Chapter 17
Molecular Regulators of Entomopathogenic Nematode–Bacterial Symbiosis

Ioannis Eleftherianos and Christa Heryanto

Abstract Entomopathogenic nematodes are parasitic organisms with an exceptional capacity to infect rapidly and efficiently a wide range of insect species. Their distinct pathogenic properties have established entomopathogenic nematodes as supreme biocontrol agents of insects as well as excellent models to simulate and dissect the molecular and physiological bases of conserved strategies employed by parasitic nematodes that cause infectious diseases in humans. The extreme infectivity of entomopathogenic nematodes is due in part to the presence of certain species of Gram-negative bacteria that live in mutualistic symbiosis during the infective juvenile stage, which forms the central part of the nematode life cycle. Both nematodes and their mutualistic bacteria are capable of interfering and undermining several aspects of the insect host innate immune system during the infection process. The mutualistic bacteria are also able to modulate other biological functions in their nematode host including growth, development, and reproduction. In this review, we will focus our attention on the mutualistic relationship between entomopathogenic nematodes and their associated bacteria to discuss the nature and distinct characteristics of the regulatory mechanisms, and their molecular as well as physiological components that control this specific biological partnership.

Keywords Entomopathogenic nematodes · Bacteria · Symbiosis · Parasitism · Pathogenicity · Gene expression

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17.1   Introduction

Bacteria–host interactions are ubiquitous in nature (Ruby 2008). They form complex relationships that influence critical biological processes such as the nutrition, development, and immunity of plants and animals (Hentschel et al. 2000; Ochman and Moran 2001). Relationships range from being ancient, stable, and beneficial mutualisms, as exemplified by the origin of the mitochondria and chloroplasts from the endosymbiosis, to those which are more recent, dynamic, and highly pathogenic, such as the evolution of *Yersinia pestis*, the causative agent of plague, from a relatively benign ancestor (Sagan 1967; Parkhill et al. 2001). Although the outcomes of these interactions have very different consequences for their hosts, there is increasing evidence that common mechanisms regulate the ability of bacteria to act as either mutualists or pathogens. Both lifestyles are thought to have evolved from living in close proximity to their hosts and both require the ability to circumvent host immunity and modulate the host environment (Dale and Moran 2006). The diversity of these associations and their importance to medicine and agriculture define them as a key area for research (Ochman and Moran 2001; Maurelli 2007). Recent works have adopted model organisms to determine the overlap between the nature of molecular signaling pathways and their specific genes that are necessary for regulating mutualism and pathogenicity lifestyles in bacteria and their invertebrate hosts. Understanding the genetic mechanisms and dictating the outcomes of bacteria–host interactions will ultimately allow us to determine how microbes switch from one lifestyle to another, thus shedding light on the evolution of complex multiorganism relationships.

Insight into the delicate balance between mutualism and pathogenicity requires a system that allows for the direct study of both interactions (Chaston and Goodrich-Blair 2010). Entomopathogenic nematodes are microscopic worms that target and naturally infect a diverse range of insect hosts, and therefore, they have been implemented in modern agricultural practices as promising biological control agents and alternatives to chemical insecticides for managing destructive insect pests of plants and deleterious vectors of infectious diseases. Due to their remarkable pathogenic properties toward various insect stages, and their unique life cycle that involves mutualistic cooperation with specific bacterial species, entomopathogenic nematodes have been employed in recent years in biomedical research as outstanding and simultaneously environmentally safe tools for unraveling the molecular and physiological basis of mutualistic relationships in animals, and resolving pathogenicity mechanisms in nematode–bacterial complexes, in relation to the host innate immune function. The mutualistic bacteria of entomopathogenic nematodes perform critical biological tasks that are not restricted only to promoting pathogenicity and compromising the insect immune system during infection, but they also protect their nematode host by producing antimicrobial molecules to support the growth of other competitive bacteria. In addition, they promote nematode dispersal and development, growth, and reproduction by supplying nutrients from the bioconverted insect

tissues and organs, as well as by acting themselves as a rich food source (Herbert and Goodrich-Blair 2007a).

Application of entomopathogenic nematodes in the field requires careful analysis of various traits of parasites and their associated bacteria acting together as a complex or as a separate one, during the distinct phases of their life cycle. Due to the lack of understanding of the molecular and physiological determinants that control the harmonious coordination between the two mutualistic players, it is considered imperative to invest future efforts and resources on deconstructing the life cycle of different entomopathogenic nematode species to expose the exact elements that enhance or diminish the interaction with the insect host. This approach would, in turn, provide us with the necessary knowledge to validate the attributes that increase the performance of entomopathogenic nematodes, and improve their stability and efficiency in the field. Such information would ultimately enable us to make convenient interventions to refine biocontrol programs that would reduce pesticide use and improve food safety and production.

17.2 Entomopathogenic Nematode–Bacterial Complexes

Nematode–bacterial complexes with insect pathogenic properties are formed specifically in the soil nematodes of the genera *Heterorhabditis* and *Steinernema*, which develop mutualistic relationship with the proteobacteria *Photorhabdus* spp. and *Xenorhabdus* spp., respectively. The nematodes together with their associated bacteria undergo a complex life cycle that comprises two stages; a mutualistic stage that takes place in the nematode gut, during which the bacteria are vectored by their cognate nematode and a pathogenic stage that occurs in the insect host during infection, and involves the manipulation of humoral and cellular innate immune defenses by both partners that lead to the accelerated insect death. Although entomopathogenic nematode life cycles exhibit similar characteristics, variation especially in certain features of nematode reproduction and population growth rate, as well as in host range and phase variants of mutualistic bacteria, can be observed among different genera and species (Forst et al. 1997).

Heterorhabditis nematodes from the Heterorhabditidae family act as 'cruiser' parasites, a behavior that involves active seeking out of suitable insect hosts by burrowing into the soil. *Heterorhabditis* parasitic nematodes form a mutually beneficial symbiotic relationship with the entomopathogenic Gram-negative bacteria from the genus *Photorhabdus*, which belong to the Enterobacteriaceae family (Waterfield et al. 2009). The bacteria are found in the gut of the infective juvenile stage (Ciche et al. 2006; Ciche 2007). The infective juvenile is an obligate stage in the nematode life cycle and is required for the infection of larval stages of mainly lepidopteran insects (Ciche 2007; Kaya and Gaugler 1993) (Fig. 17.1). This stage is analogous to the *C. elegans* dauer stage and the developmentally arrested infective third-stage larva (L3) of many important parasitic nematodes. Infective juveniles gain entry to the insect through natural openings (anus, spiracles, and mouth) or by
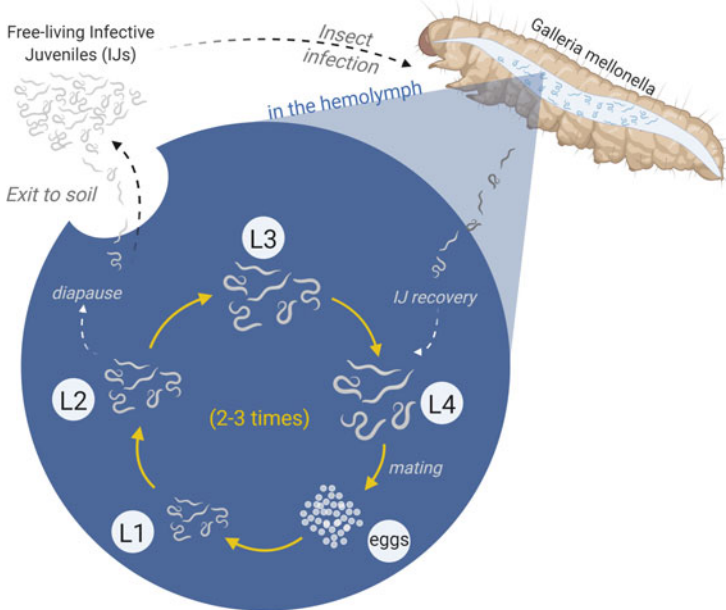
Fig. 17.1 Life cycle of the entomopathogenic nematode *Heterorhabditis bacteriophora*. The infective juveniles (IJs) form the only free-living stage of this parasite. Nematode mating, reproduction, and development occur within the hemolymph of the infected insects (e.g. larvae of the greater wax moth, *Galleria mellonella*) in the presence of high titers of their mutualistic bacteria *Photorhabdus luminescens*. Depending on the amount of resources in the dead insect, two or three generations may take place within the insect cadavers. L1, L2, L3, and L4: Larval molts. Images are made using Biorender graphic software (https://biorender.com)

abrading the insect cuticle using a dorsal tooth (Ciche 2007). Once inside the insect, the infective juveniles expel a small number of *Photorhabdus* cells into the hemolymph where the bacteria begin to divide exponentially. After two to three days of bacterial growth, the insect succumbs to the infection due to septicemia with the concomitant conversion of the internal organs and tissues into bacterial biomass. This bioconversion is facilitated by the production of a wide range of toxins and hydrolytic enzymes by the bacteria (Ffrench-Constant et al. 2007; Eleftherianos 2009; Bode 2009). The worms feed on the bacterial biomass, and subsequent nematodes' growth and development require the presence of high-density *Photorhabdus* bacteria (Ciche and Ensign 2003). The infective juvenile nematodes mature to first-generation hermaphrodite females, which give rise to the second generation of amphimictic males and females (cross-fertilization) and to the self-fertile hermaphrodite females and infective juveniles. Nematodes reproduce and the progeny develops through four juvenile stages (L1, L2, L3, and L4) to adults. Nematode reproduction continues over two to three generations until the nutrient status of the cadaver deteriorates, whereupon adult development is suppressed, and the infective juvenile stage accumulates. These non-feeding infective juveniles enter

the soil where they may survive for several months in the absence of a suitable host. The transmission of mutualistic bacteria by infective juveniles is essential for the nematodes to reproduce (Goodrich-Blair and Clarke 2007).

Similar to *Heterorhabditis*, *Steinernema* nematodes are found free in the soil where they gain access to insect larvae through natural body openings but they lack a dorsal tooth that facilitates penetration through the cuticle. However, in contrast to *Heterorhabditis*, the *Steinernema* nematodes exhibit 'ambushing' behavior, which involves waiting and attacking sensitive insect hosts in their vicinity. *Steinernema* nematodes follow a similar life cycle to *Heterorhabditis* that mainly differs in the initial stage of recovery during which amphimictic reproduction occurs. This means that *Steinernema* infective juveniles develop into reproductive males and females. Interestingly, this infective juvenile behavior also takes place during the first- and second-generation offspring. In addition, third-generation females produce eggs all of which develop through the 'endotokia matricida' process that occurs due to the cessation of egg laying and involves intra-uterine birth causing maternal death, a relatively common phenomenon in entomopathogenic nematodes induced in response to the low food supply. As opposed to *Heterorhabditis*, the resulting juvenile stages of *Steinernema* develop into infective juveniles after they exit the mother nematode (Kooliyottil et al. 2013).

17.3 Mutualism Regulators in *Photorhabdus* Bacteria

Recent progress in quantitative proteomic techniques has been started to contribute to the identification and preliminary examination of the factors that control symbiotic processes between animal hosts and microbes, including entomopathogenic nematodes and their related bacteria. To determine the identity of bacterial proteins that underlie symbiotic specificity in the entomopathogenic nematodes *Heterorhabditis*, 2D-gel electrophoresis followed by mass spectrometry were used to analyze and compare the proteomic profiles of two *P. luminescens* subspecies (*P. luminescens* ssp. *laumondii* and *P. luminescens* ssp. *akhurstii*), each occupying a distinct *Heterorhabditis* nematode species (*H. bacteriophora* and *H. indica*, respectively) (Kumar et al. 2016). Results from the proteomic and bioinformatic analyses revealed that either bacterial subspecies expresses several unique proteins, a subset of which (e.g. outer membrane proteins, proteins regulating secondary metabolites, and hypothetical proteins) may define nematode specificity. The functional characterization of certain candidate proteins will undoubtedly provide clues on the evolutionary and mechanistic basis of host–symbiont associations (Fig. 17.2).

Proteomic analysis coupled with bacterial genetics has further explored the role of the *rpoB* gene in the symbiosis between *P. luminescens* LN2 bacteria and their *H. bacteriophora* H06 nematode vectors (Qiu et al. 2012). Gene *rpoB* codes for the bacterial beta subunit of RNA polymerase and interestingly rifampicin prevents the initiation of transcription by repressing the *rpoB* gene. This research showed that certain rifampicin-resistant *P. luminescens* LN2 mutant strains, which surprisingly
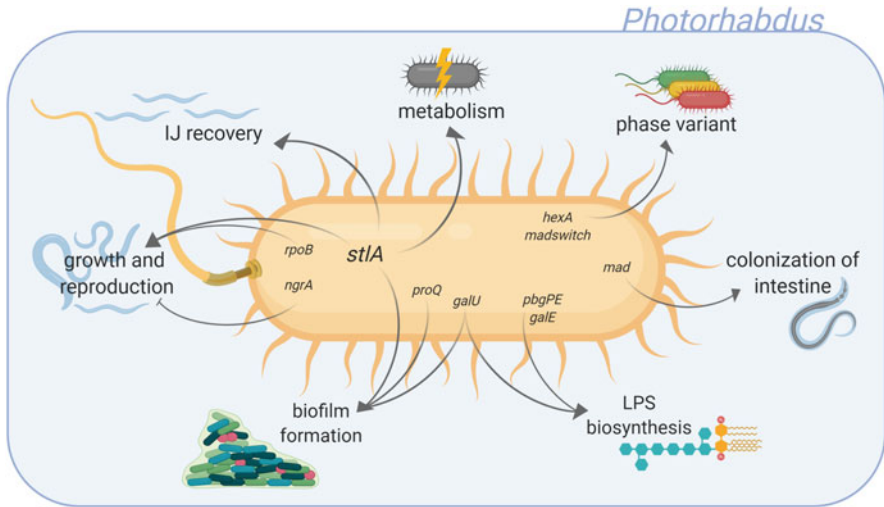
Fig. 17.2 *Photorhabdus* molecular regulators of symbiosis. Growth and reproduction of *Heterorhabditis bacteriophora* nematodes are controlled by genes *rpoB*, *ngrA*, and *stlA* in *P. luminescens*. Recovery of nematode infective juveniles (IJ) and bacterial metabolism are controlled by *P. luminescens* gene *stlA*. The *madswitch* promoter and gene *hexA* regulate the two distinct forms (mutualistic and pathogenic) in *P. luminescens* and *P. temperata* bacteria, respectively. The fimbrial locus *mad* in *P. luminescens* participates in initiating symbiosis through bacterial colonization of the posterior maternal intestinal cells in *H. bacteriophora*. LPS biosynthesis is modulated by genes *galE*, *galU*, and *pbgPE* in *P. luminescens*. Biofilm formation in *P. luminescens* is modulated by genes *galU*, *proQ*, and *stlA*. Images are made using Biorender graphic software (https://biorender.com)

also contained mutations in the *rpoB* gene, were able to support the growth of *H. bacteriophora* H06 infective juveniles. It was further demonstrated that mutations in the *rpoB* gene reconstitute the bacteria as the nutrient source for sustaining nematode reproduction; however, without conferring the ability of the bacteria to colonize the nematode intestines during the infective juvenile stage. Rifampicin selection of *P. luminescens rpoB* mutant strains supporting nematode growth may provide an elegant approach for increasing the production of *H. bacteriophora* in order to achieve more efficient insect pest control in the field.

Genetic analysis of the nematode–bacterial symbiotic relationship using a transposon mutagenesis and screening approach identified a single mutant strain of *P. luminescens* that was deficient in providing growth and reproduction to the *H. bacteriophora* nematode vector (Ciche et al. 2001). Characterization of the mutation localized the transposon insertion into gene *ngrA* encoding the enzyme Ppant transferase, which is involved in the biosynthesis of the siderophore enterobactin. Although this mutation also conferred an inability of the bacteria to produce antibiotics and siderophores, and probably interrupted the biosynthesis of fatty acids or lipids, these deficiencies were not attributed to the nematode defects. Instead, the assumption is that the inactivation of the *ngrA* gene possibly affects the

biosynthesis of hormones, polyketides, or other secondary metabolites that are produced by *P. luminescens* when *H. bacteriophora* is also present, and act as signal molecules to promote the nematode's growth and development.

A subsequent study continued this work to examine whether gene *ngrA* encoding a putative phosphopantetheinyl transferase (PPT) that is involved in the biosynthesis of siderophore forms a determining factor for *P. luminescens* to support the growth and reproduction of *H. bacteriohora* nematodes (Ciche et al. 2003). Following a mini-Tn5 mutagenesis approach, *P. luminescens* mutant strain NS414 with a deficiency in producing measurable siderophore activity was first isolated, and then its properties were characterized. The results showed that the mutant bacteria were not able to grow normally in media depleted of iron, but they were capable of promoting the growth and reproduction of their nematode hosts, as well as their transmission by *H. bacteriophora* infective juveniles. Interestingly, the transposon was found to be inserted into gene photobactin synthetase (*phbH*) encoding a putative peptidyl carrier protein, which is covalently modified by PPTase for siderophore production. As *phbH* is not essential for nematode symbiosis, these findings signify that failure of the *ngrA* mutants to support nematode symbiosis was not due to their inability to produce functional siderophore but rather due to their incapacity to synthesize another currently unknown peptide that performs this function.

An interesting feature of *Photorhabdus* is that the bacteria can exist in two forms, the primary and secondary, which are morphologically distinct and are associated with the different phases of the pathogen's lifestyle. Only the primary form bacteria can colonize the intestinal tract of their associated nematode host and promote its growth and development due to the production of extracellular enzymes and antibiotic compounds that support the interaction between the two symbiotic partners during the infection of a suitable insect (Waterfield et al. 2009). Remarkably, it has been previously shown that inactivation through transposon insertion of gene *transcriptional regulator LrhA* (*hexA*) in the secondary phase of *P. temperata* bacteria results in the suppression of nematode colonization, and concomitantly, the mutant bacteria can foster growth and development of the host nematodes *H. downesi* (Joyce and Clarke 2003). These findings provide proof that *hexA* in the secondary phase of *P. temperata* bacteria encodes a molecule that confers direct or indirect repressive effects on symbiotic factors, which are normally expressed in the primary phase variants.

Another library screen of GFP-labeled *P. luminescens* transposon mutants, involving symbiotic assays to examine the qualitative ability of the mutant bacteria to colonize the gut of the infective juvenile stage of *H. bacteriophora* nematodes, further aimed at identifying bacterial genes, and their encoded factors responsible for the symbiotic collaboration between the two organisms (Easom et al. 2010). This work showed that mutations in a subset of genetic loci (e.g. *pbgPE* operon and genes *galE* and *galU*) involved specifically in the biosynthesis of lipopolysaccharide (LPS) and assembly and maintenance of LPS structure, as well as of other bacterial cell surface components, conferred substantially reduced transmission frequency of the mutant bacteria to associate with their nematode host. In addition, the *P. luminescens* mutant for genes *proQ* (encoding an RNA chaperone) and *galU* were also defective

in biofilm formation as shown through testing the ability of the mutant bacteria to attach to an abiotic surface. This information highlights further the vital role of cell surface molecules in *P. luminescens*, and probably in other entomopathogenic bacteria, in adjusting the symbiotic outcome of bacterial–nematode partnerships.

Using a similar random transposon mutagenesis screening approach, the transmission ability of GFP-labeled *P. luminescens* mutants in the intestine of *H. bacteriophora* nematode parasites was analyzed in detail. The genetic analysis detected that the *maternal adhesion defective* (*mad*) fimbrial locus in *P. luminescens* has an essential role in initiating symbiosis through the bacterial colonization of the posterior maternal intestinal cells in *H. bacteriophora*. This process facilitates bacterial symbiont transmission from the maternal nematodes to the infective juveniles. Importantly, this is a specialized function because *mad* is required for symbiosis but not for insect pathogenesis, and the effect is regulated by bacterial phase variation in the wild type bacteria but not in the *mad* mutants (Somvanshi et al. 2010). These are the findings of particular significance because although fimbriae are known colonization factors that were previously shown to promote animal tissue or cell colonization by various bacterial pathogens through receptor recognition events, this was the first time that these adhesive organelles were assigned a similar function in modulating nematode–bacteria mutualistic symbiosis.

A previous study identified the production of crystalline inclusion proteins containing high levels of essential amino acids by *P. luminescens* bacteria to assist nematode reproduction (Bintrim and Ensign 1998), and a more recent work linked the two distinct forms of *P. luminescens* (M, initiating nematode Mutualism and P, initiating insect Pathogenicity) with the expression of the *mad* fimbrial locus, which occurs after the inversion of the *madswitch* promoter (Somvanshi et al. 2012). More precisely, it was demonstrated that during the first stage of mutualism in *H. bacteriophora*, *P. luminescens* bacteria switch to the M-form in the posterior intestine of the maternal nematodes, while the P-form bacteria are temporarily present in the intestines. The M-form cells then occupy the intestines of the new generation of infective juveniles before turning into the P-form cells to provide nematodes with bacteria possessing properties that promote infection of susceptible insects. Strikingly, the M-form bacteria are smaller than the P-form bacteria; they grow slower and exhibit decreased bioluminescence, virulence, and ability to secrete secondary metabolic compounds. Therefore, the biological implication of these findings underlines the importance of *madswitch* promoter orientation, which defines not only the phenotypic appearance of *P. luminescens* cells but also influences the lifestyle of the bacteria.

Additional factors have been associated with the persistence of *P. temperata* in the intestine of *H. bacteriophora* nematodes, as demonstrated by the drastic changes in the transcriptional profile of the bacteria during mutualism in the non-feeding infective juvenile stage (An and Grewal 2010). To determine strategies adapted by the bacteria to strengthen their persistence in the parasite through diminishing nutritional reliance on the nematode host, the number and identity of the differentially expressed genes in *P. temperata* was explored using the selective capture of the transcribed sequences technique. Analysis of the results displayed a large number of

differentially regulated genes in *P. temperata* when the bacteria reside in the nematode intestine compared to the bacteria being cultured in vitro or being present in the insect host hemolymph. The differentially expressed genes denote modifications in physiological functions when residing in the nematodes' intestine including the activation of the pentose phosphate pathway, alteration in amino acid metabolisms, modification in LPS, induction of intracellular acidification and urea cycle mechanisms, proton transport and biofilm formation, as well as processes involving bacterial replication, transcription, and translation. These findings provide an exciting pool of potential molecular regulators of bacterial symbiosis in parasitic nematodes and future work awaits to dissect the specific mechanistic roles of these symbiosis factors and whether (and how) they are interconnected to enable the close biological link between the two organisms.

P. luminescens is a bacterium that is able to produce the antibiotic 3,5-dihydroxy-4-isopropylstilbene (stilbene, ST) during insect infection. The production of this antibiotic compound eliminates non-symbiotic micro-organisms that colonize insect tissues to compete for resources and nutrients, it prevents decay of the insect carcass and therefore, provides a favorable environment to their *H. bacteriophora* nematode vectors to grow, replicate, and complete their life cycle (Hu and Webster 2000). ST functions as a signal for the nematodes by stimulating the recovery of infective juveniles to adult hermaphrodites. This was demonstrated by the finding that *P. luminescens* mutants deficient in ST production were also unable to support *H. bacteriophora* growth and development (Joyce et al. 2008). The biosynthesis of ST involves the non-oxidative deamination of phenylalanine that leads to the synthesis of cinnamic acid by the enzyme phenylalanine-ammonium lyase, which is encoded by the gene *stlA* (Williams et al. 2005). Interestingly, it was subsequently shown that *stlA* expression is temporally controlled during growth, and it can be regulated by nutrient limitation (Lango-Scholey et al. 2013). This gene regulatory mechanism is further controlled by three transcriptional regulators; LysR-type TyrR, which is absolutely essential for *stlA* expression, as well as *Leucine-responsive regulatory protein* (*Lrp*) and RNA polymerase Sigma factor (*rpoS*), which are also required for normal *stlA* expression under suitable environmental conditions. These findings signify the molecular players that modulate secondary metabolism, and as a consequence, the mutualism of a potent entomopathogenic bacterium with its nematode host. Recently, additional findings provided evidence for the role of *P. luminescens* stilbene in the biology of the bacteria and their association with *H. bacteriophora* nematodes (Hapeshi et al. 2019). Exogenous ectopic addition of stilbene to *P. luminescens stlA* mutants reduces biofilm formation and downregulates the transcriptional expression of genes participating in the secondary metabolism, and basic cellular processes. These findings illustrate that stilbene cannot be only produced but also be detected by *P. luminescens* and plays a modulatory role by possibly acting as a signal for the bacteria to regulate the symbiotic phase of their life cycle through promoting the production of other molecules that are important for the recovery of nematode infective juveniles. This is a crucial process because it facilitates bacterial transmission to the next insect host following parasitic nematode infection.

17.4 Mutualism Regulators in *Xenorhabdus* Bacteria

Similar to *Photorhabdus*, *Xenorhabdus* bacteria are considered to have evolved distinct physiological and metabolic mechanisms that facilitate the close association with their cognate nematode hosts, as revealed by a previous study comparing the genome sequences of the two entomopathogenic bacterial symbionts (Chaston et al. 2011). The current speculation is that the two bacteria most likely share a single progenitor and as a result of multiple selective pressure events following differentiation, they have acquired unique factors to support the close relationship with their related nematode vectors.

Transposon mutagenesis to detect factors in *X. nematophila* that promote symbiosis with *Steinernema carpocapsae* nematodes led to the isolation of a mutant strain that was able to produce certain phospholipases such as lecithinase, but was unable to produce antibiotics and the mutant bacteria failed to grow and emerge normally from their nematode host (Volgyi et al. 2000) (Fig. 17.3). The transposon mutation was detected in the gene *var1* encoding a protein that is involved in the formation of the variant cell type. Deficient growth of the mutant bacteria may suggest a negative effect on the survival of this variant type of cells in the nematode intestines or the inability of the bacteria to swarm properly, which may delay their exit from the nematode. Although the specific physiological and biochemical bases of this
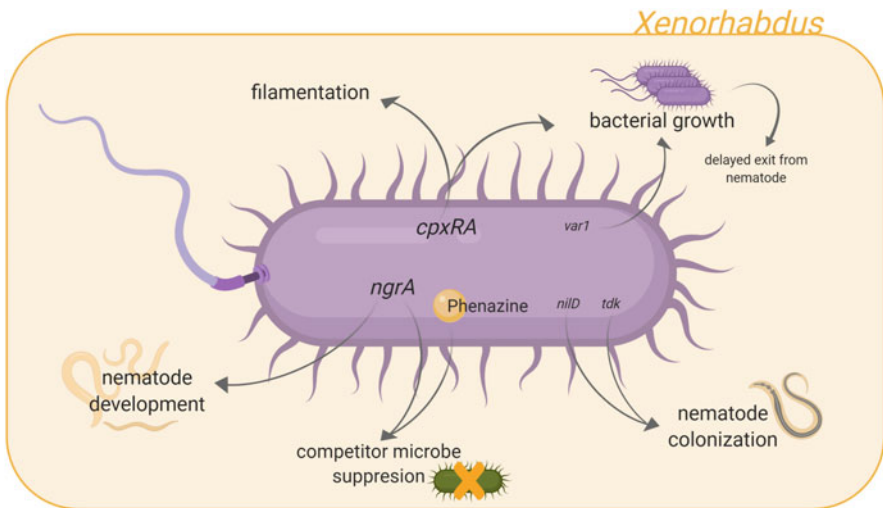

Fig. 17.3 *Xenorhabdus* molecular regulators of symbiosis. Filamentation and replication in *X. nematophila* are regulated by gene *cpxRA*. Bacterial growth and survival are controlled by gene *var1*. Colonization of *Steinernema carpocapsae* infective juveniles by *X. nematophila* is controlled by the bacterial genes *nilD* and *tdk*. Growth of competitor microbes in the dead insect is suppressed by phenazine compounds produced by *X. nematophila* as well as by gene *ngrA*. The latter also modulates the development and emergence of *S. carpocapsae* nematodes. Images are made using Biorender graphic software (https://biorender.com)

phenotype in *X. nematophila* is unclear, further elucidation of the processes and the particular factors modulating the switch from primary to secondary phase cells will enhance our knowledge regarding the type of features that are required to support the symbiotic interactions between entomopathogenic nematodes and their affiliated bacteria.

During the symbiotic relationship with *S. carpocapsae*, the *X. nematophila* uses nutrients from their nematode host; however, the exact identity of these compounds required for bacterial growth during this interaction is not well-determined. Since bacteria can utilize salvaged nucleosides as a supplement to endogenous nucleotides for DNA synthesis, they are able to form nitrogen sources, they can participate in the activation of signal transduction, and they are involved in the construction of cell structures. Therefore, it was previously hypothesized that the regulation of pyrimidine salvage pathways might constitute a process that facilitates the *X. nematophila*-nematode interplay (Orchard and Goodrich-Blair 2005). Curiously, it has been shown that the *X. nematophila* mutant for gene *tdk* encoding the enzyme deoxythymidine kinase, which synthesizes the pyrimidine nucleotide deoxythymidine monophosphate from deoxythymidine, are deficient in nematode colonization in vitro but not when present in the insect host. This defect is also fully restored by the addition of the wild copy *tdk* allele to the mutant strain. Such a mechanism could represent a broader strategy for entomopathogenic bacteria to associate with their nematode hosts.

An important aspect to consider in host–microbe interactions is the ability of the organisms involved in symbiotic relationships to sense and respond to external environmental changes. The two-component regulatory system CpxRA in *X. nematophila* consists of a sensor histidine kinase (CpxA) and a cytoplasmic response regulator (CpxR). It forms a signaling pathway, which in other bacteria, such as *E. coli*, regulates the function of structural components that permit interaction with the host. They might also act as a transducer to transmit internal signals inside the cells for initiating signaling pathways that would generate a cellular response (Herbert and Goodrich-Blair 2007b). Testing the symbiotic competence of *X. nematophila cpxR1* mutant bacteria, in which expression of both *cpxA* and *cpxP* genes is abolished, revealed that their ability to associate with *S. carpocapsae* infective juveniles is markedly impaired, and this effect is not due to a reduced survival ability of the mutants. Alternatively, this effect is primarily associated with modifications in cell morphological features in *cpxR1* mutants that might alter cell division dynamics or cause filamentation, which in turn could lead to interference in the symbiotic partnership between the bacteria and their nematodes, as well as with the decreased expression of genes that participate in nematode colonization. The regulatory role of CpxRA in *X. nematophila* is not unique, as other genes including *Lrp* (Leucine responsive regulatory protein) have also been shown to possess regulatory properties (Cowles et al. 2007). Overall, these findings imply the presence of certain genes in the *X. nematophila* genome performing multiple activities to promote the symbiotic connection of the bacteria with their affiliated entomopathogenic nematode partners.

It is given that molecules with antimicrobial activity may also modulate molecular and physiological processes by acting as signals in certain bacteria, previous efforts have focused on the role of bacterial secondary metabolites in promoting symbiotic relationships. This is because the *P. luminescens ngrA* gene, which encodes various secondary metabolites, is important for the growth and reproduction of *H. bacteriophora* nematodes, the involvement of *X. nematophila ngrA* in establishing or maintaining symbiosis with *S. carpocapsae* nematodes was also analyzed (Singh et al. 2015). Results from this research indicated that the number of nematode progenies from *Manduca sexta* caterpillars inoculated with *S. carpocapsae* infective juveniles containing the *X. nematophila ngrA* mutant were remarkably decreased compared to nematode progeny containing *X. nematophila* wild-type bacteria. These findings support the notion of a dual role for *ngrA*-derived compounds in not only combating competitor microbes in the insect cadaver to facilitate nematode development but also acting as signals for accelerating nematode development and emergence from the infected insect host.

Other molecules with multiple activities in *Xenorhabdus* that might serve as crucial factors for providing efficient bacteria–nematode symbiotic cooperation are the phenazines, which are commonly found in Gram-negative bacteria (Shi et al. 2019). It was recently tested whether phenazine compounds derived from *X. szentirmaii* influence the symbiotic ability between the bacteria and their *S. rarum* nematode vectors. Although the exact function of these molecules in regulating bacteria–nematode interactions is currently obscure because phenazines possess broad-spectrum as well as specific antibiotic activity, the current working hypothesis is that these compounds target a variety of competitor soil microbes present in the insect carcass. Elimination of these competitor microbes enables the *S. rarum* parasitic nematodes to complete and maintain their life cycle together with their closely related *X. szentirmaii* bacteria.

Further efforts to detect bacterial molecular factors promoting nematode colonization have included a signature-tagged mutagenesis screen in *X. nematophila*, an approach that identified a transposon mutant that lost its ability to colonize infective juveniles of *S. carpocapsae* nematodes (Veesenmeyer et al. 2014). Of note, the transposon was found to be inserted into gene *nilD* that codes for a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) element. CRISPR associated sequences (Cas) are widely found in bacteria and perform several cellular functions including the regulation of gene expression and DNA repair mechanisms as well as bacterial behavior and resistance to foreign nucleotide sequences (Li and Peng 2019; Hampton et al. 2020). The investigators were able to demonstrate elegantly that the *nilD* CRISPR sequence is sufficient to support the colonization of *S. carpocapsae*, as well as *S. anatoliense* and *S. websteri* nematodes. The *nilD* RNA is expressed in a Cas6e-dependent manner under in vitro growth conditions and during nematode symbiosis, but in the latter case only within a specific genetic background of *X. nematophila*. These exciting new findings open novel avenues of investigation for designing strategies to decode the precise mechanism of CRISPR bacterial systems in modulating symbiotic interdependence with entomopathogenic nematodes.

17.5 Development of Tools for Identifying Nematode Regulators of Mutualism

Although most information currently available on the number and nature of genes and their products acting as regulators of mutualism with entomopathogenic nematodes has been obtained from bacteria, knowledge of analogous molecules in parasites playing a central or complementary role to the evolution, and stability of this process, is missing so far. This, at least till now, was mostly attributed to the lack of availability of genetic and genomic tools in entomopathogenic nematodes, which has impeded progress with dissecting the molecular basis of the mutualistic interaction between the two partners. To this end, recent efforts have mainly focused on developing whole-genome sequencing approaches and molecular procedures in *H. bacteriophora* to genetically manipulate the vector nematode in a similar way to the *C. elegans* model. Earlier work identified species-specific satellite DNA motifs in *H. bacteriophora* and *Steinernema glaseri* nematodes, and employed DNA reassociation kinetics to determine the genome size and complexity in *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* (Grenier et al. 1996, 1997). At the same time, the expressed sequence tags were generated in *H. bacteriophora* to elucidate their involvement in various biological functions and more recently RNA-sequencing studies were carried out to understand the molecular basis of nematode parasitism (Sandhu et al. 2006; Bai et al. 2007, 2009; Vadnal et al. 2017). A tremendous breakthrough in entomopathogenic nematode research, including the nematode–bacterial mutualistic symbiosis, came about with the complete sequencing of the *H. bacteriophora* genome, the annotation which was recently improved (Bai et al. 2013; Vadnal et al. 2018; McLean et al. 2018). In terms of genetic techniques, the development of gene silencing RNAi interference through soaking and microinjection in *H. bacteriophora* has substantially upgraded the research value of this model organism (Ciche and Sternberg 2007; Ratnappan et al. 2016). Such advances are particularly significant because not only they promise to uncover previously unknown players of the interrelationship between the nematodes and their associated bacteria, but also to reveal the exact contribution of the parasites to the infection process of insects.

17.6 Concluding Remarks

Entomopathogenic nematodes are spectacular organisms that have received particular attention due to their complex life cycle that involves the mutualistic interdependence with specific bacteria that act as symbionts for the parasites and potent pathogens for the invaded insects. This extremely efficient relationship provides a fascinating model system for studying the interactions between invertebrates and their mutualistic microbes in relation to the host immune system (Ffrench-Constant et al. 2003; Joyce et al. 2006; Clarke 2008). Appreciating the details of

the participation of the molecular processes and the specific genes or their products in fine-tuning the relationship between entomopathogenic nematodes and their associated bacteria is essential for understanding mutualistic interactions in other invertebrate organisms including beneficial insects, and devastating vectors of infectious diseases. It is also important for deciphering the conserved mechanisms that regulate similar types of interconnection between microbes and vertebrate animals, including humans. Such knowledge will significantly advance our biological interpretation of a wide range of host–microbial interplays that occur not only in the lab but also in different environmental settings.

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