACTGGTAGGC	19	62.0°C
nuoN	Forward	CATCGTCCAGACCAACTTCAA	21	62.0°C
	Reverse	CGTAGGTCAGCATGTAGAACAG	22	62.0°C
16S rRNA	Forward	GGATTAGATACCCTGGTA	18	46.0°C
	Reverse	CCGTCAATTCCTTTGAGTTT	20	50.9°C

Table S3.2. Primers for generation of gene knock-out mutants.

Gene	Sequence (5' → 3')
BB0096-UF	CCTATGCTAGGGCGGCCGCACTAGGGTCGATCTGGATTTCGAAGTGCAGGC
BB0096-intUR	GAGCACGCCATGGTACGACCGTAGCCCGTCGACGCCTGTTTCCATGACTTG
BB0096-intDF	CAAGTCATGGAAACAGGCGTCGACGGGCTACGGTCGTACCATGGCGTGCTC
BB0096-DR	GCAGGTCCGGATCTGTACACCTAGGAGCTGCGCGAGTTCGACCTGCAGC
BB1908-UF	CCTATGCTAGGGCGGCCGCACTAGTCTGTGGATCGGCTTGCTTGGATGAG
BB1908-intUR	GGCTTATTGCAGAACGATGTTGGCCTGGAGCAGTTGCAAACCTTTGCGTAGCAT
BB1908-intDF	ATGCTACGCAAAGGTTTGCAACTGCTCCAGGCCAACATCGTTCTGCAATAAGCC
BB1908-DR	GCAGGTCCGGATCTGTACACCTAGGGGATGGATCAGGTAGATGCCGAACGTGAGC
BB1948-UF	CCTATGCTAGGGCGGCCGCACTAGTCGAGCAGTATTCGGAATCGTCGTCGTCTTCCCAGT
BB1948-intUR	CGCTTAGTCGATCTTCACGTTGGCTTCACGACGTTGCATGGTTTCCCCTGTAGGAAG
BB1948-intDF	CTTCCTACAGGGGAAACCATGCAACGTCGTGAAGCCAACGTGAAGATCGACTAAGCG
BB1948-DR	GCAGGTCCGGATCTGTACACCTAGGGCCTATGTACTTCTTGACGGTCTCTGGCGTTGC
BB1999-UF	CCTATGCTAGGGCGGCCGCACTAGGTCGTTCGATAACGCCGTG
BB1999-intUR	TGGCCTGCTTGAAGACCAACCTGAAGCCGATCTTCG
BB1999-intDF	ATCGGCTTCAGGTTGGTCTTCAAGCAGGCCACGTTC
BB1999-DR	GCAGGTCCGGATCTGTACACCTAGGGCCTGCATCTGCTCGAAGGC
pSS4245-Forward	CTAGTGCGGCCGCCCTAGCATAGG
pSS4245-Reverse	CCTAGGTGTACAGATCCGGACCTGC

Red: primer region complementary to pSS4245

Blue: primer region complementary to the corresponding intUR/intDF primer

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CHAPTER 4

INSIGHTS IN THE EVOLUTION AND CONSERVATION OF INTRACELLULAR SURVIVAL IN THE GENUS BORDETELLA AND THE IMPLICATION IN CURRENT STRATEGIES AGAINST PERTUSSIS DISEASE

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Abstract

The classical bordetellae possess several partially characterized virulence mechanisms that are studied in the context of a complete extracellular life cycle in their mammalian hosts. Yet, classical bordetellae have repeatedly been reported within dendritic cells and alveolar macrophages in clinical samples, and *in vitro* experiments convincingly demonstrate that the bacteria can survive intracellularly within mammalian phagocytic cells, an ability that appears to have descended from ancestral progenitor species that lived in the environment and acquired the mechanisms to resist unicellular phagocytic predators. Many pathogens, including *Mycobacterium tuberculosis*, *Salmonella enterica*, *Francisella tularensis*, and *Legionella pneumophila* are known to parasitize and multiply inside eukaryotic host cells. This strategy provides protection, nutrients, and the ability to disseminate systemically. While some work has been dedicated at characterizing intracellular survival of *Bordetella pertussis*, there is limited understanding of how this strategy has evolved within the genus *Bordetella* and the contributions of this ability to bacterial pathogenicity, evasion of host immunity and systemic dissemination. Here, we explore the mechanisms that control the metabolic changes accompanying intracellular survival and how these have been acquired and conserved throughout the evolutionary history of the *Bordetella* genus and discuss the possible implications of this strategy in the persistence and reemergence of *B. pertussis* in recent years.

Genus *Bordetella*

The bordetellae are gram negative coccobacilli of the class betaproteobacteria known to cause disease in a wide range of animals including small mammals and humans. Despite widespread vaccination, *Bordetella pertussis* –the etiological agent of whooping cough or Pertussis, still affects 24.1 million with 160,700 deaths in children younger than 5 years (1). Characterized by paroxysmal cough accompanied by an inspirational whooping sound (hence the name), Pertussis can last for months and cause severe respiratory complications and even death particularly in infants and adults with underlying health conditions. *B. pertussis* is closely related to *Bordetella parapertussis* and *Bordetella bronchiseptica*, and the three species are known as the "Classical bordetellae". In contrast to the human-restricted *B. pertussis*, *B. parapertussis* has two major lineages, one of which, *B. parapertussis*$_{hu}$, causes whooping cough-like disease in children, and the other lineage, *B. parapertussis*$_{ov}$ causes pneumonia in sheep (2, 3), while *B. bronchiseptica* is a respiratory pathogen of diverse mammals that causes a variety of pathologies ranging from chronic and often asymptomatic infection to acute bronchopneumonia such as kennel cough in dogs (4).

In contrast to the closely related classical bordetellae, several *Bordetella* species with broader genetic diversity have been identified, collectively referred to as "non-classical" *Bordetella*. Members of the non-classical bordetellae are also known to be animal-specific pathogens. The emerging human pathogen *B. holmesii*, initially isolated from the blood of septicemic patients (5) has since increasingly been isolated from patients with pertussis-like respiratory infections (6, 7). *B. avium* causes respiratory infections in poultry and wild birds (8). The other 'avian' species, *B. hinzii*, colonizes the respiratory tracts of poultry and was shown to

cause disease during experimental infection in turkeys (9, 10) and has also been isolated from immunocompromised humans with respiratory disease and septicemia (11, 12). The closely related *B. pseudohinzii* was identified as a pathobiont in mouse breeding colonies of commercial vendors (13, 14). *B. trematum*, an opportunistic human pathogen, can cause severe skin disease and chronic otitis media (15). The environmental species *B. petrii*, originally isolated from an anaerobic bioreactor enriched with river sediment (16), has subsequently been isolated from soil samples and also from immunocompromised patients (17, 18). These *Bordetella* species share many phenotypic characteristics that makes them successful animal pathogens.

Genome sequencing and Multi-locus Sequence Typing of the classical bordetellae revealed that *B. parapertussis* and *B. pertussis* independently evolved from a *B. bronchiseptica*-like ancestor (19, 20). Despite differences in host range and disease, the classical bordetellae are over 98% similar on the sequence level and share many important virulence factors, including the well-known toxins such as adenylate cyclase toxin, pertussis toxin and dermonecrotic toxin, and putative adhesins such as pertactin (19). Since these virulence factors are present in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, but absent from the non-classical bordetellae, genes encoding these factors are believed to have been acquired before the divergence of the classical bordetellae (3, 21). Overall, gain and loss of multiple genes, including those encoding bacterial toxins, protein secretion systems and other virulence-associated factors, appeared to have shaped the diversification and speciation in the genus. Gene loss was more frequent than gene gain throughout the evolution, and loss of hundreds of genes was associated with the origin of several host-restricted species, including the recently evolved human pathogens *B. pertussis*, *B. parapertussis* and *B. holmesii* (19, 21).

Old Tricks New *Bordetella*

The genus *Bordetella* has largely been considered host-restricted pathogens with variable host-specificity. However, the recent discovery of several environmental species and 16S meta-

analysis studies have revealed that the genus likely arose from an environmental origin (22). In addition, *B. bronchiseptica* was found to infect and persist within the amoeba *Dictyostelium discoideum*, to utilize the amoeba's life cycle to translocate to the amoeba fruiting bodies, and to disseminate along with amoeba spores (23). These observations suggest that amoeba may represent an environmental niche for this, and possibly other, animal-pathogenic *Bordetella* species (24). Similar to *B. bronchiseptica*, many bacteria have been reported to form an endosymbiotic relationship with environmental reservoirs such as amoeba (25-27). This interaction provides protection against external danger, and possibly a competitive advantage against other bacteria, while enhancing bacterial dissemination along with the amoebic host. During interaction with amoeba, *B. bronchiseptica* undergoes phenotypic modulation inducing the expression of genes involved in chemotaxis, motility and growth (e.g. flagella *flhD* and chemotaxis gene *cheZ*), while suppressing the expression of virulence factors such as the adenylate cyclase toxin (encoded by *cyaA*) and filamentous hemagglutinin encoded by *fhaB* (23). Thus, in addition to possessing mechanisms to colonize a wide range of mammals including swine, rats, rabbits, sheep, dogs and cats, *B. bronchiseptica* is able to establish a successful symbiotic relationship with amoeba in the environment outside a mammalian host.

The ability to adapt to profoundly different environments requires the capacity to sense and respond to changes in the surroundings. *Bordetella* species have evolved tools to rapidly modulate transcriptional gene expression in order to response to these changes. Activation of virulence in *Bordetella* is largely controlled by BvgAS two-component system, which consists of a sensor protein, BvgS, a transcriptional activator, BvgA, and a transcriptional repressor, BvgR. During growth at temperatures at and below 25ºC, the sensor protein is unphosphorylated and inactive, and the bacteria are in the so-called Bvg minus (Bvg^-) phase in which transcription of virulence genes is repressed. When receiving inducing signals such as temperature of 37ºC, which mimics presence in a mammalian host, the BvgS sensor protein autophosphorylates, goes through a phosphorylation

cascade and subsequently transfers a phosphor group to BvgA. Upon phosphorylation by BvgS, BvgA binds to the promoter regions of the Bvg-activated genes and induces the transcription of virulence genes in response to temperature. This culminates in downstream activation of virulence proteins, such as the type six secretion system (T6SS), type three secretion system (T3SS), pertactin (PRN), filamentous hemagglutinin (FHA), adenylate cyclase toxin (ACT), pertussis toxin (PTX), and others (3 refs).

The virulence-repressed Bvg$^-$ phase and the environment

Under laboratory conditions, the classical *bordetellae*, including *B. bronchiseptica*, can respond to different environmental stimuli by switching between two distinct lifestyles. When cultured at 37°C in the so-called Bvg$^+$ phase, *in vitro* conditions that mimic the infectious phase at temperatures in the mammalian host (28), expression of genes associated with colonization and virulence is up-regulated (29, 30). Activation of the Bvg$^+$ phase is necessary and sufficient to facilitate bacterial colonization during infection. While in this phase, flagella and chemotaxis genes are repressed, and the bacteria are non-motile (31). However, when cultured at 25°C, mimicking environmental conditions, *B. bronchiseptica* adopts a second lifestyle during which gene expression of virulence-related factors is repressed, while transcription of a large alternative set of genes is activated, the so-called Bvg$^-$ phase. However, an actual role for the Bvg$^-$ phase has long been hypothetical, mainly due to the lack of a clear role for the virulence-repressed state during *in vivo* studies. Several authors have speculated that activation of virulence-repressed genes might serve a role during environmental persistence (refs). Since many of the Bvg$^-$ phase transcribed genes are predicted to be metabolic enzymes and transport proteins, they are suspected to enhance acquisition of nutrients, growth and proliferation in environmental settings. In addition, the transcriptionally active genes in the Bvg$^-$ phase include chemotaxis and flagella synthesis genes, suggesting bacterial motility.

Even though *B. pertussis* possess the ability for phenotypic modulation between the two Bvg states (29, 30, 32), to this date, no evidence of an outside-host or environmental niche has been found for this human restricted pathogen. This has led to the hypothesis that genes induced during the Bvg^- phase are likely the vestigial remnants of an important phenotype of ancient *Bordetella*. However, contrary to what is to be expected under this hypothesis, the Bvg regulon in *B. pertussis* is genetically conserved, demonstrating that the system has been under purifying selective pressure. In addition, the ability to switch between life styles seems to be conserved among the bordetellae, as *bvgA* and *bvgS* gene homologs have been found in the genomes of animal-associated species as well as of the environmental *B. petrii* (21, 33-35), contradicting the "vestigial" hypothesis. Thus, it appeared likely that the virulence-repressed state indeed plays an active and important role in the biology of host-pathogenic *Bordetella*.

Then, indirect evidence and experimental data supported a biological relevance of the Bvg^- phase in pathogenic *Bordetella*. First, in addition to the identification of numerous *Bordetella*-like bacteria among 16S rRNA sequences and metagenomes from soil samples (22, 36, 37), the animal-pathogenic *B. hinzii* and *B. bronchiseptica* were shown to grow efficiently in soil extract at 25°C (22). And second, the Bvg^- phase was shown to mediate *B. bronchiseptica* interactions with the soil amoeba *D. discoideum*. Tens of thousands *B. bronchiseptica* wild-type and Bvg^- phase-locked bacteria were isolated from the amoeba fruiting bodies, in contrast to only one hundred Bvg^+ phase-locked *B. bronchiseptica*, indicating that the Bvg^- phase is essential for bacterial survival inside amoeba trophozoites and fruiting bodies (23, 24). Interestingly, *B. bronchiseptica* not only survived, but multiplied inside amoeba fruiting bodies, as indicated by significantly increasing bacterial numbers over time, and were then disseminated along with amoeba spores by wind and animals such as insects (23). The ability to switch between the Bvg^+ and Bvg^- life styles appears to be conserved amongst the bordetellae (perhaps with the exception of *B. ansorpii*) as *bvgA* and *bvgS* gene homologs have been identified in the genomes of animal-associated species as well as

of the environmental *B. petrii* (21, 33, 34, 38). Together, these observations suggest the presence of potential environmental reservoirs for many, if not most, animal-pathogenic and human-pathogenic *Bordetella* species. Although many *Bordetella* species are adapted to mammals, in which the Bvg^+ phase is active, they still conserved the ability to respond to changes such as temperature fluctuations (39) by switching to the ancient Bvg^- phase for proliferation in environmental conditions.

Intracellular Survival and Persistence.

In vitro studies have shown that, upon entering human alveolar epithelial cell line A549, a significant portion of intracellular *B. pertussis* evades phagolysosomal fusion and remains viable in nonacidic compartments by a mechanism that is dependent on microtubule assembly, lipid raft integrity, and the activation of a tyrosine-kinase-mediated signaling (40). Extended uptake of bacteria resulted in an increase of viable intracellular bacteria from on average one after 3 hours to over five per macrophage after 24 hours. During this stage, *B. pertussis* secretes a wide range of proteins involved in stress response, iron uptake, metabolism, and regulation, which allow the bacteria to reside and persist within host cells (40). Interestingly, intracellular survival of appears to be dependent on the type of host cell as viable *B. pertussis* were found to persist for 3 days in human macrophages and epithelial cells (41, 42) but less than 24 hours in mouse dendritic cells (43). *B. parapertussis* and *B. bronchiseptica* were also found to persist inside host phagocytes, emphasizing that the ability to survive intracellularly is not unique to *B. pertussis* (43-45).

One aspect that is commonly overlooked is the role of the Bvg^- phase during intracellular persistence of *Bordetella* inside host phagocytic cells. Spontaneous mutants lacking the *bvgS* gene, which encodes the sensor component of the BvgAS regulon, and the parental wildtype *B. bronchiseptica* displayed similar viability in dendritic cells for over 72 hours post infection. These data indicated that intracellular survival is Bvg-independent or perhaps involves genes that are actively transcribed in the Bvg^- phase (43, 46). Indeed, assessment of *B. bronchiseptica* gene

expression during intracellular survival in macrophages supported these observations. Upon uptake by macrophages, *B. bronchiseptica* activates the expression of genes involved in protein repair, DNA repair, oxidative stress response, pH homeostasis, chaperone functions, and activation of specific metabolic pathways. In contrast, the expression of genes involved in bacterial virulence, which is a hallmark of Bvg-based modulation of gene expression in the mammalian host, is suppressed. None of the known virulence factors, including toxins, type 3 and type 6 secretion systems, and the adhesins pertactin and filamentous hemagglutinin, were transcriptionally active (47), and neither were any of the 205 genes that were previously identified to encode proteins secreted under Bvg^+ conditions (28). These observations suggest a critical role of the Bvg^- phase in modulating gene expression during bacterial interaction within host phagocytes.

The ability to survive inside macrophages spans beyond the classical bordetellae (47). Comparative genome analyses between *B. bronchiseptica* and non-classical *Bordetella* species revealed the conservation of genes involved in intracellular persistence; approximately 80% of the 318 transcriptionally upregulated *B. bronchiseptica* genes during intracellular persistence were present in the genomes of non-classical *Bordetella*, in contrast to ~50% of the total of 4,981 evaluated *B. bronchiseptica* genes. Phenotypic analyses validated the significance of this observation by demonstrating that both the classical and the non-classical *Bordetella* spp. can persist inside RAW 264.7 macrophages (47). The ability for intracellular persistence and evolutionary conservation of the genes suspected to be involved in it strongly suggest that intracellular survival represents an ancestral trait the origin of which precedes speciation in the genus. As such, this feature must have evolved at some point during evolution of *Bordetella* from environmental bacteria to mammalian respiratory pathogens. Since *B. bronchiseptica* and also the sheep-associated *B. parapertussis*$_{ov}$ can resist digestion and utilize the life cycle of the ubiquitous soil amoeba *D. discoideum* (23, 24) to multiply and disseminate, the ancient interaction with these and potentially other environmental phagocytes could have served as an important evolutionary

milestone on the way to intracellular survival in phagocytic cells. Thus, intracellular persistence in environmental phagocytes may have been a "training ground" – as some authors named it – for the subsequent evolution of bacteria, including *Bordetella* spp., to animal pathogens (24, 48-50). While this point of view of an ancient evolutionary adaptation preceding speciation suggests that many, if not all, animal-pathogenic *Bordetella* species can interact with amoeba, this prediction remains to be evaluated.

Metabolic changes during intracellular survival.

Internalization by macrophages triggered a strong general SOS response (51) in *B. bronchiseptica*, characterized by suppression of cell division *via* downregulation of the *fts* locus and by upregulation of DNA repair genes, of protein chaperone genes, of oxidative stress response and of acid tolerance genes (47). As expected under microaerophilic/hypoxic conditions inside macrophages, transcription of the *nuo* genes that encode the oxidative respiratory chain was strongly suppressed. In contrast, genes of the glyoxylate cycle displayed elevated expression, including the gene *glcB* encoding malate synthase G and its transcriptional activator *glcC*, malate dehydrogenase gene *mdh*, citrate synthase gene *gltA*, and aconitase gene *acnB* (47). The glyoxylate cycle is important in the utilization of acetate or fatty acids as the main carbon source and may be essential for nucleotide and amino acid biosynthesis under intracellular conditions (52). The avian pathogen *B. avium* is the only animal-adapted *Bordetella* species that lacks malate synthase transcriptional regulator *glcC*, and this species was severely impaired in its ability to persist in macrophages. Indeed, deletion of this gene in *B. bronchiseptica* resulted in a significant reduction in intracellular survival, and in-trans complementation of the knockout mutant with a plasmid-borne gene copy restored the wildtype phenotype, confirming a critical role during intra-cellular persistence (47). Besides the glyoxylate cycle, several genes of fatty acid synthesis pathways such as 3-oxoacyl-ACP reductase BB4150, long chain fatty acid Co-A ligase BB0233, outer membrane protein OmpA, and ABC transport protein encoded by BB1556, were found to be strongly induced,

suggesting increased membrane biosynthesis, and an increase in expression of genes involved in amino acid biosynthesis and transport, of *de novo* nucleotide biosynthesis as well as of numerous ribosomal protein genes were indicative of general extensive metabolic activity in the bacterial cell in response to internalization by macrophages (47).

Notably, the array of genes expressed during intracellular survival holds similarity to those expressed in response to low pH exposure (pH less than 4.0) following acid adaptation (pH at 5.5) (53). Synthesis of ribosomal proteins (ribosomal protein L1, L4, L5), molecular chaperones (GroEL, HSP 90, DnaK), proteases (BB3293, BB1248), metabolic related proteins (Succinate-semialdehyde dehydrogenase, Aconitate hydratase and Argininesuccinate synthase) and LPS modification have been reported to contribute to *B. bronchiseptica* acid tolerance response. Furthermore, acidic conditions (pH less 4.0) the avirulent phase or Bvg- strains displayed greater resistance to lethal acid challenge and thus increased survival rate. These similarities suggest that upon entry in host macrophages *B. bronchiseptica* modulates the transcription of genes involved in the acid tolerance response while suppressing the expression of virulence genes as a mechanism to enhance resistance against stringent acid conditions.

Interestingly, the activation of an acid tolerance response is one of the many features utilized by pathogens known to cause human infection and persist inside host phagocytes, including macrophages and dendritic cells (54). For example, *Salmonella enterica* serovar Typhimurium can subvert macrophage mediated killing and survive within the acidic environment of macrophages by altering lysosomal pH. Several regulatory proteins involved in reduction of acidic pH and activation of the stress response have been previously identified for intracellular *S. enterica*. These includes the environmental response regulator proteins sigma factor RpoH, OmpR, and Ferric uptake regulator protein (Fur), and stress response proteins alkyl hydroperoxide reductase (AhpC), DNA protection during starvation protein (Dps), recombinase A (RecA), and $Mg(2^{+})$ transport ATPase protein C (MgtC). (55, 56). Expression of gene homologs encoding for RpoH, OmpR, Fur,

AhpC, Dps, and RecA have been previously identified for intracellular *B. bronchiseptica* (47), while MgtC have been reported to contribute to *B. pertussis* persistence inside macrophages (57, 58).

Similar to *B. bronchiseptica*, *Franciscella tularensis*— a facultative intracellular pathogen and the causative agent of tularemia, can infect and proliferate within mammalian phagocytes in addition to amoeba (59-61). Upon infection, intracellular *F. tularensis* subverts host defenses by escaping the phagosome (62) or by synthesizing proteins that promotes persistence inside macrophages, such as macrophage growth locus protein MglA, stringent starvation protein A (SspA), chaperone HtpG, Carbamoyl-phosphate synthase (CarA), and iron binding rubredoxin (RubA) (63-68). Previous work has demonstrated that intracellular *B. pertussis* can escape the phagosome compartment and replicate inside macrophage within 48 hours post infection [Lamberti, 2010]. In addition, upregulation of genes encoding for MglA (1.7 fold-change), AroG (2.7 fold-change), HtpG (4.2 fold-change), CarA (1.5 fold change), RubA (2.1) and to a lesser degree for SspA (1.2 fold change) have been reported for intracellular *B. bronchiseptica* at 2 post infection of RAW 246.7 macrophages (47).

For intracellular pathogens the ability to persist inside host-phagocytes is contingent upon adjustment to the intracellular environment, otherwise lethal and normally deprived of nutrients. Lethal pH, resource starvation, and oxidative burst are among the threats intracellular bacteria will face upon entry in host cells, which often result in damage to protein and DNA integrity and nutrient depravation. Mechanisms commonly employed by intracellular bacteria such as *Listeria monocytogenes*, *Salmonella enterica* and *Clostridium spp.* include the synthesis of protein complexes that promote bacterial cell homeostasis under demanding environmental conditions (55). Synthesis of molecular such as recombinase RecA, chaperones DnaK, GroEL, GroES, Hsp90, and protease Clp, promotes DNA repair and folding of critical enzymes and removal of damage proteins, thus maintains cell integrity upon stress. Like in many intracellular facultative pathogens,

the presence of genes encoding for these proteins in the *Bordetella* genome, and it elevated expression following survival inside mouse-derived macrophages serves as evidence that the bacteria possess the mechanisms to cope with the hazardous condition often present inside host phagocytic cells (47).

Impact of Intracellular Survival during Infection *in vivo*.

Host response to *Bordetella* infections has largely been studied using the mouse and non-human primate models upon challenge with *B. pertussis* or it's close relative *B. bronchiseptica*. Upon infection *B. pertussis* secretes a wide array of virulence factors that among other functions, mediates adherence to host epithelial cells and promotes bacterial survival in the host respiratory track.

For instance, *B. pertussis* internalized by host cells can persist intracellularly within the ciliated epithelial cells and alveolar macrophages. Studies in murine models have shown that innate immune cells, and antimicrobial peptides help to control the infection, while complete bacterial clearance requires cellular immunity mediated by T-helper type 1 (Th1) and Th17 cells. During the early stages of infection, local and innate immune cells including macrophages, dendritic cells (DC), neutrophils, and natural killer (NK) cells are recruited and largely accountable for the control and reduction of *B. pertussis* (69). Among the effectors that promote bacterial reduction, early secretion of interferon-gamma (INF-γ) by NK cells, DC, and T-helper type 1 (Th1) cells greatly enhance macrophage-mediated killing of *B. pertussis*, as research have shown that mice lacking INF-γ developed a lethal infection after challenge with *B. pertussis* [REF].

The adaptive immune system plays a crucial role in clearance of *B. pertussis* infection. Activation and recruitment of a Th1 and Th17 adaptive immune response mediates bacterial clearance from the lower and upper respiratory track of mice. Modulation of cellular Th1 immunity results in the secretion of IL-12 and INF-γ, which in turn promotes neutrophil recruitment and

enhances macrophage phagocytic activity. It is largely recognized that optimal immunity to primary and secondary *B. pertussis* infections is conferred by the priming and activation of Th1 cells. Similar to convalescent immunity, vaccination with whole cell pertussis (wP) –which is based on heat-killed *B. pertussis* bacteria– promotes the secretion of Th1 derived cytokines and enhances macrophage activity, resulting in increased killing of phagocytosed bacteria. Protection conferred by wP has been widely studied, and the rapid decline in the number of Pertussis cases following its introduction is strong and compelling evidence for its effectiveness. However, while whole cell pertussis vaccines (wP) were highly effective at clearing the infection and providing long-term protection, several cases of vaccine reactogenicity were reported, which led to its replacement in many countries by acellular vaccines (aP) that are composed of three to five purified immunogenic antigens.

Despite wide vaccination coverage, the incidence of pertussis is increasing, which prompted the National Institute of Allergy and Infectious Diseases (NIAID) to add *B. pertussis* to the list of priority Emerging Infectious Diseases/Pathogens in 2015. In contrast to wP, it has been shown that even though acellular pertussis vaccine is effective at clearing *B. pertussis* infection from the lungs and protecting against disease pathology, vaccination with aP does not provide protection against bacterial colonization of the upper respiratory tract of mice and fails to prevent transmission among non-human primates (70, 71). As outlined above, infection with *B. pertussis* naturally induces a significant Th1-type T-lymphocyte cytokine response in mice that is characterized by high levels of IL-2, IFN-γ, and TNF-α, a type of immune response that characteristic for infection by intracellular pathogens (ref). While protective immunity generated by wP also promotes a Th1 immune response, the less efficacious aP induces a strong antibody Th2 response. Its plausible that a Th2 humoral response to the aP vaccine could fail to efficiently target intracellular *B. pertussis*, resulting in suboptimal vaccine protection. Inability to clear intracellular bacteria could allow *B. pertussis* to evade host immunity during survival inside phagocytic cells,

and protect bacteria from antibodies, complement activation, and bactericidal activity. Thus, given that the number of Pertussis cases have been on the rise following the widespread use of aP vaccines, it seems plausible that an inadequate immune response to intracellular *B. pertussis* may have contributed to the reemergence of whooping cough.

Pathogenic members of the genus *Bordetella* have adapted to colonize, replicate and transmit in animal hosts. The environmental origin of the species suggests that the ability to survive and persist in environmental phagocytes could have protected the bacteria from external dangers while ensuring transmission to novel environments and animal hosts. A once critical survival strategy could potentially impact the bacterial success during host infection. Therefore, evaluation of the ability to reside inside host cells and the impact of this strategy during infection can provide valuable insight in the fight against the reemergence of *Bordetella pertussis*.

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CHAPTER 5

CONCLUSIONS

Bordetella infections have been historically studied following the premisse of a complete extracellular life cycle in their mammalian hosts. Contrary to this assumption, a compelling body of evidence has shown that the bacteria can enter and persist inside mammalian phagocytes. Moreover, recent studies suggest that the ability to survive inside eukaryotic cells is an ancient strategy that have been conserved throughout the evolution of *bordetella*. While some work has been dedicated at characterizing intracellular survival in the human-pathogen *B. pertussis*, there is limited knowledge on the evolution of this strategy within the genus, and its contribution to bacterial pathogenicity and dissemination. Here we identified the transcriptional basis for to intracellular adaptation and explored how these genes have been acquired and conserved throughout the divergent evolutionary histories of *Bordetella* species.

Specific Aim 1. Evaluate *B. bronchiseptica* ability to persist inside macrophages. We had earlier observed, using antibiotic protection assays, that *B. bronchiseptica* strain RB50 (Bb) can enter and survive within murine macrophage-like cell line RAW 264.7 *in vitro*. To determine the number and proportion of bacteria entering these macrophages, we performed an assay of macrophages infected with *B. bronchiseptica* at multiplicities of infection (MOI) of 100, 10 and 1 for 1 hour. The

percentage of recovery ranged from 0.7 to 1% of the original inoculum at all three MOIs, indicating that a relatively constant fraction of bacteria entered and resisted digestion by macrophages. The observation that the ratio of bacteria to macrophage did not affect survival rate suggested that this is not simply macrophages being overwhelmed or overcome by bacterial numbers. Electron microscopy, confocal microscopy and z-stack images taken after 2 hours incubation confirmed the presence of bacteria within phagocytic vacuoles. Once inside the RAW 264.7 cells, bacterial numbers remained relatively stable and decreased only slowly over time. Bacterial CFUs recovered at 4 and 8 hours showed no significant change in numbers for any of the MOIs used, and even at 24 and 48 hours intracellular *B. bronchisptica* were recovered in substantial numbers. In contrast to *Bordetella bronchiseptica*, *Klebsiella aerogenes* failed to persist in RAW 264.7 cells and was recovered at numbers over two orders of magnitude lower. These results demonstrate that similar to *B. pertussis*, *Bordetella bronchiseptica* is able to enter and persist inside mouse-derive macrophages RAW 264.7 cells.

Specific Aim 2. Identify *B. bronchiseptica* transcriptional changes during growth inside murine-derived macrophages. The apparent localization of the intracellular *B. bronchiseptica* within phagosomes implies that the bacteria are rapidly exposed to a variety of bactericidal conditions, including low pH, microbicidal factors and resource starvation. To survive such harsh conditions many pathogenic bacteria have evolved mechanisms to maintain cell homeostasis and prevent DNA damage and cell death. Based on our observation of *B. bronchiseptica* surviving intracellularly, it becomes apparent that the bacterium would also require mechanisms to survive these conditions.

We hypothesized that to survive within professional phagocytic cells, *B. bronchiseptica* would require distinct groups of genes to be transcriptionally modulated once the bacteria reached the intracellular niche. To examine this transcriptional response, we analyzed the mRNA profile of intracellular *Bordetella bronchiseptica* at 2 hours post inoculation and compared it to that of bacteria grown *in vitro.* Principal Component Analysis (PCA) of the normalized mRNA read distribution revealed a clear difference in the global gene expression between intracellular and in vitro grown *Bordetella bronchiseptica*, with clustering of the replicates and separation of the two groups along the first principal component (PC1), indicating a distinct transcriptional response to the intracellular environment.

Functional analysis of the transcriptionally upregulated genes revealed major changes at the functional levels of metabolic process, of cellular process, regulation and response to stimuli. Gene ontology evaluation showed enrichment for genes whose products are involved in cellular processes including DNA repair, protein folding and repair, oxidative stress response, and pH homeostasis, as well as enrichment for metabolic processes such as nutrient assimilation.

During survival inside macrophages we observed the transcriptional hallmarks of a general SOS response, characterized by suppression of cell division via downregulation of the *fts* locus and by upregulation of DNA repair genes. In addition, protein chaperone genes and *B. bronchiseptica* homologs (*rpoH*, *fur*, *risA*) of the *E. coli* acid tolerance genes *rpoS,* fur and *phoP* were also upregulated. Expression of *recA*, *dnaB*, *dps,* and *dksA*, all implicated in the activation of the SOS response and DNA repair, was upregulated upon internalization by macrophages. Likewise, genes for protein folding

and recycling such as molecular chaperones *groES*, *groEL*, and *htpG*, and protease genes *hslV* and *hslU*, were highly upregulated, as was expression of several other osmotic and heat shock response genes, including *clpB*, *grpE*, *dnaK*, and *dnaJ*. Expression of genes that promote resistance against oxidative stress and low pH was upregulated intracellularly. This includes transcription factor *iscR* and adjacent genes *iscS*, *hscB*, and *fdx*, and the transcription regulators *slyA*, *risA*, and *fur*. Additionally, RNA polymerase sigma factor genes *rpoH*, *rpoN*, and *rpoE* were highly upregulated as was expression of the RNA chaperone gene *hfq*, which is shown to increase resistance against killing by macrophages.

Intracellular persistence was also accompanied by metabolic changes. As expected under micro-aerophilic/hypoxic conditions inside macrophages, expression of the *nuoF – nuoN* genes that encode the oxidative respiratory chain was strongly suppressed. In contrast, genes of the glyoxylate/TCA cycle showed elevated expression levels, including malate synthase G gene *glcB* and its transcriptional activator *glcC*, malate dehydrogenase *mdh*, citrate synthase *gltA*, and aconitase *acnB*. The glyoxylate cycle is important in the utilization of acetate or fatty acids as the main carbon source and may be essential to provide hexoses for nucleotide and amino acid biosynthesis under intracellular conditions. Consistent with this observation, genes involved in biosynthesis of nucleotides, amino acids and fatty acids were significantly upregulated. Fatty acid synthesis pathways such as 3-oxoacyl-ACP reductase BB4150, long chain fatty acid Co-A ligase BB0233, outer membrane protein *ompA*, and ABC transport protein encoded by BB1556, were found to be strongly induced. Also observed was an increase in expression

of genes involved in amino acid biosynthesis and transport, including BB4592, *carA*, *argC*, and *argG*, and of de novo nucleotide biosynthesis (*ndk*, *pyrH*, *cmk*, and *nrdA*).

Surprisingly, despite conducting our experiment at 37°C—a temperature known to induce phosphorylation of bvgA, which in turn induces expression of virulence factors, *B. bronchiseptica* displayed strong suppression of most virulence factors. The *cya* and *dnt* genes encoding the adenylate cyclase (ACT) and dermonecrotic toxins respectively, were among the most downregulated genes. In agreement with previous studies, expression of pertussis toxin (PTX) and the associated type IV secretion system (T4SS) was barely detectable under either condition. The type III secretion system (T3SS) encoded by the *bsc* locus, displayed strong suppression of both the apparatus-related and the secretion-related components. In addition to toxins and secretion systems, expression of the O-Antigen–encoding wbm locus (wbmO – bplJ), was significantly downregulated.

Many intracellular pathogens such as *Burkholderia pseudomallei* employ protein secretion systems to facilitate replication and spread inside their hosts. However, in our result, *B. bronchiseptica* displayed strong suppression of the type III secretion system (T3SS) encoded by the *bsc* locus, resulting in decreased expression of both the apparatus-related and the secretion-related components.

Taken together, *B. bronchiseptica* responded to the internalization within macrophages by rapid changes in its transcriptional profile that were marked by suppression of growth and virulence, and a strong activation of the bacterial stress response, including DNA and protein repair, and pH homeostasis. Although many intracellular pathogens such as *Burkholderia pseudomallei* employ protein secretion systedms to facilitate replication and spread inside their hosts, interestingly, we identified

strong suppression of virulence during intracellular persistence of *B. bronchiseptica*. Past research has shown that the avirulent stage is required for survival, persistence and replication of *B. bronchiseptica* within amoeba. Our observations strongly suggests that repression of virulence within the intracellular environment is part of an ancient conserved stress response in the genus.

Specific Aim 3. Compare the genes involved in intracellular survival within the *Bordetella* genus. *In vitro* experiments convincingly demonstrated that the classical bordetellae can survive intracellularly within mammalian phagocytic cells, an ability that appears to have descended from ancestral progenitor species that lived in the environment and acquired the ability to resist phagocytic killing by amoebae that are ubiquitous environmental predators. While many non-classical bordetellae are also human and animal pathogens, their ability to persist inside phagocytic cells has not been evaluated. Therefore, using the genes identified in Aim 2, we evaluated the conservation of genes upregulated during intracellular survival among the non-classical Bordetella species.

While many non-classical bordetellae are also human and animal pathogens, their ability to persist inside phagocytic cells has not been evaluated. Therefore, we screened for the presence (or absence) of the upregulated genes observed in *B. bronchiseptica* among the genomes of non-classical *Bordetella* species, including the bird pathogens *B. hinzii* and *B. avium*, the mouse pathogen *B. pseudohinzii*, the human opportunistic pathogen *B. trematum*, and the environmental species *B. petrii*. We calculated the protein similarity (H value) for 318 genes previous identified (Aim 2), and their corresponding homologs in the non-classical bordetellae. An average of 77–81% of the 318 upregulated genes were present in the non-classical species with 95 (30%) of the genes displaying

similarity values of $H \geq 0.9$. In contrast, only 46–55% of the total of 4,981 evaluated *B. bronchiseptica* genes were identified in the genomes of the non-classical species ($P < 0.0001$), where only 448 (9%) of the genes reached protein similarity scores of $H \geq 0.9$. This high evolutionary conservation of genes that are upregulated in *B. bronchiseptica* during intracellular survival in phagocytic cells strongly suggests that the non-classical bordetellae would successfully persist inside macrophages. To further test this hypothesis, the non-classical species were assessed for intracellular survival in RAW 264.7 macrophages for 2 and 4 hours. All examined Bordetella species were recovered, with the exception of *B. avium*. The inoculated *B. pseudohinzii*, *B. hinzii*, *B. trematum* and *B. petrii* bacteria survived internalization by macrophages at similar rates to *B. bronchiseptica*. In contrast, *B. avium* was severely impaired in its ability to persist inside macrophages. Only 0.001% of the inoculum was recovered after 2 h and no viable bacteria were detected after 4 h. Conservation of these genes throughout the genus and the demonstrated ability of non-classical species, including the environmental *B. petrii*, to persist inside macrophages suggests that this response to phagocytes is not confined to the commonly studied classical bordetellae. It appears to represent an ancient pathway that preceded speciation in the genus and thus likely arose from a common ancestor.

<u>Specific Aim 4.</u> Assess the survival and persistence of knockout-mutants inside macrophages. We predicted that genes highly expressed by intracellular *B. bronchiseptica* play a key role in bacterial persistence. To test this hypothesis, we generated deletion mutants of genes identified in (Aims 2 and 3) using *B. bronchiseptica* RB50 and assess bacterial fitness inside macrophages and the impact of these genes during early and long-term survival inside RAW 264.7 macrophages. Deletion of two of

these genes (BB0096 and BB1908) resulted in a significant reduction in intracellular survival. Complementation of these knock-out mutants with plasmid-borne gene copies restored the wildtype phenotype in both mutants, confirming that loss of malate synthase transcriptional regulator *glcC* (BB0096) or the tripartite tricarboxylate transporter BB1908 negatively impacts intracellular persistence in macrophages.

Collectively this dissertation shows that upon internalization by macrophages a certain proportion of bordetellae are killed, but thousands of bacteria can adapt and modulate gene expression to cope with this new environment. Rapid transcriptional adaptation was marked by what can be considered a general stress response against professional phagocytes that included increased expression of genes involved in DNA and protein repair, acid tolerance and metabolism. Conservation of these genes throughout the genus and the demonstrated ability of non-classical species, including the environmental *B. petrii*, to persist inside macrophages suggests that this response to phagocytes is not confined to the commonly studied classical bordetellae. It appears to represent an ancient pathway that preceded speciation in the genus and thus likely arose from a common ancestor. The two independent but interconnected transmission cycles of *B. bronchiseptica* in environmental amoebae and in mammalian hosts lead us to speculate that early interaction with these environmental phagocytes may have played a role in the origin of this response, which subsequently facilitated the adaptation to higher animals and thus the evolution of Bordetella from environmental microbes to animal and human pathogens.