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1 **The endosymbiont and the second bacterial circle of**
2 **entomopathogenic nematodes**

3

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9 **Abstract**

10 Single host-symbiont interactions should be reconsidered from the perspective of the
11 pathobiome. We revisit here the interactions between entomopathogenic nematodes (EPNs)
12 and their microbiota. We first describe the discovery of these EPNs and their bacterial
13 endosymbionts. We also consider EPN-like nematodes and their putative symbionts. Recent
14 high-throughput sequencing studies have shown that EPNs and EPN-like nematodes are also
15 associated with other bacterial communities, referred to here as the second bacterial circle of
16 EPNs. Current findings suggest that some members of this second bacterial circle contribute
17 to the pathogenic success of nematodes. We suggest that the endosymbiont and the second
18 bacterial circle delimit an EPN pathobiome.

19

20 **Key-words**

21 entomopathogenic nematodes, pathobiome, next-generation sequencing

22 **Rethinking host-microorganism interactions: from Koch's postulates** 23 **to the notion of a “pathobiome”**

24

25 Each era has its trends. In the biology of host-microorganism interactions, the 1990s focused
26 on the basic mechanisms of these interactions, leading to a trend towards reductionism, the
27 controllability of “synthetic” systems and advances towards deciphering the molecular
28 mechanisms of microbe infection processes [1]. For example, the molecular infection biology
29 of *Salmonella*, a bacterium pathogenic to both humans and animals, has been described in
30 detail over the last 40 years. The type 3 secretion system (T3SS) was identified as the key
31 determinant of all pathogenic *Salmonella* strains, underlying their ability to invade non-
32 phagocytic host cells [2].

33 Currently, microbiologists are revisiting the field of microbial ecology, with the aim of
34 integrating various dimensions of complexity: genotypic, functional and environmental [1].
35 Moreover, the conceptual framework of mechanistic studies based on **Koch’s postulates** (see
36 Glossary), is being challenged by several new concepts.

37 Firstly, it has been shown that social interactions within a bacterial population isolated from
38 a single host must be taken into account in the infectious diseases caused by some pathogens.
39 In the example cited above, phenotypic heterogeneity in *Salmonella* leads to bi-stable
40 expression of the T3SS locus and to the existence of slow-growing virulent and fast-growing
41 avirulent subpopulations. This division of labor leads to bet-hedging, with slower growth of
42 the T3SS⁺ subpopulation associated with a greater tolerance of antimicrobial drugs. Both the
43 division of labor and bet-hedging result in host manipulation, through an induction of
44 inflammation, leading to the exclusion of the commensal microbiota from the host [3].

45 Secondly, from an evolutionary perspective, **virulence** is now considered to be only one of the
46 parameters affecting microbial spread in a host population. The fitness of the parasite
47 throughout its life cycle is the key to understanding pathogenesis as a whole [3,4]. For
48 example, in the entomopathogenic nematode *Steinernema*, which acts together with the
49 symbiotic bacterium *Xenorhabdus* to kill insects, it has been shown that not only is this
50 association crucial for host mortality, but its specificity is a determining factor in the
51 maintenance of the symbiosis over multiple parasitic cycles and, therefore, over an
52 evolutionary time scale [5].

53 Thirdly, if we take an even more holistic view of host-microorganism interactions, we must
54 also consider the **host microbiota**. The multi-organism comprising the host and its microbiota
55 may be considered an **holobiont** [6]. This concept encompasses various interactions from
56 mutualism, where the association between two individuals benefits both partners, to
57 pathogenicity or parasitism, where the association is deleterious to the host. In deleterious
58 interactions, the pathogenic agent is no longer considered to be an isolated entity, but is
59 instead seen in the context of the broader microbial community to which it belongs, which is
60 known as the **pathobiome** [7]. For example, in Pacific oyster mortality syndrome, which
61 affects juveniles of *Crassostrea gigas*, the pathobiome consists of *Ostreid herpesvirus* OsHV-1
62 μ Var, which triggers an immunocompromised state in the host, and opportunistic bacteria,
63 that subsequently cause bacteremia [8]. In plants, agro-ecological research is strongly guiding
64 efforts towards the identification of microbiomes that are protective against phytopathogens,
65 the opposite of the pathobiome. Many studies have highlighted the preponderant role of
66 bacterial communities in pathogen control in the phyllosphere or rhizosphere (see for
67 example [15,16]) .

68 Any interaction previously described as a unique host-microbe relationship can, therefore, be
69 reviewed in light of these concepts. Our objective is to revisit, from this new angle, the
70 interactions between entomopathogenic nematodes (**EPNs**) and their microbiota. To this end,
71 we relate the history of the discovery of complexes between canonical EPNs and their
72 endosymbionts, *Xenorhabdus* and *Photorhabdus*, and between putative entomopathogenic
73 nematodes (**EPN-like nematodes**) and *Serratia* strains. We describe several studies in which
74 EPNs and EPN-like nematodes were found to be associated with diverse bacterial
75 communities. We also explore several hypotheses and avenues for determining the putative
76 roles of these bacterial communities in entomopathogenicity and nematode fitness. We
77 propose that these two bacterial circles — the endosymbiotic bacteria, which were first
78 described about 60 years ago, and the less stringently associated bacterial community
79 referred to here as the **second bacterial circle** — delimit the EPN pathobiome.

80 **The endosymbiotic bacteria of canonical EPNs**

81

82 The canonical EPNs belong to the genera *Steinernema* and *Heterorhabditis*. The first specimen
83 of *Steinernema kraussei* was described in the 1920s [11] (Figure 1) and EPNs were first used

84 in biological control programs in the 1930s, when *Steinernema glaseri* was used to control the
85 Japanese cockchafer [12]. However, despite reports of associations between bacteria and
86 non-feeding, infective juveniles (IJs) of *Steinernema* as early as 1937 [13], no other specific
87 connections between *Steinernema* and a bacterial species were identified until the 1960s.
88 The initial model was *Neoplectana carpocapsae* (= *Steinernema carpocapsae*), investigated
89 by Poinar and Thomas, who showed that this nematode was the vector of the bacterium
90 *Achromobacter nematophilus* (= *Xenorhabdus nematophila*), which was pathogenic to insects
91 infested with the nematode or following direct injection into the hemolymph [14](Figure 1).
92 *Xenorhabdus nematophila* was not pathogenic by ingestion and had never been isolated from
93 the environment. The authors therefore assumed that it must be inoculated into the insect by
94 the nematode, leading to the induction of septicemia and providing ideal conditions for the
95 reproduction of the nematode within the insect cadaver [14]. This parasitism phenomenon
96 was thought to result from a mutualistic partnership between the nematode and its
97 bacterium, *X. nematophila*, acting together to kill the insect host. However, Weiser and
98 coworkers were unable to isolate *X. nematophila* from *S. carpocapsae*; they instead isolated
99 a microbial population consisting principally of pseudomonads [15]. Finally, Boemare's group
100 isolated *X. nematophila* and other Enterobacteriaceae and Pseudomonadaceae from *S.*
101 *carpocapsae* [16,17], reconciling the findings of Poinar's team in the US and Weiser's team in
102 Czechoslovakia. All these bacteria were isolated from the IJ, the only stage occurring freely in
103 nature. Their frequency was variable, except for *X. nematophila*, which was almost always
104 present. Bird and Akhurst then showed that *X. nematophila* was maintained within a special
105 intestinal vesicle in the free-living form of the nematode [18], subsequently renamed the
106 **receptacle** [19] (Figure 2.A). Both this isolation within the organism and the specialized
107 structure dedicated to housing *X. nematophila* made it seem likely that *X. nematophila* was
108 the only endosymbiont in *S. carpocapsae*. Bacterial isolations from the IJs of other
109 *Steinernema* species systematically led to the identification of other *Xenorhabdus* species,
110 further supporting the concept of a symbiotic relationship [5].
111 *Heterorhabditis*, another EPN genus, and its endosymbiotic bacterium, *Photorhabdus*
112 *luminescens*, initially named *Xenorhabdus luminescens*, were then described [20,21] (Figure
113 1). Unlike *Steinernema*, the nematodes of *Heterorhabditis* have no specialized receptacle to
114 house their symbiotic bacteria, which are instead diffusely spread throughout the intestinal
115 lumen of the anterior digestive tract [22] (Figure 2.B).

116 Numerous taxonomic studies were conducted on the *Xenorhabdus* symbionts of *Steinernema*
117 and the *Photorhabdus* symbionts of *Heterorhabditis* [23,24]. By 2020, about 100 validated
118 species of *Steinernema* and 21 of *Heterorhabditis* had been described [25]. Interestingly, each
119 nematode species is associated with a single species of bacterium, although there are some
120 exceptions to this rule, probably due to host changes (see for example [26]). In *Xenorhabdus*
121 and *Photorhabdus*, two variants were distinguished on morphological and biochemical
122 criteria: the **primary variant**, which converted into the **secondary variant** during long-term
123 stationary phase culture and, sometimes, during infection [27,28].

124 In this “endosymbiotic bacterium-focused view”, the dogma of natural monoxenicity between
125 the nematode and the endosymbiotic bacterium has become widely accepted as a rule in the
126 scientific community. In practice, the procedures used to isolate *Xenorhabdus* and
127 *Photorhabdus* were adapted to ensure the systematic elimination of the external bacterial
128 microflora by surface decontamination of the IJs (see below). Consequently, the role played
129 by the bacterial endosymbionts, *Xenorhabdus* and *Photorhabdus*, in the main steps of the EPN
130 life cycle came to predominate in studies over the last 20 years [5,29] (Figure 3.A).

131

132 **The putative symbionts of EPN-like nematodes**

133

134 Interestingly, since 2010, several bacterivorous nematodes have been isolated in *ex-vivo*
135 *Galleria* traps. The first was the *Caenorhabditis briggsae* KT0001 nematode [30]. *Serratia* sp.
136 strain SCBI, isolated from this nematode, is entomopathogenic when directly injected into
137 *Galleria* [30,31]. A second putative EPN, *Oscheius chongmongensis*, formerly
138 *Heterorhabditoides chongmongensis*, was recovered from a *Galleria* trap in East China, and
139 three bacterial taxa, *Serratia*, *Proteus* and *Acinetobacter*, were isolated by plating crushed
140 nematodes [32]. Only the *Serratia nematodiphila* strain [33] isolated from this nematode was
141 found to be entomopathogenic, and to enable the nematode to undergo sexual reproduction
142 *in vitro* [32,33]. Other *Oscheius* species have since been described as putative
143 entomopathogenic nematodes [34–39].

144

145 *Caenorhabditis briggsae* and *Oscheius* are often associated to the genus *Serratia*, which
146 facilitates pathogenicity (Table 1; criterion 1) [30,32,37,39,40]. Strains of this genus are known

147 to have entomopathogenic properties with a broad host spectrum [41]. The genomes of the
148 putative symbionts of *C. briggsae* KT0001 and *O. chongmingensis* — *S. marcescens* SCB1 and
149 *S. nematodiphila* DSM21420, respectively — harbor substantial numbers of genes encoding
150 secreted proteases, lipases, and hemolysins common to *Photorhabdus* and *Xenorhabdus*
151 [31,42]. Based on the current state of knowledge, these *Serratia* may be considered putative
152 endosymbiotic bacteria associated with *C. briggsae* and *Oscheius* sp..

153 In 2012, the definition of entomopathogenicity for a nematode was clarified, distinguishing
154 this property from parasitism on the basis of two criteria [43]. For entomopathogenicity, there
155 must be a stable symbiotic relationship between the bacteria and the nematode facilitating
156 pathogenesis (criterion 1). Insect death must occur sufficiently rapidly (within five days of
157 infection) to be unequivocally distinguishable from phoretic, necromenic or parasitic
158 associations (criterion 2). The *Steinernema-Xenorhabdus* and *Heterorhabditis-Photorhabdus*
159 pairs meet both criteria. When these criteria and their derived sub-criteria were applied (Table
160 1), the putative EPNs could not unequivocally be considered to be entomopathogenic,
161 because not all the criteria were satisfied, tested or validated in all studies. A recent
162 comprehensive comparative study of *O. chongmingensis* and *Steinernema* even concluded
163 that the former is a scavenger rather than an entomopathogenic nematode, which does not
164 exclude that it may be on an evolutionary trajectory leading to entomopathogenic life style
165 [44]. We therefore consider these nematodes to be EPN-like and *Serratia* bacteria their
166 putative endosymbiont (Figure 1).

167

168 **A second bacterial circle sporadically detected on culture, but** 169 **recently validated by NGS**

170

171 For many years, the powerful prevailing reductionist tendency in interpretations of
172 microorganism-host relationships led to bacteria other than endosymbionts being regarded
173 as environmental surface contaminants. We propose here a rethink of this assumption.

174 Despite frequently being ignored by the pioneers describing EPNs, bacteria other than
175 endosymbionts have actually often been detected by Pasteurian isolation methods on culture
176 media. As far back as the 1960s, the presence of several bacterial species regularly associated
177 with the IJs *S. carpocapsae* was described, and similar results were obtained during

178 investigations on other EPN species (Table 2). These bacteria were isolated from IJs or EPN-
179 infested cadavers and most were Proteobacteria. Depending on the study concerned and the
180 IJ washing method used (bleach solution, streptomycin and penicillin, merthiolate), these
181 bacteria were still detected after surface washing [16,17,45–48] or were not detected [49,50].
182 These findings led some authors to suggest that bacteria other than symbionts might reside
183 in the gut lumen of the nematodes. Moreover, bacteria between the two cuticles enveloping
184 *Steinernema scapterisci* IJs were observed by microscopy [51]. As bleach disinfection leads to
185 elimination of the second cuticle, it was suggested that non-symbiotic bacteria might be
186 located between the two cuticles [50,51]. Similar bacterial associates have been detected with
187 *Heterorhabditis* (Table 2). In this nematode genus, dixenic associations were detected with
188 *Ochrobactrum* spp. [52], *Providencia rettgeri* and *Paenibacillus* spp. [53].

189 The rapid development of **NGS** over the last decade has increased the capacity of researchers
190 to characterize entire microbial communities in complex samples rapidly, to detect
191 unculturable microorganisms, to discover new organisms and to explore the dynamic nature
192 of microbial populations. Interestingly, these approaches supported previous Pasteurian
193 descriptions of a microbiota associated with EPNs. Metabarcoding techniques were used to
194 monitor bacterial dynamics in the cadaver of insect larvae *Galleria mellonella* after infestation
195 with *Heterorhabditis*. Bacteria of the genus *Stenotrophomonas* were found to be abundant in
196 the insect cadaver, through their ability to grow in the presence of antibiotics (stilbene)
197 produced by the endosymbionts [54]. The IJs carried *Stenotrophomonas* spp. on their external
198 surfaces. The authors therefore suggested that *Stenotrophomonas* is probably introduced into
199 the insect larva via the nematode. The metabarcoding method was recently used
200 simultaneously with two taxonomic markers to describe the bacterial communities associated
201 with *S. carpocapsae* reared in different laboratories (France, USA) [55,56]. The authors
202 identified: (i) a core microbiota composed of the endosymbiont *X. nematophila*; (ii) a subset
203 of about ten OTUs called **FAM** (frequently-associated microbiota), (iii) a more variable
204 microbiota. The FAM includes Proteobacteria from the genera *Pseudomonas*,
205 *Stenotrophomonas*, *Achromobacter* and *Alcaligenes*, and the family Rhizobiaceae
206 (*Ochrobactrum*, *Pseudochrobactrum*) [56]. These molecular results were confirmed by
207 repeated isolation of bacteria from these genera such as *Pseudomonas protegens* from *S.*
208 *carpocapsae*, *S. glaseri*, *Steinernema weiseri* and *S. feltiae* [56,57]. Almost all the members of
209 the FAM were detected in a nematode freshly collected in the field, confirming that they were

210 not artifacts of laboratory rearing [56]. To distinguish them from the bacterial endosymbionts,
211 we refer to these other EPN bacterial communities as the second bacterial circle (Figure 1). In
212 the EPN-like nematodes, a bacterial consortium in addition to *Serratia* has also been described
213 (Table 1 and Figure 1) [40,58,59]. Second bacterial circle status requires further validation by
214 a metagenomic study in a more diverse range of EPN-like isolates.

215 The primary variant forms of *Xenorhabdus* and *Photorhabdus* can produce a huge repertoire of
216 different interbacterial competition systems and antimicrobial molecules (see for example
217 [60–64]). Is the second bacterial circle resistant to the antibiotics produced by the
218 endosymbiont? Several results obtained *in vitro* have suggested that co-adaptation between
219 the endosymbiont and some members of the second bacterial circle can occur. Hence, in
220 dioxenic *Photorhabdus* spp./ *Paenibacillus* spp. associations with *Heterorhabditis*, the
221 nematode-associated *Paenibacillus* spp. were found to be resistant to *Photorhabdus*
222 antibiotics *in vitro*, whereas phylogenetically close strains of *Paenibacillus* spp. not associated
223 with nematodes were not [53]. Stilbene, the antibiotic produced by *Photorhabdus* in *Galleria*
224 cadavers after *Heterorhabditis* infestation, affects insect-associated *Enterococcus* growth *in*
225 *vitro* but has no effect on the nematode-associated *Stenotrophomonas* spp. also present in
226 the insect cadaver [54]. Some second bacterial circle isolates from the genera
227 *Stenotrophomonas* and *Pseudomonas* also display strong antimicrobial activity against the
228 endosymbiont *in vitro* [54,56]. The cohabitation between the different variants of the
229 bacterial endosymbiont and the members of the second circle therefore seems to be depend
230 on fine-tuning based on the timed succession or spatial compartmentalization of the different
231 bacteria producing antimicrobial molecules.

232 Most of the genera of the second bacterial circle of EPNs (*Pseudomonas*, *Stenotrophomonas*,
233 *Ochrobactrum*) are also known to be associated with the free-living nematode *Caenorhabditis*
234 *elegans* [65–67], and to a lesser extent with the gut microbiota of some insects such as
235 lepidopteran or coleopteran larvae [68,69]. Interestingly, these worms and insects share
236 similar biotopes, soils, plants and decomposing plants on soils [68,70], that could shape a
237 common microbiota. However, further functional correlations would require more accurate
238 taxonomical descriptions of these different microbiota at the species or lineage scale, as well
239 as genomic comparisons to identify potential common functions.

240 **Is the second bacterial circle involved in the EPN pathobiome?**

241 The role of the second bacterial circle in the fitness of the nematode remains a matter of
242 debate. *Erwinia agglomerans*, *Serratia liquefaciens*, and *Pseudomonas fluorescens* isolated
243 from *S. carpocapsae* enable the reproductive success of the axenic nematode in the insect,
244 but not *in vitro* [16]. However, axenic cultures of *S. carpocapsae* were unable to grow in the
245 presence of *S. marcescens* carried on IJ surfaces, and IJ emergence rates are very low when *S.*
246 *marcescens* is abundant in the EPN-infected cadaver [71]. Moreover, intercuticular bacteria
247 present in *S. scapterisci* nematodes were found to have a negative impact on the
248 entomopathogenicity and reproductive success of the nematodes [51]. One can speculate
249 that these associations would not be sustainable over time. By contrast, we assume that the
250 contribution of the second bacterial circle members repeatedly isolated from IJs over the past
251 60 years by cultural approaches (Table 2) or described as core EPN microbiota by NGS
252 approaches through the many successive reproductive cycles on insects [56] is neutral or
253 positive (mutualistic). In some cases, especially when members of the second bacterial circle
254 are highly resistant to antimicrobial compounds produced by the bacterial endosymbiont (see
255 above *Paenibacillus* spp. and *Stenotrophomonas* spp.), we cannot conclude if they contribute
256 to the EPN fitness or if they are just passively present in the insect cadaver to benefit from the
257 public good (nutrient resource provided by the decomposing insect cadaver), as cheaters do
258 [72]. In the rest of this section, we consider the potential positive contribution of the second
259 bacterial circle to the infectious process and to completion of the main phases of the EPN
260 lifecycle (Figure 3.B).

261

262 ***Entry into the living insect, causing infection and death***

263 The IJ rapidly loses its outer cuticle after entering the insect intestine [73]. Members of the
264 second bacterial circle located between the two cuticles might therefore be released early
265 into the insect gut, where they could protect the nematode by producing factors enabling the
266 nematode to escape the insect immune system and or by secreting molecules (e.g. chitinase,
267 proteases, pore-forming toxins) destabilizing the intestinal epithelium. For example, *P.*
268 *protegens* and *Pseudomonas chlororaphis*, associated with several *Steinernema* species
269 [56,57], secrete the Fit insecticidal toxin, which has been shown to be responsible for
270 entomopathogenicity when ingested, into the insect gut [74]. Once in the hemolymph,

271 organisms from the second bacterial circle may also participate in the killing of the insect.
272 Some bacterial isolates, from *P. fluorescens*, *Serratia* sp., *P. rettgeri*, *Alcaligenes faecalis*, and
273 *P. protegens*, have been shown to display entomopathogenic activities after direct injection
274 into the hemolymph of several lepidopteran species [15,56,57,71,75,76].

275 In a few entomopathogenic pairs, the endosymbiont has attenuated virulence properties
276 when directly injected into insect larvae, as observed for *Xenorhabdus poinarii* associated with
277 *S. glaseri* [77,78] or *Xenorhabdus bovienii* CS03 associated with *S. weiseri* 583 [79]. At the time,
278 two hypotheses were put forward to explain why these nematode-symbiont pairs succeeded
279 in completing their reproductive cycle in insect larvae: a specialized host range or an
280 entomopathogenicity that relied more on the nematode partner than on the bacterial
281 endosymbiont [78,80]. These nematode-symbiont pairs may also live as scavengers rather
282 than insect pathogens, as previously described when some EPNs compete with saprophagous
283 organisms in soil [81]. Following the description of the second bacterial circle and its putative
284 belonging to the EPN pathobiome, we propose an additional hypothesis for those
285 entomopathogenic pairs: some entomopathogenic members of the second bacterial circle
286 complement the entomopathogenic functions of the symbiont, contributing to the success of
287 the EPN parasitic cycle.

288 The bacterial symbiont may be less dominant in IJs living in soil than in IJs multiplying in
289 optimal laboratory conditions. For example, on rare occasions, the endosymbiont bacteria
290 have been difficult to detect or to isolate from the first generation of IJs just after their capture
291 in the environment [47,82]. In *Tenebrio molitor* larvae reared in soils and infected with *S.*
292 *carpocapsae*, the symbiont does not dominate the bacterial community [83]. Finally, a
293 decrease in symbiont load has been observed in batches of IJs subjected to extreme
294 temperatures (>35°C) (Pagès S., personal communication). We can therefore speculate that,
295 in natural environments in which conditions are unfavorable (low or high temperature,
296 drought, etc.), the second bacterial circle may be necessary for successful completion of the
297 EPN cycle.

298

299 ***Nematode reproduction in the insect cadaver***

300 The insect hosts may be co-infected by an EPN and another entomopathogenic agent as well
301 as by several EPNs. During dual coinfection with *Bacillus thuringiensis* (Bt), competitive
302 interaction exists between Bt and the endosymbiont for food resources [84]. During co-

303 infection between *S. affine* and *S. feltiae*, it has been shown that the *S. affine* endosymbiont
304 directly kills reproductive stages of *S. feltiae* [85]. One could envisage that such modulations
305 of competition are also dependent on some members of the second bacterial circle.
306 The second bacterial circle could play indirect roles in nematode reproduction. For example,
307 it could provide the nematode with nutrients, by decomposing the insect cadaver through the
308 secretion of extracellular enzymes. *Pseudomonas* and *Stenotrophomonas* species, which are
309 frequently associated with EPNs, are known to produce various enzymes, such as proteases,
310 lipases, and chitinases [74,86]. The second bacterial circle may also protect nematodes against
311 pathogens, and prevent putrefaction of the cadaver. The strong antimicrobial activities of
312 members of the second bacterial circle observed *in vitro* may help to eliminate microbial
313 competitors during nematode multiplication in the cadaver [54,56].

314

315 ***Transmission and dissemination of the parasitic complex in soils***

316 The transmission of the bacterial second circle over generations remains a key question. The
317 main steps leading to the colonization of the *S. carpocapsae* IJ receptacle by *X. nematophila*
318 have been described. Symbiosis region 1 (SR1) genes provide the genetic basis for the
319 specificity of this transmission [5]. The IJ receptacle has been detected in several *Steinernema*
320 species [87], but the process of specific transmission by other *Steinernema* species has been
321 little studied.

322 The *S. carpocapsae* FAM has been conserved over generations, for 40 years, in various
323 laboratories [56]. Are there specific mechanisms of recognition and recruitment between the
324 nematode and certain members of the microbiota? It is conceivable that some members of
325 the second bacterial circle have also developed specific colonization factors or recognition
326 traits enabling them to colonize the surface of IJs, the intercuticular space or the IJ gut in insect
327 cadavers.

328 For dissemination and survival in the soil until the next encounter with an insect larva,
329 nematodes may rely on abundant progeny, but also on the development of IJ defense
330 strategies against soil biotic agents. IJs can be negatively affected by soil bacteria, such as
331 *Paenibacillus* that exploit them for their own dispersal [88]. In the same way that isolates of
332 the *P. fluorescens* subgroup belonging to the *C. elegans* microbiota protect the worms against
333 infection by *B. thuringiensis* via metabolite synthesis [89], the second bacterial circle could
334 provide a defense function for the IJ against such deleterious bacteria. Nematophagous fungi

335 are the most important and well-studied pathogens affecting EPNs [88]. The second-stage
336 cuticle protects the third stage IJs from fungal infection [84]. There is currently no evidence to
337 suggest that this may involve microbial action, but it may be relevant to investigate the
338 antagonistic properties of intercuticular bacteria from the second bacterial circle against these
339 nematophagous fungi.

340

341 ***Consequences for biocontrol application of EPNs***

342 EPNs are used as biocontrol agents for insects. The ecological risks of EPN application have
343 long been assessed and the impact of EPNs on non-target organisms (e.g. earthworms, toads,
344 mice, chickens, rabbits and guinea pigs) is limited or non-existent [90]. However, many
345 members of the second bacterial circle belong to genera, such as *Pseudomonas*,
346 *Stenotrophomonas* and *Ochrobactrum*, which encompass a few animal and human pathogens.
347 The taxonomy of some of these species is still unclear, because of their high genotypic and
348 phenotypic variability, host ranges and symbiotic abilities [86,91]. Following this new
349 polyxenic view of the EPN life cycle, further taxonomic characterization should be therefore
350 carried out to provide an accurate risk assessment survey concerning EPN soil applications
351 On the other hand, some of the species of this second circle are reported to have beneficial
352 properties for plant health. For example, the rhizospheric isolates of the species *P. protegens*
353 and *P. chlororaphis* stimulate plant growth and express antagonistic properties towards plant
354 pathogens [74]. The association between EPN and such members of the second bacterial circle
355 could expand their areas of application in the agricultural domain.

356

357 **Concluding remarks and future perspectives**

358 EPNs have long been seen as a highly specific entomopathogenic association between the
359 nematode and an endosymbiotic bacterium, but recent studies based on NGS technology have
360 shown that EPNs are associated with more complex bacterial communities (second bacterial
361 circle).

362 Could the second bacterial circle improve the fitness of nematodes and contribute to the EPN
363 pathobiome? A role for the second bacterial circle in killing insects seems likely, as some
364 members are entomopathogenic. The roles of these bacteria in other phases of the EPN
365 lifecycle remain unclear. Here, we suggest a paradigm shift in the description of EPN

366 pathogenesis, from a tripartite model (insect-nematode-endosymbiont) to a more complex
367 model taking into account the whole EPN microbiota (bacterial endosymbiont and second
368 bacterial circle). This paradigm shift accompanies the transition from Koch's postulates to an
369 enlargement of the pathobiome concept.

370 Many questions remain to be answered to validate this paradigm shift (see Outstanding
371 questions). To clarify these issues, a big challenge is the development of appropriate
372 techniques. For example, gnotobiological experiments should be performed with germ-free
373 nematodes, obtained by disinfecting nematode eggs and creating associations with bacteria
374 of the endosymbiont and the second bacterial circle. The fitness of gnotobiotic EPN should be
375 assessed all along the cycle in microcosms (soil-mimicking conditions, presence of predators,
376 etc.). Also, bacterial monitoring assays (imaging, molecular quantitative measure, etc.) should
377 be developed for following EPN-associated bacterial community into its two main habitats, the
378 IJ and the insect cadaver.

379

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381

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651

652 **Glossary**

653

654 **Endosymbiont:** the bacteria *Xenorhabdus*, *Photorhabdus* and the putatively *Serratia* that are
655 regularly associated with the EPNs *Steinernema*, *Heterorhabditis* and the EPN-like nematodes,
656 respectively

657 **EPNs:** entomopathogenic nematodes *Steinernema* and *Heterorhabditis* that are in symbiotic
658 relationship with bacterial taxa *Xenorhabdus* and *Photorhabdus*, respectively, and whose
659 entomopathogenicity, facilitated by endosymbiont occur sufficiently rapidly (within five days
660 of infection)

661 **EPN-like nematodes:** bacterivorous nematodes isolated by insect-baiting that are not
662 canonical EPNs, but may be an evolutionary trajectory leading to an entomopathogenic
663 lifestyle in the future

664 **FAM:** frequently associated microbiota described in 60 isolates of *Steinernema carpocapsae*
665 which were present in more than 70% of the samples and did not originate from the insect
666 microbiota or the laboratory environment

667 **Holobiont:** unit of biological organization with its hologenome— the sum of the genomes of
668 the host and its microbiota — that is a comprehensive genetic system subject to the rules of
669 genetic and evolution

670 **Host microbiota:** eukaryotic, prokaryotic (Eubacteria or Archaea) and/or viral microorganisms
671 associated with a host

672 **IJs:** infective juveniles are the free-living and soil-dwelling larval forms of EPN

673 **Koch's postulates:** postulates that serve as guidelines for the assessment of causality in
674 infectious diseases, established at the end of the 19th century by Robert Koch, and which
675 could be summarized by the following sentence, a particular pathogenic bacterium is the
676 cause of a particular disease

677 **NGS:** next-generation sequencing that allow the increasing description of entire microbial
678 communities in complex samples

679 **OTU:** the operational taxonomic unit is a taxonomic cluster in DNA metabarcoding studies,
680 based on the similarity of DNA sequences of a genetic marker, often a variable region of the
681 16S rRNA gene

682 **Pathobiome:** the pathogenic agent in the context of the broader microbial community to
683 which it belongs

684 **Primary and secondary variants:** wild-type bacterial endosymbiont or the primary variant, is
685 converted into the secondary variant during long-term stationary phase culture and,
686 sometimes, during infection; a common feature of the secondary variants is their weak *in vitro*
687 antimicrobial activity

688 **Receptacle:** special intestinal compartment in the free-living form of the nematode
689 *Steinernema* containing the bacterial endosymbiont

690 **Second bacterial circle:** bacterial community less stringently associated to EPNs and EPN-like
691 nematodes than the endosymbiont

692 **Virulence:** the relative capacity of a microorganism to cause damage in a host

693

694 **Figure Legends**

695 **Figure 1. Historical changes in the view of the mutualistic symbiotic interaction between**
696 **EPNs and associated bacteria.**

697 Over the last century, knowledge of EPN-bacteria interactions has progressively moved from
698 a monoxenic (entomopathogenic endosymbiont) to a polyxenic view (entomopathogenic
699 endosymbiont + second bacterial circle). Taxa belonging to the second bacterial circle
700 identified by cultural approaches since the early 1960s are indicated at the top of the figure.
701 Taxa belonging to the second bacterial circle characterized by NGS approaches as of 2016 are
702 indicated on the right-hand side of the figure.

703

704

705 **Figure 2. Location of the bacterial endosymbionts or putative endosymbionts in IJs.**

706 **A.** The GFP-labeled endosymbionts *Xenorhabdus nematophila* in intestinal receptacle of the IJ
707 stage of *Steinernema carpocapsae* (confocal micrographs from [92]).

708 **B.** The GFP-labeled endosymbionts *Photorhabdus luminescens* in the intestines of
709 *Heterorhabditis bacteriophora* IJs located anterior to the nematode basal bulb (differential
710 interference contrast and epifluorescence microscopy micrographs from [22]).

711 **C.** The natural fluorescent putative endosymbionts *Serratia nematodiphila* in the *Oscheius*
712 *chongmingensis* gut (fluorescence microscopy micrographs from [32]).

713

714 **Figure 3. The three steps of the EPN lifecycle**

715 **A.** The central role of the endosymbiotic bacteria in the parasitic success of EPNs is
716 summarized, from the infectious process to specific re-association with IJs. The
717 endosymbionts are colored in red.

718 **B.** Complementing the role of the endosymbiont, putative functions of the second bacterial
719 circle in the parasitic success of EPNs are proposed, from the infectious process to specific re-
720 association with IJs. The bacteria of the second circle are colored in blue.

721 **1**, Insect infection; **2**, Nematode reproduction in cadaver; **3**, IJ dissemination in soils

722

723

Table 1. EPN-like nematodes and their associated bacteria

Nematode species	Putative bacterial symbiont ¹	Criterion 1: symbiotic relationship between bacteria and the nematodes facilitates pathogenicity			Criterion 2: insect death should be sufficiently rapid and significant ²		Other bacteria isolated ¹	Reference
		1a: facilitates insect death	1b: facilitates offspring emergence	1c: new offspring carries the symbiont	2a: IJs kill in less than 5 days (50% mortality)	2b: putative symbiont kills by injection in less than 5 days (50% mortality) ³		
<i>Caenorhabditis briggsae</i> KT0001	<i>Serratia marcescens</i> SCBI ^N	YES	nd	nd	NO	YES	nd	[30]
<i>Oscheius (Heterorhabditoides) chongmingensis</i>	<i>Serratia nematodiphila</i> DZ0503SBS1 ^{N, I}	YES	YES	nd	VAR	YES	<i>Proteus</i> sp. ^{N, I} <i>Acinetobacter</i> sp. ^{N, I} <i>Ochrobactrum tritici</i> ^{N, I, *} <i>Bacillus cereus</i> ^{N, I, *}	[32,33,44,58,93]
<i>Oscheius carolinensis</i>	<i>Serratia marcescens</i> ^{N, I}	YES	YES	nd	YES	YES (but by topical application)	<i>Achromobacter xylosoxidans</i> ^{N, I} <i>Enterococcus mundtii</i> ^{N, I} <i>Providentia rettgeri</i> ^{N, I}	[34,40]
<i>Oscheius (Heterorhabditoides) rugaoensis</i>	<i>Serratia nematodiphila</i> ^{N, I}	YES	nd	YES	YES	nd		[37]
<i>Oscheius gingeri</i>		nd	nd	nd	YES	nd		[35,36]
<i>Oscheius onirici</i>		nd	nd	nd	VAR	nd	unidentified rod-shaped bacteria ^M	[38,94,95]
<i>Oscheius myriophila</i>	<i>Serratia marcescens</i> MC5-R ^{N, I}	nd	nd	nd	nd	YES		[39]

¹ taxa are identified after isolation by culture on culture media, from nematodes (N) or infested insect cadavers (I); an asterisk (*) indicates identification by metabarcoding; M indicates observation by microscopy

² death assessed after insect infestation on filter paper, a standard pathological assay common to all laboratories; VAR: variable results according to the studies

³ bacterial dose injected <10⁵ according to Bucher's definition for entomopathogenic bacteria [96]

YES: criterion is validated; NO: criterion is not validated; VAR: criterion validated in some, but not all studies; nd: not determined; FAM: frequently associated microbiota

Table 2. The cultivable second bacterial circle of EPNs

EPN species	Bacteria isolated from		Author comments	References
	Infective juvenile nematodes (L3)	EPN-infested cadavers		
<i>Steinernema carpocapsae</i>		Seven bacterial species		[97]
	<i>Xenorhabdus nematophila</i>	<i>Alcaligenes</i> sp., <i>Aerobacter</i> sp., <i>Proteus</i> sp. and <i>Pseudomonas aeruginosa</i>	Non-symbiotic isolates are contaminants from the insect gut.	[98]
	<i>Pseudomonas fluorescens</i> , <i>Alcaligenes odorans</i> , <i>Pseudomonas odorans</i> , <i>Pseudomonas maltophilia</i> , <i>Pseudomonas alcaligenes</i> and <i>Acinetobacter</i> sp.			[15]
	<i>Pseudomonas aureofaciens</i> , <i>Pseudomonas fluorescens</i> , <i>Erwinia agglomerans</i> , <i>Serratia proteomaculans</i> and <i>Serratia liquefaciens</i>			[16,17]
	<i>Enterobacter gergoviae</i> , <i>Pseudomonas</i> sp., <i>Salmonella</i> sp., <i>Serratia marcescens</i> , <i>Xenorhabdus nematophila</i>		The non-symbiotic bacteria are probably located in the intercuticular space.	[50]
	<i>Serratia marcescens</i> and <i>Xenorhabdus nematophila</i> were isolated from hemolymph of dead <i>Galleria mellonella</i> <i>Acinetobacter junii</i>	Proliferation of <i>S. marcescens</i> in EPN-infested cadavers (reddish coloration of <i>G. mellonella</i>)	<i>S. marcescens</i> was superficially carried by the IJs. The emergence of IJs is considerably reduced when <i>S. marcescens</i> is abundant in the cadaver.	[71]
<i>Steinernema scapterisci</i>	<i>Xenorhabdus</i> sp., <i>Ochrobactrum anthropi</i> , <i>Paracoccus denitrificans</i> , <i>Xanthomonas maltophilia</i> , <i>Pseudomonas aureofaciens</i> numerous bacteria (cocci and rods) located into the intercuticular space		Bacteria were isolated from hemolymph and crushed IJs	[46]
				[45,99]
			Intercuticular bacteria were contaminants because they were	[51]

		detrimental to nematode reproduction in <i>G. mellonella</i> .	
<i>Steinernema riobrave</i>	Gram-negative bacteria (presumably from the nematode gut or cuticular surface) grew in the cadaver (10 ⁹ cells/larvae at 168 hours post infestation)		[100]
	<i>Burkholderia cepacia</i> , <i>Flavobacterium</i> sp., <i>S. marcescens</i> , <i>Xanthomonas maltophilia</i> , <i>Xenorhabdus</i> sp.	Probable intercuticular location	[50]
<i>Steinernema feltiae</i>	<i>Burkholderia cepacia</i> , <i>Flavobacterium indologenes</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas fluorescens</i> , <i>Salmonella</i> sp., <i>Xenorhabdus bovienii</i>	Probable intercuticular location	[50]
	<i>Pseudomonas protegens</i> , <i>Delftia acidovorans</i> (no isolation, but detection by PCR amplification)	<i>X. bovienii</i> remained undetected	[47]
	<i>Stenotrophomonas maltophilia</i> , <i>Alcaligenes faecalis</i>	<i>X. bovienii</i> remained undetected	[46]
	<i>P. protegens</i>	The association of <i>P. protegens</i> with <i>S. feltiae</i> seems robust, as supported by its repeated isolation from both surface-sterilized IJs and insect larvae infected	[57]
<i>Steinernema monticulum</i>	<i>Serratia</i> sp., <i>Acinetobacter calcoaceticus</i> , <i>Pseudomonas aeruginosa</i> , <i>Delftia acidovorans</i>	<i>Xenorhabdus</i> was not detected	[48]
<i>Steinernema glaseri</i>	<i>Stenotrophomonas pavanii</i>	Non-symbiotic bacteria were isolated from hemolymph and crushed IJs	[46]
<i>Steinernema thermophilum</i>	<i>Providencia vermicola</i> , <i>Xenorhabdus indica</i> , <i>Leucobacter iarius</i>	<i>Providencia</i> , <i>Xenorhabdus</i> and <i>Leucobacter</i> were isolated from surface sterilized and crushed IJs	[101–103]
<i>Steinernema diaprepesi</i>	<i>Paenibacillus</i> sp., bacterial spores adhere to cuticles of third-stage IJs (phoretic association)	Host specificity of <i>Paenibacillus</i> isolates to <i>S. diaprepesi</i> , but bacteria were not entomopathogenic	[104]

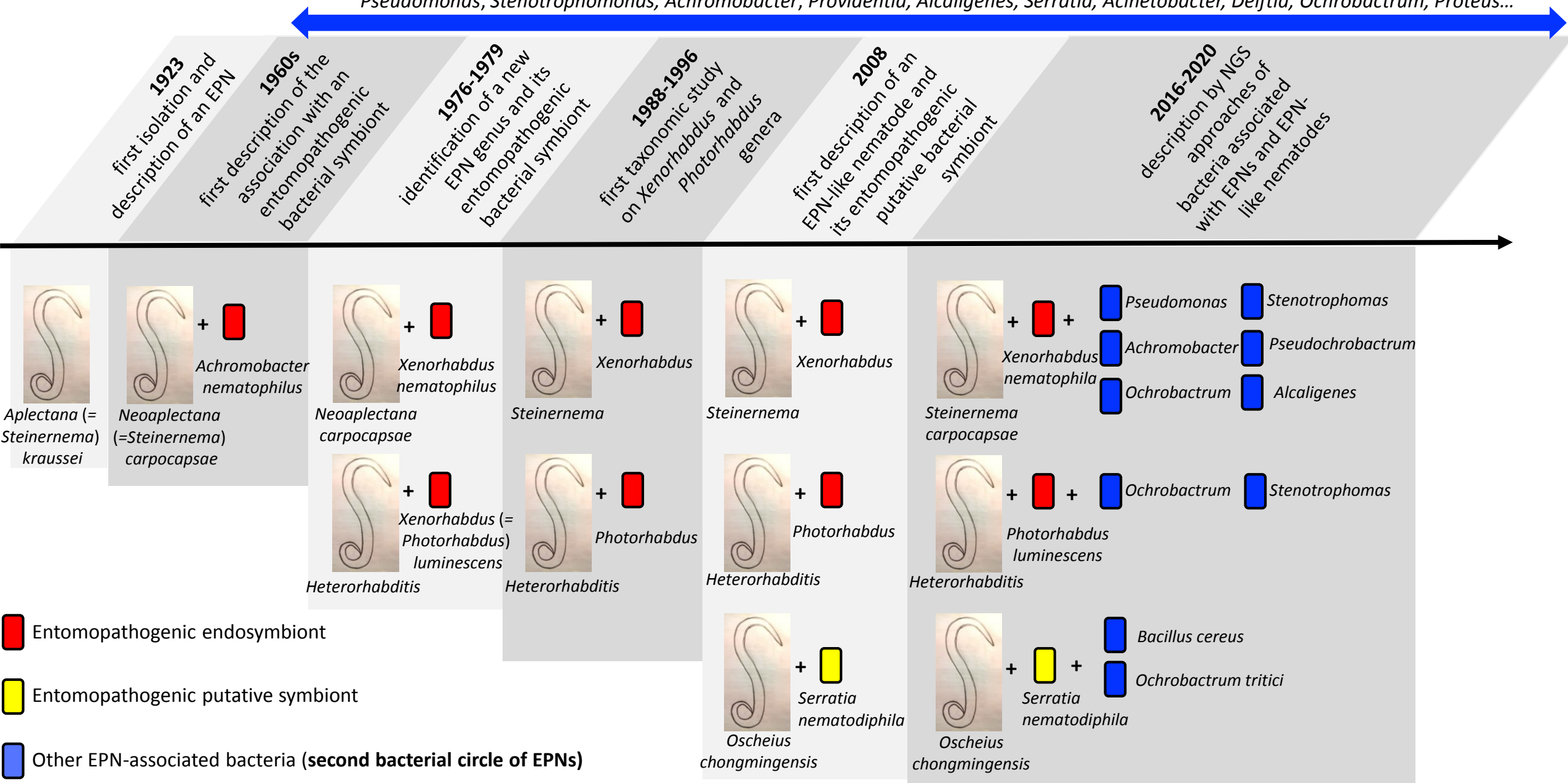
<i>Steinernema feltiae</i> , <i>Steinernema carpocapsae</i> , and <i>Heterorhabditis bacteriophora</i>	Microscopic analyses revealed that <i>Alcaligenes faecalis</i> was located in the esophagus and intestine of the nematodes	<i>A. faecalis</i> was isolated from the hemolymph of a <i>G. mellonella</i> larva cadaver found in the soil of Tenango (Santa Ana), Morelos, Mexico	<i>A. faecalis</i> were strongly pathogenic to <i>G. mellonella</i> (96% mortality 24h post infestation, 2.4×10^4 cells/larvae)	[76]
<i>Heterorhabditis spp.</i>	<i>Photorhabdus spp.</i> , <i>Providentia rettgeri</i>		Dixenic associations	[75]
	<i>Photorhabdus spp.</i> , <i>Paenibacillus spp.</i> (three strains), the sporangia of which adhere to the IJ surface during the free-living stage of the nematode in soils		Co-adaptation between <i>Paenibacillus spp.</i> and <i>Heterorhabditis spp.</i>	[53]
<i>Heterorhabditis bacteriophora</i>	<i>Photorhabdus luminescens</i> and <i>Stenotrophomonas sp.</i>	<i>P. luminescens</i> , <i>Stenotrophomonas spp.</i> , <i>Achromobacter sp.</i> , <i>Alcaligenaceae</i>	<i>Stenotrophomonas</i> bacteria could be introduced into the insect cadaver via the nematode	[54]
	<i>Alcaligenes faecalis</i>			[46]
<i>Heterorhabditis indica</i>	<i>Photorhabdus akhurstii</i> , <i>Ochrobactrum spp.</i>		Dixenic associations in 33% of native IJs freshly collected without any laboratory transfer	[52]
	<i>Pseudomonas aeruginosa</i>		<i>Photorhabdus</i> was not detected	[105]

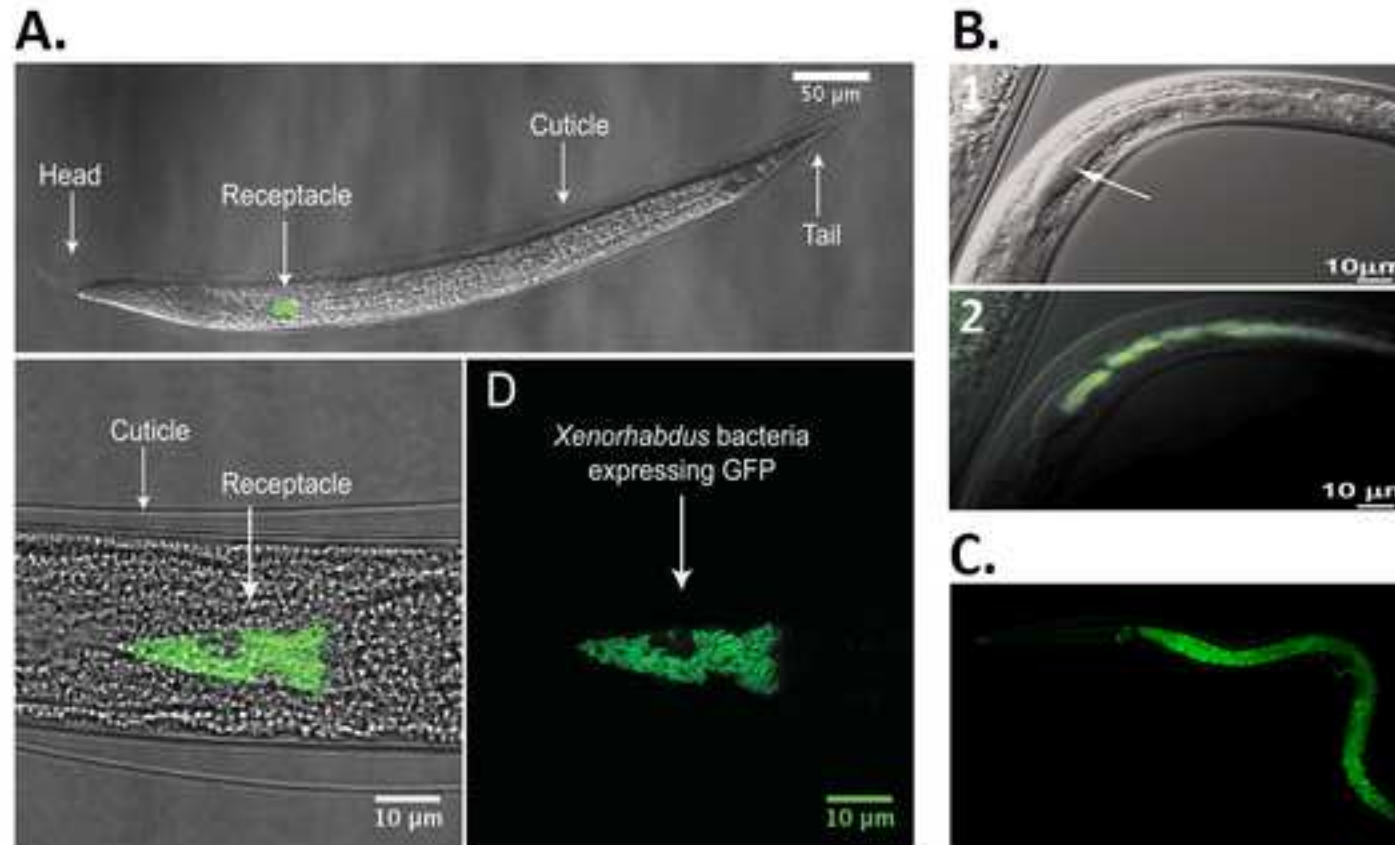
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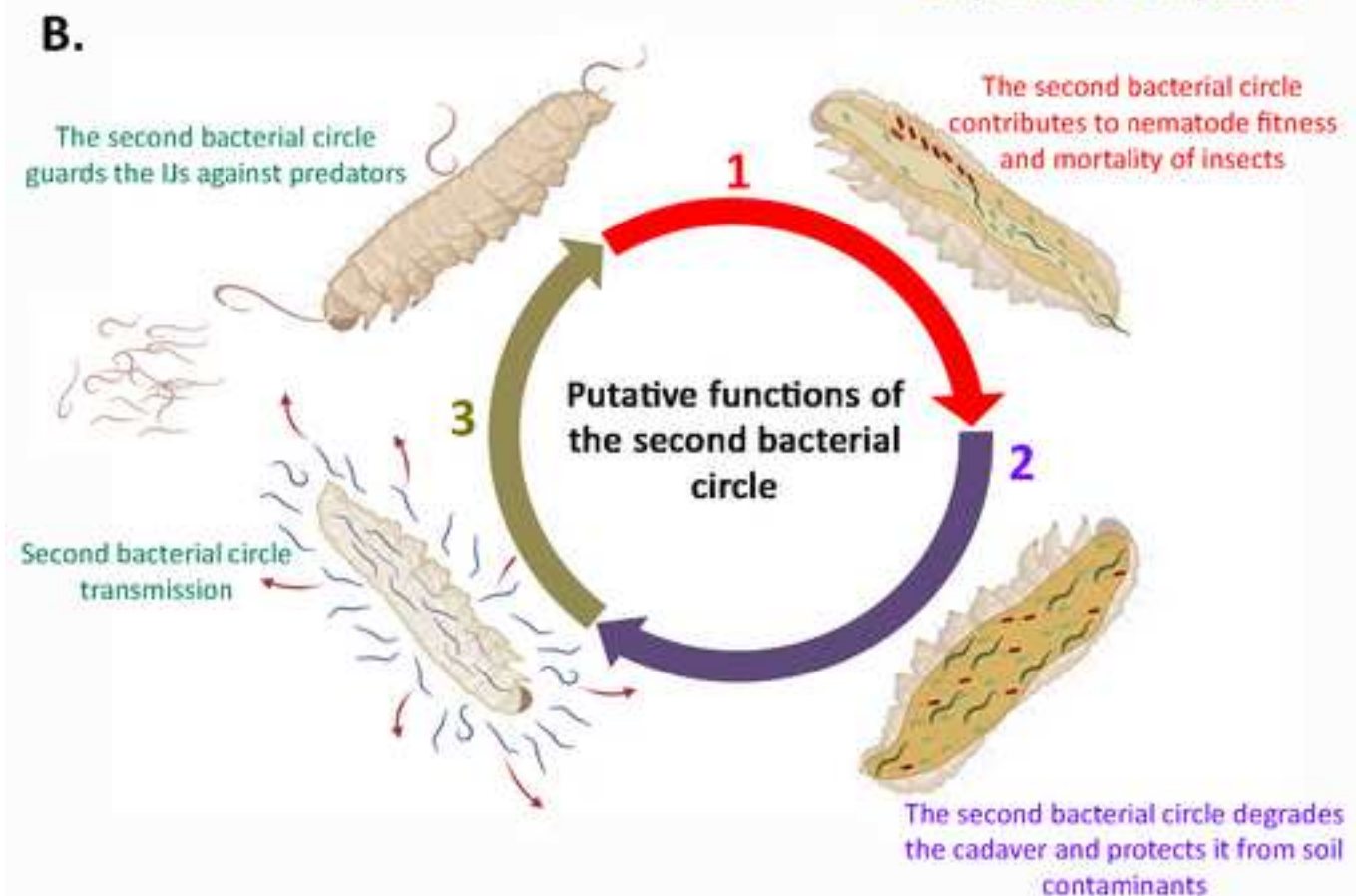
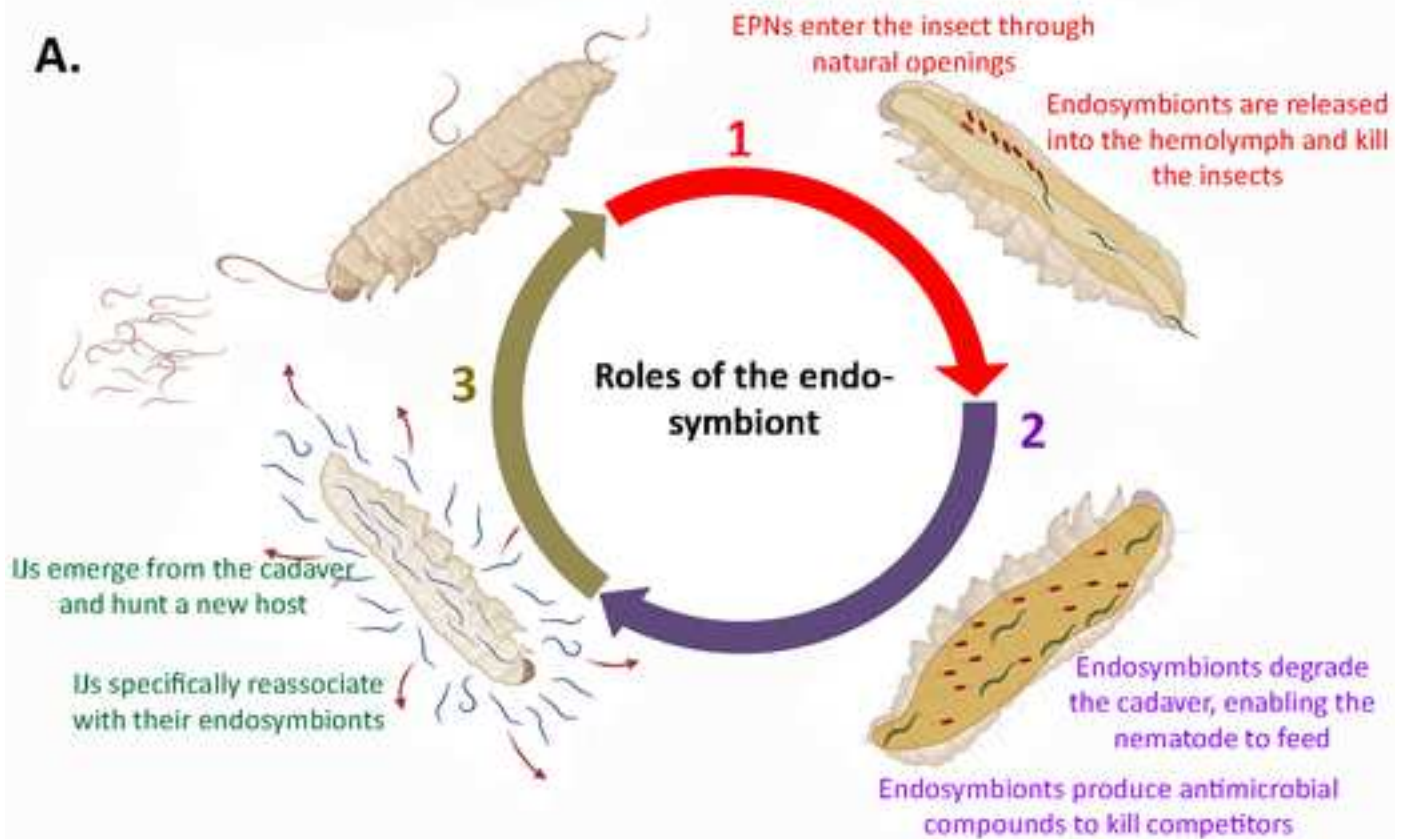
- Could the second bacterial circle improve the overall fitness of nematodes, particularly in unfavorable natural soil environments that might be expected to be less favorable than standardized laboratory conditions?
- At which offstage in the parasitic lifecycle of the EPN does the second bacterial circle plays a critical role?
- Does completion of the parasite lifecycle depend on keystone species or keystone functions within the second bacterial circle?
- In which tissues or organs of the nematodes are the second circle bacteria located (gut, intercuticular space, surface) and do molecular supports for specific association occur within these tissues?
- What is the mode of transmission of second bacterial circle, and is this transmission vertical, horizontal, or pseudohorizontal?
- What kinds of social relations (antagonism, cooperation, cheating, bet-hedging) exist between the members of the first and second bacterial circles and within the second bacterial circle?

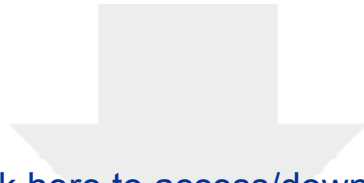
Figure 1

1962-now: cultural description of other EPN and other EPN-like nematode-associated bacteria, long observed but only recently studied: *Pseudomonas*, *Stenotrophomonas*, *Achromobacter*, *Providentia*, *Alcaligenes*, *Serratia*, *Acinetobacter*, *Delftia*, *Ochrobactrum*, *Proteus*...

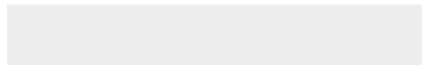
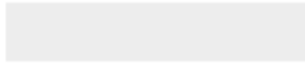


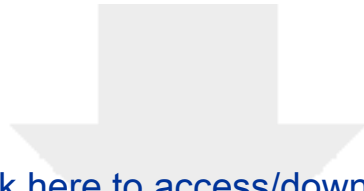




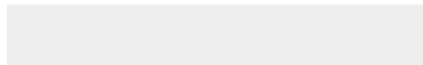


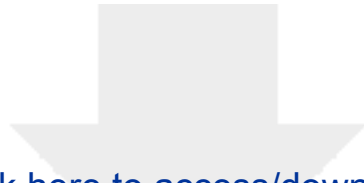
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