IHF regulates host colonization factors in the bee gut symbiont *Frischella perrara*

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Abstract

Bacteria colonize specific niches in the animal gut. However, the genetic basis of these associations is often unclear. The protobacterium *Frischella perrara* colonizes the hindgut of honey bees, where it causes a characteristic immune response leading to the formation of the scab phenotype. Genetic determinants required for the establishment of this specific association are unknown. Here, we isolated three point mutations in the genes encoding the DNA-binding protein integration host factor (IHF). All three mutants formed larger colonies than the wild type *in vitro* and did not produce an aryl polyene metabolite, which confers a yellow color to *F. perrara* colonies. Inoculation of microbiota-free bees with one of the mutants drastically decreased gut colonization of *F. perrara* and abolished scab development. Using RNAseq we find that IHF affects the expression of potential colonization factors, including a colibactin biosynthetic gene cluster, two Type 6 secretion systems, pili genes, and the aryl polyene biosynthesis pathway. Gene deletions of these components revealed different colonization defects. While some mutants were abolished in their capacity to colonize the gut, others colonized but did not trigger the scab phenotype. IHF is conserved across many bacteria and may thus regulate host colonization also in other animal symbionts.

35 **Introduction**

36 The digestive tract of many animals is colonized by specialized gut symbionts that occupy
37 distinct physical niches and utilize diverse nutrients. Owing to the availability of genetic tools,
38 gnotobiotic animal models and multi-omics approaches, we can now study the genetic features
39 that allow gut symbionts to colonize various animal hosts including mammals, fishes, and
40 insects (1-10).

41 The Western honey bee, *Apis mellifera,* is a particularly interesting model to characterize
42 colonization factors of bacterial gut symbionts, due to its agricultural importance and the
43 tractability of its gut microbiota. Honey bees harbor relatively simple yet highly specialized gut
44 microbiota composed of 8-10 bacterial genera (11, 12). The wide distribution of this community
45 across social bees suggests long evolutionary associations with the host (13). Moreover,
46 different members of the bee gut microbiota colonize distinct physical niches along the gut.
47 Lactobacilli and Bifidobacteria predominate in the posterior hindgut (rectum), while
48 Gammaproteobacteria (*Frischella perrara* and *Gilliamella* species) and a betaproteobacterium
49 (*Snodgrassella alvi*) preferentially colonize the anterior hindgut (ileum and adjacent pylorus, i.e.,
50 the transition zone between midgut and ileum) (11, 14-16). The partitioning of these bacteria
51 into distinct gut compartments suggests the existence of specific bacterial mechanisms that
52 facilitate colonization.

53 Most bacteria of the bee gut microbiota can be cultured and experiments with gnotobiotic bees
54 have been established (17-21). Moreover, genomic analyses have provided important insights
55 about the functional potential of bee gut symbionts and their adaptation to the gut environment
56 (21-29). Yet, little is known about which genes are directly involved in establishing colonization
57 in the bee gut and how these genes are regulated. The only symbiont that has been extensively
58 studied in this respect is *S. alvi.* Using transposon sequencing and transcriptome analysis,
59 Powell et al. determined genome-wide host colonization factors in *S. alvi* (7). Most genes with
60 strong fitness effects were found to belong to three major categories: extracellular interactions,
61 metabolism, and stress response. In particular, genes for attachment and biofilm formation were

62 highly beneficial for colonization, which is in agreement with the observation that this gut
63 symbiont adheres to the host epithelium of the ileum and forms a multispecies biofilm with
64 *Gilliamella*.

65 The honey bee gut symbiont *Frischella perrara* belongs to the recently described family
66 Orbaceae within the Gammaproteobacteria (30). It is taxonomically close to the bee gut
67 symbiont *Gilliamella* and also shows a similar metabolism as *Gilliamella* (31). However,
68 compared to the other members of the bee gut microbiota*, F. perrara* shows a rather distinctive
69 colonization phenotype*.* While *S. alvi* and *Gilliamella* have been reported to colonize the host
70 epithelium of the entire ileum (32, 33), *F. perrara* preferentially colonizes the transition zone
71 between the midgut and the ileum, i.e., the pylorus. Moreover, colonization with *F. perrara* leads
72 to the appearance of a brown to black material on the luminal side of the epithelial surface
73 between the cuticle layer of the host tissue and the adherent *F. perrara* cells (34). This so–
74 called scab phenotype forms after 5-7 days post-colonization and has so far not been reported
75 to be triggered by any other gut symbiont than *F. perrara*. Transcriptome analysis of the host
76 showed that *F. perrara* elicits a specific immune response which includes the upregulation of the
77 host melanization pathway likely responsible for the formation of the scab phenotype (17).

78 Genome sequencing of the type strain of *F. perrara* and comparison with other genomes of the
79 Orbaceae family revealed the presence of several genomic islands that may be involved in the
80 specific interaction of *F. perrara* with the host (35). These include a biosynthetic gene cluster for
81 the production of the genotoxic metabolite colibactin (Clb), two distinct Type VI Secretion
82 systems (T6SSs), and associated effector proteins, Type I secretion systems, and fimbrial low-
83 molecular-weight protein (Flp) pili genes. However, currently no genetic tools are available for
84 *F. perrara* precluding studies about the role of these genetic factors in gut colonization or the
85 induction of the scab phenotype*.*

86 Here, we report the isolation of a spontaneous mutant of *F. perrara* that possesses a strong
87 colonization defect *in vivo*. Resequencing of the mutant revealed a single nonsynonymous point
88 mutation in the gene encoding the alpha subunit of the DNA-binding protein integration host

89 factor (IHF). Using a combination of gnotobiotic bee experiments, transcriptomics, and
90 metabolite analyses, we characterized the genes regulated by IHF. We then established a gene
91 deletion strategy for *F. perrara,* which allowed us to knockout some of the IHF-regulated genes
92 and show that they impact gut colonization and scab development to different extent.

93 ## Results

94 **Isolation of spontaneous IHF mutants affecting growth and colony morphology of *F.***
95 ***perrara***

96 Culturing *F. perrara* type strain PEB0191 on modified tryptone yeast glucose (mTYG) agar
97 resulted in the formation of yellow colonies. However, we occasionally observed the appearance
98 of larger white colonies among the yellow ones (**Figure 1A**). Restreaking white colonies on
99 fresh mTYG agar usually resulted in yellow colonies again. However, three white colonies that
100 we identified in independent experiments did not change their appearance anymore, suggesting
101 that we had isolated stable 'white' variants of *F. perrara* PEB0191 (**Figure 1B**). Genome
102 sequencing of the white variants revealed the presence of three different non-synonymous point
103 mutations in the genes encoding the integration host factor (IHF). IHF is a widely distributed
104 DNA-binding protein consisting of the IhfA/B heterocomplex (36-38). Strikingly, two point
105 mutations were identical to each other, but occurred in the different subunits of IHF (*ihfA* and
106 *ihfB*) resulting in a proline to lysine change at amino acid position 82 and 83, respectively
107 (**Figure 1C**). The third point mutation resulted in a lysine to serine change at position 38 of IhfA
108 (**Figure 1C**). Homology modelling showed that these amino acids are located in the region
109 interacting with DNA, suggesting that the three mutations impact the DNA-binding properties of
110 IHF (**Figure 1 D**, (39)). As two of the isolated mutants occurred when generating gene deletion
111 of *F. perrara,* they harbored additional genetic modifications (see Methods). Therefore, we
112 focused further characterization of IHF on the mutation Pro83Lys (hereafter *ihfA**) that occurred
113 in the wild type (wt) background of *F. perrara*. While *IhfA** consistently formed larger colonies
114 than the wt strain on mTYG agar (**Figure 1A and 1B)**, there was no significant difference in
115 growth in liquid culture (Permutation test, $p = 0.097$; **Supplementary Figure 1A**). However, light
116 microscopy showed that cells of the mutant strain were on average slightly longer than cells of
117 the wt (Kolgomorov-Smirnov test $p<0.0001$, **Supplementary Figure 1B and 1C**).

118

119 ***F. perrara* produces an aryl polyene secondary metabolite that is responsible for the**
120 **yellow colony morphotype**
121 *F. perrara* PEB0191 encodes a genomic island that is homologous to aryl polyene (APE)
122 biosynthetic gene clusters present in other Gammaproteobacteria (**Figure 2A**). APEs are
123 polyunsaturated carboxylic acids conferring a yellow pigmentation to bacterial cells (40, 41). To
124 assess if *F. perrara* wt, but not the *ihfA** mutant, produces an APE, we cultivated both strains,
125 extracted the pellets, and analyzed the extracts by liquid chromatography coupled to heated
126 electrospray ionization high-resolution mass spectrometry (HPLC-HES-HRMS). The data
127 revealed a strongly UV-Vis-absorbent ion peak at *m/z* 323.1647 $[M+H]^+$, which had a suggested
128 molecular formula of $C_{21}H_{23}O_3$ (**Figure 2B** and **2C**). In the *ihfA** mutant, this ion was only present
129 at trace amounts (**Figure 2C**). To characterize the metabolite in greater detail a larger pellet of
130 *F. perrara* PEB0191 wt cultures was extracted and purified by several HPLC runs. Mass
131 spectrometry- (MS) and UV-Vis-guided fractionation yielded an enriched extract that was
132 analyzed by nuclear magnetic resonance (NMR) spectroscopy. The characteristic ions detected
133 in MS-MS fragmentation experiments (**Supplementary Figure 2**), the UV-Vis spectrum with an
134 absorption maximum at 415 nm (**Supplementary Figure 3**) in conjunction with NMR data
135 (**Supplementary Figure 4-10, Supplementary Table 1**) suggest an aryl polyene structure
136 identical to that reported (42, 43) (**Figure 2D**). Unfortunately it was not possible to connect the
137 NMR substructures because the central methines could not be assigned to chemical shifts
138 (**Figure 2D**, **Supplementary Table 1)**. Comparison of the organic extracts of *F. perrara* wt and
139 *E. coli* CFT073 provided further evidence that both produce the same compound
140 (**Supplementary Figure 11**). Combined, these results suggest that the APE pathway is
141 responsible for the yellow color of the wt colonies of *F. perrara* and is suppressed in the *ihfA**
142 mutant.

143

144 **The *ihfA** mutant of *F. perrara* has a colonization defect and does not cause the scab**
145 **phenotype**
146 As APEs have been shown to increase protection from oxidative stress and contribute to biofilm
147 formation (42, 43), we sought to test if the *ihfA** mutation impacts bee gut colonization. We
148 mono-associated microbiota-free bees with either *F. perrara* wt or *ihfA**. Colonization with the wt
149 strain resulted in a visible scab in 50% and 80% of all bees after five and ten days of
150 colonization, respectively (n=18 and n=36 for both treatments for day 5 and day 10,
151 respectively, **Figure 3A and 3B**). In contrast, none of the bees colonized with the *ihfA** mutant
152 developed a visible scab phenotype. To determine whether this difference was due to a general
153 colonization defect of *ihfA**, we quantified the colonization levels of *F. perrara* at day 5 and day
154 10 post colonization using colony forming units (CFUs). While there was a trend towards lower
155 colonization levels (fewer CFUs and more bees without detectable colonization) for the *ihfA**
156 mutant at day 5 post colonization, the difference was not statistically significant (**Figure 3C**,
157 Wilcoxon rank-sum test p-value=0.076). However, at day 10 post-colonization, bees colonized
158 with the wt strain showed significantly higher CFUs than the *ihfA** mutant (**Figure 3C**, Wilcoxon
159 rank-sum test p-value<0.0001). In fact, in 50% of all bees (n = 36) the colonization levels of
160 *ihfA** were below the detection limit of 500 CFUs (colony forming units) (**Figure 3C**).
161
162 As the quantification of *F. perrara* was based on CFUs obtained for whole-gut tissue, we carried
163 out a second experiment, in which we specifically assessed the colonization levels in the pylorus
164 and the ileum region of the honey bee gut, using both CFUs and quantitative PCR
165 (**Supplementary Figure S12**). The results were comparable to those obtained for the whole
166 gut: at day 10 post colonization, there was a significant difference in the colonization levels of
167 the wt and *ihfA** in both the pylorus and the ileum (Wilcoxon rank-sum test p-value<0.05).
168
169 To obtain a better understanding of the colonization dynamics of *F. perrara* wt and the *ihfA**
170 mutant, we conducted a third gnotobiotic bee experiment in which we inoculated microbiota-free
171 bees with one of the two strains and followed the colonization levels over 12 timepoints from day

172 0 (i.e. 4 h post inoculation) until day 22 post inoculation (**Figure 3D**). We used the same optical
173 density of the two strains for inoculation. However, this corresponded to fewer CFUs for the wt
174 compared to the *ihf** mutant. From the first time point at 4 h post inoculation until day 4 post
175 inoculation, the bacterial levels were below the detection limit (i.e., below 500 CFUs) at both
176 conditions. Between day 4 and day 8 post inoculation, the abundance of *F. perrara* wt increased
177 rapidly to about 10^6 CFUs per gut and then steadily further to 10^7 CFUs per gut until the last
178 timepoint. In contrast, the levels of the *ihfA** mutant remained low until day 10 post colonization
179 and reached on average no more than 10^5 CFUs per gut until the last timepoint at day 22 post
180 colonization. In summary, these results show that *ihfA** has a colonization defect. It has a
181 delayed colonization dynamics as compared to the wt, does not reach the same bacterial loads,
182 and does not cause the scab phenotype.
183

184 **Genes involved in symbiotic interactions are upregulated in *F. perrara* wt relative to the**
185 ***ihfA** mutant**

186 IHF may not have a direct effect on gut colonization, but rather regulate the gene expression of
187 host colonization factors. To test this, we assessed the transcriptional differences by RNA
188 sequencing (RNA-seq) between the wt and *ihfA** mutant when grown *in vitro*. We found that 358
189 out of 2,337 genes encoded in the genome of *F. perrara* PEB0191 were differentially expressed
190 with a log2-fold change >|2| between the two strains (Fisher's exact test with $p<0.05$ and
191 FDR<5%). Of those, 237 and 121 genes were up- and down-regulated in *F. perrara* wt versus
192 *ihfA**, respectively (**Figure 4A and 4B**, **Supplementary Dataset 1**). Among the genes
193 upregulated in the wt, 'Intracellular trafficking, secretion, and vesicular transport' (COG U),
194 'Extracellular structure' (COG W), 'Lipid transport and metabolism' (COG I), 'Mobilome:
195 prophages and transposases' (COG X), and 'Secondary metabolites biosynthesis, transport and
196 catabolism' (COG Q) were significantly enriched (Fisher's exact test, BH-adjusted p-value <
197 0.01; see **Supplementary Dataset 2**). Genes belonging to these three categories include
198 different subunits and effectors of the two T6SSs of *F. perrara*, the Clb biosynthesis gene

199 cluster, various components of the Flp pili, and a RTX (repeats in toxin) toxin belonging to the
200 Type I secretion system family (**Supplementary Dataset 1**). Also, the genes of the APE
201 biosynthesis gene cluster were among the upregulated genes, which is in line with the
202 production of the corresponding metabolites in the wt but not in the mutant strain (**Figure 2**).
203 Interestingly, a relatively large proportion of the upregulated genes encoded hypothetical or
204 poorly characterized proteins. In fact, genes without COG annotation were also enriched relative
205 to the entire genome of *F. perrara* (Fisher's exact test, BH-adjusted p-value < 0.01; see
206 Supplementary **Dataset 2**). Many of the upregulated genes were organized in genomic islands,
207 with the largest one including the biosynthesis gene cluster of Clb and many hypothetical
208 protein-encoding genes (**Figure 4C**). T6SS and Clb biosynthesis genes were among the genes
209 with the highest fold changes relative to *ihfA** mutant (**Figure 4B**, 28 of 32 genes with log2-fold
210 change >6). Moreover, 64% of the upregulated genes (152/237) belonged to the *F. perrara*-
211 specific gene content as based on our previously published genome comparison of *F. perrara*
212 PEB0191 with four other strains of the family Orbaceae (three of the genus *Gilliamella* and one
213 of the genus *Orbus*, **Supplementary Dataset 1)** (35).

214 Among the 121 down-regulated genes, only COG category O ('Posttranslational modification,
215 protein turnover, chaperones') was statistically enriched (Fisher's exact test, BH-adjusted p-
216 value < 0.01, see **Supplementary Dataset 2**). Moreover, only a small fraction (12%) belonged
217 to the "*F. perrara*-specific genes", and fewer genes were organized into genomic islands. A
218 more detailed inspection of the annotation revealed that a large number of the down-regulated
219 genes were involved in transport and metabolism (40 genes), transcriptional regulation (10
220 genes), and protein folding (8 genes), highlighting clear differences in the functional roles of the
221 up- and down-regulated genes. The two genes with the highest fold change (log2-fold change <-
222 5) both encoded transcriptional regulators. One of them, *dksA* (Fpe_01158), is located upstream
223 of the *mrsA*/*mrsB* antioxidant system (Fpe_01159 to Fpe_01162), which was also among the
224 down-regulated genes. The other one is part of the two-component regulator system *basS*/*basR*
225 (Fpe_02097 and Fpe_02098), which has been reported to act as an iron- and zinc-sensing

226 transcriptional repressor and activator in *E. coli* (44, 45). Taken together, these results show that
227 many accessory genes known to be involved in symbiotic interactions (colibactin, Flp pili, T6SS)
228 are upregulated in *F. perrara* wt as opposed to *ihfA**, providing a list of candidate genes
229 responsible for the colonization defect of the *ihfA** mutant.

230

231 **T6SS, pili, APE biosynthesis and Clb biosynthesis genes are expressed during bee gut**
232 **colonization**

233 To test if the genes upregulated *in vitro* in the wt relative to *ihfA** were expressed *in vivo*, we
234 determined the transcriptome of *F. perrara* wt at day 5 and day 10 post colonization. A total of
235 260 (149 up and 111 down) and 298 (162 up and 136 down) genes were differentially
236 expressed at day 5 and day 10 post colonization relative to growth *in vitro* (log2-fold change
237 >|2|, quasi-likelihood F-test with $p<0.05$ and FDR<5%, **Supplementary Dataset 3**). There was a
238 considerable overlap of the genes differentially regulated in vivo relative to in vitro between the
239 two timepoints (115 and 80 shared up- and down-regulated genes, respectively). At both
240 timepoints, the COG category 'Carbohydrate transport and metabolism' (COG G) was
241 significantly enriched among the genes upregulated *in vivo* relative to the entire genome
242 (**Supplementary Dataset 4**). In addition, at timepoint day 10, also the COG category (P)
243 'Inorganic ion transport and metabolism' was enriched (P adj < 0.01, Fisher's exact test,
244 **Supplementary Dataset 4**). Genes belonging to these two categories encoded transporters for
245 different sugars (Phosphotransferase systems), iron, and transferrin (**Supplementary Figure**
246 **13, Supplementary Dataset 3**). In addition, a catalase gene and several genes for the
247 biosynthesis of the amino acid tryptophan were upregulated at both timepoints. However, only
248 14 and 19 genes of the ones upregulated *in vitro* in the wt relative to the *ihfA** mutant (see
249 **Figure 5**), were also upregulated *in vivo* at day 5 and day 10 post-colonization, respectively
250 (**Supplementary Dataset 3**). In fact, several of the T6SS, APE, and Clb biosynthesis genes
251 remained unchanged or were slightly downregulated *in vivo* relative to *in vitro* (**Supplementary**
252 **Dataset 3 and Supplementary Figure 13**). This was not unexpected, because the *in vitro*

253 RNAseq analysis our reference had shown that these genes are already expressed when grown
254 on mTYG agar, which we used as a condition for the *in vivo* analysis. Indeed, when comparing
255 count-normalized gene expression (as measured by TPM) across the different conditions, we
256 found that most of the T6SS machinery, APE biosynthesis, pilus, and iron uptake genes were
257 expressed at similar levels at both timepoints *in vivo* as during *in vitro* growth (**Figure 5,**
258 **Supplementary Figure 14**). Only the Clb genomic island and some of the VgrG-like T6SS
259 effector genes had clearly lower TPM values *in vivo* than *in vitro*, yet higher than in *ihfA** mutant
260 (**Figure 5C and 5F**). These results suggest that most of the genes upregulated in the wt relative
261 to *ihfA* in vitro* were also expressed at high level at both timepoints *in vivo*.
262
263
264 **Gene deletions of IhfA-regulated genes result in impaired gut colonization and/or abolish**
265 **scab development**
266 To test the direct impact of IhfA-regulated genes on host colonization and/or scab development
267 we established a gene deletion strategy for *F. perrara* based on a two-step homologous
268 recombination procedure (see methods). This allowed us to create six different non-polar in-
269 frame gene deletion mutants of potential colonization factors regulated by IHF (Supplementary
270 Table S2). We deleted an essential gene of the colibactin biosynthesis pathway (Δ*clbB*), both
271 *hcp* genes of the two T6SSs, either separately or as double mutant (Δ*hcp1,* Δ*hcp2,* and
272 Δ*hcp1*/Δ*hcp2*), the gene encoding the major Flp pili subunit (Δ*pilE*), and the entire APE
273 biosynthesis gene cluster (Δ*apeA-R*). Deletion mutants were confirmed by genome re-
274 sequencing. The Δ*apeA-R* mutant was the only strain not forming yellow colonies anymore,
275 which is consistent with the idea that the aryl polyene pathway is responsible for the yellow
276 color. To compare the six gene deletion strains to *F. perrara* wt and the *ihfA** mutant in terms of
277 their ability to colonize the honey bee gut and induce the scab phenotype, each strain was
278 inoculated into microbiota-free bees and CFUs assessed in the pylorus/ileum region ten days
279 post inoculation. As expected, *F. perrara* wt successfully colonized the pylorus/ileum region of

280 all analyzed gnotobiotic bees (median = $9.56*10^6 \pm 5.06*10^6$ CFUs per gut; n=18 bees, two
281 independent experiments) and induced the scab phenotype in 16 of 18 bees (**Figure 6**). In
282 contrast, *F. perrara ihfA** colonized poorly compared to the wt (median=$1.46*10^4 \pm 3.19*10^5$
283 CFUs per gut, n= 20 bees, Wilcoxon rank-sum test $P<0.001$), and not a single bee developed
284 the scab phenotype. Of the six tested gene deletion mutants, only the mutant of T6SS-1 (Δ*hcp1*)
285 reached wt colonization levels and also induced the scab phenotype in most of the bees (15 out
286 of 20). The other five mutants all exhibited lower colonization levels than the wt strain. However,
287 the severity of the colonization defect varied between the mutants, and while some of the
288 mutants still caused the scab phenotype, others did not. For example, the Clb biosynthesis gene
289 cluster mutant Δ*clbB* induced the scab phenotype in all but one bee, despite the fact that the
290 colonization levels were about two-fold lower than for the wt (median=$4.24*10^6 \pm 2.87*10^6$ CFUs
291 per gut). The gene deletion mutant of the T6SS-2 (Δ*hcp2*) and the double mutant of both T6SSs
292 (Δ*hcp1*/Δ*hcp2*) reached similar colonization levels as the Δ*clbB* mutant. Yet, none of the bees
293 colonized with these two mutants developed the scab phenotype not even those that had very
294 high bacterial counts (**Figure 6**). The deletion mutant of the APE biosynthesis pathway (Δ*apeA-*
295 *R*) showed again a different colonization phenotype: for some bees no colonization was
296 detected, while others showed similar colonization levels as the wt of *F. perrara*. However, in
297 contrast to the Δ*hcp2* and the Δ*hcp1*/Δ*hcp2* mutants, the Δ*apeA-R* mutant still induced the scab
298 phenotype, but only in the bees that had high bacterial loads. Finally, the Δ*pilE* mutant had the
299 strongest impact on colonization. Only five out of 20 bees had detectable levels of *F. perrara* in
300 the gut (limit of detection: 75 CFUs per gut) and none of the bees developed the scab
301 phenotype. These results confirm that IHF regulates various host colonization factors that when
302 deleted cause distinctive colonization defects.
303

304 **Discussion**

305 In this study, we identified genetic factors that allow the gut symbiont *F. perrara* to colonize its
306 specific niche in the pylorus of the honey bee gut and to induce the scab phenotype. In
307 summary, we found that a nonsynonymous mutation in the gene encoding the alpha subunit of
308 the integration host factor (IhfA) leads to a strong colonization defect of *F. perrara* characterized
309 by reduced bacterial loads in the gut and the absence of the scab phenotype. Using RNAseq,
310 we showed that the *ihfA* mutation (*ihfA**) causes the downregulation of diverse genes known to
311 be involved in symbiosis, including two T6SSs and associated effector proteins, the Clb
312 biosynthetic gene cluster, Flp pili, and an RTX toxin locus. One of the IHF-regulated biosynthetic
313 gene clusters encoded an APE, which we structurally characterized using specialized metabolite
314 analysis, and which seems to be responsible for the yellow appearance of *F. perrara* colonies
315 when grown on mTYG agar. Most of the genes regulated by IhfA were expressed during gut
316 colonization, and gene deletion analysis revealed that the colibactin biosynthesis gene cluster,
317 one of the T6SSs, the Flp pili, and the APE biosynthetic gene cluster impact gut colonization
318 and in part also scab development.

319 These results advance our understanding of the genetic factors that facilitate symbionts to
320 colonize niches in the animal gut. Specifically, we find that the DNA-binding protein IhfA plays
321 an important role in gut colonization. IhfA is a histone-like, nucleoid-associated protein (NAP)
322 that forms a heterodimer together with IhfB. IHF binds to and bends DNA in a sequence specific
323 manner (37-39, 46), thereby facilitating the recombination of mobile DNA elements (37, 38) and
324 influencing gene expression. We identified three different mutations in IHF, all located in the
325 region of the protein interacting with the DNA. All three IHF mutants formed larger colonies than
326 the wt strain on mTYG agar. Together, this suggests that the identified mutations change the
327 DNA-binding properties of IHF resulting in broad transcriptional changes in *F. perrara* that
328 provide a growth advantage in vitro relative to the wt strain.

329 Like other microbiota members inhabiting the pylorus/ileum region of the honey bee gut
330 (*Gilliamella* and *S. alvi*), *F. perrara* colonizes the epithelial surface, where it adheres to the

331 cuticular lining and forms a thick biofilm-like layer (34). In case of the human pathogen
332 *Haemophilus influenzae*, IHF has been shown to bind to extracellular DNA, which increases
333 biofilm stability (47). Therefore, it is possible that IHF has a direct impact on gut colonization of
334 *F. perrara* by stabilizing the biofilm formed on the host epithelium of the pylorus/ileum. However,
335 based on our gene expression data and the results of the gene deletion experiments, it seems
336 more likely that IHF influences gut colonization by regulating downstream genes involved in host
337 interaction. Several of the genes regulated by IHF are known to play important roles in the
338 adhesion to surfaces or the formation of biofilms. For example, pili are key factors for adhesion
339 across many bacteria (48-51) and have already been shown in the case of the bee gut symbiont
340 *S. alvi* to be beneficial for gut colonization and biofilm formation (7). Furthermore, several of the
341 deletion mutants of IHF-regulated genes showed colonization defects in our gnotobiotic bee
342 experiments providing further evidence that IHF impacts gut colonization through its effect on
343 gene expression rather than through a direct role in biofilm formation.

344 The pili mutant (Δ*pilE*) had the strongest colonization defect of the tested gene deletion strains,
345 likely because pili mediate the adhesion to the host epithelium, and therefore allow *F. perrara* to
346 persist and replicate in the pylorus/ileum region of the honey bee gut. The deletion mutant of the
347 APE biosynthesis pathway (Δ*apeA-R*) also showed a clear colonization defect. However, while
348 some of the bees inoculated with this mutant had no detectable levels of *F. perrara* in the gut,
349 others reached similar levels as bees colonized with the wt strain and also developed the scab
350 phenotype. Aryl polyenes can protect bacteria from reactive oxygen species (42, 52) which are
351 produced by insects as part of the host immune response in the midgut (53, 54). Our time
352 course experiment revealed that in the first few days of colonization the bacterial numbers of *F.*
353 *perrara* in the gut are much lower than in the initial inoculum (**Figure 2**) suggesting that the
354 bacteria experience a significant population bottleneck at the beginning of the colonization
355 process. It is possible that the Δ*apeA-R* mutant is impaired in its ability to resist reactive oxygen
356 species (or other physicochemical stressors) when passing through the anterior sections of the
357 honey bee gut and therefore reaches its niche in the pylorus in only a fraction of the inoculated

358 bees. However, APEs have also been implicated in other functions such as biofilm formation
359 (52). Therefore, future studies will be needed to elucidate how the APE biosynthetic pathway of
360 *F. perrara* impacts honey bee gut colonization. Given the widespread occurrence of these
361 biosynthetic genes throughout different bacteria, this might help to understand their role in host
362 colonization in a much wider context (43).

363 A somewhat unexpected result of our mutant analysis was that the deletion of one of the
364 essential NRPS/PKS enzymes of the Clb biosynthetic gene cluster did not affect scab
365 development and had only a weak impact on gut colonization (2-fold lower levels than wt).
366 Studies on *E. coli* have shown that the Clb biosynthesis pathway induces DNA damage in
367 eukaryotic cells (55) and contributes to tumorigenesis in the mammalian gut (56, 57). We have
368 previously shown that *F. perrara* also induces DNA damage in eukaryotic cells and that this is
369 dependent on a functional Clb biosynthesis pathway (35). Therefore, we had speculated that the
370 genotoxic activity of colibactin may trigger the local melanization response and the development
371 of the scab phenotype upon colonization with *F. perrara* (17, 34). However, the results
372 presented here show that colibactin is not causing the scab phenotype. Therefore, other specific
373 characteristics of *F. perrara* must explain why this bacterium but no other honeybee gut
374 symbiont is causing the scab phenotype.

375 Our gnotobiotic bee experiments showed that the development of the scab phenotype depends
376 to a large extent on the bacterial loads of *F. perrara* in the gut. Bees that had $<10^6$ bacteria in
377 the gut did not develop visible scab at day 10 post colonization (**Figure 3 and Figure 7**), which
378 suggests that a certain number of bacteria is needed to elicit this characteristic host response.
379 On the contrary, not all bees with high levels of colonization developed a scab phenotype
380 indicating that high loads are necessary but not sufficient to trigger the development of a visible
381 scab phenotype. Specifically, the *Δhcp2* mutant of T6SS-2 and the *Δhcp* double mutant of
382 T6SS-1 and T6SS-2 both reached relatively high colonization levels, yet not a single bee
383 developed the scab phenotype. T6SS are usually involved in interbacterial warfare by injecting
384 toxins into neighboring bacterial cells (58-60). However, there is an increasing number of

385 studies showing that certain T6SS effectors can also target eukaryotic cells and modify diverse
386 eukaryotic processes, including adhesion modification, stimulating internalization, cytoskeletal
387 rearrangements and evasion of host innate immune responses (61). While *F. perrara* is not the
388 only bee gut symbiont harboring T6SS, the effector protein repertoires differ tremendously
389 between different species and even strains of the same species (62). Thus, it is possible that *F.*
390 *perrara* carries effector proteins, which target the host rather than other bacteria eliciting the
391 melanization response and scab phenotype in the bee gut.

392 We did not carry out a genome-wide screen for host colonization factors, and only generated
393 deletion mutants of a few of the IHF-regulated genes. There are probably many other factors
394 that also contribute to gut colonization. For example, our *in vivo* RNA-seq experiment indicated
395 that metabolic genes involved in tryptophan biosynthesis, sugar transport, and iron uptake are
396 upregulated during gut colonization. This is in line with the TnSeq screen performed in *S. alvi,*
397 which revealed that genes of essential amino acid biosynthesis pathways and iron uptake are
398 important colonization factors of the honey bee gut (63). It is however remarkable that *F. perrara*
399 only upregulates the tryptophan biosynthesis pathway, but no other amino acid biosynthesis
400 genes, which suggest a specific demand for the production of this specific amino acid during gut
401 colonization.

402 While our study revealed that IHF is important for regulating host colonization factors in the
403 honey bee gut, it remains to be elucidated whether IHF is a direct regulator of the identified
404 genes. In *Vibrio fluvialis*, IHF binding sites were identified upstream of several T6SS gene
405 clusters, indicating that direct transcriptional regulation of similar genes by IHF exists in other
406 bacteria (64). Alternatively, IHF may act upstream of another regulator that controls the
407 expression of the identified genes. In the plant pathogen *Dickeya zeae,* IHF was suggested to
408 positively regulate different virulence factors through binding to the promoter region of a
409 diguanylate cyclase gene, increasing the production of the secondary messenger c-di-GMP
410 (65). In *F. perrara,* we found that the transcriptional regulators BasR and DksA were
411 substantially downregulated in the wt versus the *ihfA** mutant. Hence, it is possible that at least

412 some of the differentially regulated genes may not be under the direct control of IHF but
413 regulated via BasR or DskA. In the future, chromatin immunoprecipitation sequencing (ChIP-
414 Seq) could be applied to experimentally identify the specific binding sites of IHF in *F. perrara*
415 and to understand how IHF regulates the gene expression of host colonization factors across
416 bacteria.

417 In conclusion, we identified important gut colonization factors of the bee gut symbiont *F. perrara*
418 and show that they are regulated by IHF. The wide occurrence of these genes in host-
419 associated bacteria suggests similar roles in other environments and calls for a more detailed
420 functional characterization. Our approach to create clean gene deletion mutants expands the
421 available genetic toolbox for bee symbionts (33) and will help to dissect the molecular
422 mechanisms of the identified gut colonization factors in this animal model.

423

424 ## Material and methods

425 ### Bacterial cultivation

426 *F. perrara* strains were cultivated on modified tryptone yeast extract glucose (mTYG) medium
427 (0.2% Bacto tryptone, 0.1% Bacto yeast extract, 2.2 mM D-glucose, 3.2 mM L-cysteine, 2.9 mM
428 cellobiose, 5.8 mM vitamin K, 1.4 µM $FeSO_4$, 72.1 µM $CaCl_2$, 0.08 mM MgSO4, 4.8 mM
429 NaHCO3, 1.36 mM NaCl, 1.8 µM Hematine in 0.2 mM Histidine, 1.25% Agar adjusted to pH 7.2
430 with potassium phosphate buffer) or Columbia Blood Agar (CBA) containing 5% defibrinated
431 sheep blood (Oxoid)) and incubated in anaerobic conditions at 35 °C (8% H_2, 20% N_2, 78% CO_2
432 in a Vinyl Anaerobic Chamber, Coy Lab). Fresh bacterial cultures were used for each
433 experiment. To this end, pre-cultures streaked out from glycerol stocks were grown for 48 hours
434 and re-inoculated onto fresh plates for 16-24 hours of growth. For liquid cultures, pre-cultures
435 streaked out from glycerol stocks were grown on TYG or BHIA plates for 48 hours and then
436 inoculated into fresh liquid TYG or BHI. Cultures were incubated at 34 °C in a ThermoMixer C
437 (Eppendorf) at 800 rpm for 16-24 hours of growth. Strains of *F. perrara* used in this study are
438 listed in **Supplementary Table S2**.

439 ### Rearing and experimental colonization of honey bees

440 Microbiota-depleted bees were generated as described by (18). For experimental mono-
441 colonization, bees were starved for 1-3 hours by removal of the sugar water solution. Then,
442 bees were cooled down to 4 °C in a refrigerator or on ice to transfer them (head side first) into
443 1.5 ml microfuge tubes with a hole at the bottom. Tubes with bees were kept at room
444 temperature. For inoculation, each bee was fed 5 µl of *F. perrara* resuspended in sugar
445 water:PBS (1:1 v/v) through the hole at the bottom of the microcentrifuge tube. The bacterial
446 inocula were adjusted to an OD_{600} of 0.01 or 0.1 depending on the experiment. Colonized bees
447 were kept at 30 °C with 70% humidity while having access to sugar water and sterilized bee
448 pollen *ad libitum* until sampling.

449

450 **Tissue dissection and bacterial quantification from colonized honey bees**

451 Bees were anesthetized by exposure to CO_2. The whole gut or desired gut tissue, e.g., pylorus
452 or ileum was dissected using a scalpel. Malpighian tubules were removed from the gut tissue
453 and the presence of a scab was documented using a dissection stereomicroscope (Leica) as
454 described in (14, 17). The tissues were placed into 2 ml screw-cap tubes containing glass beads
455 (0.75-1 mm diameter, Roth) and 500-1000 µl PBS depending on the experiment.
456 Homogenization of the sample was done by bead beating (FastPrep-24 5g MP Biomedicals) for
457 40 seconds at a speed of 7.5 m/s. Serial dilutions (1:10) were performed for each homogenate
458 and plated onto mTYG or BHI agar. Single colonies were counted to determine the total number
459 of bacteria per gut tissue by multiplying with the dilution factor.

460

461 **RNA sequencing of bacterial *in vitro* cultures**

462 Fresh *F. perrara* cultures were prepared on TYG plates, harvested, and directly transferred into
463 a tube containing TRI reagent (Sigma-Aldrich, Merck) and silica beads (0.1 mm diameter, Roth).
464 Samples were immediately snap frozen in liquid nitrogen and stored at -80 °C until RNA
465 extraction. RNA was extracted using a modified TRI reagent protocol. Samples were
466 homogenized by bead beating with a FastPrep instrument with CoolPrep adapter (FastPrep-24
467 5G, cooled with dry ice) for two cycles of 45 seconds at speed of 6 m/s, including a 30 seconds
468 break between each cycle. Samples were kept at room temperature for 5 min, subsequently
469 extracted using chloroform and RNA was precipitated using isopropanol overnight at -20 °C.
470 Precipitated RNA was dissolved in RNase-free water (Gibco) and incubated with DNase (NEB)
471 to degrade remaining DNA. RNA samples were cleaned up using NucleoSpin RNA clean up kit
472 (Machery-Nagel) according to manufacturer's protocol, eluted in RNAse free water and stored at
473 -80 °C until further use. RNA quality was assessed using Nanodrop, Qubit RNA HS kit
474 (ThermoFisher Scientific) and Bioanalyzer instrument (Agilent). High quality RNA samples were
475 sent to Lausanne Genomic Technology Facility (GTF, University of Lausanne) for RNA
476 sequencing. Samples were depleted of 16S rRNA using RiboZero reagent (Illumina) and Truseq

477 stranded-RNA Zero libraries were generated (Illumina) before sequencing on an Illumina
478 HiSeq2500 generating single-end 100 bp reads.

479

480 **RNA sequencing of bacteria during honey bee gut colonization**

481 Microbiota-depleted bees were colonized with freshly grown *F. perrara*. A fraction of the
482 inoculum was directly transferred into 2 ml tube containing TRI reagent (Sigma-Aldrich, Merck)
483 and silica beads (0.1 mm diameter, Roth). These samples were immediately snap-frozen in
484 liquid nitrogen and stored at -80 °C until RNA extraction, which was carried out in the same way
485 as described in the previous section. For the *in vivo* RNA samples, bees were colonized with *F.*
486 *perrara* as described above and sampled at day 5 and day 10 post inoculation. Guts were
487 removed from anesthetized bees, the pylorus and first part of the ileum was dissected, and the
488 presence of the scab was recorded. For each sample, 10 pylori and ileums from bees coming
489 from the same cage were pooled in a 2 ml screw cap tube containing 750 µl of TRIreagent
490 reagent (Sigma-Aldrich, Merck), glass beads (0.75-1 mm diameter, Roth), and silica beads (0.1
491 mm diameter, Roth). Immediately after collection, samples were snap frozen in liquid nitrogen
492 and stored at -80 °C until RNA extraction. RNA was extracted as described in the previous
493 section. At each time point, four biological replicate samples were used for RNA sequencing,
494 i.e., bees for one sample came from independent cages of gnotobiotic bees. The GTF at the
495 University of Lausanne generated the libraries for the sequencing of the *in vivo* RNA samples.
496 Poly-A depletion was performed in order to enrich for bacterial mRNA and Ribo-zero rRNA
497 depletion was performed in order to remove prokaryotic and eukaryotic rRNAs. Then TruSeq
498 stranded mRNA libraries (Illumina) were generated. The eight libraries were sequenced on an
499 Illumina HiSeq 2500 to obtain single-end 125 bp reads. Libraries of the *in vitro* RNA samples
500 were generated as described before. The four libraries corresponding to the *F. perrara* inocula
501 used for colonizing the bees of the four replicates were sequenced on an Illumina MiniSeq at the
502 Department of Fundamental Microbiology (125 bp single-end reads). Two *in vivo* samples

503 (sample identifier D10_3M and D5_1M) sequenced on the HiSeq 2500 at the GTF were
504 included in the MiniSeq run which confirmed that the two runs gave comparable results.

505

506 **Differential gene expression and gene enrichment analysis**

507 Raw FASTQ files provided by the GTF containing all reads and corresponding tags indicating
508 whether they were accepted or filtered out according to the CASAVA 1.82 pipeline (Illumina).
509 Only reads tagged as accepted were kept for further analysis. FASTQC
510 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to control the quality of
511 the data, followed by Trimmomatic (Trimmomatic-0.35) to trim adapters. Filtered and trimmed
512 reads were mapped onto the *F. perrara* genome using Bowtie (bowtie2-2.3.2*)*. Mapped reads
513 were quantified using HTseq (Version 0.7.2). Differential gene expression analysis was done
514 with the Bioconductor package EdgeR (66) using R scripts. For the in vitro RNAseq analysis,
515 negative binomial models were fitted to the data and quantile-adjusted conditional maximum
516 likelihood (qCML) common and tagwise dispersion were estimated. The conditional distribution
517 for the sum of counts in a group was used to calculate the significantly differently expressed
518 genes using an Exact test with a false discovery rate <5%. Only genes detected in all samples
519 with at least 1 count per million were used for the analysis. For the in vivo RNAseq analysis, we
520 followed the recommendations specified in the EdgeR user guide for generalized linear models.
521 In short, read counts were normalized by trimmed mean of M-values (67) producing scaling
522 factors used by EdgeR to determine effective library sizes. Then, negative binomial generalized
523 linear models were fitted for each condition and quasi-likelihood F-tests for each defined
524 contrast (i.e., pairwise comparison between conditions) was used to assess the significance of
525 differentially expressed genes. As for the in vitro RNAseq analysis, only genes with mapped
526 reads with at least 1 count per million in all replicates and conditions were used for the analysis.
527 Genes were considered as differentially expressed upon fulfilling the following criteria: (i) log2
528 fold change ≥ 2 (ii) a p-value of <0.05 and (iii) a false discovery rate <5%. Transcripts per million
529 (TPM) were calculated as follows (68):

$$TPM\ of\ a\ gene = \frac{number\ of\ mapped\ reads * read\ length * 10^6}{total\ number\ of\ transcripts\ sampled * gene\ length\ in\ bp}$$

530 Coverage plots in Figure 2A and Figure 4C were generated with bam2wig.pl contained in the
531 Bio-ToolBox-1.68 (https://github.com/tjparnell/biotoolbox) and visualized with the Integrated
532 Genome Browser (69). Scripts for analyzing the RNAseq data can be found under the following
533 Switchdrive link: https://drive.switch.ch/index.php/s/kCNTp4g7n60ffMi.
534

535 **DNA extraction of dissected gut tissue homogenates**

536 DNA/RNA extraction was performed using an adapted hot phenol protocol from (18) consisting
537 of sample thawing on ice, homogenization by bead beating at 7.5 m/s for 40 seconds (FastPrep-
538 24 5g MP Biomedicals), two phenol extractions, and an ethanol precipitation overnight at -80 °C.
539 DNA/RNA mixture was pelleted and eluted in RNase free water (Gibco). Samples were treated
540 with RNaseA and DNA was purified using a Gel and PCR purification kit (Machery-Nagel).

541

542 **Bacterial quantification by qPCR**

543 Bacterial absolute abundances were determined using quantitative PCR (qPCR) assays
544 targeting the 16S rRNA gene of *F. perrara*. Normalization was based on the number of host
545 actin gene copies as described in (18, 70). Primer sequences and other primer characteristics
546 are given in **Supplementary Table S3**. qPCR was conducted on a StepOne Plus instrument
547 (Applied Biosystems) with the following run method: a holding stage consisting of 2 min at 50 °C
548 followed by 2 min at 95 °C, 40 cycles of 15 sec at 95 °C, and 1 min at 65 °C. A melting curve
549 was generated after each run (15 sec at 95 °C, 20 sec at 60 °C and increments of 0.3 °C until
550 reaching 95 °C for 15 sec) and used to assess specificity of PCR products. qPCR reactions
551 were performed in 10 µL reactions in triplicates in 96-well plates, and each reaction consisted of
552 1 µL of DNA, 0.2 µM of forward and reverse primer and 1x SYBR green "Select" master mix
553 (Applied Biosystems). For each target, standard curves were generated for absolute
554 quantification using serial dilutions (from 10^7 to 10 copies) of the target amplicon cloned into the

555 plasmid vector. Absolute abundance of bacteria was calculated by using the standard curve and
556 were normalized by the median actin copy number per condition (to account for differences in
557 gut size) and by the amount of 16s rRNA copies per genome. For the calculation we used the
558 following formula:

559 $$Absolute\ bacterial\ abundance = 10^{\left(\frac{Ct_{target} - Intercept}{Slope}\right)} * dilution\ factor\ \div number\ of\ 16S\ rRNA\ copies \div$$
560 $$(median\ of\ actin\ copies\ per\ condition)$$

561 Here, Ct_{target} is the cycle threshold of the target bacterium and intercept and slope correspond to
562 the values calculated for the standard curve of the target bacterium.

563

564 **Single cell microscopy**

565 *F. perrara* was freshly grown from stock on plate. Subsequently, liquid overnight cultures
566 starting at an OD_{600} 0.1 were inoculated in TYG. Liquid cultures were grown overnight 16-24
567 hours with shaking at 34 °C in a ThermoMixer C (Eppendorf) in anaerobic atmosphere. The
568 OD_{600} was measured and adjusted to 0.1 – 0.01 and cells were distributed onto small agar
569 patches on a microscopy slide (Milan S.A., Menzel-Gläser). Cells were observed under 1000
570 times magnification using a ZEISS imager M1 microscope with phase contrast (PH3)
571 condenser. Pictures were acquired randomly on the slides with VisiView software, 8-bit images
572 were corrected with ImageJ and the contrast was set at min-value of 0 and max-value of 4000.
573 A 10 µM scale bar was added with ImageJ (71). MicrobeJ plugin for ImageJ was used to
574 measure cell size and area using the acquired pictures (72).

575

576 **Isolation and identification of aryl polyene compounds**

577 *F. perrara* was streaked from the -80 °C glycerol stock onto mTYG plates. Plates were placed in
578 an anaerobic tank with a pack of AnaeroGel 3.5 L (AN0035A Thermo Scientific) at 37 °C and
579 incubated. Under aerobic sterile conditions, colonies were picked and 5 mL of mTYG medium
580 were inoculated in a Hungate tube. After purging the liquid for 1 h with gas (83% N_2, 10% CO_2,
581 7% H_2 v/v) bacteria were grown at 37 °C. After one day of growth, *F. perrara* wt and the *ihfA**

582 mutant were individually cultivated in Hungate tubes containing 30 mL mTYG for three days.
583 Pellets were harvested, extracted with dichloromethane and methanol, then extracts were
584 analyzed by HPLC-HESI-HRMS. The data revealed a strongly UV-Vis absorbent ion peak at
585 *m/z* 323.1647 $[M+H]^+$. To enrich this compound, 400 mL of mTYG were inoculated with 1 mL of
586 pre-culture in a Hungate tube and purged as described above. After 5-10 days all but a few mL
587 of medium were harvested and 400 mL of fresh mTYG was added under sterile aerobic
588 conditions, purged with anaerobic gas and incubated at 37 °C. A total of 3 L of mTYG were used
589 to obtain 6.53 g of cells, which were stored at -80 °C. Light exposure was minimized during all
590 following steps of the aryl polyene enrichment procedure. A mixture of 340 mL dichloromethane
591 and 170 mL of methanol was added to *F. perrara* cell pellets. After 2 h stirring at room
592 temperature (RT), the suspension was filtered and 250 mL KOH (0.5 M) were added. After an
593 additional 1 h of stirring at RT the pH was set to 6 using H_2SO_4 (1 M). The organic layer was
594 washed twice with 500 mL water, once with 250 mL brine, dried over Na_2SO_4 and concentrated.
595 The extract was separated by reverse-phase high performance liquid chromatography (RP-
596 HPLC , Phenomenex Luna 5 µm C18, φ 21.2 x 250 mm, 15.0 mL/min, λ=420 nm) with water
597 +0.1% formic acid (solvent A) and MeCN +0.1% formic acid (solvent B). Solvent compositions of
598 5% B for 4 min, a gradient to 95% B for 18 min, 95% B for 9 min and 5% B for 4 min were used.
599 Fractions around 24 min exhibited strong UV absorption at 420 nm and were further purified by
600 RP-HPLC (Phenomenex Luna 5µ Phenyl-Hexyl, φ 10 x 250 mm, 2.0 mL/min, λ=420 nm) with
601 80% B for 5 min and a gradient of 80% B to 100% B for 30 min. Peaks around 17 min were
602 combined, concentrated and analyzed by NMR spectroscopy. HPLC-HESI-HRMS was
603 performed on a Thermo Scientific Q Exactive mass spectrometer coupled to a Dionex Ultimate
604 3000 UPLC system. NMR spectra were recorded on a Bruker Avance III spectrometer equipped
605 with a cold probe at 500 MHz and 600 MHz for ^{1}H NMR and 125 MHz and 150 MHz for ^{13}C
606 NMR at 298 K. Chemical shifts were referenced to the solvent peaks of DMSO-d_6 at δ_H
607 2.50 ppm and δ_C 39.51 ppm.

608

609 **Structural characterization of the aryl polyene**

610 ^{1}H NMR in conjunction with HSQC data suggested approximately 15 methines and aromatic
611 protons, one methoxy group and one methyl group (**Supplementary Figure 3-6**). From the
612 COSY spectrum, four methines of the conjugated double bond system, and two of the aromatic
613 protons could be connected (**Supplementary Figure 7, 8**). HMBC correlations from the methyl
614 group to an aromatic carbon at δ_C 156 ppm placed it in *ortho*-position of a hydroxy group and
615 next to a singlet aromatic proton. The singlet of the methoxy-group was connected to a carbonyl
616 group by HMBC correlations (**Supplementary Figure 9**).

617

618 **Construction of gene deletion mutants**

619 Targeted in-frame gene deletions of *F. perrara* were constructed using a two-step homologous
620 recombination procedure (see **Supplementary Figure 14**). In a first step, suicide plasmids were
621 constructed harboring two adjacent 700-800 bp-long homology regions matching the up- and
622 downstream region of the gene or gene cluster to be deleted. To this end, the two homology
623 regions were amplified using 2 Phanta Max Master Mix (Vazyme) and cloned into pKS2 using
624 Golden Gate assembly. pKS2 is a derivative of pSEVA312 (73) which contains a sfGFP
625 expression cassette flanked by two BsaI restriction sites in the multiple cloning site. All clonings
626 were done in *E. coli* DH5a/λpir. To validate successful cloning of the two homology regions,
627 PCR and Sanger sequencing was performed. The plasmids derived from pKS2 containing the
628 homology regions were shuttled into the diaminopimelic acid (DAP)-auxotroph *E. coli* JKE201
629 and delivered to *F. perrara* wt via conjugation using bi-parental mating. To this end, *E. coli*
630 JKE201 carrying the plasmid of interest was cultivated for 16-20 hours at 37 °C in LB with
631 500µM DAP (Sigma, LB-DAP) and 30 µg/ml Chloramphenicol. Sub-cultures were made by
632 diluting the culture at a ratio of 1:20 into fresh medium and incubated until exponential growth
633 was reached. A fresh culture of *F. perrara* was prepared by growing it for 36 h on mTYG agar.
634 *F. perrara* and *E. coli* were harvested in PBS and the OD_{600} adjusted to 10. Equal quantities of

635 both bacteria were mixed and spotted onto a cellulose acetate membrane filter (0.2 µm, 25 mm,
636 Huberlab, Sartorius) placed on BHI agar with 500 µM DAP, followed by incubation at 35 °C for 6
637 hours under microaerophilic condition. Filters with bacterial mixtures were removed from the
638 plates with a forceps, placed into a tube with PBS. Bacteria were removed from the filter by
639 pipetting, vortexing and agitation for 10 min at 1500 rpm (Thermomixer C, Eppendorf). The
640 bacterial suspension was centrifuged at 8000x g for 10 minutes, the supernatant discarded to
641 remove dead bacteria and the bacterial pellet was washed once with 1000 µl PBS before
642 resuspending in a reduced volume, i.e. 100 µl PBS. The bacterial mixture was plated onto CBA
643 and TYG with selection antibiotic and incubated at 35 °C for 5 days under anaerobic condition.
644 Colonies were picked after 4-5 days of incubation and expanded to fresh plates. DNA was
645 isolated and five different PCRs performed to check for successful integration of the plasmid into
646 the chromosomal region of *F. perrara* targeted for deletion. Positive clones (loop-in strains) were
647 stocked in liquid mTYG containing 20% (v/v) glycerol at -80 °C until further usage. To select for
648 bacteria that have lost the integrated plasmid, *F. perrara* loop-in strains were grown on mTYG
649 agar and subsequently transformed with plasmid pYE1. pYE1 is a derivative of pBZ485 (74)
650 containing the restriction enzyme *I-SceI* under the control of the IPTG-inducible *lac* promoter.
651 The activity of the restriction enzyme *I-SceI* will negatively select bacteria containing pKS2-
652 derivatives as two *I-SceI* sites are flanking the multiple cloning sites on pKS2. To generate
653 electrocompetent *F. perrara* cells, the bacteria were cultivated from glycerol stocks on mTYG
654 agar and incubated at 35 °C for 2 days under anaerobic condition. Bacteria from plate were
655 inoculated in BHI broth with a starting OD_{600} of 0.1 and grown anaerobically at 40 °C for 12-16
656 hours. Subsequently, growth was stopped by placing cultures onto ice for 15 minutes. Cells
657 were made electrocompetent by washing twice with ice-cold MOPS solution supplemented with
658 20% glycerol, with decreasing volumes per wash. Electrocompetent cells were mixed with >500
659 ng plasmid DNA and incubated for 15 minutes on ice before transferring into an electroporation
660 cuvette. Electroporation was performed applying a voltage of 2.5 kV with resistance of 200 Ω
661 and capacitance of 25 µF. Bacteria were immediately resuspended in BHI and incubated
662 anaerobically for 6 hours at 35 °C to allow phenotypic expression. Bacteria were plated on CBA,

663 mTYG and/or BHIA plates with 25 µg/ml Kanamycin and 100 µM IPTG to select for pYE1 and
664 induce the restriction enzyme *I-SceI*. Plates were incubated in anaerobic atmosphere at 35 °C.
665 After 3 to 5 days of incubation, bacterial colonies were replica plated onto fresh plates
666 containing 15 µg/ml chloramphenicol (selection marker of pKS2-derivatives) or 25 µg/ml
667 kanamycin (selection marker of pYE1) to identify clones that cannot grow on chloramphenicol
668 anymore as a consequence of the loss of the integrated pKS2-derivative. DNA was extracted
669 and a PCR screen performed with primers amplifying the gene targeted for deletion as well as
670 the overspanning region of the deletion. All clones possessing the gene deletion were
671 subsequently expanded on mTYG agar and stocked in glycerol as described above

672

673 **Genome re-sequencing**

674 All gene deletion strains and the three white variants of *F. perrara* PEB091 were subjected to
675 Illumina genome re-sequencing at Novogene (2x150bp). Mutations in the re-sequenced
676 genomes relative to the genome of the wt strain of *F. perrara* were analyzed with *breseq* (75).
677 The detailed results of this analysis can be found:
678 https://drive.switch.ch/index.php/s/kCNTp4g7n60ffMi.

679

680 **Statistical analysis**

681 Statistical analysis was performed using GraphPad Prism v6.01 or R. Results of all statistical
682 tests can be found in **Supplementary Dataset 5**. Normality was analyzed using D'Agostino-
683 Pearson and/or Shapiro-Wilk test. For comparison with two conditions, t-test was used for
684 parametric data and Wilcoxon sum-rank test or Kolgomorov-Smirnov test for non-parametric
685 data. For comparison of the growth curves of *F. perrara* wt and *ihfA**, we used a permutation
686 test 'compareGrowthCruves' included in the statmod package of R (76).

687

688 **Data availability**

RNA sequence datasets are available under NCBI Gene Expression Omnibus ID GSE189728. Code, scripts and numeric data files of experimental data have been deposited on Switchdrive: https://drive.switch.ch/index.php/s/kCNTp4g7n60ffMi.

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Figures

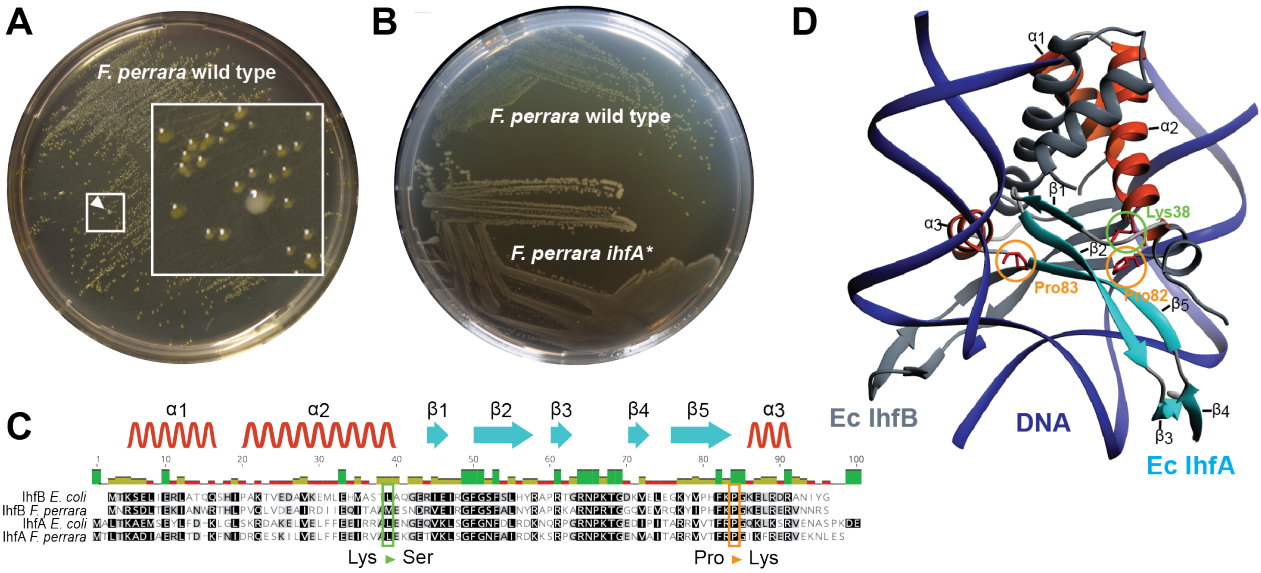


Figure 1. Isolation of a spontaneous ihfA* mutant of *F. perrara* displaying an altered colony morphotype. (A) Colonies of *F. perrara* PEB0191 (wt) after 48h of growth on mTYG agar. Arrowhead points at a larger white colony in between many yellow colonies. The area in the white square is magnified. (**B**) Colony morphology of *F. perrara* wt and the isolated white *ihfA** mutant after growth on mTYG for 48h. (**C**) Protein sequence comparison of IhfA and IhfB of *F. perrara* wt and *E. coli*. The mutated lysine and proline residues in IhfA and IhfB are outlined with a green and orange rectangle and the amino acid change due to the point mutations indicated. Secondary structures are depicted above as ribbons (α-helix) and arrows (β-sheet) and are numbered according to their appearance in the protein. (**D**) Three-dimensional structure of *E. coli* IhfA/B heterocomplex with DNA (source protein databank NDB: PDT040). DNA is depicted in blue and IhfB in dark grey. IhfA is colored according to secondary structure: α-helix orange, β-sheet light blue and the rest in light grey. α-helices and β-sheets are numbered. The mutated Pro83 and Lys38 residues of *F. perrara ihfA* and the Pro82 residue of *ihfB* are marked with a orange and green circle, respectively.

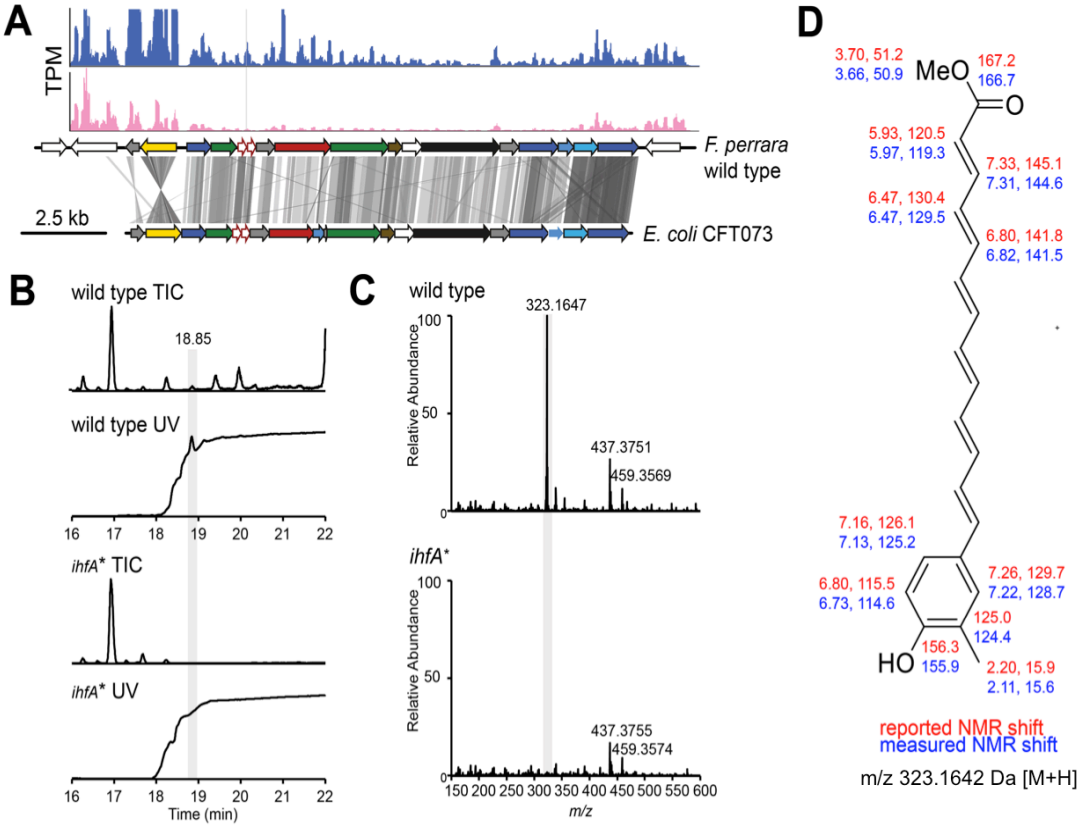


Figure 2. Metabolite analysis of *F. perrara* wt and the *ihfA mutant. (A)** Comparison of gene synteny and sequence similarity of the genomic islands of *F. perrara* PEB0191 (top) and *E. coli* CFT073 (bottom) encoding the aryl polyene (APE) biosynthesis genes. Grey lines indicate homologous regions based on tblastx analysis. Plots were generated with genoplotR (77). Transcripts per million (TPMs) are shown on top of the genomic island for one RNAseq replicate of each *F. perrara* wt (blue) and the *ihfA** mutant grown in vitro. Coverage plots were generated with the Integrated Genome Browser v9 (69). (**B**) Total ion chromatogram (TIC) and UV trace (λ=420 nm) of wt and *ihfA**. A peak highly abundant in the wt was discovered at 18.85 min. Its high UV absorbance at λ=420 nm indicated a conjugated carbon double bond system. (**C**) The normalized mass spectrum at 18.85 min reveals the ion *m/z* = 323.1647 Da to be approximately 50-fold more abundant in the wt compared to *ihfA**. (**D**) Enrichment of the ion containing fraction by HPLC followed by NMR experiments suggest a structure identical to that reported by Cimermancic *et. al.*. Reported (red) and observed (blue) ^{1}H and ^{13}C chemical shifts are shown. Central methenes could not be assigned.

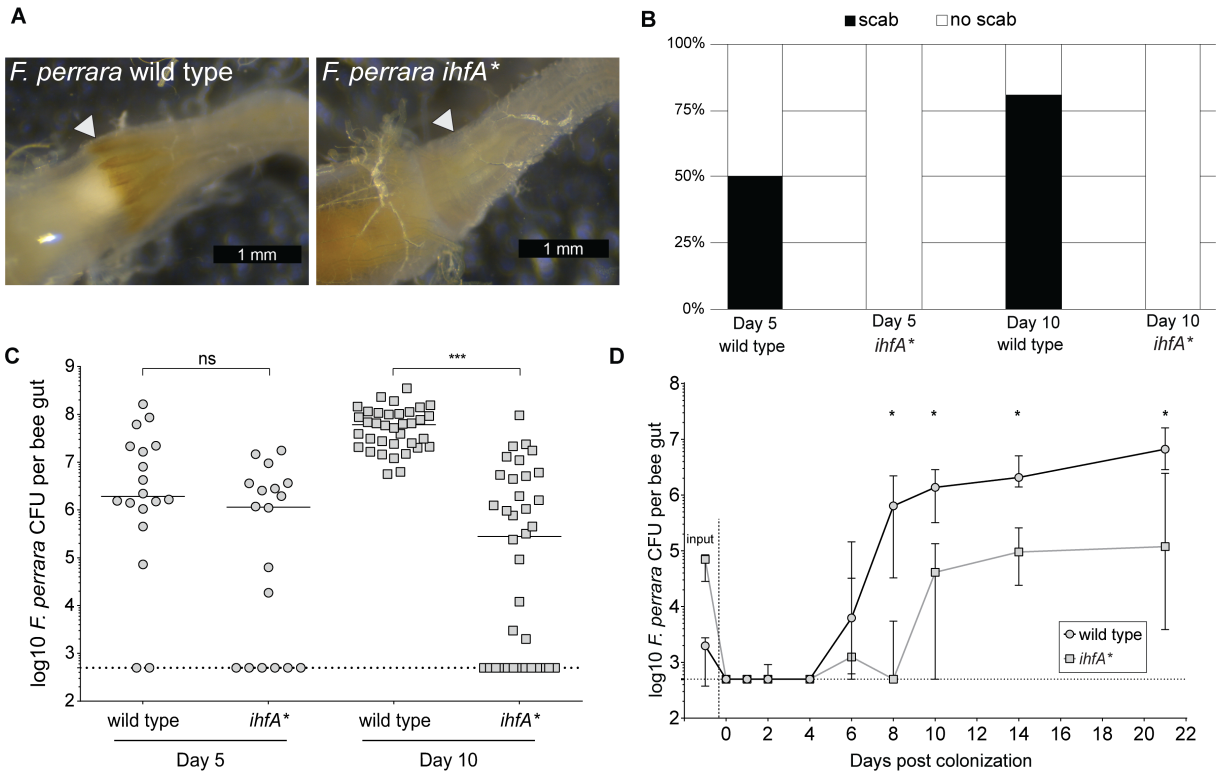


Figure 3. *F. perrara ihfA mutant displays a colonization defect. (A)** Light microscopy pictures of pylorus region of bees colonized with *F. perrara* PEB0191wt or *ihfA** 10 days post colonization. (**B**) Quantification of scab phenotype of bees 5 and10 days post colonization with n=18 and n=36 per treatment, respectively. (**C**) Quantification of colonization levels are measured by colony forming units (CFU) 5 (n=18) and 10 (n=36) days post colonization. Wilcoxon rank-sum test was used to assess significant differences. (**D**) Time-course experiment of bees colonized with *F. perrara* wt or *ihfA**. Colonization levels were measured by CFUs every second day until Day 10 and following at Day 14 and Day 21. n=12 per time point per treatment. Wilcoxon rank-sum test was used to assess significant differences per time point. Error bars represent median and interquartile range. Data from three independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

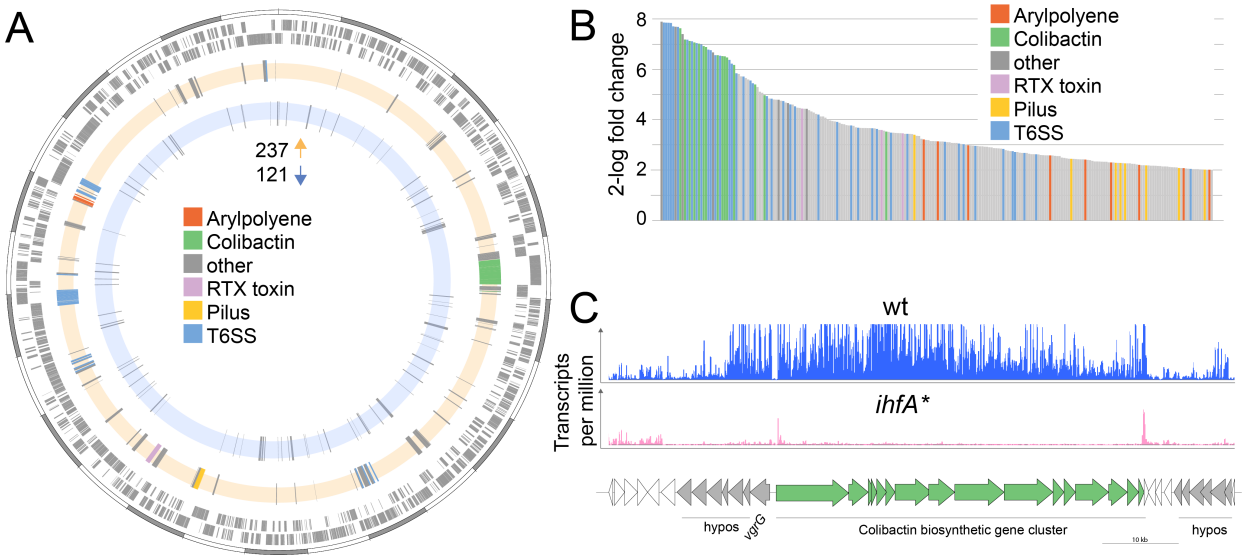
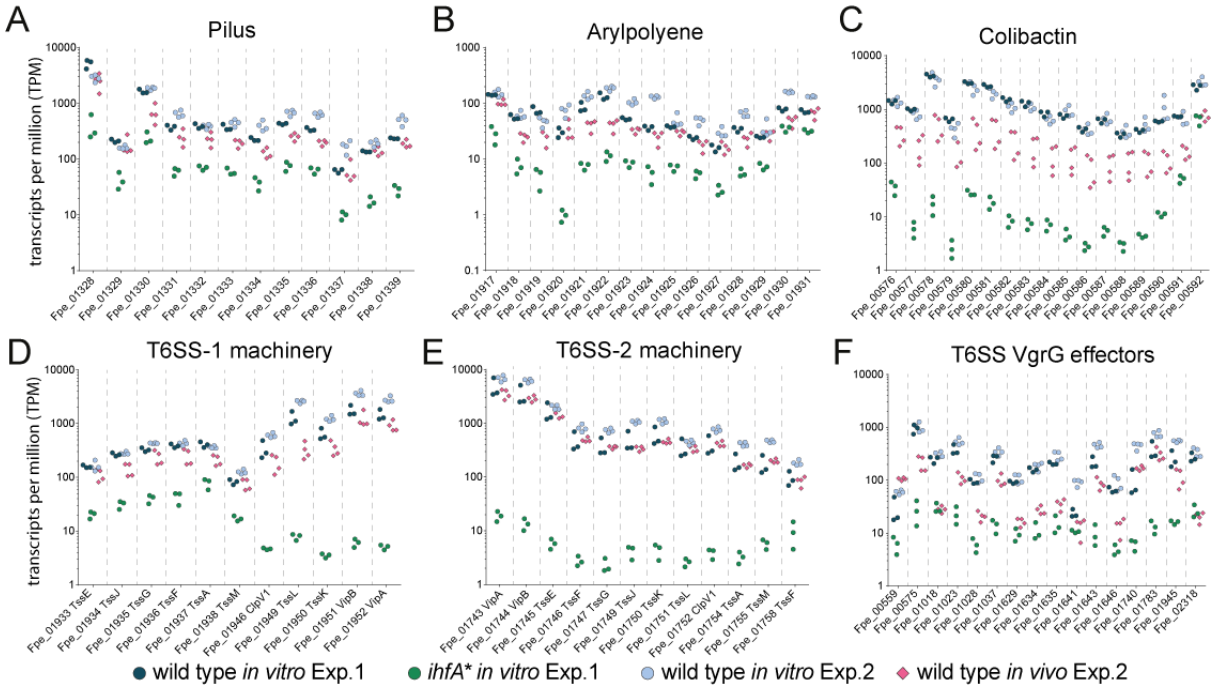


Figure 4. Differential gene expression between *F. perrara* wt and *ihfA mutant during *in vitro* growth**. (**A)** Chromosomal localization of all genes significantly differentially expressed (2-log fold change = |2|, Fisher's exact test p value <0.05, FDR of <0.05) between *F. perrara* wt and the *ihfA** mutant. Starting from outside, the first circle shows the scale of the genome representation of *F. perrara* in gray and white steps of 100 kb. The second and third circles (gray) depict the genes on the plus and minus strands of *F. perrara*. The fourth (beige) and fifth (light blue) circle depicts genes upregulated and downregulated in wt compared to *ihfA**. Genomic islands are highlighted by coloration. (**B)** Bar plot of the genes differentially expressed between *F. perrara* wt and *ihfA** with a log2-fold change >2 (Fisher's exact test p value <0.05, FDR of <0.05). (**C)** Comparison of the transcriptional profile of the genomic location encoding the colibactin biosynthetic gene cluster between *F. perrara* wt and the ihfA* mutant. Transcripts per million were visualized using the Integrative Genome Browser (69). The colibactin operon is schematically depicted below (green arrows).



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765

766 **Figure 5. Gene expression of Ihf-regulated genes of *F. perrara* ten days post-inoculation**
767 **of gnotobiotic honey bees.** Transcripts per million were calculated for all replicates of the *in*
768 *vitro* and the *in vivo* RNAseq experiments. For the in vitro experiment (Exp. 1), all three
769 replicates of the wt and the *ihfA** mutant are shown. For the *in vivo* experiment (Exp. 2), the four
770 replicates of the day 10 time-point and the in vitro reference condition are shown. Data for day 5
771 time-point in comparison to day 10 time-point is shown in **Supplementary Figure14**.

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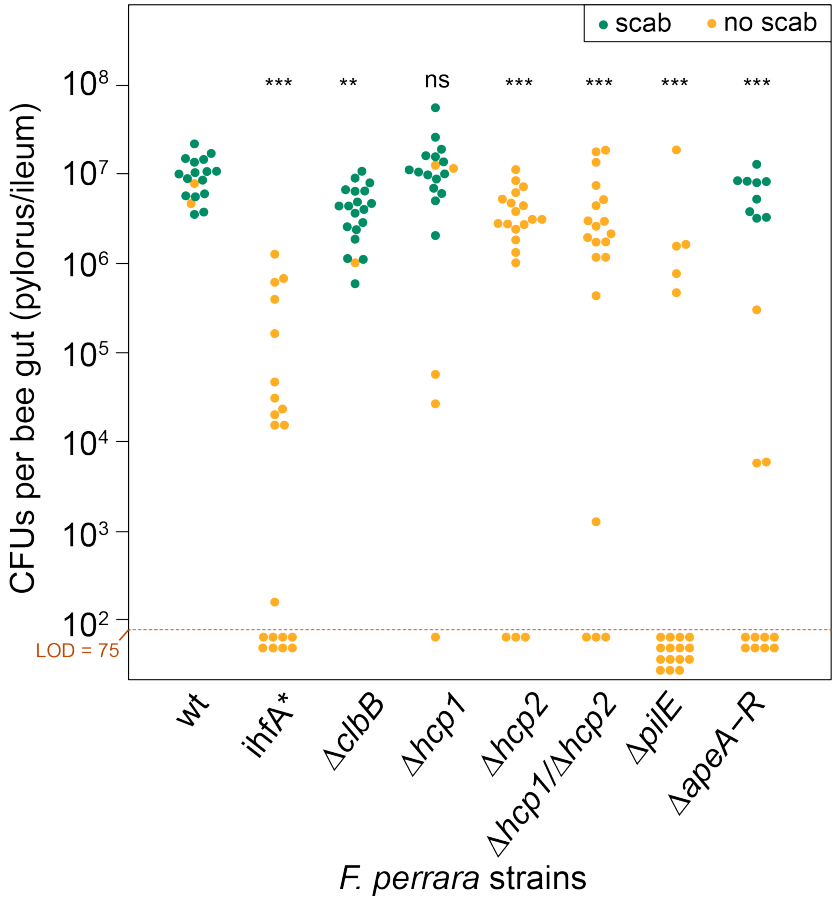

Figure 6. Colonization levels of T6SSs, Clb, Flp pili, and ape gene deletion mutants of *F. perrara.* Colonization levels were assessed 10 days after inoculation by counting colony-forming units (CFU) in dilutions of homogenized bee guts plated on BHI agar. Only the pylorus and ileum section of the gut were analyzed. Limit of detection (LOD) corresponds to the lowest colonization level detectable in our assay, i.e. points below the LOD correspond to bees for which no CFUs were detected. Statistically significant differences of the colonization levels of each mutant relative to the wt of *F. perrara* were determined using the Wilcoxon rank-sum test with BH correction. Bees were inoculated with an OD_{600} of 0.01. Data come from two independent experiments. **Supplementary Figure 15** shows the data points by experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Filled circle colors indicate whether a scab was detected during dissection (green = scab; yellow = no scab).

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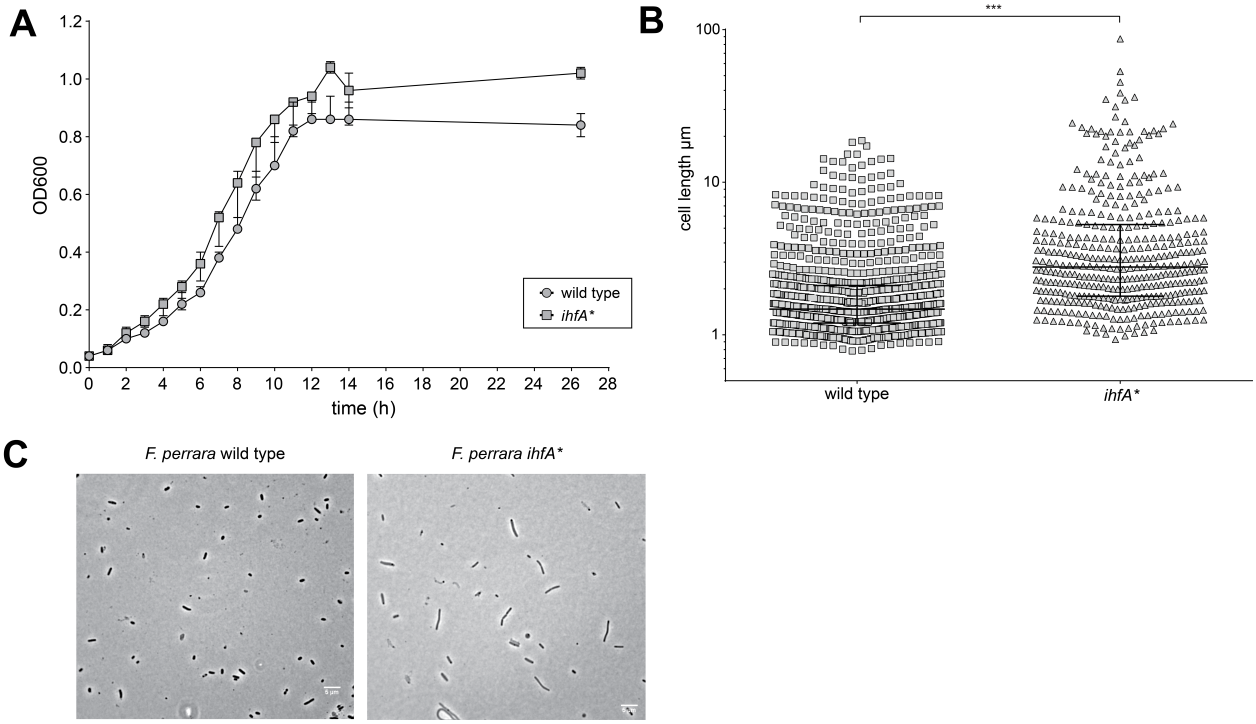
790 ## Supplementary Figures

791



792

793 **Supplementary Figure 1. *In vitro* characterization of *F. perrara ihfA**. (A)** Growth curve of *F.*

794 *perrara* PEB0191 wt and *ihfA** in liquid TYG. The optical density (OD600) was measured every

795 hour for 14 hours (Permutation test, p = 0.097). (**B)** Quantification of single cell length of *F.*

796 *perrara* wt PEB0191 and *ihfA** from the single cell microscopy experiment shown in C.

797 Significance was tested using Kolgomorov-Smirnov test, stars depict significance: *** p<0.0001.

798 **(C)** Single cell light microscopy of *F. perrara* wt and *ihfA**. The scale bar (5µm) is depicted in the

799 right lower corner.

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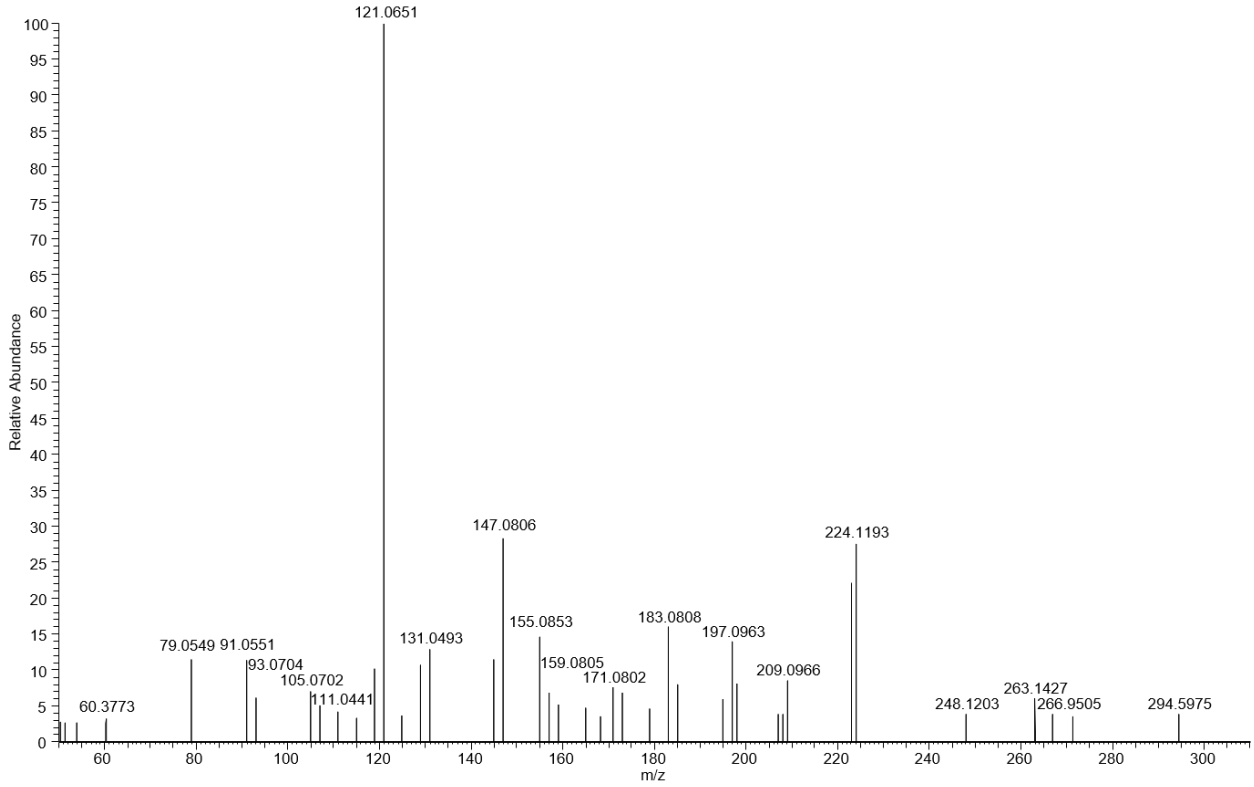

Supplementary Figure 2. Ms-Ms fragmentation spectrum of *m/z* = 323.1647 in extracts of *F. perrara* wt. Many of the prominent ions were reported from aryl polyenes by **(42)**: *m/z*= 121.00, 131.00, 145.00, 147.08, 171.08, 183.00, 197.08, 209.08, 223.08. The spectrum was recorded at 30 kV collision energy.

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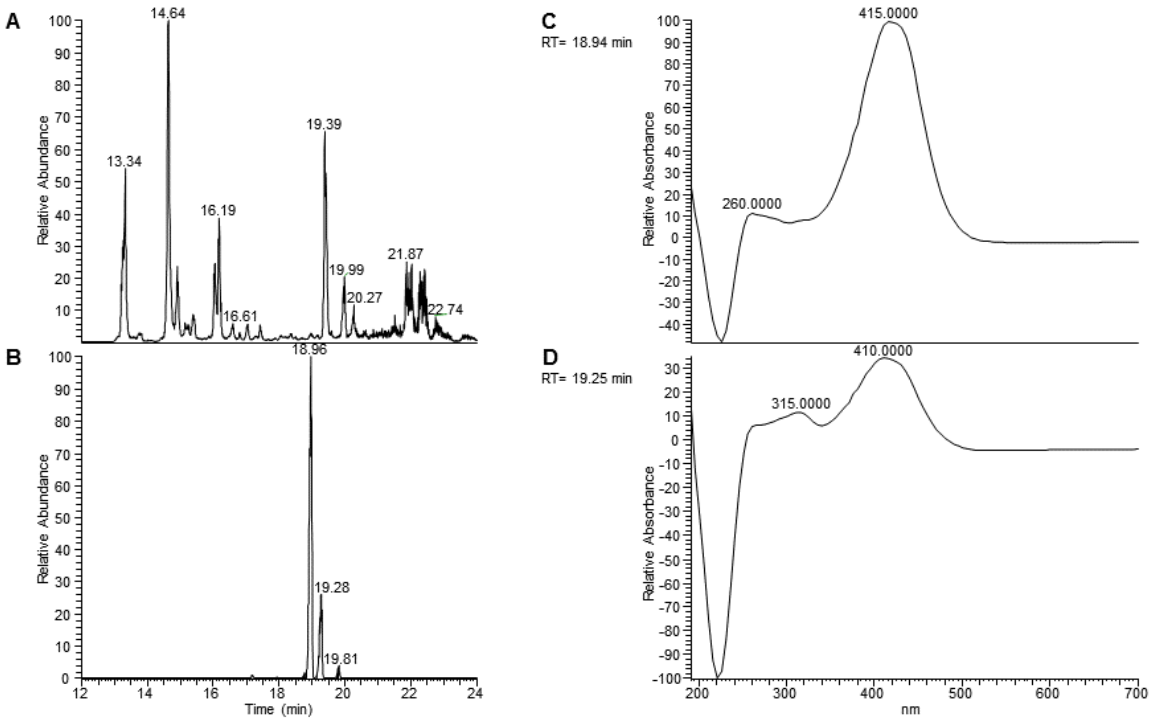

808

809 **Supplementary Figure 3**. **UV-spectrum of the extracts indicates isomerization of the aryl**
810 **polyene.** UV spectrum of the extracts indicates isomerization of the aryl polyene. **A** Total ion
811 chromatogram of extracts of *F*. *perrara* wt. **B** Extracted ion chromatogram of *m/z* = 323.1640 Da
812 (+/- 5ppm). Storage led to the formation of an additional peak at 19.28 min, possibly by
813 isomerization. **C** The UV-spectrum at 18.94 min has the maximum around 415 nm, similar to
814 that reported by (Cimermancic et al., 2014) **D** The UV-spectrum at 19.25 min shows an
815 additional maximum at 315 nm, characteristic for a *trans-cis* conversion of a double bond.

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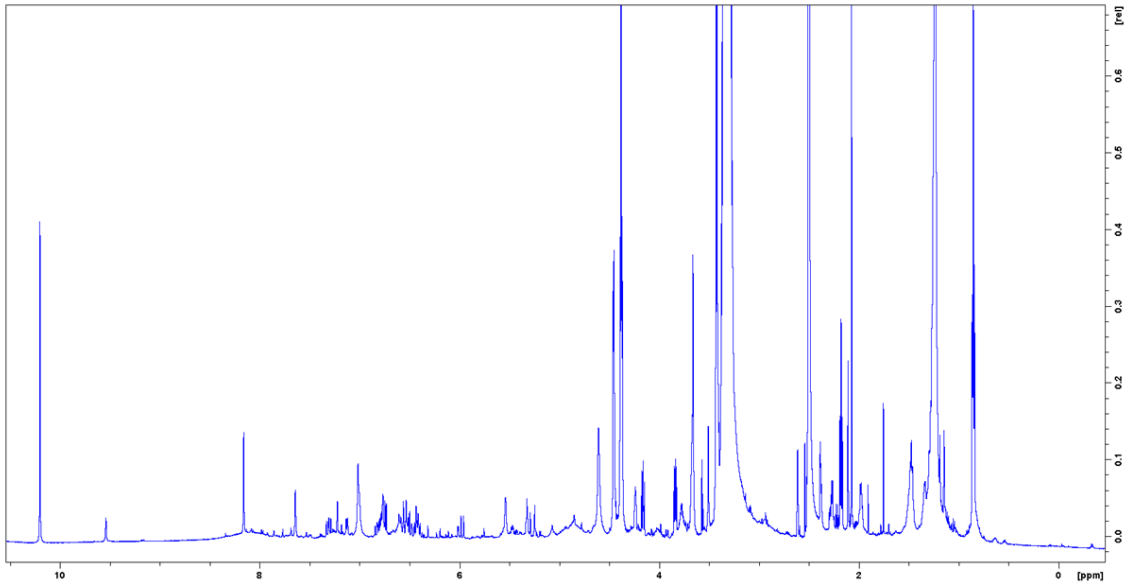

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819 **Supplementary Figure 4.** ^{1}H NMR spectrum of enriched aryl polyene in DMSO–δ_6 at 298 K.

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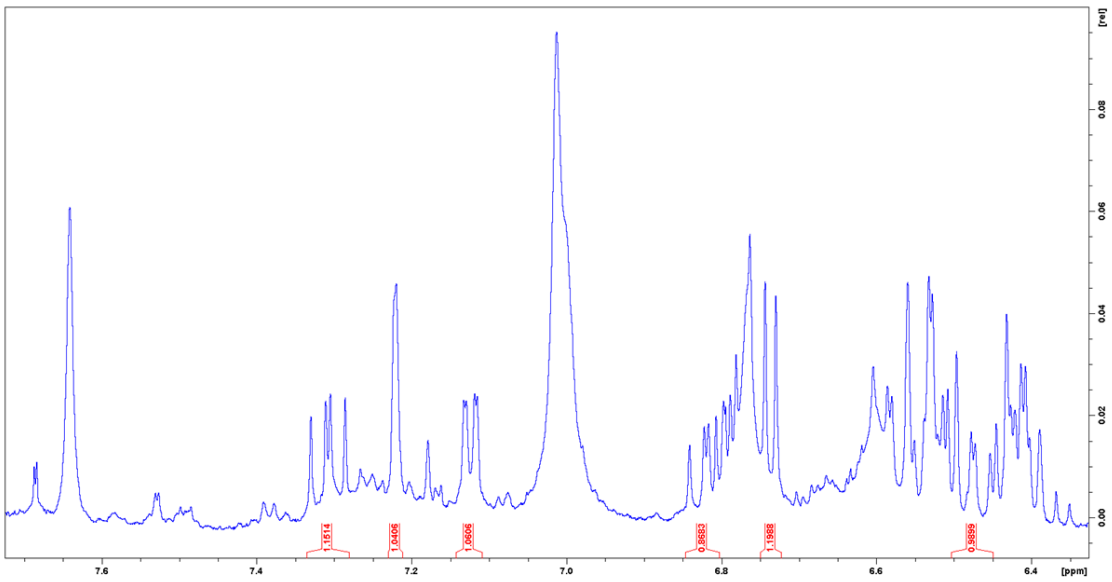

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822 **Supplementary Figure 5. ^{1}H NMR spectrum of enriched aryl polyene in DMSO–δ_6 at 298 K.**

823 Enhanced view of the aromatic region and integrals of assigned peaks.

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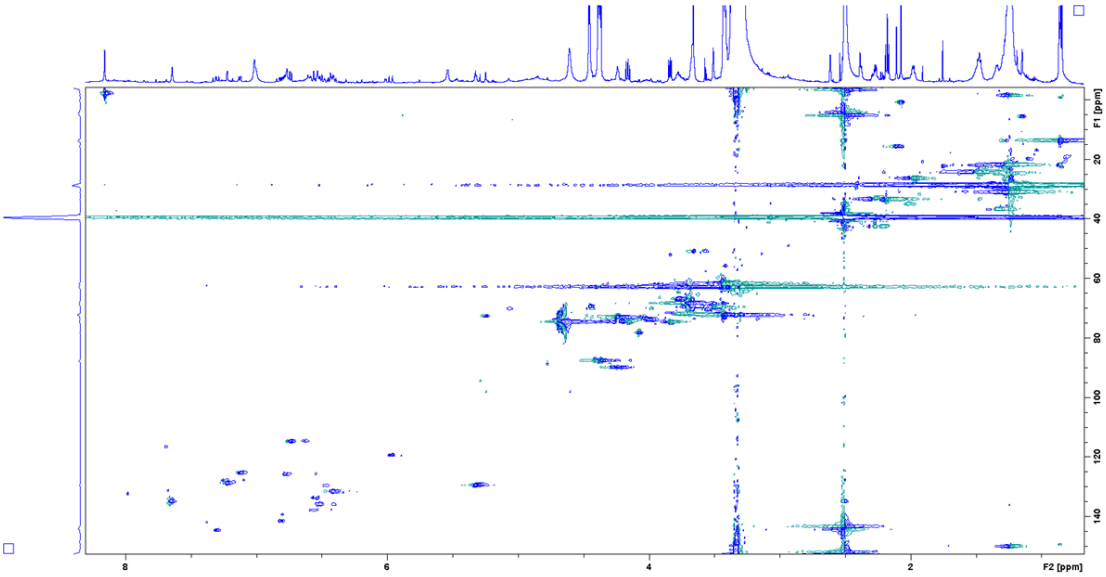

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826 **Supplementary Figure 6.** HSQC spectrum of enriched aryl polyene in DMSO–δ_6 at 298 K.

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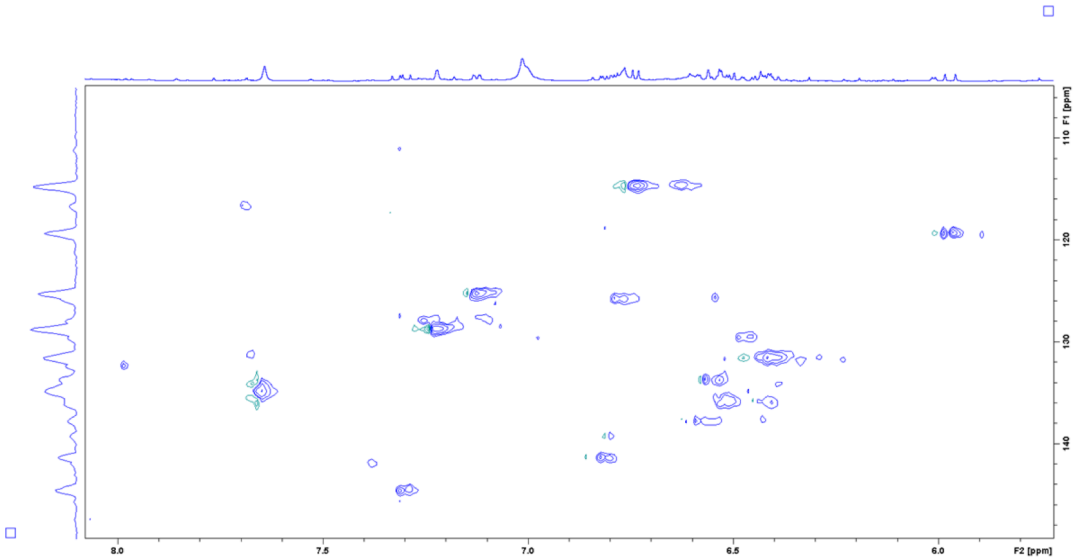

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829 **Supplementary Figure 7**. HSQC spectrum of enriched aryl polyene in DMSO–δ_6 at 298 K.

830 Enhanced view of the aromatic region.

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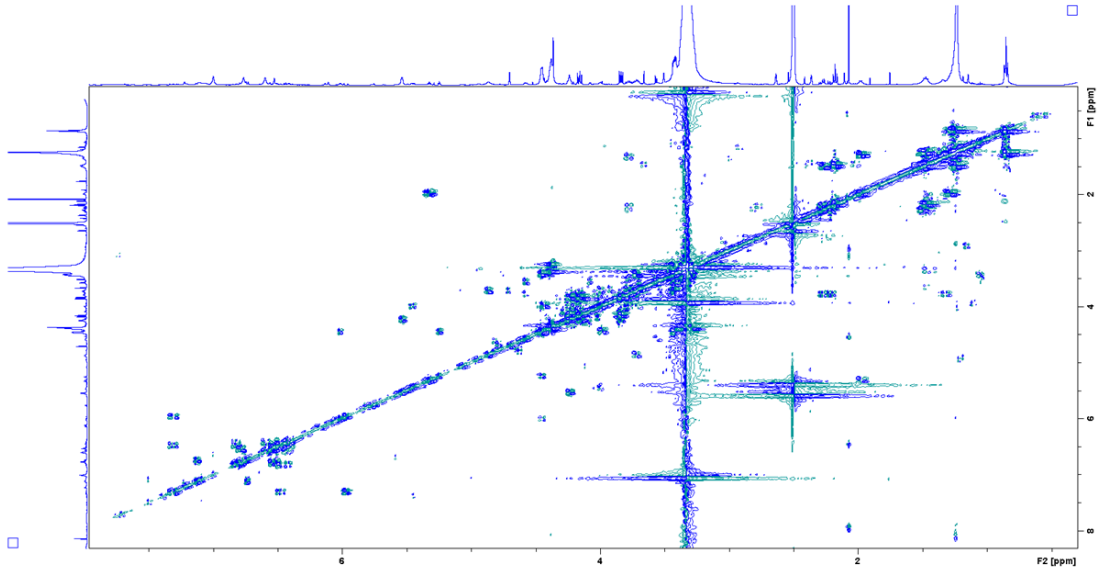
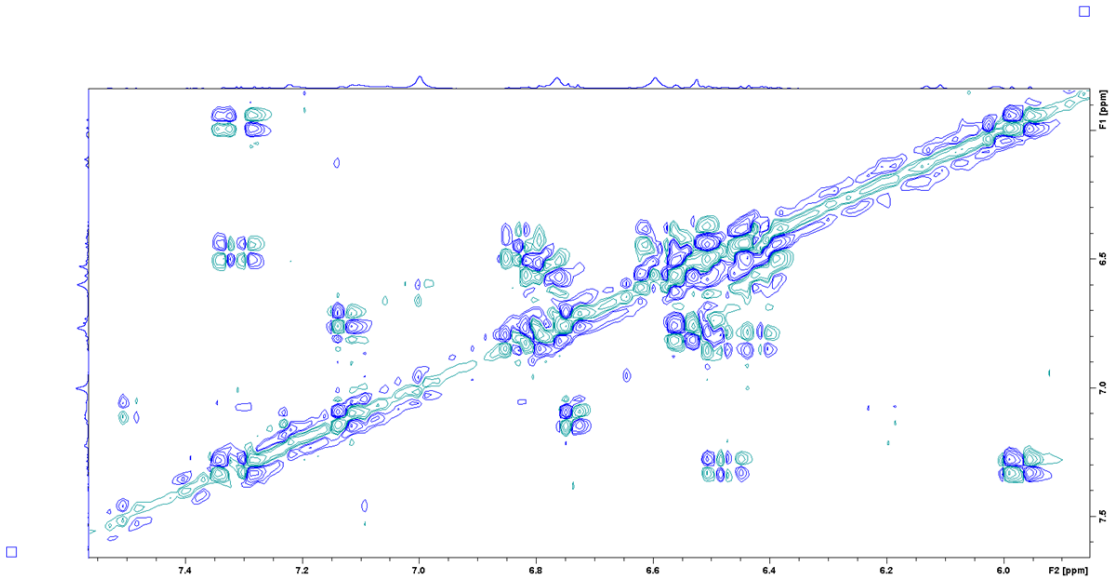
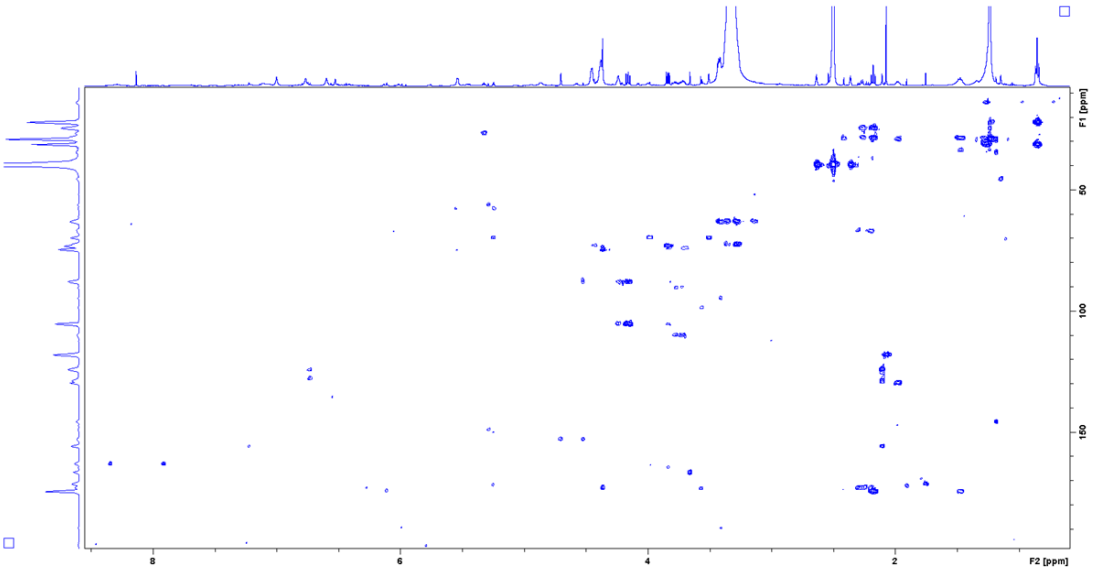


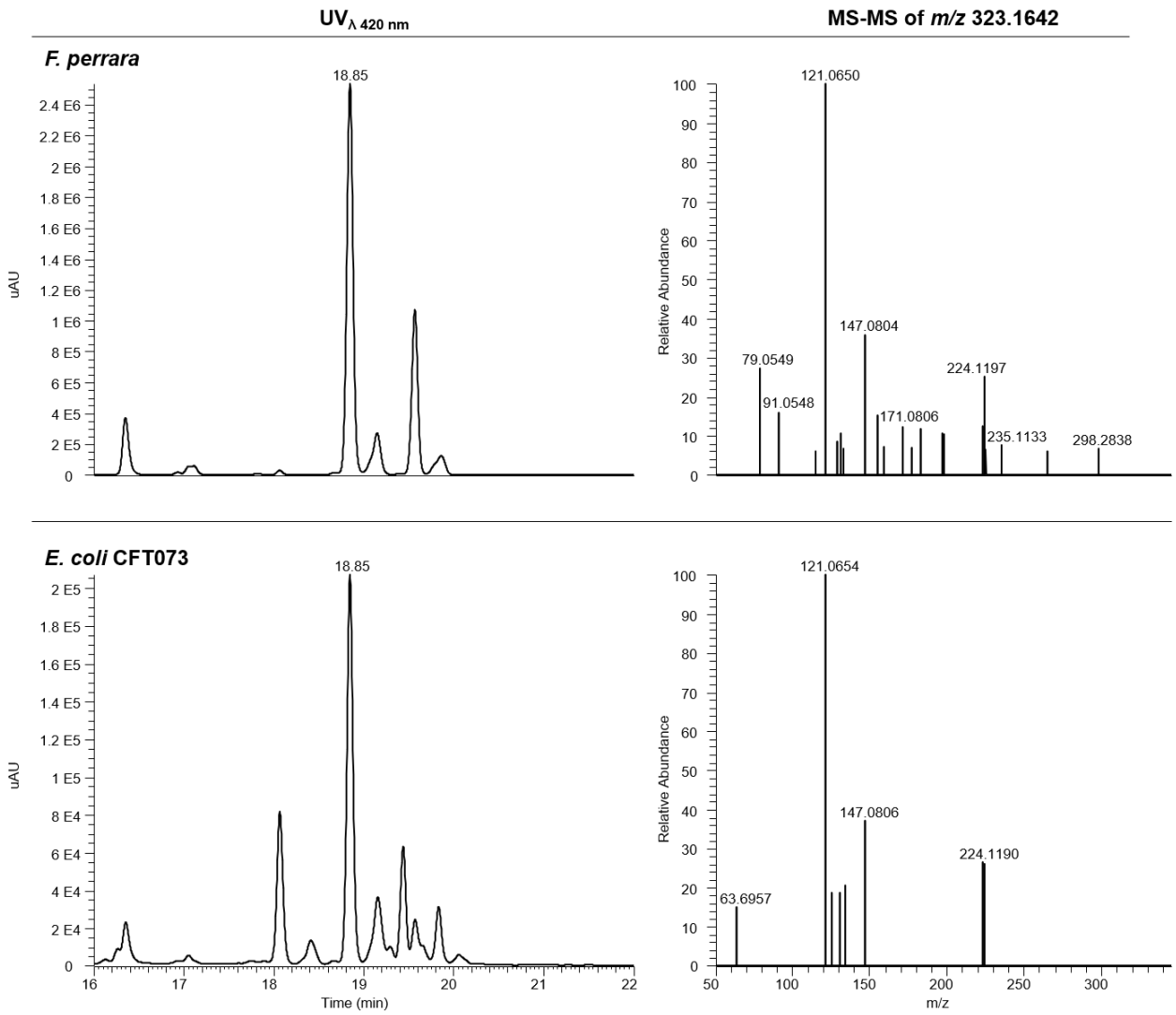
Supplementary Figure 8. COSY spectrum of enriched aryl polyene in DMSO–δ_6 at 298 K.



Supplementary Figure 9. COSY spectrum of enriched aryl polyene in DMSO–δ6 at 298 K. Enhanced view of the aromatic region.



Supplementary Figure 10. HMBC spectrum of enriched aryl polyene in DMSO–δ6 at 298 K.



Supplementary Figure 11. HPLC-HRESIMS analysis of *F. perrara* and *E. coli* CFT073. At 18.85 min both extracts show strong absorbance at λ= 420 nm. The MS-MS fragmentation pattern of the ion *m/z* 323.1642 at that retention time shows a similar pattern in extracts from both strains.

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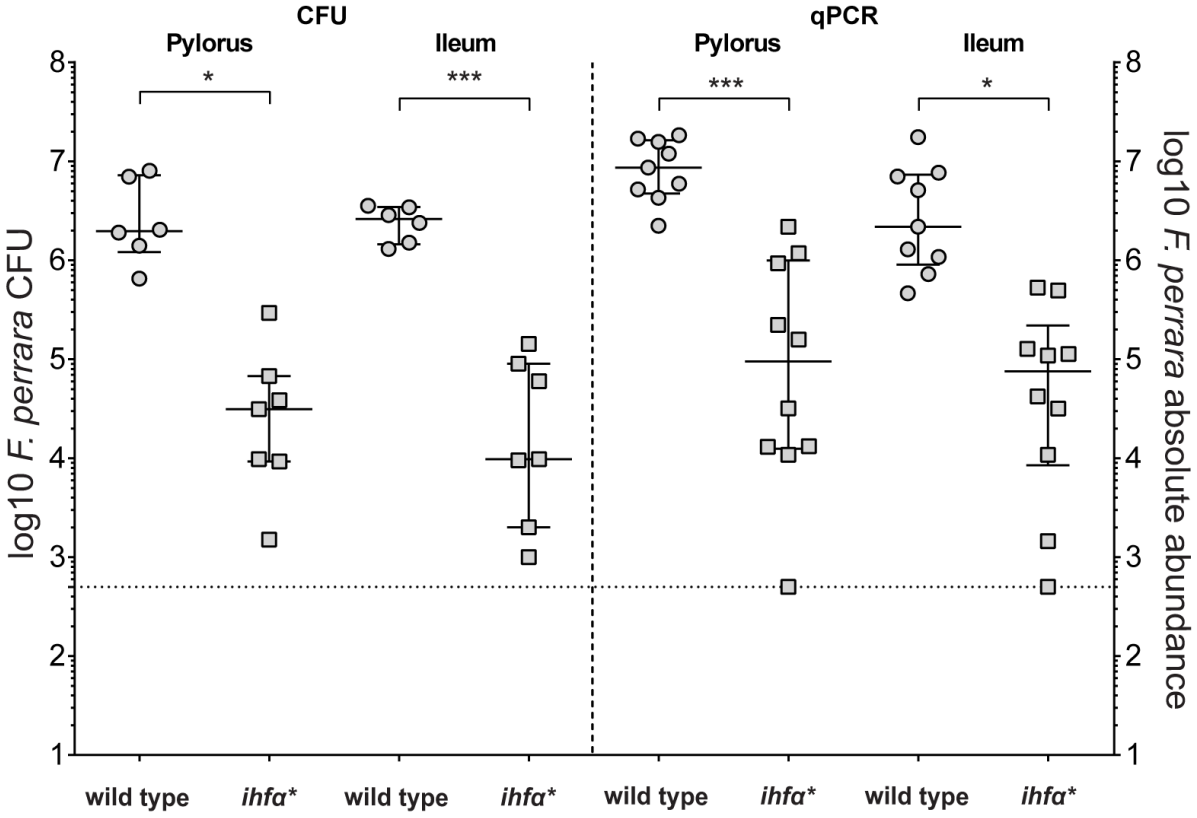

849

850 **Supplementary Figure 12. Quantification of *F. perrara* wt and *ihfA** in pylorus and ileum.**

851 Mono-colonization of bees with *F. perrara* wt and *ihfA**. Colonization levels are measured by

852 colony forming units (CFU) and quantitative PCR (qPCR) 10 days post colonization in the

853 specified gut regions: pylorus and ileum. Wilcoxon rank-sum test was used to assess the

854 statistical significance of differences. n=6.

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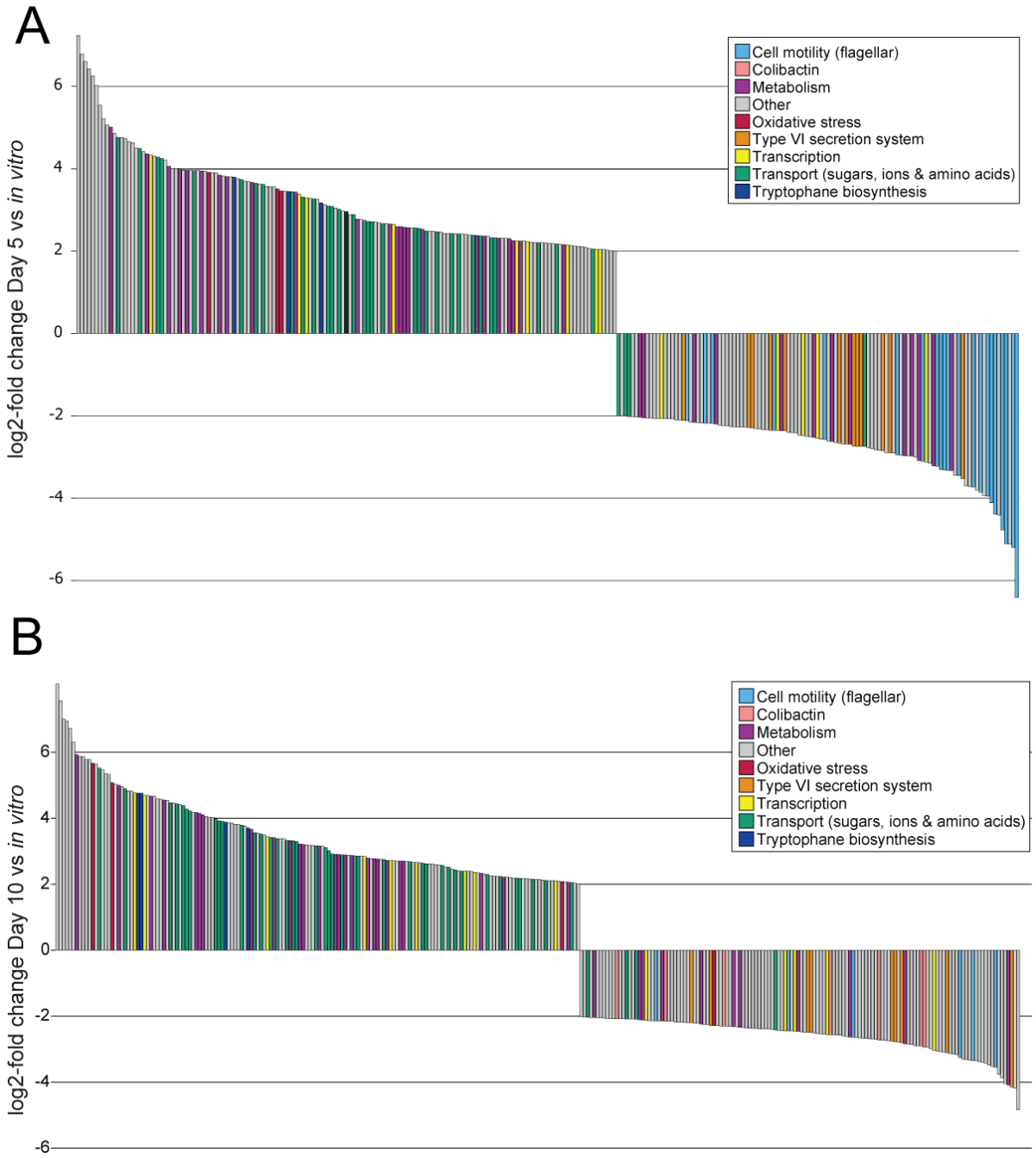

Supplementary Figure 13. RNAseq comparison of *F. perrara* during *in vivo* colonization compared to growth *in vitro*. Significantly differentially expressed genes with a |log2-fold change| >|2| of *F. perrara* PEB0191 wt 5(**A**) and 10 (**B**) days post colonization in comparison to growth *in vitro*. Genes are colored according to their category. The experiment was performed in quadruplicates, all genes displayed were significantly differentially expressed with $p<0.05$ and a FDR of <0.05 (Exact test).

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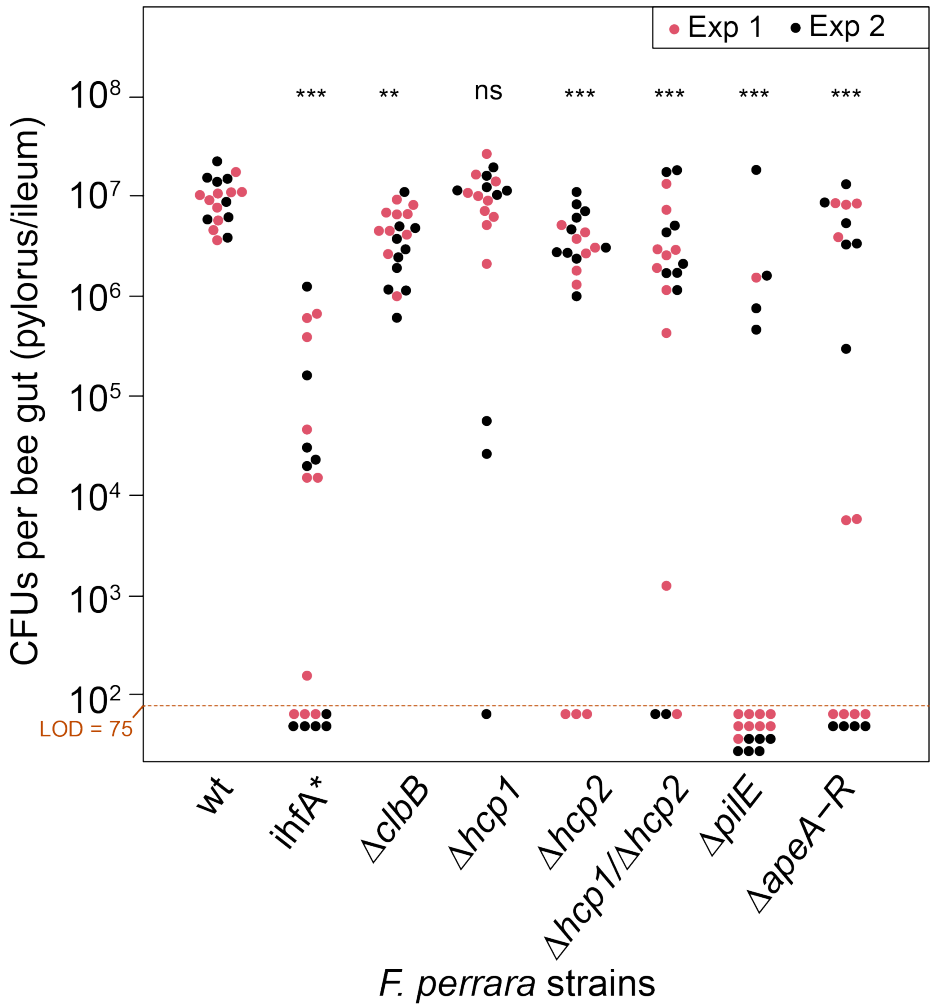

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869 **Supplementary Figure 14**. Same graph as shown in Figure 7 but datapoints are labelled by

870 experiment.

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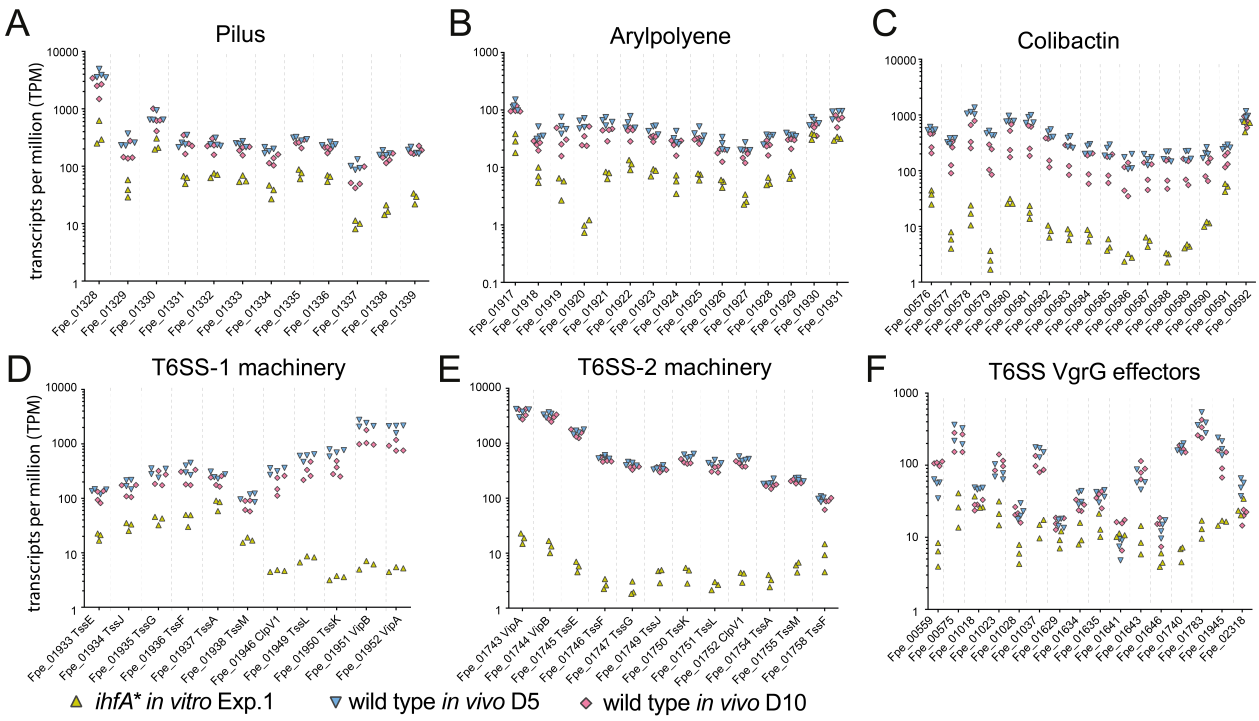
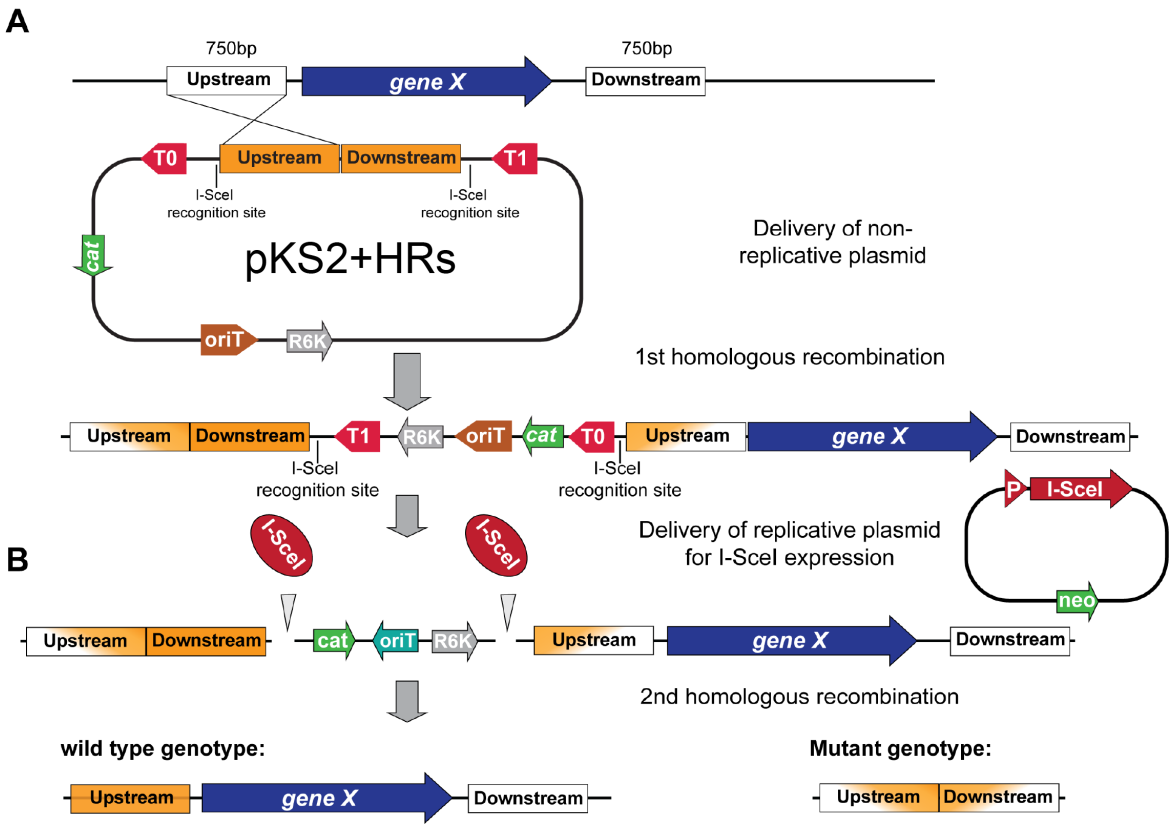


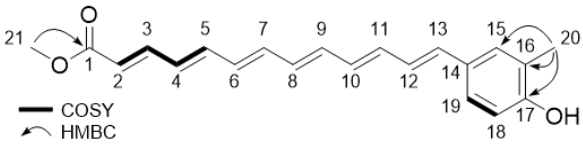
Supplementary Figure 15. **Gene expression of Ihf-regulated genes of *F. perrara* five days post-inoculation of gnotobiotic honey bees**. Same as Figure 5, but only time point Day 5 and Day 10 are depicted in comparison to *F. perrara ihfA* in vitro*.



Supplementary Figure 16. **Scheme of the gene deletion strategy based on a two-step homologous recombination procedure. A**, A non-replicative plasmid pKS2 integrates via homologous recombination of one of the two cloned 'homology regions' (HRs) upstream or downstream of the gene that is targeted for deletion. **B**, In a second step, a replicative plasmid harboring the restriction enzyme I-SceI is transformed. I-SceI targets corresponding recognition sites located on pKS2 resulting in the selection of either revertant (wt genotypes) or mutant genotypes that underwent a second homologous recombination event in the region targeted for deletion. PCR screening and replica plating on different selective allows to identify to correct clones.

Supplementary Tables

Supplementary Table 1. Chemical shifts of enriched aryl polyene in DMSO–δ6 at 298 K. Key COSY and HMBC correlation of the enriched polyene.



No	δ_C [ppm]	δ_H[ppm], mult.	coupling constant	integral
1	166.7			
2	119.3	5.97, d	*J*=15.1 Hz	0.5
3	144.6	7.33, dd	*J*=11.6 Hz *J*=15.1 Hz	1.2
4	129.5	6.47, dd overlap	*J*=11.6 Hz *J*=14.4 Hz	1.0
5	141.5	6.82, dd overlap	*J*= 11.4 Hz *J*= 14.5 Hz	0.9
6				
7				
8				
9				
10				
11				
12				
13				
14				
15	128.7	7.22, d	*J*=1.7 Hz	1.0
16	124.4			
17	155.9			
18	114.6	6.73, d	*J*=8.3 Hz	1.2
19	125.2	7.13, dd	*J*=1.9 Hz *J*=8.3 Hz	1.1
20	15.6	2.11, s		2.8
21	50.9	3.66, s overlap		3.5

Supplementary Table 2. Strains used in this study.

Species	Strain	Genotype*	Mutated gene	Reference	Parent strain
F. perrara	PEB0191/ ESL0157	wild type (wt)	None	(31)	Primary isolate
F. perrara	ESL0158	P83L (CCA→CTA) in *ihfA*	Fpe_00769	This study	PEB0191
F. perrara	ESL0910	P82L (CCA→CTA) in *ihfB;* Δ*apeO*	Fpe_00769; Fpe_01928	This study	PEB0191
F. perrara	ESL0922	L38S (TTA→TCA) in *ihfA;* Δ*pilE**	Fpe_00769; Fpe_00791*; Fpe_01328	This study	PEB0191
F. perrara	ESL0654	Δ*hcp1**	Fpe_01742	This study	PEB0191
F. perrara	ESL0655	Δ*hcp2*	Fpe_01947	This study	PEB0191
F. perrara	ESL0656	Δ*hcp1*, Δ*hcp2*	Fpe_01742, Fpe_01947	This study	PEB0191
F. perrara	ESL0888	Δ*clbB**	Fpe_ 00576	This study	PEB0191
F. perrara	ESL0921	Δ*pilE**	Fpe_01328	This study	PEB0191
F. perrara	ESL0957	Δ*apeA-R**	Fpe_01915-Fpe_01932	This study	PEB0191

*re-sequencing of the genomes revealed that some of the strains (indicated with an asterisk) carried an additional point mutation. However, these mutations were located in random genes and included synonymous as well as non-synonymous base substitutions unlikely to have an effect on the observed phenotypes. The genomic analysis of the re-sequenced strains can be found on the Switchdrive: https://drive.switch.ch/index.php/s/kCNTp4g7n60ffMi.

904 **Supplementary Table 3.** Primers used for qPCR quantification

Primer name	Target	Sequence 5' to 3'	Tm (°C)[a]	Amplicon size (bp)	# Gene loci per genome	Standard curve			
						Primer Efficiency, R^2	Slope, Intercept	LOD Cq[b] (#copies)	Reference
prPEN0013	Actin AB023025 *A. mellifera*	TGCCAACACTGTCCTTTCTG	58.4	156	-	1.896 = 89.6 %, 1.0	-3.6, 37.57	33.7 (10)	(78)
prPEN0014		AGAATTGACCCACCAATCCA	56.4						
prLK-Frisch-042-F	*F. perrara* 16S rRNA gene	GGAAGTTATGTGTGGGATAAGC	60.1	185	4	1.946 = 94.6 %, 0.995	-3.45, 38.04	31.7 (100)	(18)
prLK-Frisch-043-R		CTATTCTCAGGTTGAGCCCG	60.5						

905 a - Melting temperatures were calculated with the online tool described in (Kibbe 2007)

906 b - LOD refers to the limit of detection of primer sets, expressed here as the lowest number of

907 plasmid copies reliably detected by qPCR when standard curves were performed

908

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