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ORIGINAL ARTICLE
Genomic changes associated with the evolutionary transition of an insect gut symbiont into a blood-borne pathogen

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Introduction

Many host-restricted bacterial pathogens causing chronic infectious diseases have evolved from free-living environmental ancestors through a stepwise evolutionary process of host adaptation (Toft and Andersson, 2010; McCutcheon and Moran, 2012). Broadly, two major genomic changes accompany the evolution of pathogens: (i) genome reduction due to population bottlenecks and nutrient-rich host environments and (ii) acquisition of virulence factors for host invasion and persistence (Ochman and Moran, 2001; Pallen and Wren, 2007). Investigating the genomic changes associated with the transition of lifestyle is key to understand pathogen ecology and evolution (Parkhill et al., 2003; Rohmer et al., 2007; Langridge et al., 2015). To this end, comparative genome analysis of pathogens and closely related non-pathogenic environmental bacteria can provide valuable insights (Reuter et al., 2014; Bentley and Parkhill, 2015).

The alphaproteobacteria provide excellent subjects for comparative studies due to the large diversity of lifestyles within this class (Ettema and Andersson, 2009). Intracellular animal-associated pathogens emerged in several alphaproteobacterial lineages, each time associated with an overall reduction of genomic content and the acquisition of genes for the adoption of specific host infection strategies (Batut et al., 2004; Ettema and Andersson, 2009).

A striking example represents the genus Bartonella belonging to the Rhizobiales. This alphaproteobacterial order is dominated by plant and soil-associated bacteria. In contrast, Bartonella species include host-restricted facultative intracellular pathogens that have evolved a unique stealth infection strategy (Harms and Dehio, 2012): They colonize the erythrocytes of mammalian reservoir hosts resulting in acute or chronic, often asymptomatic, bloodstream infections. Transmission between hosts is ensured by blood-sucking arthropods. The evolutionary success of this life cycle is reflected by the adaptive radiation of this pathogen (Engel et al., 2011; Guy et al., 2013): More than 30 pathogenic Bartonella species have
been described to date (Kešnerová et al., 2016), infecting a wide variety of mammalian host species.

The divergent adaptation of the genus Bartonella to distinct hosts has been linked to the horizontal gene transfer of several factors for host interaction (Saenz et al., 2007; Engel et al., 2011; Guy et al., 2013), including Type IV secretion systems, auto-transporters and adhesins (Saenz et al., 2007; Engel et al., 2011; Guy et al., 2013). Moreover, bartonellae evolved a gene transfer agent, which mediates the transfer of genomic DNA between strains thereby facilitating diversification of genes for host interaction and enabling host adaptation (Guy et al., 2013).

Despite the acquisition of these genetic elements, the overall genomic evolution of Bartonella has been dominated by gene loss (Boussau et al., 2004). Bartonella genomes range in size from 1.4 to 2.6 Mb, which is small compared with soil or plant-associated Rhizobiales (Batut et al., 2004; Ettema and Andersson, 2009). The closest related family, the Brucellaceae, is phylogenetically already quite distant from the Bartonellaceae. Therefore, previous comparative analyses (Alsmark et al., 2004) provided limited insights into the evolutionary history of the genus Bartonella, especially in regard of the genomic makeup and ecology of its last common ancestor (LCA). However, microbial community analyses have recently indicated that honey bees and diverse ant species possess gut bacteria that are more closely related to the genus Bartonella than the family Brucellaceae (Cox-Foster et al., 2007; Martinson et al., 2011; Hu et al., 2014; Sanders et al., 2014). These insect-associated bacteria form deeply rooted sister lineages of the pathogenic Bartonella species (Kešnerová et al., 2016). Their symbiotic functions have so far remained elusive. However, several lines of evidence suggest that in ants these bacteria might play a role in nitrogen uptake by either fixing atmospheric nitrogen or recycling excreted insect waste products (Feldhaar et al., 2007; Russell et al., 2009; Anderson et al., 2012; Sapountzis et al., 2015). The symbiotic relationship between the Rhizobiales bacteria and the honey bee is so far unexplored.

We have recently cultivated several strains of the Rhizobiales gut symbiont of honey bees. On the basis of 16S rRNA sequence similarity (>95%), this bacterium belongs to the genus Bartonella and accordingly was named Bartonella apis (Kešnerová et al., 2016). The closest related Bartonella species to date is Bartonella tamiae, a pathogen that was isolated from the blood of three human patients in Thailand (Kosoy et al., 2008) and since then, B. tamiae-like DNA has been detected in various blood-sucking arthropods (Billeter et al., 2008; Kabeya et al., 2010; Leulmi et al., 2016). The close phylogenetic relationship with pathogenic Bartonella species renders B. apis promising to elucidate the evolutionary history of the unique infection strategy of the genus Bartonella. Here we sequenced the genomes of six divergent strains of B. apis. Our phylogenomic and comparative analyses shed light on the ancestral genomic state of the genus Bartonella providing novel insights into the evolutionary origin of these pathogenic strains.

### Materials and methods

#### Genome sequencing, assembly and annotation

Six cultured strains of B. apis were chosen for sequencing. Three strains (PEB0122, PEB0149 and PEB0150) originated from a honey bee colony (Apis mellifera) in West Haven, CT, US. Three other strains (BBC0122, BBC0178 and BBC0244) were isolated from a colony in Lausanne, Switzerland. The US strains were sequenced with Illumina MiSeq technology (2 × 250 bp reads) and assembled with Spades v3.6 (Bankevich et al., 2012). The Swiss strains were sequenced with SMRT technology (Pacific Biosciences, Menlo Park, CA, USA) and assembled with HGAP (Chin et al., 2013). All six genomes were annotated with the Integrated Microbial Genomes system (Markowitz et al., 2014). Additional details regarding DNA extraction and genome assembly are available in the Supplementary Methods and in Supplementary Table 1.

#### Determining orthologous gene families

For phylogenomic analyses, we determined orthologous gene families between the six strains of B. apis, two strains of B. tamiae (Bartonella initiative, Broad Institute) and 14 species representing the radiating lineage within the genus Bartonella (Supplementary Table 2). The latter group will be referred to as the eubartonellae in accordance with a previous study (Zhu et al., 2014). As outgroup taxa, we included the genome of the recently sequenced ant gut symbiont Ca. Tokpelaia hoeldobleri (Neuvonen et al., 2016) and five other Rhizobiales. An all-against-all BLASTP analysis of the proteomes of these 28 strains identified candidate orthologs. BLAST hits with an e-value >10⁻⁵ and for which the query and the hit sequence had less than 50% overlap of their gene length were excluded. Clusters of orthologous gene families were created using OrthoMCL (Li et al., 2003) with recommended settings (abc -I 1.5), resulting in a total of 7192 gene families.

#### Inferring phylogenetic trees

Genome-wide phylogenies were inferred from 589 orthologous gene families based on protein and DNA sequence alignments. Tree topologies and branch support values were inferred with maximum likelihood and Bayesian inference methods using RAxML v8.0.0 (Stamatakis, 2014) and PhyloBayes v4.1 (Lartillot et al., 2009), respectively. Additional details regarding the phylogenetic analyses are available in the Supplementary Methods.
Comparison of genome structure, genome divergence and gene content
To compare and visualize genomic regions we used the R-package genoPlotR (Guy et al., 2010). TBLASTX comparison files were created with DoubleACT (www.hpa-bioinfotools.org.uk). For comparison of specific regions, we generated pairwise comparison files with command-line TBLASTX. To estimate sequence divergence between genomes, we calculated pairwise average nucleotide identity (ANI) with JSpecies (Richter et al., 2015). We further estimated the number of synonymous substitutions per synonymous site (dS) with the Nei and Gojobori (1986) method in PAML-4.8 (Yang, 2007) for the 589 genes used in the phylogenetic analysis. The dS values were averaged over all genes to obtain genome-wide values for species comparisons. Pan genomes of \textit{B. apis}\textit{,} \textit{B. tamiae}\textit{,} and the eubartonellae were based on the gene families identified with OrthoMCL.

Inferring gene gain and gene loss
To identify at which branch of the genome-wide phylogeny genes have been gained and lost, and to infer the gene content of the LCA of the genus \textit{Bartonella}, we performed a gene flux analysis in PAUP v4.0 (Wilgenbusch and Swoford, 2003). We followed the method used in previous publications (Boussau et al., 2004; Guy et al., 2013); with generalized parsimony the 7192 protein families were mapped onto the phylogenetic tree. A cost-matrix was used which penalizes gene gain with 12, gene loss with 5, and gene duplication with 1 and copy changes with 0.2 units. These parameters were based on previous publications (Guy et al., 2013; Tamarit et al., 2015), except that the gene gain penalty was increased from 10 to 12 based on empirical testing of a range of parameters.

Analysis of functional gene contents
Gene contents were categorized based on COG functions. We used the COG database, which was updated in 2014 (www.ncbi.nlm.nih.gov/COG). Analysis of amino acid and cofactor biosynthesis pathways were based on KEGG pathway maps and EC (Enzyme Commission) numbers.

Table 1
| Strain | Size* (Mb) | Contigs* | GC %* | CDS* | rRNA loci* | tRNAs genes* | ANI* | dS** |
|--------|------------|----------|-------|------|------------|--------------|------|------|
| \textit{B. apis} PEB0122 | 2.60 | 16 | 45.5 | 2174 | 2 | 46 | – | – |
| \textit{B. apis} PEB0149 | 2.53 | 7 | 45.5 | 2113 | 2 | 46 | 98.3 | 0.03 (589) |
| \textit{B. apis} PEB0150 | 2.58 | 12 | 45.6 | 2196 | 1 | 43 | 97.5 | 0.05 (589) |
| \textit{B. apis} BBC0178 | 2.60 | 1 | 45.3 | 2228 | 2 | 47 | 85.8 | 0.77 (584) |
| \textit{B. apis} BBC0244 | 2.64 | 1 | 45.2 | 2264 | 2 | 46 | 85.8 | 0.77 (586) |
| \textit{B. apis} BBC0122 | 2.91 | 1 | 45.7 | 2396 | 2 | 47 | 85.1 | 0.82 (585) |
| \textit{B. tamiae} Th239 | 2.25 | 10 | 38.9 | 1983 | 2 | 44 | 69.9 | 1.61 (408) |
| \textit{B. tamiae} Th307 | 2.25 | 10 | 38.9 | 1977 | 2 | 44 | 69.8 | 1.62 (407) |
| \textit{B. australis} NH1 | 1.60 | 1 | 41.8 | 1265 | 2 | 42 | 68.4 | 1.24 (284) |
| \textit{B. bacilliformis} KS83 | 1.45 | 1 | 38.2 | 1322 | 2 | 44 | 68.6 | 1.48 (359) |
| \textit{B. bovis} 91-4 | 1.62 | 1 | 37.3 | 1379 | 2 | 43 | 68.5 | 1.58 (379) |
| \textit{B. clarridgeiae} 73 | 1.52 | 1 | 35.7 | 1326 | 2 | 59 | 68.6 | 1.48 (368) |
| \textit{B. henselae} Houston-1 | 1.93 | 1 | 38.2 | 1611 | 2 | 44 | 68.6 | 1.56 (374) |

*For previously published genomes, values are taken from the integrative microbial genomes (IMG) data management and analysis system (Markovitz et al., 2014).†Pairwise ANI and average number of synonymous substitutions per site (dS) are in respect to \textit{B. apis} PEB0122.‡dS values could not be calculated for each of the 589 genes due to saturation of sequence divergence. The numbers in brackets indicate the number of genes for which a dS value was obtained.
Ellegaard et al., 2015). Despite the marked degree of sequence divergence, the genomic organization between the B. apis strains is conserved (Figure 1). A high degree of genome structure conservation also exists between B. apis and B. tamaiae, with a few larger rearrangements present around the terminus of replication. In contrast, eubartonellae display a lower degree of structural conservation and many more rearrangements (Figure 1).

B. apis and B. tamaiae are sister clades that diverged before the radiation of the eubartonellae

The phylogenetic analysis of the concatenated protein alignments using maximum likelihood and Bayesian methods grouped B. apis and B. tamaiae into a monophyletic clade that diverged before the radiation of the eubartonellae (Figure 2 and Supplementary Figure 3a). In contrast, maximum likelihood and Bayesian trees inferred from aligned DNA sequences suggests that B. tamaiae branched off earlier (Supplementary Figures 3 and 4). The DNA analysis, however, was strongly affected by the similar GC content of B. tamaiae and the eubartonellae. When only considering the first and second codon position for the maximum likelihood analysis (Supplementary Figure 4), the monophyletic group of B. tamaiae and the eubartonellae was no longer supported by bootstrap analysis. On the basis of these findings and the fact that protein sequences are more reliable for divergent taxa (see high dS and low ANI values, Table 1), we decided to use the protein tree for further analyses. Sampling of additional taxa, closely related to B. apis or B. tamaiae, will help to resolve the phylogenies further in the future. Independent of the slightly different topologies, both phylogenies show that B. apis and B. tamaiae are sister clades that diverged before the radiation of the eubartonellae.

This suggests that B. tamaiae has a different evolutionary history than the eubartonellae despite the fact that both colonize the bloodstream of mammals and can cause human illness. Moreover, monophyly of B. apis and B. tamaiae would suggest that pathogenicity in the genus Bartonella has evolved twice, once in an ancestor of the eubartonellae and independently in the lineage of B. tamaiae.

B. apis and B. tamaiae share a large number of genes which are absent from the eubartonellae

The close phylogenetic position and conserved genomic synteny of B. apis and B. tamaiae prompted us to analyze the gene content which they have in common with the eubartonellae (Figure 3a). We found that the three groups share a relatively large fraction of their pan genomes (1081 genes) of which 805 genes were present in all 22 analyzed genomes, thus representing the core genome of the genus Bartonella. Surprisingly, B. apis and B. tamaiae share an additional 551 genes, which are absent from the eubartonellae. This represents a large fraction of their total genetic content, for example, 28% of the genome of B. tamaiae strain Th239. In contrast, the eubartonellae only share 111 and 46 genes with B. apis and B. tamaiae, respectively (Figure 3a). These results show that in terms of gene content, B. apis is more similar to B. tamaiae than the eubartonellae, providing further evidence that the two pathogenic groups of the genus Bartonella have distinct evolutionary histories.

The accessory gene content of B. tamaiae and B. apis was vertically inherited from the LCA of the genus Bartonella

Analysis of the genomic structure revealed no clustering of the 551 genes exclusively shared by B. apis and B. tamaiae providing little evidence for

**Figure 1** Comparative genome alignments of different Bartonella strains. Bartonella genomes are shown as gray horizontal lines. Genes specific to B. apis are shown in blue. Genes shared between B. apis and B. tamaiae, B. apis and eubartonellae and B. tamaiae and eubartonellae are shown in green, black and red, respectively. TBLASTX hits between genomes are shown in gray (bit score cutoff = 600).
horizontal gene transfer en bloc (Figure 1). Using a gene flux analysis, we inferred when genes were gained and lost along the species tree shown in Figure 2. In agreement with previous studies (Boussau et al., 2004; Guy et al., 2013), this analysis revealed an extensive gene loss before and after the split of the families of Brucellaceae (Brucella melitensis and Ochrobactrum anthropi) and Bartonellaceae (Ca. T. hoelldobleri and genus Bartonella) (Figure 3b). This general trend of genome reduction
continued within the genus Bartonella. Our analysis predicted that an additional 737 gene families were lost after the divergence of B. apis and B. tamiae, on the ancestral branch toward the eubartonellae (Figure 3b, Supplementary Table 3). Strikingly, 456 of the 551 shared genes by B. tamiae and B. apis belong to these 737 gene families (Figure 3c), indicating that a large fraction (83%) of the genes exclusively shared by these two species were vertically inherited from the LCA of the genus Bartonella. A BLASTP analysis of these 551 genes against the non-redundant database corroborated these findings yielding mainly hits to other Rhizobiales (Supplementary Figure 5).

Among the remaining genes that were lost by the eubartonellae are 214 genes present only in B. apis (Figure 3c). Apparently, these vertically inherited genes were also lost by B. tamiae and only retained by B. apis. Conversely, only 60 genes were retained in B. tamiae, but lost by B. apis (Figure 3c). Moreover, only 31 genes were lost by B. tamiae and B. apis, but retained by the eubartonellae (Figure 3b). These findings show that B. tamiae and B. apis harbor a markedly larger set of ancestral gene functions than the eubartonellae and that B. apis has retained the largest number of such ancestral gene families. Consequently, based on its functional potential, B. apis seems to resemble most the LCA of this genus. Considering that all bartonellae colonize insect hosts, it is conceivable that the LCA of the genus Bartonella was already an insect-associated gut symbiont, from which mammalian pathogens evolved. In support of this scenario is the existence of a deeply rooted sister lineage comprising gut symbionts of diverse ant species (including Ca. Tokpelaia hoelldobleri, Figure 2) (Stoll et al., 2007; Russell et al., 2009). Similar to this suggested evolutionary trajectory for the eubartonellae, vertebrate pathogens of the genus Rickettsia are thought to have emerged from insect symbionts. Pathogenic rickettsiae are transmitted by blood-feeding arthropods. However, the largest part of the currently known diversity of this genus consists of non-pathogenic arthropod associated strains (Perlman et al., 2006; Weinert et al., 2009). Also like B. apis, rickettsiae seem to be mainly facultative symbionts of their invertebrate hosts (Perlman et al., 2006). Possibly, a larger diversity of Bartonella-like bacteria is still to be discovered among insects.

The LCA of the genus Bartonella encoded amino acid and cofactor biosynthesis pathways
One hundred ten of the 456 ancestral gene families that were retained in the genomes of B. apis and B. tamiae, but lost in the eubartonellae belong to the COG categories ‘Amino acid transport and metabolism’, and ‘Coenzyme transport and metabolism’ (Figure 4a; Supplementary Table 3), suggesting that core metabolic functions were lost by the eubartonellae. For example, B. tamiae and B. apis both
encode complete biosynthesis pathways for all amino acids except Asn and Ala (Supplementary Table 4). According to our gene flux analysis, these pathways were vertically inherited from the LCA of Bartonella. The eubartonellae, however, have experienced substantial gene loss in these pathways (Figure 4b). Only six pathways were found to be complete in all analyzed genomes of the eubartonellae (Lys, Asp, Glu, Gln, Pro and Gly) (Supplementary Table 4). Although most genes were lost on the ancestral branch of the eubartonellae, we noted that further gene loss must have occurred within sublineages, as some species were missing more genes than others (Figure 4b and Supplementary Table 4).

A similar pattern of gene loss was found for cofactor biosynthesis (Figure 4b). All strains of B. apis and B. tamiae encode ancestral pathways for the biosynthesis of cofactors including heme, vitamin B12, vitamin B6, molybdopterine and tetrahydrofolate (Supplementary Table 5). It remains elusive whether B. apis and B. tamiae are completely autonomous in producing these cofactors, because some necessary enzymes could not be identified in the analyzed genomes. However, in the eubartonellae, these pathways are practically absent with most genes predicted to have been lost on the ancestral branch preceding the radiation (Supplementary Table 5).

The eubartonellae likely exploit the host for amino acid and cofactor acquisition, which makes the corresponding biosynthetic pathways superfluous. Indeed, genome-wide experimental screens have previously revealed that amino acid transporters and heme-binding proteins and transporters are essential for eubartonellae to colonize and persist in the mammalian bloodstream (Mavris et al., 2005; Saenz et al., 2007). These findings are in line with the absence of biosynthesis capabilities in other (facultative) intracellular pathogens (Zhang and Rubin, 2013).

In contrast, the honey bee gut symbiont B. apis does not seem to rely on amino acids and cofactors from the host. Its extracellular lifestyle, the competition with other gut bacteria and the nitrogen-limited plant diet of the host may impose strong selective pressure to retain the vertically inherited biosynthetic capabilities. Accordingly, these pathways were also found to be conserved in other honey bee gut symbionts (Kwong and Moran, 2016).

The conservation of the vitamin B12 biosynthesis pathway is particularly interesting. This pathway involves over 30 different steps, but only a few enzymes require vitamin B12 as cofactor, including methylmalonyl CoA mutase (MCM, EC 5.4.99.2) and methionine synthase H (MetH, EC 2.1.1.13). Both enzymes are encoded in the genomes of B. apis and B. tamiae, but are absent from the eubartonellae. MetH catalyzes the last step of the methionine biosynthesis. Thus, the conservation of the vitamin B12 and the methionine biosynthesis pathways is likely coupled. The second vitamin B12-dependent enzyme, MCM, catalyzes the last step in the degradation of propionate. This pathway is present in all six B. apis strains, but in none of the other Bartonella (Supplementary Figure 6). As in mammals or termites (den Besten et al., 2013; Brune, 2014), propionate may be an end product of bacterial fermentation in the honey bee hindgut, which could be utilized by B. apis for energy production.

The presence of ancestral biosynthetic pathways in B. apis suggests that the LCA of the genus Bartonella was a metabolically self-reliant bacterium. However, it was rather surprising to find that the pathogen B. tamiae has retained most of these ancestral pathways. In line with the evolution of the eubartonellae, adaptation to the mammalian bloodstream should have resulted in the loss of these pathways. Possibly, the primary ecological niche of B. tamiae is in the gut of hematophagous insects and colonization of mammals only happens incidentally. Thus, the genome of B. tamiae may not be streamlined to a host-restricted intracellular lifestyle as in the case of the eubartonellae. Indeed, to date little is known about a possible mammalian reservoir host of B. tamiae. Only three studies have so far detected B. tamiae in mammals, two in humans (Kosoy et al., 2008; 2010) and one in bats (Leulmi et al., 2016).

Bartonella apis-specific gene contents
We analyzed the B. apis-specific gene content to obtain insights into possible functional roles of this symbiont in the honey bee gut, but also to better understand the functional capabilities of the inferred ancestor of the entire genus. Out of 1241 B. apis-specific genes (Figure 3a), 289 are present in all six strains suggesting key functions for the ecology of this bacterium. On the basis of the gene flux analysis, 158 genes were vertically inherited from the LCA and 131 genes were acquired by horizontal gene transfer.

Anaerobic respiration via nitrate reduction. Interestingly, a large fraction of the B. apis-specific core genes are predicted to be involved in nitrogen metabolism. All six strains of B. apis encode a nitrate reductase gene cluster for anaerobic respiration (Supplementary Figure 7a). Two nitrate/nitrite antiporter genes are located upstream of the reductase genes and four to five ABC-type nitrate/taurine transporters are encoded elsewhere in the six genomes. According to the gene flux analysis and phylogenetic trees, the reductase genes were vertically inherited, whereas the ABC-type transporters were acquired by horizontal gene transfer (Supplementary Figure 7b and 7c). The acquisition of these additional transporters suggests that B. apis has a high demand for nitrate, possibly as an electron acceptor to acquire energy via anaerobic respiration. Previous experiments have indeed shown that B. apis,
in contrast to other bartonellae, reduces nitrate to nitrite in vitro (Kešnerová et al., 2016).

Recycling of nitrogenous waste products. For several herbivorous insects, it was proposed that gut bacteria may recycle nitrogenous waste products into amino acids (for example, Anderson et al., 2012). Strikingly, all six strains of B. apis encode a vertically inherited urease gene cluster to degrade urea into ammonia, which in turn can be converted into glutamine and glutamate (Supplementary Figure 8). Urea may derive from uric acid, the major waste product released by insects into the hindgut (McNally et al., 1965; Gullan and Cranston, 2009). Accordingly, a gene cluster for uric acid degradation is encoded in all six strains of B. apis. However, genes for two key enzymes are missing, suggesting that B. apis is not capable of converting uric acid on its own (Supplementary Figure 9). Interestingly, the related ant gut symbiont Ca. Tokpelaia hoelldobleri has retained the urease gene cluster suggesting that recycling of urea may be an important function of rhizobial gut symbionts that was also present in the LCA of the genus Bartonella. In contrast, neither the analyzed eubartonellae, nor the two B. tamiae strains encode the urease gene cluster. Thus, it was recently hypothesized that the loss of this function may have been a critical step toward the adaptation to hematophageous insect hosts (Neuvonen et al., 2016).

Degradation of plant secondary metabolites. Among the B. apis-specific gene content were also genes for the degradation of secondary plant metabolites. Four of the six strains encode the complete protocatechuate pathway for the degradation of 4-hydroxybenzoate, a common plant-derived aromatic compound (Figure 5). In addition, all six B. apis strains encode two quinone-dependent quinate dehydrogenases. One of these genes and the genes for the biosynthesis of the cofactor pyrroloquinoline quinone are located upstream of the gene cluster of the protocatechuate pathway (Figure 5). Quinate is a plant-derived cyclic polyol that is degraded by bacteria into shikimate and hippoc acid and subsequently used for amino acid biosynthesis (Teramoto et al., 2009). The protocatechuate pathway and the two quinate dehydrogenase genes were likely acquired by horizontal gene transfer, as the gene trees are incongruent with the species phylogeny (Supplementary Figures 10 and 11).

As plant secondary metabolites are present in pollen and nectar, they could be used by bee gut symbionts for energy and amino acid production. Likewise, these functions may have been beneficial for a herbivorous insect-associated ancestor of the genus Bartonella. However, at which point in evolution these genes were acquired, whether in a common ancestor of the entire genus or specifically in the lineage of B. apis, cannot be concluded from the current data.

Acquisition and expansion of virulence factors in the eubartonellae after divergence from B. apis and B. tamiae
To learn more about the evolutionary origin of the genus’ pathogenicity, we analyzed which virulence factors of the eubartonellae are present in B. apis and B. tamiae. We searched for 88 virulence factors (that is, genes important for host interaction or establishment of an intraerythrocytic infection) of which 69

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Figure 5 Genomic region encoding genes for the degradation of aromatic compounds in B. apis. The genomic regions of the six B. apis strains are compared with the corresponding region in B. tamiae strain Th239. Genes are depicted as arrows in different colors according to the legend. TBLASTX hits between genomes are shown as bands, with gray intensity reflecting the percentage identity of a given hit (e-value cutoff = 10^-15). In strain BBC0122 and PEB0150, the gene cluster of the protocatechuate pathway (that is, 4-hydroxybenzoate degradation) is interrupted by the insertion of a gene cluster coding for several dehydrogenase genes.
were classified as conserved (that is, present in alphaproteobacterial outgroup species) and 19 as Bartonella-specific (Supplementary Table 6). Except for two genes of unknown functions, none of the Bartonella-specific virulence factors are present in the genomes of B. apis and B. tamiæ (Figure 6a) including the well-characterized trw and virB T4SS and most autotransporter genes (Harms and Dehio, 2012). Consequently, these virulence factors must have been acquired after the divergence of B. apis and B. tamiæ, consistent with the hypothesis that they were key innovations for the radiation of the eubartonellae, facilitating the adaptation to different mammalian host species (Engel et al., 2011; Guy et al., 2013).

The majority of the conserved virulence factors have orthologs in B. apis and B. tamiæ (Figure 6a). Interestingly, both strains of B. tamiæ encode orthologs of the YadA-like trimeric autotransporter family (TAA) (Supplementary Figure 12). Representatives of this adhesin gene family, for example, BadA of B. henselæ, mediate important interactions of the eubartonellae with their mammalian hosts (Riess et al., 2004; Zhang et al., 2004; Saenz et al., 2007; Lu et al., 2013). Both homologs of B. tamiæ contain a 600-bp N-terminal extension including a signal peptide. Future experimental studies need to verify whether the TAA of B. tamiæ play similar roles for mammalian host interaction as their homologs in the eubartonellae.

Another group of conserved virulence factors, which show distinct patterns of evolution within the three major lineages of Bartonellae are hemin-binding proteins (Hbp). Although both B. apis and B. tamiæ encode two ancestrally duplicated paralogs (one is pseudogenized in B. tamiæ), this gene family has substantially expanded and diversified in the eubartonellae. Certain strains encode up to eight

![Figure 6](image-url)

**Figure 6** Bartonella virulence factors in B. apis and B. tamiæ. (a) Distribution of 88 virulence factors in B. apis and B. tamiæ categorized according to their evolutionary history (conserved in Rhizobiales versus Bartonella-specific). (b) The YadA-like trimeric autotransporter gene *badA* of *B. henselæ* Houston-1 (GenBank accession CAF26961) compared with three homologous genes or gene fragments identified on different contigs in the draft genome of *B. tamiæ* Th239. Protein domains are shown in different colors according to the legend and based on Riess et al., (2004). Contig names are indicated below the gene representations. AIMB01000003 and AIMB01000004 are adjacent contigs suggesting that the two open reading frames may present the N- and C-terminal part of the gene. In strain Th239, but not in strain Th307, the gene on contig AIMB01000003 has a frameshift in a homopolymeric G stretch as indicated by an asterisk. Vertical lines show contig ends. Diagonal dashes indicate that the contig would continue. TBLASTX hits are shown between the homologs of *B. henselæ* and *B. tamiæ*, with gray intensity reflecting the percentage identity of a hit (e-value cutoff = 10^-5). (c) Maximum likelihood phylogeny (model: WAG) of the hemin-binding proteins (Hbps) of *Bartonella* and corresponding homologs identified in outgroup species. The tree is based on the protein alignment (273 aa) of the conserved C-terminal region. The alignment was stripped of alignment positions with less than 50% coverage. Colors indicate different *Bartonella* species. A tree including the Hbps identified in all analyzed *Bartonella* species is shown in Supplementary Figure 13a. Bar indicates number of substitutions per site.
copies of *hbp* genes. Phylogenetic analysis suggests that this expansion occurred after the divergence of *B. apis* and *B. tamiae* via repeated duplication events (Figure 6c). Moreover, paralogs of this gene family show higher levels of sequence divergence in the eubartonellae than in *B. tamiae* and *B. apis* (Supplementary Figure 13b). Hbps are outer membrane proteins involved in the acquisition of heme and iron from the environment (Lee, 1992). Thus, the expansion of this family may have complemented the loss of the heme biosynthesis genes in the eubartonellae (Figure 4b). Expression of a diverse set of Hbps may increase the efficiency to absorb and uptake heme from the blood. Alternatively, the binding properties of the Hbps may lead to an extracellular heme coat that could serve as a nutritive reservoir for *Bartonella* during passage through the arthropod host, as shown for *Y. pestis* (Hinnebusch et al., 1996), or may act as antioxidant barrier against reactive oxygen species (Harms and Dehio, 2012). Independent of the precise role of the Hbps, our data strongly suggests that the expansion of this gene family is an adaptation to the specific infection strategy of *Bartonella*.

**Conclusion**

In this study, we sequenced the first six genomes of the honey bee gut symbiont *B. apis*, the closest known relative of the pathogenic members of the genus *Bartonella*. Our results show that *B. apis* has retained a large ancestral gene pool, which allowed us to reconstruct the metabolic capabilities of the *Bartonella* LCA. This provided compelling new insights into the genomic changes associated with the evolution of the characteristic intraerythrocytic infection strategy of *Bartonella*.

We conclude that the LCA of *Bartonella* was likely an amino acid and cofactor self-reliant gut symbiont that recycled nitrogenous waste products from its insect host. This is corroborated by the fact that a deeply rooted lineage of the genus *Bartonella* comprises a large group of ant-associated Rhizobiales with presumably similar functional capabilities (for example, Stoll et al., 2007; Russell et al., 2009; Neuvonen et al., 2016). Our hypothesis that the mammalian pathogens of the genus *Bartonella* derived from an insect-associated gut symbiont is in contrast to the evolution of other vector-borne pathogens. For example, *Y. pestis* is believed to have been adapted to the mammalian host before it evolved insect-vector transmission (Chain et al., 2004). It is similar, however, to the emergence of pathogens in the genera *Rickettsia* (Perlman et al., 2006; Weinert et al., 2009) and *Coxiella* (Duron et al., 2015). Bacterial strains of the genus *Coxiella* have so far only been found in ticks and among them are maternally inherited endosymbionts that form a basal lineage to the mammalian pathogen *Coxiella burnetti* (Duron et al., 2015). Genome analyses of these endosymbionts suggest that they provision their tick hosts with vitamins and cofactors (Gottlieb et al., 2015; Smith et al., 2015). A key step in the evolution of the pathogenic *Bartonella, Coxiella* and *Rickettsia* was probably the colonization of blood-sucking arthropods. In *Bartonella*, this transition could have happened twice, once in the ancestor of the eubartonellae, and another time in the lineage of *B. tamiae*. Because our phylogenetic analyses based on amino acid and DNA sequences disagree on the placement of *B. tamiae*, discovery of more *Bartonella*-like bacteria could further resolve the evolution of the genus.

In contrast to the eubartonellae, *B. tamiae* has retained ancestral amino acid and cofactor biosynthesis pathways and lacks most *Bartonella*-specific virulence factors. This indicates that *B. tamiae* may have a different ecology than the eubartonellae. Possibly, *B. tamiae* presents an intermediate state of the evolutionary transition from a gut symbiont to a mammalian parasite. Although it is adapted to colonize hematophageous insects, it may not be adapted to a mammalian reservoir host and only causes opportunistic infections. Future experimental studies focusing on the infection course of *B. tamiae* in mammals will shed light on the stepwise evolution of virulence in the genus *Bartonella*. For example, it is currently unknown whether *B. tamiae* can enter erythrocytes and persist in the bloodstream, as in the case of the eubartonellae (Harms and Dehio, 2012).

Finally, our study shows that genome reduction in host-restricted bacterial pathogens may be counteracted by the expansion of vertically inherited gene families. Besides the acquisition of dedicated virulence factors, we find that the eubartonellae have compensated the loss of the heme biosynthesis pathway by the expansion of an ancestral family of Hbps. This may allow pathogenic *Bartonella* to efficiently acquire heme and iron from the mammalian host and overcome nutritional shortages during passage through the insect vector.

Bartonellae are experimentally amenable and in vivo colonization models are established for both honey bee gut symbionts (Engel et al., 2015) and *Bartonella* mammalian pathogens (Harms and Dehio, 2012). Thus, our comparative genomic analysis sets the ground for future experimental studies to understand how changes in gene content affect host interactions during the evolutionary transition from an insect-associated gut symbiont to a mammalian pathogen.

**Conflict of Interest**

The authors declare no conflict of interest.

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### Sequence deposition

The genome sequences of *Bartonella apis* PEB0122, PEB0149, PEB0150, BBC0122, BBC0178, and BBC0244 are deposited in GenBank with accession numbers LXYU00000000, LXYT00000000, LXYZ00000000, CP015625, CP015820 and CP015821.

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