RESEARCH ARTICLE

The Bacterium *Frischella perrara* Causes Scab Formation in the Gut of its Honeybee Host

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ABSTRACT **Honeybees harbor well-defined bacterial communities in their guts. The major members of these communities appear to benefit the host, but little is known about how they interact with the host and specifically how they interface with the host immune system. In the pylorus, a short region between the midgut and hindgut, honeybees frequently exhibit scab-like structures on the epithelial gut surface. These structures are reminiscent of a melanization response of the insect immune system. Despite the wide distribution of this phenotype in honeybee populations, its cause has remained elusive. Here, we show that the presence of a common member of the bee gut microbiota, the gammaproteobacterium *Frischella perrara*, correlates with the appearance of the scab phenotype. Bacterial colonization precedes scab formation, and *F. perrara* specifically localizes to the melanized regions of the host epithelium. Under controlled laboratory conditions, we demonstrate that exposure of microbiota-free bees to *F. perrara* but not to other bacteria results in scab formation. This shows that *F. perrara* can become established in a spatially restricted niche in the gut and triggers a morphological change of the epithelial surface, potentially due to a host immune response. As an intermittent colonizer, this bacterium holds promise for addressing questions of community invasion in a simple yet relevant model system. Moreover, our results show that gut symbionts of bees engage in differential host interactions that are likely to affect gut homeostasis. Future studies should focus on how these different gut bacteria impact honeybee health.**

IMPORTANCE **As pollinators, honeybees are key species for agricultural and natural ecosystems. Their guts harbor simple communities composed of characteristic bacterial species. Because of these features, bees are ideal systems for studying fundamental aspects of gut microbiota-host interactions. However, little is known about how these bacteria interact with their host. Here, we show that a common member of the bee gut microbiota causes the formation of a scab-like structure on the gut epithelium of its host. This phenotype was first described in 1946, but since then it has not been much further characterized, despite being found in bee populations worldwide. The scab phenotype is reminiscent of melanization, a conserved innate immune response of insects. Our results show that high abundance of one member of the bee gut microbiota triggers this specific phenotype, suggesting that the gut microbiota composition can affect the immune status of this key pollinator species.**

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The honeybee, *Apis mellifera*, presents a reservoir for a multitude of microbes that affect health and disease in this key pollinator species (1–4). Transmission, dissemination, and persistence of bee-associated microbes are facilitated by the host's social lifestyle, by sharing foraging sites with other insects, and by mixing and distributing populations in the bee-keeping industry across the world. Some bee-associated microbes are agents of emerging infectious diseases, including several viruses transmitted by mites and the microsporidian *Nosema ceranae* (5–7). These pathogens present severe threats to honeybee health and, in part, are responsible for the elevated losses of honeybee populations reported across the world in recent years (4, 5, 8, 9). In addition, honeybees are colonized by a highly characteristic gut microbiota dominated by only eight bacterial species (10, 11). A subset of these bacteria is also found in guts of other *Apis* spp. and related bumblebee species (genus *Bombus*) (12, 13), suggesting longstanding associations between gut bacteria and social bees. There is accumulating evidence that the most conserved members of these communities engage in mutualistic interactions with the host, with roles in nutrition or pathogen defense (14, 15). However, some members could be opportunistic parasites or commensals that occasionally invade and colonize the bee gut to exploit this nutrient-rich environment. Evidence from microbial community analyses shows that certain bee gut bacteria have sporadic occurrence and variable abundance (11, 13, 16, 17). As yet, little is known about which niches these bacteria colonize in the gut and how they interact with the host.

A particularly interesting region for studying gut bacterium-host interaction and community invasion is the ileum, the anterior part of the bee hindgut. Here, *Gilliamella apicola* (*Gammaproteobacteria*) and *Snodgrassella alvi* (*Betaproteobacteria*)

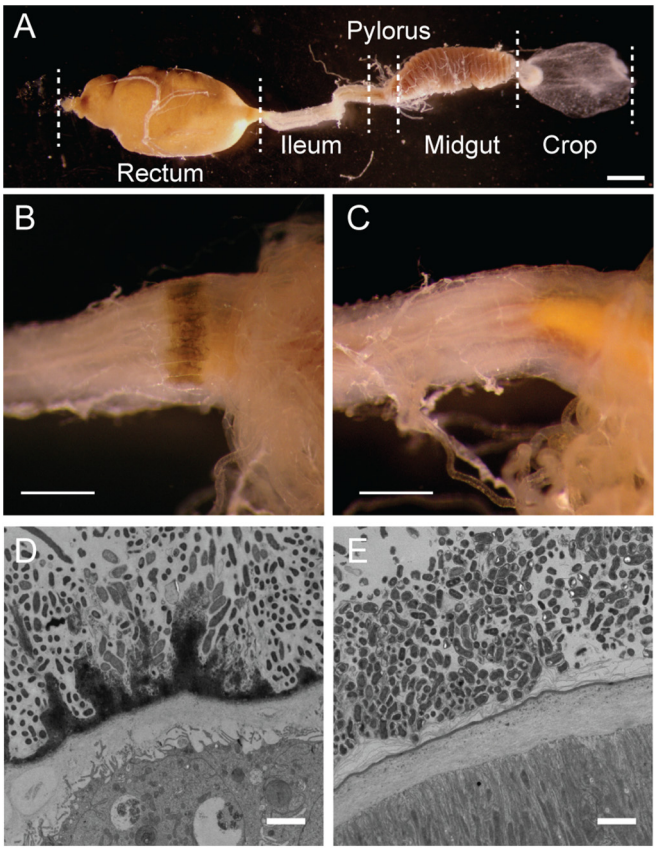

FIG 1 Scab phenotype in the pylorus of honeybees. (A) Dissected gut of an adult honeybee. Different gut regions, including the region defined as the pylorus, are outlined with dashed lines. The rectum is filled with pollen. Scale bar, 1 mm. (B and C) Pylorus region with scab and without scab, respectively. Scale bars, 0.5 mm. (D and E) Electron micrographs of the pylorus epithelium with scab and without scab, respectively. A cuticle layer separates the gut bacteria from the epithelial cell envelope. Scale bar, 2 μm. In panel D, an electron-dense material presenting the dark deposits of the scab structures is located between the cuticle layer and the bacteria in the lumen.

predominate and colonize the host epithelium in a thick biofilm-like layer (18). These two bacteria are common members of the bee gut microbiota, being present in various *Apis* spp. and *Bombus* spp. (10–13, 16, 17, 19). In the European bumblebee, *Bombus terrestris*, both species have been suggested to play a role in protection against the protozoan parasite *Crithidia bombi* (15), and in honeybees, *G. apicola* produces enzymes that may contribute to pollen digestion (14). *G. apicola* and *S. alvi* appear to partition metabolic resources in the ileum and thus may cooperate rather than compete for nutrients (20). A third member, *Frischella perrara* (*Gammaproteobacteria*), is closely related to *G. apicola* and has similar metabolic capabilities, gaining energy from anaerobic fermentation of carbohydrates (21). Two recent studies provided evidence that *F. perrara* also colonizes the ileum and therefore could directly compete with *G. apicola* for resources (18, 22). However, its abundance varies across honeybees and often is much lower than that of *G. apicola* or *S. alvi* (11, 16, 17, 22). In bumblebees, *F. perrara* has so far not been detected.

In the transition zone from the midgut to the ileum, a region also referred to as the pylorus (Fig. 1A), honeybees frequently show a peculiar morphology on the gut epithelium characterized by dark, scab-like depositions of an unknown material (shown in Fig. 1B). These depositions are located on the luminal surface of the gut epithelium and typically appear as a yellow to brown to black band restricted to one side of the gut perimeter. In early microscopy studies, it was observed that these structures are found only in a subset of bees of a given colony and that bacteria as well as the protozoan *Crithidia mellificae* colonize the top of these structures (23, 24). However, neither the underlying cause nor the role of these structures has been elucidated previously.

In this paper, we show that the frequency of bees with scab phenotypes differs among bee colonies. We monitored the development of the scab phenotype in newly emerged bees over time and analyzed its correlation with bacterial colonization of the pylorus, using quantitative PCR (qPCR), fluorescence in situ hybridization (FISH) microscopy, and gut colonization experiments. Our data suggest that the scab phenotype presents a putative host melanization response caused by the specific colonization of the pylorus with the bacterium *F. perrara*. This highlights the need for a better understanding of how different microbiota members impact the health of this important pollinator species.

RESULTS

Characterization of the scab phenotype in the pylorus of honeybees. We identified pylorus scabs in adult honeybees from all colonies analyzed in Switzerland (four colonies, $n = 25$ to 50) and in the United States (two colonies, $n = 21$ to 140). The percentage of bees with scabs varied among colonies, ranging from 24% to 82% (see Fig. S1 in the supplemental material). The scab phenotype was always restricted to the same posterior region of the pylorus (Fig. 1A), located about 0.5 mm behind the attachment sites of the Malpighian tubules at the beginning of the ileum (Fig. 1B and C). As previously reported (24), the scab most often appeared as a relatively regular band that rarely surrounded the entire gut circumference. Its coloration varied from yellow-brown to dark-brown to black (see Fig. S2 in the supplemental material). Electron microscopy analysis of pylorus cross sections revealed that the scab material is located on the luminal surface of the gut epithelium between the cuticle layer and the bacteria colonizing the gut lumen (Fig. 1D and E).

In a time series experiment, we analyzed the development of the scab phenotype in adult honeybees (Fig. 2A). To this end, 20 age-controlled bees were sampled within a colony at each of 11 time points over a period of 29 days. Newly emerged bees (day 0) did not exhibit any scab structures in the pylorus. However, 5 days after emergence, 20% of the bees had developed weak scab structures. At day 7, the percentage of bees with scabs increased to 70% and then stayed more or less constant (70 to 85%) until the last sampling time point at day 29. Scab structures tended to be darker and covered larger surface areas in older than in younger bees (see Fig. S2 in the supplemental material), suggesting a continuous deposition of new scab material. A second time series experiment conducted in a different colony in a different year yielded similar results (see Fig. S3 in the supplemental material), though the percentage of scab-positive bees was lower. Notably, when removed from the hive before pupal eclosion and kept under clean laboratory conditions, bees did not exhibit pylorus scabs at any of the analyzed time points ($n = 23$ [see Fig. S3 in the supplemental material] or $n = 45$ [see Fig. 6]).

Occurrence of scab phenotypes correlates with high abundance of the gut symbiont *F. perrara*. Using fluorescence in situ

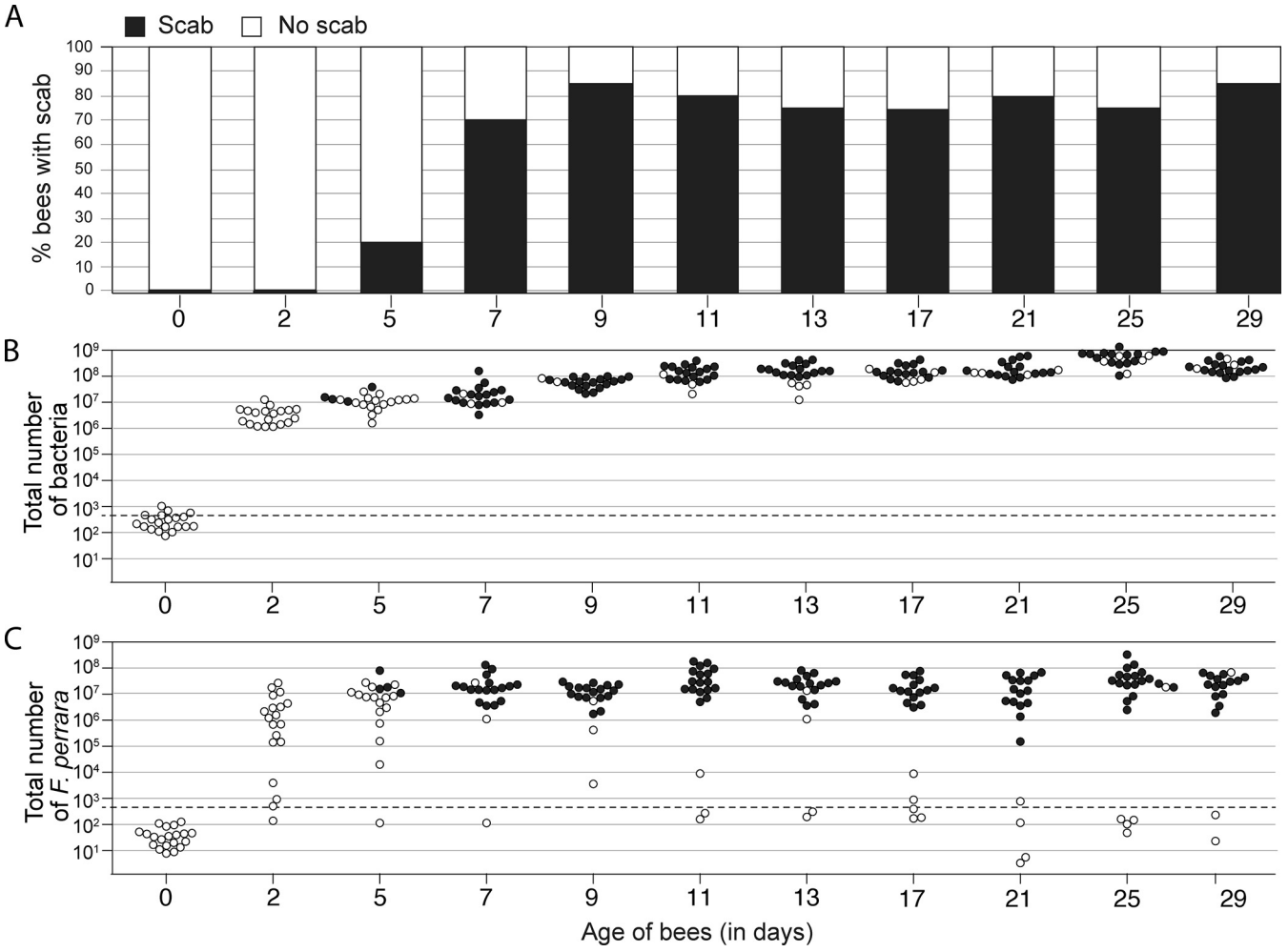

FIG 2 Scab development and abundance of bacteria over time in the pylorus of adult honeybees. (A) Percentages of bees with and without scab at different sampling time points. Twenty bees were analyzed per time point. (B) Total number of bacteria in the pylorus of adult honeybees of different ages, determined by absolute quantification of 16S rRNA gene copies. (C) Number of *F. perrara* bacteria in the pylorus of adult honeybees of different ages, determined by absolute quantification of *rpoE* gene copies. For panels B and C, values were adjusted by dividing by the number of actin gene copies in the sample and multiplying by the average number of actin gene copies per pylorus. Filled and empty circles indicate bees with and without scab, respectively. The dashed lines depict the detection limit of the qPCR method.

hybridization (FISH) experiments, we found that bacteria colonizing the posterior regions of the pylorus belong to the three dominant *Proteobacteria* in the honeybee gut: *G. apicola*, *F. perrara*, and *S. alvi* (Fig. 3; also, see Fig. S4 in the supplemental material). Bacterial colonization was typically restricted to regions of the epithelium that showed surface ruffling. Along these surfaces, we detected strong yellow autofluorescent signals (Fig. 3; also, see Fig. S4) originating from the scab material (see Fig. S5A in the supplemental material). Strikingly, we found *F. perrara* (magenta signal in Fig. 3 and Fig. S4) to colonize right at the edge of these scab structures, while *G. apicola* and *S. alvi* mostly formed a second layer on top (turquoise signals in Fig. 3 and Fig. S4). In most samples from bees without scab, we did not detect *F. perrara* in the pylorus (see Fig. S5B in the supplemental material), suggesting a link between the presence of this bacterium and the occurrence of the scab phenotype.

To test this hypothesis, we quantified the abundance of *F. perrara* in the pylorus of forager bees with and without scab ($n = 15$ each) using qPCR. Strikingly, most bees with scab showed a 4- to 5-log-higher abundance of *F. perrara* in the pylorus than bees without scab (randomization test, $P < 0.0001$) (Fig. 4). Only three out of the 15 analyzed bees without visible scab revealed high levels of *F. perrara*. In the other 12 bees, levels of *F. perrara* were close to the limit of detection. In contrast, levels of *G. apicola* were high in all analyzed samples (Fig. 4), and no significant difference in *G. apicola* abundance was detected between bees with and without scab (randomization test, P value $=0.179$). To confirm these results, we determined absolute numbers of bacteria in the pylorus of all bees from our time series experiment. General bacterial colonization (Fig. 2B) and specifically colonization with *F. perrara* (Fig. 2C) preceded scab development. As in our analysis of forager bees (Fig. 4), there was a significant difference in the abundance of *F. perrara* between bees with and without scab (considering bees older than 5 days, $n = 40$; Mann-Whitney, $P < 0.0001$): bees with scab showed high levels of *F. perrara* in the pylorus, while most bees without scab revealed low levels of *F. perrara*. In the latter

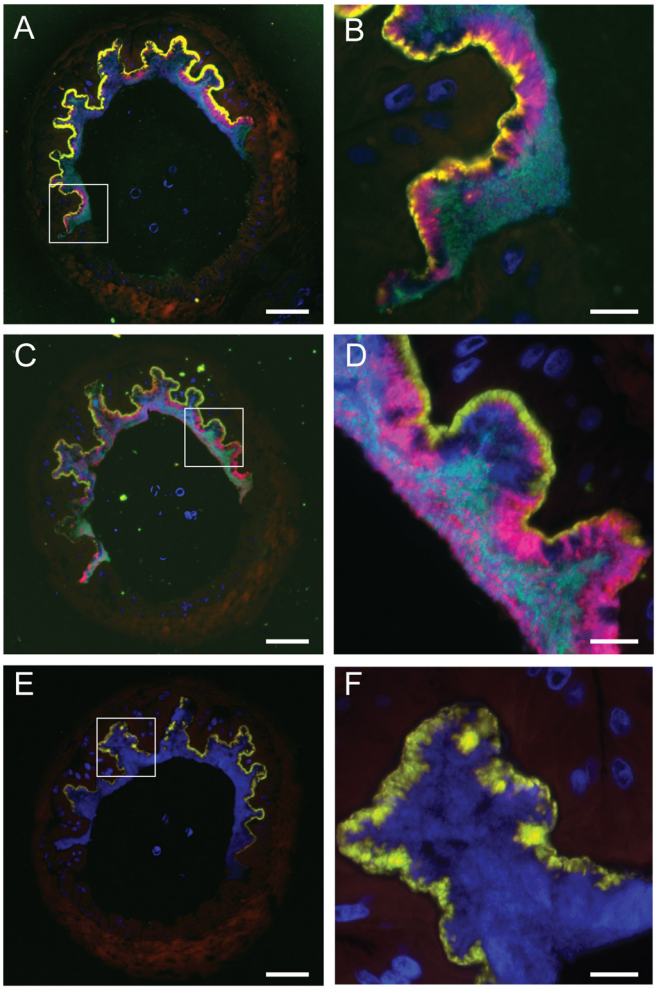

FIG 3 Fluorescence in situ hybridization of cross sections of a pylorus with scab phenotype. (A and B) Hybridizations with probes specific for *F. perrara* (magenta) and *G. apicola* (turquoise). (C and D) Hybridizations with probes specific for *F. perrara* (magenta) and *S. alvi* (turquoise). (E and F) Hybridizations without probes to show the autofluorescent signal (yellow) originating from the scab material on the host epithelial surface. Hoechst counterstaining of host nuclei and bacteria is shown in blue. Panels A, C, and E show the entire cross section. Scale bars, 100 μm. Panels B, D, and F show magnifications of the boxed regions in panels A, C, and E. Scale bars, 20 μm.

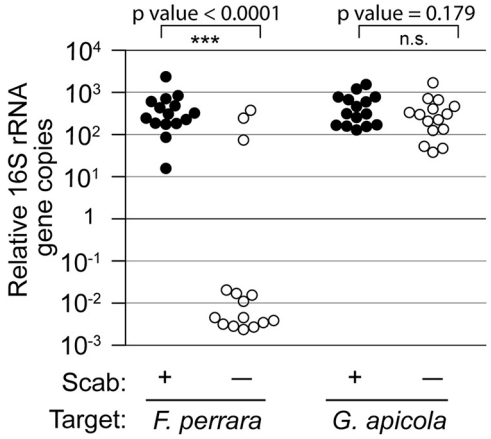

FIG 4 Correlation between *F. perrara* abundance and presence of the scab phenotype. 16S rRNA gene copies of *F. perrara* and *G. apicola* in the pylorus of forager bees with and without scab were normalized by dividing by actin gene copies of *Apis mellifera*. Fifteen bees both with and without scab were analyzed. Filled circles represent bees with scab, and empty circles represent bees without scab. *P* values for randomization tests are indicated.

group, most *F. perrara* counts were close to or even below the detection limit (Fig. 2C). However, as in the analysis of forager bees (Fig. 4), there were a few individuals with high levels of *F. perrara* but no detectable scab phenotype.

Despite the difference in *F. perrara* colonization, there was no significant difference in total bacterial numbers between bees with and without scabs (considering bees older than 5 days, $n = 40$; Mann-Whitney, $P = 0.065$). *F. perrara* abundance seemed to level off 7 days after emergence, while general bacterial counts still increased by ~10× from day 7 to day 13 (Fig. 2B and C). According to our qPCR analyses, *F. perrara* was the dominant bacterium (>50%) between day 2 and day 7, while it declined at later time points to <25% of the bacterial community in the pylorus.

***F. perrara* specifically colonizes the pylorus.** By using qPCR, we analyzed the abundance of *F. perrara* in different gut regions of forager bees with and without scab ($n = 5$ each). All data were normalized for differences in tissue size to the size of the pylorus based on average number of actin gene copies. In bees with pylorus scabs, *F. perrara* abundance was lowest in the anterior part of the gut, i.e., the crop (average = 7.12×10^3 bacteria) and the midgut (average = 1.45×10^4 bacteria) (Fig. 5A). The highest abundance of *F. perrara* was found in the pylorus (average = 7.29×10^7 bacteria), followed by the ileum, with ~10×-lower abundance (average = 7.67×10^6 bacteria; Mann-Whitney, $P < 0.05$), and the rectum, with ~100×-lower abundance (average = 9.34×10^6 bacteria; Mann-Whitney, $P < 0.01$), than in the pylorus. Overall bacterial load was also highest in the pylorus (average = 1.14×10^9 bacteria) (Fig. 5B). However, differences with respect to the ileum (average = 2.83×10^8 bacteria, Mann-Whitney, $P < 0.05$) and the rectum (average = 3.70×10^8 bacteria, Mann-Whitney, $P = 0.056$) were not as pronounced as for *F. perrara*. Notably, in bees without scab, *F. perrara* abundance was close to the detection limit in all gut regions (Fig. 5C), while the total number of bacteria was in the same range as in bees with scab (Fig. 5D).

Experimental colonization of MF bees with *F. perrara* induces the scab phenotype. We exposed newly emerged microbiota-free (MF) bees to *F. perrara* or *S. alvi*. Ten days after exposure, we analyzed bees for scab development and confirmed colonization using qPCR and bacterial culturing. Neither MF bees ($n = 45$) nor *S. alvi*-colonized bees ($n = 43$) developed pylorus scabs within 10 days after emergence (Fig. 6). However, 93% of all bees colonized with *F. perrara* ($n = 45$) revealed scab phenotypes in the pylorus resembling those found in bees sampled from colonies (Fig. 1; also, see Fig. S2 in the supplemental material). Abundance of *F. perrara* in the pylorus of colonized laboratory bees (average = 3.03×10^7 bacteria) (see Fig. S6 in the supplemental material) was in the range for bees of similar age in the colony: in our time series experiments (Fig. 2C), 9-day-old bees had slightly lower counts (average = 1.30×10^7 bacteria) than 10-day-old bees colonized in the laboratory, while 11-day-old bees had slightly higher counts (average = 4.90×10^7 bacteria).

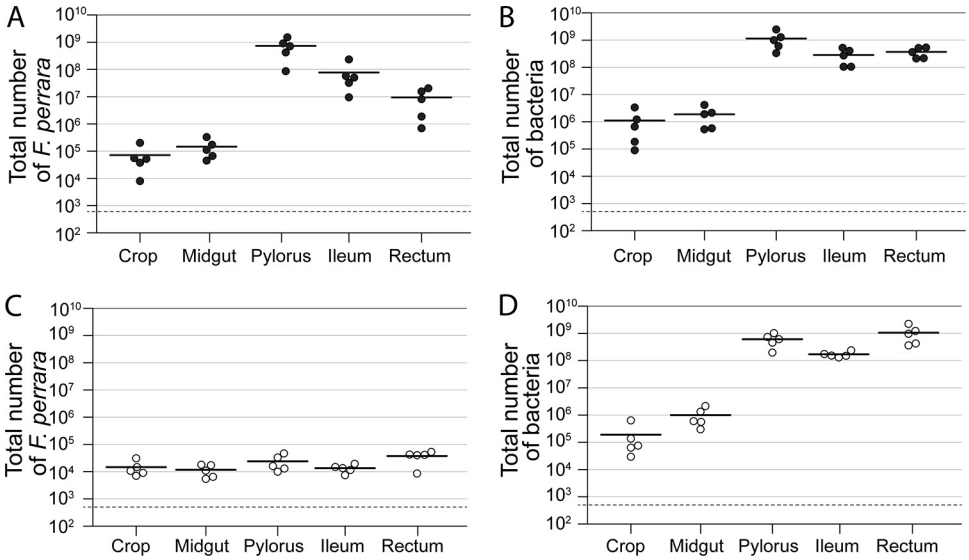

FIG 5 *F. perrara* preferentially colonizes the pylorus region of adult honeybees. (A and B) Absolute quantification of total bacteria and *F. perrara* bacteria in different gut regions of five adult forager bees with scabs. (C and D) Absolute quantification of total bacteria and *F. perrara* bacteria in different gut regions of five adult forager bees without scabs. Filled and empty circles indicate bees with and without scabs, respectively. Dashed line depicts the detection limit of the qPCR method. Numbers were adjusted as in Fig. 2B and C.

DISCUSSION

The scab phenotype was first described in 1946 in honeybees from Switzerland and other European countries (23). At the time, light microscopy revealed that resting stages of the flagellate *Crithidia mellificae* as well as many bacteria colonize the epithelial surfaces covered with the characteristic scab structures. Despite its wide distribution in bee populations and its peculiar appearance as a brown to black band along the gut circumference (Fig. 1), the scab phenotype has never been studied further. In the present study, we show that *F. perrara* causes this phenotype. First of all, we observed a strong positive correlation in adult bees between the presence of a pylorus scab and a high abundance of *F. perrara* (Fig. 2 and 4). Second, our FISH microscopy experiments revealed that dense bacterial layers colonize the corresponding region in the pylorus with *F. perrara* located right at the edge of the scab structures (Fig. 3). Third, we showed that experimental colonization of microbiota-free bees with *F. perrara* results in scab development (Fig. 6).

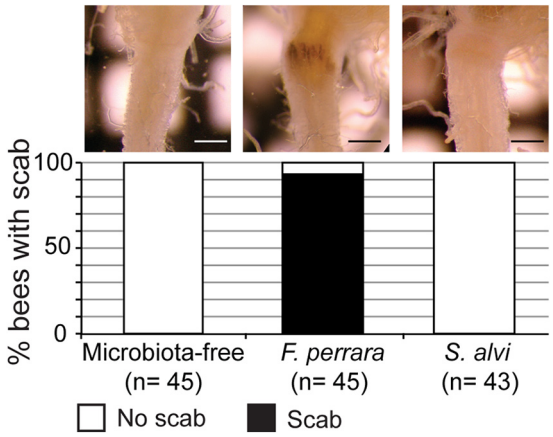

FIG 6 Experimental colonization of microbiota-free (MF) bees with *F. perrara* causes scab development. Data are the percentage of 10-day-old bees with scab phenotypes after exposure to *F. perrara* or *S. alvi* or when left microbiota-free. *n*, number of animals analyzed. Only bees that were successfully colonized were included in the analysis. Data come from three independent experiments. *S. alvi* colonization was tested in only two of the three replicate experiments. Images of representative samples from the three tested conditions are shown above the graph. Absolute quantification of *F. perrara* and *S. alvi* in the pylorus of colonized bees is shown in Fig. S6 in the supplemental material.

The scab phenotype is likely to present a melanization response of the host, triggered by *F. perrara* colonization. Melanin is a complex and heterogeneous biopolymer resulting from the oxidation of phenols (25). In insects, melanization is involved in the hardening process of the cuticle and plays an important role in the innate immune response (26). It is involved in wound healing processes after tissue damage, exhibits encapsulation activity against parasites, and is a major cause of oxidative stress. The scab material in the pylorus has the typical dark coloration of melanin (27–29). In UV light, no fluorescence was observed, but strong autofluorescence appeared in the green and red channels after fixation, which is a characteristic previously reported for oxidized melanin (30). In our electron micrographs (Fig. 1), the scab material appeared as electron-dense matter that resembles melanin deposits found in other insects after tissue damage (28, 31–33). The material is located on the cuticle layer of the gut epithelium from where it protrudes into the lumen (Fig. 1), suggesting secretion from the host across the cuticle layer rather than production by the bacteria in the lumen. Frequently, we found the scab material to encompass the gut bacteria, which is reminiscent of the encapsulation activity of melanin.

In insects, melanin is primarily produced by hemocytes (blood cells) in the hemolymph (34). However, melanization can also occur in the hindgut independent of hemocyte recruitment, presumably by melanin production from specific epithelial cells (28, 31, 35). Vacuoles have been found in the epithelial cells of melanized gut regions (31). We observed similar structures in the

cellular tissue and cuticle layer underlying the scab structures, indicating the secretion of cellular compounds into the lumen (Fig. 1).

Melanization is a localized and relatively fast response occurring immediately after host tissue damage. However, the development of the scab phenotype in honeybees takes several days. In hive bees, high abundance of *F. perrara* was already detected 2 days after pupal emergence, while scabs became visible only 5 to 7 days after emergence (Fig. 2). This delay suggests either that very high levels of *F. perrara* are necessary or that a certain host developmental stage must be reached to trigger the local melanization response in the gut. Other factors, such as diet, host genotype, and absence/presence of other bacteria, could promote or inhibit scab development, because a few bees in our experiments were older than 7 days and contained high levels of *F. perrara* but showed no obvious scab phenotype (Fig. 2).

Two remarkable characteristics of the scab phenotype are its specificity to *F. perrara* colonization (Fig. 2 to 6) and its restriction to a small region in the pylorus (Fig. 3 and 5). Other bacteria, including the closely related *G. apicola* and *S. alvi*, colonize the same region of the host epithelium (Fig. 3) but seem not to trigger scab formation. Possibly, *F. perrara* causes tissue damage or activates the insect immune system via a specific mechanism that results in a melanization response. It will be important to identify the bacterial factors involved in this process in order to understand whether *F. perrara* has pathogenic characteristics and whether this influences gut homeostasis. Genome analysis of *F. perrara* revealed the presence of several factors that could be involved in the interaction with the host. For example, a homolog of the biosynthetic colibactin gene cluster was found, and the product was shown to cause DNA damage in eukaryotic cells (36). This gene cluster is also present in certain *Escherichia coli* strains isolated from the human gut (37, 38), and these *E. coli* strains were shown to cause DNA damage in epithelial cells in a murine gut model (39). Future studies will show whether *F. perrara* also causes DNA damage *in vivo* in the bee gut and whether this contributes to the observed host response in the pylorus.

Based on bacterial community analyses, *F. perrara* is widely distributed in honeybee populations: it has been detected in samples collected in Europe, North America, and Asia (1, 11, 19, 40). We found scab phenotypes in all colonies analyzed in both Switzerland and the United States (see Fig. S1 in the supplemental material), and scabs were also reported in other countries in Europe (23, 24). However, negative effects associated with the presence of this bacterium have so far not been reported. Potentially, the melanization response provides a mechanical resistance against tissue damage or regulates growth and expansion of the bacterium via oxidative stress. We could isolate *F. perrara* from bees with scabs, demonstrating that the bacteria are not killed by this response. However, *F. perrara* abundance increased only until day 5 to day 7 after colonization and then stayed more or less constant, while overall bacterial numbers increased further until later time points (Fig. 2). A recent study of colonization dynamics of honeybee microbiota showed a similar pattern: *F. perrara* colonizes the ileum region and is the most abundant member of the community in young adult workers, whereas *G. apicola* and *S. alvi* dominate in the ilea of older workers (22). The arrest of *F. perrara* proliferation coincides with scab development, suggesting that the response could control *F. perrara* colonization. Alternatively, other bacteria might simply outcompete *F. perrara* and impede its proliferation at later time points.

Our qPCR experiments on different gut regions showed that *F. perrara* is by far most abundant in the pylorus (Fig. 5). This may explain why scab formation is limited to this particular region in the gut. The uniform appearance of the phenotype across bees could also suggest that a specific cell type present in this region is needed for inducing the response. Possible reasons why *F. perrara* may preferentially colonize the posterior part of the pylorus could be (i) competition with other bacteria, (ii) physicochemical properties, and (iii) access to specific nutrients. Ingested pollen often accumulates in the pylorus before it is passed through the ileum into the rectum (23). In addition, the Malphigian tubules excrete nitrogenous waste and electrolytes into the pylorus (41). This creates a nutrient-rich environment in which *F. perrara* and other bacteria can thrive by colonizing the adjacent gut walls.

F. perrara shows a widespread but irregular occurrence in honeybee populations, is confined to a small region in the gut that is heavily colonized by other gut bacteria, and elicits a phenotype that may represent an immune response. Furthermore, *F. perrara* has so far not been detected in bumblebees, although bumblebees do contain *G. apicola* and *S. alvi* (13). Possibly, *F. perrara* has invaded the gut community of honeybees relatively recently and established itself in a specific niche. The *F. perrara*-host interaction illustrates that gut microbial communities are evolutionarily dynamic. Invasive or parasitic organisms that elicit strong immune responses can potentially evolve into commensals and sometimes into mutualists. The simple gut communities of social bees present ideal model systems to investigate the underlying evolutionary and genetic processes of such interactions. Future studies will elucidate the precise relationship between *F. perrara* and bees, determining the bacterial factors involved and the impact of the interaction on bee health and gut homeostasis.

MATERIALS AND METHODS

Bee experiments and generation of microbiota-free bees. Bees used in this study originated from *Apis mellifera* colonies kept at Yale University, West Haven, CT, and at the University of Lausanne, Lausanne, Switzerland. To sample forager bees, we collected bees returning with pollen on their legs on the landing boards of the hives. To sample age-controlled hive bees, frames with emerging brood were kept overnight in humidified insect chambers at 35°C. On the next day, newly emerged bees were marked (time point day 0), put back into the colony, and sampled at later time points within the colony. To generate microbiota-free bees, black-eyed pupae were removed from brood frames in the lab and kept in clean cages in humidified insect chambers at 35°C. After ~48 h, newly emerged adult bees were distributed into experimental cages, provided with 1:1 (wt/wt) sucrose-water and gamma-irradiated pollen, and kept in an insect chamber at 35°C.

Bee colonization experiments. *F. perrara* PEB0191 (21) was inoculated from frozen glycerol stocks on brain heart infusion agar and grown at 37°C under anaerobic conditions. *S. alvi* wkB2 (42) was inoculated from frozen glycerol stocks on tryptic soy agar and grown at 37°C in 5% CO_2. Two days later, bacteria were restreaked on fresh agar plates and grown for another 1.5 to 2 days. Bacteria were harvested in 1× PBS, diluted in PBS plus sucrose-water (1:1), and 3×10^8 to 9×10^8 bacterial cells were spread over the gamma-irradiated pollen in the cages containing the microbiota-free bees.

DNA extraction from gut tissue. Bees were anesthetized by chilling on ice or by exposure to CO_2. Midguts and hindguts were dissected in 1× PBS, and the pylorus region was cut out with a sterilized scalpel. A first cut was done immediately after the attachment sites of the Malpighian tubules

and a second cut about 1 to 2 mm further downstream. DNA was extracted from this gut fragment using a previously published cetyltrimethylammonium bromide (CTAB)/phenol extraction protocol (22). The DNA samples were resuspended in 50 μl nuclease-free water and used for quantitative PCR analysis.

Quantitative PCR to estimate bacterial abundance. Diagnostic 16S rRNA gene primers were used to determine the abundance of *G. apicola*, *S. alvi*, and *F. perrara* and total numbers of bacteria in DNA extracted from gut tissues. For quantification of *F. perrara*, we also used *rpoE* gene primers. For normalization of DNA samples, the *A. mellifera* actin gene was used. All primers are summarized in Table S1. Quantitative PCR was carried out on an Eppendorf Mastercycler ep realplex (data in Fig. 2 and 4), an ABI Prism 7000 (data in Fig. 5), or a BioRad CFX96 thermal cycler (data in Fig. S6 in the supplemental material). Triplicate reactions with 10-μl mixtures were performed with 5 μl KAPA SYBR Fast 2× master mix (KAPA Biosystems), 1 μl each primer at 5 μM, 2 μl H_2O, and 1 μl template DNA. The cycling conditions consisted of 95°C for 3 min and 40 cycles of two step PCR at 95°C for 3 s and 20 s at 60°C. We used a randomization test (2,000 iterations) as implemented in the REST 2009 software (43) to compare relative gene copy numbers of *G. apicola* and *F. perrara* between forager bees with and without scabs (Fig. 4). For all other experiments, absolute quantification was based on standard curves from amplification of the cloned target sequence in a pGEM-T vector (Promega). Samples with copy numbers below the range of detection of the standard curve were assigned a value of 5×10^2 copies corresponding to the lower limit of detection. To adjust for differences in tissue size, bacterial numbers were adjusted by dividing by the actin gene copy number of the sample and multiplying by the average actin gene copy number of all samples in the experiment. If not otherwise stated, values of 16S rRNA gene copies were divided by 4 to account for the four 16S rRNA gene copies present in the genomes of *G. apicola*, *S. alvi*, and *F. perrara* (the three dominant species in the pylorus). Values were log-transformed for statistical tests. Mann-Whitney U tests were carried out in R (44). Plots were generated in R using the beeswarm function (45).

FISH. Tissue sections and FISH experiments were performed as previously described (46, 47). In short, the pylorus and ileum of forager bees were dissected as described above and fixed for 5 days in Carnoy's solution (ethanol-chloroform-acetic acid, 6:3:1 [vol/vol]). Fixed tissue samples were washed three times for 1 h in absolute ethanol, then incubated three times for 20 min in xylene, and finally infiltrated with paraffin three times for 1 h at 60°C under a vacuum. Samples were placed into molds containing melted paraffin and then hardened by placing them into an ice slurry. Paraffin-embedded tissues were cut into serial 5-μm sections with a microtome (Leica), placed on coated microscopy slides, and cleared from paraffin with xylene. Sections were then rehydrated and hybridized overnight with fluorochrome-labeled oligonucleotide probes targeting the 16S rRNA of *G. apicola*, *S. alvi*, or *F. perrara*. The specificity of the FISH signals was validated by performing control experiments with an excess of unlabeled oligonucleotides (50:1) and with the addition of RNase during hybridization. Oligonucleotide sequences of probes are listed in Table S1.

Electron microscopy. Scab regions were dissected in fixation solution (2.5% gluteraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) in a fume hood. Tissue embedding and sectioning was carried out by the electron microscopy facility of the Yale School of Medicine following their standard protocol for tissue samples. After 1 h of fixation, samples were rinsed three times for 20 min in 0.1 M sodium cacodylate buffer, followed by a postfix in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature in a fume hood. Samples were washed three times for 5 min in water before they were dehydrated in a series of ethanol washes from 50% to 100% ethanol. Ethanol was replaced with propylene oxide for 10 min and then incubated on a rotating wheel in propylene oxide-Epon mix (1:1 [vol/vol]) for 1 h, followed by two pure Epon mixes for 2 h. Samples were transferred into fresh Epon in molds and cured in the oven overnight at 60°C. Ultrathin sections were cut and stained with uranyl acetate and viewed on a Zeiss EM900 transmission electron microscope.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00193-15/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 1.2 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 0.8 MB.
Figure S5, TIF file, 2.5 MB.
Figure S6, TIF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.

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