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The endosymbiont and the second bacterial circle of entomopathogenic nematodes

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Abstract

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

Key-words

entomopathogenic nematodes, pathobiome, next-generation sequencing

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

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

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

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

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

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

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

The endosymbiotic bacteria of canonical EPNs

The canonical EPNs belong to the genera *Steinernema* and *Heterorhabditis*. The first specimen of *Steinernema kraussei* was described in the 1920s [11] (Figure 1) and EPNs were first used in biological control programs in the 1930s, when *Steinernema glaseri* was used to control the Japanese cockchafer [12]. However, despite reports of associations between bacteria and non-feeding, infective juveniles (**IJs**) of *Steinernema* as early as 1937 [13], no other specific connections between *Steinernema* and a bacterial species were identified until the 1960s.

The initial model was *Neoaplectana carpocapsae* (= *Steinernema carpocapsae*), investigated by Poinar and Thomas, who showed that this nematode was the vector of the bacterium *Achromobacter nematophilus* (= *Xenorhabdus nematophila*), which was pathogenic to insects infested with the nematode or following direct injection into the hemolymph [14](Figure 1). *Xenorhabdus nematophila* was not pathogenic by ingestion and had never been isolated from the environment. The authors therefore assumed that it must be inoculated into the insect by the nematode, leading to the induction of septicemia and providing ideal conditions for the reproduction of the nematode within the insect cadaver [14]. This parasitism phenomenon was thought to result from a mutualistic partnership between the nematode and its bacterium, *X. nematophila,* acting together to kill the insect host. However, Weiser and coworkers were unable to isolate *X. nematophila* from *S. carpocapsae*; they instead isolated a microbial population consisting principally of pseudomonads [15]. Finally, Boemare's group isolated *X. nematophila* and other Enterobacteriaceae and Pseudomonadaceae from *S. carpocapsae* [16,17], reconciling the findings of Poinar's team in the US and Weiser's team in Czechoslovakia. All these bacteria were isolated from the IJ, the only stage occurring freely in nature. Their frequency was variable, except for *X. nematophila*, which was almost always present. Bird and Akhurst then showed that *X. nematophila* was maintained within a special intestinal vesicle in the free-living form of the nematode [18], subsequently renamed the **receptacle** [19] (Figure 2.A). Both this isolation within the organism and the specialized structure dedicated to housing *X. nematophila* made it seem likely that *X. nematophila* was the only endosymbiont in *S. carpocapsae*. Bacterial isolations from the IJs of other *Steinernema* species systematically led to the identification of other *Xenorhabdus* species, further supporting the concept of a symbiotic relationship [5].

*Heterorhabditi*s, another EPN genus, and its endosymbiotic bacterium, *Photorhabdus luminescens,* initially named *Xenorhabdus luminescens,* were then described [20,21] (Figure 1). Unlike *Steinernema*, the nematodes of *Heterorhabditis* have no specialized receptacle to house their symbiotic bacteria, which are instead diffusely spread throughout the intestinal lumen of the anterior digestive tract [22] (Figure 2.B).

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

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

The putative symbionts of EPN-like nematodes

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

Caenorhabditis briggsae and *Oscheius* are often associated to the genus *Serratia*, which facilitates pathogenicity (Table 1; criterion 1) [30,32,37,39,40]. Strains of this genus are known to have entomopathogenic properties with a broad host spectrum [41]. The genomes of the putative symbionts of *C. briggsae* KT0001 and *O. chongmingensis* — *S. marcescens* SCB1 and *S. nematodiphila* DSM21420, respectively —harbor substantial numbers of genes encoding secreted proteases, lipases, and hemolysins common to *Photorhabdus* and *Xenorhabdus* [31,42]. Based on the current state of knowledge, these *Serratia* may be considered putative endosymbiotic bacteria associated with *C. briggsae* and *Oscheius* sp..

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

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

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

Despite frequently being ignored by the pioneers describing EPNs, bacteria other than endosymbionts have actually often been detected by Pasteurian isolation methods on culture media. As far back as the 1960s, the presence of several bacterial species regularly associated with the IJs *S. carpocapsae* was described, and similar results were obtained during investigations on other EPN species (Table 2). These bacteria were isolated from IJs or EPN-infested cadavers and most were Proteobacteria. Depending on the study concerned and the IJ washing method used (bleach solution, streptomycin and penicillin, merthiolate), these bacteria were still detected after surface washing [16,17,45–48] or were not detected [49,50]. These findings led some authors to suggest that bacteria other than symbionts might reside in the gut lumen of the nematodes. Moreover, bacteria between the two cuticles enveloping *Steinernema scapterisci* IJs were observed by microscopy [51]. As bleach disinfection leads to elimination of the second cuticle, it was suggested that non-symbiotic bacteria might be located between the two cuticles [50,51]. Similar bacterial associates have been detected with *Heterorhabditis* (Table 2). In this nematode genus, dixenic associations were detected with *Ochrobactrum* spp. [52], *Providencia rettgeri* and *Paenibacillus* spp. [53].

The rapid development of **NGS** over the last decade has increased the capacity of researchers to characterize entire microbial communities in complex samples rapidly, to detect unculturable microorganisms, to discover new organisms and to explore the dynamic nature of microbial populations. Interestingly, these approaches supported previous Pasteurian descriptions of a microbiota associated with EPNs. Metabarcoding techniques were used to monitor bacterial dynamics in the cadaver of insect larvae *Galleria mellonella* after infestation with *Heterorhabditis.* Bacteria of the genus *Stenotrophomonas* were found to be abundant in the insect cadaver, through their ability to grow in the presence of antibiotics (stilbene) produced by the endosymbionts [54]. The IJs carried *Stenotrophomonas* spp. on their external surfaces. The authors therefore suggested that *Stenotrophomonas* is probably introduced into the insect larva via the nematode. The metabarcoding method was recently used simultaneously with two taxonomic markers to describe the bacterial communities associated with *S. carpocapsae* reared in different laboratories (France, USA) [55,56]. The authors identified: (i) a core microbiota composed of the endosymbiont *X. nematophila*; (ii) a subset of about ten OTUs called **FAM** (frequently-associated microbiota), (iii) a more variable microbiota. The FAM includes Proteobacteria from the genera *Pseudomonas, Stenotrophomonas*, *Achromobacter* and *Alcaligenes*, and the family Rhizobiaceae (*Ochrobactrum*, *Pseudochrobactrum*) [56]. These molecular results were confirmed by repeated isolation of bacteria from these genera such as *Pseudomonas protegens* from *S. carpocapsae*, *S. glaseri*, *Steinernema weiseri* and *S. feltiae* [56,57]. Almost all the members of the FAM were detected in a nematode freshly collected in the field, confirming that they were not artifacts of laboratory rearing [56]. To distinguish them from the bacterial endosymbionts, we refer to these other EPN bacterial communities as the second bacterial circle (Figure 1). In the EPN-like nematodes, a bacterial consortium in addition to *Serratia* has also been described (Table 1 and Figure 1) [40,58,59]. Second bacterial circle status requires further validation by a metagenomic study in a more diverse range of EPN-like isolates.

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

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

Is the second bacterial circle involved in the EPN pathobiome?

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

Entry into the living insect, causing infection and death

The IJ rapidly loses its outer cuticle after entering the insect intestine [73]. Members of the second bacterial circle located between the two cuticles might therefore be released early into the insect gut, where they could protect the nematode by producing factors enabling the nematode to escape the insect immune system and or by secreting molecules (e.g. chitinase, proteases, pore-forming toxins) destabilizing the intestinal epithelium. For example, *P. protegens* and *Pseudomonas chlororaphis*, associated with several *Steinernema* species [56,57], secrete the Fit insecticidal toxin, which has been shown to be responsible for entomopathogenicity when ingested, into the insect gut [74]. Once in the hemolymph, organisms from the second bacterial circle may also participate in the killing of the insect. Some bacterial isolates, from *P. fluorescens*, *Serratia* sp., *P. rettgeri*, *Alcaligenes faecalis,* and *P. protegens*, have been shown to display entomopathogenic activities after direct injection into the hemolymph of several lepidopteran species [15,56,57,71,75,76].

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

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

Nematode reproduction in the insect cadaver

The insect hosts may be co-infected by an EPN and another entomopathogenic agent as well as by several EPNs. During dual coinfection with *Bacillus thuringiensis* (Bt), competitive interaction exists between Bt and the endosymbiont for food resources [84]. During coinfection between *S. affine* and *S. feltiae*, it has been shown that the *S. affine* endosymbiont directly kills reproductive stages of *S. feltiae* [85]. One could envisage that such modulations of competition are also dependent on some members of the second bacterial circle.

The second bacterial circle could play indirect roles in nematode reproduction. For example, it could provide the nematode with nutrients, by decomposing the insect cadaver through the secretion of extracellular enzymes. *Pseudomonas* and *Stenotrophomonas* species, which are frequently associated with EPNs, are known to produce various enzymes, such as proteases, lipases, and chitinases [74,86]. The second bacterial circle may also protect nematodes against pathogens, and prevent putrefaction of the cadaver. The strong antimicrobial activities of members of the second bacterial circle observed *in vitro* may help to eliminate microbial competitors during nematode multiplication in the cadaver [54,56].

Transmission and dissemination of the parasitic complex in soils

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

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

For dissemination and survival in the soil until the next encounter with an insect larva, nematodes may rely on abundant progeny, but also on the development of IJ defense strategies against soil biotic agents. IJs can be negatively affected by soil bacteria, such as *Paenibacillus* that exploit them for their own dispersal [88]. In the same way that isolates of the *P. fluorescens* subgroup belonging to the *C. elegans* microbiota protect the worms against infection by *B. thuringiensis* via metabolite synthesis [89], the second bacterial circle could provide a defense function for the IJ against such deleterious bacteria. Nematophagous fungi are the most important and well-studied pathogens affecting EPNs [88]. The second-stage cuticle protects the third stage IJs from fungal infection [84]. There is currently no evidence to suggest that this may involve microbial action, but it may be relevant to investigate the antagonistic properties of intercuticular bacteria from the second bacterial circle against these nematophagous fungi.

Consequences for biocontrol application of EPNs

EPNs are used as biocontrol agents for insects. The ecological risks of EPN application have long been assessed and the impact of EPNs on non-target organisms (e.g. earthworms, toads, mice, chickens, rabbits and guinea pigs) is limited or non-existent [90]. However, many members of the second bacterial circle belong to genera, such as *Pseudomonas*, *Stenotrophomonas* and *Ochrobactrum*, which encompass a few animal and human pathogens. The taxonomy of some of these species is still unclear, because of their high genotypic and phenotypic variability, host ranges and symbiotic abilities [86,91]. Following this new polyxenic view of the EPN life cycle, further taxonomic characterization should be therefore carried out to provide an accurate risk assessment survey concerning EPN soil applications

On the other hand, some of the species of this second circle are reported to have beneficial properties for plant health. For example, the rhizospheric isolates of the species *P. protegens* and *P. chlororaphis* stimulate plant growth and express antagonistic properties towards plant pathogens [74]. The association between EPN and such members of the second bacterial circle could expand their areas of application in the agricultural domain.

Concluding remarks and future perspectives

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

Could the second bacterial circle improve the fitness of nematodes and contribute to the EPN pathobiome? A role for the second bacterial circle in killing insects seems likely, as some members are entomopathogenic. The roles of these bacteria in other phases of the EPN lifecycle remain unclear. Here, we suggest a paradigm shift in the description of EPN pathogenesis, from a tripartite model (insect-nematode-endosymbiont) to a more complex model taking into account the whole EPN microbiota (bacterial endosymbiont and second bacterial circle). This paradigm shift accompanies the transition from Koch's postulates to an enlargement of the pathobiome concept.

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

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Glossary

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Figure Legends

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

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

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

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

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

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

Figure 3. The three steps of the EPN lifecycle

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

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

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

Table 1. EPN-like nematodes and their associated bacteria

		Criterion 1: symbiotic relationship between bacteria and the nematodes facilitates pathogenicity			**Criterion 2:** insect death should be sufficiently rapid and significant [2]			
Nematode species	**Putative bacterial symbiont** [1]	**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]	**Other bacteria isolated**[1]	**Reference**
***Caenorhabditis briggsae* KT0001**	***Serratia marcescens* SCBI** ^{N}	**YES**	nd	nd	**NO**	**YES**	nd	[30]
Oscheius (Heterorhabditidoides) chongmingensis	***Serratia nematodiphila* DZ0503SBS1** $^{N, I}$	**YES**	**YES**	nd	**VAR**	**YES**	*Proteus* sp. $^{N, I}$ *Acinetobacter* sp. $^{N, I}$ *Ochrobactrum tritici* $^{N, I, *}$ *Bacillus cereus* $^{N, I, *}$	[32,33,44,58,93]
Oscheius carolinensis	***Serratia marcescens*** $^{N, I}$	**YES**	**YES**	nd	**YES**	**YES** (but by topical application)	*Achromobacter xylosoxidans* $^{N, I}$ *Enterococcus mundtii* $^{N, I}$ *Providentia rettgeri* $^{N, I}$	[34,40]
Oscheius (Heterorhabditidoides) rugaoensis	***Serratia nematodiphila*** $^{N, I}$	**YES**	nd	**YES**	**YES**	nd		[37]
Oscheius gingeri		nd	nd	nd	**YES**	nd		[35,36]
Oscheius onirici		nd	nd	nd	**VAR**	nd	unidentified rod-shaped bacteria ^{M}	[38,94,95]
Oscheius myriophila	***Serratia marcescens* 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 $<10^5$ 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

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

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

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

Outstanding_Questions_Box

- 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

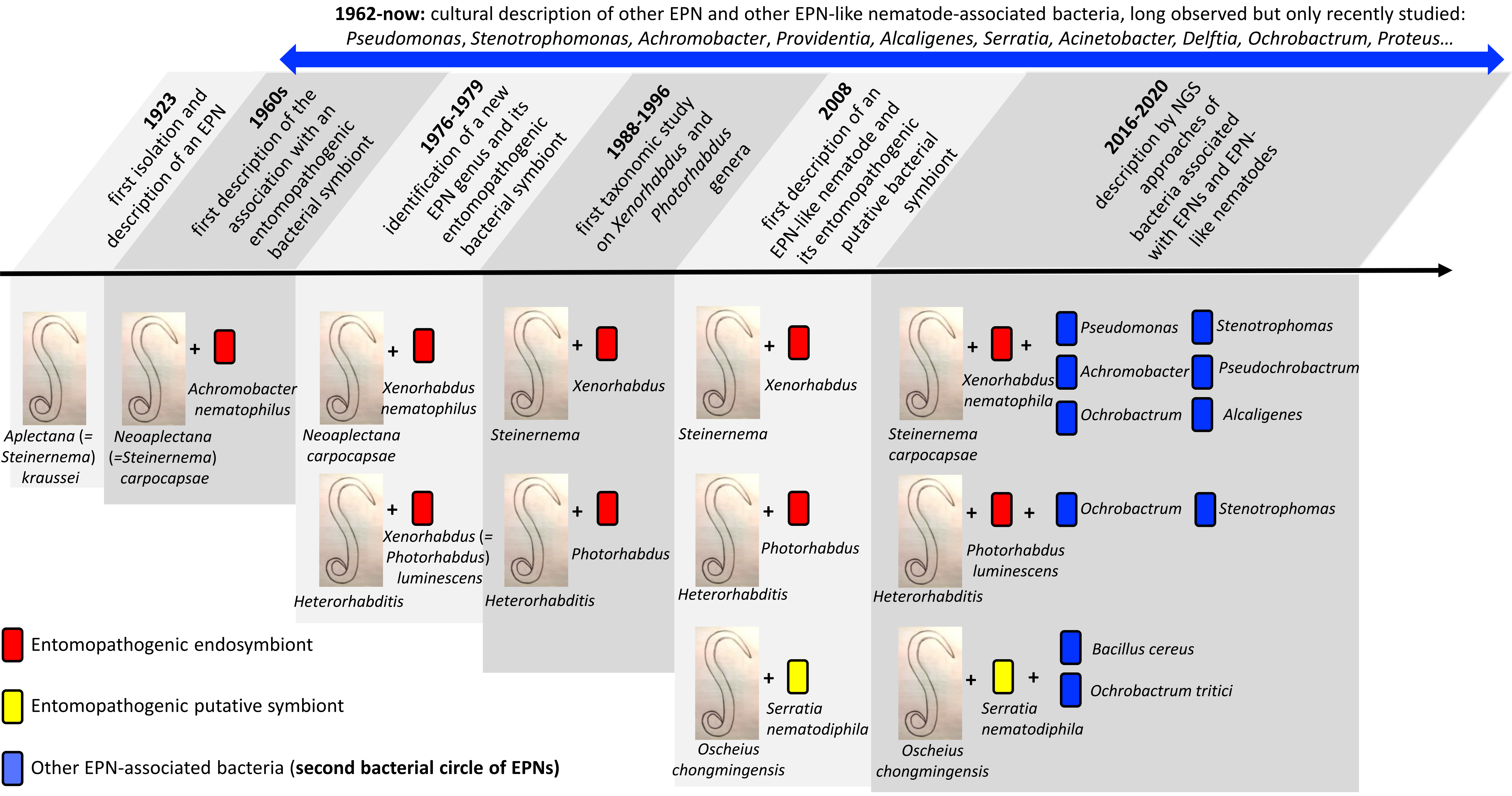

1962-now: cultural description of other EPN and other EPN-like nematode-associated bacteria, long observed but only recently studied:
Pseudomonas, Stenotrophomonas, Achromobacter, Providentia, Alcaligenes, Serratia, Acinetobacter, Delftia, Ochrobactrum, Proteus…
1923 first isolation and description of an EPN
1960s first description of the association with an entomopathogenic bacterial symbiont
1976-1979 identification of a new EPN genus and its entomopathogenic bacterial symbiont
1988-1996 first taxonomic study on *Xenorhabdus* and *Photorhabdus* genera
2008 first description of an EPN-like nematode and its entomopathogenic putative bacterial symbiont
2016-2020 description by NGS approaches of bacteria associated with EPNs and EPN-like nematodes
Aplectana (= Steinernema) kraussei
+
Achromobacter nematophilus
Neoaplectana (=Steinernema) carpocapsae
+
Xenorhabdus nematophilus
Neoaplectana carpocapsae
+
Xenorhabdus (= Photorhabdus) luminescens
Heterorhabditis
+
Xenorhabdus
Steinernema
+
Photorhabdus
Heterorhabditis
+
Xenorhabdus
Steinernema
+
Photorhabdus
Heterorhabditis
+
Serratia nematodiphila
Oscheius chongmingensis
+
Xenorhabdus nematophila
+
Pseudomonas
Stenotrophomas
Achromobacter
Pseudochrobactrum
Ochrobactrum
Alcaligenes
Steinernema carpocapsae
+
Photorhabdus luminescens
+
Ochrobactrum
Stenotrophomas
Heterorhabditis
+
Serratia nematodiphila
+
Bacillus cereus
Ochrobactrum tritici
Oscheius chongmingensis
🟥 Entomopathogenic endosymbiont
🟨 Entomopathogenic putative symbiont
🟦 Other EPN-associated bacteria (**second bacterial circle of EPNs**)

Figure 2

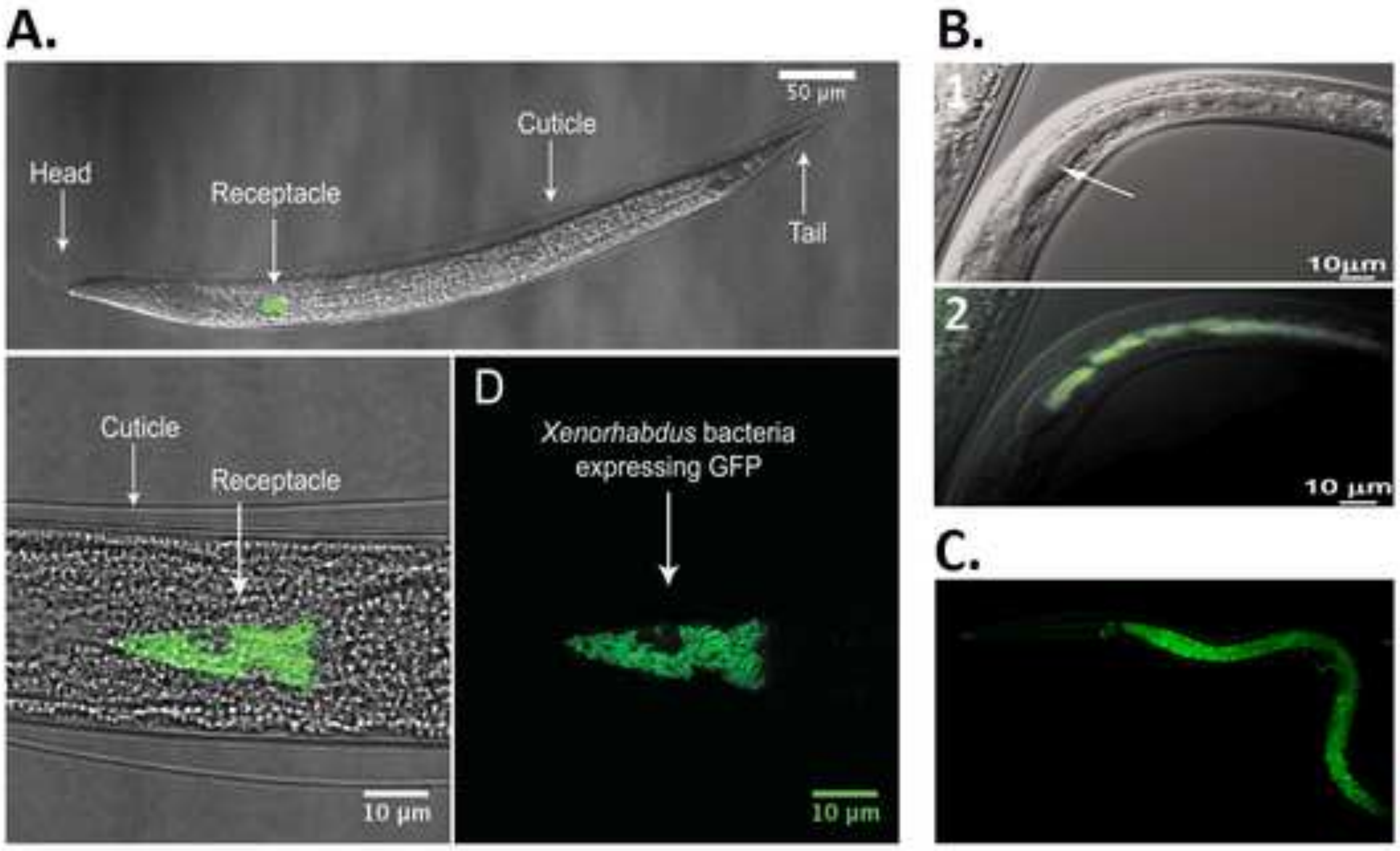
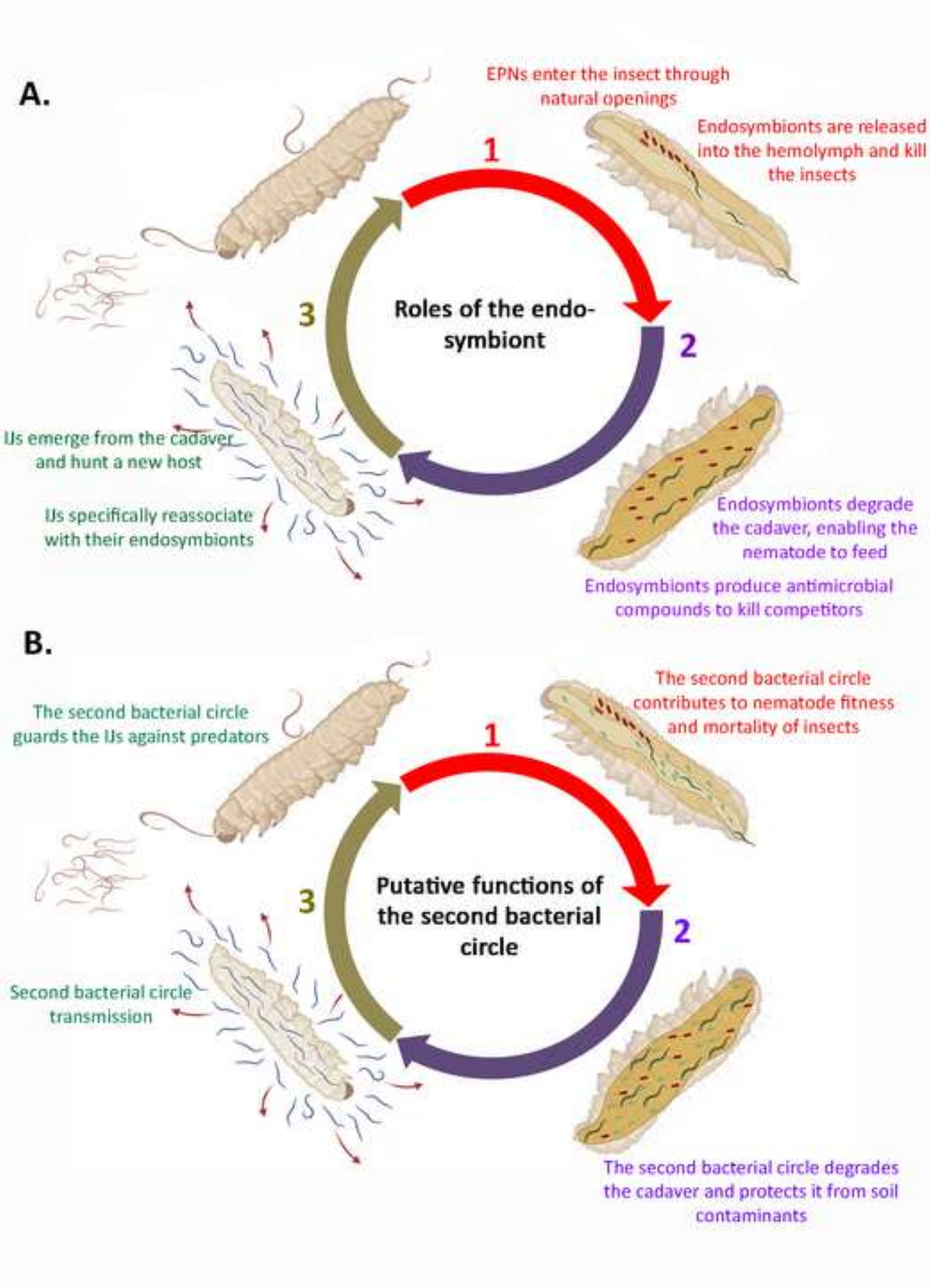

A.
50 µm
Head
Receptacle
Cuticle
Tail
Cuticle
Receptacle
10 µm
D
Xenorhabdus bacteria
expressing GFP
10 µm
B.
1
10µm
2
10 µm
C.

Figure 3


A.
EPNs enter the insect through natural openings
Endosymbionts are released into the hemolymph and kill the insects
1
Roles of the endo-symbiont
3
2
IJs emerge from the cadaver and hunt a new host
IJs specifically reassociate with their endosymbionts
Endosymbionts degrade the cadaver, enabling the nematode to feed
Endosymbionts produce antimicrobial compounds to kill competitors
B.
The second bacterial circle guards the IJs against predators
The second bacterial circle contributes to nematode fitness and mortality of insects
1
Putative functions of the second bacterial circle
3
2
Second bacterial circle transmission
The second bacterial circle degrades the cadaver and protects it from soil contaminants

Copyright Fig2A

Copyright Fig2B

Copyright Fig2C