Chapter – 2

Review of Literature
II. Review of Literature

Brief account of Entomopathogenic nematodes (EPN)

The nematodes belonging to the two families steinernematidae (Chitwood and Chitwood, 1937) and heterorhabditidae (Poinar, 1976) of the super family rhabditoidea, order rhabditida and phylum Nematoda, are small parasitic round worms that infect and often quickly kill insects and are known as entomopathogenic nematodes (EPN). These nematodes are also referred to as beneficial (insecticidal) nematodes. EPN are found under diverse ecological conditions including cultivated fields, forests, grasslands, deserts and beaches of oceans (Hominick et al., 1996). EPN have great potential as biological control agents against insect pests (Ehlers, 2005).

Rhabditids as a monophyletic group include two taxa. The strongylids are a group of vertebrate parasites traditionally considered outside of rhabditids. Molecular analyses have placed strongylids clearly within the eurhabditis group of rhabditids and as a sister group of the insect pathogenic heterorhabditis (Blaxter et al., 1998; Sudhaus and Fitch, 2001; De Ley and Blaxter, 2002). The Oscheius genus comprises two main subclades dolichura and insectivora groups. One distinctive character between these two clades appears to be the smaller size of the animals in the dolichura clade (that includes oscheius tipulae) (Flemming et al., 2000). The dolichura group comprises Oscheius guentheri, which is a species with a reduced posterior-gonadal arm (Sudhaus and Hooper, 1994), plus two subgroups, of which two representative species are Oscheius tipulae and Oscheius dolichura, respectively. Historically, Oscheius dolichura (Schneider, 1866) was one of the first nematode species (before C. elegans) described to have an androdioceous mode of reproduction (Maupas, 1900).

Survey of EPN has been conducted in Japan since late 1980's. Six described species (Steinernema carpocapsae, Steinernema feltiae, Steinernema kraussei, Steinernema kushidai, Steinernema litorale and Steinernema monticolum) and six undescribed putative species (Steinernema spp. MY3, MY4, MY5, MY6, MY7, and MY8) have been isolated thus far (Mamiya and Ogura, 1990; Yoshida et al., 1998; Mamiya et al., 2001; Yoshida, 2003a). Isolation of S. carpocapsae in Japan has been reported by Mamiya and Ogura (1990), but the species has never been reisolsted. The
putative species were categorized by their morphological differences and PCR-RFLP patterns (Yoshida et al., 1998; Yoshida 2003b).

Steinernematidae and heterorhabditidae are strongly virulent against a wide range of insects. Gaugler and Kaya (1990) showed that they were non-pathogenic to mammals and suggested that they could be raised for use in biological control of insect pests. All Steinernema spp. carry in their gut symbiotic bacteria belonging to the genus Xenorhabdus (Akhurst and Boemare, 1988; Thomas and Poinar, 1979; Vivas and Goodrich-Blair, 2001; Forst and Clarke, 2002; Poinar, 1990 and all Heterorhabditis spp. carry symbiotic bacteria belonging to the genus Photorhabdus (Akhurst et al., 1996; Boemare et al., 1993; Fischer-Le Saux et al., 1999; Khan and Brooks, 1977). The symbiotic association plays an important role in both reproduction and pathogenicity of the nematodes (Boemare et al., 1996; 1997). Lysenko and Weiser (1974) isolated bacteria such as Alcaligenes, Pseudomonas and Acinetobacter spp. from Steinernema carpocapsae. When S. carpocapsae was raised in the laboratory for extended periods (e.g. 15 years), bacteriological investigations indicated the presence of other associated bacteria such as Pseudomonas aureofaciens, Pseudomonas fluorescens, Enterobacter agglomerans and Serratia liquefaciens (Boemare, 1983). Similar observations were reported for Steinernema scapterisci, which was transferred from South America and subcultured many times in Florida. This nematode was associated with Ochrobactrum anthropi, Paracoccus denitrificans, Pseudomonas maltophilia and Xenorhabdus spp. (Aguillera et al., 1993; Aguillera and Smart, 1993). The isolates associated with Photorhabdus luminescens subsp. Akhurstii, symbionts of H. indica (Fischer-Le Saux et al., 1999) were identified by using conventional phenotypic tests, restriction fragment length polymorphism and sequence analysis of PCR-amplified 16S rRNA genes (16S rDNAs).

EPN- Bacteria

Xenorhabdus and Photorhabdus are motile, Gram-negative rod shaped bacteria that are highly pathogenic to insects (Burnell and Stock, 2000). Xenorhabdus occurs naturally in a special intestinal vesicle of Steinernema infective juveniles (Bird and Akhurst, 1983). Photorhabdus are mainly located in the anterior part of
Heterorhabditis infective juveniles (Boemare et al., 1996). Xenorhabdus sp. secrete proteinaceous toxin complexes that are similar to the insecticidal complexes produced by the sister taxa, Photorhabdus sp. (Bowen et al., 1998; Bowen, 1995; Ensign et al., 1990).

The nematodes enter the digestive tract of the larval stage of diverse insects and subsequently penetrate into the hemocoel of the host insect. The nematode can also gain access to the hemocoel via the respiratory spiracles or by penetrating directly through the insect cuticle (Akhurst and Dumphy, 1993; Poiar, 1990). Upon entry into the hemocoel, the nematodes release the bacteria into the hemolymph. Together, the nematodes and the bacteria rapidly kill the insect larva, although in most cases the bacteria alone are highly virulent (Akhurst and Dumphy, 1993). Within the hemocoel of the larval carcass, the bacteria grow to stationary-phase conditions while the nematodes develop and sexually reproduce. Nematode reproduction is optimal when the natural symbiont (Xenorhabdus or Photorhabdus sp.) dominate the microbial flora, suggesting that the bacteria can serve as a food source and/or provide essential nutrients that are required for efficient nematode proliferation (Akhurst and Boemare, 1990; Akhurst and Dumphy, 1993; Poinar, 1990).

Both Xenorhabdus spp. and Photorhabdus spp. can be grown as free-living organisms under standard laboratory conditions. Growth in vitro is probably supported by the rich nutrient supply of the laboratory growth media and the lack of competition that normally exists in the soil environment. As the bacteria enter the stationary phase of their growth cycle, they secrete several extracellular products, including lipase(s), phospholipase(s), protease(s) and several different broadspectrum antibiotics (Akhurst, 1982; Akhurst and Boemare, 1990; Boemare and Akhurst, 1988; Nealson et al., 1990) that can be assayed in the culture media. Cytoplasmic inclusion bodies, composed of highly expressed crystalline proteins, are also produced by both bacteria during stationary-phase growth (Couche and Gregson, 1987; Couche et al., 1987).

Serratia nematodiphila sp. nov., a red-pigmented, non-sporeforming, fluorescent strain, designated DZ0503SBS1T, was isolated from the intestine of the nematode Heterorhabditidoides chongmingensis. The novel strain was associated

**Phylogeny of Xenorhabdus and Photobrdhus**

Both oligonucleotide cataloging (Ehlers *et al.*, 1988) and 16S rRNA sequence analysis (Rainey *et al.*, 1995) have placed the *Xenorhabdus/Photobrdhus* group within the gamma subdivision of the purple bacteria (proteobacteria). By oligonucleotide cataloging, it was estimated that *Xenorhabdus/Photobrdhus* constituted a taxonomic unit equivalent to and possibly distinct from, the family enterobacteriaceae, into which it has been placed after phyletic analysis by several authors. 16S rRNA data recently published by Rainey *et al.* (1995) support this contention, showing a close relationship with other members of the gamma proteobacteria, which, interestingly include many other organisms specialized as symbionts and/or pathogens of eukaryotes. Thus while the placement of the genera *Xenorhabdus* and *Photobrdhus* within the enterobacteriaceae seems firm, it should be noted that some uncertainty still exists with regard to this issue. Rainey *et al.* (1995) discussed the appropriateness of including the group within the family enterobacteriaceae, while on the other hand, Janse and Smits (1990) on the basis of fatty acid analyses suggested that the differences are sufficient for them to be separated from the enterobacteriaceae. Farmer (1984) and Farmer *et al.* (1989) have pointed out that the inability of *Xenorhabdus* sp. to reduce nitrate, coupled with their catalase-negative property, is a reason to question their inclusion in the enterobacteriaceae. They also note that *Photobrdhus* species are nitrate reduction negative and they produce characteristic yellow or red pigments not common in the enterobacteriaceae, and, of course, that they are bioluminescent, a property that is otherwise unknown in the enterobacteriaceae.

**Taxonomy of Xenorhabdus and Photobrdhus**

The taxonomy of the *Xenorhabdus/Photobrdhus* group has had a brief and somewhat confusing history since the naming of the organisms in the mid-1960s. From the early studies, it is concluded that these bacteria are closely related to the family enterobacteriaceae and share many properties with their enteric neighbours (Forst and Nealson, 1996). One of the first published discussions of bacteria
associated with EPN appeared in 1959 (Dutky, 1959) although the bacteria were neither fully characterized nor named. According to the recommendation of Hendrie et al. (1974) the genus Achromobacter was disallowed, and the symbionts were left without a name when some of the existing type strains of symbiotic luminous bacteria, such as Hb (ATCC 29999) and NC-19 (ATCC 29304), were isolated (Khan and Brooks, 1977). This situation continued until the proposal of the genus name Xenorhabdus by Thomas and Poinar (1979) to accommodate both the non-luminous (X. nematophilus) and the luminous (X. luminescens) symbionts. It was recognized at that time that the luminous symbionts were obtained only from heterorhabditid nematodes, while the non-luminous X. nematophilus isolates were found associated with neoaplectanid nematodes (Thomas and Poinar, 1979). Xenorhabdus was placed in the family enterobacteriaceae on the basis of extensive phyletic analysis, although some traits were notably different from those of the other members of the enterobacteriaceae. A quick look at the description of the species in Bergey’s Manual (Farmer, 1984) reveals that Xenorhabdus sp. is much less metabolically diverse than many of their enterobacterial neighbours.

Although molecular methods (16S rRNA sequence comparisons) have not yet completely resolved the taxonomic issues, it has been possible to develop group-specific probes of several types. 16S rRNA probes were developed for the four Xenorhabdus spp. and for P. luminescens (Putz et al., 1990).

The genus Photorhabdus

The genus Photorhabdus comprises the species of Photorhabdus luminescens, P. temperata and P. asymbiotica. Although all three are highly pathogenic to insects, P. asymbiotica is originally isolated from human wounds and its nematode vector has only recently been identified (Gerrard et al., 2006). Photorhabdus forms Gram-negative, asporogenous, rod-shaped (2-6 × 1-1.4 μm) peritrichious cells. All strains are mesophilic bacteria with an optimal growth temperature at 28-30°C, only some strains exhibiting an even broader temperature range between 16-38°C (Fischer-Le Saux et al., 1999; Peel et al., 1999). The fact that Photorhabdus cells can not reduce nitrate but can produce iso-branched fatty acids is very uncommon to enterobacteriaceae and keeps them detached from other genera in this family (Janse
and Smits, 1990; Suzuki et al., 1990). *Photorhabdus* is also marked by another laboratory relevant trait. One can observe a variation in colony forms after prolonged subculturing, a primary and a secondary form which are also referred to as phase I and phase II forms. The primary form is displayed by pigmentation, the adsorption of dyes like bromothymol blue or neutral red, the ability to constitute inclusion bodies and to bioluminesce, but also to produce lipases, phospholipases and proteases. The secondary form has lost these characteristics and can be further discriminated by a decreased support of nematode growth and antibiotic production. It has been shown that isolates from nematodes are always in primary form and the conversion is unidirectional from primary to secondary form. (Gerritsen et al., 1992; Wang et al., 2007).

In general all *Photorhabdus* isolates are obtained from infected insects or their associated nematode host and no free living strains have been detected yet. However, some isolates isolated from human wounds draw attention as clinical relevant strains causing invasive soft tissue and disseminated bacteremic infections. It is the first time that the bacterium is not recognized to be associated with a nematode and according to this the strain is given the name *P. asymbiotica*. But the epithet turned out to be a misnomer, as Gerrard et al. (2006) succeeded in identifying the nematode symbiont. For all that, the reports of *Photorhabdus asymbiotica* isolates from human wounds revealed that this strain represents an opportunistic human pathogen, in which the nematode part during infection is still unclear (Farmer et al., 1989; Gerrard et al., 2003). *Photorhabdus luminescens* is first isolated from a light emitting insect cadaver infected by entomogenous nematodes (Khan et al., 1976; Poinar 1975).

**The genus *Xenorhabdus***

The genus *Xenorhabdus* comprises of twenty highly diverse species *X. nematophila* (Thomas and Poinar, 1965), *X. bovienii*, *X. poinarii*, *X. beddingii*, *X. japonica* (Nishimura et al., 1994), *X. budapestensis*, *X. ehlersii*, *X. innexi*, *X. szentirmai* (Lengyel et al., 2005), *X. cabanillasi*, *X. doucetiae*, *X. griffiniae*, *X. hominickii*, *X. koppenhoeferi*, *X. kozodoii*, *X. mauleonii*, *X. miraniensis*, *X. romanii*, *X. stockiae* and *X. indica* (Somvanshi et al., 2006). The genus *Xenorhabdus* is highly isolated in the family of enterobacteriaceae. There is only a 4% DNA/DNA
relatedness to the type species of the type genus of enterobacteriaceae. In addition they are unable to reduce nitrate and lack the enzyme catalase, which both are positive characteristics of other genera in this family (Farmer, 1984). Nevertheless, the affiliation to the family of enterobacteriaceae is confirmed by phylogenetic analyses based on 16S rDNA and the existence of the enterobacterial common antigen (Brunel et al., 1997; Ramia et al., 1982).

In *Xenorhabdus* as well as in *Photorhabdus*, insecticidal toxins contribute to the pathogenicity against insects. The cytotoxin of *Xenorhabdus* α-xenorhabdolysin was purified from *X. nemataphila* and showed an apoptotic and haemolytic activity. This cytotoxin is encoded by two genes *xaxA* and *xaxB* and homologues thereof can also be found in other entomopathogenic bacteria like *Photorhabdus* and *Pseudomonas entomaphila* (Vigneux et al., 2007). Insight into the mechanism of how the bacteria overcome the insect immune response is delivered by the observation that a bacterial compound, presumably benzylideneacetone inhibits the insect phospholipase A2 (Kwon and Kim, 2008). The phospholipase plays an important role in the insect immune system as it turns on the eicosanoid biosynthesis pathway by hydrolyzing arachidonic acids from cellular phospholipids, which then mediate phagocytosis and nodulation (Park et al., 2003; 2004).

**Molecular characterization of Bacteria**

The ribosomal DNA (rDNA) unit is particularly popular for phylogenetic analyses because it includes highly conserved regions as well as highly variable regions, used to reconstruct phylogenetic relationships among organisms with varying degrees of relatedness (Hillis and Dixon, 1991). Taxonomic studies of entomopathogenic nematodes and their symbiotic bacteria have revealed the specificity that nematodes possess to their symbiont, *Photorhabdus* with *Heterorhabditis* and *Xenorhabdus* with *Steinernema* (Boemare et al., 1997). Using the technique of PCR based ribotyping of 16S rRNA gene, a number of *Photorhabdus* and *Xenorhabdus* isolates from the Caribbean region have been identified (Brunel et al., 1997; Fischer-Le Saux et al., 1998). Recently, Fischer-Le Saux et al. (1999) proposed a polyphasic classification of *P. luminescens* derived from various geographical regions. They reported a correlation between the bacterial samples from
different ecological regions and their maximum growth temperatures. For the taxonomy and systematics of bacteria, 16S rRNA gene sequences have been used to distinguish between groups of *Photorhabdus* bacteria (Fischer-Le Saux *et al*., 1999; Boemare, 2002).

With the advent of the polymerase chain reaction (PCR), even minute amounts of specific target DNA can be exponentially amplified for restriction fragment length polymorphism (RFLP) analysis. The presence of polymorphic bands reflects the amount of DNA sequence divergence between the compared populations (Nasmith *et al*., 1996). Comparison of 16S rRNA gene sequences has proved to be extremely useful for determining phylogenetic relationship among eukaryotic and prokaryotic organisms (Woese, 1987) and has been used in determining the relatedness of the genera *Photorhabdus* and *Xenorhabdus* (Rainey *et al*., 1995).

Morphological and biochemical approach is widely used in bacterial identification, whereas, with the development of molecular biology techniques, homology analysis based on 16S rRNA gene sequences have become the "golden index" in the field of bacterial identification (Shen and Feng, 2004). 16S rRNA gene is the skeleton of the small subunit ribosome and the necessary location for protein biosynthesis, its coding gene existence in all bacteria. In a long period of time, 16S rRNA gene select pressure is bigger, the sequence variation is slower; 16S rRNA gene have the feature of molecular chronometers, it can span the perspective of evolution of human life; 16S rRNA gene sequences have different evolution rate region, so it can be used in systems classification study of different evolution extent organism (Ochman and Wilson, 1987). Because of the length of nucleotide sequence is moderate and the structure is complete, the 16S rRNA gene was the common section in the classification study of bacteria. Recently the 16S rRNA gene sequence with the size of 1.5 Kb is considered and widely used in bacterial taxonomy. It contains high conservation region which have variable nucleic acid region in different species (Kox *et al*., 1995). Furthermore, the most important is that 16S rRNA gene can be sequenced easily. By combining the molecular phylogeny with traditional approaches, such as morphological, physiological and biochemical characteristics, bacteria identification can be carried out more accurately (Li *et al*., 2006; Mo *et al*., 2003).
The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include its presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are more accurate measure of time (evolution); and the 16S rRNA gene (1500 bp) is large enough for informatics purpose (Patel, 2001). Although it has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score of <97% represents a new species, the meaning of similarity scores of >97% is not as clear (Petti, 2007). The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less with regard to species (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing (Drancourt et al., 2000; Mignard and Flandrois, 2006; Woo et al., 2003). Lagace et al. (2004) studied the identification of bacterial community by using ARDRA analysis and 16S rRNA gene sequencing. Hayashimoto et al. (2005) reported about the genetic diversity on 16S rDNA sequence and phylogenetic tree analysis of bacteria.

Although 16S rRNA gene sequencing is highly useful in regards to bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera (Bosshard et al., 2006; Mignard and Flandrois, 2006) and DNA relatedness studies are necessary to provide absolute resolution to these taxonomic problems. In the case of Bacillus the type strains of B. globisporus and B. psychrophilus share > 99.5% sequence similarity with regard to their 16S rRNA genes, and yet at the DNA level exhibit only 23 to 50% relatedness in reciprocal hybridization reactions (Fox et al., 1992). The overall quality of nucleotide sequences deposited in public databases years ago is questionable, since many depositions are of poor quality (Heikens et al., 2005; Petti, 2007).

Molecular characterization of Nematodes

Duncan et al. (1999) analyzed some species of Pratylenchus as well as tree isolates of P. loosi, isolate T from Srilanka (original description by Loof, 1960) and isolates N1 and N2 from central Florida, USA describing P. loosi (Inssera et al.,
1996) by using D2-D3 LSU rDNA expansion segment sequence and found that there is substantial D2-D3 28S rDNA sequence difference between them. Among coding regions in rDNA, the D2-D3 expansion segments (part of the LSU) have evolved the most rapid changes. This region is readily amenable to the determination of DNA sequences. It has proved useful for resolving closely related taxa (Al Banna et al., 1997; Duncan et al., 1999; Kanzaki and Futai, 2002; Nadler, 2002).

The D2-D3 expansion segments of the 28S rDNA subunit (D2-D3 LSU-rDNA) are the longest expansion fragments in the LSU and are the most rapidly evolving coding region of the rDNA genes (De Ley et al., 2002; Kaplan et al., 2000; Al Banna et al., 2004; Subbotin et al., 2005). In the recent years, comparative analysis of the D2/D3 28S rDNA expansion segment sequence has become a popular tool to differentiate cryptic species which are morphologically identical (or with some overlapped morphological variation) but genetically distinct (Subbotin et al., 2005).

Over the last few years, an impressive number of molecular data-based papers have been published that focused on specific taxonomic groups. In the basal part of the nematode tree, the resolution among dorylaimida, remarkably poor with SSU rDNA data, was substantially improved by using the 5' region of the LSU rDNA (Holterman et al., 2008a). The under-representation of marine nematodes in the phylogenetic overview presented so far was, to some extent, lifted by SSU rDNA-based papers from Meldal et al. (2007) and Holterman et al. (2008a). Nadler et al. (2007) greatly increased our insight into the relationships among animal-parasitic nematodes by analysing 113 SSU rDNA sequences. Bert et al. (2008) investigated relationships within the suborder tylenchina (covering four infraorders, namely panagrolaimorpha, cephalobomorpha, drilonematomorpha and tylenchomorpha) by combining SSU rDNA data and morphological information on the female gonoduct. Holterman et al. (2009) concentrated on the tylenchomorpha (mainly insect and plant parasites) and revealed phylogenetic relationships among some of the major plant parasites based on 116 SSU rDNA sequences.

**Secondary metabolites of Photorhabdus and Xenorhabdus**

More than thirty compound classes have been isolated from Photorhabdus and Xenorhabdus in the last three decades denoting a high structural biodiversity and
specificity among these compounds (Brachmann et al., 2008). Four classes of compounds are specifically described in the genera Photorhabdus. Derzelle et al. (2002) identified a gene cluster in Photorhabdus responsible for the biosynthesis of carbapenems, a class of β-lactam antibiotics that have a carbon in place of sulphur in the 5-membered ring system. This antibiotic class showed activity against a few Gram-negative bacteria (Derzelle et al., 2002). The first isolated secondary metabolites from Photorhabdus are stilbenes viz. 2-isopropyl-5-[(E)-2-phenylethenyl] benzene-1, 3-diol (IPS) and 2-ethyl-5- [(E)-2-phenylethenyl] benzene-1,3-diol (ES), (Paul et al., 1981). An epoxidized form of IPS 2-isopropyl-5-(3-phenyloxiranyl)benzene-1,3-diol (eIPS) is isolated recently from infected insect larvae and showed activity against many bacteria and even against a drug-resistant clinical strain of Staphylococcus aureus. Photorhabdus produces a catecholate siderophore named photobactin which contributes to the antibiosis in the insect cadaver (Ciche et al., 2003). Benzylideneacetone acts as phospholipase A2 inhibitor which mediates immunosuppression in the insect and consequentially might enhance the virulence of the bacteria and nematode complex (Kwon and Kim, 2008). Following the identification of hydroxystilbenes and indoles as antibiotics in cultures of Photorhabdus and Xenorhabdus, respectively, by Paul et al. (1981), more antimicrobial compounds are reported, such as xenorhabdins (McInerney et al., 1991a), xenocoumacins (McInerney et al., 1991b), xenorxides (Li et al., 1998), xenomins (unpublished) and nematophilin (Li et al., 1997). Strain A2 of X. bovienii is unusual in the diversity of small-molecule antimicrobial compounds as xenomins and xenorxides, several xenorhabdins, including three new ones and four indoles have been isolated from this strain alone (Chen, 1996). These compounds showed strong activity against Gram-positive bacteria, yeast and many fungal species.

Xenorhabdins are dithiopyrrolone derivatives, a class of compounds that is isolated initially from Streptomyces species and are active against a variety of fungi, amoebae and bacteria (Celmer and Solomons, 1955). Xenorhabdins have significant antibacterial activity against Gram-positive bacteria but have little effect against Gram-negative bacteria (McInerney et al., 1991a). Xenorhabdins are isolated from X. nematophila and X. bovienii, whereas the oxidized xenorxide derivatives are only known from the latter. Several xenorhabdin derivatives have been described to confer
antibacterial, antifungal and insecticidal activity (Li et al., 1998; McInerney et al., 1991). The mechanism of antimicrobial action of dithiolopyrrolone derivatives, such as thiolutin, is primarily through inhibition of RNA and protein synthesis, as has been shown in yeast (Jimenez et al., 1973; Tipper, 1973).

Xenocoumacins belong to the same class of compounds, with regard to structure and pharmacological activity, as the amicoumacins that are isolated initially from Bacillus pumilus (Itoh et al., 1982). Compounds of this class are normally active against Gram-positive bacteria, including Staphylococcus and Streptococcus species, and some Gram-negative bacteria, such as Escherichia coli (McInerney et al., 1991b). However, most enterobacteria and pseudomonas aeruginosa are resistant to xenocoumacins, as are the drug-resistant strains of S. aureus, xenocoumacin 1 is also active against the fungal species Aspergillus and Trichophyton and the yeasts Candida and Cryptococcus (McInerney et al., 1991b).

The xenocoumacins 1 and 2 are the major antibiotics produced by X. nematophila. They exhibit a broad biological spectrum as they show antibacterial activity against many Gram positive bacteria and bear a strong antiulcer activity. In addition, xenocoumacin 1 also exhibits an antifungal activity (McInerney et al., 1991). Very recently the biosynthesis gene cluster of xenocoumacins is identified and revealed several independent transcriptional units. This seems consistent with the detection that xenocoumacin 2 is derived biosynthetically from xenocoumacin 1 in a much later phase of growth (Starr et al., 1996; Reimer, 2008; Reimer et al., 2009).

Recently xenematide and xenortides have been identified as the first peptides from X.nematophila with xenematide showing weak insecticidal activity (Lang et al., 2008). The compounds produced by the three Xenorhabdus sp. (X. nematophila, X. budapestensis and X. szentirmaii) were powerful in bioassay against mastitic Staphylococcus aureus, Escherichia coli and Klebsiella pneumoniae isolated from dairy cows but the sensitivity of the isolates differed from each other (Furgani et al., 2008). Ribeiro et al. (2003) purified the flh DC-dependent cytotoxin, alpha-xenorhabdolysin which targets the plasma membrane of insect hemocytes and of mammalian red blood cells. The alpha-xenorhabdolysin (Xax) triggers apoptosis in both insect and mammalian cells (Vigneux et al., 2007).
Production of Antibiotics

Dutky et al. (1964) suggested that the bacteria which live as symbionts of the EPN Steinernema carpocapsae produce an antibiotic which was confirmed by Akhurst (1982) who showed that two of these bacteria viz. Xenorhabdus spp., and Photorhabdus luminescens produce antibiotics that inhibit the growth of many bacteria and yeast species. Several antibiotics and antmycocotics from Xenorhabdus and Photorhabdus have been isolated and characterized (Paul et al., 1981; Nealson et al., 1990; McInerney et al., 1991a; 1991b) and among these compounds Xenocoumacin 1, isolated from X. nematophila is active against animal and human pathogenic fungi (McInerney et al., 1991b). Xenorhabdus species produce two forms of colony on agar media (Akhurst, 1980). Several invertebrate groups, including nematodes (Aballay and Ausubel, 2001; Gallagher and Manoil, 2001; Garsin et al., 2001; O’Quinn et al., 2001) and insects (Daborn et al., 2001; De- Gregorio et al., 2001; Wu et al., 2001), are being developed as model systems for the study of bacterial virulence.

The production of secondary metabolites with antibiotic properties is a characteristic common to many bacterial species. The study of Xenorhabdus spp. and Photorhabdus spp. both in vitro (Paul et al., 1981; Akhurst, 1982; Li et al., 1995a; 1995b; 1997) and in vivo (Maxwell et al., 1994; Jarosz, 1996; Hu et al., 1997; 1998; 1999) has increased our understanding not only of the chemistry of the metabolites and their possible role in the biology of the symbiosis, but also of their production techniques.

Despite promising results and patents, published information on the commercial use of Xenorhabdus antibiotics has not been realized. Interestingly, the antibacterial activity of the complete cell free media are much stronger (Brachmann et al., 2006) than any of the isolated, identified or patented compounds e.g. nematophin (Li et al., 1997). The antibiotically active, non-purified, cell-free liquid cultures, of Xenorhabdus strains are effective against a large spectrum of invaders, viz bacteria, fungi and protozoa. Recently, Boszormenyi et al. (2009) showed that secondary metabolite(s) produced by Xenorhabdus budapestensis effectively reduced fire blight indexes on apple trees in greenhouse conditions and also exerted strong toxicity on
both zoospores and cystospores of Phytophthora nicotianae. While there are advantages of using two or more Xenorhabdus strains/species simultaneously intraspecific/inter-generic competition must be considered. Different EPN/EPB complexes have been shown to invade the same insect. Inter-species competition involves bacteriocins and xenocins (Boemare et al., 1992) phage derived bacteriocins (Thaler et al., 1995; 1997) and colicin E3 type killer proteins (Singh and Banerjee, 2008). Sicard et al. (2006) monitored experimental inter-specific competition between two EPN species, S. carpocapsae and S. scapterisci and their respective EPB symbionts, X. nematophila and X. innexi, within an experimental insect-host (Galleria mellonella). Furgani et al. (2008) compared antibacterial compounds from some of the Xenorhabdus strains against Gram-positive and Gram-negative mastitis isolates.

Insect-nematode-bacterium tripartite associations such as those involving Xenorhabdus species provide attractive systems for both discoveries of new natural products, identification of novel compounds involved in inter kingdom signaling and antibiotics (Park et al., 2009). The evolution of the entomopathogenic Xenorhabdus bacteria has resulted in a broad, inter-specific, diversity of compounds with antimicrobial activity. These antagonize related or non-related competitors in the insect cadaver in the soil (Sicard et al., 2006). To compete successfully with invaders, EPB species produce several watersoluble and non-polar compounds with antibiotic activity (Paul et al., 1981; McInerney et al., 1991a; Sztaricskai et al., 1992; Sundar and Chang, 1993; Webster et al., 1996; 2002; Li et al., 1998). The water-soluble peptide antimicrobial compounds xenocoumacin 1 (Xcn1) and 2 (Xcn2), both of which are hybrids of amino and carboxylic acid moieties, are the major antibiotics produced in broth culture by X. nematophila strain (McInerney et al., 1991a). Both Xcn1 and Xcn2 were also shown to be produced in the haemocoel of Xenorhabdus infected insect cadavers (Maxwell et al., 1994). Xcn1 is active against Gram-positive and Gram-negative bacteria and several fungal species, while Xcn2 is less active against the bacteria and inactive against the fungal species examined (McInerney et al., 1991b). Recent molecular genetic analysis has identified a 14 gene complex involved in the biosynthesis of Xcn1 and conversion to Xcn2 (Park et al., 2009).
Antimicrobial activity

Akhurst (1982) demonstrated the antibiotic activity of cultures of *Xenorhabdus* spp. against a wide variety of microorganisms, including the Gram-positive *Micrococcus*, *Staphylococcus* and *Bacillus*, the Gram-negative *Escherichia*, *Shigella*, *Enterobacter*, *Serratia*, *Proteus*, *Erwinia*, *Flavobacterium* and *Pseudomonas*, and the yeasts *Candida*, and *Saccharomyces*, although the chemical nature of the antibiotic substances in the cultures was not known. Chen *et al.* (1994) observed a strong antimycotic activity in cultures of the symbiotic bacteria on a range of fungi including *Botrytis cinerea, Fusarium oxysporum, Fusarium solani, Mucor piriformis, Pythium coloratum, Pythium ultimum, Penicillium* spp., *Rhizoctonia solani, Trichoderma pseudokoningii* and *Verticillium dahliae*.

More than 30 bioactive secondary metabolites, belonging to diverse chemical classes, have been reported from cultures of *Xenorhabdus* and *Photorhabdus*. These include puromycin and madumycin 11 (unpublished), both of which were previously identified from cultures of *Streptomyces* (Suhadolnik, 1970; Tavares *et al.*, 1996). Following the speculation of Dutky (1959) as to the antimicrobial activity of the bacterial symbiont of *Steinernema* (*Neoaplectana*), there was 'relative silence' on the subject for two decades until Paul *et al.* (1981) isolated and identified several new antibacterial compounds produced by *Xenorhabdus* spp. Thereafter, there has been a steady flow of publications reporting the chemical nature and bioactivity of the products derived from both *Xenorhabdus* and *Photorhabdus* species (Frost and Nealson, 1996; Li *et al.*, 1998; Webster *et al.*, 1998). An important break through was the discovery of a large-molecule, insecticidal toxin from cultures of *Photorhabdus* (Ensign *et al.*, 1990) and this is reported upon elsewhere. However, it is the range of small-molecule compounds with antibiotics, insecticidal and nematicidal properties that has continued to intrigue researchers as they seek to identify a role for these substances in the biology of the symbiosis. Examination of *in vitro* cultures of the symbiotic bacteria has revealed secondary metabolites that have antibiotic activity against a wide range of bacterial and fungal species including human pathogenic fungi and yeasts (McInerney *et al.*, 1991a; 1991b; Li *et al.*, 1997) and multi-drug resistant, human pathogenic bacteria (Chen, 1996). As well, antineoplastic activity has been reported (Webster *et al.*, 2000) and further medicinal potential is foreseen.
Chen et al. (1994) demonstrated the effect of *Xenorhabdus* and *Photorhabdus* on growth of some pathogenic and non-pathogenic fungi *in vitro* and Vagelas et al. (2004) achieved suppression of *Fusarium oxysporum* f.sp. *lycopersici* with the bacterium *Flavimonas oryzihabitans* in pot experiments.

Bacterial proteases are mainly involved in providing peptide nutrients for the microorganisms. In fact, some authors regard bacterial proteases as the main virulence factors present among the extracellular factors (Secades and Guijarro, 1999; Miyoshi and Shinoda, 2000; Secades et al., 2001; Farto et al., 2002). Although extracellular proteases produced by entomopathogenic bacteria play a role in insect toxicity, their physiological importance is highly conflicting. Certain literatures acknowledge that proteases might have a role in insect toxicity by analogy with proteases produced by other pathogens (Jarosz et al., 1991). Alkaline proteases have been estimated in different species of entomopathogenic bacteria of the genera *Xenorhabdus* (Kucera and Mracek, 1989; Abu Hatab et al., 1998; Caldas et al., 2002) and *Photorhabdus* (Abu Hatab et al., 1998; Bowen et al., 2000; Cabral et al., 2004; Marokhazi et al., 2004). Laboratory studies were undertaken with the symbiotic bacteria isolated from some of the indigenous *Steinernema* and *Heterorhabditis* spp. (Nagesh et al., 2002) in order to test the bioefficacy of bacterial cells and cell-free culture filtrates from these bacteria against 2nd instar larvae of *Helicoverpa armigera* (Hubner), *Phthorimaea operculella* (Zeller), *Plutella xylostella* (Linnaeus) and *Spodoptera litura* (Fabricius) and 3rd instar larvae of *H. armigera* and *S. litura*.

The literature on peptide-like molecules (Thaler et al., 2001; Badosa et al., 2007) with antibiotic potential is extensive, but there is no overall view concerning structure activity relations. The known peptides of antimicrobial activity are completely different from bicornutin-A. They did not contain arginine, but leucine at or close to the N-terminal position. Several arginine-containing peptides, such as colicins are known as 'delivery vectors' (Tung and Weissleder, 2003).

Extracellular alkaline proteases produced by different species of *Photorhabdus*, a symbiont of *Heterorhabditis* sp. (Bowen et al., 2000; 2003; Waterfield et al., 2001) and *X. nematophila* strain *kraussei*, a symbiont of the *Steinernema carpocapsae* (Kucera and Mracek, 1989) have been purified,
characterized and classified as alkaline metalloproteases. Recently, they have been also purified and characterized from different species of *Bacillus* (Prakash *et al*., 2005; Miyaji *et al*., 2006), *Pseudomonas* (Gupta *et al*., 2005) and *Stenotrophomonas* (Miyaji *et al*., 2005).

**Insecticidal activity**

EPN have also been shown to have potential for controlling field populations of the sweetpotato weevil, *Cylas formicarius* (Fabricius) (Jansson *et al*., 1990; 1991; 1993). *Heterorhabditid* nematodes were superior to steinernematids at reducing weevil populations and their concomitant damage to storage roots (Jansson *et al*., 1990; 1993). Welch and Briand (1960) found that aqueous applications of *Steinernema carpocapsae* (Weiser) DD136 strain and those made using infected greater wax moth, *Galleria mellonella* (L.), cadavers, were equally effective at controlling cabbage root maggot, *Hylemya brassicae* (Bouche). More recently, Jansson *et al*., (1993) showed that application of *G. mellonella* cadavers infected with heterorhabditid nematodes were efficacious for controlling field populations of *C. formicarius* and their concomitant damage; however, nematode application via infected cadavers were not compared with those made via aqueous suspension.

The efficacy of cell free filtrate of *Photorhabdus* sp. against insects depends on insecticidal toxins produced by the bacteria. Bowen *et al*. (1998) and Jarrett *et al*. (1997) demonstrated that, when cultured in liquid medium, *Photorhabdus* and *Xenorhabdus* species secrete highly virulent insecticidal toxins. The cell free filtrate of *P. luminescens* and *X. bovienii* were more lethal to both *G. mellonella* and *H. philanthus* than were similar filtrates of *X. poinarii*. As injections of *P. luminescens* and *X. bovienii* were very lethal to the insects and injections of *X. poinarii* had only limited impact, it is obvious that symbiotic bacteria act against *G. mellonella* and *H. philanthus* via the toxins they produce. The toxic effect of *Xenorhabdus* sp. filtrate against insects is poorly documented. Some studies demonstrate the existence of a toxic factor in *X. nematophilus* (Jarrett *et al*., 1997) and other *Xenorhabdus* species (Palha *et al*., 1998). Hu *et al*. (1999) and Grewal *et al*. (1999) demonstrated the toxicity of cell free culture filtrate of *X. nematophilus* and *P. luminescens* against second stage juveniles of *Meloidogyne incognita*. ffrench-Constant and Bowen (1999)
suggested that insecticidal activities of *P. luminescens* could be eliminated by heat treatment.

The bacterial symbionts of *X. nematophila* and its secretion are lethal to *G. mellonella* larvae when applied in sand media rather than filter substrate, thus confirming the results of Gotz *et al.* (1981). Similar results were also reported in fire ant (Dudney, 1997) and the beet army worm (Elawad *et al*., 1999) when *X. nematophila* bacterium was applied in sand media to control these pests. These studies considered that symbiotic bacteria may effectively act without the need of vector. Bacterial concentration of $4 \times 10^6$ cells/ml in broth caused 100% mortality of *Galleria* and indicated the harmful effect of *S. carpocapsae* penetration into the haemocoel of this insect. Similar bacterial dose caused 100% mortality in root-knot nematode, *Meloidogyne javanica* within 24 hr of application (Samaliev *et al*., 2000).

Fractions resolved by ion exchange chromatography (10 µl) were injected aseptically, using a 1-ml syringe with a -26-G needle, into the hemocoel of 5th instar *S. litura* and *Galleria mellonella* larvae. Five insects were used to assay each fraction, and care was exercised to eliminate cross contamination of proteins from different fractions. Following injection, insects were observed for 48 hr for viability and other morphological changes. If at least four or five insects injected with the fraction died in 48 hr, the fraction was considered insecticidal (Rajagopal and Bhatnagar, 2002).

Entomopathogenic nematode, *S. carpocapsae* carries specific symbiotic bacterium, *X. nematophila* which is pathogenic to a wide range of agriculturally important insect pests (Poinar, 1979). Toxic secretion of *X. nematophila* in broth worked rapidly and caused 95% mortality after 96 hr of application while the cells suspension took 48 more days to kill 93% larvae. Many researchers also approved the use of bacterial toxin obtained from *X. nematophila* to control various insect pests (Bowen and Ensign, 1998; ffrench-Constant and Bowen, 1999; Ensign *et al*., 2002).

When bacterial cells in broth were applied against the *Galleria*, they penetrated into the insect body within 4 hr, whereas bacterial cells in water gave similar results after 16 hr of application (Mahar *et al*., 2005). In another study, 100% cells penetration of *X. nematophila* in broth and water suspension was observed in larvae and pupae of diamondback moth after 4 hr and 8 hr, respectively (Mahar,
The role of the bacterial cell, as a parasite, is to produce the toxins which break down the immunity of the insect. *X. nematophila* recovered from the insect body indicated that the bacteria entered into the haemocoel of the *G. mellonella* larvae from different body openings (spiracles, cuticle, mouth or anus). However, the passage through which these bacterial cells gain entry to the haemocoel is unclear but both species of bacterial symbiont exhibit swarming motility when grown on suitable solid media (Mohan *et al.*, 2003; Forst and Nealson, 1996; Boemare *et al.*, 1997; Elawad, 1998). A possible point of entry for motile cells under moist conditions is the spiracle, the only organ other than the mouth or anus directly open to the external environment.

EPN demonstrate great variation in their pathogenicity to insects; some of the species or strains are highly specific (Georgis and Manweiler, 1994). It has been reported that on their own, *Photorhabdus* and *Xenorhabdus* display virulence against insects only after injection of bacterial cells or bacterial preparations directly into the insect hemocoel (Dunphy, 1995). Nematode-based commercial products have become available for use against several pests worldwide during the past decade (Grewal and Smith, 1996). These nematode–bacteria complexes are highly virulent to insects and are considered as one of the best non-chemical insect pest control alternatives. As a result, many surveys to isolate these organisms have been reported and research is ongoing worldwide to study EPN–bacteria taxonomy, phylogenetics, biogeography, biology, genetics, physiology, biochemistry and ecology as well as commercial production, formulation, and application technologies (Adams *et al.*, 2006; Kaya *et al.*, 2006). However, relatively few studies have emphasized the biological characterization of the symbiotic bacteria. Burnell (2002) proposed the utility of such studies to improve our understanding of factors that modulate the efficacy of nematode–bacteria complexes. Recent studies of the distribution and ecology of EPN in Mediterranean areas, including isolation of the symbiotic bacteria, provide valuable knowledge about the nematode–bacteria complexes (Emelianoff *et al.*, 2008; Stock *et al.*, 2008).