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BACTERIOLOGY (N BOREL, SECTION EDITOR)

Bacterial Diseases in Honeybees

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Abstract
Purpose of Review American foulbrood (AFB) and European foulbrood (EFB) are widely distributed and highly infectious bacterial diseases of honeybee brood causing colony losses and considerable economic strain on apiculture globally. In this review, we synthesize the most recent discoveries and achievements made towards understanding the pathogenesis and epidemiology of these two bacterial diseases and present current efforts in finding ways to combat them.
Recent Findings Advancements in molecular methods, such as next-generation sequencing, have provided high-resolution insight into the epidemiological parameters and factors of virulence for the foulbroods of honeybees.
Summary The recently gained detailed knowledge of the diversity, biogeography, and relatedness of strains and sub-types of the causative bacteria of AFB and EFB provides a background to study their epidemiology at many scales. Such information will help provide a more global perspective on honeybee disease epidemiology for an increasingly international beekeeping industry.

Keywords American foulbrood · *Apis mellifera* · European foulbrood · *Melissococcus plutonius* · *Paenibacillus larvae*

Introduction

American foulbrood (AFB) and European foulbrood (EFB) are two well-known and widely distributed bacterial brood diseases of honeybees causing colony losses and considerable economic strain on apiculture globally [1]. These pathogens affect the honeybee brood, i.e. the larval and pupal stages of the bee, causing an eponymous foul smell and weakening of the colony that can lead to colony death [2]. Both AFB and EFB are classified as epizootic and in many countries are notifiable, i.e. must be reported by law to the relevant government authorities. In most European countries, AFB and EFB are controlled through burning of symptomatic colonies and through the use of beekeeping management techniques to avoid the spread of the infectious agent to uninfected hives. Current legislation does not allow European beekeepers to use antibiotics to control AFB or EFB since there is no maximum residue limit (MRL) set for the safe amount of such substances in honeybee products used for human consumption, such as honey. In the USA, Canada, and several other countries, antibiotics are common and frequently used as a precautionary measure for these diseases. However, the use of antibiotics as a control strategy against AFB and EFB is unsustainable since this treatment only masks the symptoms and does not eliminate the bacterial spores that drive the spread of the disease. It has been estimated that in areas where antibiotics are used, 10 to 20% of AFB-infected colonies would succumb to the disease if the antibiotic treatment ceased or became ineffective due to the development of antibiotic resistance [3]. Beekeeping management techniques that avoid the spread of the disease to other colonies and areas, supplemented by the destruction of clinically symptomatic honeybee colonies, appear to be a more sustainable way to control AFB and EFB [4]. It is therefore crucial to understand the epidemiology and spread of these diseases in order to develop sustainable control measures and improve management techniques that prevent disease outbreaks. Furthermore, there are vast differences in distribution patterns, diversity, pathogenesis, and virulence between AFB and EFB that require clear understanding for

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establishing good monitoring and control of these diseases. In recent years, huge advances in molecular techniques have provided new insight on AFB and EFB pathogenic mechanisms and epidemiology. These new insights, on two previously poorly understood but economically important honeybee diseases, are presented in this review.

American Foulbrood

The Gram-positive bacterium *Paenibacillus larvae* is the etiological agent of the most serious brood disease of honeybees, American foulbrood (AFB), causing considerable losses of honeybee colonies in temperate and subtropical regions throughout the world [2]. *P. larvae* produces extremely tenacious spores that are the only infectious form of this bacterium [5]. Young honeybee larvae ingest the *P. larvae* spores with the food that is provisioned to them by the adult nurse bees in the colony. Only the young bee larvae become diseased and are most susceptible for infection during the first 12–36 h after hatching [6, 7]. At these stages, a dose of ten spores or less are sufficient to infect a larva and cause disease [8]. The spores of *P. larvae* germinate and proliferate in the midgut, invading the larval tissue where it continues to proliferate and produce billions of spores [9]. The infected larva eventually dies and is degraded by *P. larvae* to a brownish, semi-fluid glue-like colloid usually known as a "ropy mass"-the primary clinical symptom for diagnosis of AFB. Brood combs of infected colonies show a patchy brood pattern, and the capping of cells containing diseased honeybee larvae appear darkened and sunken with a greasy look and abnormal perforations. The semi-fluid glue-like colloid eventually dries down to a hard scale tightly adhering to the lower cell wall.

To confirm a disease suspicion or to monitor the prevalence of *P. larvae*, various products from the honeybee hive (e.g., honey, bees, wax, pollen, debris) can be sampled for laboratory analysis [10]. Disease diagnosis using samples of honey and adult bees have a higher prognostic value compared to the detection of the bacteria in wax, pollen, and debris samples [11, 12]. Regular disease monitoring is important because if AFB is not detected and treated, it will lead to the loss of the infected hive [13] and serve as a major source for infections to neighboring colonies. Control methods for AFB differ across the world. While the burning of diseased colonies is considered to be the most effective control method [2], infected colonies can also be treated by the widely practiced "shook swarm" method where essentially all brood is removed and the adult bees are given new material and new wax foundation [14]. The excessive use of broad-spectrum antibiotics has resulted in bacterial resistance and residues in bee products and is legally banned in several countries. The increased demand for alternative, natural strategies for the prevention and control of AFB has led to extensive studies on the application of essential oils, plant extracts, propolis, royal jelly, nonconventional natural molecules, probiotics, prebiotics, fatty acids, bacteria, and bacteriocins for this purpose. However, the extremely resilient nature of the *P. larvae* spores may decrease the efficacy of these various approaches. A detailed description of different alternative, natural products and strategies for AFB control can be found in Alonso-Salces et al. [15] and Kuzyšinová et al. [16]. Another recently evaluated strategy is bacteriophage therapy, which was reported to be an effective treatment or prophylaxis against AFB when used as a cocktail of multiple phages in larvae diet [17–19]. However, the host range of the used phages must be investigated to ensure that all relevant *P. larvae* strains are targeted and to reduce dysbacteriosis of the midgut microbiota as well as any ecological risks [20].

The natural spread of *P. larvae* between colonies occurs through horizontal transmission where individual honeybees carrying bacterial spores drift between colonies or when robbing bees, stealing resources from weaker sick colonies, pick up spores and carry them back to their own colony [21]. The transmission of this disease is therefore density dependent, and AFB outbreaks occur more frequently in areas with high colony densities typical of apiculture. The detection of low numbers of *P. larvae* sequences outside of an AFB outbreak zone reveals the spatial enzootic occurrence of the pathogen [22, 23] suggesting other transmission pathways. Common beekeeping practices, like the transport and reuse of hive material and the transfer of bees between colonies, accelerate the spread of AFB and are actually much more important routes of transmission than natural drifting or robbing [24, 25]. Additionally, the global trade in honeybees and honeybee products facilitates long distance movement of infected material [2, 26•].

In the past, the lack of solid approaches to classify different strains of *P. larvae* limited epidemiological studies to a local or regional basis [2]. However, recent developments in the molecular epidemiology of AFB, using multi locus sequence typing (MLST) [27•, 28] and more recently core genome (cg) MLST [26•], are enhancing our understanding of the epidemiological relationship among *P. larvae* isolates of different origin. Such methods represent useful tools in future studies for high-resolution tracing of AFB outbreaks.

Four different ERIC-genotypes (I–IV) of *P. larvae* have been described using enterobacterial repetitive intergenic consensus sequence (ERIC) primers [29]. High-resolution methods, including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [30], MLST [27•], multiple locus variable number of tandem repeat analysis (MLVA) [31], and recently whole-genome sequencing (WGS) [26•], have confirmed and extended the ERIC typing. Of these four genotypes, only ERIC I and ERIC II are of practical importance as they both are regularly isolated from diseased honeybee colonies

worldwide [27•, 30]. In contrast, ERIC III and ERIC IV exist only in strain collections. The genotypes ERIC I and ERIC II differ in virulence from one another at both the individual and the colony level [8, 32]. At the individual level, ERIC I strains lead to 100% mortality of infected larvae in about 12 days, while bacteria of the ERIC II genotype kill infected individuals in about 7 days [8, 29]. The more virulent genotype at the individual level, ERIC II, actually has a reduced virulence at the colony level since the larvae are killed before capping and can be easily detected and removed by the social hygienic behaviors of adult bees. The reverse is true for bacterial strains of the ERIC I genotype, where infected larvae survive to be capped and elude early detection and removal. Thus, the infection of the ERIC I genotype proliferates in the sealed brood cell with spore production and ultimately higher virulence at the colony level [32].

The understanding of *P. larvae* pathobiology is constantly progressing and has recently been thoroughly reviewed by Ebeling et al. [33]. Midgut bacteria that might compete for organic nutrients inside the larval gut are potentially eliminated by non-ribosomal peptides produced by *P. larvae* [34–38]. Chitin-degrading enzymes attack the peritrophic membrane [39], and several toxins produced by bacterial strains of ERIC I genotype are known to attack the gut epithelium [40]. In contrast, an S-layer protein is involved in the attachment of *P. larvae* to the midgut epithelium in bacteria of ERIC II type [41].

Full genome sequencing of *P. larvae* strains DSM25719 and DSM25430 allowed the first comparison of ERIC I and ERIC II genomes [42••]. The results showed a higher genome size and gene number in the ERIC I strain. BLAST comparison (Fig. 1a) confirmed that most of the additional regions in the ERIC I sample are prophage sites. The comparison also showed a high copy number of transposases belonging to the mutator superfamily IS256 in the analyzed ERIC II strain (Fig. 1b). In addition, *P. larvae* genomes contain long genomic repeats which could challenge full-length sequencing [43]. Overall, these studies suggest that the *P. larvae* genome is frequently rearranged and plastic. Further work is required to test if *P. larvae* genome characteristics and differences between ERIC types could be extended to more strains.

The study by Djukic and collaborators [42••] also enabled identification of the genes potentially involved in pathogenesis and secondary metabolite production. Four different polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) clusters were identified in the sequenced genomes [42••], as well as four dedicated non-ribosomal secondary metabolites [37]. The non-ribosomal tripeptide sevadicin was shown to have antibacterial activity [34], the iturin lipopeptidas paenilarvins to have antifungal activities [38], and the penilamicin both antibacterial and antifungal activities [35]. However, the synthesized bacillibactin, a catechol-type siderophore, is not regarded to be involved in *P. larvae* virulence. Larval infection assays comparing wild-type strains and gene manipulated strains of *P. larvae* genotypes ERIC I and ERIC II showed that neither larval mortality nor disease progression (cumulative larval mortality) differed as a function of presence or absence of bacillibactin [36]. For more detailed information on secondary metabolites, see the review by Müller et al. [37]. Functional identification of virulence factors was recently investigated in an unbiased way in an ERIC I strain, and several loci previously reported to encode for virulence factors in other bacteria were found, such as *CirD* and *gbpA* [44]. Further discoveries of *P. larvae* virulence factors and secondary metabolites will contribute to the understanding of the pathogenesis of AFB.

European Foulbrood

The bacterial disease European foulbrood (EFB) occurs in honeybees throughout the world. It is usually not as devastating as AFB, although it may lead to serious losses of brood and to the weakening and losses of host colonies. In many areas, the disease is endemic with occasional, seasonal outbreaks and spontaneous recovery. However, dramatic increases in EFB incidence have been seen during the last decades in the UK and Switzerland and large disease outbreaks have been recorded from countries thought to be disease-free (e.g., Norway) [45–47]. High numbers of clinical cases of the disease have also recently been reported from Finland, France, Greece, Holland, Italy, and Czech Republic [48, 49]. The disease appears to be benign in some areas and increasingly severe in others. Apiculture may locally agglomerate extremely high densities of honeybee populations thus promoting transmission of the pathogen [50]. Bees suffering from the disease die during the larval stage. and death may occur at any time from the fourth day up to pupation. However, the defining characteristic of EFB is the death of brood during the feeding stage in uncapped cells. The general symptoms a beekeeper may observe in a colony is patchy and erratic brood pattern that is sometimes accompanied by an unpleasant odor. The individual larvae die displaced in their cells, and the color of the larvae changes from pearly white to yellow, brown, and grayish black. Treatment strategies for EFB are similar to that of AFB. The use of antibiotics is restricted in many countries, and the "shook swarm" method is recommended for EFB control. However, the burning of colonies with severe disease symptoms is widely used [50].

EFB is caused by the Gram-positive lanceolate coccus *Melissococcus plutonius* [51, 52]. The bacterium is isolated not only from the European honeybee, *Apis mellifera*, but also from *Apis cerana* and *Apis laboriosa* [53–55]. EFB is an intestinal infection of honeybee larvae initiated by the consumption of contaminated feed provided by nurse bees [56]. Early studies show that *M. plutonius* multiplies in the food mass and

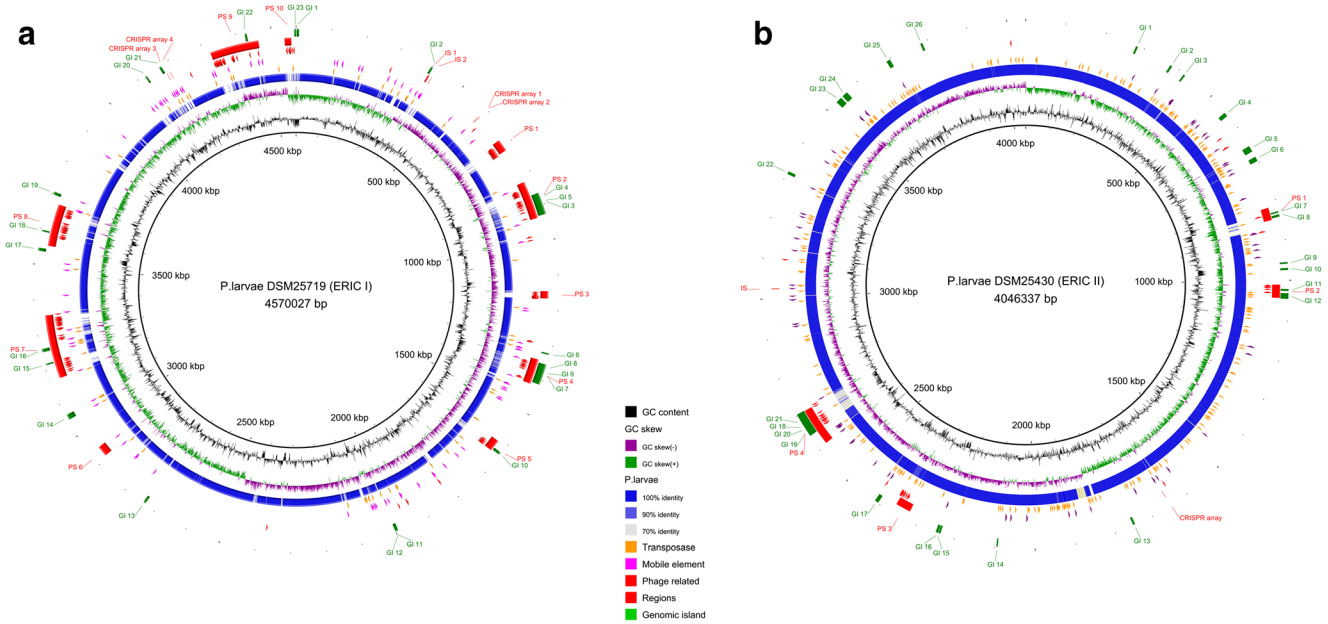

Fig. 1 Comparison of ERIC I (DSM25719) and ERIC II (DSM25430) reference genomes. Genomes were compared using BLAST Ring Image Generator [73]. **a** Represents the comparison of ERIC I versus ERIC II. **b** The opposite comparison. The blue ring corresponds to the percent identity of the BLAST results. Transposases, phage-related genes, and mobile elements (arrowheads) were positioned onto each genome. Red rectangles correspond to regions (PS prophage site, IS Insertion sequence, CRISPR array), and green rectangles represent genomic islands

the peritrophic membrane interface and is considered to kill the host before it or any other bacteria associated with EFB invades the larval tissue [56, 57]. A recent study confirms that the infection is essentially confined to the digestive tract, but diffusion of *M. plutonius*-derived substances into the larval tissue was observed [58•]. The factors leading to the second step of infection, tissue damage and the phase of overt symptomatology, remain enigmatic.

Unlike bee larvae that die from AFB infection containing exclusively the causative agent, *P. larvae*, bee larvae that die from EFB usually contains secondary bacteria [59, 60]. Secondary bacteria may have a supplementary pathogenic effect on *M. plutonius* in diseased larvae but their role in disease development is unclear and debated. Bacteria such as *Enterococcus faecalis*, *Brevibacillus laterosporus*, *Bacillus pumilis*, *Paenibacillus alvei*, and *Paenibacillus dendritiformis* have been isolated from symptomatic larvae together with *M. plutonius* [59, 61, 62, 63•, 64], and some are even considered as presumptive evidence of EFB [65]. The classification of the bacterium *Achromobacter eurydice*, frequently found together with *M. plutonius* in larvae with symptoms of EFB, has recently been revised [63•]. The bacterium *A. eurydice* was morphologically and biochemically characterized more than a century ago [57], and very few studies have investigated its biological relevance in EFB. Moreover, the only available reference strain deposited by White (ATCC 39312) was recently re-classified as *Kurthia* sp. and a comparative literature search provides circumstantial evidence that two fructophilic lactic acid bacteria (*Lactobacillus kunkeii* and *Fructobacillus fructosus*) could indeed be the bacterium earlier described as *A. eurydice* [63•]. However, one should be careful to diagnose EFB based on observation of disease symptoms and the presence of secondary bacteria without confirming the presence of *M. plutonius*. Any bacteria present in the honeybee colony could potentially invade a dead larva, which died for other reasons than *M. plutonius* infection, then take advantage of the available nutrients, multiply, and cause symptoms similar to those described typical for EFB.

Early reports have suggested that *M. plutonius* exhibit extremely low levels of genetic diversity although some differences were observed in biochemical and physical characteristics among bacterial isolates [66, 67]. More recently, a *M. plutonius* "atypical" subtype from Japan showing phenotypic and genetic differences from previously described "typical" bacterial isolates were reported suggesting more genetic variation. Using pulsed-field gel electrophoresis (PFGE), the typical and atypical isolates grouped into two genetically distinct clusters [68•]. A higher resolution multilocus sequence typing (MLST) scheme for *M. plutonius* was able to further distinguish strains of this bacterium into sequence types (STs) grouped into three genetically distinct groups or clonal complexes (CCs), CC3 and CC12 and CC13 [69•, 70••]. The MLST scheme was used to analyze international isolates of *M. plutonius*, and isolates from Brazil, the UK, the USA, and the Netherlands were found identical or similar to the Japanese atypical genome [71] suggesting that the so-called atypical strains are distributed globally. Strains included in CC13 and CC3 (including the *M. plutonius* type strain, LMG 20360) belong to the typical *M. plutonius* subtype while CC12 (including the Japanese atypical strain DAT561) belong

to the atypical subtype. The MLST was further applied to *M. plutonius* isolates sampled across England and Wales, and the results suggest that CC3 is an established, endemic complex whereas CC12 and CC13 were introduced more recently, possibly through intercontinental trading in honeybees and their products [70••]. To facilitate an international picture of disease movements, the authors developed a public MLST database for the deposition and administration of genetic data on *M. plutonius*, collective data that will provide insight into the global epidemiology of the pathogen in the future.

The virulence of *M. plutonius* is known to reduce rapidly after culturing [61], and the attenuation rate differ between *M. plutonius* cultures [68•]. Data from Budge and coworkers provide evidence that *M. plutonius* from different CCs may differ in virulence at both brood and colony level, and that severe cases of disease might be correlated with particular CCs [70••]. This study is the first to compare direct observations of virulence in the field, and the data suggest that pathogen variation could explain regional, national, and international variation in disease impact.

Concluding Remarks

Combating bacterial diseases of honeybees is critical for developing strategies towards sustainable and economically viable beekeeping. Successfully combating foulbrood diseases starts with cutting-edge detection methods for laboratory diagnosis and epidemiological studies. Other key factors include understanding the pathobiology of the causative agents, the host-pathogen interactions during infection, and the role of virulence factors and secondary metabolites in the pathogenesis of *P. larvae* and *M. plutonius.* The virulence factors of *P. larvae* identified to date have already immensely improved our understanding of its pathogenesis, and future discoveries will widen this picture.

The understanding of foulbrood disease epidemiology has been fundamentally improved by the ability to distinguish between genetically related pathogens within a bacterial species. Now, with the development of internationally harmonized typing methods, scientists are able to determine the relation between outbreak areas in which bacterial diseases cluster over time [72]. Further developments in the use of high-resolution whole-genome sequencing methods will be important for epidemiological studies and establishing continuous monitoring of both AFB and EFB.

Improving current control strategies for honeybee bacterial diseases begins with understanding the spread of disease and its distribution at a local level, which are both strongly influenced by management practices. However, apiculture is a global industry with a steady movement of bees and hive products around the world. An international perspective on honeybee bacterial disease is therefore necessary to help identify and eventually eliminate the major mechanisms of the spread of these diseases and to ensure safe trade worldwide.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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