

1 **Characterization of *Paenibacillus larvae*-directed**
2 **bacteriophage HB10c2 and evaluation of its application in**
3 **honey bee larvae suffering from American Foulbrood**

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21 **Keywords**

22 *Paenibacillus larvae*, American Foulbrood, phage therapy, *Apis mellifera*, bee disease,
23 Siphoviridae

24

25 **ABSTRACT** *Paenibacillus larvae* is the causative agent of American Foulbrood (AFB), the
26 most serious bacterial disease of honey bee brood. We isolated and characterized *P. larvae*-
27 directed bacteriophages and developed criteria for a safe phage therapy. Whole genome
28 analysis of a highly lytic virus of the family of Siphoviridae (HB10c2) provided a detailed
29 safety profile and uncovered its lysogenic nature and a putative beta-lactamase-like protein.
30 To rate the antagonistic activity against the targeted pathogens and to specify potentially
31 harmful effects on the bee population and the environment, the *P. larvae* genotypes ERIC I-
32 IV, representatives of the bee gut microbiota and a broad panel of *Bacillales* were analyzed
33 for phage HB10c2 induced lysis. Breeding assays with infected bee larvae revealed that the
34 observed *in vitro* phage activity was not predictive for the real-life scenario and the
35 therapeutic efficacy. Based on the disclosed *P. larvae*-bacteriophage co-evolution, we
36 discuss the future prospects of AFB phage therapy.

37

38 **Introduction**

39 The widespread collapse of honeybee (*Apis mellifera*) colonies has led to serious concerns
40 worldwide. Although the exact causes are not fully understood, infectious agents obviously
41 pose a significant threat to bee health and may contribute to the decline of this major
42 pollinator of crops, fruit and wild plants. Despite increased bee mortalities, adequate or
43 authorized medical products to treat bacterial and viral bee infections are missing. This also
44 holds true for the American Foulbrood (AFB), which is the most contagious and destructive
45 bacterial infection affecting honey bees. The aetiological agent is *Paenibacillus larvae*, a
46 Gram-positive, rod-shaped, spore-forming bacterium. The extremely resilient and long-lived
47 endospores are the infectious form and only bee larvae at an age of less than 30 h are
48 susceptible (1). Oral uptake of about ten spores is sufficient to initiate a fatal infection in bee
49 larvae. After germination *P. larvae* proliferates in the midgut, breaches the epithelium and
50 invades the haemocoel of bee larvae. This invasion coincides with the death of infected
51 larvae, which turn into a brown glue-like liquid that contains vast amounts of *P. larvae*

52 spores. Killed larvae are detected by nurse bees, which clean the cells and prepare them for
53 new brood. During this cleaning they incorporate spores of *P. larvae* and feed those to
54 healthy larvae that subsequently become infected (2).

55 Different methods for treating AFB infected colonies are known, including burning down the
56 colonies, artificial swarming and decontaminating of the hives with NaOH (2). In some
57 countries the antibiotics oxytetracycline and tylosin are applied prophylactically or to treat
58 symptoms (3). However, all known strategies exhibit severe disadvantages for beekeepers,
59 bee colonies and the environment. The registered use of antibiotics e.g. has been withdrawn
60 in many countries since antibiotic residues appear within the honey. The European
61 Community legislation (EC Regulation 2377/90) limits the presence of antibiotics in honey
62 excluding its use for AFB therapy. Moreover, the accumulation of antibiotic resistances in the
63 gut microbiota of bees has been detected. As proposed recently, these resistances might
64 additionally lead to bee colony collapses (4).

65 Taken together, it is obvious that new strategies for the control of AFB are urgently needed
66 but difficult to develop. The introduction of new antibiotics in the multidrug-resistance era
67 may only reproduce known drawbacks. The application of antagonistic bacteria combating
68 *P. larvae* seems not to be in sight. Also, essential oils have not proven to be effective enough
69 in practical use (2). In addition, they may have disadvantageous side effects on bees or
70 honey. Since bacteriophages, viruses that infect and lyse bacteria, have already proven
71 great efficacy in controlling bacterial infections in humans and animals, phage therapy seems
72 to be a compelling alternative for the following reasons (5, 6). First, phages as bactericidal
73 agents can easily be discovered and have been employed for treatment of human infections
74 since the early 1900s. Second, phages are auto dosing at the site of infection and show a
75 negligible inherent toxicity (7). Third, due to their host specificity, phages usually exhibit
76 minimal disruption of the normal flora. Fourth, as phages eliminate bacteria by mechanisms
77 that differ from those of antibiotics, cross-resistances are not observed (8). Fifth, since
78 phages are isolated from the environment they can be regarded as natural products with only

79 an irrelevant environmental impact compared to antibiotic drugs causing sustainable
80 resistance loads (7).

81 Nonetheless, the safety profile of therapeutic phages needs to be addressed. It is important
82 to ensure that the selected phages do not exhibit generalized transduction, nor possess gene
83 sequences with significant homology to antibiotic resistance genes and genes for other
84 bacterial virulence factors (9, 10). Moreover, it is critical that the therapeutic phages lyse
85 relevant strains of the targeted bacteria and relieve bacteria of the bee microbiome and the
86 habitat of the hives.

87 In this study, we isolated several *P. larvae*-specific bacteriophages and rigorously
88 characterized the novel phage HB10c2 with respect to morphology, genomic potential and
89 host range. Moreover, we analyzed the potential of HB10c2 as a therapeutic agent in co-
90 cultivation assays with *P. larvae*, and in *in vitro* breeding assays in which infected bee larvae
91 were treated with this bacteriophage. Finally we discuss prerequisites for a successful and
92 safe phage therapy of AFB in honey bees.

93

94 **Methods**

95

96 **Bacterial strains and cultivation conditions.** All bacterial strains used in this study are
97 listed in Table 1. Growth media and cultivation conditions were as described elsewhere
98 (www.dsmz.de). *P. larvae* strains were cultivated on Columbia sheep blood agar (BD) at
99 37 °C with 5% CO₂. For liquid culture *P. larvae* strains were grown in Brain-Heart-Infusion
100 medium (37 g BHI (Roth), 3 g yeast extract (BD), 1 L H₂O) at 37 °C and 200 rpm. Field
101 isolates of *P. larvae* ERIC I and II were derived from honey samples by the LAVES Institute
102 for Bee Research, Celle, Germany. Plaque assays were performed using the Small Drop
103 Plaque Assay System (33).

104

105 **Isolation of phage HB10c2.** Bacteriophage HB10c2 was isolated from the glue-like liquid of
106 a bee hive with clinical symptoms caused by *P. larvae* ERIC I. The combs of the hives were
107 stored at the LAVES Institute for Bee Research in Celle, Germany at 4 °C. The glue-like
108 liquid was taken out of the comb, transferred into 50 mL H₂O and dissolved for 48 h at 4 °C.
109 To kill contaminants 5% (v/v) CHCl₃ (VWR) was added followed by centrifugation at
110 4,000 x g for 15 min. The supernatant was mixed with the same volume 2 x BHI medium and
111 inoculated with *P. larvae* DSM 16116 representing ERIC II. The culture was grown for 3 d at
112 37 °C and 200 rpm. After incubation with 5% (v/v) CHCl₃ bacteria were sedimented by
113 centrifugation at 4,000 x g for 15 min. Phages were purified from the supernatant by
114 ultracentrifugation at 100,000 x g for 1 h at 4 °C. Bacteriophages were dissolved in 500 µL
115 SM-buffer (100 mM NaCl (Roth), 8 mM MgSO₄ x 7 H₂O (Roth), 50 mM Tris (pH 7,5) (Roth),
116 0.002% (w/v) Gelatin (Roth)).

117

118 **Enrichment of phages.** *P. larvae* was grown on Columbia Blood Agar (Oxoid) at 37 °C and
119 5% CO₂ for 16 h. Bacteria were suspended in sterile H₂O and centrifuged for 5 min at
120 5,000 x g. Sedimented bacteria were resuspended in double distilled H₂O to an OD₆₀₀ of 15.
121 For enrichment of bacteriophages 1 volume of the OD₆₀₀-suspension was added to
122 0.5 volumes phage lysate and transferred into 1 volume 2 x BHI. After adsorption for 15 min
123 at room temperature the complete sample was added to 400 volumes of BHI and incubated
124 over night at 37 °C and 200 rpm. To lyse the bacteria 15 volumes of CHCl₃ were added. The
125 mixture was inverted and centrifuged at 3,000 x g for 5 min. Supernatant was transferred into
126 sterile tubes with a drop of CHCl₃ to inhibit bacterial growth. The derived lysate was used for
127 further experiments.

128

129 **Determination of viral burst size.** A volume of 400 mL BHI was inoculated with 5 x 10⁸ cells
130 of *P. larvae* (DSM 25430) and 1.25 x 10⁷ PFU of phage HB10c2. The culture was grown at
131 37 °C and agitated at 200 rpm. A culture without phage HB10c2 was taken as control culture.

132 After incubation aliquots of each culture were taken and the number of bacterial cells was
133 determined. For phage isolation the cultures were mixed with 5% (v/v) CHCl₃ and shaken for
134 15 min at 37 °C. The aqueous phase was centrifuged at 12,000 x *g* for 15 min and the
135 supernatant was used for enumeration of bacteriophages in titration assays by using the
136 Small Drop Plaque Assay System (33). The specific burst size of bacteriophage HB10c2 was
137 calculated using the following equation:

$$138 \quad B_{\text{HB10c2}} = \lg(\text{pfu}_0/\text{pfu})/\lg(G_{P. \textit{larvae}}),$$

139 where the burst size (*B*) was calculated by the PFU at time zero (*pfu*₀) and the PFU after the
140 incubation time (*pfu*) with regard to the number of host generations (*G*).

141

142 **Electron microscopy analysis.** Phage HB10c2 was enriched in a total volume of 400 mL
143 BHI. Bacterial DNA was hydrolyzed by incubation with 10 U DNase I (Macherey-Nagel) and
144 40 µg RNase A (Sigma) for 30 min at room temperature. After adding NaCl (Roth) to a final
145 concentration of 0.5 M and incubation on ice for 15 min, the phages were separated from
146 bacterial debris by centrifugation at 8,000 x *g* for 20 min at 4 °C. Phages were enriched by
147 PEG 3,000 (Roth) purification (34). The enriched phages were concentrated by CsCl
148 equilibrium centrifugation at 100,000 x *g* for 12 h at 4 °C after adding 10 g CsCl (Roth) to the
149 PEG purified phages. Phages were absorbed onto carbon film, rinsed with TE-buffer (10 mM
150 Tris-HCl, pH 8.0 (Roth), 2 mM EDTA (Sigma)), followed by rinsing in double distilled H₂O,
151 and negatively stained with 2% (w/v) aqueous uranyl acetate (Merck). Carbon films were
152 then collected from the uranyl acetate drops with copper grids (300 mesh) and blotted dry
153 with filter paper. After air-drying samples were examined in a Zeiss TEM 910 at an
154 acceleration voltage of 80 kV. Images were recorded digitally with a Slow-Scan CCD-
155 Camera (ProScan, 1024 x 1024) with ITEM-Software (Olympus Soft Imaging Solutions).
156 Contrast and brightness of images were adjusted with Adobe Photoshop.

157

158 **Phage sequencing.** Phages derived from CsCl equilibrium centrifugation were used for
159 isolation of DNA via phenol/chloroform extraction followed by chloroform extraction. DNA
160 was precipitated with ethanol by adding 300 μ L 3 M NaAc (Roth). SMRTbell™ template
161 libraries were prepared according to instructions from Pacific Biosciences, Menlo Park, CA,
162 USA, following the Procedure and Checklist for “Low-Input 10 kb Template Preparation and
163 Sequencing” using C2-C2 Chemistry as described previously (35).

164

165 **Genome analyses.** Phage genome assemblies were performed using the
166 “RS_HGAP_Assembly.3” protocol included in SMRTPortal version 2.2.0. applying standard
167 parameter. For all phages one final contig could be obtained, which was linearized due to
168 recognition of distinct start and end points in the phage assemblies. A quality check of the
169 final phage genomes regarding overall coverage as well as SNPs was performed using
170 SMRT View and IGV (36). All phage genomes were annotated using PROKKA 1.8 (PMID
171 24642063) with subsequent manual curation in Artemis (16).

172

173 **Phage host range determination.** Bacterial susceptibility to phage HB10c2 was assayed by
174 the small Drop Plaque Assay System (see above) and optical density measurements in liquid
175 culture. In brief, pre-cultures of *P. larvae* were grown over night in BHI medium. For host
176 range determination 2.4×10^7 bacterial cells in 50 mL BHI were inoculated with 4×10^9 PFU
177 of phage HB10c2. Infected cultures were incubated at 37 °C and agitated at 200 rpm for
178 24 h. Optical density at 600 nm (OD_{600}) was determined every hour. Growth curves were
179 depicted by non-linear regression using the following model (37):

$$180 \quad f(\text{time}) = (N_0 * K * e^{r \cdot \text{time}}) / (K + N_0 * e^{r \cdot \text{time} - 1}),$$

181 where N_0 is the OD_{600} at time (t) zero, K is the maximum OD_{600} (after 24 h) and r is the
182 expansion rate.

183 Field isolates of *P. larvae* ERIC I and ERIC II were grown on Columbia Sheep Blood Agar
184 (CSA) and phage susceptibility was tested in plaque assays. The species identity and the
185 ERIC-genotypes of all field isolates were determined by 16S rRNA-PCR and rep-PCR as
186 described elsewhere (23).

187 The gut microbiota of honey bee larvae and adult bees were grown in their respective
188 standard culture media as described at www.dsmz.de. After resuspending in H₂O,
189 1 x 10⁶ bacterial cells were used for inoculation of 4 mL BHI top agar in plaque assays.
190 Plaque formation by phage HB10c2 was monitored for two days.

191

192 **Phage therapy of *A. mellifera*.** The *in vivo* therapeutic effect of phage HB10c2 on *P. larvae*
193 ERIC I and ERIC II infected bee larvae was analyzed in breeding assays as described
194 previously (38, 39). Larvae at the age of less than 24 h were transferred on 20 µL Diet A
195 (50% royal jelly, 50% aqueous solution containing 2% yeast extract, 12% glucose, 12%
196 fructose) (d1). At day 3 (d3) the larvae were fed 20 µL Diet B (50% royal jelly, 50% aqueous
197 solution containing 3% yeast extract, 15% glucose, 15% fructose). Increasing amounts
198 (30 µL, 40 µL, 50 µL) of Diet C (50% royal jelly, 50% aqueous solution containing 4% yeast
199 extract, 18% glucose, 18% fructose) were fed from day 4 (d4) to day 6 (d6), respectively.
200 Three replicates of 48 larvae from different, non related queens were used for infection. Each
201 larvae was infected with 20 µl of diet A containing *P. larvae* spores (500 cfu/larvae
202 *P. larvae* ERIC I DSM 7030 or *P. larvae* ERIC II DSM 25430). To confirm *P. larvae* as
203 causative agent of the observed bee mortality, dead bee larvae were homogenized and
204 plated on blood agar. The identity of *P. larvae* colonies was confirmed by 16S rRNA PCR
205 (22). To ensure that phage HB10c2 has no effect on the mortality of developing *A. mellifera*,
206 50,000 PFU of phage HB10c2 were fed with Diet A. For phage therapy diet A containing
207 *P. larvae* spores (500 cfu/larvae) and bacteriophages (50,000 PFU/larvae) were fed.
208 Numbers of killed insects were used for statistical analysis. Significance of changes in the
209 mortality rates was calculated by unpaired Student's t-test.

210

211 **Genome accession number.** The genome sequence of bacteriophage HB10c2 was
212 deposited in Genbank under accession number KP202972.

213

214 **Results**

215 **Phage isolation, propagation and host cell lysis.** *P. larvae*-specific phages were isolated
216 from infected bee hives by plaque formation on lawns of *P. larvae* DSM 16116 (genotype
217 ERIC II). Phage HB10c2, the most active isolate with regard to plaque size and clarity,
218 originated from the glue-like larval remains of a bee hive with clinical symptoms of AFB
219 caused by *P. larvae* genotype ERIC I. Lytic activity of phage HB10c2 was further
220 characterized after enrichment and sub cultivation of phage lysates by titration assays (Fig.
221 1A). The calculated burst size of bacteriophage HB10c2 (B_{HB}) after infection of *P. larvae*
222 ERIC II (DSM 25430) was 8.34. According to previous studies (11, 12) the particle-to-plaque
223 forming unit ratio is about circa 15 bacteriophages per plaque forming unit (PFU), which
224 suggests the release of approximately 125 phage particles per infected host cell. In addition
225 to plaque assays and burst size calculation, the influence of phage HB10c2 on the growth of
226 the four *P. larvae* genotypes ERIC I-IV was determined in liquid culture medium, respectively
227 (Fig. 1B). The growth of *P. larvae* ERIC I and IV in co-culture with HB10c2 was completely
228 inhibited, whereas ERIC II and III exhibited a 50% reduction in cell density (OD_{600}) compared
229 to the control without bacteriophages.

230

231 **Electron microscopy reveals B2 morphotype of phage HB10c2.** For a first classification
232 of the *P. larvae*-specific phage HB10c2 transmission electron microscopy of negatively-
233 stained phages was applied. After phage purification by CsCl equilibrium centrifugation of *P.*
234 *larvae*-lysates, the morphology of the virions could be determined. Figure 2 shows
235 representative intact phages with a capsid (length: 98.54 nm \pm 0.54 nm, diameter: 46.79 nm

236 ± 0.56 nm), a siphon (length: 165.34 nm ± 19.09 nm, diameter: 10.32 nm ± 0.21 nm), and a
237 base plate (diameter: 22.77 nm ± 0.62 nm). According to these morphological properties,
238 especially the length-to-width ratio, bacteriophage HB10c2 exhibited the B2 morphotype of
239 the *Siphoviridae* family (13, 14).

240

241 **Whole genome analysis of *P. larvae* phage HB10c2.** For further characterization with
242 respect to gene content and genomic organization, the complete genome of phage HB10c2
243 was sequenced using the *PacBio* RS II system. The analysis revealed that the genome
244 contains linear, double-stranded DNA with a length of $35,644$ bp and a G+C content of
245 41.8% . We performed an automatic annotation using PROKA (15) for the positioning of
246 coding sequences (CDS) on three different start codons (ATG, GTG and TTG) and
247 identification of putative Shine Dalgarno sequences upstream of the coding regions.
248 Accordingly, we determined 56 CDS for phage HB10c2 (52 ATG, 2 GTG and 2 TTG)
249 resulting in a coding percentage of 91.3% . Automatic annotation was manually refined using
250 ARTEMIS (16) and BLASTP. Hereby, the genome could be divided into different functional
251 clusters with genes e.g. for structural proteins, replication, host lysis or lysogeny (Fig. 3).
252 Notably, we identified a gene encoding for a putative beta-lactamase-like protein in phage
253 HB10c2 which demonstrates the importance of genome sequencing for the risk assessment
254 of putative therapeutic phages.

255 At the right end of the phage chromosome we identified a cluster of genes similar to
256 *Paenibacillus* phage phiIBB_PI23 (GenBank Acc. No. KP296792) that partly show similarities
257 to genes involved in replication. Amongst them were genes for a helicase (HB_00044), an
258 NTP-PPase (HB_00047) and a RuvC-like protein (HB_00050). Apart from these genes,
259 HB_00046 revealed similarities to a Cytosine-C5 specific DNA methylase which might help to
260 protect the phage DNA against degradation by the restriction system of the bacterial host.
261 Aside from these genes, we could not identify genes for a DNA polymerase, a primase or a

262 single-stranded binding protein that are essential for replication and may therefore be
263 provided by the phage host.

264 As in most phage genomes, the genes for the small and large terminase (HB_00002) subunit
265 of phage HB10c2 could be clearly identified upstream the cluster for structural genes. In
266 HB10c2, this cluster was identified at the left end of the chromosome. BLASTP analysis
267 revealed the conserved domains Terminase_4 (pfam05119) and COG4626, respectively.
268 Both proteins are identical to those from *Paenibacillus* phage phiIBB_PI23. This cluster for
269 DNA packaging is followed by the gene cluster for structural proteins. All these genes also
270 show high similarities to genes from *Paenibacillus* phage phiIBB_PI23 or even are identical.
271 Amongst others this cluster contains genes for a portal protein, a Clp-protease like protein
272 (HB_00004), a tail length tape-measure protein (HB_00014) and several structural proteins
273 for the phage tail and head (HB_00014 - HB_00017).

274 Analysis with TMHMM Server v. 2.0 (17) revealed that the deduced amino acid sequences of
275 HB_00020 and HB_00022 contain one and two transmembrane regions, respectively.
276 Therefore HB_00022 could be assigned as a class II holin (18). Between the two putative
277 holin genes we identified a gene that is identical to a gene for an N-acetylmuramoyl-L-
278 alanine amidase from *Paenibacillus larvae* ERIC I strain DSM 25719.

279 Downstream the lysis cluster of phage HB10c2 we determined some genes located on the
280 other strand that show no similarities to *P. larvae* phage phiIBB_PI23, but to genes of
281 *P. larvae* ERIC I strain DSM 25719 (GenBank Acc. No. PRJNA42203) with unknown
282 functions. Downstream those genes we identified CDS for an integrase (HB_00029), an
283 excisionase-like protein (HB_000333), anti-repressor proteins (HB_00032 and HB_00037)
284 and some genes that might play a role in the further regulation of the lysogenic state
285 (HB_00030, HB_00031 and HB_00034). Taken together the results of the genome
286 sequencing suggest the potential of phage-mediated horizontal gene transfer.

287

288 **Lytic activity of phage HB10c2 against different *P. larvae* genotypes and field isolates,**
289 ***Bacillales* and gut microbiota of *A. mellifera*.** To analyze the host range of phage HB10c2
290 and to exclude harmful effects on the bee gut microbiota or the environment, a broad panel
291 of bacteria was tested for their susceptibility to phage HB10c2 induced lysis by plaque
292 assays. In addition to the four different known genotypes of *P. larvae* (ERIC I: DSM 7030,
293 ERIC II: DSM 25430, ERIC III: LMG 16252, ERIC IV: LMG 16247) 20 ERIC I and II field
294 isolates were tested. As shown in Table 1 all 40 tested isolates were sensitive to phage
295 HB10c2 induced lysis.

296 In order to explore health implications for honey bees and possible ecological effects after an
297 assumptive therapy with phage HB10c2, representative culturable members of the larval and
298 adult gut microbiota of *A. mellifera* (19) and different *Bacillales* were analyzed in plaque
299 assays (Tab. 1). In general, bacteriolytic activity of phage HB10c2 was not observed for
300 Gram-negative bacteria. All species which allowed plaque formation belonged to the
301 *Bacillales* and most of them are part of the genus *Paenibacillus*. Interestingly, nine
302 environmental *Bacillales* (*B. brevis*, *P. phyllosphaerae*, *P. soli*, *P. humicus*, *P. cookii*, *P. filicis*,
303 *P. lactis*, *P. alvei*, *P. castaneae*) were susceptible to phage HB10c2 mediated lysis. This
304 reveals, that HB10c2 is a phage with a rather broad host range.

305

306 **Treatment of *P. larvae* infected bee larvae with phage HB10c2.** Since phage HB10c2
307 exhibited a strong lytic effect on *P. larvae* on agar plates and in liquid medium, we analyzed
308 the *in vivo* therapeutic effect utilizing *P. larvae* ERIC I and II infected larvae of *A. mellifera*
309 respectively (Fig. 4). After feeding with a larval diet containing spores of *P. larvae* and phage
310 HB10c2, the mortality of the developing bee larvae was analyzed for 14 days on a daily
311 basis. In control experiments, bee larvae were only fed with larval diet or with larval diet
312 containing phage HB10c2.

313 Larvae fed with the larval diet alone exhibited a mortality rate of 25.00% \pm 3.61%. The
314 addition of phage HB10c2 to uninfected larvae led to a mortality rate of 31.25% \pm 8.33%.

315 Since the mortality of the two control groups is not significantly different ($p = 0.2992$), we
316 concluded that phage HB10c2 has no harmful impact on the survival of bee larvae. In
317 contrast, larvae infected with either *P. larvae* ERIC I or II revealed a mortality rate of 100%
318 after 14 d. This significant difference to the respective control groups ($p = 3.56 \times 10^{-6}$)
319 demonstrates the devastating effect of this pathogen. Larvae, which were infected with
320 *P. larvae* ERIC I or ERIC II and treated with phage HB10c2 showed a mortality rate of
321 $99.31\% \pm 1.20\%$ and $98.61\% \pm 2.41\%$, respectively. Both values are not significantly different
322 from the mortality rate of infected bee larvae without phage therapy ($p = 0.4226$ and
323 $p = 0.6770$). Thus, we conclude that the application of phage HB10c2 did not exhibit
324 therapeutic effects on bee larvae suffering from AFB.

325

326 Discussion

327 The successful use of lytic phages in preventing and treating diverse bacterial infections in
328 humans and animals prompted us to investigate the therapeutic potential of a phage isolate,
329 which shows strong lytic activity against *P. larvae* ERIC I-IV, the causative agents of AFB. At
330 first sight, phage therapy appears as ideal alternative to antibiotics, since it represents an
331 auto dosing biological pest control (5, 7). Based on experience with therapeutic phages for
332 almost a century and due to the fact that phages are abundant in natural ecosystems, they
333 are regarded as inherently safe (5). However, rigorous research is required to determine the
334 efficacy of respective therapeutic phages and to confirm their anticipated safety (20). Critical
335 issues which have to be taken into consideration are the following. Phages can convert
336 nontoxic bacteria to toxic strains by lysogenic conversion (9, 21). The pathogenicity of e.g.
337 *Vibrio cholerae*, Shiga toxin-producing *Escherichia coli*, *Corynebacterium diphtheriae*, and
338 *Clostridium botulinum* depends on specific prophage-encoded toxins. Moreover,
339 *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Salmonella enterica* serovar
340 Typhimurium harbor a multitude of prophages and each phage-encoded virulence or fitness
341 factor makes an incremental contribution to the fitness of the lysogen. Other ways by which
342 temperate phages may affect bacterial fitness are as anchor points for genome

343 rearrangements, via gene disruption, by protection from lytic infection, by lysis of competing
344 strains through prophage induction, and via the introduction of new fitness factors (21). In the
345 treatment of AFB the amplification and application of broad-range phages may also have an
346 impact on the gut microbiota of adult honey bees, bee larvae and the entire microbial
347 ecosystem of the hive. Therefore, to avoid collateral damage by phage therapy, it is
348 important to explore the spectrum of bacteria, which are susceptible for the respective
349 therapeutic phage. Finally, for the therapeutic success and to avoid resistances it is also
350 important that the selected phage is sufficiently antagonistic against the bacteria being
351 targeted.

352 In the present study we thoroughly characterized a potential therapeutic phage and
353 developed criteria for a safe and efficient phage therapy of AFB. To avoid the potentially
354 hazardous introduction of new infectious agents into the ecosystem by phage therapy we
355 isolated phages from bee habitats, namely from the glue-like larval remains of a bee hive
356 with clinical symptoms of AFB. Plaque assays were used to select highly lytic isolates
357 against *P. larvae* genotypes ERIC I-IV. Phage HB10c2 was isolated from typical AFB glue-
358 like liquid of a honey comb and the natural bacterial host was identified as *P. larvae*
359 genotype ERIC I by 16S rRNA PCR (22) and by rep-PCR using ERIC primers (23). ERIC I
360 causes the most cases of AFB worldwide (23), whereas ERIC II seems to be restricted to
361 Europe. The former subspecies *P. larvae* subsp. *pulvificiens*, represented by ERIC III and
362 ERIC IV are only rarely isolated.

363 Purified HB10c2 phages were subjected to transmission electron microscopy. This
364 morphological characterization revealed a long, flexible siphon, a capsid and a base plate
365 and indicates that phage HB10c2 belongs to the order Caudovirales and the family
366 *Siphoviridae* with a B2 morphotype.

367 To obtain a deeper insight into the biology of phage HB10c2, to identify potential risks, and
368 as a basis for analyzing and comparing newly isolated phages that infect *P. larvae*, we
369 sequenced and annotated the complete genome of phage HB10c2. Prior to this study, 15
370 phages of *P. larvae* were described and phage HB10c2 exhibits structural similarities to them

371 (24). According to recent developments and further classification of phages at the genomic
372 level in the ICTV (International Committee on Taxonomy of Viruses) phage HB10c2 could be
373 grouped into the proposed genus "Divavirus".

374 Compared to complete *Paenibacillus larvae* genomes sequenced so far (44-45% GC content
375 (25)) the G+C content of the phage genome is lower and similar to those in other
376 *Paenibacillus* phage genomes (26, 27). Sequence analysis with tRNAscan-SE v.1.21 (28) did
377 not reveal any genes for tRNAs in the genome, which is also consistent with other analyzed
378 *Paenibacillus* phage genomes. We determined 56 CDS for phage HB10c2 resulting in a
379 coding percentage of 91.3%. Analyses of deduced amino acid sequences revealed that 37 of
380 56 gene products are similar or identical to gene products of *Paenibacillus* phage
381 philBB_P123 (27). Further similarities could be identified to a putative prophage in *P. larvae*
382 ERIC I strain DSM 25719. Whereas *P. larvae* phage philBB_PI23 harbors a gene for a
383 putative toxin, phage HB10c2 does not encode for a comparable gene. However,
384 importantly, we identified a gene encoding for a putative protein of the beta-lactamase
385 superfamily in phage HB10c2. This is a critical point since it should be avoided to transfer
386 antibiotic resistances horizontally by phage therapy.

387 BLASTP analysis revealed the conserved domains Terminase_4 (pfam05119) and
388 COG4626, which are responsible for packing DNA into phage capsids. Both proteins are
389 identical to those from *Paenibacillus* phage philBB_PI23 (27). Terminases are highly
390 conserved and phylogenetic analyses with other terminases with different known DNA
391 packaging strategies can give first hints on the mechanisms that are used for the packaging
392 (29). Recently, Merrill et al. 2014 performed a phylogenetic analysis of all terminases from all
393 known *P. larvae* phages. The phages that belong to the *Myoviridae* seem to use a headful
394 packaging system, whereas Siphovirus philBB_PI23 likely has 3' cohesive ends. As the
395 deduced amino acid of the terminase of HB10c2 is identical to that of *Paenibacillus* phage
396 philBB_PI23, we presume that phage HB10c2 also contains *cos* sites and might form

397 concatemers, which was confirmed by pulsed-field gel electrophoresis of genomic DNA of
398 HB10c2 (data not shown).

399 Most phages lyse their hosts in order to release the newly produced phage progeny into the
400 environment. For this, they often use two different kinds of proteins, so-called holins and
401 endolysins. The holins are membrane proteins that assemble in the cytoplasmic membrane
402 and form a pore. The endolysin is a cell wall-degrading enzyme that uses this pore to get into
403 the periplasmic space where it degrades the peptidoglycan, which leads to the burst of the
404 bacterial cell (30, 31). Downstream the cluster for structural genes, we identified the lysis
405 cluster consisting of three genes that encode two putative holins and an endolysin.
406 Comparison with other *Paenibacillus* phages showed that they indeed also harbor either a
407 gene for N-acetylmuramoyl-L-alanine amidase, e.g. phage Emery, or a gene for an endo-
408 beta-N-acetylglucosamidase, e.g. phage Davies (26). In particular, endolysins of phages with
409 Gram-positive hosts often act as endopeptidases or amidases and reveal a highly specific
410 activity (32). Therefore, endolysins from *Paenibacillus* phages could be a further option for
411 the treatment of bee hives.

412 Downstream these genes we determined a cluster of genes that might be the lysogeny
413 module that is responsible for the integration of the phage genome into the host genome and
414 its further regulation. Amongst others we identified genes for an integrase (HB_00029), an
415 excisionase-like protein (HB_00033), and anti-repressor proteins (HB_00032 and
416 HB_00037). Therefore, phage HB10c2 can clearly be determined as a temperate phage,
417 doubting the suitability for therapeutic use.

418 To prevent ecological damage by using HB10c2 as a therapeutic agent against AFB, the
419 bacteriophage was also tested for its bacteriolytic activity against environmental and bee-
420 related *Bacillales* and members of the larval and adult gut microbiome of honey bees (19).
421 Bacterial lysis was detected in nine species within the Gram-positive group of Bacillales,
422 mostly the genus *Paenibacillus*. The lytic activity of HB10c2 on other bacterial hosts in
423 addition to the pathogen *P. larvae* is a critical point. On the one hand this could lead to

424 dysbiosis in the gut of honey bees, the alteration of a healthy microbiota towards a
425 compositional and functional imbalance. On the other hand, the presence of phage HB10c2
426 in other than *P. larvae* species could be beneficial, since it may pre-arm the microbiota
427 against a pathogenic attack. Since the identified alternative bacterial hosts for phage HB10c2
428 in the gut microbiota are ubiquitous, they may be constantly replaced from the environment
429 by food and water intake of the bees. Experimental data to answer the question whether a
430 prophylactic treatment and the establishment of a therapeutic phage within a healthy
431 microbial gut community have detrimental or beneficial effects are not available. However, for
432 the development of a phage therapy of AFB this issue seems to be of great importance.

433 Our breeding assays with *P. larvae* infected bee larvae showed no significant effects of
434 phage therapy with phage HB10c2. This result confirms other analyses, which showed that
435 lytic phage activity in liquid or on solid media is not always predictive for the *in vivo*
436 therapeutic efficacy (33). The reasons for this discrepancy are still elusive but may be due to
437 differences in the accessibility of bacterial host cells or the effective phage doses in the
438 respective systems. Moreover, *P. larvae* is known to sporulate massively in infected bee
439 larvae which is not the case in the artificial media used (1). *P. larvae* spores may help the
440 bacterial cells to resist lytic phages and by this means could represent a reservoir for
441 perpetual re-infections. This highlights a general problem in the treatment of spore forming
442 pathogens either by antibiotics or by phage therapy and should be taken into consideration in
443 the development of a future AFB therapy.

444

445 **Conclusion**

446 This study on *P. larvae* specific bacteriophages revealed that rigorous and comprehensive
447 research is required to explore the true potential of phage therapy for treating AFB. This
448 includes the selection of efficient non-lysogenic phages, whole genome analyses and
449 representative animal studies, which closely simulate the real-life scenario. The missing
450 efficacy of phage HB10c2 in bee larvae and the potential risk of resistances developments of

451 the targeted pathogen suggests that phage cocktails may be required, particularly in
452 preventive applications. From the genome analysis of phage HB10c2 we conclude that the
453 risk assessment for a putative therapeutic phage should include a data inventory of
454 undesirable virulence and antibiotic resistance genes. Moreover, the host range of the
455 respective therapeutic phage needs to be investigated to ensure that all relevant pathogenic
456 strains are targeted and to minimize dysbiosis of the microbiota and ecological risks. Thus,
457 although it appears relatively simple to isolate phages with activity against the pathogen
458 *P. larvae*, it seems to be a great challenge to find isolates that meet all efficacy and safety
459 criteria. This may be partly due to the source of the current isolates, since the analyzed
460 phages originated from *P. larvae* infected bee hives which could not cure themselves. The
461 bird's eye view of the whole genomes of *P. larvae* and *P. larvae* phages suggest that phages
462 like HB10c2 contributed to shape the genome of this pathogen. This co-evolutionary
463 interaction may have led to a rather balanced than an antagonistic interaction. Accordingly, it
464 may be promising to isolate and characterize phages from other settings or geographical
465 areas.

466

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470

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610 **Tab. 1:** Lytic activity of bacteriophage HB10c2 against *P. larvae*, microbiota and
 611 environmental *Bacillales*.

Organism	Reference	Gram staining	Lytic activity of phage HB10c2
<i>Paenibacillus larvae</i> ERIC I	DSM 7030	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 5	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 9	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 11	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 15	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 22	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 24	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 25	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 26	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 29	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 145	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 146	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 174	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 148	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 153	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 155	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 157	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 159	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 160	+	+
<i>Paenibacillus larvae</i> ERIC I	Isolate 162	+	+
<i>Paenibacillus larvae</i> ERIC II	DSM 16116	+	+
<i>Paenibacillus larvae</i> ERIC II	DSM 25430	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 1	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 3	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 6	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 7	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 17	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 20	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 23	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 27	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 28	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 135	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 137	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 144	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 149	+	+

<i>Paenibacillus larvae</i> ERIC II	Isolate 152	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 156	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 158	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 161	+	+
<i>Paenibacillus larvae</i> ERIC II	Isolate 168	+	+
<i>Bacillus licheniformis</i> ¹	LMG 7559	+	-
<i>Bacillus megaterium</i> ¹	LMG 7127	+	-
<i>Bacillus pumilus</i> ¹	LMG 3455	+	-
<i>Bacillus subtilis</i> ¹	LMG 2099	+	-
<i>Bacillus borstelensis</i> ¹	DSM 6347	+	-
<i>Delftia acidovorans</i> ¹	LMG 1226	-	-
<i>Enterococcus faecalis</i> ¹	DSM 20376	+	-
<i>Janthinobacterium lividum</i> ¹	LMG 2892	-	-
<i>Kocuria rosea</i> ¹	DSM 20447	+	-
<i>Pseudomonas fluorescens</i> ¹	DSM 6147	-	-
<i>Staphylococcus pasteurii</i> ¹	DSM 30868	+	-
<i>Streptococcus salivarius</i> ¹	DSM 20067	+	-
<i>Streptomyces griseus</i> ¹	DSM 1471	+	-
<i>Comamonas denitrificans</i> ^{1,2}	LMG 21602	-	-
<i>Salmonella enterica</i> ^{1,2}	DSM 11320	-	-
<i>Gluconobacter oxydans</i> ²	DSM 2003	-	-
<i>Lactobacillus crispatus</i> ²	LMG 9479	+	-
<i>Pedobacter africanus</i> ²	LMG 10345	-	-
<i>Planomicrobium okeanokoites</i> ²	DSM 15489	+	-
<i>Ralstonia pickettii</i> ²	LMG 5942	-	-
<i>Saccharibacter floricola</i> ²	LMG 23170	-	-
<i>Paenibacillus amylolyticus</i> ³	DSM 11730	+	-
<i>Brevibacillus brevis</i> ³	DSM 30	+	+
<i>Brevibacillus laterosporus</i> ³	DSM 25	+	-
<i>Paenibacillus pabuli</i> ³	DSM 3036	+	-
<i>Paenibacillus peoriae</i> ³	DSM 8320	+	-
<i>Paenibacillus phyllosphaerae</i> ³	DSM 17399	+	+
<i>Paenibacillus polymyxa</i> ³	DSM 36	+	-
<i>Paenibacillus popilliae</i> ³	DSM 22700	+	-
<i>Paenibacillus xylanilyticus</i> ³	DSM 17225	+	-
<i>Paenibacillus soli</i> ³	DSM 21316	+	+
<i>Paenibacillus humicus</i> ³	DSM 212785	+	+
<i>Paenibacillus apiarius</i> ⁵	DSM 5581	+	-
<i>Paenibacillus cookii</i> ³	DSM 16944	+	+
<i>Paenibacillus elgii</i> ⁵	DSM 22254	+	-
<i>Paenibacillus filicis</i> ³	DSM 23916	+	+

<i>Paenibacillus fonticola</i> ³	DSM 21315	+	-
<i>Paenibacillus lactis</i> ³	DSM 15596	+	+
<i>Paenibacillus lautus</i> ³	DSM 3035	+	-
<i>Paenibacillus nanensis</i> ³	DSM 22867	+	-
<i>Paenibacillus alvei</i> ³	DSM 29	+	+
<i>Paenibacillus castaneae</i> ³	DSM 19417	+	+

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613 ¹Microbiota of bee larvae; ²microbiota of adult bees; ³environmental *Bacillales*

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636 **Figures**

637

638 **Fig. 1: Plaque formation and host cell lysis by *P. larvae*-specific bacteriophage**

639 **HB10c2.** (A) Titration assay to determine the lytic activity of phage HB10c2 against *P. larvae*

640 genotype ERIC II on top agar. (B) Growth of *P. larvae* ERIC I-IV (continuous lines) and effect

641 of phage HB10c2 on bacterial growth (dotted lines) in BHI liquid medium. Phage HB10c2

642 revealed a significant effect (unpaired Student's t-Test, $p < 0.05$) on the growth of ERIC I ($p =$

643 1.91×10^{-10}), ERIC II ($p = 3.06 \times 10^{-7}$), ERIC III ($p = 1.72 \times 10^{-8}$) and ERIC IV ($p = 7.12 \times 10^{-4}$).

644

645 **Fig. 2: Transmission electron micrograph of bacteriophage HB10c2.** Morphology of

646 phage HB10c2 after negative staining and transmission electron microscopy. According to

647 the average size of capsid, siphon and base plate phage HB10c2 exhibits the B2

648 morphotype of the *Siphoviridae* family. The scale bar indicates 100 nm.

649

650 **Fig. 3: Comparison of the genomic structure of phages HB10c2 and phiBB_P123.**

651 Functional cluster for DNA packaging is marked in orange, structural genes in red, genes for

652 replication in blue, genes for host lysis in yellow and genes involved in lysogeny in green.

653 Figure was generated using Easyfig with amino acid sequence comparison. Level of amino

654 acid identity range is shown via the gradient scale.

655

656 **Fig. 4: Treatment of *P. larvae* infected *A. mellifera* larvae with phage HB10c2.** Bee

657 larvae infected with *P. larvae* genotype ERIC I (DSM 7030) and ERIC II (DSM 25430)

658 exhibited a mortality of 100% after 14 d. Uninfected bee larvae (control) showed a

659 significantly lower mortality compared to infected larvae (unpaired Student's t-Test, $p < 0.05$).

660 Uninfected larvae treated with phage HB10c2 revealed a mortality which is not significantly

661 different compared to uninfected bee larvae without phage treatment ($p = 0.2992$). The

662 mortality of *P. larvae* ERIC I and II infected bee larvae did not decline significantly after

663 phage treatment respectively ($p = 0.4226$ and $p = 0.677$).







