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THE COMPLEX AND MULTIFORM RELATIONSHIP BETWEEN NEMATODA AND BACTERIA

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Clausi M., Rappazzo G., Vinciguerra M.T. – The complex and multiform relationship between Nematoda and Bacteria.

The relationship between nematodes and bacteria has taken place in different ways, from the simple trophic relation to the most sophisticated forms of symbiosis. Such symbiosis is not a primitive condition but it appeared and was established, being advantageous, during the evolution of different nematode taxa, independently and with different, sometimes complex, modalities ranging from mutualism to parasitism. The most significant modalities of mutualism are exposed here with particular stress on the highly specific relationship between entomopathogenic nematodes of the families Heterorhabditidae and Steinernematidae and their symbionts. This relationship, fruit of co-evolution between the nematodes and their bacteria, is treated from different points of view and at the light of the most recent genetic research.

KEY WORDS: Entomopathogenic nematodes, Evolution, *Photorhabdus*, Symbiosis, *Xenorhadus*.

There are no ecosystems, aquatic or terrestrial, without rich populations of bacteria, organisms of a primary ecological role due to their ability in decomposing organic molecules to inorganic ones, again utilizable by living organisms. Due to their abundance and diffusion in nature, they constitute the elective food for an enormous number of species, belonging to virtually all the phyla of animals, which feed on them either from the substratum or filtering from water. Nematodes do not constitute an exception and numerous are the taxa feeding on bacteria, often developing complex relationship patterns with them.

POTTS (1910) was the first to hypothesize that the free-living nematodes without a buccal stylet, which were found in great number in decaying organic matter, were not saprophagous but fed on the bacteria which there were abundant. This hypothesis was then confirmed by STEINER (1914), who observed the intestinal content of a species of *Rhabditis*; it was later demonstrated experimentally by MCCOY (1929), who grew larvae of *Ancylostoma* on bacterial cultures, and mainly by NIELSEN (1949), who grew numerous species of Rhabditida, Plectida, Araeolaimida, Monhysterida, Chromadorida and Enoplida. BRIGGS (1946) observed that a given species of nematodes can feed only on some species of bacteria. To this purpose very relevant for the comprehension of the relationship between nematodes and bacteria were some experimental works conducted by CAYROL & B'CHIR (1973) and CAYROL & DREYFUS (1975). These authors tried to grow some species of *Rhabditis* on the same organic substrate, both after having disinfected them of the microorganisms possibly present in their bodies and without such disinfection. In the former case the nematodes did not survive for more than a couple of days, while in the latter they proliferated quickly and an abundant bacterial population grew up in the substrate. It apparently happens because, for a given species of nematodes, only some bacteria possess all the nutrients needed by that nematode to develop and to reproduce quickly; other bacteria allow only a very slow and poor development, while others do not consent at all the nematode survival and development. For this reason these nematodes keep inside their intestine the bacterial complexes useful to their survival, which they disseminate in the organic substrate so to obtain a great quantity of the nutrients needed. It was also observed that these nematodes released in the substrate substances stimulating bacterial growth and proliferation. The same authors, moreover, showed that each nematode species tested was associated to a definite bacterial complex, different for each of them, even though all the species were coming from the same environment.

The existence of such mutualistic symbiosis between nematodes and bacteria points out the importance of the nematode role in the biological processes of soil. Disseminating their bacteria and favoring their proliferation, nematodes improve the organic matter decomposition process; moreover, they can play either a positive or a negative role if they disseminate bacteria useful or noxious to a given culture.

Coming back to the nature of the relationship between nematodes and the bacteria on which they feed, it is evident that such symbiosis is not a primitive condition but it appeared and was established, being advantageous, during the evolution of different nematode taxa, independently and with different, sometimes complex, modalities ranging from mutualism to parasitism. We will expose here only the most significant modalities of mutualism.

The mutualistic symbiosis between nematodes and bacteria can be characterized by different degrees of specificity. Some Rhabditidae and Diplogasteridae defined "necromenic" (SUDHAUS & SCHULTE, 1988) live as parasites of soil invertebrates: the Dauer Juvenile (DJ) enters its host through body openings or when ingested with the food; it remains quiescent in the host until the latter dies and its body is invaded by saprophytic bacteria; then the DJ resumes development and growth and reproduces thanks to the presence of the bacteria associated with the decaying insect. A further example of non-specific symbiosis concerns some species of *Phasmarhabditis*, parasites of snails: no specific bacterium has been isolated systematically from their larvae and the nematodes can survive *in vitro* also in axenic environment, but in such a case they loose virulence against the mollusks. Though the life cycle can be complet-

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ed with several species of bacteria, still the highest degree of virulence is achieved in association with *Moraxella oslensis* (TAN & GREWAL, 2001).

A highly specific interaction has evolved in the entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae and in their symbiotic bacteria. In such a case a very strict relationship has developed between the three partners, nematode, bacterium and insect, where the bacterium has a relation of mutualistic symbiosis with the nematode and of parasitism with the insect. No other nematodes seem to have a symbiotic relation so complex and perfect as that showed by them.

Though Steinernematidae and Heterorhabiditidae are not closely related (BLAXTER *et al.*, 1998), they share many features, including the association with insect-pathogenic bacteria. These similarities are presumed to have arisen through convergent evolution. In both families there is a single free-living not feeding stage, the infective juvenile (IJ), that carries in its gut bacteria of the genus *Xenorhabdus* and *Photorhabdus* respectively. It, encountering a suitable insect, enters through the mouth, anus or spiracles and reaches the haemocoel. In both genera the IJ releases in the haemocoel its bacterial symbionts which proliferate in the nutrient-rich insect haemolymph, causing the insect death in 24-48 h from septicemia or toxemia (FORST *et al.,* 1997). In the insect the IJs start feeding on the proliferating bacteria and digested host tissues; they develop to the adult stage and then reproduce. The symbionts occupy two different ecological niches in their life cycle: the first is a phoretic state where the bacteria are retained in the intestine of IJ apparently without any significant multiplication. The second stage is a vegetative one when the bacteria overcome the insect host's defense system and multiply in its haemolimph. Therefore the bacteria live both in the nutrient-lacking nematode intestine and in the insect cadaver very rich in nutrients. The bacteria are carried monoxenically in the IJ.

Both nematodes and bacteria can be cultured separately, but their specific association allows a high degree of sinergy: the nematodes reproduce with success if their natural symbionts are present and the bacteria do not survive for long without their nematode. Both partners take an advantage of the association: the bacteria, killing rapidly the insect, provide the nutrients suitable to the growth and reproduction of the nematodes and suppress the competitors by producing antibiotics; the nematode protects the bacteria, carries them inside the insect haemolymph and, in some cases, inhibits the insect defence system. In *Heterorhabditis, Photorhabdus* colonizes the proximal part of IJ intestine; it has been observed that such colonization is transmitted by the mother through *endotokia matricida*, a process where the juvenile develops consuming mother's body. Before such process the mother rectal glands are infected by cells of *Photorhabdus* which will be inoculated into the new generation of IJs; these then reproduce in the nematode intestine giving origin to a mature bacterial population (CICHE and ENSIGN, 2003). Thus, in the colonization of the *Heterorhabditis* IJ by its symbiont there are three stages: 1) colonization of the mother's rectal glands, 2) colonization of IJ intestine and 3) reproduction. It is probable that the antimicrobial compounds produced by the operone pbgPE of *Photorhabdus* species cause a positive selection in the specificity *Heterorhabditis - Photorhabdus*. So antimicrobial barriers may play an important role in protecting the specificity of the symbiosis. As regards *Steinernema*, the mechanisms of the bacterial colonization have not yet been investigated; also in this case, however, the IJ colonization is realized by one or two bacterial cells which later multiply and settle in a special vesicle at pharynx base, distinct from intestine.

The relationship nematode-bacterium in the EPN is characterized by exceptional events: it is exceptional, for example, that the insect cadaver does not putrefy due to the intestinal bacterial flora, as usual. Moreover, in *Xenorhabdus*, the bacterial cells are preserved from being digested by their location in the special intestinal vesicle. This appears to be the result of co-evolution.

Recent studies have shown how in *Photorhabdus* and *Xenorhabdus* there may have happened a shifting from a pathogenic to a mutualistic relation, due to a regulatory control. Tests conducted on mutants of *Photorhabdus* have shown the role of the gene *HexA* in regulating the transition from pathogenic to symbiotic. *HexA* is a symbiosis repressor; it inhibits the transcription of at least 100 genes, many of which involved in the control of the stationary phase (typical of symbionts) and some mutants of this gene cause attenuation of virulence. This results suggest that the two molecular ways which cause the selection between the stationary (in the symbiont) and virulent (in the insect infection) phase are coincident at least in part.

The relationship nematode-bacterium in the EPN shows a very high degree of specificity: in the intestine of each nematode species of *Steinernema* and *Heterorhabditis* only a single symbiotic species has been found, with the exception of *H. bacteriophora*, which can be associated to two species of *Photorhabdus*. Otherwise, more species of nematodes can be associated to the same bacterium species: *X. bovienii, X. poinarii* and *X. kozodoi* are symbionts each of more species of nematodes. Actually, the symbiosis between nematodes and bacteria is a fascinating model of co-evolution, which we will try to resume here.

Morphological, paleontological and molecular studies have demonstrated that steinernematids and heterorhabditids do not belong to a monophyletic taxon but started independently their relationship with Arthopoda and with enteric bacteria gram-negative (like Enterobacteriaceae) about 350 millions years ago, during Paleozoic. *Heterorhabditis*, both from physiological and biogeographical studies, seems to have evolved from a *Pelliodites* stem group, free-living bacterial feeders, in a coastal environment (POINAR, 1993). Their associated bacteria are very similar to the luminescent marine genus *Lucibacterium*. As to steinernematids, POINAR (1993, 2003) suggested that *Steinernema* shares a common ancestor with *Rhabditophanes* (Alloionematidae). In some fossil ambers from Mexico going back to 22-26 millions years ago nematodes *Rhabditophanes*-like have been found. These nematodes are not associated with bacteria and only their adults or pre-adults develop inside insects. During evolution a *Rhabditophanes*-like ancestor would have acquired symbiotic bacteria related to *Xenorhabdus* and introduced them in body cavity of an insect. In such new location the bacteria would have reproduced favoring in turn the nematode development. In Poinar's hypothesis the bacteria should have been Enterobacteriaceae near to the genus *Proteus*. ADAMSON (1986) suggested that rhabditids parasite both of vertebrates and invertebrates took origin from free-living bacterial feeding nematodes. According to SUDHAUS (1993) heterorhabditids and steinernematids would have evolved from necromenic nematodes which developed a symbiotic association with entomopathogenic bacteria. In any case the bacteria associated to the two genera must have lost their capacity of giving origin to forms able to live freely in the environment, adapting to the protect life in the nematode or insect body.

The hypothesis that *Xenorhabdus/Photorhabdus* constitute a phyletic line belonging to Enterobacteriaceae is supported by data concerning 16S rRNA (RAINEY *et al*, 1995). Otherwise, JANSE e SMITS (1990), analyzing the production of fat acids, pointed out significant differences from Enterobacteriaceae. However, even accepting, with the majority of Authors, their origin from Enterobacteriaceae, it is common opinion that these genera show peculiar characteristics, both chemotaxonomical (such as the incapacity of reducing nitrates) and genotypic (as the sequences of the genes 16S rRNA) which indicate that they belong to a monophyletic group arising at the base of Enterobacteriaceae, that is with a significant divergence from this taxon, and that the most related genus is *Proteus*. The molecular analysis, however, reveals also a significant divergence between the two genera.

To define the association between EPN and bacteria in terms of co-evolution is the fundamental key to understand that the subject-object of the evolution are the two organisms together, due to a functional specialization of each EPN strain together with its symbiont. Being each EPN strain to be considered as a peculiar specialized mutualistic association, aim of the most recent research was to find valid tools to determine such association.

In our experience, the analysis of the sequence of the locus ITS1 of nematodes has revealed to be useful in most cases (mainly for *S. feltiae*) to realize a *fingerprint* of each isolate of a species. This locus is so variable that even small evolutionary distances can be revealed, even though these divergences must be used with some caution in the phylogenetic reconstruction.

The genome of the symbiotic bacteria is less known, except for *X. nematophila, X. bovienii, P. asymbiotica* and *P. luminescens*, which have been completely sequenced. The most used locus is the 16S rRNA, which however in bacteria is very conservative; for this reason the few variations observed cannot be "weighed" in the same way as the locus ITS1 of EPN. It would therefore be advisable not to use such locus any more for molecular phylogeny at intraspecific or even interspecific level. Alternative methods have been used by TAILLIEZ *et al.* (2006) and by LEE and STOCK (2010). In the former case primers for ERIC (Enterobacteriaceae Repetitive Intergenic Consensus) sequences and casual primers for RAPD experiments have been used in parallel so to obtain specific profiles able to distinguish species of the genus *Xenorhabdus*. Otherwise, LEE and STOCK (2010) used loci (recA, serC and 16S rRNA) known for evolving with constant speed and therefore more suitable to measure reliable evolutionary distances between the bacterial species.

The attribution of a same bacterium species to different EPN species (TAILLEZ *et al.*, 2006) can also be explained, at least in part, in terms of co-evolution between EPN and bacterium. During the research carried out in our laboratory, for example, we have identified the species *X. kozodoii* in two species of *Steinernema* of the *glaseri* group: the new species *S. vulcanicum* and *S. apuliae*, the ITS1 of which was not yet known; moreover, the sequences of further species of the same group which share the same symbiont were obtained from public database. It was interesting to note, reconstructing the phylogenetic tree of these *Steinernema* species, that the species sharing the same symbiont *X. kozodoi* belong to a same monophyletic clade and share a European geographical distribution. Also, the strains of *X. kozodoi* found in the above species are not genetically identical but they belong to a same monophyletic clade, even though not completely superimposable to that of the species host. Then, there is a host-symbiont specificity even when a same species of bacterium is common to more species of hosts. It might be hypothesized that the strains of *X. kozodoi* are still differentiating and have not yet reached the species level, taking also into account that the species definition in bacteria is rather problematic.

A very important objective of further research on these aspects will be the individuation of the characters which make each association unique. The identification of such characters (or markers) would make it easier to understand what is the key of such mutualistic relations.

As it can be inferred from the present, though very partial, review of the relations between nematodes and bacteria, the role that these play in nematode evolution is various and complex but always of major importance in the life strategy acquired by the different nematode taxa. Such interactions have been achieved many times and independently in the various phyletic lines, probably starting from the more common and simple trophic relationship. Only recently the possibility of sequencing the genome of these organisms has thrown new light on some aspects of this relationship and this research line, promising both for achieving new information on the phylogeny of nematodes and bacteria and for the possible applications, will certainly give interesting results in the near future.

RIASSUNTO

LA COMPLESSA E MULTIFORME RELAZIONE FRA NEMATODI E BATTERI

La relazione fra nematodi e batteri si realizza in modi assai diversi, dalla semplice relazione trofica alle più sofisticate forme di simbiosi. Tale simbiosi non è una condizione primitiva ma è apparsa e, in quanto vantaggiosa, si è affermata più volte durante l'evoluzione di diversi taxa di nematodi con modalità differenti, talvolta assai complesse, che vanno dal mutualismo al parassitismo. Qui vengono illustrate le modalità più significative di mutualismo, con particolare riferimento alla relazione altamente specializzata fra i nematodi entomopatogeni delle famiglie Heterorhabditidae e Steinernematidae e i loro simbionti. Questa relazione, frutto di coevoluzione tra i nematodi e i loro batteri, è sviluppata da differenti punti di vista anche alla luce delle ricerche genetiche più recenti.

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