Genotypic and phenotypic adaptation of pathogens: lesson from the genus *Bordetella*

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Purpose of review
To relate genomic changes to phenotypic adaptation and evolution from environmental bacteria to obligate human pathogens, focusing on the examples within *Bordetella* species.

Recent findings
Recent studies showed that animal-pathogenic and human-pathogenic *Bordetella* species evolved from environmental ancestors in soil. The animal-pathogenic *Bordetella bronchiseptica* can hijack the life cycle of the soil-living amoeba *Dictyostelium discoideum*, surviving inside single-celled trophozoites, translocating to the fruiting bodies and disseminating along with amoeba spores. The association with amoeba may have been a 'training ground' for bacteria during the evolution to pathogens. Adaptation to an animal-associated life style was characterized by decreasing metabolic versatility and genome size and by acquisition of 'virulence factors' mediating the interaction with the new animal hosts. Subsequent emergence of human-specific pathogens, such as *Bordetella pertussis* from zoonoses of broader host range progenitors, was accompanied by a dramatic reduction in genome size, marked by the loss of hundreds of genes.

Summary
The evolution of *Bordetella* from environmental microbes to animal-adapted and obligate human pathogens was accompanied by significant genome reduction with large-scale gene loss during divergence.

Keywords
amoebic predation, bacterial pathogen, *Bordetella*, environmental origin, host specialization

INTRODUCTION

Genomic plasticity has enabled bacteria to rapidly adapt to ever-changing environments. The imprints of these adaptive changes are often retained in the genome and provide a blueprint on how bacteria adapt to, and evolve, within newly established niches. These imprints can often be identified by comparative study of closely related bacterial genomes, providing insight on the subtle genetic changes that accompany phenotypic adaptations. The bordetellae are ideally suited for these studies, owing to the broad environmental niches they occupy, the diverse life styles they have and their phylogenetic relatedness.

The genus *Bordetella* belongs to the *Betaproteobacteria* and contains several pathogenic species that include the so-called 'classical' bordetellae consisting of *B. bronchiseptica*, *B. pertussis* and *Bordetella parapertussis*. *B. pertussis* is the etiological agent of whooping cough, which is known for the characteristic symptoms of paroxysmal cough, whooping and posttussive vomiting, and kills hundreds of thousands of children annually [1]. *B. parapertussis* is the common name of two different lineages: a human-adapted lineage that causes whooping cough-like disease in children [2], hereafter referred to as Bpp_{hu}, and an ovine-adapted lineage that causes pneumonia in sheep, referred to as Bpp_{ov} [3]. The third species, *B. bronchiseptica*, has a broader host range, causing respiratory diseases that can be mild and chronic or acute and severe: kennel cough in dogs, bronchopneumonia or atrophic rhinitis in pigs [4]. These three species can be considered subspecies as their shared genes possess over 98% nucleotide identity. Multi locus sequence typing and whole genome sequences revealed that *B. pertussis* and *B. parapertussis* independently evolved from a *B. bronchiseptica*-like ancestor [5–7], human-specific

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KEY POINTS

- Environmental *Bordetella* species have significantly larger genetic diversity than animal-associated and human-associated samples.
- Association with amoebic hosts may have been an intermediate step in the evolution from environmental microbes to pathogenic bacteria.
- Host specialization was accompanied by significant genome reduction.
- Anthropogenic factors enable closed life cycles in populous host species, with pathogenesis-mediating efficient transmission between hosts.

pathogens emerging from likely zoonoses of the broader host range progenitor.

In addition to the 'classical' bordetellae, the genus contains numerous other, phylogenetically distinct species collectively referred to as 'nonclassical'. The emerging, human-restricted *Bordetella holmesii* was first isolated in 1983 [8], and has since been isolated from blood of septicemic patients and from nasopharyngeal samples of patients with pertussis-like disease [9–12]. *Bordetella avium* causes respiratory disease in poultry with the clinical symptoms known as coryza or bordetellosis [13] and also infects other wild and domesticated birds [14]. *Bordetella hinzii* is generally regarded as a commensal in the respiratory tract of poultry but causes disease during experimental infection of turkey chicks [15] and opportunistic infection in immunocompromised patients [16,17]. The closely related *Bordetella pseudohinzii* naturally infects laboratory raised mice [18,19] and was also identified in a wild rat in Malaysia [20▪]. *Bordetella trematum* and *Bordetella ansorpii* are rare and generally found associated with infected wounds of immunocompromised patients [21–23], and *Bordetella bronchialis*, *Bordetella sputigena* and *Bordetella flabilis* have recently been isolated from respiratory samples collected from patients with cystic fibrosis [24]. *Bordetella petrii* shares the most ancient common ancestor and also has been recovered from quite diverse environmental settings, including soils and immunocompromised patients [25,26], providing some perspective on the natural history of the bordetellae, an intriguing 'origin story' explored herein.

In a whole genome-based phylogenetic tree, *Bordetella* species form three main clades (Fig. 1) [7]. One clade contains the three 'classical' species *B. bronchiseptica*, *B. parapertussis* and *B. pertussis*, by

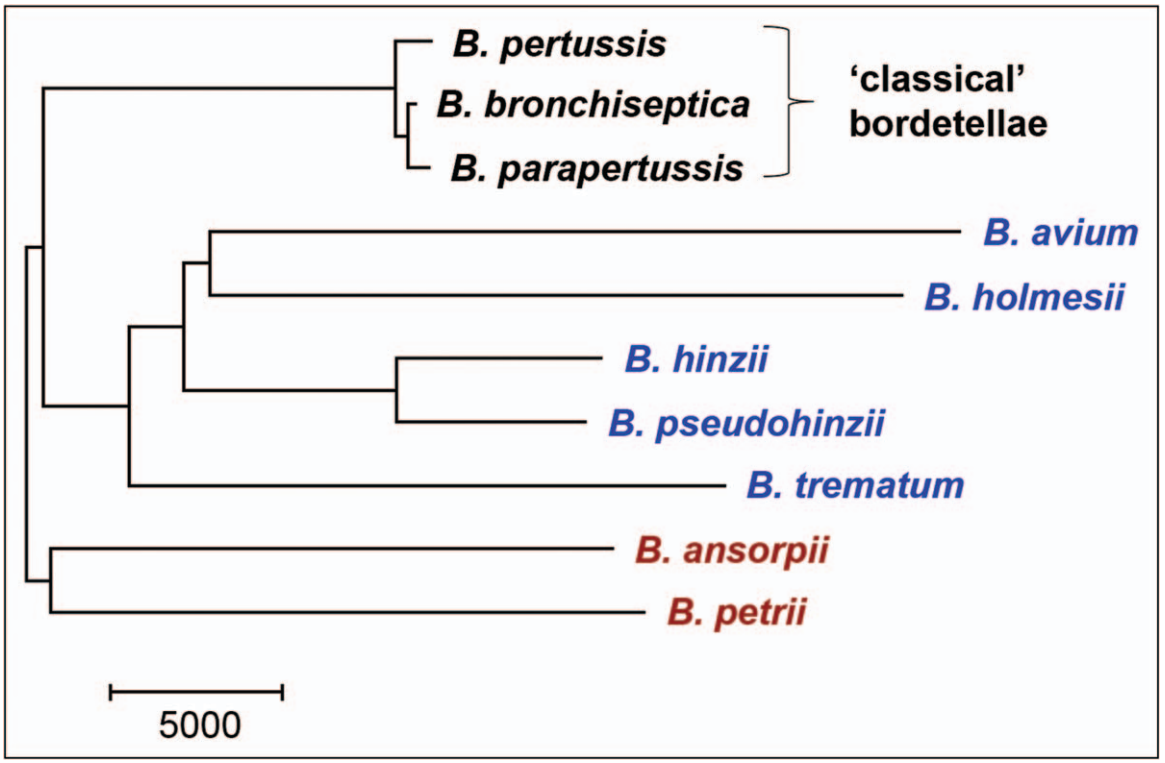

FIGURE 1. Whole genome phylogeny of 10 *Bordetella* species based on a genome-wide sequence alignment. The Neighbor-joining tree shows three clades of species that consist of *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* (circles), of *B. avium*, *B. holmesii*, *B. hinzii*, *B. pseudohinzii* and *B. trematum* (squares) and of *B. ansorpii* and *B. petrii* (triangles). The tree was rooted according to Linz *et al.* [7].

far the best studied species. A second clade consists of *B. trematum*, *B. hinzii*, *B. pseudohinzii*, *B. avium* and *B. holmesii,* and the third clade includes *B. petrii* and *B. ansorpii* [7,19]. The genomes of the classical bordetellae are closely related and show a low between-species genetic diversity, which reflects the relatively recent emergence of *B. parapertussis* and *B. pertussis* from *B. bronchiseptica*-like ancestors [7]. Indeed, *B. pertussis* is a very young species. A global genome-based phylogeny of a worldwide collection of *B. pertussis* strains revealed two deep branches that coalesce about 2300 years ago [27]. Subsequently, one of the branches in the phylogenetic tree, which contains over 98% of all analyzed strains, appears to have started to expand about 500 years ago [27], which coincides with the first descriptions of whooping cough outbreaks in Persia and Europe [27,28].

ENVIRONMENTAL ORIGIN OF *BORDETELLA* SPECIES

For most of the last century *Bordetella* species were considered to be host-restricted pathogens, with variable host specificity. However, in 2001, *B. petrii* was isolated from a mixed anaerobic, dechlorinating culture enriched with river sediment [25]. In 2015, three new species named *Bordetella muralis*, *Bordetella tumulicola* and *Bordetella tumbae* were isolated from the plaster wall surface of 1300-year-old mural paintings inside the stone chamber of the Takamatsuzuka Tomb in Japan [29]. Since then, bordetellae have been identified in several metagenomic analyses of environmental samples: A microbial ecosystem in a bioreactor degrading thiocyanate and cyanide contained a *Bordetella*-like organism [30], and a metagenomic analysis of a consortium of biphenyl-degrading soil bacteria revealed *Bordetella* as a key player in the degradation of benzoate [31▪▪]. Sequences from numerous *Bordetella*-like organisms were found among environmental sources by probing 16S ribosomal RNA sequences available in the NCBI database [32]. A phylogenetic comparison of 16S rRNA sequences from animal-associated species and from strains recovered from various sources, including soil and water, provided evidence for an environmental origin of the bordetellae. First, the genetic diversity of the environmental samples was significantly larger than the diversity of the animal-associated and human-associated samples. And second, sequences from environmental sources were present in all 10 phylogenetic clades in the tree, including sequence clades at the root of the tree, whereas sequences from animal samples were only found in four clades near the top of the tree. The branching order in the phylogenetic tree indicated that the genus *Bordetella* originated from an environmental source [32].

A detailed genome analysis of *B. petrii* strain DSMZ12804 has identified a core set of 2049 genes that are shared with the genomes of both *B. bronchiseptica* (strain RB50) and *B. avium* (strain 197N) and thus are likely part of the ancestral genome inherited from a common ancestor [33]. Among these are genes encoding the central metabolic pathways that are shared between *B. petrii* as an example of an environmental species and the animal-pathogenic *Bordetella* species, including the TCA cycle and synthesis of amino acids, fatty acids and nucleotides. Interestingly, *B. petrii* has a large set of 1825 unique genes, many of which encode auxiliary pathways that may be involved in the metabolic breakdown of plant material and other organic components. In this regard, *B. petrii* is remarkably versatile. It possesses genes to utilize gluconate, other plant products such as pectin, cyanate and a vast variety of aromatic compounds, including phthalate, phenylacetate, benzoate, benzylalcohol, chlorobenzenes and phenol [33]. *B. petrii* harbors numerous peripheral pathways to preprocess and channel those and other aromatic compounds into at least eight different central metabolic pathways. Some of these pathways are encoded by genes on genomic islands with an atypical GC content, indicating an important role of horizontal gene transfer in the metabolic versatility. In addition, several of the central pathways are represented by multiple gene paralogs, such as three sets of chlorocatechol pathway genes for the degradation of chlorobenzenes [33]. Likewise, the genome of a biphenyl-degrading *Bordetella* isolate from soil possesses genes of three central pathways for the degradation of benzoate, with most of the genes present as 2–6 distinct copies, emphasizing the vast metabolic diversity [30]. In contrast, most of the pathways for the degradation of aromatic compounds are not present in the genome of the animal-pathogenic bordetellae. Still, both *B. bronchiseptica* and *B. hinzii* are known to associate with animal hosts, yet are able to grow efficiently in soil and thus to survive under environmental conditions [32].

Comparative genomics suggested that *B. petrii* may be well equipped for competition with other microbes. The *B. petrii* genome harbors two different type 6 secretion systems (T6SS) [7], which encode a syringe-like apparatus that mediates injection of effectors into both bacterial competitors and eukaryotic host cells and are commonly found in many soil bacteria [34–36]. One T6SS was also found in the genomes of *B. bronchiseptica*, *B. parapertussis* and *B. ansorpii*, suggesting that it may have been present in the *Bordetella* ancestor. In contrast, the second T6SS

was uniquely present in the genome of the environmental *B. petrii*, implying gene acquisition or repeated loss in all other divergent lineages [7]. Together, these findings suggest that a high genome plasticity has provided the tools to cope with environmental challenges, allowing acquisition of nutrients and competition against other bacteria.

ADAPTATION FROM THE ENVIRONMENT TO ANCIENT HOSTS

The ability to persist, replicate and disseminate can provide critical advantages in competitive soil/water environments. Numerous bacteria have been reported to form endosymbiotic relationships with amoeba, including *Escherichia coli* O157, *Francisella tularensis*, *Legionella pneumophila* and *Mycobacterium* spp. [37–41]. Apart from providing protection against external dangers and an apparent competitive advantage against other bacteria, symbiosis with amoebae can enhance bacterial dissemination along with the amoebic host, some of which have evolved highly effective mechanisms of travel. Our recent study showed that the animal-pathogenic *B. bronchiseptica* can hijack the life cycle of the soil-living amoeba *Dictyostelium discoideum* [42], resisting digestion by single-celled trophozoites and translocating to the fruiting bodies to spread along with its spores. This relationship is stable over multiple amoeba life cycles without affecting *B. bronchiseptica*'s ability to subsequently infect a mammalian host. By not just residing but growing within the amoeba fruiting body, the bacteria can be disseminated along with the spores to new geographic locations, where they can either maintain a stable association with the amoebic host or infect a new mammalian host, revealing two independent but interconnected life cycles of *B. bronchiseptica* in protozoan and mammalian hosts [42]. The bacteria and spores can be spread by wind and passing animals, such as ants and flies, and a recent metagenomic study indeed detected *B. bronchiseptica* and *B. hinzii* sequencing reads in the microbiome of flies [43].

The similarities between amoeba trophozoites and mammalian phagocytic cells have led some authors to speculate that the interaction between bacterial pathogens and amoebic hosts could have served as a 'training ground' for bacteria before they became animal pathogens [40]. Our recent examination of the potential environmental origin of the *Bordetella* genus [32] and description of complex *B. bronchiseptica* interaction with *D. discoideum* [42] also supported the view that bacterial interaction with amoebae may have enabled members of this genus to evolve from environmental microbes to become pathogenic [41].

SPECIES-SPECIFIC VIRULENCE AND HOST SPECIALIZATION

Bordetella species have evolved and successfully adapted to infect and transmit in animal hosts. But what did it take to become animal pathogens? The paucity of genomes from environmental sources makes this a challenge to address. A comparison of the relatively small number of genomes available [7] indicated that the potential ancestor of the pathogenic bordetellae likely possessed several protein secretion systems, including a T2SS, a T3SS and one or two T6SS, but lacked the best studied *Bordetella* toxins, including pertussis toxin, adenylate cyclase toxin and dermonecrotic toxin (DNT). The genes encoding those toxins appear to have been acquired by the ancestor of the 'classical' bordetellae, with DNT being independently acquired by *B. avium* at a different chromosomal location. Species-specific putative adhesins, such as autotransporters, appear to have been acquired both vertically and horizontally. All pathogenic bordetellae possess heme receptors, but those are absent in the genome of the environmental *B. petrii* and likely represent an adaptation to an animal-associated life style. In general, gain and loss of multiple genes, including many encoding bacterial toxins, protein secretion systems and other virulence factors, accompanied the diversification and speciation in the genus. The loss of so many genes associated with pathogenesis in specialized lineages reveals that there were many more of these in the apparent progenitor than are necessary for the remarkable success of some lineages, including the most prominent human pathogens [7].

B. bronchiseptica, the apparent progenitor of the classical *Bordetella* species, can infect the respiratory track of a wide range of mammals, including dogs, cats, sheep, rabbit, swine and mice [4]. Yet, it retains the ability to establish a successful symbiosis with amoeba. Other classical *Bordetella* spp. have specialized at causing disease and spreading within a single mammalian host. For example, the ovine-specific *B. parapertussis* (Bpp_{ov}) successfully colonizes and persists in the respiratory track of sheep but is not observed in other animals and is severely defective in its ability to infect mice [3]. Although accumulation of mutations in genes involved in O-antigen production has rendered Bpp_{ov} highly susceptible to complement mediated killing in mice [44], loss of O-antigen is also well described in human restricted pathogen *B. pertussis*, suggesting that the lack of O-antigen confers a selective advantage in their respective hosts, although it may also limit them to that particular host. Another example of adaptation to a particular mammalian host is the recently discovered *B. pseudohinzii*, which was isolated from

laboratory raised mice with no obvious clinical symptoms [18,19]. Interestingly, all *B. pseudohinzii* strains to date have been isolated from mice [18,19,20▪,45–48] and a wild rat [20▪], but the sister species *B. hinzii* can infect poultry, rabbits and immunocompromised humans [16,17].

GENOME REDUCTION DURING HOST SPECIALIZATION

Several *Bordetella* species have evolved and adapted to infect only humans, undergoing large-scale gene loss and inactivation in the process [5–7], similar to the genome reduction associated with host-specialization of other pathogens [49]. During speciation, *B. pertussis* and Bpp_{hu} experienced genome reduction of about 22 and 9%, respectively, which is reflected in the much smaller genome size of *B. pertussis* (~ 4.1 Mb) and *B. parapertussis* (~ 4.8 Mb) compared to *B. bronchiseptica* (~ 5.3 Mb). *B. pertussis* genome reduction was mediated by homologous recombination between identical copies of insertion sequence (*IS*) element *IS*481, which effectively deleted large parts of the genome and resulted in loss of over 1000 genes [5]. Genome reduction was less severe in *B. parapertussis*, as genome comparisons of Bpp_{hu} strain 12822 and Bpp_{ov} strain Bpp5 against *B. bronchiseptica* strain RB50 showed large colinear regions between the genomes, with a limited number of chromosomal breakpoints flanked by *IS*1001 [5,6]. Interestingly, the genome of *B. holmesii*, the only human-restricted species of the 'nonclassical' bordetellae, also appears to have undergone substantial reduction in size, mediated by *IS*407, *IS*481 and *IS*L3 [7]. As a result, the genome of *B. holmesii* (3.7 Mb) is much smaller than that of its closest relative *B. hinzii* (4.9 Mb), as is the genome of the poultry-specific pathogen *B. avium* (3.7 Mb). Thus, it appears that host specialization of several *Bordetella* species is associated with acquisition, expansion and subsequent recombination between *IS* elements mediating large-scale gene loss. In addition, the extremely low genetic diversity within these species suggests that these speciation events were associated with severe population bottlenecks that drastically reduced the genetic diversity, followed by expansion of a few successful clones [5,7,27].

GENOME SIZE AND LIFE CYCLE

Environmental species need to possess genomes that can facilitate adaption to changing availability of various carbon and nitrogen sources by encoding multiple metabolic pathways. *B. petrii*'s versatile pathways for the degradation for aromatic compounds [31▪▪,33] are likely to provide a valuable fitness advantage in these competitive environmental microbial communities. Diverse metabolic pathways provide a substantial advantage in naturally variable environments but require relatively large genomes. In contrast, the environment and nutrient sources within the airways of hosts are relatively stable and do not require such metabolic versatility. However, opportunistic pathobionts and host-restricted pathogens need other sets of genes that mediate their interactions with the host, including adhesins and virulence factors to manipulate the host's immune response. Interestingly, the genomes of these animal-adapted *Bordetella* species are substantially smaller than those of environmental species (Fig. 2). Just like other host-restricted bacteria, host-specialized bordetellae appear to have undergone dramatic genome reduction during speciation that led to the loss of hundreds of genes and resulted in genomes that may be more than 1 Mb smaller than those of their inferred ancestors. These results are consistent with the explanation that a sustained, closed life cycle in a specific host allows for the loss of the many genes involved in success outside of that host.

Relatively frequent loss or mutation of genes required in the extra-host environment is likely to result in host-restricted pathogens. But such spontaneous mutants would be rapidly lost from nature when they cannot sustain a stable chain of transmission that lasts over extended evolutionary time. *Bordetella* species that are observed in a single host must have established a successful medium to long-term strategy, which is only possible in a host population that is large and connected enough to sustain transmission for long periods. Interestingly, several *Bordetella* species appear to have emerged as host-restricted in the relatively recent Anthropocene era, when human activities have had a major impact on various animal populations. Thus, *B. pertussis* and Bpp_{hu} are highly successful in large human populations. Similarly, *B. avium* and Bpp_{ov} are highly successful in poultry and sheep populations, respectively, that human husbandry has allowed to reach sizes and densities not present before the Anthropocene. Together, these observations support speculation that the emergence of these host restricted *Bordetella* species is anthropogenic; human changes to host populations provided an environment in which these species could emerge with host-specific closed life cycles and persist over time.

ONGOING CHANGES IN CLINICAL *B. PERTUSSIS* GENOMES

All *Bordetella* species are continuing to evolve. In the best studied of these, there is regular speculation

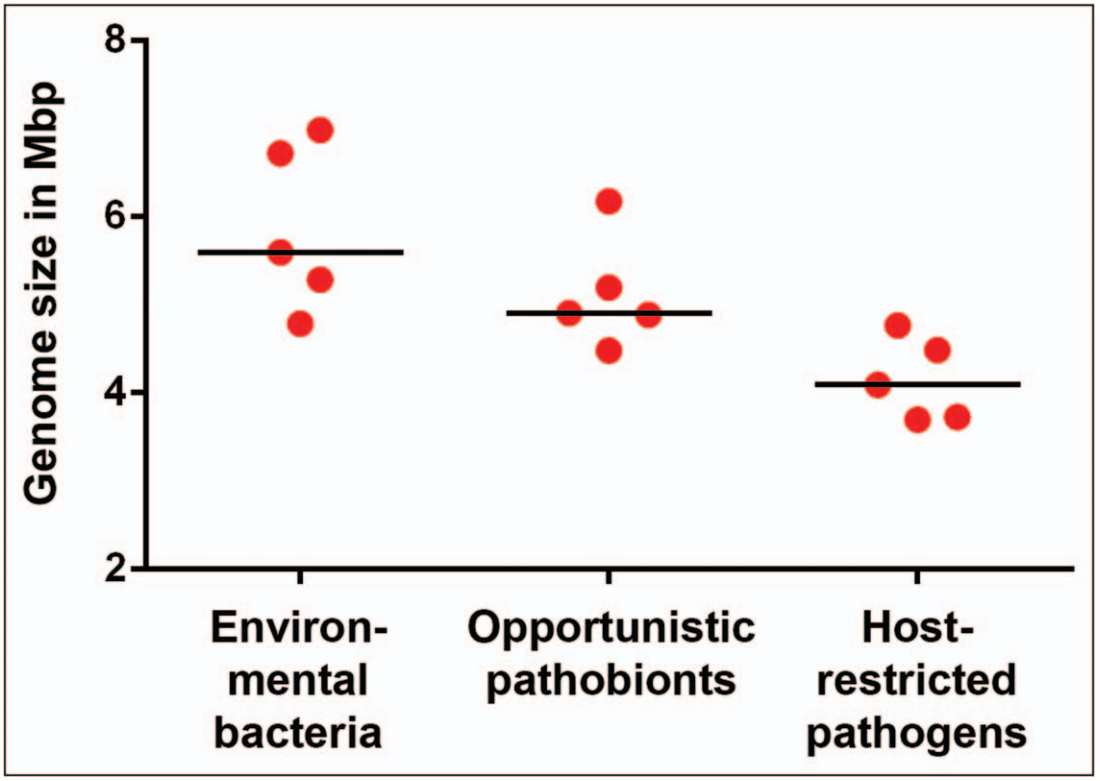

FIGURE 2. Genome size of environmental bacteria, pathobionts and host-restricted pathogens in the genus *Bordetella*. *Bordetella sp.* SCN 68-11 (accession: MEFS00000000), *B. sp.* SCN 67-23 (MEDQ00000000), *B. sp.* BFMG2 (PKCD00000000), *B. petrii* (NC_010170) and *B. sp.* N (NZ_CP013111), isolated 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.

about the pressures shaping this evolution. Recent *B. pertussis* isolates that are deficient in expression of components of the current acellular pertussis (aP) vaccines have led to extensive speculation about 'vaccine-driven evolution'. Precisely, because this is such a compelling and scary idea, it is particularly important to remain impartial and skeptical, to consider other explanations, to look for all types of evidence for and against and to put genotypic and phenotypic changes in the context of recent evolution of the genus. Increasing numbers of isolates lacking a particular factor, such as Pertactin, correlate with increased aP vaccine use in some populations, but these are observations of correlations, and are quite emphatically NOT evidence of vaccine-driven evolution. The evolutionary history of the genus demonstrates substantial genome reduction in several lineages, especially *B. pertussis*, resulting in the loss of a large proportion of genes during speciation, including several autotransporter genes. In addition, the observed switches in allele frequencies of aP vaccine component genes cannot be directly linked to aP vaccines, because a comparative genome analysis of 343 *B. pertussis* strains isolated between 1920 and 2010 revealed that the new alleles were already present in the prevaccine era (*ptxA1*) or in the whole cell vaccine era (*ptxP3*, *prn2*, *fim2-2*, *fim3-2*) and thus did not originate under aP vaccine pressure [27]. The argument that loss of one of five antigens would somehow allow escape from all five antigens in the vaccines is tenuous. In addition, a true vaccine-escape mutant would be expected to sweep across aP vaccinated populations across the globe, resulting in substantially decreased vaccine efficacy and newly increased epidemics in many countries simultaneously, which is not the prevailing pattern. In the absence of compelling evidence of such a sweep, Ocham's Razor requires that we consider a simpler explanation; *B. pertussis* continues to undergo rapid and profound genome reduction associated with its relatively recent loss of an environmental reservoir and commitment to a closed life cycle in humans. It is critical to better understand the genotypic and phenotypic variation among these species as they diverge, evolve and adapt to different ecological niches. This will, in

turn, provide the broader context in which to consider and understand their ongoing evolution.

CONCLUSION

Recent isolates from environmental sources provide a new perspective on the natural history of *Bordetella*, showing that pathogenic bordetellae likely evolved from ancestors in soil. The evolutionary pressure to evade amoebic predators may have selected for adaptations that enable survival of phagocytosis by either protists or mammalian immune cells. As more genomes from environmental sources become available, comparative genomics and metabolomics will reveal the genomic changes that accompany bacterial adaptation to eukaryotic hosts, which paved the way for the emergence of pathogens. Subsequent specialization of some lineages to a closed host-specific lifecycle involved substantial genome reduction, resulting in the loss of genes required only in the environmental reservoir.

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Conflicts of interest

There are no conflicts of interest.

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