# Characterisation of nematode symbiotic bacteria and the *in vitro* liquid culture of *Heterorhabditis zealandica* and *Steinernema yirgalemense*

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## Abstract

Entomopathogenic nematodes have the potential to be outstanding biocontrol agents against agricultural pest insects. Combined with their bacterial symbionts, these biocontrol agents have proven to be very effective against numerous pests. The nematodes belong to the families Steinernematidae and Heterorhabditidae, and are ideal to be used in, and integrated with, pest management systems. There is a dire need for new and innovative methods to control agricultural pests, as numerous pest insects have developed resistance against broad-spectrum insecticides. Together with the environmental impact of these insecticides and the safety aspect regarding humans and animals, the need to develop new technologies, including entomopathogenic nematodes for pest management, is high. In this study, the associated symbiotic bacteria of three entomopathogenic nematodes species were isolated, and the potential of two nematode species to be successfully mass cultured in liquid medium was evaluated.

Regarding the symbiotic bacteria, results from the study showed that bacteria species from all three nematode species, *Heterorhabditis noenieputensis*, *Steinernema khoisanae* and *Heterorhabditis zealandica*, were novel. *Heterorhabditis noenieputensis* was isolated in the Mpumalanga province during a previous survey conducted in citrus orchards. The bacterium isolated from this nematode belongs to the genus *Photorhabdus,* and bear closest similarity (98.6%) to the type strain of *P. luminescens* subsp *laumondii* (TT01$^{T}$). *Photorhabdus luminescens* subsp. *noenieputensis* subsp. nov., derives its name from the area where the nematode was sourced, namely the farm Springbokvlei, near the settlement Noenieput close to the Namibian border. Thus far, 85 *Steinernema* spp. have been described worldwide, including *S. khoisanae* which was isolated in the Western Cape province of South Africa. Four *S. khoisanae* strains, namely SF87, SF80, SF362 and 106-C, were used for characterisating the new bacteria from different localities in South Africa. Using the neighbor-joining method, all the strains were aligned with 97% homology to the 16S rRNA sequences of several *Xenorhabdus*- type strains, indicating that they belonged to the same genus. The multigene approach was used to distinguish between the *Xenorhabdus* spp. and partial *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences of the various strains were analysed. The bacterium species was named *Xenorhabdus khoisanae* sp. nov. after the nematode from which it was isolated. The results showed that the third bacterium species, which was isolated from *H.*

*zealandica,* was new. The sequence of the bacteria strain clustered with the type strains of *P. temperata* and *P. asymbiotica,* indicate that it belonged to the genus *Photorhabdus*. This is the first study to show that *H. zealandica* associates with a luminescent *Photorhabdus* species, rather than with the known non-luminescent *P. temperata*.

The potential of *H. zealandica* and *Steinernema yirgalemense* mass culture in liquid was investigated. Results illustrated that *H. zealandica* and its *P. luminescens* symbiont can be successfully cultured in liquid. However, two generations occurred during the process time, instead of the desirable one-generation. The growth curve of the symbiotic bacteria during the process time was measured, in order to determine when the stationary phase was reached, with the results showing this to occur after 36 h. Therefore, the optimum amount of time required for inoculating the IJs and for aiding in maximum infective juvenile (IJ) recovery is 36 h for adding the nematodes post pre-culturing of the bacteria. Future research goals should be to increase the percentage recovery in liquid culture, which would increase the number of nematodes produced per ml, which would, therefore, reduce the processing time significantly.

The results from mass culturing the second nematode species, *S. yirgalemense,* indicated an asynchronous nematode development in the first generation. Growth curves were performed with the symbiotic bacteria that showed the exponential phase of *Xenorhabdus* started after 15 h, and that, after 42 h, the stationary phase was reached, with an average of $51 \times 10^7$ cfu·ml$^{-1}$. Bioassays were performed to compare the virulence between *in vitro*- and *in vivo*-produced nematodes, with the results showing that the *in vitro*-produced nematodes were significantly less virulent than were the nematodes produced *in vivo*. The success obtained with the production of *S. yirgalemense* in liquid culture can serve as the first step in the optimising and upscaling of the commercial production of nematodes in industrial fermenters.

The last aim of the current study was to determine when *Xenorhabdus* reached the stationary phase, when it is grown in a 20-L fermenter, as this would be the optimum time at which to add the IJs of *S. yirgalemense*. Such characteristics as the effect of stationary phase conditions on the bacterial cell density and on the $DO_2$ rate in the fermenter were investigated. The results showed that the stationary phase of *Xenorhabdus* was reached after 36 h at 30˚C, which took 6 h less than did the same procedures followed with the *Xenorhabdus* sp. cultured in Erlenmeyer flasks on orbital shakers. This is the first step

toward the liquid mass culturing of *S. yirgalemense* in industrial-size fermenters. Data from this study indicated the optimum amount of time that is required for adding nematodes to the bacterial culture in the fermenter, and for ensuring the optimum recovery of IJs, as well as a subsequent high yield of nematodes within a minimum processing time.

This is the first report of its kind to investigate comprehensively the successful liquid culture of two South African entomopathogenic nematode species for the sole purpose of evaluating potential commercialisation. Results emanating from this study could be used as groundwork in future, in combination with similar research such as culturing nematodes intensively in large fermenters.

## Opsomming

Entomopatogeniese nematodes het die potensiaal om as doeltreffende biologiese beheeragente teen sleutelplaaginsekte gebruik te word. Elke nematood werk interaktief met ‘n spesifieke bakterium. Entomopatogeniese nematodes, behorende tot die families Steinernematidae en Heterorhabditidae, is ideale kandidate vir gebruik in ‘n geïntegreerde plaagbestuurprogram. Tans is daar ’n behoefte vir nuwe metodes vir die beheer van plaaginsekte, omdat meeste insekte reeds weerstand opgebou het teen bestaande plaagdoders. As gevolg van die negatiewe impak van plaagdoders op die omgewing, asook kommer oor veiligheid vir die mens en diere, is die ontwikkeling en gebruik van alternatiewe plaagbeheermiddels noodsaaklik.

In die eerste deel van die studie word drie nuwe bakterie spesies geïsoleer en beskryf. Resultate van hierdie studie het aangetoon dat die bakterië spesies vanuit die nematode spesies, *Heterorhabditis noenieputensis*, *Steinernema khoisanae*, en *Heterorhabditis zealandica*, tot dusver onbeskryf was. Eersgenoemde, *H. noenieputensis,* is afkomstig van ’n sitrusboord in die Mpumalanga Provinsie. Die bakterie hieruit geïsoleer behoort tot die genus *Photorhabdus* en is biologies verwant (98.6%) aan *P. luminescens* subsp *laumondii* ($TT01^T$). Die bakterie is benaam as *Photorhabdus luminescens* subsp. *noenieputensis* nov. en is na die nematood waaruit dit geïsoleer is vernoem. Tot dusver is wêreldwyd 82 spesies van *Steinernema* spp. beskryf, insluitende *S. khoisanae* van die Weskaap provinsie. Vier bakterie isolate is van *S. khoisanae*, SF87, SF80, SF362 en 106-C geïsoleer. Die buur-koppeling metode was gebruik om te bepaal dat hierdie bakterie isolate tot 97% ooreenstem met verskeie isolate van *Xenorhabdus* se 16S rRNA DNS volgordebepalings. Om tussen *Xenorhabdus* spp. te onderskei is ’n multi-geen benadering gebruik deur gedeeltelike *recA*, *dnaN*, *gltX*, *gyrB* en *infB* DNS basispaar volgordebepalings van die verskeie isolate te bepaal. Hierdie bakterie isolaat is soortgelyk ook vernoem as, *Xenorhabdus khoisanae* sp. nov., na die nematood waaruit dit geïsoleer is. Die derde onbekende bakteriële spesie is uit *H. zealandica* geïsoleer. Die DNS basispaar volgordebepaling van die 16S geen van SF41 toon aan dat dit in dieselfde groep as *P. temperata* en *P. asymbiotica* val en sodoende aan die genus *Photorhabdus* behoort. Hierdie is die eerste studie met die bevinding dat *H. zealandica* ook met ’n ander bakterie spesie geassosieer kan word buiten die normale *P. temperata* spesie.

Die tweede deel van die studie gaan oor die teling van twee nematood spesies, *H. zealandica* en *Steinernema yirgalemense*, en hulle is geëvalueer vir hulle potensiaal om geteel te word in 'n vloeibare medium. Die resultate het gewys dat *H. zealandica* met sy *P. luminescens* simbiont suksesvol in vloeistof aangeteel kan word, ten spyte van die feit dat daar twee generasies ontwikkel het, in plaas van die meer ideale enkel generasie. Die groeikurwe van die simbiotiese bakterie was gemonitor om te bepaal wanneer die stasionêre fase bereik word. Die resultate toon dat hierdie fase na 36 uur bereik was. Dus was die infektiewe nematode larwes eers na 36 uur tot die vloeibare medium waarin die bakterie geteel was bygevoeg. Navorsing in die toekoms moet dus gefokus wees om die persentasie herwinning van die infektiewe larwes te verhoog. Dit sal daartoe lei dat meer nematodes per ml geproduseer kan word en ook die prosesseringstyd van die nematodes verminder.

'n Tweede nematode spesie, *S. yirgalemense,* was ook in vloeistof geteel. Hier het 'n asinkroniese ontwikkeling in die eerste generasie plaasgevind wat problematies is. Groeikurwes is bepaal van die bakteriële simbiont en die resultate het gewys dat die groeifase van *Xenorhabdus* na 15 uur in aanvang geneem het en dat die stasionêre fase bereik was na 42 uur met 'n gemiddelde van $51 \times 10^7$ selle·ml$^{-1}$. Die virulensie van nematodes wat *in vitro* geteel is, is vergelyk met die virulensie van nematodes wat *in vivo* geteel is en die resultate het getoon dat die *in vitro* geteelde nematodes minder virulent was. Die teling van *S. yirgalemense* in vloeistof was oor die algemeen meer suksesvol as die teling van *H. zealandica* in dieselfde medium.

Die doelwit van die laaste gedeelte van hierdie studie was om te bepaal wanneer *Xenorhabdus* die stasionêre fase bereik wanneer dit in 'n 20-L fermenter gekweek word. Dit bepaal sodoende die optimale tyd wanneer die infektiewe larwes van *S. yirgalemense* bygevoeg behoort te word. Die uitwerking van die stasionêre fase op die bakteriële selle, asook die $DO_2$-konsentrasie in die fermenter, was geëvalueer. Resultate het gewys dat die stasionêre fase van *Xenorhabdus* na 36 uur bereik was, wat 6 uur korter is as toe dit gekweek is in Erlenmeyer flesse. Hierdie studie is die eerste stap om die massa teling van *S. yirgalemense* in industriële fermenters suksesvol te bemeester. Die data wat verkry was, het aangedui wat die ideale tydsduur sal wees om die bakteriegetalle te vermeerder voordat die nematode bygevoeg word.

Hierdie is die eerste studie wat die teling van twee Suid-Afrikaanse nematode spesies omvattend in vloeistof evalueer het. Die hoof doelwit is om die potensiaal van hierdie nematode spesies, met die oog op kommersiële gebruik, te meet. Die resultate van hierdie studie kan gekombineer word met toekomstige studies in hierdie spesifieke navorsingsveld.

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## Table of Contents

## List of Tables

## Table of Figures

# CHAPTER 1

## Literature review

## *Xenorhabdus* and *Photorhabdus*, bacterial symbionts of the entomopathogenic nematodes *Steinernema* and *Heterorhabditis* and their *in vitro* liquid culture

### Introduction

Annually, numerous insect pests cause damage to fruit and vegetables that are grown as food crops all over the world (Wyniger, 1962). These insect pests are a serious economic burden on agriculture in South Africa. Control methods that are highly specific to the target pests and that are, in addition, environmentally friendly, such as biological control agents, should constitute a major component of integrated pest management systems.

Entomopathogenic nematodes (EPNs) represent an important part of the spectrum of potential biological control agents. Previous research in South Africa has shown that two local nematodes, *Heterorhabditis zealandica* Poinar, 1990 and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005, in particular, have great insecticidal potential (De Waal *et al.*, 2011a,b, 2012; Malan *et al.*, 2011; Van Niekerk & Malan, 2012). Therefore, the ability to mass culture these two nematode species in liquid medium, using *in vitro* technology, is an important step toward their application as biocontrol agents on a commercial scale against key insect pests. However, for *in vitro* technology to be successful, the nematode-bacteria interaction needs to be understood. Bacterial symbionts of EPNs need to be isolated and characterised, with the life cycle of the nematode in culture requiring to be understood to be able to optimise for maximum nematode yield in liquid culture.

### *Xenorhabdus* and *Photorhabdus*

EPNs of the families Steinernematidae and Heterorhabditidae share a mutualistic relationship with bacteria of the genera *Xenorhabdus* Thomas & Poinar, 1979 and *Photorhabdus* Boemare, Akhurst & Mourant, 1993, respectively. The bacteria belong to the family Enterobacteriaceae, whose characteristics include being gram-negative and having non-fermentative rods (Koppenhöfer, 2007). *Photorhabdus* and *Xenorhabdus* are a unique group, as they are phenotypically (Holt *et al*., 1994) and genotypically (Brenner & Farmer, 2005) similar to no other genera grouped in this family. Both of them produce the enterobacterial common antigen that is present among the species of Enterobacteriaceae (Ramia *et al*., 1982). Said nematode bacterial symbionts are pathogenic to insects. There is, however, an exception to the rule, as one species, *Photorhabdus asymbiotica* Fischer-Le Saux, Viallard, Brunel, Normund & Boemare, 1999 has been found to be an opportunistic pathogen to humans (Farmer *et al*., 1989; Peel *et al*., 1999).

The bacterial symbionts are carried by their associated nematodes and released into the haemolymph of a host insect. Once inside the insect, the symbiotic bacteria overcome the immune system of the host and release endo- and exotoxins. Septicaemia develops, with the death of the host insect usually occurring within one to two days (Poinar, 1990a; Forst & Clarke, 2002). The bacterial symbionts contribute to the symbiotic relationship with EPNs by creating conducive conditions for nematode growth and reproduction in the host (Boemare *et al*., 1997b). Nutrients, as well as antimicrobial substances, are provided that inhibit the growth of a wide range of micro-organisms (Akhurst, 1982) in and on the insect cadaver. They also excrete substances preventing scavenging nematodes and insects from utilising the cadaver as a food source (Zhou *et al*., 2002). Up to three generations of nematodes can be produced per host, depending on the size of the insect host (Kakouli-Duarte & Hague, 1999). In smaller hosts, only one or two generations are produced (Ferreira, 2010; Van Niekerk & Malan, 2012). As soon as the food in the host cadaver is depleted, a new cohort of infective juveniles (IJs) enters the environment, with their bacterial symbionts enclosed in the digestive system of the nematode. The bacteria have not so far been reported as occurring freely in nature, but only in association with the nematode (Akhurst *et al*., 2004; Hazir *et al*., 2004; Lengyel *et al*., 2005; Tailliez *et al*., 2006).

Previous research has been aimed at screening and assaying the insecticidal properties of several of the symbiotic bacteria. Currently, there are three described species of *Photorhabdus* and 15 subspecies and 23 species of *Xenorhabdus*. Since 2004, three new subspecies of *Photorhabdus* (Akhurst *et al*., 2004; Hazir *et al*., 2004) and 14 new species of *Xenorhabdus* (Lengyel *et al*., 2005; Tailliez *et al*., 2006) have been described. However, many bacterial isolates from previously described nematode species still require characterisation.

*Life cycle of* Xenorhabdus *and* Photorhabdus

The Heterorhabditidae and Steinernematidae families of nematodes are obligate insect pathogens. The only way in which such nematodes can persist outside the insect host is as specialised third-stage IJs. Their bacterial symbionts are contained in the intestinal tract of the IJs. The bacterial symbiont of *Heterorhabditis*, which is *Photorhabus*, mainly colonises the anterior region of the intestine just posterior to the basal bulb. However, it is also to varying degrees located throughout the remainder of the intestine (Endo & Nickle, 1991; Ciche & Ensign, 2003). *Xenorhabdus* is the bacterial symbiont of *Steinernema*, with the nematodes having a specialised bilobed intestinal vesicle that becomes colonised by the bacteria (Bird & Akhurst, 1983; Martens *et al*., 2003). In both of the nematode symbionts, the bacteria are in a dormant state inside the IJ, apart from in the case of *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, where limited bacterial growth takes place until the intestinal vesicle is colonised by *Xenorhabdus nematophila* (Poinar & Thomas, 1965; Thomas & Poinar, 1979; Martens *et al.*, 2003).

Once the IJ enters the haemocoel of a susceptible insect host, the nematode resumes development and releases its bacterial symbiont. Inside the insect gut, *X. nematophila* cells are released from the vesicle into the nematode's intestine (Sicard *et al*., 2004) by means of defecation (Poinar & Thomas, 1966; Wouts, 1984; Martens *et al*., 2004; Sicard *et al.*, 2004). *Photorhabdus* are released through the mouth of the nematode, in an action resembling regurgitation (Ciche & Ensign, 2003).

The nematodes and bacteria work together to overcome the immune response of the host, thus allowing the bacteria to proliferate (Koppenhöfer, 2007). *Steinernema carpocapsae* secretes proteins suppressing the immune response of the insect host and this may aid the release of their symbionts (Gotz

*et al*., 1981; Simoes, 1998). It is unknown whether similar proteins are released by *Heterorhabditis* (Forst & Clarke, 2002). Both genera produce haemolysin activity (Brillard *et al*., 2001, 2002). While the bacteria develop in the insect host, they produce toxins and exo-enzymes. This results in the septicaemia of the insect host and the cadaver, which provides nutrition for the developing nematodes (Forst & Clarke, 2002). The above takes place early on in the infection and preceding the insect's death (Sicard *et al.*, 2004). Likewise, *Photorhabdus* reproduce in the haemocoel of *Manduca sexta* Linnaeus, 1763, destroying the immune system. Toxins are released by the bacteria late in the infection, destroying the epithelium of the midgut (Bowen *et al*., 1998; Silva *et al*., 2002).

Towards the end of bacterial growth, the symbionts produce a range of antimicrobial compounds that protect the cadaver from colonisation by other organisms. The compounds include bacteriocins, which are active against closely related bacteria. In *P. luminescens*, the bacteriocins are also active against distantly related bacterial taxa (Thaler *et al*., 1995; Sharma *et al*., 2002). Antibiotics are other compounds that are produced by the bacteria, which are active against fungi, yeasts and other bacteria (Akhurst, 1982; Boemare *et al*., 1997a; Webster *et al*., 2002).

Developing nematodes feed on a mixture of bacteria and bioconverted host tissue, enabling them to produce one to three generations until the food resources in the cadaver are depleted. As soon as depletion takes place, the nematodes develop a new generation of a special third generation of IJs enclosing the symbiotic bacterial cells, which exit the cadaver in search of a new susceptible insect host (Koppenhöfer, 2007).

*Phenotypic variation*

Phenotypic variants are produced by both *Xenorhabdus* and *Photorhabdus.* The primary form, which is called form I, is associated with the nematodes. The secondary form of the bacteria (Form II cells) arises abruptly when in artificial culture, and seldom occurs in the insect host during the later stages of nematode reproduction (Akhurst, 1980). The two forms of bacteria mentioned differ both morphologically and physiologically.

Form I cells are larger, as well as motile, as the result of peritrichous flagella, which form II cells do not have (Givaudan *et al*., 1995). Form I cells are able to absorb certain dyes and to produce crystalline inclusion bodies, antibiotics, lipase, and protease, while certain strains of *Photorhabdus* are also bioluminescent (Akhurst, 1980, 1982; Couche *et al*., 1987; Boemare & Akhurst, 1988; Forst *et al.*, 1997). All of these mentioned characteristics are reduced or missing in the variant cells. Form II cells in *X. nematophila* do not produce a stationary-phase outer membrane protein called OpnB (Volgyi *et al*., 2000). *Xenorhabdus* form II colonies have little or no pigment on nutrient agar, while *Photorhabdus* form II colonies depend on the strain or species, which differ (Akhurst, 1983; Akhurst & Boemare, 1988; Boemare & Akhurst, 1988; Boemare *et al.*, 1997a).

Higher levels of respiratory enzyme activity are present in form II cells for both *X. nematophila* and *P. luminescens*, and such cells are also more capable of taking up nutrients than are other cells (Smigielski *et al*., 1994). Differences in pathogenicity exist between the phenotypic variants of *X. nematophila* in lepidopteran hosts, but the overall pathogenicity is maintained in form II cells. Form I and form II cells are both pathogenic against *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Akhurst, 1980). *Photorhabdus* form II cells cannot support the growth and reproduction of *Heterorhabditis* (Gerritsen & Smits, 1993, 1997). On the contrary, form II cells of *X. nematophila* can support nematode reproduction both *in vitro* (Ehlers *et al*., 1990; Volgyi *et al.*, 2000) and *in vivo* (Sicard *et al*., 2005). In *S. carpocapsae*, the production of xenorhabdicin is maintained in form II cells, but, when antagonistic bacteria are present, they are not sensitive to xenorhabdicin, with form II cells providing less protection than do form I cells to their nematode host (Sicard *et al.*, 2005). Producing antibiotics is costly with regard to metabolism, and, without this function, a great deal more nutrient uptake can occur than with it. The above ensues with increased proliferation of the bacteria and, as a result, they become more adaptive for survival (Smigielski *et al.*, 1994; Sicard *et al.*, 2005). Phenotypic change and the mechanisms that drive it are in the process of becoming understood. For example, form I characteristics in *P. luminescens* are negatively regulated by HexA (Joyce & Clarke, 2003), with, in *X. Nematophila*, such characteristics being positively regulated by Lrp (Park *et al*., 2007). Mutualism and pathogenesis are both affected by said bacterial regulators, and will occasionally revert to form I bacteria in *Xenorhabdus*, which has, however, not yet been documented for *Photorhabdus* (Givaudan *et al*., 1995; Forst & Clarke, 2002).

*Taxonomy and systematics*

According to Stackebrandt *et al*. (2002), the current definition of bacterial species, although arbitrary and artificial, is still universally operational. A genomically consistent group of individual isolates sharing a high degree of similarity is regarded as a species. The degree of similarity should be present in numerous independent features, as well as be diagnosable by means of the presence of a discriminative phenotype (Rossello-Mora & Amann, 2001; Stackebrandt *et al*., 2002; Adams *et al*., 2006). The main criterion for the description of bacterial species continues to be DNA : DNA homology. Strains within a species should preferably have a DNA : DNA relatedness value of 70% or higher and a $\Delta T_m$ of 5˚C or lower (Wayne *et al*., 1987; Rossello-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). Such values, however, are not absolute for the description of a new species (Rossello-Mora & Amann, 2001), as additional molecular techniques are encouraged when the degree of similarity of DNA : DNA reassociation is adequate (Stackebrandt *et al*., 2002). As a result of the low DNA : DNA relatedness values of earlier studies, and the differences between 16S rRNA gene sequences (Boemare *et al*., 1993; Nishimura *et al*., 1994), previous *Xenorhabdus* species have been described without considering DNA : DNA reassociation (Lengyel *et al.*, 2005; Somvanshi *et al*., 2006). According to Tailliez *et al*. (2006), a consistent alternative to DNA : DNA hybridisation is a combination of randomly amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) sequences.

Multi-locus sequence typing (MLST) is a method that holds great promise for the delineation of species. Partial sequences of internal fragments from multiple housekeeping genes are used. Based on the number of different loci, the evolutionary distance between isolates is subsequently quantified (Maiden *et al*., 1998; Adams *et al.*, 2006). The method concerned can easily be replicated and, furthermore, there are publicly accessible databases containing sequences and software to use for comparing specific isolates (see http://www.mlst.net and http://pubmlst.org). The genomic association of strains can be determined with more confidence using MLSTs than with the use of DNA : DNA reassociation (Lan & Reeves, 2001; Adams *et al.*, 2006). When using MLSTs the concatenation of several of these unlinked gene sequences have the ability to yield more robust phylogenetic trees (Rokas *et al*., 2003; Wertz *et al*., 2003) when compared to single-gene phylogenies. According to Tailliez *et al* (2010), creating a resolved phylogeny of these bacteria is necessary in order to study their co-evolution

with their nematode hosts. Analysis of the topology of single-gene phylogenetic trees (Doolittle, 1999) can be used to identify plausible lateral gene transfers (LGT) between species, which can have implications for the classification of strains and new isolates in the genera *Photorhabdus* and *Xenorhabdus* (Tailliez *et al*., 2010).

A preferred method to DNA similarity or molecular methodology is a polyphasic approach for species description. The description of species should include an almost complete 16S rDNA sequence, the G + C mol% content of the type strain of the type genus, the phenotype and also the chemotaxonomic characters (Stackebrandt *et al.*, 2002). Standard systems can easily be used to obtain phenotypic data. The description of *Xenorhabdus* and *Photorhabdus* species has been done in toto using API® substrate panels, which were designed to illustrate the carbohydrate metabolism. Recently, Biolog GN™ has been used for species description and bacterial identification, in order to illustrate substrate utilisation (Hazir *et al.*, 2004; Lengyel *et al.*, 2005; Gouge & Snyder, 2006; Somvanshi *et al.*, 2006). As phenotypes that are described by metabolism only are regarded as insufficient, supplementary chemotaxonomic characters should be considered (Rossello-Mora & Amann, 2001).

The considerable differences in biochemical reactions for *Xenorhabdus* and *Photorhabdus* that have been reported (Holt *et al.*, 1994; Brenner & Farmer, 2005) have complicated the comparing of species, with the variation probably being a result of using different bacterial strains and/or phenotypic variants (Akhurst & Boemare, 1988). Other possibilities for the variations may include weak and slow reactions, and the media type that is used for biochemical characterisation (Holt *et al.*, 1994).

The first bacterial symbiont that was isolated from the DD-136 strain of *S. carpocapsae* was described as a new bacterium species, namely *Achromobacter nematophila* Poinar & Thomas, 1965, by Poinar & Thomas (1965). The genus *Achromobacter* was later rejected (Hendrie *et al*., 1974) and reassigned to a different genus. The authors decided to create a new genus, *Xenorhabdus*, as *A. nematophila* did not closely enough resemble any of the accepted genera. The new genera included *X. nematophilus*, which is a symbiont of a *Steinernema* species, and *X. luminescens*, which is a symbiont of a *Heterorhabditis* species (Thomas & Poinar, 1979). A distinct difference could be discerned between *X. luminescens* and other *Xenorhabdus* strains, both in terms of their phenotypic (Akhurst, 1983; Akhurst &

Boemare, 1988; Boemare & Akhurst, 1988) and their molecular characteristics (Grimont *et al*., 1984; Farmer *et al.*, 1989; Suzuki *et al*., 1990). Grimont *et al*. (1984) placed *X. luminescens* into a different group through the use of s1 nuclease and hydroxyapatite methods for determining DNA : DNA similarity. *Photorhabdus* was proposed as a new genus, as a result of insufficient DNA homology to other *Xenorhabdus* species (Boemare *et al.*, 1993). Even though *Photorhabdus* is quite species poor in comparison to *Xenorhabdus*, it is still more homogenous than is the *Xenorhabdus* group (Akhurst *et al*., 1996). Most of the bacterial symbionts of recently isolated EPNs must still be described.

*Xenorhabdus* and *Photorhabdus* belong to the family Enterobacteriaceae (Rahn, 1937) Ewing, Farmer & Brenner, 1980, as well as the gamma subdivision of the Proteobacteria. Characteristics that said bacteria have include: the possession of gram-negative rods; motility by means of peritrichous flagella or non-motility; facultative anaerobism; negativity for oxidase; and asporogenous, non-acid fast, chemoorganic heterotrophs, with respiratory and fermentative metabolisms (Brenner, 1999; Brenner & Farmer, 2005). When considering the phenotypical characteristics of *Xenorhabdus* and *Photorhabdus*, they can be seen to be out of character, compared to other members of the Enterobacteriaceae family (Holt *et al.*, 1994).

When comparing *Xenorhabdus* and *Photorhabdus*, two main differences are that the latter are catalase-positive, with the majority being bioluminescent. *Xenorhabdus* isolates are negative for both characteristics concerned (Poinar *et al*., 1980; Farmer, 1984; Boemare & Akhurst, 1988). Distinguishing the two groups of bacteria clearly is the fact that *Photorhabdus* contains unique sequences in the 16S small subunit rDNA, which *Xenorhabdus* lacks. The sequence TTCG of *Xenorhabdus* is at the 208–211 position (in terms of *E. coli* numbering), while *Photorhabdus* contains the longer TGAAG sequence instead (Szallas *et al*., 1997).

By assessing bacterial diversity and identification through the polymorphism of the gene coding for the ribosomal RNA subunit, laborious phenotypic characterisation can be avoided. A method, such as the restriction fragment analysis of PCR-amplified gene products, has been used successfully for such purposes. The identification of *Xenorhabdus* and *Photorhabdus* can accurately be identified by means of restriction fragment length polymorphisms (RFLP) of the 16S rRNA gene sequence (Brunel *et al*., 1997;

Fischer-Le Saux *et al*., 1998; Bonifassi *et al*., 1999). Distinguishing between *Xenorhabdus* and *Photorhabdus* is also practical when using 16S rRNA sequences (Liu *et al*., 2001; Sergeant *et al*., 2006).

### *Heterorhabditis* and *Steinernema*

More than 30 nematode families are known to parasitise, or are associated with, insects (Nickle, 1972; Maggenti, 1981; Poinar, 1983, 1990b; Kaya & Stock, 1997). As a result of biocontrol potential, more research has been undertaken into seven families, including Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae, Rhabditidae, Steinernematidae and Heterorhabditidae. The last two families have received the most attention so far, as they can be cultured and formulated, and can be used to control a wide range of insect pests within a short space of time (Lacey *et al*., 2001).

*Phasmarhabditis hermaphrodita* (Schneider) is a member of the family Rhabditidae. It is known to suppress numerous slug and snail species, and has been developed as a biological molluscicide (Wilson *et al*., 1993; Glen & Wilson, 1997; Wilson & Gaugler, 2000). Such potential biocontrol agents of plant-parasitic nematodes and plant pathogens as predatory mononchids, dorylaimids, nygolaimids, diplogasterids and the fungal-feeding nematode, *Aphelenchus avenae* Bastian, have also been studied (Kasab & Abel-Kader, 1996; Lootsma & Scholte, 1997; Matsunaga *et al*., 1997; Choudhury & Sivakumar, 2000), without much success.

## Biology and behaviour of entomopathogenic nematodes

EPNs of the family Steinernematidae and Heterorhabditidae are lethal pathogens of insects. In nature, they play a role in regulating the natural population of insects, but their main point of interest is their inundative application as biocontrol agents. The unique partnership between the nematode and the lethal insect-pathogenic bacterium has helped to ensure their success as biocontrol agents (Griffin *et al*., 2005).

Even though *Heterorhabditis* and *Steinernema* have adopted the same lifestyle, they belong to different families (Heterorhabditidae and Steinernematidae respectively) (Blaxter *et al*., 1998). Similarities include their association with insect-pathogenic bacteria, in addition to which they are thought to have originated through convergent evolution (Poinar, 1993). Both *Steinernema* and *Heterorhabditis* have a

single free-living stage, the IJ stage, which carries bacteria from the genus *Xenorhabdus* and *Photorhabdus*, respectively, in its gut (Boemare *et al.*, 1993). The IJ can enter the insect through its mouth, anus or spiracles and move towards the haemocoel. Some species have the ability to penetrate through the insect cuticle (Bedding & Molyneux, 1982; Peters & Ehlers, 1994). For example, *Heterorhabditis* are able to do so by means of their anterior dorsal tooth (Bedding & Molyneux, 1982).

Once the IJ is inside the haemocoel of the host, the nematode releases the cells of its bacterial symbiont from its intestine. The insect's haemolymph is extremely nutrient-rich, and the bacteria multiply exponentially, causing insect death within 24-48 h. IJs feed on the proliferating bacteria and digested host tissue after they recover from their arrested state. Nematodes develop to the fourth larval stage, and subsequently to adult stages in order to reproduce. Depending on the available nutrients and resources, more than one generation can occur (Dix *et al*., 1992).

Steinernematids and heterorhabditids have a different mode of reproduction. The first generation for the latter consists of self-fertilising hermaphrodites, with males and females developing in subsequent generations (Dix *et al*., 1992). For steinernematids, all the generations can reproduce through amphimixis (i.e. cross-fertilisation involving males and females) (Poinar, 1990a). Recently, however, a *Steinernema* sp. was found, of which most of the individuals were self-fertilising hermaphrodites, with a small portion of the population in each generation being males (Griffin *et al*., 2001). Therefore, in general, when only a single IJ invades a host insect, a heterorhabditid is able to reproduce and develop, while most steinernematids require two individuals, one male and one female, to be present before they can reproduce (Griffin *et al.*, 2005).

At first, eggs are laid by the females or hermaphrodites, and, in older females or hermaphrodites, the eggs hatch in the uterus, with the parental tissue being consumed by the juvenile. The process concerned is known as '*endotokia matricida*' (Johnigk & Ehlers, 1999). Parental tissue makes for an extremely efficient conversion from insect biomass to IJ biomass. When there is an adequate food supply, the juveniles develop into adults. When the conditions inside the host insect are crowded, and there are limited resources, the IJs do not develop further. In a large insect host, hundreds of thousands of IJs can

develop, emerging from the insect cadaver over a period of days, whereupon they then begin to search for a new insect host (Griffin *et al.*, 2005).

When an IJ has newly emerged from its insect host, it retains the second-stage cuticle as a sheath. The sheath assists with the prevention of desiccation, freezing and fungal pathogen infection, especially in heterorhabditids (Timper & Kaya, 1989; Campbell & Gaugler, 1991b; Wharton & Surrey, 1994). Steinernematids lose their sheath quite easily as they move through the soil, while the heterorhabditid sheath is not so easily lost, due to it being more tight-fitting (Campbell & Gaugler, 1991a; Dempsey & Griffin, 2003).

## ***In vitro* culturing of nematodes**

The production of nematodes *in vitro* requires a detailed understanding of the biology and behaviour of the nematode species being mass produced. The first axenically liquid culture was concocted by Stoll (1952), using raw liver extract shaken in flasks. The use of bioreactors to culture nematodes was first attempted and described in 1986 by Pace *et al*. When said researchers cultured the nematodes in a standard 10-l bioreactor (Braun Biostat E), the main finding was that an impeller tip velocity of 1 m/s or more led to the disruption of adult females, leading to them recommending that the shear be less than 0.3 m/s, in order to produce maximum yield. Pace *et al*. (1986) used a kidney homogenate-yeast extract medium in which they inoculated *X. nematophila* 24 h before the inoculation of 2000 IJs/ml of *S. carpocapsae*.

Liquid culture technology was first made commercially available by the company Biosys, Palo Alto, California in 1992. The nematode produced was *S. carpocapsae*, which was upscaled to an 80 000-l fermenter. Currently, the majority of nematodes are produced in liquid culture by only a few companies, such as e-nema Gmbh (www.e-nema.de) in Germany, Koppert B.V. (www.koppert.nl) in The Netherlands, and Becker Underwood (www.beckerunderwood.com) and Certis (www.certisusa.com) from the United States (Ehlers & Shapiro-Ilan, 2005).

*Liquid culture process technology*

The long process time required for nematode cultures, combined with the need for an even distribution of fluids and organisms, result in the cultures being extremely vulnerable to contamination. Any non-symbiotic micro-organism that is present in the culture will lead to a reduction in nematode yield. Monoxenicity of nematode and bacterial cultures must be ensured from the outset in inoculum production.

Although symbiotic bacteria can be isolated from insect larvae infected with nematodes (Boemare & Akhurst, 1988), what is more laborious, however, is the establishing of bacteria-free nematodes. IJs cannot only be surface-sterilised as such a procedure does not exclude all contaminants. The preferred method requires the establishment of a monoxenic culture, by obtaining nematode eggs from gravid female stages (Lunau *et al*., 1993; Han & Ehlers, 1998). Monoxenic cultures of the nematode and bacteria together can be stored for months until they are inoculated into the bioreactor by shaking them at 20 rpm at 4°C.

*Photorhabdus* and *Xenorhabdus* have the ability to metabolise almost any kind of protein-rich medium. For this reason, selecting a specific medium for the nematode culture will depend mostly on economic considerations. A typical medium should have a carbon source (e.g. glucose or glycerol), various proteins of animal and plant origin, yeast extract, and, lastly, lipids of animal or plant origin (Pace *et al.*, 1986; Friedman *et al*., 1989; Han *et al*., 1995; Surrey & Davies, 1996; Ehlers *et al*., 1998). Possible ways of increasing nematode yield entail either improving or adapting the liquid medium used (Ehlers, 2001).

Medium requirements vary between different nematode species. For example, *S. carpocapsae* require proteins of animal origin, without which they cannot reproduce (Yang *et al*., 1997). Production of offspring in a liquid medium without the addition of lipids is possible for *H. bacteriophora*, however (Han & Ehlers, 2001), because *P. luminescens* provides and metabolises the necessary lipids. *S. glaseri* is the only nematode for which essential amino acid requirements have been defined (Jackson, 1973). Nematodes can metabolise sterols from numerous steroid sources (Ritter, 1988), such as from lipids of either animal or plant origin. As a rule, though, lipids should always be added in order to increase the total

IJ fat content. The lipid composition affects the fatty acid composition of the bacteria and IJ (Abu Hatab *et al*., 1998), with a reduction in efficacy as a result of the low fat content in IJs (Patel *et al*., 1997a, 1997b).

Conventional equipment used in biotechnology, such as bioreactors with flat-blade impellers, bubble columns, and airlift, and internal-loop bioreactors, have been tested, with the latter consistently yielding higher IJ concentrations than have the other types (Ehlers & Shapiro-Ilan, 2005). Before the IJs are added, the medium is always inoculated and pre-incubated for 24–36 h with the nematode species specific symbiotic bacterium, which is normally between 0.5% and 1% of the total culture volume. The nematode inoculum usually forms between 5% and 10% of the total culture volume (Ehlers & Shapiro-Ilan, 2005).

Specific optimum culture temperature varies, depending on the nematode species and medium composition being used. The optimum temperature for the growth of a bacterial symbiont should always be defined before mass culturing of the nematode is attempted, as deviation from the optimum temperature can potentially induce a switch to the secondary phase, impeding nematode reproduction (Ehlers *et al*., 2000). When the culture medium is started, the pH should ideally be between 5.5 and 7, with the oxygen saturation rate being kept above 30%, as doing so will prevent the bacteria from switching to the secondary phase (Ehlers & Shapiro-Ilan, 2005).

One of the important parameters for an *in vitro* liquid culture is the aeration rate. Strauch and Ehlers (2000) compared yields of *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, with one culture being aerated at 0.3 vvm and another at 0.7 vvm. They found a significantly higher number of adults 8 days after IJ inoculation, and a higher final yield in the culture aerated at a higher rate. Increasing the aeration rate often leads to increased foaming, which silicon oil can be used to prevent, but high concentrations of such oil can affect the nematodes negatively. The use of long-chain fatty acids to control foaming was found to affect *H. bacteriophora* negatively (Ehlers & Shapiro-Ilan, 2005).

Numerous authors have reported final IJ yields from liquid culture (Pace *et al.*, 1986; Bedding *et al*., 1993; Surrey & Davies, 1996; Han, 1996; Ehlers *et al.*, 1998; Strauch & Ehlers, 2000). A negative correlation has been found to exist between the body length of the IJ and its yield. Body length is genetically defined and rather stable within a species, although it can differ according to strain and culture

condition. Ehlers *et al*. (2000) recorded > 500 000 IJs/ml for *H. indica*, which has one of the smallest IJs, with a body length of 528 µm. However, it has been proven to be more economically feasible to produce IJs with shorter body lengths (Ehlers & Shapiro-Ilan, 2005).

*Developmental biology of nematodes in liquid culture*

Nematodes experience a very different environment in liquid culture than they do in nature. When *Steinernema* infest insect cadavers, the inside of the insect becomes liquid. *Heterorhabditis,* however, turn the inside of the insect to a viscous texture (Ehlers & Shapiro-Ilan, 2005). When nematodes are in a bioreactor, they are continuously moved around by impellers or air bubbles, with the artificial environment having consequences for nematode feeding, development and copulation.

The population dynamics of the nematode need to be understood in order to ensure a successful liquid culture, as there are critical phases during the process of developing a culture that can be optimised to obtain maximum IJ yield. The main principle that drives development is the availability of food. Low food concentration induces the formation of IJs, whereas high concentration induces the development of additional generations, or the recovery of IJs. The IJ, which is a developmentally arrested third-stage juvenile, can be stored in this stage until it is needed.

When *Heterorhabditis* IJs are inoculated into the bacterial culture of their symbiont, they recover development. Their development then recommences through to where they are fourth-stage juvenile, and then into where they are automictic (i.e. self-fertilising) hermaphrodites. The density of the hermaphrodites, together with their body length, can be used to predict their final yield (Ehlers & Shapiro-Ilan, 2005). A positive correlation exists between the food supply and the length of the hermaphrodites, as well as the number of eggs laid. The hermaphrodites first lay eggs in the surrounding medium, and then, after 12 h of first-stage juvenile hatch, the male phenotypes become identifiable. Female phenotypes can be distinguished after another 12 h (Johnigk & Ehlers, 1999).

Amphimictic adults of *Heterorhabditis* can copulate and produce further generations of insects on solid media, but not in liquid media (Ehlers & Shapiro-Ilan, 2005). The *Heterorhabditis* male is unable to attach itself to the female in order for insemination to take place (Strauch *et al*., 1994). Development

subsequently ends at this stage, with the females being identifiable as containing unfertilised eggs, which can be distinguished by the enlarged nucleus. For *Heterorhabditis*, the only offspring that are able to continue their life cycle in liquid are the hermaphrodites resulting from the initial IJ inoculation. Development into either amphimictic adults or into IJs occurs during the first stage, and depends on the concentration of available food, as high concentrations of food induce amphimictic adults and low concentrations IJ formation. The development applies to both the genera of nematodes.

When the parental *Heterorhabditis* hermaphrodites are no longer laying eggs, the juveniles will hatch within the uterus, with the term '*endotokia matricida*' (i.e. intrauterine birth causing maternal death) being used to describe such occurrence, as has been described above in relation to the biology and behaviour of EPNs. High food concentrations delay the start of *endotokia matricida*, and subsequently lead to an increase in the number of eggs laid (Johnigk & Ehlers, 1999). The number of offspring in the uterus depends on the length of the hermaphrodite. First-stage juveniles that hatch out earliest immediately feed on the sperm, the non-fertilised eggs and oogonia, and when *endotokia matricida* subsequently starts, no further offspring can develop. Due to the combination of a low-concentration food source and a high number of nematodes, IJ formation is induced in the uterus. When the juveniles destroy the uterus and intestinal tissue, a change occurs in the food supply. They then have access to the body content of the adult, as well as to the cells of the symbiotic bacteria that they retain in their intestines. The body content of the hermaphrodite tends to be just enough to feed the number of offspring that reside in the uterus. The subsequent IJs are of excellent quality, with good fat reserves (Johnigk & Ehlers, 1999). *Endotokia matricida* in amphimictic females is also observed in insects and solid cultures. The IJs that emerge are a result of the IJs that developed from laid eggs or from *endotokia matricida.*

The life cycle of *Steinernema* is similar, except that amphimictic adults developing from the inoculated IJs have the ability to copulate in liquid. Males of *Heterorhabditis* have a 'bursa copulatrix', which is a fan that is supplied with sensory receptors. The bursa copulatrix enables the male to attach to the female at the vulval region and to copulate with her, forming a lambda or 'y' with the female. Males of *Steinernema* wind themselves around the female, as they lack said structure, and the copulation behaviour concerned is achievable in liquid culture. The type of mating behaviour involved has important implications for a liquid culture, as *Heterorhabditis* can, in effect, have only one generation time in which

to produce IJs from hermaphrodites, while *Steinernema* can go though one or more generations (Ehlers *et al.*, 1998; Strauch & Ehlers, 2000).

Yields from different genera cultured in the same medium can vary quite substantially (Ehlers *et al.*, 1998; Strauch & Ehlers, 2000). The population dynamics of the species of nematode is extremely important, especially from a commercial production point of view. Up to a hermaphrodite density of 4000/ml on day 3, IJ yield is positively correlated to hermaphrodite density. This means that an inoculation density of > 4000 IJs/ml should be sufficient to obtain maximum yields. The challenge, however, lies in obtaining said hermaphrodite density, as IJ recovery is highly variable in liquid culture. Liquid culture lacks a food signal that could trigger recovery, whereas basically 100% of the IJs recover within a day after they enter the haemocoel of an insect. The symbiotic bacteria produce such food signals, and therefore preculturing the symbiotic bacteria is the key to the success of nematode *in vitro* production. The levels of recovery are, however, variable, and can range from 18% to 90% within a period of several days (Strauch & Ehlers, 1998).

Unpredictable IJ yield is mainly due to an unsynchronised, low IJ recovery rate. The latter also impedes population management, which is required to maximise yield and to shorten the process time. A low hermaphrodite density is the result of a low IJ recovery rate. At a low density, the high concentration of food causes the hermaphrodite to lay many eggs, from which most develop into amphimictic adults instead of IJs. Such development is acceptable when culturing steinernematids, as it only serves to prolong the process time. The amphimictic adults of *Steinernema* can copulate in liquid culture and produce an F2 offspring generation (Strauch *et al.*, 1994). When copulation occurs in a heterorhabditid culture, it can result in process failure, as the F1 amphimictic adults cannot produce offspring.

Another problem with amphimictic adults is that they consume much of the bacterial culture that is needed for the hermaphrodites to develop from second-stage juveniles (J2d), or *endotokia matricida*. In some cases, high yield can be obtained from low hermaphrodite densities, due to the adaptability of the hermaphrodite. They can respond by increasing their body length, and, therefore, by increasing their number of offspring. Such a response is, however, only observable when synchronous IJ recovery takes place. When there are high numbers of hermaphrodites (> 2000/ml), the bacterial concentration becomes

very low, as a result of their feeding. Thus, less offspring develop into amphimictic adults, and many develop into IJs. The yield increases, and the hermaphrodites stop laying eggs. Most of the offspring at that stage originate from *endotokia matricida*. Said scenario results in high yields of high-quality IJs within a minimum processing time. The number of IJs per hermaphrodite is then reduced, as a result of limited food supply (Strauch & Ehlers, 1998).

*Increasing recovery in liquid culture*

*Steinernema* production is not as vulnerable to reduced recovery, and IJs normally respond well to the food signals of *Xenorhabdus*. There is, however, a key to the industrial-scale production of *Heterorhabditis*, which is a synchronised, reproducible and high IJ recovery rate. This goal can be reached by attaining an optimum number of parental hermaphrodites. Recovery can be influenced by preculturing the bacteria, as higher bacterial density creates a higher food signal. Therefore, nematodes should only be inoculated when the bacteria have reached the stationary growth phase, as the food signal will then be the strongest (Strauch & Ehlers, 1998). There is a significant drop in the respiration coefficient and a drop in the pH at the moment when conditions are favourable for the nematodes to be added (Ehlers & Shapiro-Ilan, 2005).

The main source of variability is, however, the IJs themselves (Strauch & Ehlers, 1998; Jessen *et al*., 2000). Response to food signals can vary significantly from one batch to another, and it has been hypothesised that this can be as a result of the difference in the fat reserves of the IJs, and, therefore, IJ with low energy reserves have a higher need for recovery. According to Ehlers & Shapiro-Ilan (2005), however, this hypothesis was not supported.

In conclusion, endemic EPNs are not currently commercially produced in South Africa. Therefore, the next logical step would be to use local nematode species, proven in previous research to perform the best against local key pest insects, and to assess their performance when they are mass cultured in liquid. This is a very important step, as the most suitable nematode isolate would not necessarilly be the most virulent, but must also be successfully cultured in high numbers, and formulated with a long shelf life. It is also imperative that the optimum growth parameters and general characteristics be known for the bacterial symbiont of any nematode isolate that might have the potential to be mass cultured for

commercial application. Research into the characterisation and identification of the symbiotic bacteria of EPN worldwide is still in its infancy. In South Africa, no bacterial symbiont associated with local EPNs has previously been described.

## Aims and objectives of the study

The overall aim of this study was to develop an *in vitro* mass culture technique for two local EPNs, identified as promising biocontrol agents against key South African insect pests.

The specific objectives of the study were to:

1. Successfully isolate and characterise the associated symbiotic bacteria from these nematodes;
2. Successfully mass culture *H. zealandica* in a liquid medium, using *in vitro* technology;
3. Successfully mass culture *S. yirgalemense* in a liquid medium, using *in vitro* technology; and
4. Determine the commercial potential of *Xenorhabdus* by upscaling to a 20 L reactor, enabling the investigation of the growth characteristics of the bacteria, including when the stationary growth phase was reached.

**The chapters of this study have been written as separate publishable papers, and, for this reason, some repetition in the different chapters has been unavoidable.**

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# CHAPTER 2

## Description of *Photorhabdus luminescens* subsp*. noenieputensis* subsp. nov., a symbiotic bacterium associated with a new *Heterorhabditis* species related to *Heterorhabditis indica**

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### Abstract

The bacterial symbiont AM7$^T$, isolated from a new entomopathogenic nematode species of the genus *Heterorhabditis*, displays the main phenotypic traits of the genus *Photorhabdus* and is highly pathogenic to *Galleria mellonella.* Phylogenetic analysis based on a multigene approach (16S rRNA, *rec*A, *gyr*B, *dna*N, *glt*X and *inf*B) confirmed the classification of isolate AM7$^T$ within the species *Photorhabdus luminescens* and revealed its close relatedness to *Photorhabdus luminescens* subsp. *caribbeanensis*, *Photorhabdus luminescens* subsp. *akhurstii* and *Photorhabdus luminescens* subsp. *hainanensis.* The five concatenated protein coding sequences (4197 nucleotides) of strain AM7$^T$ revealed 95.8%, 95.4% and 94.9% nucleotide identity (NI) with sequences of *P. luminescens* subsp. *caribbeanensis* strain HG29$^T$, *P. luminescens* subsp. *akhurstii* strain FRG04$^T$ and *P. luminescens* subsp. *hainanensis* strain C8404$^T$, respectively. The NI values concerned were less than the threshold of 97% proposed for classification within one of the existing subspecies of *P. luminescens.* Unlike other strains described for *P. luminescens*, strain AM7$^T$ produces acid from adonitol, sorbitol and xylitol, assimilates xylitol, and has no lipase activity on medium containing Tween 20 and Tween 60. Strain AM7$^T$ is differentiated from *P. luminescens* subsp. *caribbeanensis* by the assimilation of N - acetyl glucosamine

and the absence of hemolytic activity. Unlike *P. luminescens* subsp. *akhurstii*, strain AM7$^T$ does not assimilate mannitol and is distinguished from *P. luminescens* subsp. *hainanensis* by the assimilation of trehalose and citrate, the inability to produce indole from tryptophan, and the presence of acetoin production and urease activity. Strain AM7$^T$ (ATCC BAA- 2407$^T$, DSM 25462$^T$) belongs to a new subspecies and is proposed as the type strain of *Photorhabdus luminescens* subsp. *noenieputensis* sp. nov.

## Introduction

Bacteria of the genus *Photorhabdus* are symbiotically associated with entomopathogenic nematodes of the genus *Heterorhabditis* and are highly pathogenic to insects (Boemare *et al.,* 1993). The genus *Photorhabdus* belongs to the family Enterobacteriaceae and contains three recognized species, i.e. *P. luminescens*, *P. temperata* and *P. asymbiotica*. *Photorhabdus luminescens* is further divided into *P. luminescens* subsp. *caribbeanensis* and *P. luminescens* subsp. *hainanensis* (Tailliez *et al*., 2010), *P. luminescens* subsp. *kleinii* (An & Grewal, 2011), *P. luminescens* subsp. *akhurstii*, *P. luminescens* subsp. *laumondii, P*. *luminescens* subsp. *luminescens*, (Fischer-Le Saux *et al*., 1999), *P. luminescens* subsp. *kayaii* and *P. luminescens* subsp. *thracensis* (Hazir *et al*., 2004). *Photorhabdus luminescens* subsp. *thracensis* was reclassified as *P. temperata* subsp *thracensis* comb. nov. (Tailliez *et al*., 2010). *P. temperata* is separated into *P. temperata* subsp. *temperata* (Fischer-Le Saux *et al*., 1999), *P. temperata* subsp. *cinerea* (Tóth & Lakatos, 2008), *P. temperata* subsp. *khanii* (=syn. *P. temperate* subsp. *stackebrandii,* An & Grewal, 2010) and *P. temperata* subsp. *tasmaniensis* (Tailliez *et al*., 2010). *Photorhabdus temperata* subsp. *stackebrandtii* includes strains GPS11 (DSM 23271), NC19, Habana and Meg1 (An & Grewal, 2010). Strain NC19 (=C1) was chosen as the type strain of *P. temperata* subsp. *khanii* by Tailliez *et al*. (2010) and includes Habana and Meg1. Thus, *P. temperata* subsp. *stackebrandtii* is a later heterotypic synonym of *P. temperata* subsp. *khanii*. *P. asymbiotica* is divided into *P. asymbiotica* subsp. *australis* and *P. asymbiotica* subsp. *asymbiotica* (Akhurst *et al*., 2004).

During a survey conducted in citrus orchards in the Mpumalanga province of South Africa, one population of an unknown *Heterorhabditis* species was collected (Malan *et al*., 2011). We described here

the bacterial symbiont of the genus *Photorhabdus* associated with this unique population, as no other population of this unknown species has been collected elsewhere in the world.

## Materials and methods

*Bacterial strains and growth conditions*

Bacterial symbionts from *Heterorhabditis* strain 158-C (FJ235075) (Malan *et al*., 2011) were isolated according to the procedure described by Akhurst (1980). Bacteria were streaked onto NBTA (Nutrient agar; Biolab Diagnostics, Midrand, South Africa) and the plates incubated at 25°C for 48 h. Blue and blue-green colonies were randomly selected from the plates and transferred to Tryptic soy broth (TSB) (BD, Sparks, USA). Pure cultures were stored in 40% (v/v, final concentration) sterile glycerol at -80°C. All the other bacterial strains used in the current study were from the bacterial collection at INRA (Montpellier, France) except DSM 10 and DSM 23513 which were from the DSMZ culture collection (Braunschweig, Germany).

*Genotypic characterization*

Total genomic DNA of an overnight culture of isolate AM7$^T$ 79 was extracted using the ZR fungal/bacterial DNA kit (Zymo Research Corporation, Irvine, California, USA). DNA of the 16S rRNA gene was amplified as described by Felske *et al.* (1997). The five protein coding genes *rec*A, *gyr*B, *dna*N, *glt*X and *inf*B were amplified as described by Tailliez *et al.* (2010, 2011). PCR reactions used were as described previously (Tailliez *et al*., 2010), using *TaKaRa Ex Taq*™ (Takara Bio Inc., Shiga, Japan) together with the supplied 10x *Ex Taq*™ buffer and dNTP mixture at concentrations recommended by the manufacturer. Amplified products were cleaned (QIAquick PCR purification kit, Qiagen, Valencia, USA) and sequenced (DNA Sequencing Unit, Central Analytical Facility, University of Stellenbosch) using the BigDye Teminator V3.1 sequencing kit (Applied Biosystems). Sequences were analysed using BLAST (basic local alignment search tool, National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA).

Sequences were assembled using the SeqMan module included in the DNASTAR Lasergene Software, version 7.0.0. (http://www.dnastar.com). Concatenated gene sequences were obtained using the function "concatenate" of the seaview software (http://pbil.univ-lyon1.fr/software/seaview.html). Single gene and concatenated gene sequences were aligned using ClustalW (http://www.clustal.org). Neighbour-joining distance (Saitou & Nei, 1987) and maximum likelihood (Guindon *et al*., 2010) trees were calculated using the "Phylogeny.fr" platform dedicated to phylogenetic analysis (Dereeper *et al*., 2008). The neighbour-joining distance tree using the Kimura 2-parameter model (Kimura, 1980) was used for the 16S sequences. The maximum likelihood tree was used for the five single gene sequences (*rec*A, *gyr*B, *dna*N, *glt*X and *inf*B) and the concatenated sequences. Models of evolution were selected using jModelTest to best fit with the data using the AICcriterion (Posada & Crandall, 1998). Sawyer's test for detecting recombination intervals based on the detection of shared patterns of polymorphisms (Sawyer, 1989) was performed with the computer program GENECONV (http://www.math.wustl.edu/~sawyer).

*Physiological and biochemical characterization*

Growth at different temperatures was recorded by taking optical density readings at 600 nm. Ten ml Luria Broth (Biolab) was inoculated with 100 µl of an overnight-grown culture and incubated at 26°C, 30°C, 37°C and 42°C, respectively, for 24 h. Catalase activity was determined by adding a drop of 10% (v/v) $H_2O_2$ onto a 20h-old colony on a plate (Koppenhöfer, 2007). The ability of strain AM7$^T$ to absorb dye was tested by growing the cells on NBTA (Biolab) containing bromophenol blue (Sigma-Aldrich, St. Louis, USA) and 2,3,5, triphenyltetrazolium chloride (Sigma-Aldrich), as described by Koppenhöfer (2007), and on MacConkey agar containing Neutral Red (Biolab). Bioluminescens was determined by scanning the colonies with the Xenogen *in vivo* imaging system (IVIS, Caliper Life Sciences Inc., Alameda, USA). DNAse activity was determined by streaking colonies onto DNAse test agar (20 g tryptose, 2.0 g DNA, 5.0 g NaCl, 15 g agar and 5 mg methyl green per litre distilled water). Lecithinase activity was determined using nutrient agar plates supplemented with 0.9% (w/v) NaCl and 10% (v/v) egg yolk emulsion (Oxoid, Basingstoke, United Kingdom). Hemolysis was observed by streaking strain AM7$^T$ on sheep blood agar plates (National Health Laboratory Services, Cape Town, South Africa). Lipase activity was determined by streaking cells onto peptone agar medium, supplemented with Tween 20, Tween 40, Tween 60 and

Tween 80, respectively, as described by Sierra (1957). API 20E and API 20NE characters, acid production from carbohydrate and carbohydrate assimilation using API50 CH strip were obtained as described by the manufacturer (BioMerieux, Inc., Lyon, France). Reactions were recorded after 24 h, 48 h and 10 days of incubation at 30°C in a temperature regulated growth chamber. Resistance to ampicillin was determined in duplicate in microtitre plates by growing serial dilutions of strain AM7$^T$ in LB containing 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg/ml antibiotic, respectively. Plates were incubated for 48h at 30°C. *Escherichia coli* DH5α was used as the negative control. Cell density was determined at 595 nm using a Biorad Model 680 microplate reader (Bio-Rad Laboratories, Hercules, USA). Antibiosis was determined by overlaying AM7$^T$ colonies on nutrient agar medium with 10 ml 0.8% (w/v) soft agar containing 100 µl of an overnight culture of *Bacillus subtilis* DSM 10 (Hazir *et al*., 2004).

In vivo *pathogenicity assay*

An *in vivo* pathogenicity assay was conducted with strain AM7$^T$ and strain *E. coli* DH5α as control as described by Givaudan & Lanois (2000). *Galleria mellonella* (L.) larvae were reared on artificial diet at 28°C. Ten ml LB broth were inoculated with 100 µl of a growing culture of AM7$^T$ or *E. coli* DH5α and incubated at 30°C to an optical density of 0.7 (measured at 595 nm). The bacterial cells were harvested (2 x $10^2$ cells per µl for AM7$^T$ and 2.1 x $10^4$ per µl for *E. coli* DH5α), washed with 0.8% (w/v) sterile saline, plated onto NBTA plates and the number of viable cells was determined after 24 h of incubation at 30°C (Sicard *et al.*, 2006). Twenty *G. mellonella* larvae were surface-sterilized with 70 % (v/v) ethanol and their haemocoel was injected with 20 µl containing bacterial cells of strain AM7$^T$ (4 x $10^3$ cfu) or *E. coli* DH5α (4.2 x $10^5$ cfu), using a BD Micro-Fine syringe.

## Results

*Genotypic characterization*

The accurate phylogenetic position of isolate AM7$^T$ was studied using five protein coding sequences (Fig. 2. 1). The *gyr*B and *rec*A genes have been used in several *Photorhabdus* and *Xenorhabdus* phylogenetic studies (Akhurst *et al*., 2004; An & Grewal, 2010, 2011; Lee & Stock, 2010,

Peat *et al*., 2010; Sergeant *et al*., 2006; Tailliez *et al*., 2010, 2011; Tóth & Lakatos, 2008). We also used *gltX*, *dnaN* and *infB* genes (Tailliez *et al*., 2010 & 2011) as markers with low probability of lateral gene transfer (Lerat *et al*., 2003). We investigated the *inf*B gene instead of the *gln*A gene (Peat *et al*., 2010), which was not selected by Lerat *et al.* (2003) as marker with low probability of lateral gene transfer. Nevertheless, *gln*A appeared also to be interesting in resolving many relationships within the *Photorhabdus* phylogeny (Peat *et al*., 2010) and should be included in further studies after validation on representatives of all the *Photorhabdus* species and subspecies. The *serC* gene was not used in the current study, as was done by Lee & Stock (2010), because the gene in question was clearly shown to be submitted to extensive recombination within *Xenorhabdus*, brother genus of *Photorhabdus*, by other authors (Sergeant *et al*., 2006). Whatever the protein coding gene used in our study (Fig. 2. 2 to 2. 7), the phylogenetic analysis confirmed that isolate AM7$^T$ belongs to the species *P. luminescens*. For *dna*N, *glt*X and *rec*A, AM7$^T$ shared a common ancestor with *P. luminescens* subsp. *caribbeanensis* strains HG26 and HG29$^T$ despite low bootstrap values obtained for *glt*X and *rec*A (58%). For *inf*B and *dna*N, AM7$^T$ was included in a clade consisting of *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*. At least for *gyr*B, AM7$^T$ shared a common ancestor with *P. luminescens* subsp. *luminescens* (with a bootstrap value of 70%), with the position not being in full agreement with that obtained with the four other genes described above. In the same way, we noted that the phylogenetic position (*P. luminescens* subsp. *kayaii*) of the type strain of *P. luminescens* subsp. *kleinii* (KMD37$^T$, DSM 23513$^T$), based on four gene sequences studied herein (*recA*, *dnaN*, *gltX* and *infB*) was not in agreement with that given by the *gyrB* gene sequence [HM072281, for KMD37$^T$ and JX513407 for DSM23513$^T$ are identical] (Fig. 2. 4) whatever the method of tree reconstruction used (parsimony for An & Grewal, 2011, and maximum likelihood in this study). However, for both KMD37$^T$ (DSM 23513$^T$) and AM7$^T$ strains, no recombination event was highlighted for the *gyr*B gene using the GeneConv software (Sawyer's test), in contrast to strain CIP108428$^T$ for which recombination events were detected for its *glt*X gene leading to an atypical phylogenetic position of the strain concerned (Tailliez *et al*., 2010). The authenticy of strain DSM 23513 with the type strain (KMD37$^T$) needs to be determined.

The five concatenated sequences of strain AM7$^T$ showed 95.8% nucleotide identity (NI) with those of strain *P. luminescens* subsp. *caribbeanensis* HG29$^T$ (Fig. 2. 1). Tailliez *et al*. (2010) proposed that

within the three recognized *Photorhabdus* species *P. asymbiotica*, *P. luminescens* and *P. temperata*, strains that shared less than 97% NI calculated on the four concatenated sequences (*gyr*B, *rec*A, *glt*X and *dna*N) do not belong to the same subspecies. The threshold of 97% NI applied to the concatenation of five gene-coding sequences (*gyr*B, *rec*A, *glt*X, *dna*N and *inf*B, 4197 nucleotides) is still valid for differentiating between the *P. luminescens* subspecies previously recognized (Table 2. 1.). However, the same rule should be applied with caution after detection of atypical (type) strains and isolates with incongruent single gene phylogenies which could lead to misclassifications (e.g. the five concatenated sequences of strains DSM 23513$^T$ and CIP108428$^T$ showed 96.8% NI only due to their atypical *gyr*B and *glt*X sequences, respectively). The analysis of single-gene phylogenies remains essential to avoid erroneous species assignments. Thus, based on this result, we propose to consider strain AM7$^T$ as a representative of a new *P. luminescens* subspecies, *P. luminescens* subsp *noenieputensis* subsp. nov.

The 16S rRNA gene sequence of isolate AM7$^T$ was compared with the sequences of representative strains of the different species and subspecies of the genus *Photorhabdus* (Fig. 2. 2). The sequence of isolate AM7$^T$ clustered with the type strains of *P. luminescens*, indicating that it belongs to the genus *Photorhabdus*, with the closest similarity (98.6%) to the type strain of *P. luminescens* subsp *laumondii* (TT01$^T$).

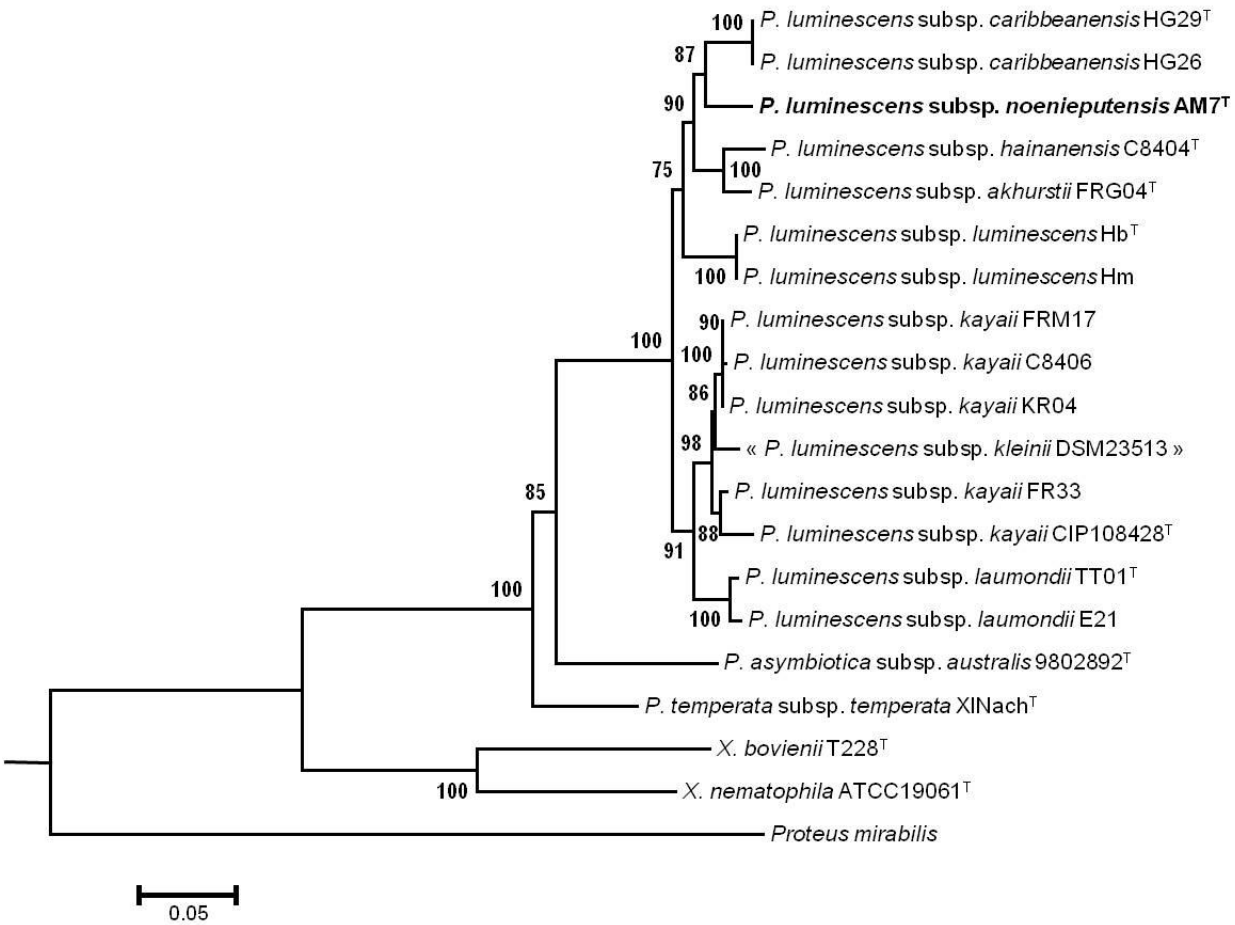


**Fig. 2.1.** Maximum likelihood (ML) phylogenetic tree of *Photorhabdus luminescens* calculated from five concatenated protein-coding sequences (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*). *Photorhabdus luminescens* subsp. *noenieputensis* AM7$^T$ belongs to a monophyletic group including *P. luminescens* subsp. *caribbeanensis*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*. The ML analysis was carried out using the General Time Reversible model of substitution with gamma-distributed rate heterogeneity and a proportion of invariant sites determined for all five protein coding sequences determined by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The concatenated sequences of *Xenorhabdus bovienii* strain T228$^T$, *Xenorhabdus nematophila* strain ATCC19061$^T$ and *Proteus mirabilis* were used as outgroups. Bootstrap values (percentages of 100 replications) (Felsenstein, 1988) of more than 50% are shown at the nodes. The bar represents 5% divergence.

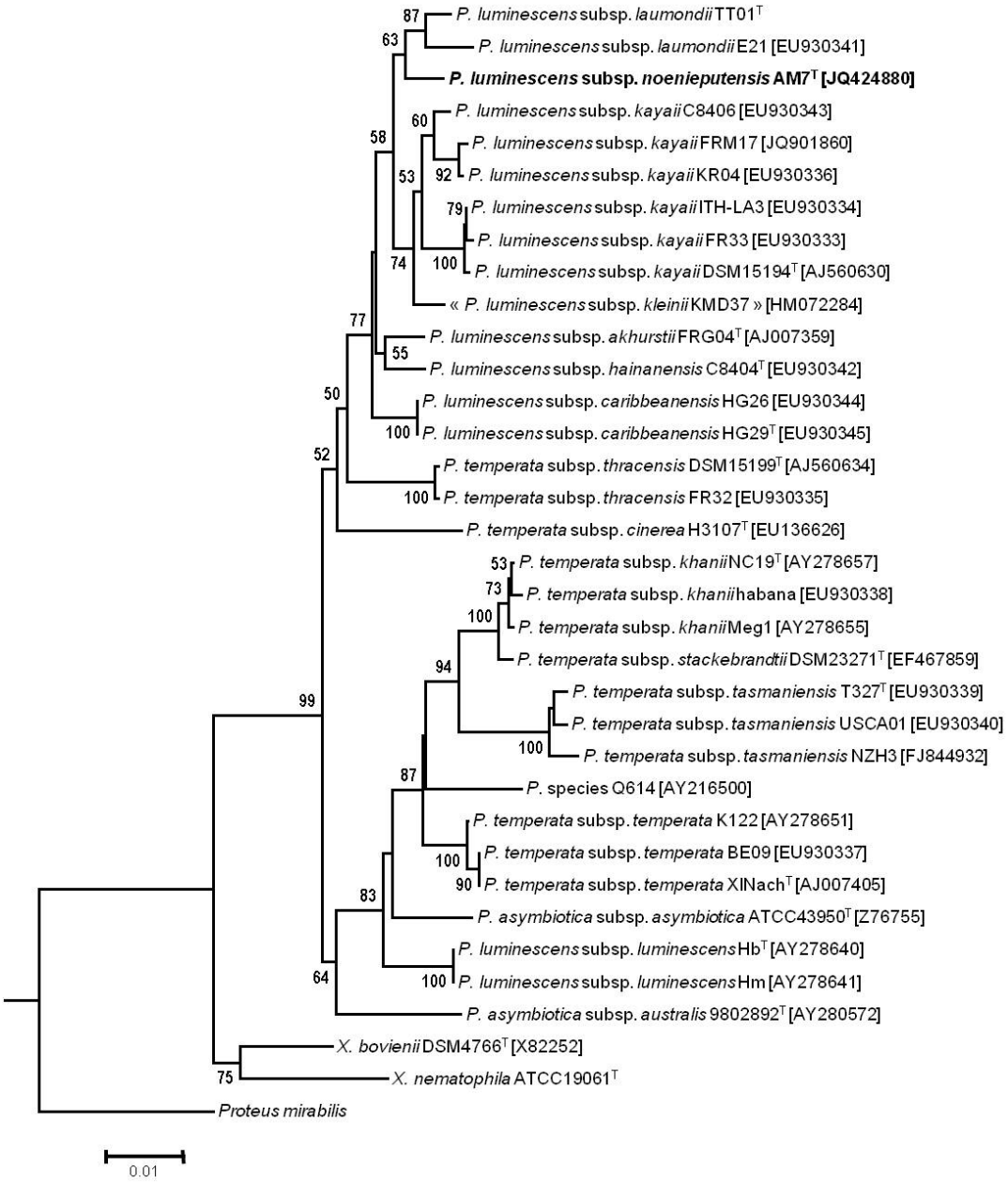


**Fig. 2.2.** Distance tree based on 16S rDNA sequences of *Photorhabdus* strains including *P. luminescens* subsp. *noenieputensis* subsp. nov. strain AM7$^{T}$. The 16S rDNA sequences of *P. luminescens* subsp. *noenieputensis* subsp. nov. strain AM7$^{T}$ and *P. luminescens* subsp. *laumondii* TT01$^{T}$ share 98.6% nucleotide identity on a length of 1159 nucleotides. The neighbour-joining tree (Saitou & Nei, 1987) was constructed using the Kimura 2-parameter model (Kimura, 1980). Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar indicates 1 % sequence divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061$^{T}$ and *P. luminescens* subsp. *laumondii* TT01$^{T}$ were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

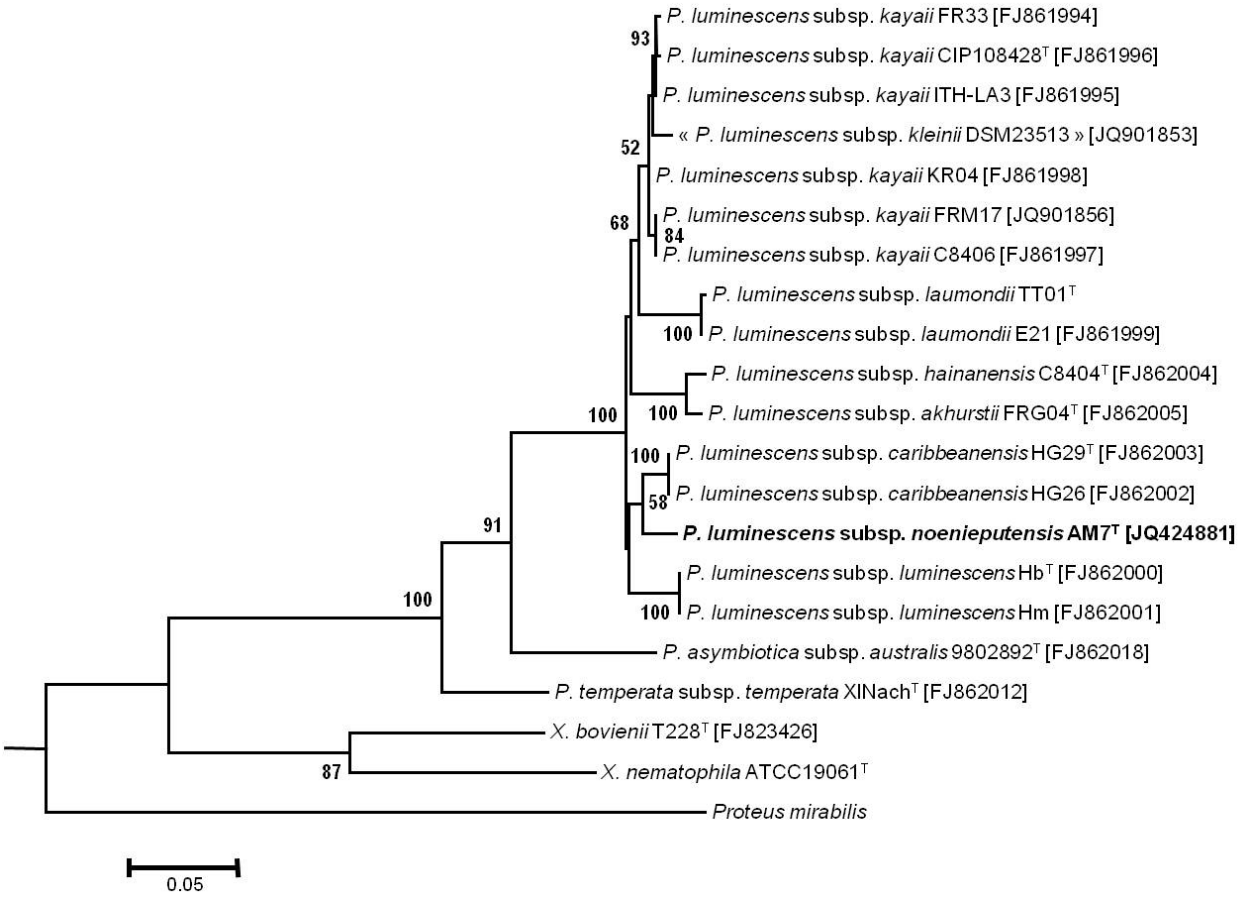


**Fig. 2.3.** ML tree based on recA sequences of *Photorhabdus luminescens* strains including *P. luminescens* subsp. *noenieputensis* AM7T. The ML analysis was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Xenorhabdus bovienii* T228T, Xenorhabdus nematophila ATCC19061T and *Proteus mirabilis* were used as outgroups. Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar represents 5% divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061T and *P. luminescens* subsp. *laumondii* TT01T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

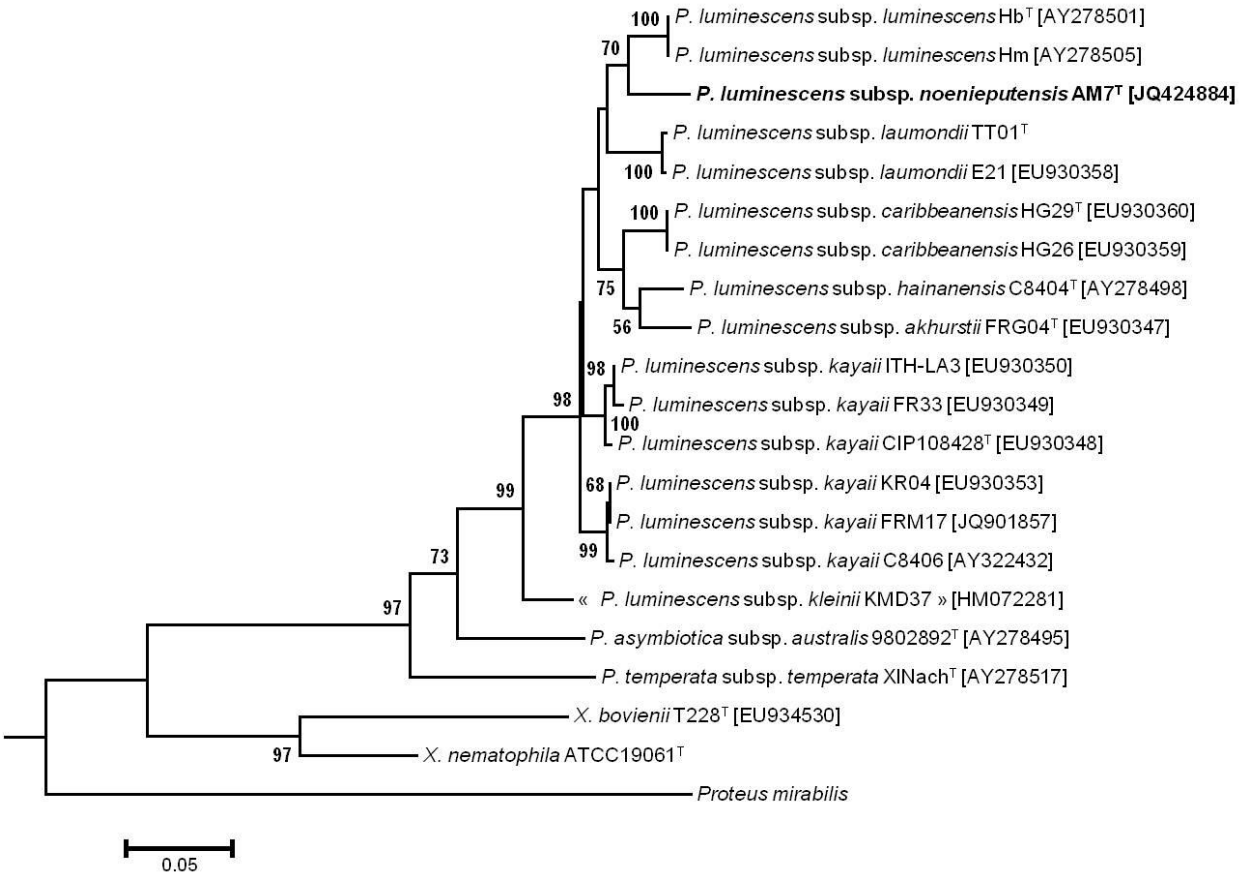


**Fig. 2.4**. ML tree based on *gyrB* sequences of *Photorhabdus luminescens* strains including *P. luminescens* subsp. *noenieputensis* AM7$^T$. The ML analysis was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Xenorhabdus bovienii* T228$^T$, *Xenorhabdus nematophila* ATCC19061$^T$ and *Proteus mirabilis* were used as outgroups. Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar represents 5% divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061$^T$ and *P. luminescens* subsp. *laumondii* TT01$^T$ were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554]. The sequence of *P. luminescens* subsp. *kleinii* strain KMD37 (= DSM23513) is that published by An & Grewal (2011).

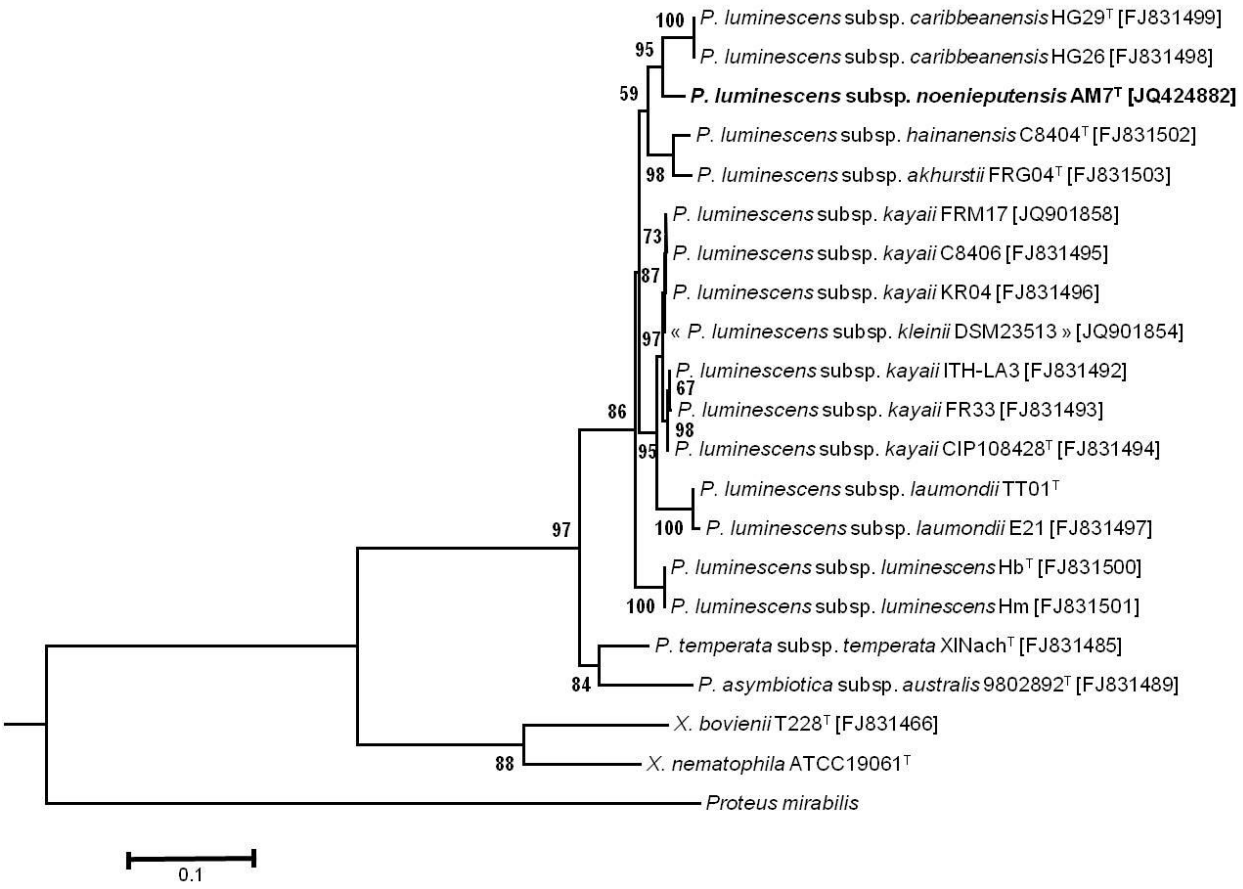


**Fig. 2.5.** ML tree based on *dnaN* sequences of *Photorhabdus luminescens* strains including *P. luminescens* subsp. *noenieputensis* AM7$^T$. The ML analysis was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Xenorhabdus bovienii* T228$^T$, *Xenorhabdus nematophila* ATCC19061$^T$ and *Proteus mirabilis* were used as outgroups. Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar represents 10% divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061$^T$ and *P. luminescens* subsp. *laumondii* TT01$^T$ were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

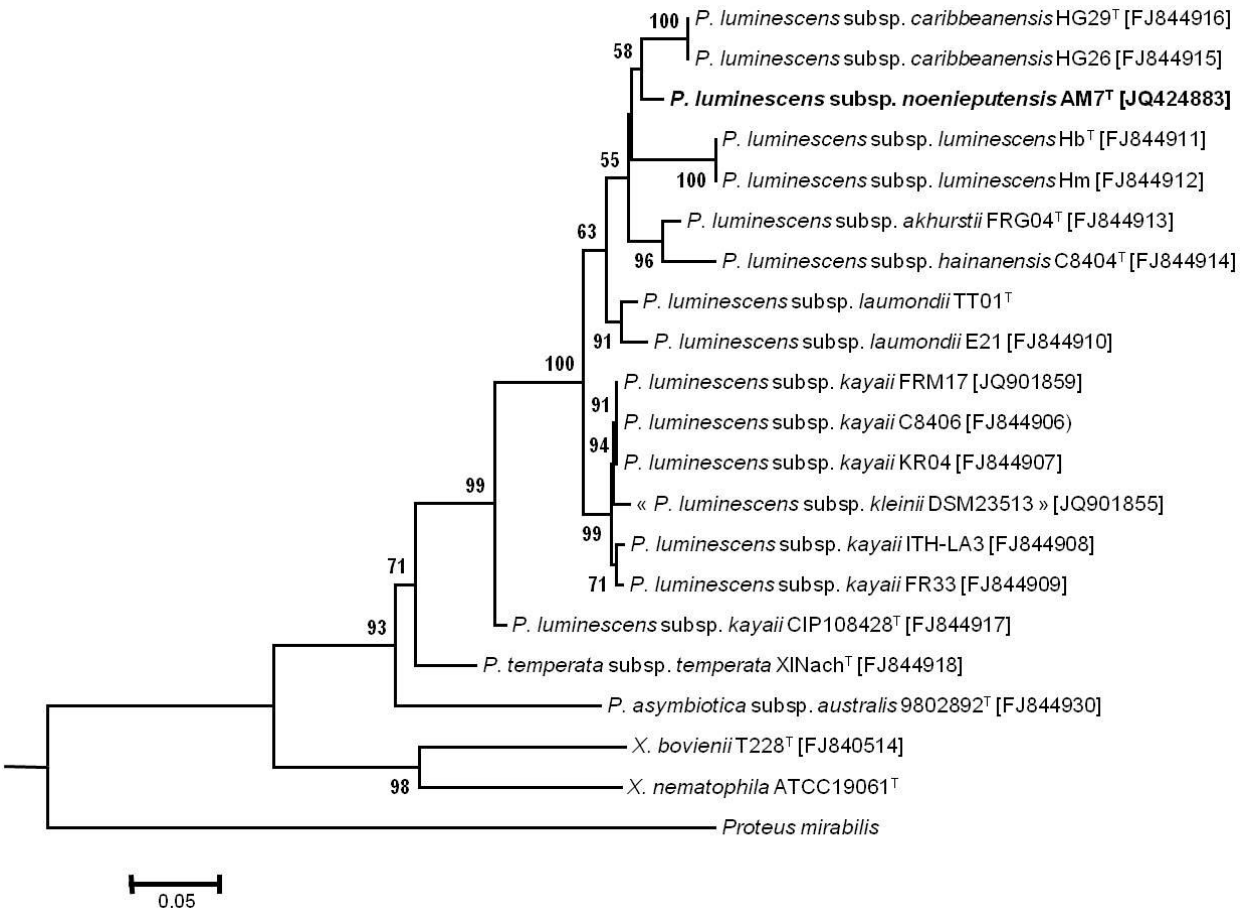


**Fig. 2.6.** ML tree based on *gltX* sequences of *Photorhabdus luminescens* strains including *P. luminescens* subsp. *noenieputensis* AM7$^T$. The ML analysis was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Xenorhabdus bovienii* T228$^T$, *Xenorhabdus nematophila* ATCC19061$^T$ and *Proteus mirabilis* were used as outgroups. Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar represents 5% divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061$^T$ and *P. luminescens* subsp. *laumondii* TT01$^T$ were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

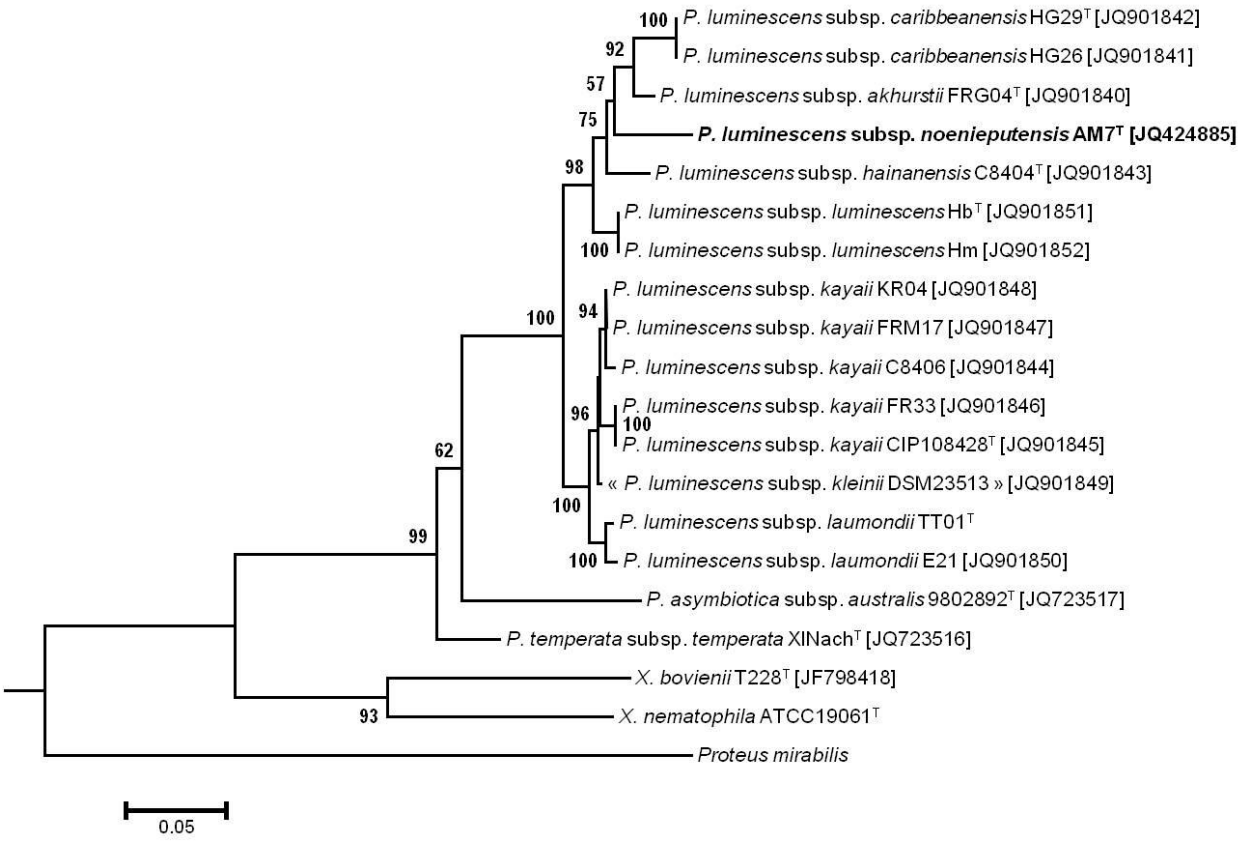


**Fig. 2.7.** ML tree based on *infB* sequences of *Photorhabdus luminescens* strains including *P. luminescens* subsp. *noenieputensis* AM7$^T$. The ML analysis was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Xenorhabdus bovienii* T228$^T$, *Xenorhabdus nematophila* ATCC19061$^T$ and *Proteus mirabilis* were used as outgroups. Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar represents 5% divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061$^T$ and *P. luminescens* subsp. *laumondii* TT01$^T$ were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

*Physiological and biochemical characterization*

The fifteen strains distributed in the different *P. luminescens* subspecies were analyzed for their main phenotypic traits (Table 2.1). Also included in this part of the study were *P. luminescens* subsp *akhurstii* strains D1 (Australia), EG1 and EG2 (Egypt), IS5 (Israel) and Tetuan (Cuba), and *P. luminescens* subsp *laumondii* strains HP88 (Utah), HV16 (Australia), NC162 (North Carolina) and K80

(Argentina) in order to increase the robustness of the analysis. These strains were described by Akhurst *et al*. (1996, 2004), Fischer-Le Saux *et al*. (1999), Marokhazi *et al*. (2003) and Peat *et al.* (2010) and were grouped by ribotyping in the laboratory of Tailliez, INRA Montpellier, France. All the *P. luminescens* strains were positive (cavaties showing photometric value above 30%) for catalase and negative (no reaction) for lecithinase, oxydase and nitrate reductase activities. All the *P. luminescens* strains were able to assimilate glycerol, ribose, glucose, fructose, D-mannose and D-maltose. Strain AM7$^T$ can be differentiated from the other *P. luminescens* strains studied by the following specific traits: weak production of acid from adonitol, sorbitol and xylitol, capability to assimilate xylitol, and lack of lipase activity on Tween 20 and Tween 60. *Photorhabdus luminescens* subsp. *caribbeanensis* HG29$^T$ and HG26 can be differentiated from strain AM7$^T$ by their annular hemolytic activity on sheep blood agar and their lack of N-acetyl glucosamine assimilation. *P. luminescens* subsp. *hainanensis* strain C8404$^T$ and the majority of the *P. luminescens* subsp. *akhurstii* strains were able to assimilate (6/6) and to produce acid (5/6) from mannitol in contrast to strain AM7$^T$. Strain AM7$^T$ can also be differentiated from *P. luminescens* subsp. *hainanensis* strain C8404$^T$ by the assimilation of trehalose and citrate, urease activity, production of acetoin and the lack of indole production from tryptophan.

**Table 2. 1**. Main phenotypic characters differentiating *Photorhabdus luminescens* subspecies.

| **Subspecies** | ***noenieputensis*** | ***akhurstii*** | ***caribbeanensis*** | ***hainanensis*** | ***kayaii*** | ***laumondii*** | ***luminescens*** |
|---|---|---|---|---|---|---|---|
| **Type strain** | AM7$^T$ | FRG04$^T$ | HG29$^T$ | C8404$^T$ | CIP108428$^T$ | TT01$^T$ | Hb$^T$ |
| **Number of strains studied** | n = 1 | n = 6 | n = 2 | n = 1 | n = 6 | n = 6 | n = 2 |
| Upper threshold temperature for growth (˚C) | 37 | 37-40 | 38-40 | 39-40 | 37-38 | 35-37 | 38-40 |
| Pigmentation | + | + | V | - | V(+) | + | + |
| DNase | - | V(+) | w | - | V(+) | V(+) | + |
| Arginine dihydrolase | w | - | V | - | V(-) | - | - |
| Simmons' citrate | + | V | + | + | V(+) | V(+) | + |
| Urease | + | V(+) | + | - | V(+) | + | V |
| Indole production | - | V(+) | V | + | V(+) | V | + |
| Voges-Proskauer | + | - | - | - | V(-) | V(-) | - |
| Esculin hydrolysis | + | + | + | + | V | V(+) | V |
| *Acid production from:* | | | | | | | |
| Glycerol | + | + | + | + | V | + | + |
| Ribose | + | + | + | + | V(+) | V(+) | V |
| Adonitol | w | - | - | - | - | - | - |
| Glucose | + | + | + | + | V(+) | + | + |
| Fructose | + | + | + | + | V | V(+) | + |
| Mannose | + | + | + | + | V(+) | V(+) | + |
| Inositol | + | V(+) | w | - | V(-) | V(-) | + |
| Mannitol | - | V(+) | + | + | - | V(-) | V |
| Sorbitol | w | - | - | - | - | - | - |
| N-acetyl glucosamine | + | V | + | + | V | V(+) | + |
| Esculine | + | + | + | + | V(+) | V(+) | V |

| **Subspecies** | ***noenieputensis*** | ***akhurstii*** | ***caribbeanensis*** | ***hainanensis*** | ***kayaii*** | ***laumondii*** | ***luminescens*** |
|---|---|---|---|---|---|---|---|
| **Type strain** | AM7$^T$ | FRG04$^T$ | HG29$^T$ | C8404$^T$ | CIP108428$^T$ | TT01$^T$ | Hb$^T$ |
| **Number of strains studied** | n = 1 | n = 6 | n = 2 | n = 1 | n = 6 | n = 6 | n = 2 |
| Salicine | w | - | - | - | V(-) | V(-) | - |
| Maltose | + | + | + | + | V(-) | + | V |
| Trehalose | + | V | V | + | V(-) | V(+) | w |
| Xylitol | w | - | - | - | - | - | - |
| L-Fucose | w | V | + | + | - | - | w |
| 5-Keto gluconate | - | V(-) | V | + | V(-) | V(-) | w |
| *Assimilation of:* | | | | | | | |
| Inositol | + | + | + | + | V(+) | + | + |
| Mannitol | - | + | + | + | - | - | + |
| N-acetyl glucosamine | + | + | - | + | + | + | + |
| Esculine | + | + | V | + | + | + | V |
| D-Trehalose | + | V(+) | + | - | V(+) | V(+) | + |
| Xylitol | + | V(-) | - | - | - | - | - |
| L-Fructose | - | V | V | - | - | - | - |
| Gluconate | + | + | + | + | + | V(+) | + |
| Caprate | - | V | - | - | V(-) | V(-) | + |
| L-Malate | - | V(+) | + | - | V(+) | V(+) | + |
| Citrate | + | V(+) | + | - | + | + | + |

+, 90% of strains positive; V(+), 50 to 89% of strains positive; V(-), 11 to 49% of strains positive; -, 0 to 10% of strains positive; V, variable; w, weak

In vivo *pathogenicity assay*

After 16 h, all *G. mellonella* larvae that had been injected with strain AM7$^T$ died, whereas larvae of the control group (which had been injected with *E. coli*) survived. This result confirms the entomopathogenic trait of *P. luminescens* subsp. *noenieputensis* strain AM7$^T$.

## Discussion

*Photorhabdus luminescens* subsp. *noenieputensis* [noe.ni.e.put.en'sis N.L. adj. *noenieputensis* from South Africa, from the farm Springbokvlei near the settlement Noenieput close to the Namibian border, was the source of the nematode (Malan *et al.*, 2011) from which the bacterial type strain was isolated. Bacterial cells from this strain are Gram-negative, oxidase negative, catalase positive and bioluminescent. Colonies are pigmented. Good growth is observed in TSB from 26°C until the upper limit of 37°C. Nitrate is not reduced and no DNAse and lecithinase activity were noted. Haemolytic reaction for this bacterial strain was, however, observed when grown on sheep blood agar plates. Lipolytic activity was also observed when grown in the presence of Tween 40 and Tween 80, but not in the presence of Tween 20 and Tween 60. The bromophenol blue in NBTA and Neutral Red in MacConkey medium were absorbed by growing bacterial cells. Assimilation and acid production from glycerol, ribose, glucose, fructose, mannose, inositol, N-acetyl glucosamine, esculin, maltose, trehalose and xylitol were also recorded for the *P. luminescens* subsp. *noenieputensis* strain. The latter also applied for acid production from adonitol, sorbitol, salicine, L-fucose and the assimilation of gluconate and citrate. The strain showed to be resistant to ampicillin concentrations up to 100 µg/ml, while growth of *B. subtilus* DSM 10 was inhibited by cells grown on nutrient agar plates. The type strain is AM7$^T$ (= ATCC BAA-2407$^T$, = DSM 25462$^T$) and GenBank accession numbers of the type strain are: JQ424880 (16S rRNA), JQ424881 (*rec*A), JQ424884 (*gyr*B), JQ424882 (*dna*N), JQ424883 (*glt*X) and JQ424885 (*inf*B).

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## CHAPTER 3

## Description of *Xenorhabdus khoisanae* sp. nov., the symbiont of the entomopathogenic nematode *Steinernema khoisanae**

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### Abstract

Strain SF87, and additional strains SF80, SF362 and 106-C, isolated from the nematode *Steinernema khoisanae*, are non-bioluminescent gram-negative bacteria that share many of the carbohydrate fermentation reactions recorded for the type strains of previously describe *Xenorhabdus* spp. Based on 16S rRNA gene sequence data, strain SF87 is 98.1% related to *Xenorhabdus hominickii*. Comparison of sequences obtained from the *recA*, *dnaN*, *gltX*, *gyrB* and *infB* genes grouped strain SF87 at 96 to 97% with *Xenorhabdus miraniensis.* However, strain SF87 shares only 52.7% DNA homology with the type strain of *X. miraniensis* (DSM17902$^T$), confirming that it belongs to a separate species. This is the first description of a bacterial symbiont, associated with the genus *Steinernema* from Africa. *Xenorhabdus khoisanae* sp. nov. is proposed as a new species of the genus *Xenorhabdus* (type strain SF87$^T$, DSM 25463$^T$, ATCC BAA-2406$^T$).

### Introduction

*Xenorhabdus* species are bound in obligate alliance of mutual benefit with specific species of entomopathogenic nematodes of the family Steinernematidae (Thomas & Poinar, 1979). Thus far 85 *Steinernema* spp. have been described worldwide, of which *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006 was isolated in the Western Cape province of South Africa (Nguyen *et al*., 2006).

Thomas and Poinar were the first to describe symbiotic bacteria in *Steinernema* as members of the genus *Xenorhabdus* in 1979 (Thomas & Poinar, 1979). Since then, 23 species in the genus *Xenorhabdus* have been described, i.e. *X. beddingii* (DSM 4764$^T$), *X. bovienii* (DSM4766$^T$), *X. budapestensis* (DSM 16342$^T$), *X. cabanillasii* (DSM 17905$^T$), *X. doucetiae* (DSM 17909$^T$), *X. ehlersii* (DSM 16337T), *X. griffiniae* (DSM17911$^T$), *X. hominickii* (DSM 17903$^T$), *X. indica* (DSM 17382$^T$), *X. innexi* (DSM 16336$^T$), *X. ishibashii* (DSM22670$^T$), *X. japonica* (DSM 16522$^T$), *X. koppenhoeferi* (DSM 18168$^T$), *X. kozodoii* (DSM 17907$^T$), *X. magdalenensis* (DSM 24915$^T$), *X. mauleonii* (DSM 17908$^T$), *X. miraniensis* (DSM 17902$^T$), *X. nematophila* (DSM 17382$^T$), *X. poinarii* (DSM 4768$^T$), *X. romanii* (DSM 17910$^T$), *X. stockiae* (DSM 17904$^T$), *X. szentirmaii* (DSM 16338$^T$) and *X. vietnamensis* (DSM 22392$^T$) (Akhurst & Boemare, 1988; Kuwata *et al*., 2012; Lengyel *et al*., 2005; Nishimura *et al*., 1994; Somvanshi *et al*., 2006; Tailliez *et al*., 2006, 2010, 2011; Thomas & Poinar, 1979). In this paper we describe a novel symbiont for South Africa, as a new species, *Xenorhabdus khoisanae* sp. nov., associated with the entomopathogenic nematode *S. khoisanae*.

## Materials and methods

### *Bacterial strains and growth conditions*

Bacterial strains SF87 (type strain), SF80, SF362 and 106-C were isolated from different local *S. khoisanae* nematode populations, as previously described (Malan *et al*., 2006, 2011). Cultures were obtained indirectly from the nematodes by drawing blood from the haemocoel of wax moth larvae (*Galleria mellonella* L; Lepidoptera:Pyralidae) which was plated onto nutrient agar (Biolab, Biolab Diagnostics, Midrand, South Africa), supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue (NBTA) according to the procedure described by Akhurst (1980). Plates were incubated in a growth chamber at 26°C for 72 h and typical blue-green colonies were randomly selected for identification and further characterization. Isolates of *Xenorhabdus* spp. were also routinely cultured in Tryptic Soy Broth (TSB; Beckton Dickinson and Company, Sparks, USA) and Luria Broth (LB; Biolab), and stored in a 1.5 ml Eppendorf tube in 40% (v/v) glycerol at -80°C. *Xenorhabdus miraniensis* DSM 17902$^T$, *X. hominickii* DSM 17903$^T$ and *Bacillus subtilis* subsp. *subtilis* DSM 10$^T$ were obtained from

Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. *Escherichia coli* transformants were cultured in LB containing 100 µg/ml ampicillin. *Bacillus subtilis* DSM $10^T$ was grown in Nutrient Broth (NB; Biolab).

*Physiological and biochemical characterization*

Phenotypic characterization included colony pigmentation, determination of optimum growth temperature, sensitivity to ampicillin, antibiosis, lipase activity on peptone agar containing Tween 20, Tween 40, Tween 60 and Tween 80, DNAse activity, haemolysis of sheep and horse blood, lecithinase activity, production of catalase and oxidase, presence or absence of bioluminescens and biochemical reactions. *Xenorhabdus miraniensis* DSM $17902^T$ and *X. hominickii* DSM $17903^T$ were included as reference strains in carbohydrate (API) reactions and agar plate phenotypic tests, except for ampicillin sensitivity.

For dye uptake, all isolates were streaked onto NBTA and McConkey Agar (Biolab), and incubated for 48 h at 30°C. To determine optimum temperature requirements for growth isolates were grown overnight in 10 ml test tubes containing LB at temperatures ranging from 26°C to 42°C (1°C increases). Growth was measured spectrophotometrically at 600 nm. Sensitivity to ampicillin in LB was determined as described by Somogyi *et al*. (2002). Antimicrobial activity was tested by overlaying 48 h-old cultures of strains SF87, SF80, SF362, 106-C, *X. hominickii* DSM $17902^T$ and *X. miraniensis* DSM $17903^T$ with an active growing culture of *B. subtilis* subsp. *subtilis* DSM $10^T$. Lipase, DNAse and lecithinase activities were determined as described by Ferreira *et al.* (2012). Haemolysis was observed by streaking the strains on agar plates containing either 10% (v/v) sheep blood or 5% (v/v) horse blood (National Health Laboratory Services, Cape Town, South Africa). Plates were incubated in a growth cabinet at 30°C for 48 h. All tests were conducted in duplicate. Biochemical properties were recorded using Biolog GN microplates (Biolog, Hayward, Canada), API 20 NE and API 50 CH test strips (BioMérieux, Marcy l'Etoile, France). Test strips were incubated at 30°C for 10 days as indicated by Boemare and Akhurst (1988). Possible presence of bioluminescens was determined by scanning the colonies with the Xenogen *in vivo* imaging system (IVIS, Caliper Life Sciences Inc., Alameda, USA).

*Genotypic characterization*

Total genomic DNA of overnight (12 h) cultures of strains SF87, SF80, SF362 and 106-C was extracted using a ZR fungal/bacterial DNA kit (Zymo Research Corporation, Irvine, California, USA). Isolates were initially identified by amplifying the 16S rRNA gene using primers as described by Brunel *et al*. (1997). Based on previous phylogenetic studies, the recombinase A *(recA),* DNA polymerase III beta chain *(dnaN),* glutamyl-tRNA synthetase catalytic subunit *(gltX),* DNA gyrase subunit B *(gyrB)* and initiation factor B (*infB)* genes were amplified with primers recA1(F) and recA2(R), 8SF_gyrB(F) and 9Rev_gyrB(R), dnaN1(F) and dnaN2(R), gltX1(F) and gltX2(R), and infB1 and infB2, respectively (Tailliez *et al*., 2010, 2011). An initial denaturation step of 94°C for 4 min was used, followed by 35 cycles of 94°C for 1 min, the appropriate temperature for each primer pair for 30 sec and 72°C for 1 min. Final extension was at 72°C for 7 min. *TaKaRa Ex Taq*, 10x *Ex Taq* buffer and dNTP mixture (Takara Bio Inc., Shiga, Japan), were used at concentrations recommended by the manufacturer. Amplified products were purified using a QIAquick PCR Purification kit (Qiagen Inc., Valencia California, USA), ligated into pGEM T-Easy vector (Promega Corporation, Madison, USA), transformed into *Escherichia coli*, and plasmid preparations sequenced (DNA Sequencing Unit, Central Analytical Facility, University of Stellenbosch) using the BigDye Teminator V3.1 sequencing kit (Applied Biosystems). Sequences were analyzed using BLAST (Basic Local Alignment Rearch Tool, National Center for Biotechnology Information, National Library of Medicine, Bethesda USA).

The 16S rRNA gene sequences of strains SF87, SF80, SF362, 106-C and related species were aligned using the program Clustal_X, ver. 1.18 (Thompson *et al*., 1997). Sequences of the closest relatives were retrieved from DNA Databank of Japan (DDBJ). The Kimura two-parameter model was used to calculate distance matrices for the aligned sequences (Kimura, 1980) and bootstrapping with 1000 replicates was done to estimate the robustness of the individual branches (Felsenstein, 1985). The neighbour-joining method (Saitou & Nei, 1987) analysed with PHYLIP ver. 3.65 (Felsenstein, 2005) were used for sequence analysis. Partial *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences were analyzed using the neighbour-joining method.

*Hybridization studies*

DNA-DNA hybridization between strain SF87 and the closest relative *X. miraniensis* DSM 17902$^T$ was performed according to Huss *et al*. (1983). Strains SF87, SF80, SF362 and 106-C were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Strain SF87 was also deposited at the American Type Culture Collection (ATCC).

## Results

*Physiological and biochemical characterization*

All strains tested absorbed dye. Colonies were dark blue-green on NBTA and light reddish brown on McConkey agar. None of the strains displayed bioluminescens. All the isolates were catalase and oxidase negative, and did not reduce nitrate. Optimum growth temperature in LB was 31-32°C, while a maximum growth temperature of 42°C was observed for strain SF87 in NB. Strains SF87, SF80, SF362 and 106-C were inhibited by ampicillin in solid medium at concentrations higher than 50 µg/ml, while slight variations in liquid medium were observed (SF87, no growth above 50 µg/ml; SF80 and 106-C, no growth at 25 µg/ml; SF362, no growth at 12.5 µg/ml). No lecithinase activity was observed for strains SF87, SF80, SF362 and 106-C, but positive reactions were recorded for the reference strains. All strains tested negative for DNAse activity. Lipase activity was not detected on plates containing Tween 20 or Tween 40, and variable results were recorded for plates containing Tween 60 and Tween 80. Total haemolysis was observed for all strains on sheep and horse blood. All strains showed strong antimicrobial activity against *B. subtilis* DSM 10$^T$.

BIOLOG GN microplate reactions indicated that strain SF87 utilized N-acetyl glucosamine, D, L-lactic acid, bromo-succinic acid, L-alanine, L-alanine-glycine, glycyl-L glutamic acid, L-histidine and L-serine (Table 3.1). This corresponds to BIOLOG GN results for most of the strains studied by Somvanshi *et al*. (2006). Strain SF87 had a weak affinity for D-mannose, uridine, ρ-hydroxy-phenyl acetic acid and D-trehalose. Reactions recorded by Somvanshi *et al*. (2006) indicated that all strains tested utilized D-mannose, most strains utilized uridine and D-trehalose, while few utilized ρ-hydroxy-phenyl acetic acid.

According to the API 50 CH system, strains SF87, SF80, SF362, 106-C, *X. miraniensis* DSM 17902$^T$ and *X. hominickii* DSM 17903$^T$ produced acid from N-acetyl-glucosamine, D-fructose, D-glucose, glycerol, D-maltose, D-mannose and ribose. Weak acid production from inositol and 5-ketogluconate were recorded. Acid production on D-trehalose was positive for strains SF87, 106-C and the control strains, but only weak positive for strains SF80 and SF362. Weak positive reactions on starch were recorded for strains SF87 and 106-C, while strains SF80 and SF362 were negative. A comparison of API results of strains SF87, SF80, SF362 and 106-C to previously published data is shown in Table 3.1. Results from API 20 NE showed that all four strains and the two reference strains assimilated glucose, mannose, N-acetyl-glucosamine, maltose and gluconate. Similar results were obtained from all strains.

**Table 3.1**. Carbohydrate reactions recorded for *X. khoisanae* (SF87, SF80, 106-C and SF362) and other *Xenorhabdus* species after 48 h of incubation at 28°C and using the BIOLOG GN microplate (Somvanshi *et al*., 2006; Tailliez *et al*., 2010).

| **Carbon substrate** | ***Xenorhabdus* species** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SF87$^T$** | **DSM 17382$^T$** | **DSM 3370$^T$** | **DSM 16522$^T$** | **DSM 4764$^T$** | **DSM 4766$^T$** | **DSM 4768$^T$** | **DSM 16336$^T$** | **DSM 16342$^T$** | **DSM 16337$^T$** | **DSM 16338$^T$** | **DSM 17903$^T$** | **DSM 17902$^T$** |
| Glycogen | − | − | + | − | − | − | − | − | − | + | − | nd | nd |
| Tween 40 | − | − | − | − | − | − | + | − | − | − | − | nd | nd |
| Tween 80 | − | − | − | − | − | − | + | + | + | − | + | nd | nd |
| *N*-acetyl-d-glucosamine | + | ++ | ++ | + | − | ++ | ++ | ++ | ++ | ++ | ++ | − | + |
| D-Fructose | − | + | ++ | ++ | + | + | − | + | + | + | + | v(−) | + |
| *m*-Inositol | − | − | − | + | − | − | − | − | − | − | − | − | − |
| *α*-Lactose | − | − | − | − | − | − | + | − | − | − | − | v(+) | + |
| Maltose | − | − | + | + | + | − | + | − | + | ++ | + | + | + |
| D-Mannose | w | ++ | ++ | + | + | + | + | ++ | ++ | ++ | + | nd | nd |
| Psicose | nd | − | − | − | − | − | − | + | − | − | − | nd | nd |
| Mono-methyl succinate | − | − | − | + | − | − | + | + | − | − | w | nd | nd |
| Acetic acid | − | − | + | + | − | − | + | − | − | + | − | nd | nd |
| Cis-aconitic acid | − | − | + | − | + | − | + | − | − | ++ | ++ | − | + |
| Citric acid | − | − | + | − | + | − | + | − | − | + | w | − | + |
| D-Gluconic acid | − | − | + | − | ++ | − | + | + | − | + | − | + | + |
| *p*-Hydroxy-phenyl acetic acid | w | w | + | − | − | − | − | − | + | + | − | nd | nd |
| *α*-Keto glutaric acid | − | − | + | − | − | − | − | − | − | + | − | nd | nd |
| D,L-Lactic acid | + | w | + | + | + | − | + | + | + | + | + | v(+) | + |
| Bromo-succinic acid | + | − | + | ++ | + | − | + | + | − | + | + | nd | nd |
| Alanin-amide | − | − | + | + | − | − | − | + | − | + | − | nd | nd |

| **Carbon substrate** | ***Xenorhabdus* species** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SF87$^T$** | **DSM 17382$^T$** | **DSM 3370$^T$** | **DSM 16522$^T$** | **DSM 4764$^T$** | **DSM 4766$^T$** | **DSM 4768$^T$** | **DSM 16336$^T$** | **DSM 16342$^T$** | **DSM 16337$^T$** | **DSM 16338$^T$** | **DSM 17903$^T$** | **DSM 17902$^T$** |
| D-Alanine | nd | − | + | + | − | − | + | + | − | + | + | w | + |
| L-Alanine | + | − | + | + | − | + | + | + | − | + | + | nd | nd |
| L-Alanyl-glycine | + | − | + | − | + | + | + | + | − | + | + | nd | nd |
| Glycyl-L-aspartic acid | − | w | + | − | − | + | + | + | + | + | + | nd | nd |
| Glycyl-L-glutamic acid | + | − | + | − | − | − | − | + | + | + | − | nd | nd |
| L-Histidine | + | w | + | + | − | + | + | + | + | + | − | − | − |
| L-Proline | − | − | + | − | + | − | + | + | + | + | + | nd | nd |
| D-Serine | + | − | + | + | + | + | + | + | + | + | − | nd | nd |
| L-Threonine | − | − | + | + | − | + | + | − | − | − | − | nd | nd |
| Uridine | w | − | ++ | ++ | − | + | + | ++ | + | ++ | + | nd | nd |
| Thymidine | − | − | + | + | − | − | − | + | + | − | + | nd | nd |
| Glucose-1-phosphate | − | + | ++ | − | + | + | ++ | + | − | ++ | w | nd | nd |
| Adonitol | − | − | − | − | − | − | − | − | − | − | − | nd | nd |
| D-Trehalose | w | w | + | + | + | + | + | + | + | + | + | − | + |
| Methyl pyruvate | nd | w | + | + | + | + | + | + | + | + | + | nd | nd |
| Succinic acid | − | − | + | + | + | + | + | + | + | + | + | v(+) | + |
| L-Aspartic acid | nd | − | + | + | + | + | + | + | + | + | + | + | + |
| L-Asparagine | nd | − | + | + | + | + | + | + | + | + | + | nd | nd |
| L-Glutamic acid | − | − | + | + | + | + | + | + | + | + | + | nd | nd |
| L-Serine | + | − | + | + | + | + | + | + | + | + | + | nd | nd |

+ = carbohydrate utilised, - = not utilised, w = weak reaction (<20% of the highest reaction after 24 and 48 h), v = variable reaction, nd = not determined.

All strains utilized dextrin, α-D-glucose, glycerol, DL-α-glycerol phosphate, inosine and glucose-6-phosphate. None of the strains utilized any of the other substrates listed in Biolog GN2. SF87$^T$ = *X. khosanensis*, DSM 17382$^T$ = *X. indica*, DSM 3370$^T$ = *X. nematophila*, DSM 16522$^T$ = *X. japonica*, DSM 4764$^T$ = *X. beddingii*, DSM 4766$^T$ = *X. bovienii*, DSM 4768$^T$ = *X. poinarii*, DSM 16336$^T$ = *X. innexi*, DSM 16342$^T$ = *X. budapestensis*, DSM 16337$^T$ = *X. ehlersii*, DSM 16338$^T$ = *X. szentirmaii,* DSM 17903$^T$ = *X. hominickii*, DSM 17902$^T$ = *X. miraniensis* (Table 3.1).

*Genotypic characterization*

According to the neighbour-joining method, strains SF87, SF80, SF362 and 106-C aligned with 97% homology to the 16S rRNA sequences of several *Xenorhabdus* type strains, clearly indicating that it belongs to the same genus (Fig. 3. 1).

In addition to 16S rRNA gene sequence analysis, several recent studies have adopted a multi gene approach to distinguish between *Xenorhabdus* spp. (Kuwata *et al*., 2012; Lee & Stock, 2010; Tailliez *et al*., 2010, 2011). Lee and Stock (2010) analysed the 16S rRNA gene and two housekeeping genes, phosphoserine aminotransferase (*serC*) and *recA*, while Tailliez *et al*. (2010, 2011) and Kuwata *et al*. (2012) used the genes *recA*, *dnaN*, *gltX*, *gyrB* and *infB*. In this study, partial *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences of strains SF87, SF80, SF362 and 106-C were analysed using the neighbour-joining method. Results obtained with all five gene sequences have shown that these strains are phylogenetically closely related to *X. miraniensis* (96 to 97% similarity; Fig. 3. 2–3. 6.). This was confirmed by groupings obtained from concatenated sequences of the six genes (96 to 97% similarity, Fig. 3.7.).

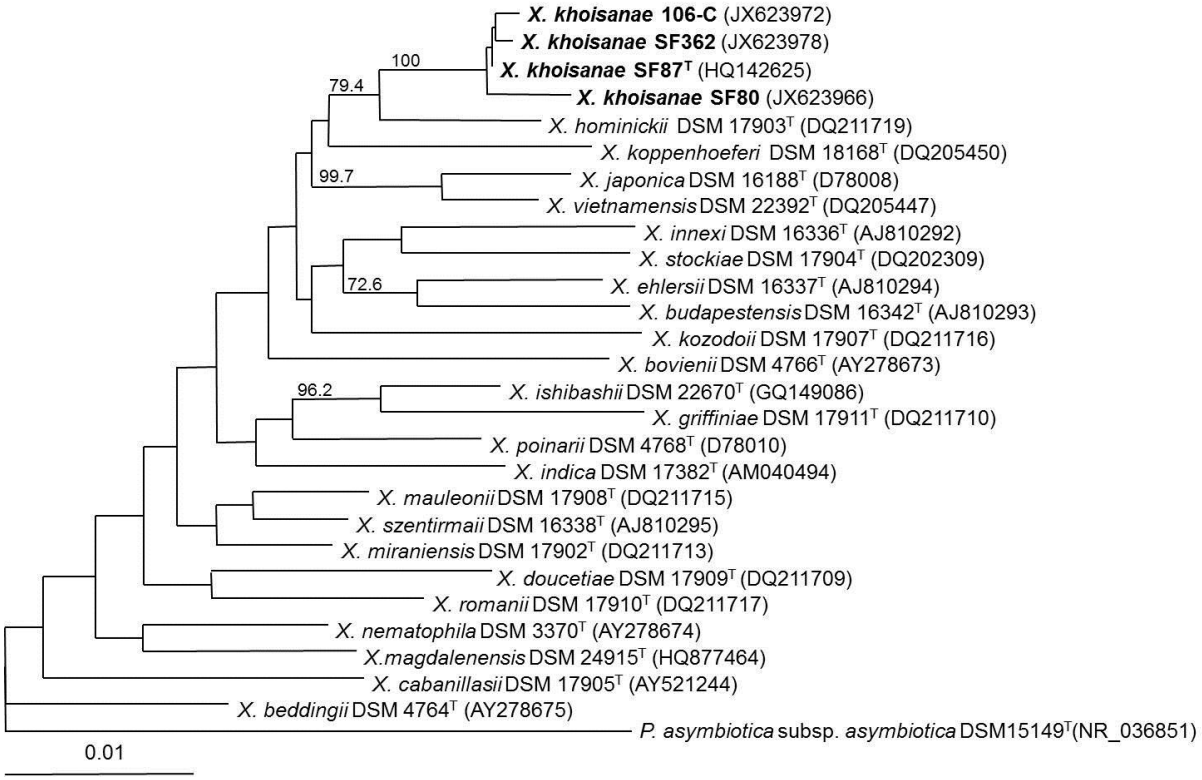


**Fig. 3.1.** Bootstrap percentages above 55% are given at branching points. Phylogenetic relationship of strain *Steinernema khoisanae* sp. nov. SF87$^T$ to known *Xenorhabdus* spp. based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

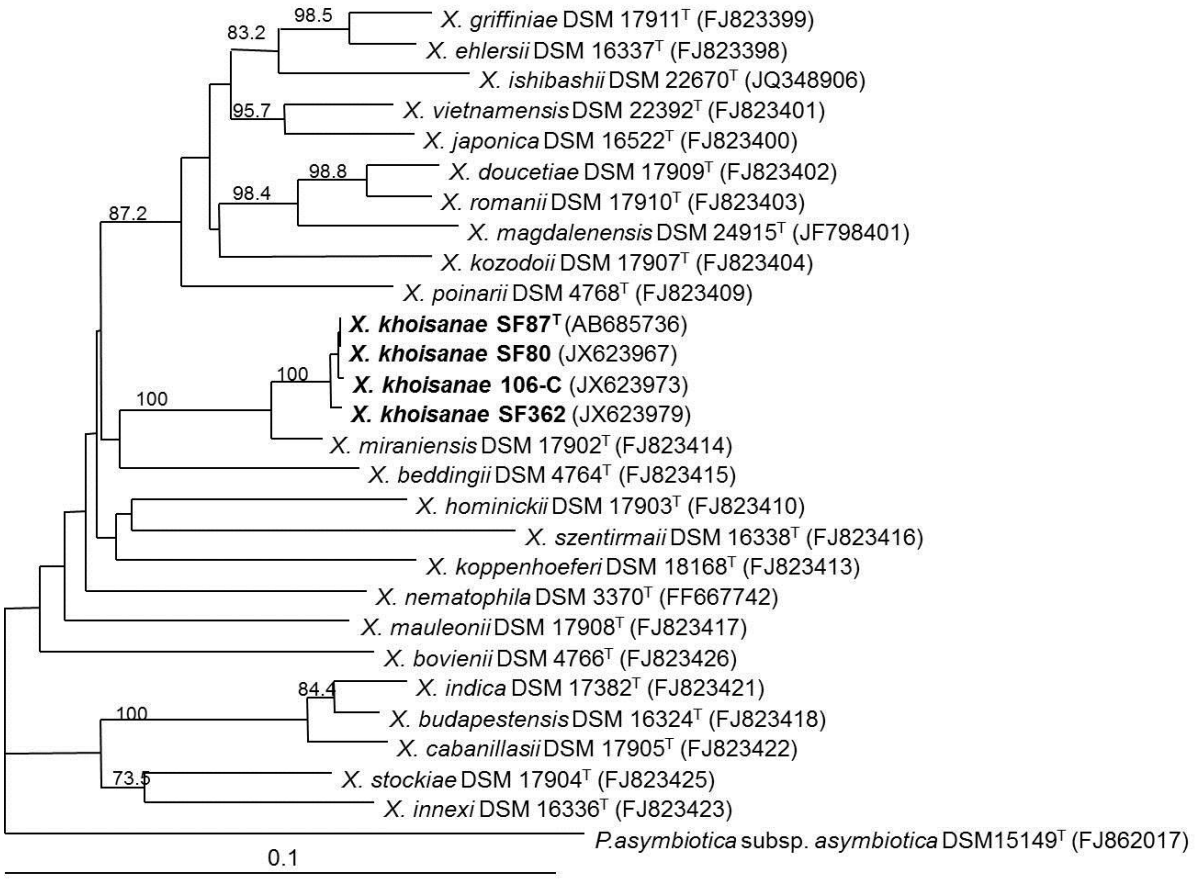


**Fig. 3.2.** Phylogenetic relationship of *Steinernema khoisanae* sp. nov. strains SF87$^T$, SF80, 106-C and SF362 to known *Xenorhabdus* spp. based on *recA* gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

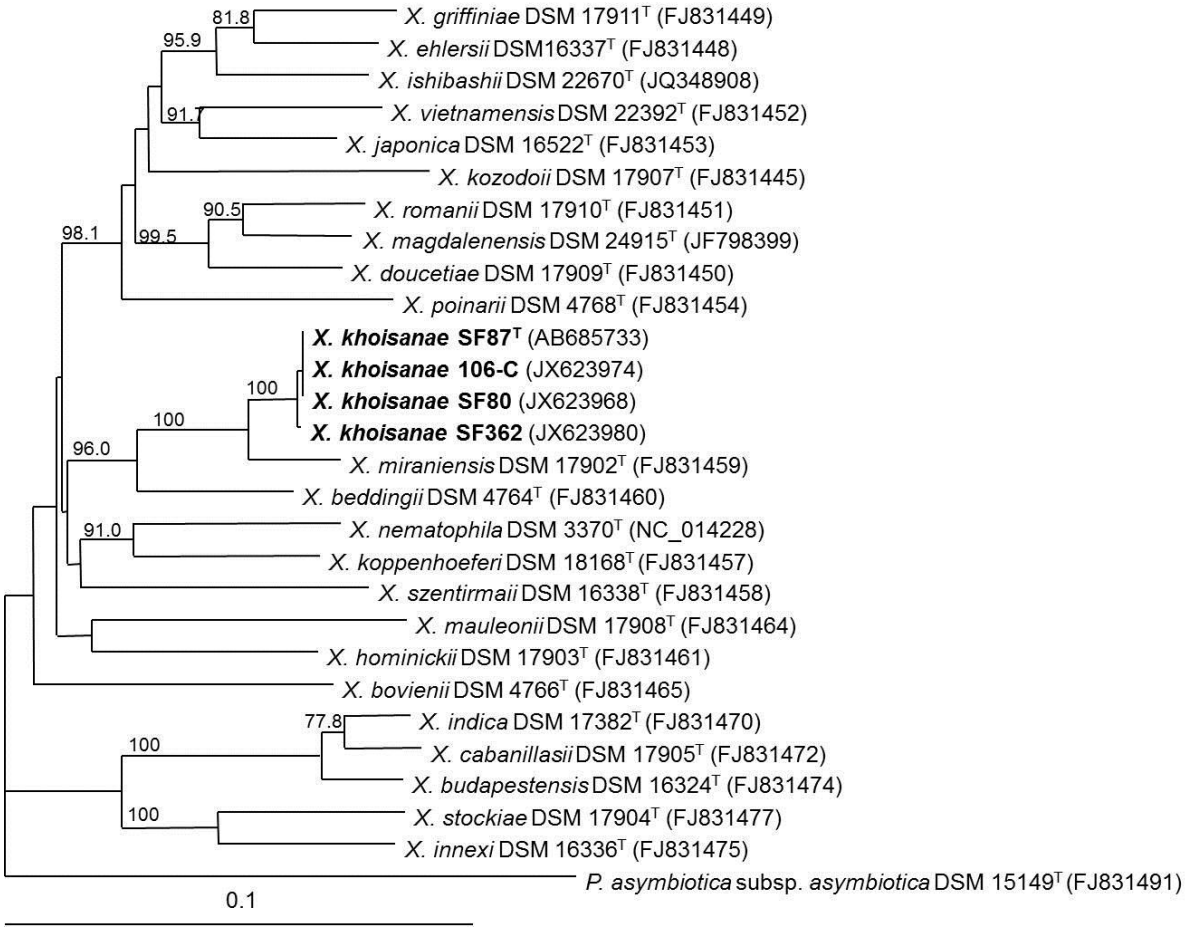


**Fig. 3.3.** Phylogenetic relationship of *Steinernema khoisanae* sp. nov. strains SF87$^T$, SF80, 106-C and SF362 to known *Xenorhabdus* spp. based on *dnaN* gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

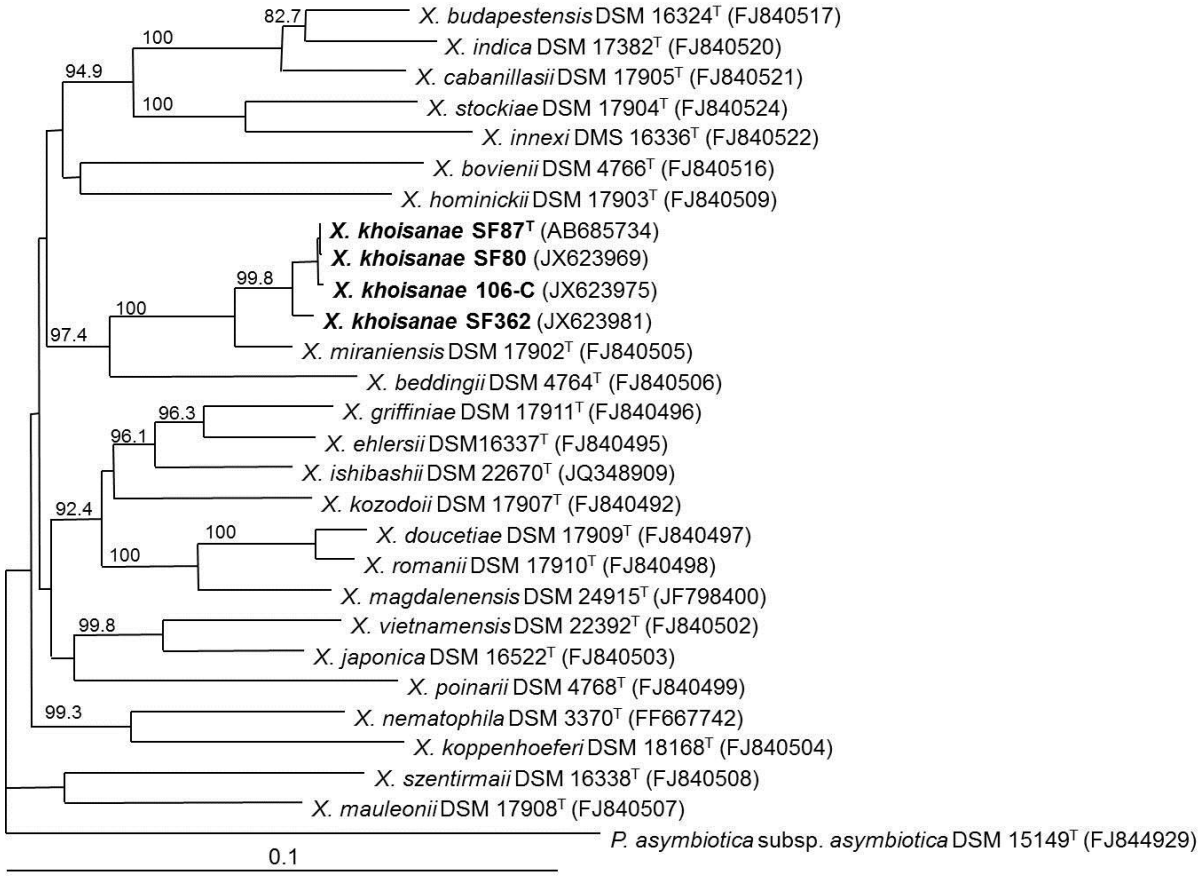


**Fig. 3.4.** Phylogenetic relationship of *Steinernema khoisanae* sp. nov. strains SF87$^T$, SF80, 106-C and SF362 to known *Xenorhabdus* spp. based on *gltX* gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

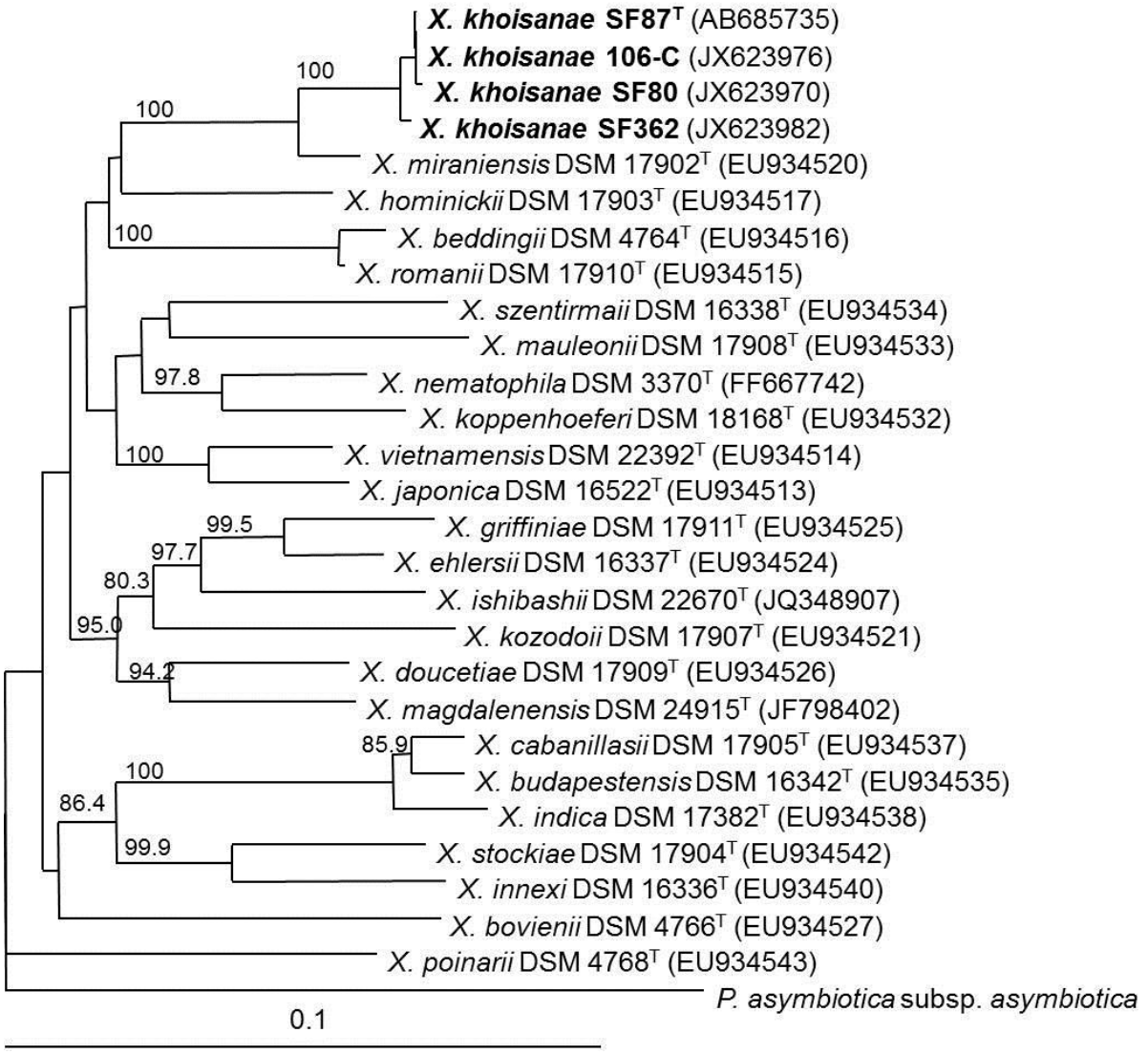


**Fig. 3.5.** Phylogenetic relationship of *Steinernema khoisanae* sp. nov. strains SF87$^T$, SF80, 106-C and SF362 to known *Xenorhabdus* spp. based on *gyrB* gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

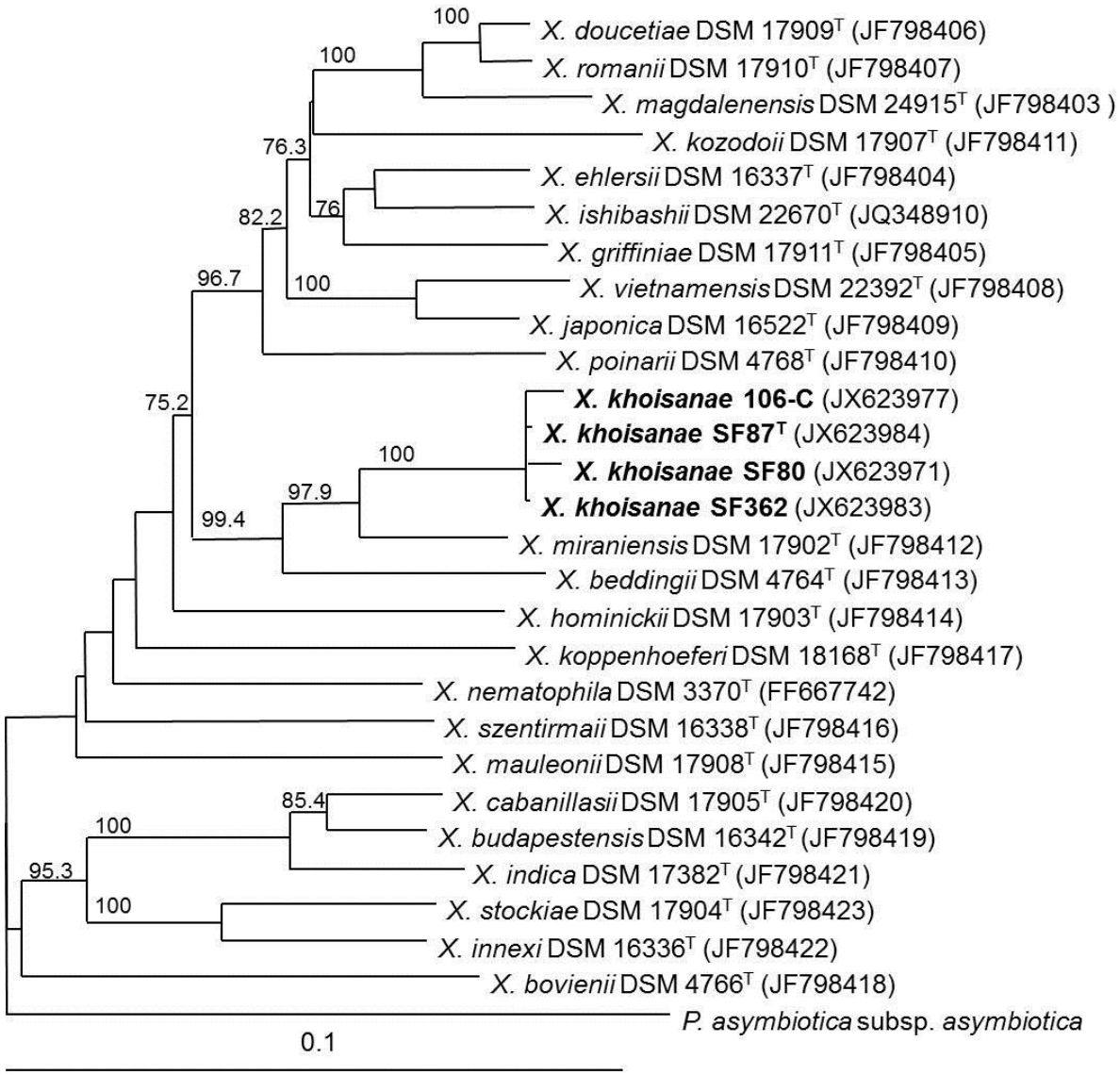


**Fig. 3.6.** Phylogenetic relationship of *Steinernema khoisanae* sp. nov. strains SF87$^T$, SF80, 106-C and SF362 to known *Xenorhabdus* spp. based on *infB* gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

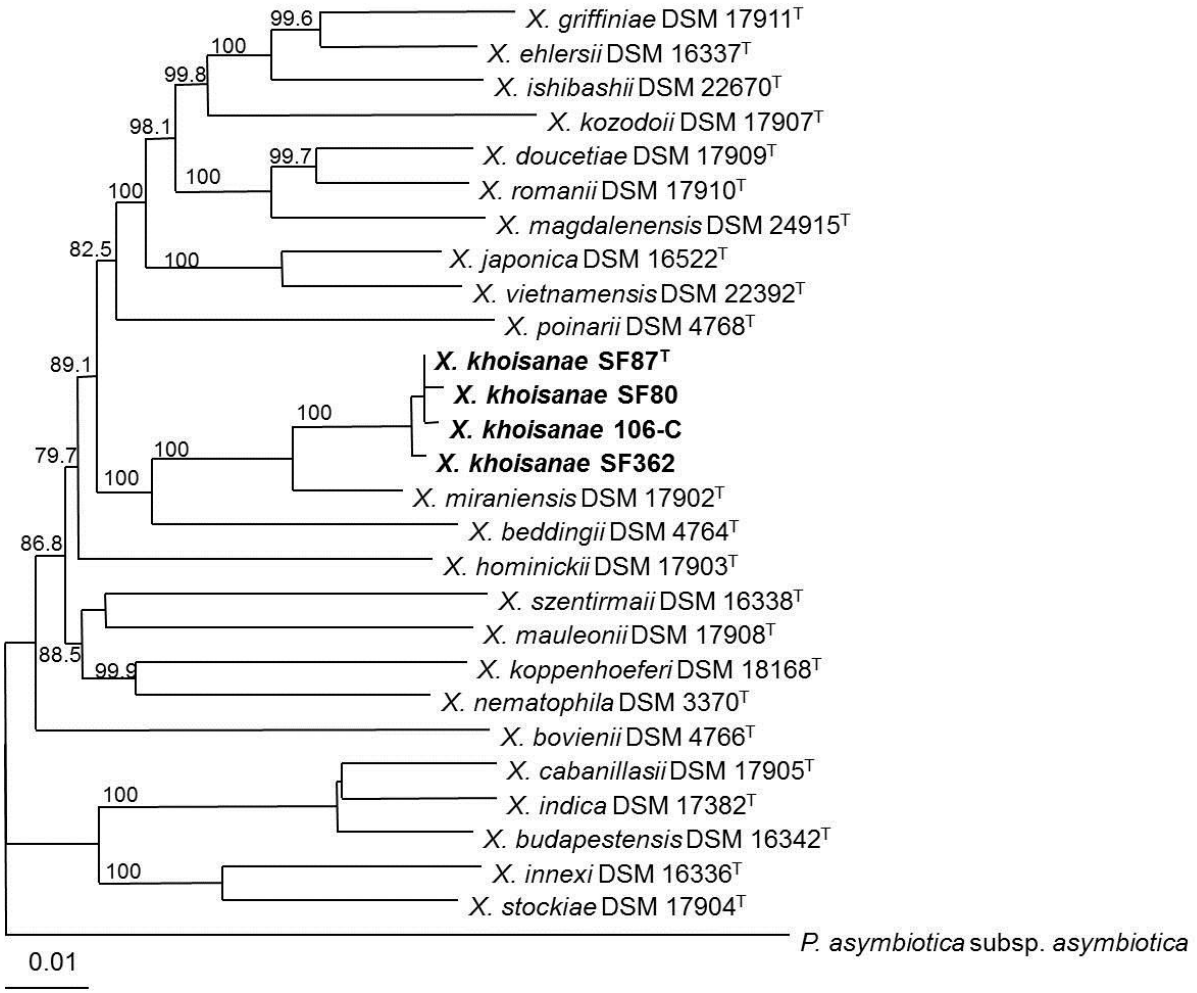


**Fig. 3.7.** Phylogenetic relationship of *Steinernema khoisanae* sp. nov. strains SF87$^T$, SF80, 106-C and SF362 to known *Xenorhabdus* spp. based on concatenated (16S rRNA, *recA*, *dnaN*, *gltX*, *gyrB* and *infB*) gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* was used as an outgroup. Bootstrap percentages above 70% are given at branching points.

*Hybridization studies*

Strain SF87 shared only 52.7% DNA homology with the type strain of *X. miraniensis* (DSM 17902$^T$). This is below the 70% DNA-DNA similarity threshold the *ad hoc* committee proposed for strains within a single species (Wayne *et al*., 1987). *Steinernema khoisanae* sp. nov. strains SF87, SF80, SF362 and 106-C are thus regarded isolates of a new species and the name *X. khoisanae* sp. nov. is proposed (type strain SF87). The name pertains to the nematode *S. khoisanae* from which the strain has been isolated.

## Discussion

*Xenorhabdus khoisanae* sp. nov., *khoisanae,* [khoi.san.ae. N.L. fem. adj. *khoisanae*] from the nematode *Steinernema khoisanae* (Nguyen *et al*., 2006). Cells are Gram-negative, catalase and oxidase negative and rod shaped (2-3 x 0.2-0.7 µm). Growth of bacterial cells of this strain is aerobic, with maximum growth temperature being recorded at 42°C in NB and 37°C in TSB. Colonies on McConkey agar are light reddish-brown. Acid is produced from N-acetyl glucosamine, D-fructose, D-glucose, glycerol, D-maltose, D-mannose and ribose. Acid production on D-trehalose and starch is variable and negative for esculin. High 16S rRNA sequence similarity (98.1%) was recorded with *X. hominickii*, but comparison of *recA, dnaN*, *gltX*, *gyrB* and *infB* gene sequences grouped the species at 96 to 97% with *X. miraniensis.* However, strain SF87 and the type strain of *X. miraniensis* (DSM 17902$^T$) share a DNA homology of only 52.7%.

Strain SF87 of *S. khoisanae* was isolated by trapping with *G. mellonella* from soil of an apple orchard on the farm Tweefontein, Villiersdorp (33º57'06S/19º24'.02E), strain SF80 from soil of grass from the farm Roodezand, Tulbagh (33º12'.33S/019º06'.57E), strain SF362 from soil of grapevine on the farm Nuutbegin, Rawsonville and strain 106-C from a citrus orchard on the farm Rooihoogte, Porterville (33º04'5.03S/18º50'.30E). The type strain is SF87$^T$ (DSM 25463$^T$, ATCC BAA-2406$^T$). The additional strains were also deposited at DSMZ (*X. khoisanae* 106-C = DSM 26373, *X. khoisanae* SF362 = DSM 26374 and *X. khoisanae* SF80 = DSM 26378).

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# CHAPTER 4

## Description of *Photorhabdus* sp. (SF41), a symbiont of the entomopathogenic nematode *Heterorhabditis zealandica*


### Abstract

The bacterial symbiont SF41 (HQ142626) was isolated from the insect pathogenic nematode *Heterorhabditis zealandica*. This isolate belongs to the genus *Photorhabdus*, which is based on the 16S rRNA gene sequence, has bioluminescent qualities and is Gram-negative. The phylogenetic position of the new isolate was analysed using a multigene approach. Strain SF41 was shown to share a common ancestor with *P. temperata* subsp. *temperata*, with *P. asymbiotica* subsp. *asymbiotica* and with *P. luminescens* subsp. *luminescens*. Several phenotypic traits, and the difference between the upper temperatures limiting growth of these four bacteria, allowed for the genetic and phenotypic differentiation of strain SF41 from its three closely related species. This is the first study to show that *H. zealandica* associates with a luminescent *Photorhabdus* species, and is not the known non-luminescent *P. temperata*. Strain SF41, therefore, represents a new species.


### Introduction

*Photorhabdus* bacteria are symbiotically associated with entomopathogenic nematodes of the genus *Heterorhabditis*, contributing actively to the biological cycle of their host. The Heterorhabditidae family of nematodes consists of obligate insect pathogens. The nematodes and bacteria work together to overcome the immune response of their insect host, thus allowing the bacteria to proliferate. Developing nematodes feed on a mixture of bacteria and bioconverted host tissue, enabling them to produce one to three generations until the food resources in the cadaver are depleted (Koppenhöfer, 2007).

Currently, three species of *Photorhabdus, P. asymbiotica (*Fischer-Le Saux, Viallard, Brunel, Normand &Boemare, 1999*, P. luminescens* (Thomas & Poinar, 1979) Boemare, Akhurst & Mourant, 1993 and *P. temperata* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare, 1999, have been described. In addition, six *P. luminescens* subspecies, three *P. temperata* subspecies, and two *P.*

*asymbiotica* subspecies are recognised. The *P. luminescens* subspecies are: *P. luminescens* subsp. *luminescens* (Thomas & Poinar, 1979) Boemare, Akhurst & Mourant, 1993; *P. luminescens* subsp. *laumondii* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare, 1999, *P. luminescens* subsp. *akhurstii* Fisher-Le Saux, Viallard, Brunel, Normand & Boemare, 1999 (Fischer-Le Saux*et al*., 1999); *P. luminescens* subsp. *kayaii* Hazir, Stakebrant, Lang, Schumann, Ehlers & Keskin, 2004; *P. luminescens* subsp. *thracensis* Hazir, Stackebrandt, Lang, Schumann, Ehlers & Keskin, 2004 (Hazir *et al*., 2004); *and P. luminescens* subsp. *kleinii* An & Grewal, 2011. The recognised *P. temperata* subspecies are: *P. temperata* subsp. *temperata* Fisher-Le Saux, Viallard, Brunel, Normand & Boemare, 1999, *P. temperata* subsp. *cinerea* (Toth & Lakatos, 2008): and *P. temperata* subsp. *stackebrandtii* An & Grewal, 2010. Two subspecies were described for *P. asymbiotica*, namely *P. asymbiotica* subsp. *australis* Akhurst Boemare, Janssen, Peel, Alfedson & Beard, 2004 and *P. asymbiotica* subsp. *asymbiotica* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare, 1999.

In this study a novel symbiont for South Africa is described, as a new species, which is associated with the entomopathogenic nematode *H. zealandica*.

## Materials and methods

*Bacterial strains and growth conditions*

Bacteria were obtained from the haemolymph of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae infected with *H. zealandica*. Isolation was done by plating on blue NBTA (Trypticase soy agar and bacteriological agar supplemented with 0.0025% (w/v) bromothymol blue) plates and incubated at 30°C. Colonies that absorbed the blue colour were submitted to 16S rRNA sequencing. The most important phenotypic characteristics of the genus *Photorhabdus* was investigated for strain SF41, using the methods of Boemare & Akhurst (1988). Strain SF41 was stored at -80°C in trypticase soy broth, containing 15% glycerol (v/v).

The shape and colour of the colonies were recorded after 72 h of incubation at 30°C on NBTA (Biolab) plates. Growth between 24°C and 42°C was determined by inoculating equal cell densities ($OD_{620nm}$ = 0.03) into Trypticase Soy Broth (TSB) (Biolab) and Nutrient Broth (NB) (Biolab), respectively. Morphology was determined using a Leica DM2000 research microscope equipped with Leica Application Suite (LAS), ver. 3.5.0.

*Physiological and biochemical characterisation*

Carbohydrate fermentation reactions were recorded using API 20NE and API 50CH E test strips (BioMérieux, Marcy l'Etoile, France). The API test strips were used according to the manufacturers' instructions. Reactions in the API 20NE and API 50CH E test strips were recorded after 48 h and 10 days of incubation at 30°C. Nitrate reduction and indole production were recorded after 48 h.

Antimicrobial activity was tested by using the spot-on lawn method (Akhurst, 1982), with *Bacillus subtilis* subsp. *subtilis* DSM 10$^T$, *Ralstonia solanacearum* DSM 9544$^T$, and *Pectobacterium carotovorum* subsp. *carotovorum* DSM 30168$^T$ as target organisms.

*Genotypic characterisation*

Phylogenetic analysis was conducted by sequencing and amplifying the16S rRNA gene, as well as the *rec*A, *gyr*B, *dna*N, *glt*X and *inf*B genes, based on a multigene approach and described previously by Tailliez *et al*. (2010). The DNA of strain SF41was extracted using a ZR fungal/bacterial DNA kit (Zymo Research Corporation, Irvine, California, USA). The partial 16S rRNA gene was amplified using primer pair 8F and 1512R, as described by Felske *et al*. (1997). The partial *infB* gene was amplified with primers *inf*B1 and *inf*B2, as described by Tailliez *et al*. (2011), and the partial *recA*, *gyrB*, *dnaN* and *gltX* genes were amplified using primers *rec*A1(F) and *rec*A2(R), 8SF_*gyr*B(F) and 9Rev_*gyr*B(R), *dna*N1(F) and *dna*N2(R), and *glt*X1(F) and *glt*X2(R), respectively, as described by Tailliez *et al*. (2010), with an Esco Swift MiniPro Thermal Cycler (Esco Micro Pte. Ltd., Singapore). An initial denaturation step of 94°C for 4 min was used, followed by 35 cycles of 94°C for 1 min, the appropriate temperature for each primer pair for 30 sec, and 72°C for 1 min. A final extension step at 72°C for 7 min was added and samples were kept at 4°C until analysed. Reaction mixtures (50 µl) containing *TaKaRa Ex Taq*™ (Takara Bio Inc., Shiga, Japan), together with the supplied 10x *Ex Taq*™ buffer and dNTP mixture were used at concentrations recommended by the manufacturer.

*Hybridisation studies*

The DNA homology shared between strain SF41 and the type strain of *Photorhabdus asymbiotica* subsp*. australis* DSM 17609$^T$was determined. A French pressure cell (Thermo Spectronic) was used to disrupt cells. DNA in the crude lysate was purified by means of

chromatography on hydroxyapatite, as described by Cashion *et al*. (1977). DNA-DNA hybridisation was performed, as described by De Ley *et al*. (1970), modified according to Huss *et al*. (1983). A Cary 100 Bio UV/VIS-spectrophometer was used, equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

In vivo *pathogenicity assay*

An *in vivo* pathogenicity assay was conducted with strain SF41 and with an *Escherichia coli* Escherich 1885, isolate that served as the control (Givaudan & Lanois, 2000). *Galleria mellonella* larvae were reared on artificial diet (Poinar, 1975) at 28°C. The bacterial cultures were prepared by inoculating 100 µl of culture into 10 ml of Luria-Bertani broth, which was then incubated in 10 ml test tubes overnight at 30°C. When an optical density of 0.7 (600 nm wavelength) was reached, the culture was centrifuged in a 1.5 ml Eppendorf tube and the cells were washed and centrifuged three times using 0.8% sodium solution. Wax moth larvae were surface sterilised with 70% (v/v) ethanol using cotton wool, prior to intrahaemocoelic injection. Then, with a BD Micro-Fine syringe, groups of 20 larvae were injected with 20 µl of bacterial cell solution. A control of the actual number of bacteria in the injected suspension was measured by plating it onto three NBTA plates (Au *et al*., 2004; Sicard *et al*., 2006). The experiment was performed on two different test dates.

## Results

*Physiological and biochemical characterisation*

Cells from the blue or blue-green colonies on the NBTA (Biolab) plates were Gram-negative and rod-shaped. The colony diameter was 1-3 mm and the pigmentation on the NBTA plates was blue-green, with a red centre. The isolates preferred aerobic growth conditions and were catalase-positive.

Biochemical properties determined by using API 20 NE and API 50CH test strips are listed in Table 4. 1. Growth in Nutrient Broth (Biolab) was recorded between 24°C and 42°C, and for Trypticase Soy Broth between 24°C and 35°C (Biolab). The optimum growth temperature in both media was 30°C (Table 4. 2).

Strain SF41 exhibited strong antibacterial activity towards *B. subtilis* subsp. *subtilis* DSM 10$^T$ and the plant pathogens *R. solanacearum* DSM 9544$^T$ and *P. carotovorum* subsp. *carotovorum* DSM 30168$^T$, corresponding to what has been described for other *Photorhabdus* spp. (Somvanshi *et al*., 2006) (Table 4.2).

**Table 4.1.** Results from API 20 NE and API 50 CH test strips for SF41, *P. asymbiotica* subsp. *australis*, *P. asymbiotica* subsp. *asymbiotica* and *P. temperata* subsp. *temperata.*

| | *P. asymbiotica* subsp. *australis* | *P. asymbiotica* subsp. *asymbiotica* | P. *temperata* subsp. *temperata* | SF41 |
|---|---|---|---|---|
| Control | | | | |
| Glycerol | + | + | + | + |
| Erythritol | | | | |
| D-Arabinose | + | | | |
| L-Arabinose | | | | |
| D-Ribose | w | + | w | w |
| D-Xylose | | | | + |
| L-Xylose | | | | |
| D-Adonitol | | | | |
| Methyl-βD xylopyranoside | | | | |
| D-Galactose | | | | |
| D-Glucose | + | + | + | + |
| D-Fructose | + | + | + | + |
| D-Mannose | + | + | + | + |
| L-Sorbose | | | | |
| L-Rhamnose | | | | |
| Dulcitol | | | | |
| Inositol | + | w | | w |
| D-Mannitol | | | | |
| D-Sorbitol | | | w | |
| Methyl-αDmannopyranoside | | | | |
| Methyl-αD-glucopyranoside | | | | |
| N-Acetyl glucosamine | + | + | + | + |
| Amygdalin | | | | |
| Arbutin | | | | |
| Esculin ferric citrate | + | + | + | + |
| Salicin | + | + | | w |
| D-Cellobiose | | | | |
| D-Maltose | + | + | w | w |
| D-Lactose | | | | |
| D-Melibiose | | | | |
| D-Saccharose | | | | |
| D-Trehalose | w | w | w | |
| Inulin | | | | |
| D-Melezitose | | | | |

| | *P. asymbiotica* subsp. *australis* | *P. asymbiotica* subsp. *asymbiotica* | P. *temperata* subsp. *temperata* | SF41 |
|---|---|---|---|---|
| D-Raffinose | | | | |
| Amidon | | | | |
| Glycogen | | | | |
| Xylitol | | | w | |
| Gentibiose | | | | |
| D-Turanose | | | | |
| D-Lyxose | | | | |
| D-Tagatose | | | | |
| D-Fucose | | | | |
| L-Fucose | w | + | + | |
| D-Arabitol | | | | |
| L-Arabitol | | | | |
| Potassium gluconate | w | | w | w |
| Potassium 2-ketogloconate | | | | w |
| Potassium 5-ketogluconate | w | w | w | w |
| Reduction of nitrates to nitrites | | | | |
| Reduction of nitrates to nitrogen | | | | |
| Indole production | | | | |
| Glucose fermentation | + | + | | + |
| Arginine dihydrolase | + | + | w | + |
| Urease | + | + | + | + |
| Hydrolysis (β-glucosidase) esculin | + | + | + | + |
| Hydrolysis (protease) Gelatin | + | + | + | + |
| B-galactosidase | | | | |
| Glucose assimilation | + | + | + | + |
| Arabinose assimilation | | | | |
| Mannose assimilation | + | + | + | + |
| Mannitol assimilation | | | | |
| N-acetyl-glucosamine assimilation | + | + | + | + |
| Maltose assimilation | + | + | + | + |
| Potassium gluconate assimilation | + | + | + | + |
| Capric acid assimilation | | | | |
| Adipic acid assimilation | | | | |
| Malic acid assimilation | + | + | | |
| Trisodium citrate assimilation | + | + | + | |
| Phenyl acetic acid assimilation | | | | |
| Oxidase | | | | |

+, 90% of strains positive; w, weak; blank, not available

**Table 4.2.** Phenotypic characters differentiating SF41, *P. asymbiotica* subsp. *australis* and *P. asymbiotica* (Somvanshi *et al*., 2006).

| **Phenotypic characterisation** | *P. asymbiotica* subsp. *asimbiotica* | *P. asymbiotica* subsp. *australis* | *P. asymbiotica* subsp. *temperata* | SF41 |
|---|---|---|---|---|
| Growth in LB 26-42°C: Optimum | | | | 30 |
| Maximum temperature | | | | 42 |
| Amp resistance 1.563-200 ug/ml | | | | 75 |
| Colony pigmentation: NBTA | | | | Yes |
| MacConkey | | | | Yes |
| Bioluminescens | Yes | Yes | Yes | Yes |
| Antibiosis against *Bacillus subtilis* | | | | ++ |
| *Pectobacterium* sp. | | | | ++ |
| *Ralstonia* sp. | | | | ++ |
| Growth in Tween 20 | | | | + |
| Tween 40 | | | | + |
| Tween 60 | | | | + |
| Tween 80 | | | | + |
| DNAse activity | | | | + |
| Type of haemolysis (total, partial, annular) horse/sheep | T | T | T | T |
| Lecithinase activity | - | - | - | + |
| Catalase | + | + | + | + |
| Oxidase | - | - | - | - |

Blank, not available

*Genotypic characterisation*

The 16S rRNA gene sequence of strain SF41 was compared with the sequences of representative strains of the different species and subspecies of the genus *Photorhabdus* (Fig. 4.1). The sequence of strain SF41 clustered with the type strains of *P. temperata* and *P. asymbiotica*, indicating that it belongs to the genus *Photorhabdus*.

In addition to 16S rRNA gene sequences analysis, several recent studies have adopted a multi gene approach to distinguish between *Photorhabdus* species, as well as subspecies (Kuwata *et al*., 2012; Lee & Stock, 2010; Tailliez *et al*., 2010, 2011). Lee and Stock (2010) analysed the 16S rRNA

gene and two housekeeping genes, phosphoserine aminotransferase (*serC*) and *recA*, while Tailliez *et al*. (2010, 2011) and Kuwata *et al*. (2012) used the genes *recA*, *dnaN*, *gltX*, *gyrB* and *infB*. In this study, partial *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences of strain SF41 were analysed, using the neighbour-joining method. Results obtained with all five gene sequences have shown that the strains are phylogenetically closely related to *P. asymbiotica* (Fig. 4.2-4.5).

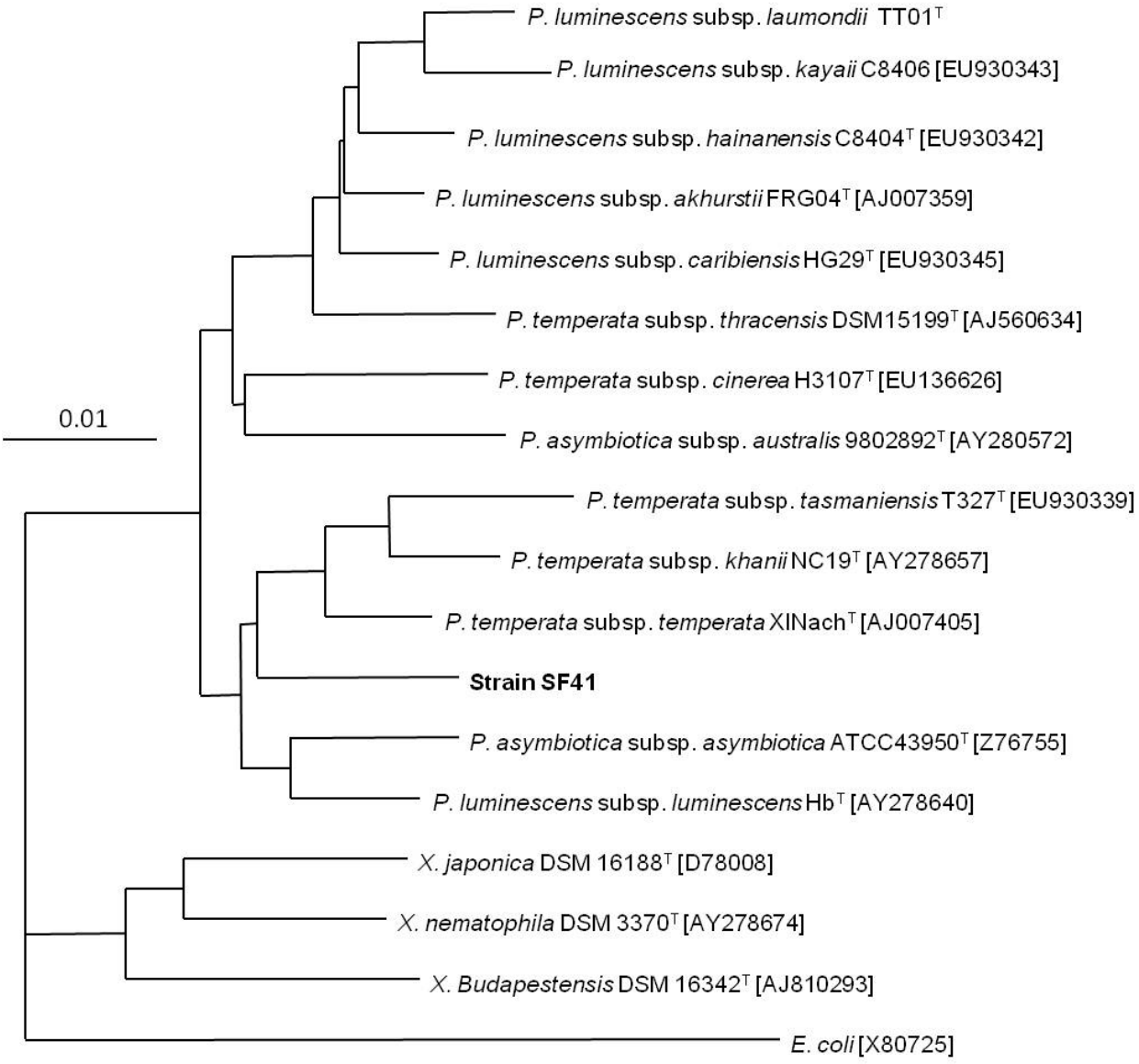


**Fig. 4.1.** Phylogenetic tree based on 16S rDNA sequences of *Photorhabdus* strains, including strain SF41. The tree was constructed using the maximum-likelihood method. *Escherichia coli* was used as an outgroup.

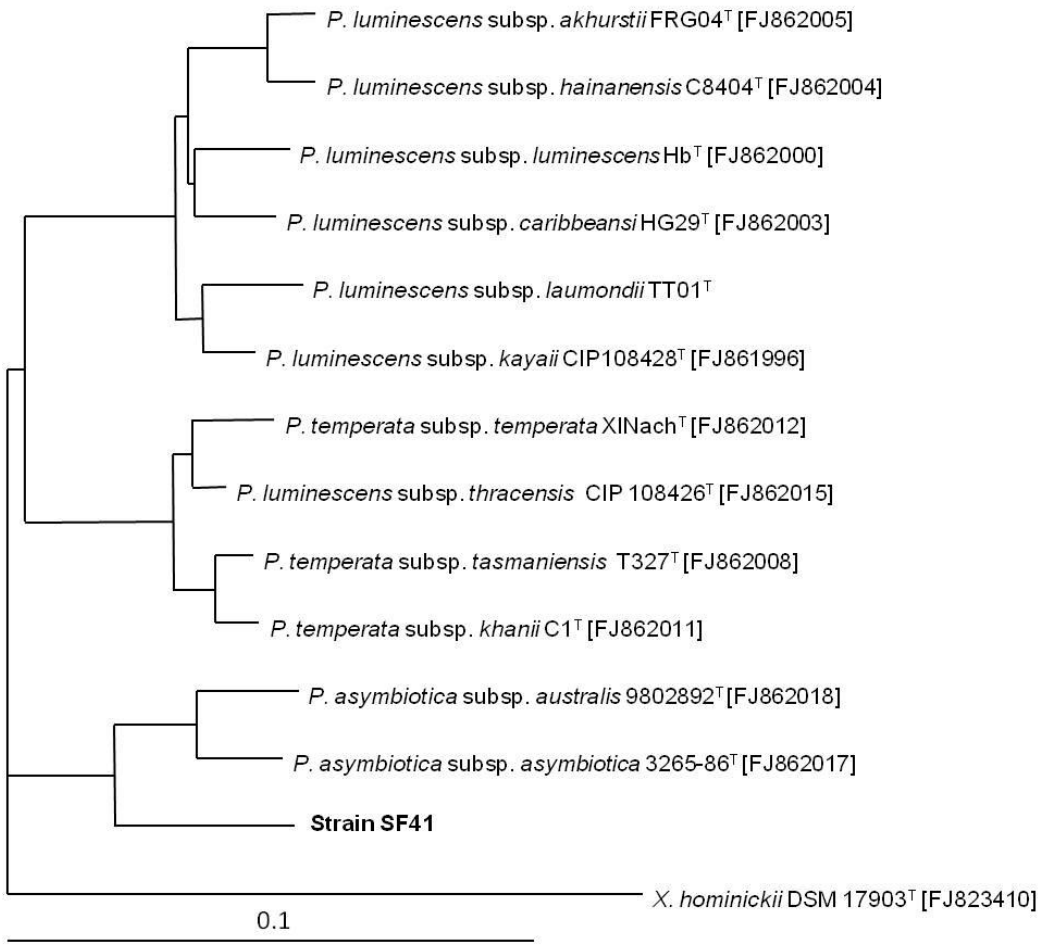


**Fig. 4.2.** Phylogenetic tree based on *recA* sequences of *Photorhabdus* strains, including strain SF41. The tree was constructed using the maximum-likelihood method. *Xenorhabdus hominickii* was used as an outgroup.

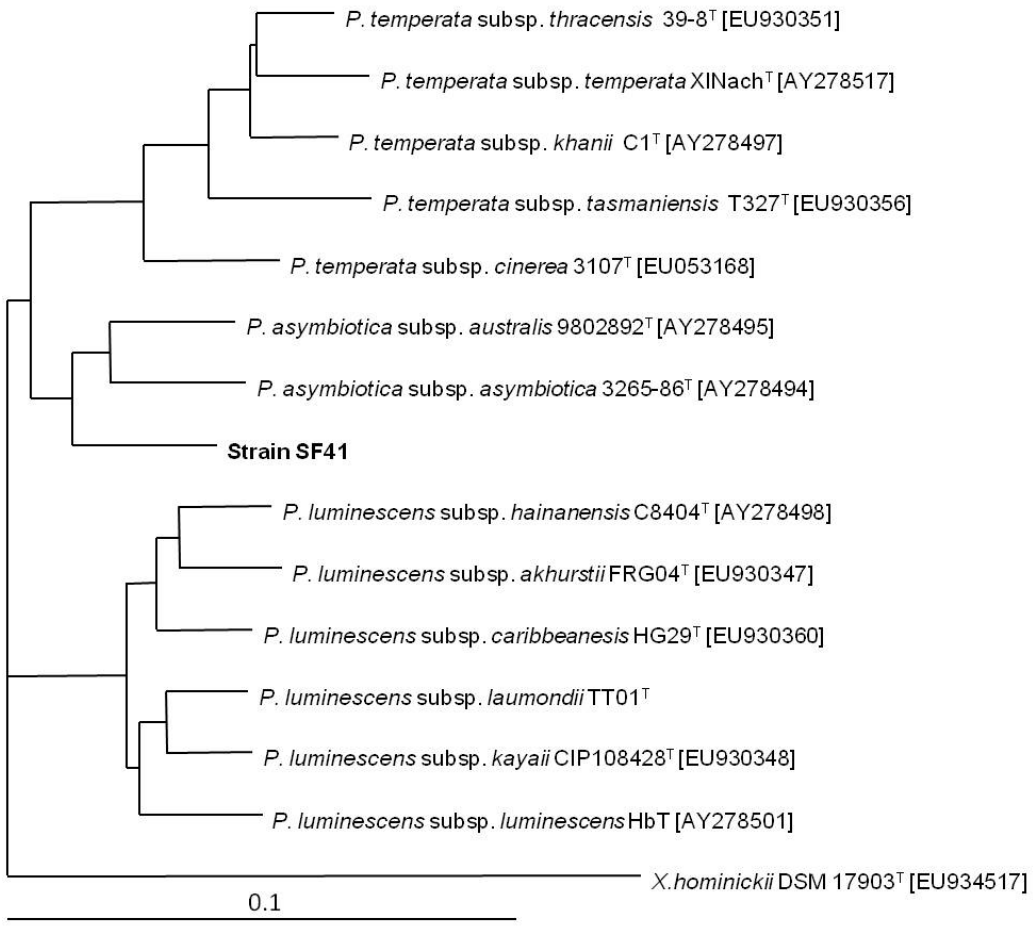


**Fig. 4.3.** Phylogenetic tree based on *gyrB* sequences of *Photorhabdus* strains, including strain SF41. The tree was constructed using the maximum-likelihood method. *Xenorhabdus hominickii* was used as an outgroup.

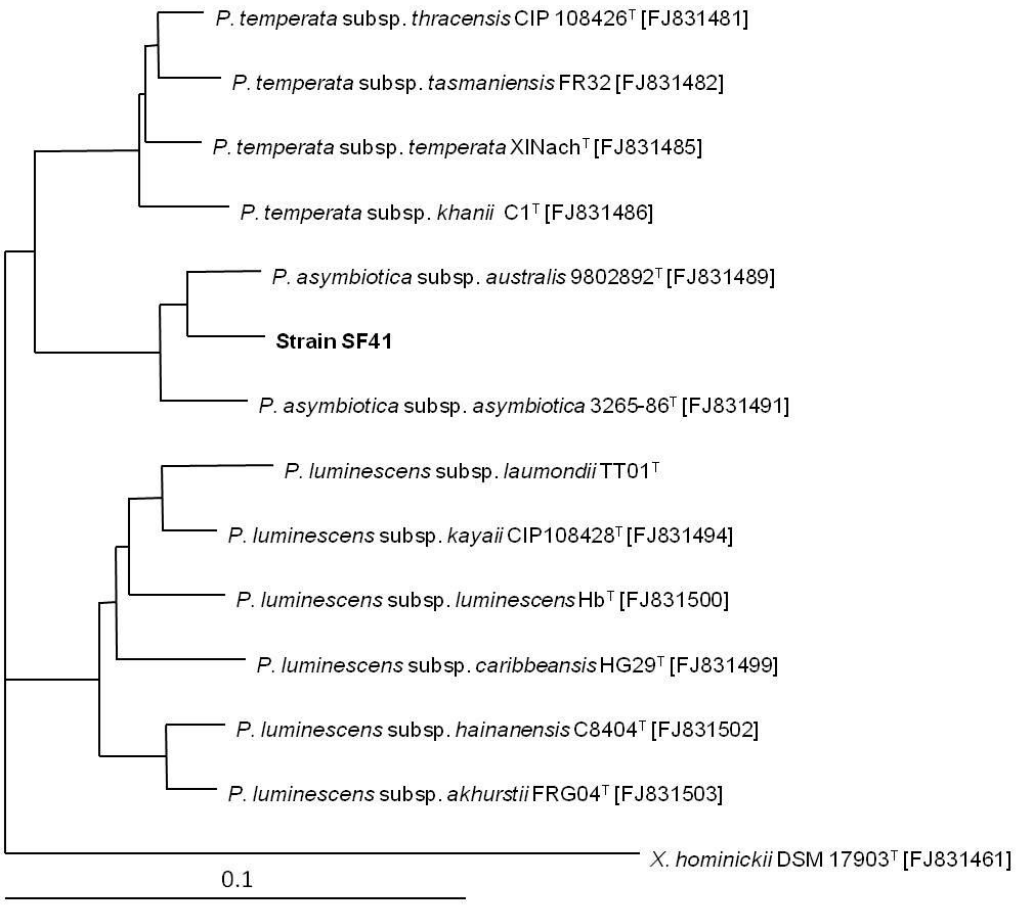


**Fig. 4.4.** Phylogenetic tree based on *dnaN* sequences of *Photorhabdus* strains, including strain SF41. The tree was constructed using the maximum-likelihood method. *Xenorhabdus hominickii* was used as an outgroup.

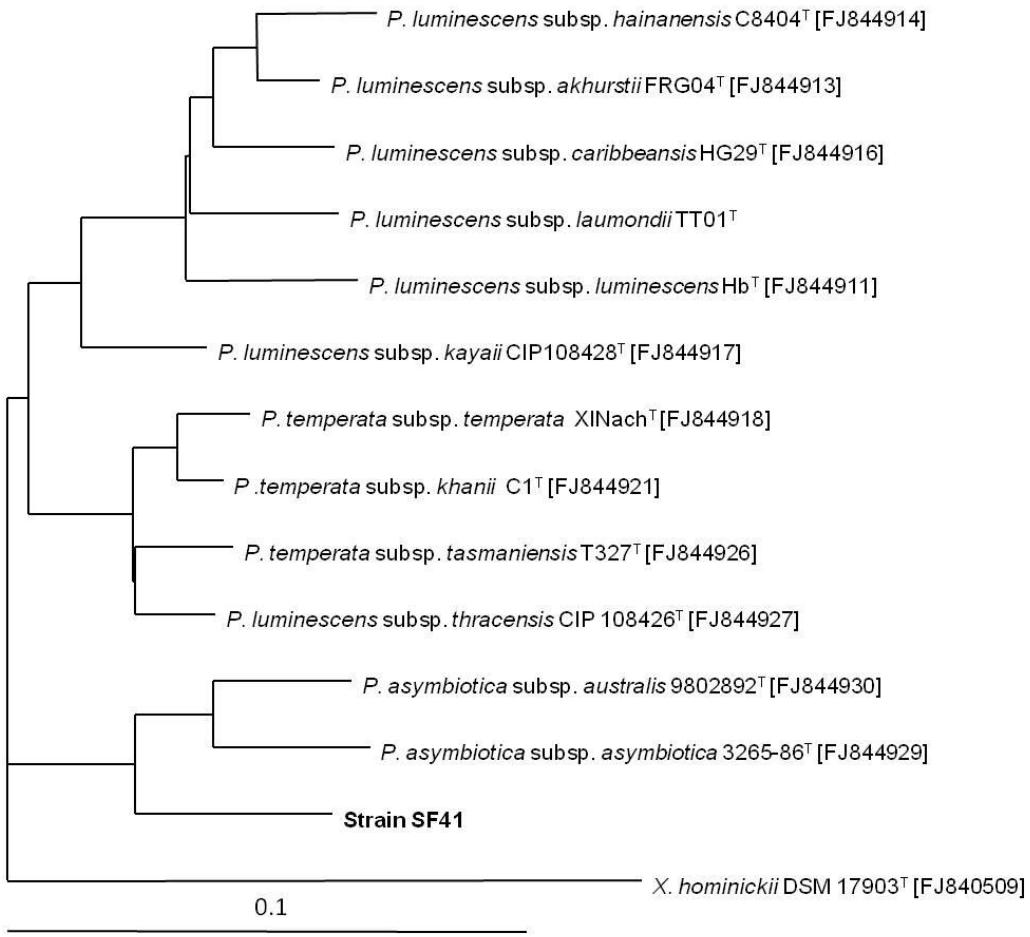


**Fig. 4.5.** Phylogenetic tree based on *gltX* sequences of *Photorhabdus* strains, including strain SF41. The tree was constructed using the maximum-likelihood method. *Xenorhabdus hominickii* was used as an outgroup.

*Hybridisation studies*

Based on DNA-DNA hybridisation, strain SF41 shares only 50.8% homology with the type strain of *P. asymbiotica* ssp. *australis* (DSM 17609$^T$). This is below the 70% DNA-DNA similarity threshold that the *ad hoc* committee proposed for strains within a single species (Thompson *et al*., 1997). Strain SF41 is, thus, regarded as a new species and the name *Photorhabdus zealandica* is proposed (strain SF41). The name pertains to the nematode *Heterorhabditis zealandica*, from which the strain has been isolated. The strain has been deposited into the Deutsche Sammlung von Mikro-organismen und Zellkulturen (DSM 25263$^T$) and the American Type Culture Collection (ATCC).

In vivo *pathogenicity assay*

After 16 h, there was 82.5% mortality of *G. mellonella* larvae injected with strain SF41, while the control group all survived. Results from the pathogenicity assay show strain SF41 is an effective insect pathogen, and has potential to be used in combination with its vector, *H.zealandica*, as a biological control organism against pest insects.

## Discussion

Bacterial cells are Gram-negative, catalase-positive and rod-shaped. Growth of these bacterial cells is aerobic, with growth temperatures of strain SF41 ranging from 24°C to 42°C in Nutrient Broth, and from 24°C to 35°C in Trypticase Soy Broth. Optimal growth in both media was recorded at 30°C. Colonies on NBTA plates were blue or blue-green. Glycerol, glucose, fructose, mannose and N-Acetyl glucosamine were utilised by the bacteria after 10 days. Results from API 20NE show that strain SF41 were positive for glucose fermentation, arginine dihydrolase, urease, hydrolysis of esculin and hydrolysis of gelatine. After 10 days assimilation of glucose, mannose, N-acetyl-glucosamine, maltose, potassium gluconate and malic acid occurred. Nitrate was not reduced to either nitrite or nitrogen by bacterial cells of this strain. Strain SF41 and the type strain of *P. asymbiotica* ssp. *australis* (DSM 17609$^T$) share a DNA homology of only 50.8%.

The bacterial strain identified in this study was isolated from the nematode *Heterorhabditus zealandica* in Patensie, in the Northern Cape province, South Africa. The type strain is SF41$^T$ and has been deposited into the Deutsche Sammlung von Mikroorganismen und Zellkulturen (25263) and the American Type Culture Collection (ATCC).

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# CHAPTER 5

## Development and population dynamics of *Heterorhabditis zealandica* and growth characteristics of the associated *Photorhabdus luminescens* symbiont in liquid culture


### Abstract

Commercial use of entomopathogenic nematodes against key insect pests of deciduous fruit, grapevine and citrus in South Africa requires massive numbers of nematodes for inundative field application. High-technology *in vitro* liquid culture requires development to mass culture and to formulate said nematodes for commercial purposes. *Heterorhabditis zealandica* was identified as a species with potential as a biological control agent against a wide range of key insect pests. The first step towards the development of *in vitro* mass culture of *H. zealandica* is the establishment of monoxenic cultures of both the nematode and its *Photorhabdus luminescens* symbiont, using *in vitro* liquid culture technology. The body length of various *H. zealandica* life stages during *in vitro* development was measured to determine the growth characteristics of *H. zealandica* in liquid culture. The growth curve of the symbiotic bacteria during the process time was measured, to determine when the stationary phase was reached, as this would indicate the optimum time required for inoculating infective juveniles (IJs) and for aiding in maximum IJ recovery. On day 15, the IJs reached a maximum density of 41100·ml$^{-1}$, while the hermaphrodites and females reached their highest density on day 16 at 9800·ml$^{-1}$ and 7700·ml$^{-1}$, respectively, after which the experiment was terminated. Bioassays using *Galleria mellonella* were performed to compare the virulence between *in vitro*- and *in vivo*-produced nematodes, which indicated *in vitro*-produced nematodes to be significantly less virulent. This study illustrates that *H. zealandica* and its *P. luminescens* symbiont can be successfully cultured in liquid. However, two generations occurred during the process time, instead of the desirable one-generation. Future research goals would be to increase the percentage recovery in the liquid culture, as doing so would increase the number of nematodes produced per ml and it would also reduce the processing time significantly.

## Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are symbiotically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, which are natural antagonists of the soil stages of insect pests (Dowds & Peters, 2002). The genus *Photorhabdus* belongs to the family Enterobacteriaceae and contains three recognised species, namely *P. luminescens* (Thomas & Poinar, 1979) Boemare, Akhurst & Mourant, 1993; *P. temperata* Fisher-Le Saux, Viallard, Brunel, Normand & Boermare, 1999; and *P. asymbiotica* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare, 1999b. Within these nematode genera, a free-living soil stage occurs that overcomes periods of starvation as an enduring third juvenile stage, the infective juvenile (IJ). In *Heterorhabditis*, this stage carries the cells of the symbiotic bacterium in its intestine and represents the infective stage (Endo & Nickle, 1991). These bacto-helminthic complexes are safe biocontrol agents for agricultural soil insect pests (Boemare *et al*., 1996; Ehlers & Hokkanen, 1996).

There is a demand for environmentally safe insecticides, with low toxicity, short-term persistence and limited effects on non-target organisms. For this reason, new insecticidal compounds are difficult to register for soil application, while many older compounds have been banned (Ehlers, 1996). EPNs, however, can be used to control insect pests such as curculionid and scarabeid larvae in ornamental plants, strawberries and turf (Kaya & Gaugler, I993, Sulistyanto & Ehlers, 1996) and have many advantages over chemical compounds. They can reproduce in the host insect and, therefore, give a persistent effect in the soil (Ehlers, 1996). When the IJ enters the haemocoel of an insect host, it releases the symbiotic bacteria and starts developing from the third stage juvenile (J3) to the fourth-stage juvenile (J4) (Poinar *et al*., 1977), with the process concerned termed "recovery" in the closely related species *Caenorhabditis elegans* (Golden & Riddle, 1984). *Heterorhabditis* always develops, during the first generation, into self-fertilising hermaphrodites that produce eggs. The first stage juvenile (J1) hatching from the egg can either develop through the J2, J3 and J4 stages into males or females, which are amphimictic cross-fertilising adults, or they stop developing at the J3, in order to form IJs. The ratio of amphimictic (males and females) to automictic (hermaphrodites and IJs) individuals depends on the nutritional conditions encountered by the J1 larvae. Favourable conditions enhance the development of amphimictic males and females, whereas depleting food resources lead to the development of IJs, which can further develop into hermaphrodites (Strauch *et al*., 1994).

When the egg production of the hermaphrodites ceases, the remaining eggs develop within the uterus. These individuals develop into IJs and enter into the surrounding medium only after the hermaphrodite has died. The process concerned is called *endotokia matricida* (Johnigk & Ehlers, 1999b). Due to their 'y', or lambda, type of copulation behaviour, the amphimictic adults of *Heterorhabditis* are unable to mate in liquid culture. In contrast, *Steinernema* males and females are able to copulate in liquid culture, because of the curling mating behaviour of the male (Strauch *et al*., 1994).

The objective of this study was the successful mass culture of *Heterorhabditis zealandica* Poinar, 1990 in liquid culture, in order to study the population dynamics of *H. zealandica* as well as the growth characteristics of the associated symbiotic bacterium *Photorhabdus luminescens*, followed throughout its culture period.

## Materials and methods

### *Source of insects and nematodes*

*Galleria mellonella* (L.) (Lepidoptera: Pyralidae), wax moth larvae, were reared on a diet containing the following ingredients: five parts brown bread flour; five parts Cerelac Nestle™ regular baby cereal; two parts wheat germ; two parts yeast; two parts glycerine; and one part honey. All the ingredients were mixed together with a beeswax comb (Bronskill, 1961; Woodring & Kaya, 1988).

Nematodes were obtained from the nematode collection of the Department of Conservation Ecology and Entomology at Stellenbosch University (Malan *et al*., 2006). *In vivo*-produced IJs were reared and harvested at room temperature, according to the procedures devised by Kaya and Stock (1997). The IJs were stored at 14˚C in filtered tap water in flat, vented, horizontally placed 500 ml volume culture flasks. The flasks contained 150 ml nematode suspension, and were shaken weekly for aeration. Nematodes were used within one month after harvesting.

### *Isolation of symbiotic bacteria*

The primary form of the symbiotic bacteria was isolated from last instar *G. mellonella* larvae infected with *H. zealandica*. Last instar *G. mellonella* larvae were surface-sterilised, 24 h after inoculation with IJs of *H. zealandica*, by being dipped into 95% (v/v) ethanol, ignited and immediately

plunged into sterile water. The cadaver was opened, using a sterile syringe and inoculating loop, the haemolymph was transferred and spread onto an NBTA plate (nutrient broth supplemented with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue (NBTA). Plates were incubated at 25-28˚C for 48 h (Akhurst, 1980; Kaya & Stock, 1997), propagated in trypticase soy broth (TSB) in 10 ml test tubes and stored in 15% glycerol at -80˚C. When required, the glycerol stocks were melted at room temperature, propagated in 10 ml TSB in 25 ml test tubes for 2 days at 30˚C, and subsequently used.

*Identification of symbiotic bacteria*

The bacterium was identified by isolating the total genomic DNA of an overnight (12 h) culture of the symbiotic bacteria. It was extracted using the ZR fungal/bacterial DNA kit (Zymo Research Corporation, Irvine, California, USA). The DNA of the 16S rRNA gene was amplified, as described by Felske *et al.* (1997). PCR reactions were done, as described by Tailliez *et al*. (2010), using *TaKaRa Ex Taq*™ (Takara Bio Inc., Shiga, Japan), together with the supplied 10 × *Ex Taq*™ buffer and dNTP mixture, at concentrations recommended by the manufacturer. Amplified products were cleaned (QIAquick PCR purification kit, Qiagen, Valencia, USA) and sequenced at the Analytical Centre of the Department of Genetics at Stellenbosch University, using BigDye chemistry (PE Applied Biosystems, Foster City, California, USA). The base-pair calls of the sequences were verified and edited, using the software CLC DNA Workbench, version 6 (http://www.clcbio/products/clc-main-workbench/). Sequences were analysed using Basic Local Alignment Search Tool (BLAST). National Centre for Biotechnology Information, National Library of Medicine, Bethesda, USA). Bioluminescens was visualised by scanning the colonies with the Xenogen *in vivo* imaging system (IVIS, Caliper Life Sciences Inc., Alameda, USA).

*Axenisation of nematodes*

To obtain nematode eggs, last instar *G. mellonella* larvae were infected with IJs of *H. zealandica*. Infected *G. mellonella* larvae were dissected 4 days after inoculation with IJs. The hermaphrodites found were isolated and transferred to a 10-ml glass tube. Pieces of razor blades were added with Ringer's solution (8 ml), which was then vortexed to cut the hermaphrodites and to release the eggs. The suspension was poured through a 25-µm sieve, allowing the eggs to pass

through, but retaining the nematode and razor blade pieces. The filtrate containing eggs was transferred to 1-ml Eppendorf tubes and centrifuged for 1 min at 2000 rpm to form an egg pellet. The supernatant was removed, and the tube was filled with 1 ml fresh Ringer's solution. Centrifugation was repeated until the supernatant remained clear. The eggs were then transferred to a sterile 1.5 ml Eppendorf tube and again centrifuged, after which the supernatant was removed. Aliquots of a mixture of 0.5 ml sodium hypochlorite, 1.5 ml NaOH (4 N) and 10 ml distilled water was subsequently added and centrifuged for 2 min at 2000 rpm. The supernatant was discarded, after which the Eppendorf tube was filled with 1 ml TSB. The eggs were transferred using a pipette to a sterile 12-multiwell plate, of which the wells were filled with 300 µl TSB, and incubated for 72 h (Ehlers *et al*., 1998).

A 250 ml Erlenmeyer flask was filled with 30 ml TSB and inoculated with the primary form of the bacterial symbiont. The culture was incubated on a platform orbital shaker at 180 rpm at 30˚C in the dark. Wout's agar plates (5.4-cm diam.) were inoculated with two drops of the *Photorhabdus* bacterial suspension, after which 50-100 J1 larvae from the sterile cell-wells was added (Wouts, 1981). The plates were sealed with Parafilm and incubated in a growth chamber and kept at 25˚C.

*Monoxenic culture protocol*

The liquid medium (complex medium) in which the nematodes were propagated consisted of 15.0 g yeast extract (Merck), 20.0 g soy powder (Nature's Choice, Meyerton, South Africa), 4 g NaCl, 0.35 g KCl, 0.15 g $CaCl_2$, 0.1 g $MgSO_4$ (Merck), 46 ml vegetable oil per L (Ehlers *et al.*, 1998). The 250 ml Erlenmeyer flasks, containing 30 ml of nematode culture medium, were inoculated with 1% of the bacterial culture and pre-cultured at 30˚C for 36 h, before inoculating IJs from the monoxenic cultures. Flasks were incubated in a dark growth chamber at 25˚C for 15 days. Samples of 1 ml were taken in sterile conditions in a laminar flow cabinet and washed with Ringer's solution through a 25-µm sieve. The nematodes were counted using 10 µl aliquotes with a stereo microscope and the concentration determined.

In order to provide a homogenous bacterial inoculum, *Photorhabdus* sp. was produced in one batch culture, and then 1 ml was added to three 250 ml Erlenmeyer flasks each (Hirao *et al*., 2010). When the bacterial pre-cultures reached a cell density of $10^7$ cells·$ml^{-1}$ (as pre-determined in TSB) 300 µl of it were transferred using a pipette to sterile 30-ml Erlenmeyer flasks and incubated on a

platform orbital shaker for 36 h at 180 rpm at 30˚C in the dark. The IJs were subsequently inoculated in the liquid medium at a density of 2000 IJs·ml$^{-1}$, and incubated on a platform orbital shaker at 180 rpm at 25˚C for 16 days in the dark. IJs were taken from monoxenic pre-cultures (Lunau *et al*., 1993). Three flask cultures were used, and the experiment was replicated on two different test dates.

*Assessment of developmental stages*

Samples of 1 ml of each of the three flasks were taken each day over a period of 16 days. Nematodes were washed with Ringer's solution through a 25-µm sieve to determine population development in liquid culture. The different juvenile stages, J1/J2 and the J3/J4, were counted. The juvenile stages were distinguished from one another by measuring the body lengths. IJs were recognised by their slender shape, the tapering of the mouth region, the loose second-stage cuticle, and the dark intestine without a visible cavity. The recovered IJs had a flat mouth region, no second-stage cuticle, and the intestinal lumen was evident (Strauch & Ehlers, 1998). The IJs were grouped and counted together with pre-infective juveniles (J2d), which were identified by the body being much darker than that of the other juvenile stages (Hirao *et al.*, 2010). The adult hermaphrodites were distinguished from female phenotypes by the fertilised eggs in their uterus (Strauch *et al*., 1994). All female phenotypes were observed carrying unfertilised eggs (counted as females), and died without producing offspring. Nematode female phenotypes with fertilised eggs, or juveniles in the uterus, were counted as hermaphrodites. Males were recognised by their spicules (Johnigk & Ehlers, 1999a). Recovery was calculated by counting inoculated IJs that recovered and developed beyond the IJ stage, which included the number of hermaphrodites and the J3/J4 stages (Ehlers *et al*., 1998).

Nematodes used for measurements were fixed in hot (85ºC) TAF (triethanolamine 2%, formalin 8%) (Courtney *et al*., 1995), and processed to pure glycerine, using the Seinhorst (1959) method, after which they were mounted in glycerine, using wax ring supports, to prevent flattening. Measurements were taken by means of a Leica DM2000 research microscope (Leica Microsystems, Wetzlar, Germany) equipped with a camera, computer and digital image software Leica Application Suite (LAS), version 3.5.0.

*Bacterial growth curve*

A bacterial growth curve was determined in order to investigate the cell density dynamics of the symbiotic bacteria over a period of 48 h, to establish the stationary phase of the bacteria (Johnigk *et al*., 2004). This experiment was conducted at a temperature of 30˚C and using TSB as a medium. A sample, containing 40 µl of the bacteria stock solution, was inoculated into 10 ml of TSB and incubated in 25 ml test tube for 2 days in the dark at 30˚C. Then 400 µl of this solution was inoculated into 400 ml of 1 L schott bottle TSB, and a sample of 1 ml was taken every 3 h for 48 h. A dilution series was conducted for each sample and streaked out in triplicate and replicated twice, using NBTA plates. Plates were incubated for 2 days in a growth chamber and colony forming units were counted (Atlas, 1988). Optical density was also measured every 3 h using a spectophotometer.

Cell density was determined once the IJs were inoculated into the liquid (Hirao & Ehlers, 2009a; Hirao & Ehlers, 2010), Samples were taken every day for 16 days, streaked out in quadruplicate and replicated twice, using NBTA plates. Plates were incubated at 30˚C in a growth chamber in the dark for 2 days. Colonies were subsequently counted again using a dilution series (Ehlers, personal communication, 2012).

*Virulence studies*

Virulence bioassays were conducted after the IJs were harvested from the flasks. The *in vitro*-produced IJs were harvested by washing them with Ringer's solution through a 25-µm sieve and the supernatant containing the nematodes washed into a 250 ml glass beaker. *Galleria mellonella* were infected with 200 IJs/50 µl of *in vitro*-produced nematodes, and compared with *H. zealandica* IJs harvested from *G. mellonella* cultured nematodes (*in vivo*).

Last instar *G. mellonella* larvae were placed in 24-well plates (flat bottom, Nunc™, Cat. No. 144530) with 10 filter paper discs (13-mm diam.) in each of the 10 wells. A concentration of 200 IJs per 50 µl was inoculated onto the filter paper discs, and one *G. mellonella* larva each was placed in each of the 10 wells. The plates were incubated in a growth chamber at 25˚C in the dark. Mortality of *G. mellonella* larvae was assessed by movement and colour change after 2 days and infection with nematodes confirmed by dissection in Ringer's solution.

*Statistical analysis*

All statistical analyses were performed by using the data analysis software program STATISTICA 10 (Statsoft Inc., T.O.U., 2011). Data were analysed using descriptive statistics. For the measurement of different nematode life stages and virulence studies, a one-way ANOVA with post-hoc comparisons of means was used. Bonferroni's method was used where residuals were not normally distributed. Data obtained from the counting of different life stages and bacterial growth curves were analysed using a factorial ANOVA with post-hoc comparisons of means, using the Tukey's HSD test.

## Results

*Identity of symbiotic bacteria*

Analyses of the 16S gene showed the associated symbiotic bacteria of *H. zealandica* to belong to *Photorhabdus luminescens*. Bioluminescence of the bacteria was visually confirmed. Further analysis with known sequences from Genbank indicated the bacteria to be a new subspecies of *P. luminescens*, with the characterisation of the species as a new subspecies having been described in Chapter 4.

*Population dynamics of* Heterorhabditis zealandica

Analyses of data obtained for the *H. zealandica* life cycle development on each day (1 to 16) showed no significant differences between the two test dates (males: $\rho = 0.93$; females: $\rho = 0.31$; hermaphrodites: $\rho = 0.5$; IJs: $\rho = 1$; J1/J2: $\rho = 0.65$; J3/J4: $\rho = 0.16$). For the rest of the analyses, the data were therefore pooled. The population development for *H. zealandica* at 25˚C, from inoculated IJs to adults in monoxenic liquid culture, is presented in Figures 5.I., 5.2. and 5.3. The first hermaphrodites occurred on day 3 and the first males and females occurred on days 6 and 7 respectively (Fig. 5.1.). The highest concentration of hermaphrodites and females was recorded on day 16, while the highest concentration of males was recorded on day 11. The sex ratio (males: females) of the amphimictic population was calculated as being 0.3 on day 7. As the males occurred one day earlier than did the females, the ratio was more than 1 on day 6. From day 10 to day 12, the ratio was calculated as being 0.8. A decrease in the number of female nematodes on day 13 was

followed by an increase in their number until the experiment was terminated on day 16. Contrary to the fluctuation in the female numbers, the male numbers remained relatively constant from day 10 to day 13, after which they decreased, until the experiment was terminated on day 16. The ratio of amphimictic to automictic nematodes on day 7 was 1:12 and the ratio on day 15 was 1:6. Since IJs exclusively develop into hermaphrodites, the IJs were counted as automictic when the ratio was calculated.

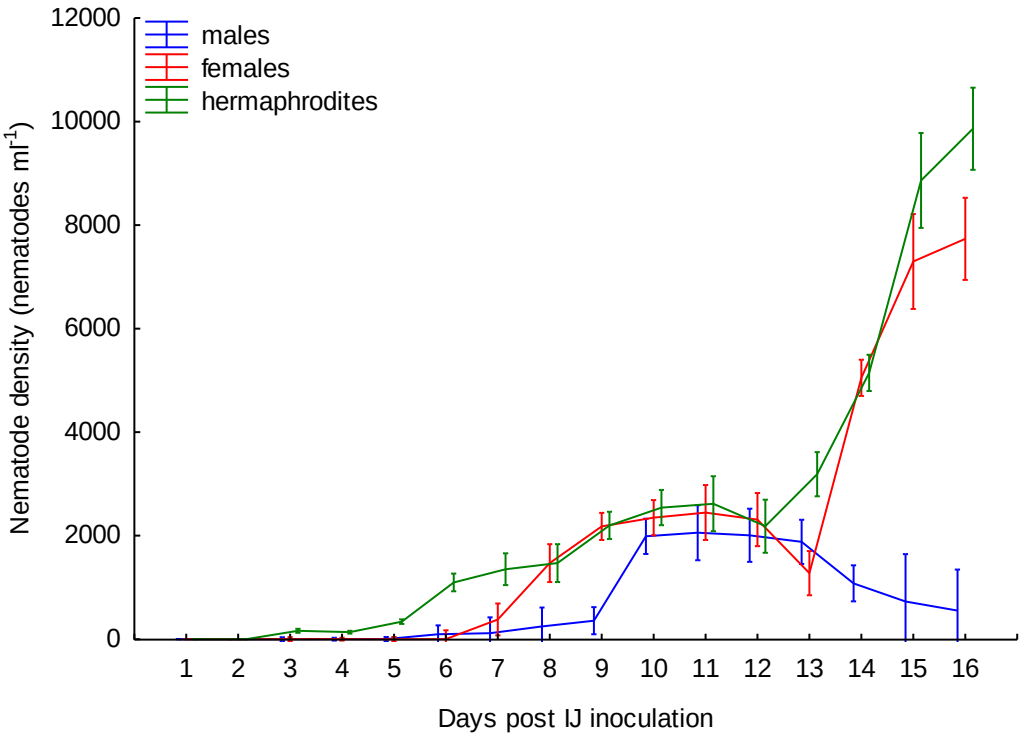


**Fig. 5.1.** Mean population density (95% confidence interval) of males, females and hermaphrodites of *Heterorhabditis zealandica* in monoxenic liquid culture at 25˚C, over a period of 16 days (one-way ANOVA; $F_{(30, 405)} = 61.81$).

The inoculum density was 2000 IJs·ml$^{-1}$, and the highest IJ concentration was reached on day 15, with 41 100 IJs·ml$^{-1}$. There was an increase in IJ density that occurred on day 7 (Fig. 5.1.). The inoculated IJs developed into hermaphrodites that reproduced, and J1 offspring occurred on day 4 (Fig. 5.2.). On day 16, the IJs decreased to 40 000 IJs·ml$^{-1}$ (Fig. 5.3.). The J1 offspring developed either into infective juveniles or adults (Fig. 5.3.). The highest concentration of J1/J2 stages was observed on day 7, with the highest concentration of J3/J4 stages occurring one day later (Fig. 5.2.). Recovery of IJs was observed as 12% on day 3, as 13% on day 4, and as 24% on day 5.

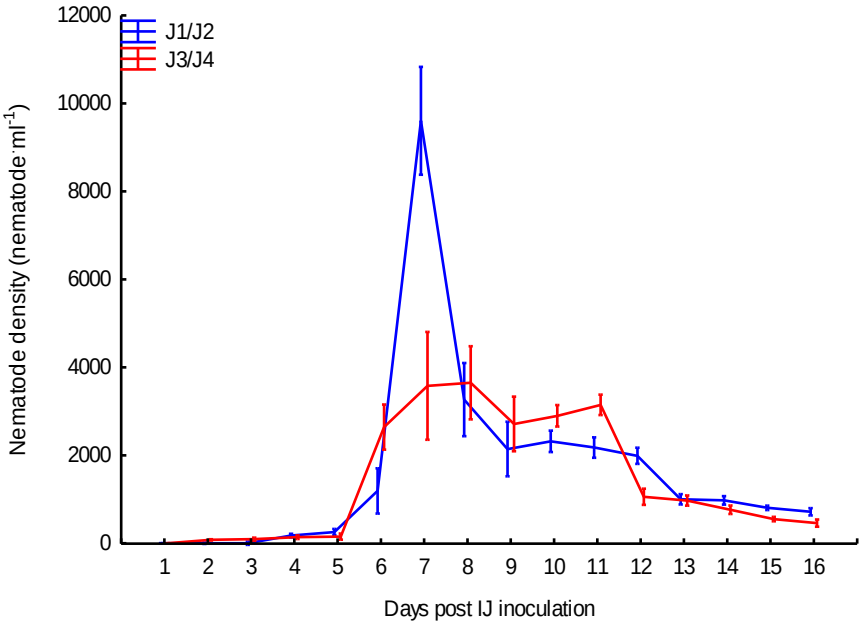


**Fig. 5.2.** Mean population density (95% confidence interval) of J1/J2 and J3/J4 larvae of *Heterorhabditis zealandica* in monoxenic liquid culture at 25˚C, over a period of 16 days (one-way ANOVA; $F_{(15, 270)} = 31.12$).

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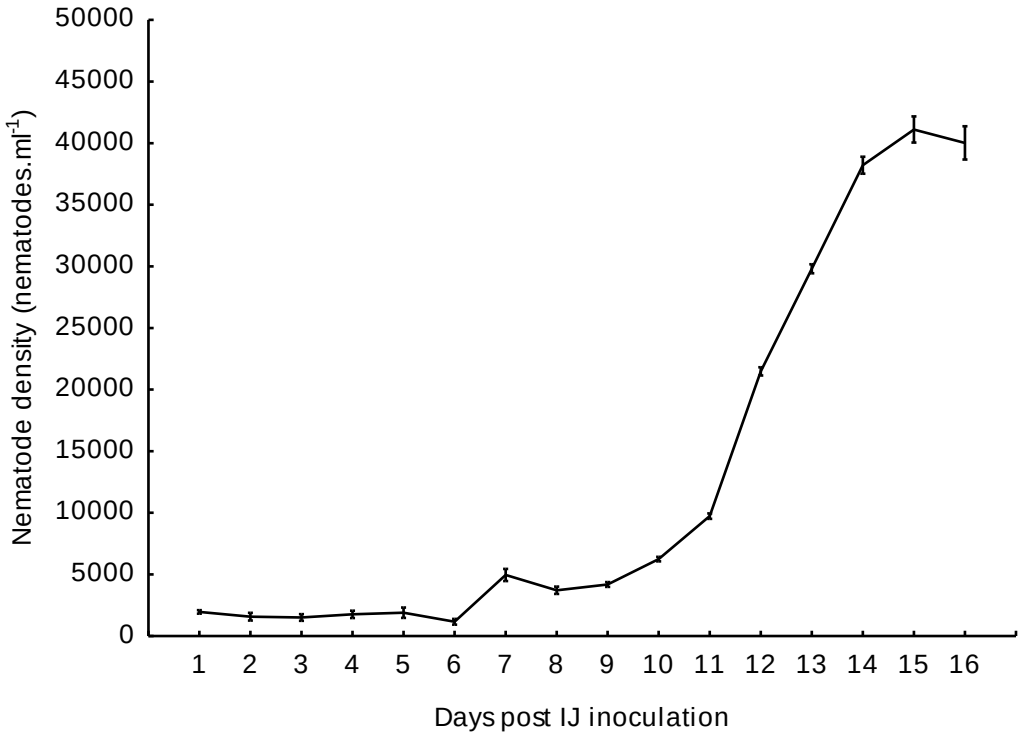


**Fig. 5.3.** Mean population density (95% confidence interval) of *Heterorhabditis zealandica* IJs in monoxenic liquid culture at 25˚C, over a period of 16 days (one-way ANOVA; $F_{(15, 135)} = 4133$).

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The different body lengths of the hermaphrodites, males and F1 juvenile stages are summarised in Table 5.1 below. Measurements of the different life stages were taken until 7 days post IJ inoculation, as F1 and F2 generations could not be distinguished from each other (Hirao *et al.*, 2010). The body length of the different larval stages of *H. zealandica* were all significantly different from each other, while the adult male and female were easily distinguishable by means of their distinctive reproductive organs. The mean length of the IJ was found to be 635 µm and ranged between 589 µm and 731 µm.

**Table 5.1.** Body length for hermaphrodites, males, J1, J2, IJ, J3 and J4 (mean ± standard error and range) (one way ANOVA; $F_{(5, 95}$ = 265.93; ρ = < 0,0001).

| Nematode stage | *n* | Body length (µm) | Significance symbols* |
|---|---|---|---|
| Hermaphrodites | 20 | 2240 ± 100 (1343 – 2836) | n/a |
| Males | 20 | 936 ± 12 (849 – 1029) | n/a |
| J1 | 20 | 209 ± 10 (152 – 310) | a |
| J2 | 20 | 482 ± 11 (376 – 531) | b |
| IJ | 20 | 635 ± 10 (589 – 731) | c |
| J3 | 20 | 611 ± 12 (532 – 694) | cd |
| J4 | 20 | 878 ± 13 (782 - 990) | e |

***Different letters indicate significant difference.

*Bacteria growth curve*

Analyses of data obtained for the cell densities and optical densities showed no significant differences between test dates (the mean cell density without nematodes: $\rho$ = 0.6; the mean optical density: $\rho$ = 0.5; the mean cell density with nematodes: $\rho$ = 0.5) and results from the two test dates were thus pooled. The lag-phase of the *Photorhabdus* production was observed to be from 0 to 12 h (Figs. 5.4., 5.5.). The exponential growth phase started after 12 h, and after 33 h the stationary phase, with an average of $23 \times 10^7$ per ml colony forming units, was reached (Fig 5.4).

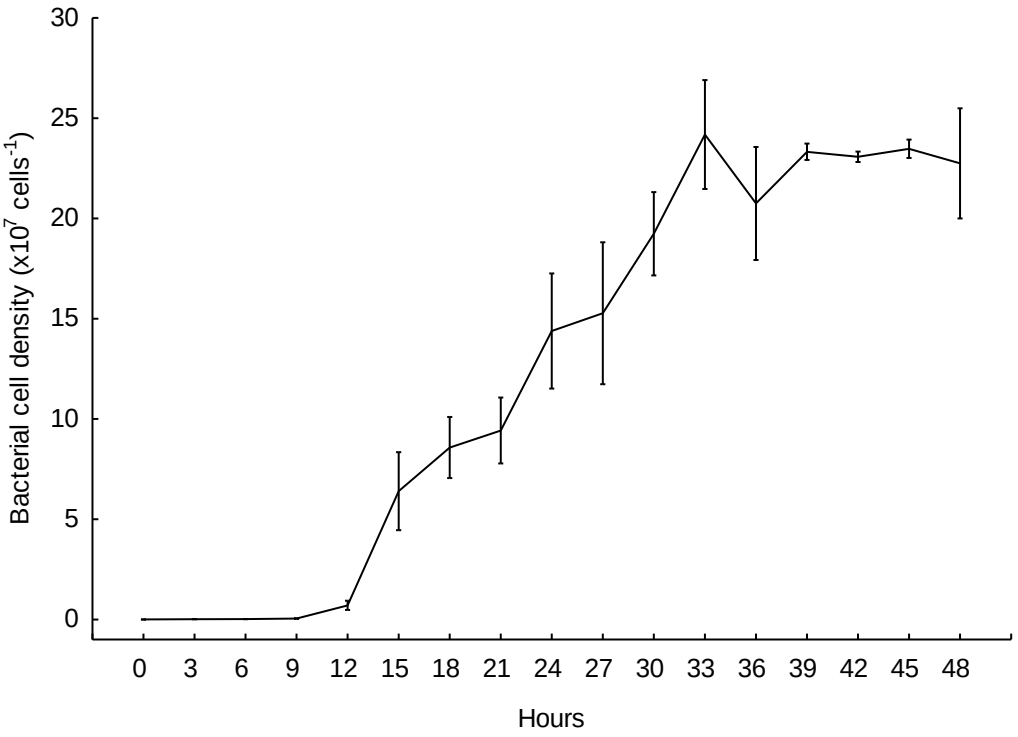


**Fig. 5.4.** The mean colony forming units per ml (95% confidence interval) of *Photorhabdus luminescens* in trypticase soy broth, over a period of 48 h, at 30˚C in the dark (one-way ANOVA; $F_{(16, 112)} = 168.4$).

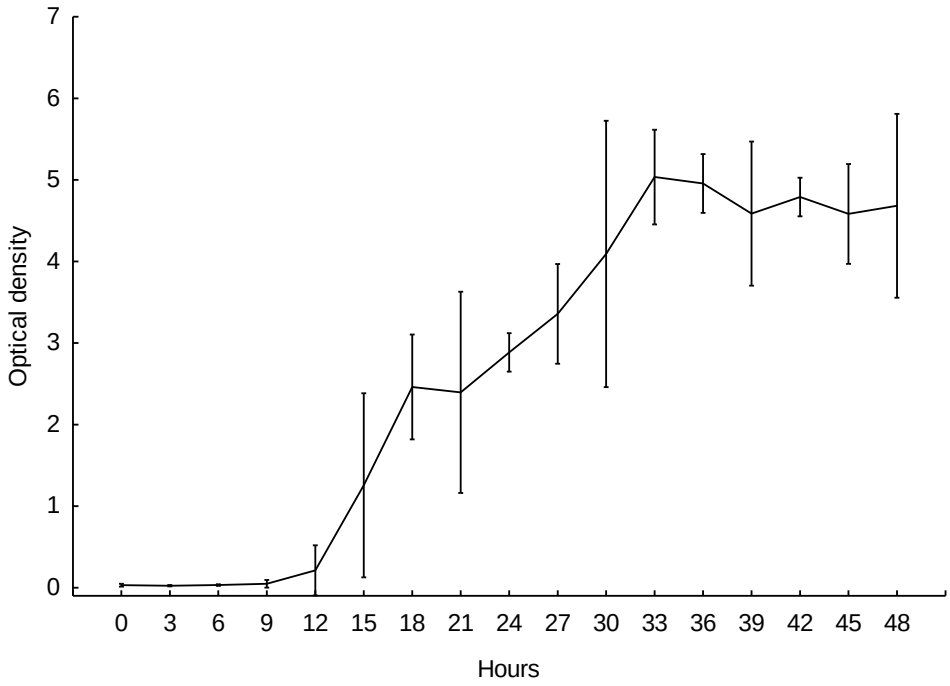


**Fig. 5.5.** The mean optical density (measured at 595 nm) (95% confidence interval) of *Photorhabdus luminescens* in trypticase soy broth, over a period of 48 h at 30˚C in the dark (one-way ANOVA; $F_{(16, 32)} = 132.5$).

.

A marked decrease occurred in bacterial cell density when nematodes were added to the liquid culture, as the IJs fed on the bacteria involved (Fig. 5.6.). The cell density was 23 × $10^7$·$ml^{-1}$, after the bacteria had been grown for 33 h (Fig. 5.4). When nematodes were added, the cell density decreased substantially, until day 6 post IJ inoculation (Fig. 5.6.). After reaching its minimum amount on day 6, the bacterial cell density increased until day 12. After day 12, the cell density started to decrease again, until day 16, when the experiment was terminated (Fig. 5.6.).

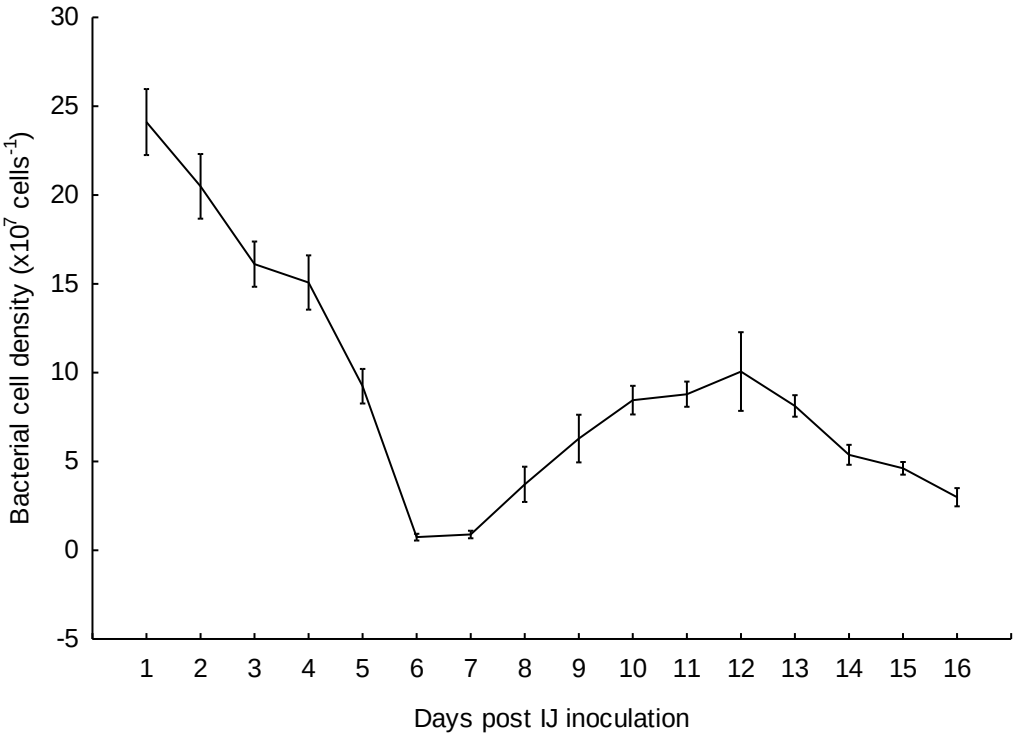


**Fig. 5.6.** The mean colony forming units per ml (95% confidence interval) of *Photorhabdus luminescens* in liquid culture over a period of 16 days, post IJ inoculation at 25˚C (one-way ANOVA; $F_{(15, 105)}$ = 184.2).

*Bioassays for virulence*

A high percentage mortality of *G. mellonella* was obtained for both *in vitro*- (80% ± 5.53%) and *in vivo*- (90% ± 5.55%) produced IJ of *H. zealandica* (Fig. 5.7.), with *in vivo*-produced nematodes causing higher percentage mortality (one-way ANOVA; $F_{(1, 68)}$ = 28.26; $\rho$ < 0.0001).

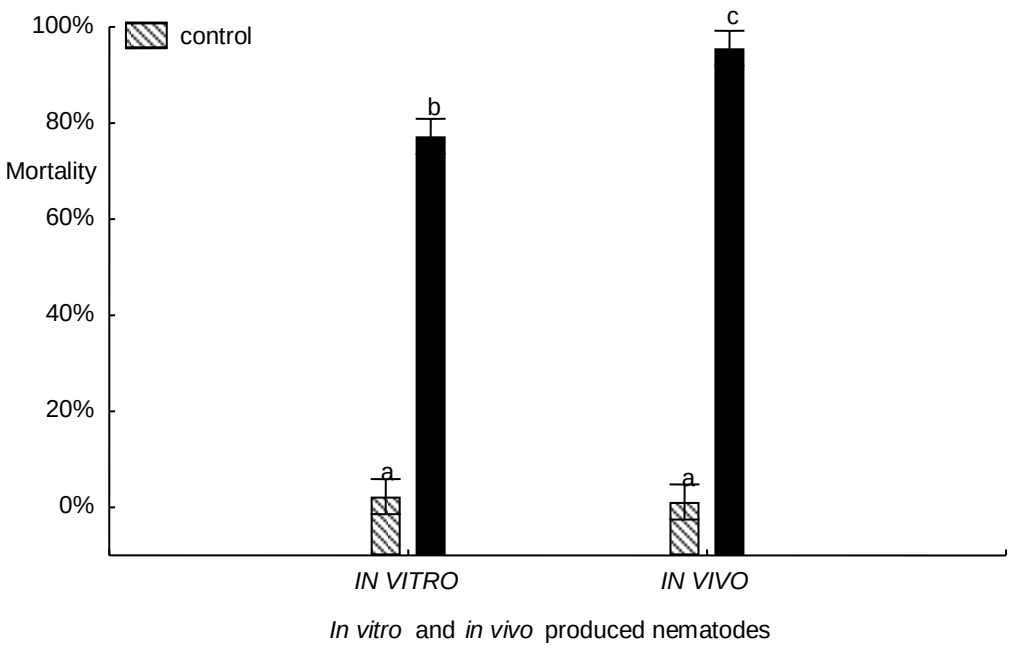


**Fig. 5.7.** The mean percentage mortality (95% confidence interval) after two days of *Galleria mellonella* larvae inoculated with 200 IJs/insect of *Heterorhabditis zealandica* (one-way ANOVA; $F_{(1, 68)} = 28.26$; $\rho < 0{,}0001$). Black bars depict mortality. Different letters indicate significant difference.

## Discussion

The bacterium associated with *H. zealandica* in this study was identified as *P. luminescens*, of an unknown subspecies (Chapter 4). In previous studies, the associated bacterium of *H. zealandica* was described as *Photorhabdus temperata*. This is the first study to show that *H. zealandica* associates with a different symbiotic bacteria rather than the known *P. temperata* association. Limited research has been undertaken with *H. zealandica*, as the distribution of said nematode is not widespread. *Heterorhabditis zealandica* has been isolated in New Zealand (Barker & Barker, 1998), and is currently also available commercially in Australia for the control of a number of turf and pasture pests.

The results obtained during this study indicate the first successful mass culture of *H. zealandica* in liquid medium. In this study, a low percentage recovery of the inoculated IJ was experienced, which

caused lower hermaphrodite numbers, and subsequently had an impact on the total number of offspring in the F1 generation. Recovery was found to be only 12% on day 3, 13% on day 4, and 24% on day 5. This low percentage recovery is comparable to that which was achieved in the research done by Ehlers *et al*. (1998) for *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, who found 16% recovery on day 7 and 15% recovery on day 8, with an increase to 23% on day 13.

Research undertaken by Strauch and Ehlers (1998) showed that the bacteria produce a food signal that induces IJ recovery in *Heterorhabditis* species, with the food signal being excreted through the culture medium. The bacterial food signal is much less efficient when compared to the food signal in an insect host, which, in said study, immediately induced the recovery of the IJs concerned (Strauch & Ehlers, 1998). This could partially explain the low percentage IJ recovery observed in this study. Another reason for low recovery could be ascribed to the lower bacterial cell density in the liquid medium during the IJ inoculation. *H. zealandica* IJs were inoculated when the bacterial symbiont reached the stationary phase of its growth curve, with a cell density of $23 \times 10^{7}$·cfu $ml^{-1}$. Research undertaken by Strauch and Ehlers (2000) showed that the bacterial symbiont of *H. megidis* was 1-5 $\times 10^{9}$·$ml^{-1}$ after only one day. The difference in bacterial cell density between the bacterial symbiont of *H. zealandica* and *H. megidis* might, to some degree, explain the low IJ recovery.

The method used in this study for determining cell density differed from that employed by Hirao and Ehlers (2010), who assessed bacterial cell density by counting cells in a Thoma chamber. For this study, a dilution series was made of each sample, and the number of colony-forming units (cfu) was determined by streaking out onto NBTA plates (Atlas, 1988; L. Dicks, personal communication, 2012). Bacteria were initially grown in TSB to determine general growth characteristics, including growth rate and optimum temperature, as no work had previously been done with the specific *P. luminescens* subspecies identified for *H. zealandica*. Thereafter, the bacteria were grown in the complex medium before inoculating the *H. zealandica* IJs. During both experiments, the cfu concerned were determined, with no difference being found between growing the bacteria in either TSB or the complex medium used. The use of the different methods, however, could explain the difference found in bacterial cell density between the results from this study, and the results from the study conducted by Hirao and Ehlers (2010). Research done by Jeffke *et al*. (2000) produced good results for analysing bacterial growth. Amongst other things, analysis of growth was determined by cell mass and cell dry weight. The lag-phase of *Photorhabdus* was observed to be overcome after 12

h in this study, compared to a lag phase of only 8 h (Jeffke *et al*., 2000) conducted with *P. luminescens*. The exponential growth phase of *P. luminescens* ended after 16 to 18 h (Jeffke *et al*., 2000), compared to the exponential phase that only ended after 33 h in this study. The methodology used by Jeffke *et al*. (2000) is likely more suitable for the analysis of bacterial growth and should be used in future research.

When IJs were inoculated into the liquid medium containing bacteria on day 1, the cell density of the *Photorhabdus* was $23 \times 10^{7} \cdot ml^{-1}$. The cell density decreased, until it reached its lowest level on day 6. This was as a result of the feeding of the inoculated IJs, and their recovery and development into hermaphrodites (Hirao & Ehlers, 2009b). After day 7, the cell density, however, increased again, with the increase preventing the starvation conditions necessary for inducing their development into IJs (Hirao & Ehlers, 2009b). These results were similar to the results obtained by Hirao and Ehlers (2009b), with whom an increase in cell density was found on days 8 and 12. In the current study, two peaks of cell density also occurred during the 16-day inoculation period, being found to take place on days 1 and 12. As a result of the cell density increasing again, the IJs that were present in the liquid culture recovered and developed into hermaphrodites (Ehlers *et al*., 1998). The increase of the bacterial cell density could be ascribed to the nematode density being too low for feeding sufficiently on the bacterium cells. The second peak of bacterial cell density coincided with the hermaphrodites also reaching their first peak around day 12. From day 12 to day 16, the bacterial cell density decreased, as the number of hermaphrodites and females increased. On day 16, the hermaphrodites reached a second peak of $9800 \cdot ml^{-1}$, originating from the F1-generation nematodes that had recovered. As a result of the high number of hermaphrodites present at this stage of the experiment, it was probably too early to terminate and harvest the IJs. The IJs recorded were offspring of the F1 hermaphrodites, thus representing the F2 generation (Ehlers *et al*., 1998). Such a two-generation process was also reported by Ehlers *et al*. (1998). In a two-generation process, the total number of offspring in the F1 generation is lower, due to a lower hermaphrodite density, as a result of the lower percentage of inoculum recovery attained (Ehlers *et al*., 1998). This has an effect on the ratio of amphimictic (male and female) to automictic (F1-hermaphrodite and IJ) nematodes.

Regarding the numbers of the larval stages, there should have been a peak in J1/J2 larvae, followed by a peak in J3/J4 larvae, and lastly followed by a peak in IJs. Theoretically every IJ would

have developed to a J1 and further on to J2, J3 and J4. This could be as a result of having lost J1-J4 stages during the sieving process.

The ratio of amphimictic to automictic nematodes is a key factor, as the amphimictic population is non-reproductive in liquid culture and should be kept low (Ehlers *et al.*, 1998). In the current study, the ratio of amphimictic to automictic nematodes was 1:12 on day 7, which is a more ideal ratio than the ratio of 1:6, which was found on day 15. The ratio of amphimictic (males and females) to automictic (F1-hermaphrodite and IJ) nematodes can have a severe impact on the final number of IJs harvested.

The sex ratio of the amphimictic population was calculated as 0.3 on day 7. On days 8 and 9, the ratio was 0.16, after which it increased to 0.8 on days 10, 11 and 12. Said results are similar to those of Johnigk and Ehlers (1999a), who had ratio between 0.7 and 0.8 until day 6, thereafter remaining constant at 0.6. Research undertaken by Strauch *et al*. (1994), however, found that their nematode sex ratio was almost 1:1. It is unusual to find an increase in females without any increase in males. This finding could be ascribed to hermaphrodites being misidentified as females from day 13 onwards.

Basic knowledge, such as that of the body length of various life stages of *H. zealandica*, is needed to monitor and to improve culture conditions for the successful production of *H. zealandica* in liquid. Therefore, in the current study, measurements were taken at each life stage. The data gleaned in this way are usable in estimating the population development and yields in commercial production, as well as in improving future culture conditions (Strauch & Ehlers, 2000).

When the virulence of IJs produced from the use of *in vivo* methods was compared to that of IJs produced through the use of *in vitro* methods, a significant difference was observed. During the current study, a concentration of 200 IJs/50µl as an inoculum was used, which is high for *G. mellonella* larvae, as they are very susceptible to EPNs. Even with the relatively high concentration, a significant difference was found between the *in vivo*- and *in vitro*-produced IJ. This could have been the result of *in vivo*- produced IJs containing increased amounts of lipids (Molyneux, 1985). The *in vitro*-produced nematodes appeared visibly more transparent, when compared to their *in vivo*-produced counterparts. This condition was generally associated with a reduction in the number of lipids, and is related to a reduction in pathogenicity against *Tenebrio molitor* L. (Coleoptera:

Tenebrionidae) larvae, according to Vanninen (1990). The *in vitro*-produced nematodes were also slightly shorter than those that were originally described as belonging to the species (Khuyen & Hunt, 2007). Research undertaken by Gaugler and Georgis (1991), in which *H. bacteriophora* Poinar, 1975, were mass cultured in liquid, achieved significantly lower mortality against the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeoidea), than those did that were reared *in vivo* (Gaugler & Georgis, 1991).

Lipids decline much more quickly in *H. bacteriophora* than they do in *S. feltiae* (Gaugler & Georgis, 1991). In the current study, a difference, though not significant, was found between the percentage mortality caused by *S. yirgalemense* compared to that caused by *H. zealandica* (Chapter 6) for *G. mellonella* larvae, where both nematode species were cultured *in vitro*.

The attempt to culture *H. zealandica* and its *Photorhabdus* symbiont was successful, even though two generations occurred, instead of the more desirable single generation. The only way, according to Ehlers *et al*. (1998), to achieve the latter is by increasing the inoculum density of the IJs. Since the IJs can only develop into hermaphrodites, the inoculum density is the key factor in the management of the population density. When there is a high concentration of J1 larvae in the F1 generation, the high density induces the majority of nematodes to develop directly into IJs, and not into amphimictic adults. This high IJ population then, subsequently, prevents the development of F1 hermaphrodites (Ehlers *et al.*, 1998). The difficulty is that IJ recovery is extremely variable, and the challenge for future research with *H. zealandica* would be to increase the percentage recovery of the IJs of *H. zealandica* in the liquid culture, as this would be a key factor in optimising their mass rearing.

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# CHAPTER 6

## Development and population dynamics of *Steinernema yirgalemense* and growth characteristics of its associated *Xenorhabdus* symbiont in liquid culture


### Abstract

Development of insect resistance to chemical insecticides has been a key driving force for changes in insect pest management. Entomopathogenic nematodes have become a valuable addition to the range of biological control agents that are available for such management, since they possess many of the attributes of an effective biocontrol agent. Therefore, the next logical step would be to mass culture a potential candidate, such as *Steinernema yirgalemense,* which has been found to be effective against numerous South Africa pest insects. The mass production of *S. yirgalemense* and the associated *Xenorhabdus* bacteria require the establishment of monoxenic cultures. Bacteria-free first-stage nematode juveniles from eggs were obtained through the alkaline lysis of gravid females, while the symbiotic bacteria of *S. yirgalemense* were isolated, cultured and identified, using molecular techniques. In this study, the population density of the various life stages of *S. yirgalemense* during the developmental phase in liquid culture was counted and measured. The recovery rate of IJs was 66%, with them reaching a maximum population density of 75 000·ml$^{-1}$ on day 13. There was an increase in adult density after 8 days, with the maximum female density being 4600·IJ ml$^{-1}$ on day 15, whereas the maximum male density was 4300·ml$^{-1}$ on day 12. Results from this study indicate an asynchronous nematode development in the first generation. Growth curves performed with the symbiotic bacteria showed that the exponential phase of *Xenorhabdus* started after 15 h and after 42 h the stationary phase was reached, with an average of 51 × 10$^{7}$ cfu·ml$^{-1}$. Bioassays were performed to compare the virulence between *in vitro*- and *in vivo*-produced nematodes. The results showed the *in vitro*-produced nematodes to be significantly less virulent than were the nematodes produced *in vivo*. The success obtained with the production of *S. yirgalemense* in liquid culture can serve as the first step in the optimising and upscaling of the commercial production of nematodes in industrial fermenters.

## Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* of the order Rhabditida in the families Steinernematidae and Heterorhabditidae respectively, are used to control a wide range of pest insects (Grewal *et al*., 2005), as they have been proven to be safe and effective biological control agents (Ehlers & Hokkanen, 1996). The control of insects is based on the interactions between the nematodes and their symbiotic bacteria (Poinar & Thomas, 1966; Poinar *et al*., 1977). The association is an obligate mutualism, in which each (nematode and symbiotic bacteria) requires the other to proliferate (Poinar & Thomas, 1966; Poinar *et al*., 1997). The humoral and cellular defences of the insect hosts are defeated by the bacteria (Han & Ehlers, 1998). Said bacteria protect the insect cadaver against saprophytic micro-organisms, bacteriovorous nematodes, and scavenging insects, as well as acting as a substrate for growth and reproduction (Han & Ehlers, 1998).

The bacteria are delivered into the insect haemocoel through the nematode that serves as the vector. The nematode also enables the bacteria to persist outside an insect host, and to be transferred to a new insect host (Poinar & Thomas, 1966; Poinar *et al*., 1997; Han & Ehlers, 1998). When nematodes lack the symbiotic bacteria association, they usually fail to cause insect death, or, if mortality takes place, the reproduction of nematodes does not occur (Poinar & Thomas, 1966; Poinar *et al*., 1977; Han & Ehlers, 1998). The bacterial symbionts can be isolated from the nematodes, and separately cultivated.

The bacterial symbionts have the ability to persist for numerous weeks within the free-living IJs, until the bacteria are again released within the insect haemocoel (Poinar, 1966; Wouts, 1984; Ciche & Ensign, 2003; Martens et al., 2004). Symbiotic bacterial cells are stored in the intestine of the IJ, and, during the recovery stage of the infective juvenile, such cells are released (Han & Ehlers, 2000). When IJ recovery takes place, the IJ develop from the arrested stage to the J4 stage (Strauch & Ehlers, 1998; Hirao & Ehlers, 2009a). The recovered juveniles develop into adults through feeding on bacterial cells and degraded host tissue. When the nutritious conditions are optimal, the nematodes produce offspring, which develop into another reproductive adult generation. In steinernematids, such adults are amphimictic, which enables them to copulate in liquid conditions. When depletion of nutrients and bacteria occurs, termination of egg-laying by the adults coincides with it. Instead, an

occurrence that is known as 'endotokia matricida' (Johnigk & Ehlers, 1999) takes place, whereby the first larval stages (J1s) hatch from eggs inside the uterus and develop into IJs. The IJs leave the insect cadaver when food resources are depleting (Strauch *et al*., 1994). The IJ is adapted to be durable and to survive in the soil environment (Susurluk & Ehlers, 2008), until it can find a suitable insect host (Lewis, 2002; Torr *et al*., 2004).

In Europe and the USA, the first steps towards an outdoor commercial biopesticide application have been taken using EPNs. Various EPN formulations against a wide range of insect pests are currently commercially available in both Europe and the USA. However, for the quantities of EPNs needed for commercial field application against pest insects, they need to be mass cultured, using *in vitro* liquid culturing techniques (Ehlers, 2001). In South Africa, endemic nematodes are not currently available as a product on the market. Therefore, *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler and Adams, 2005, which is an endemic nematode, needs to be mass cultured for future commercialisation.

The liquid culture process is extremely vulnerable to contamination, as a result of the long process time and the even distribution of fluids and organisms required during the mixing in the bioreactor (Grewal *et al*., 2005). The presence of non-symbiotic micro-organisms will result in the reduction of nematode yields, and, since the liquid culture process can last up to three weeks, it presents quite a challenge to maintain sterile conditions throughout. It is of importance that the monoxenicity of the cultures is ensured from the onset of inoculum production (Grewal *et al*., 2005). Symbiotic bacteria can be isolated from nematode-infected insect larvae, and stock cultures are obtained by mixing the bacteria with 15% (v/v) glycerol, and subsequently freezing it at -80˚C (Boemare & Akhurst, 1988). Establishing bacteria-free nematodes is more laborious (Lunau *et al*., 1993; Han & Ehlers, 1998). Monoxenic cultures can be stored at 4˚C on an orbital shaker at 20 rpm for several months. Liquid nitrogen can also be used for storing nematode strain collections (Popiel & Vasquez, 1991).

In South Africa, *S. yirgalemense* showed promise as biological control agents in laboratory bioassays against false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), larvae, pupae and emerging moths (Malan *et al.*, 2011). De Waal *et al*. (2011) also found *S. yirgalemense* effective in controlling *Cydia pomonella* L. (codling moth) (Lepidoptera: Tortricidae),

whereas Van Niekerk and Malan (2012) found *S. yirgalemense* to be more potent than was *Heterorhabditis zealandica* Poinar, 1990 in laboratory bioassays

*Steinernema yirgalemense* was found in a single sample in the Mpumalanga province during a survey for EPNs in citrus orchards (Malan *et al*., 2011). This nematode was first described by Nguyen *et al*. (2005) from Yiglemen in Ethiopia, where it was found to be the dominant species, with it later also being reported from the Central Rift Valley of Kenya (Mekete *et al*., 2005; Mwaniki *et al*., 2008). The nematode in question belongs to the *bicornutum* group of six described species, of which the unsheathed IJ has two hornlike structures in the cephalic region (Nguyen *et al*., 2004). Currently, the nematode has not been reported outside of the African continent.

The aim of the current study was to mass culture *S. yirgalemense*, using liquid culture technology. The population development of *S. yirgalamense* in liquid culture was monitored, and the growth characteristics of the associated symbiotic *Xenorhabdus* bacteria were described.

## Materials and methods

*Source of insects and nematodes*

*Galleria mellonella* (L.) (Lepidoptera: Pyralidae) larvae, were reared on a diet containing the following ingredients: five parts brown bread flour; five parts baby Cerelac Nestle™ regular cereal; two parts wheatgerm; two parts yeast; two parts glycerine; and one part honey. All the ingredients were mixed together, with a beeswax comb being added to the mixture (Bronskill, 1961; Woodring & Kaya, 1988).

Nematodes were obtained from the Department of Conservation Ecology and Entomology, Stellenbosch University collection of EPNs from previous surveys (Malan *et al*., 2011). *In vivo*-produced IJs were reared and harvested at room temperature, according to the procedures devised by Kaya and Stock (1997). The IJs were stored in vented 500 ml culture flasks placed horizontally at 14˚C in filtered tap water. The flasks containing 150 ml nematode suspension per flask were shaken weekly for aeration. Nematodes were used within one month of harvesting.

*Isolation of symbiotic bacteria*

The primary form of the associated symbiotic bacteria was isolated from last-instar *G. mellonella* larvae infected with *S. yirgalemense*. Agar plates (9.5 cm diameter petri dishes) were incubated at 25-28˚C for 48 h (Akhurst, 1980; Kaya & Stock, 1997), propagated in trypticase soy broth (TSB), and stored in 15% glycerol at -80˚C. When required, the glycerol stocks were melted at room temperature, propagated in TSB for 2 days at 30˚C, and subsequently used (Chapter 5).

*Identification of associated symbiotic bacteria*

The bacterium was identified by isolating the total genomic DNA of an overnight (12 h) culture of the bacterium, isolated from *S. yirgalemense*-infected *G. mellonella* larvae (Chapter 5). DNA of the 16S rRNA gene was amplified, as described by Felske *et al.* (1997). PCR reactions were done, as described by Tailliez *et al*. (2010).

*Axenisation of nematodes*

To obtain nematode eggs, last instar *G. mellonella* larvae were infected with IJs of *S. yirgalemense*. Infected *Galleria* larvae were dissected 4 days after inoculation, and gravid nematode females were isolated. The liquid medium (complex medium) in which the nematodes were propagated consisted of 15.0 $g \cdot l^{-1}$ yeast extract (Merck), 20.0 $g \cdot l^{-1}$ soy powder (Nature's Choice, Meyerton, South Africa), 4 $g \cdot l^{-1}$ NaCl, 0.35 $g \cdot l^{-1}$ KCl, 0.15 $g \cdot l^{-1}$ $CaCl_2$, 0.1 $g \cdot l^{-1}$ $MgSO_4$ (Merck), and 46 $ml \cdot l^{-1}$ of vegetable oil (Ehlers *et al.*, 1998). The 250 ml Erlenmeyer flasks containing 30 ml of nematode culture medium were inoculated with 1% of the bacterial culture, and pre-cultured at 30˚C for 42 h before inoculating IJs from the monoxenic cultures. The flasks were incubated in a growth chamber at 25˚C for 15 days. Samples of 1 ml were taken under sterile conditions, and washed with Ringer's solution through a 25-µm sieve. Nematodes were counted using a stereo microscope (Leica MZ75), and the concentration was determined (Chapter 5).

*Monoxenic cultures*

In order to provide a homogenous bacterial inoculum, the symbiotic bacteria were produced in one batch culture and then distributed over three Erlenmeyer flasks (Hirao *et al*., 2010). When the bacterial pre-cultures had reached a cell density of $10^7$ $cfu \cdot ml^{-1}$, 300 µl was transferred to sterile 30-

ml Erlenmeyer flasks and incubated on a platform orbital shaker, at 180 rpm at 30˚C in the dark for 42 h. The IJs were subsequently inoculated into the liquid medium in 250 ml Erlenmeyer flasks at a density of 4000 IJs·ml$^{-1}$, and incubated on a platform orbital shaker at 180 rpm at 25˚C in the dark for 15 days. IJs were taken from monoxenic pre-cultures (Lunau *et al*., 1993). Three flask cultures were used and the experiment was conducted on two different test dates (Chapter 5).

*Assessment of developmental stages*

Samples of 1 ml each containing the nematodes, of the three flasks were taken every day for 15 days. Nematodes were washed with Ringer's solution through a 25-µm sieve to determine the population development in liquid culture. The different juvenile stages, which included from the first juvenile stage (J1) through to the fourth stage (J4) and the adults (male and female), were identified and counted (Hirao *et al*., 2010). The presence of the vulva and uterus was used to identify the females, with the spicules being used to identify the males (Hirao *et al*., 2010). The difference between the J1s, J2s and J3s was determined by measuring the body length of each juvenile stage. IJs were grouped and counted together with pre-infective juveniles (J2d), which were identified by their body being much darker than that of other juvenile stages (Hirao *et al.*, 2010). Recovery was calculated by counting inoculated IJs that recovered and developed beyond the IJ stage on the second day post IJ inoculation, which included the J4 larval stages, as well as males and females (Hirao *et al*., 2010).

Nematodes used for measurement were fixed in hot TAF (2% tri-ethanolamine and 8% formalin) (85˚C) (Courtney *et al*., 1995), and processed to pure glycerine, using the Seinhorst (1959) method, after which they were mounted in glycerine, using wax ring supports to prevent flattening. Measurements were taken by means of a Leica DM2000 research microscope that was equipped with a camera, computer and digital image software Leica Application Suite (LAS), ver. 3.5.0 (Chapter 5).

*Bacterial growth curve*

A bacterial growth curve was determined in order to investigate the cell density dynamics of the bacterial symbiont over a period of 48 h. This experiment was conducted at a temperature of 30˚C and using TSB as a medium. A sample, containing 40 µl of the bacteria stock solution, was inoculated into 10 ml of TSB, and incubated for 2 days in the dark at 30˚C. Then, 400 µl of this solution was

inoculated into 400 ml of TSB, with a sample of 1 ml being taken every 3 h for 48 h. Each sample was streaked out in triplicate and replicated twice, using NBTA plates. Plates were incubated for 2 days, and colony forming units were counted (Atlas, 1988), with the optical density also being measured every 3 h (Chapter 5).

Bacterial colony forming units (cfu's) were determined once the IJs were inoculated into the liquid (Hirao & Ehlers, 2009b; Hirao *et al.*, 2010). Samples were taken every day for 15 days, streaked out in quadruplicate, and replicated twice, using NBTA plates. Plates were incubated at 30˚C in the dark for 2 days. Colonies were subsequently counted (R-U. Ehlers, personal communication, 2012) (Chapter 5).

*Virulence studies*

Virulence bioassays were conducted after IJs were harvested from flasks. *Galleria mellonella* were infected with *in vitro*-produced *S. yirgalemense* and compared with *S. yirgalemense* IJs harvested from *G. mellonella*-cultured nematodes (*in vivo*). The *in vitro*-produced IJs were harvested by washing them with Ringer's solution through a 25-µm sieve (Chapter 5).

The *G. mellonella* larvae were placed in 24-well plates (flat bottom, Nunc$^{TM}$, Cat. No. 144530) which contained 10 filter paper discs (13-mm diameter) placed in 10 of the 24 wells. A concentration of 200 IJ·50 µl$^{-1}$ was inoculated onto the filter paper discs, and one *G. mellonella* larva was placed in each of the 10 wells. The plates were incubated at 25˚C in the darkness. Insect mortality was assessed after 2 days, and infection was confirmed by dissection in Ringer's solution (Chapter 5).

*Statistical analysis*

The results were analysed by means of STATISTICA 10 (Statsoft Inc., T.O.U., 2011), using descriptive statistics. For the measurement of different nematode life stages and virulence studies, a one-way ANOVA with post-hoc comparisons of means was used. Bonferroni's method was used where the residuals were not normally distributed. Data obtained from the counting of different life stages and bacterial growth curves were analysed using a factorial ANOVA with post-hoc comparisons of means, using the Tukey's HSD test.

## Results

*Identification of the symbiotic bacteria*

The 16 rDNA sequence indicated that the bacterial symbiont associated with *S. yirgalemense* belongs to the genus *Xenorhabdus*. The phylogenetic analysis of the 16S rDNA gene sequence, with other known *Xenorhabdus* spp. sequences from Genbank, indicate the species to share a common ancestor with *X. indica* Somvanshi, Lang, Ganguly, Swiderski, Sazena & Sackebrandt, 2006 and *X. cabanillasii* Tailliez, Pagés, Ginibre & Boemare, 2006. Based on the phylogenetic relationship concerned, the bacterial symbiont associated with *S. yirgalemense* is regarded as an unknown and new species of *Xenorhabdus*.

*Population dynamics of* S. yirgalemense

Analyses of data obtained for the life cycle development on each day (1 to 15) showed no significant differences between the two test dates (males: $\rho$ = 0.12; females: $\rho$ = 0.17; *endotokia matricida* females: $\rho$ = 0.99; J1: $\rho$ = 0.98; J2: $\rho$ = 0.57; IJs: $\rho$ = 0.79; pre-adult: $\rho$ = 0.51), therefore, for the rest of the analyses, data of the different test dates were pooled. The population development for *S. yirgalemense* at 25˚C from the inoculation of IJs into adults in monoxenic liquid culture is presented in Figures 6.1, 6.2 and 6.3.

The first male and females occurred on day 2. The highest concentration of males was recorded on day 12, and the highest concentration of females on days 14 and 15 (Fig. 6.1). During the process time (15 days) concerned, the sex ratio was biased towards the females from day two to day four. From days 5-9, and then on days 12 and 13, the sex ratio was in favour of the males. On days 14 and 15, the female numbers exceeded the male numbers.

The second and third adult (males and females) generations of *S. yirgalemense* were observed and increased from day 7 onwards (Fig. 6.1), with the maximum density being observed on day 13. The pre-adult juvenile stages (J3 and J4) reached their highest concentration on day 14. *Endotokia matricida* female numbers were very low throughout the 15 days, except for an increase that took place on day 4 and then again on day 7 (Fig. 6.1). Newly hatched J1 and J2 offspring occurred simultaneously on day 4 (Fig. 6.2). The J2 concentration was low, except for a slight

increase that occurred on day 5 (Fig. 6.2). The highest concentration of J2 larvae was observed 5 days after the inoculation of the IJs.

The recovery of the IJs was observed as 67% (Fig. 6.1) on the second day post IJ inoculation and newly formed IJs were observed on day 7 (Fig. 6.3). The first offspring of the second generation occurred on day 7. The highest concentration of IJs and pre-infective juveniles occurred from day 13 onwards (Fig. 6.3).

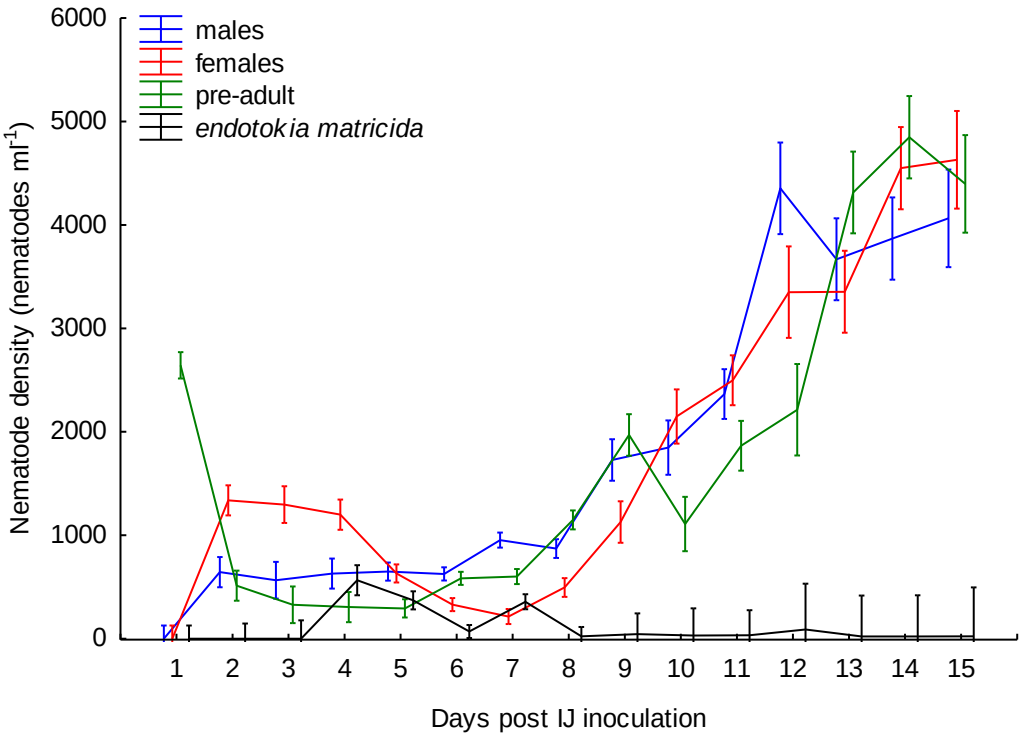


**Fig. 6.1.** Mean population density (95% confidence interval) of *Steinernema yirgalemense* in monoxenic liquid culture at 25˚C, over a period of 15 days. The density of males, females, pre-adult stage juveniles (J3 and J4 stages) and *endotokia matricida* females is indicated (one-way ANOVA; $F_{(42, 504)} = 52.12$).

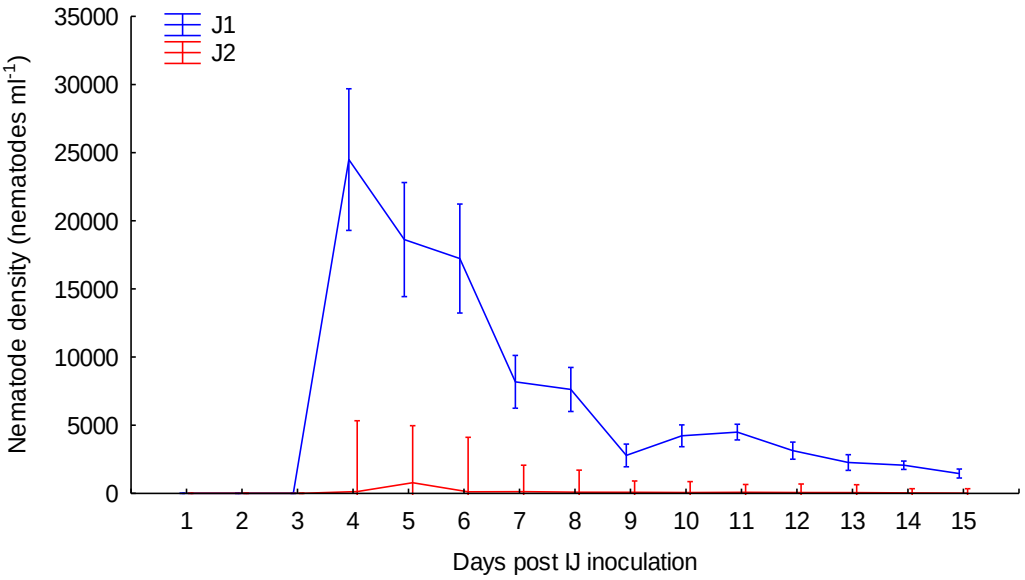


**Fig. 6.2.** Mean population density (95% confidence interval) of J1 and J2 stages of *Steinernema yirgalemense*, in monoxenic liquid culture at 25˚C, over a period of 15 days (one-way ANOVA; $F_{(14, 252)}$ = 26.89).

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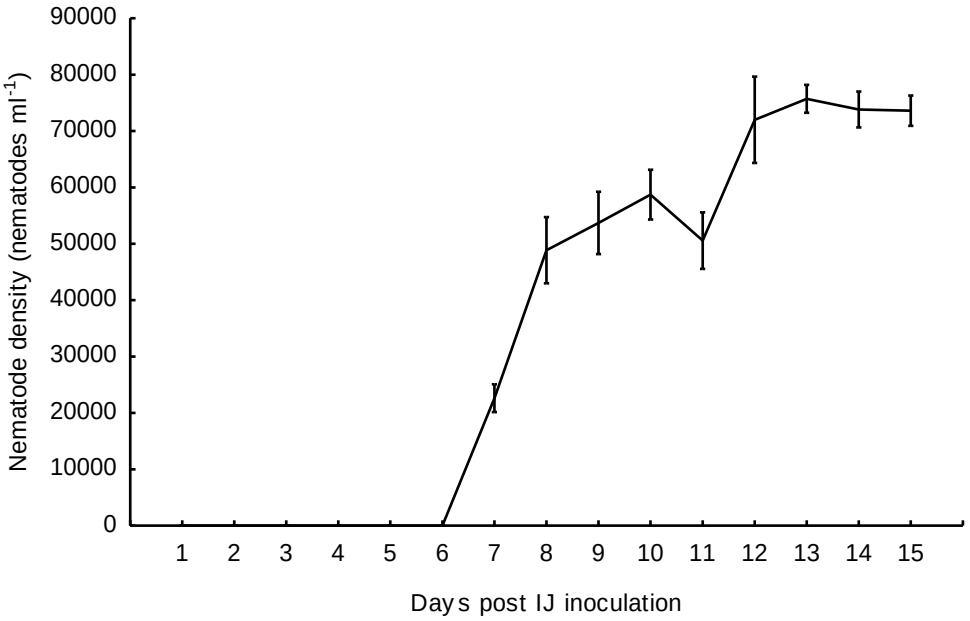


**Fig. 6.3**. Mean population density (95% confidence interval) of infective juveniles (IJ) and pre-infective juveniles (J2d) of *Steinernema yirgalemense*, in monoxenic liquid culture at 25˚C, over a period of 15 days (one-way ANOVA; $F_{(14, 126)}$ = 411.12).

The body lengths of the first generation adults and juveniles are summarised in Table 6.1 below. Differences in lengths were observed between the IJ, J1, J2, J3 and J4 stages. Measurements

of the different life stages were undertaken until 7 days post IJ inoculation, as the F1 and F2 generations could not be distinguished from each other after 7 days (Hirao *et al.*, 2010).

**Table 6.1.** Body length for females, males, IJ, J1, J2, J3 and J4 (pre-adults) (mean ± standard error and range) (one-way ANOVA; $F_{(5, 95)} = 362.09$; $\rho = < 0{,}0001$).

| Nematode stage | *n* | Body length (µm) | Significance symbols* |
|---|---|---|---|
| Females | 20 | 1987 ± 65 (1424 – 2666) | n/a |
| Males | 20 | 877 ± 18 (671 – 995) | n/a |
| J1 | 20 | 320 ± 12 (213 – 387) | a |
| J2 | 20 | 519 ± 8 (432 – 557) | b |
| IJ | 20 | 635 ± 10 (549 – 682) | b |
| J3 | 20 | 670 ± 15 (531 – 773) | c |
| J4 | 20 | 835 ± 18 (701 – 990) | e |

***Different letters indicate significant difference.

The body length of the different life stages of *S. yirgalemense* varied significantly from one another, except for the IJs that were not significantly different from the J2 larvae in length.

*Bacteria growth curve*

Analyses of data obtained for the cell densities and optical densities showed no significant differences between test dates (mean cell density without nematodes: $\rho = 0.54$; mean optical density: $\rho = 0.9$; mean cell density with nematodes: $\rho = 0.19$), and the results from the two test dates were pooled. The colony forming units of *Xenorhabdus*, measured every three h up to 48 h at 30°C , are indicated in Figure 6.4, and the optical density in Figure 6.5. The lag phase of the *Xenorhabdus* was recorded as lasting from 0 to 15 h (Fig. 6.4). The exponential phase started after 15 h (Figs. 6.4) and, after 42 h, the stationary phase was reached, with an average of $51 \times 10^7$ cfu $ml^{-1}$ (Fig. 6.4).

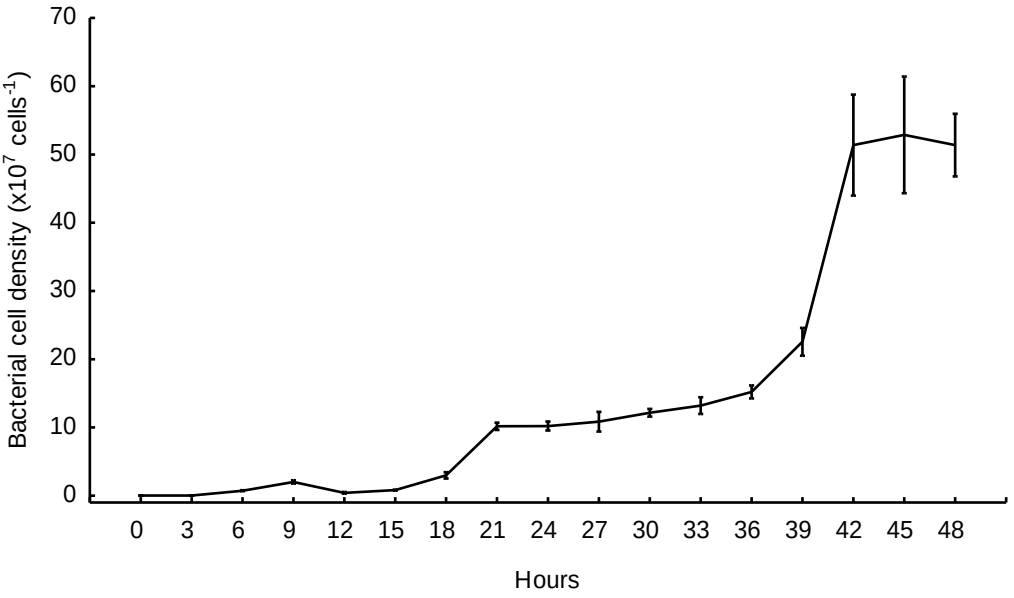


**Fig. 6.4**. The colony forming units·ml$^{-1}$ (95% confidence interval) of *Xenorhabdus* sp. in trypticase soy broth, over a period of 48 h, at 30˚C in the dark (one-way ANOVA; $F_{(16, 112)} = 206.7$).

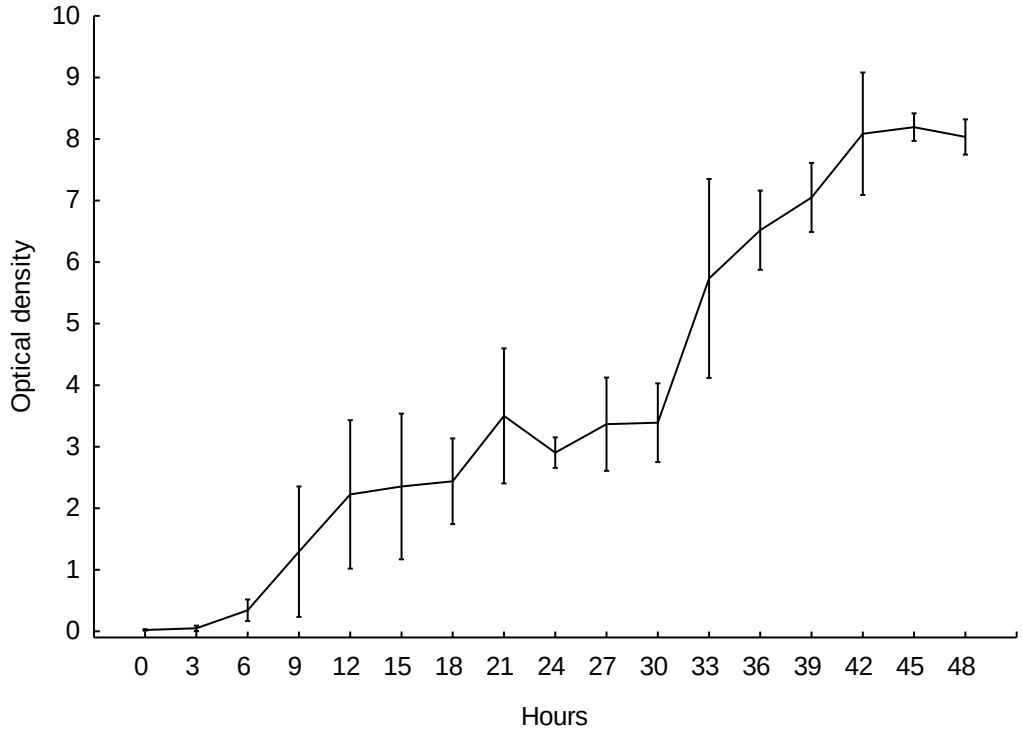


**Fig. 6.5**. The optical density (measured at 595 nm) (95% confidence interval) of *Xenorhabdus* sp. in Luria broth, over a period of 48 h, at 30˚C in the dark (one-way ANOVA; $F_{(16, 32)} = 248.1$).

The colony forming units of *Xenorhabdus*, measured over a period of 15 days, at 25°C, after inoculation of the IJs, is indicated in Figure 6.6. A marked reduction in bacterial cell density was observed until day 5 after the nematodes were added. This can be ascribed to the recovery and feeding of the nematodes on the bacteria, causing the cell density to decrease dramatically. After the bacteria had been grown for 42 h, the cell density was 50 × $10^{7}$·$ml^{-1}$. From day 5, the bacterial cell density increased until day 9. On day 10, a slight decrease in bacterial cell density was observed, after which the cell counts seemed to remain constant until day 15 (Fig. 6.6).

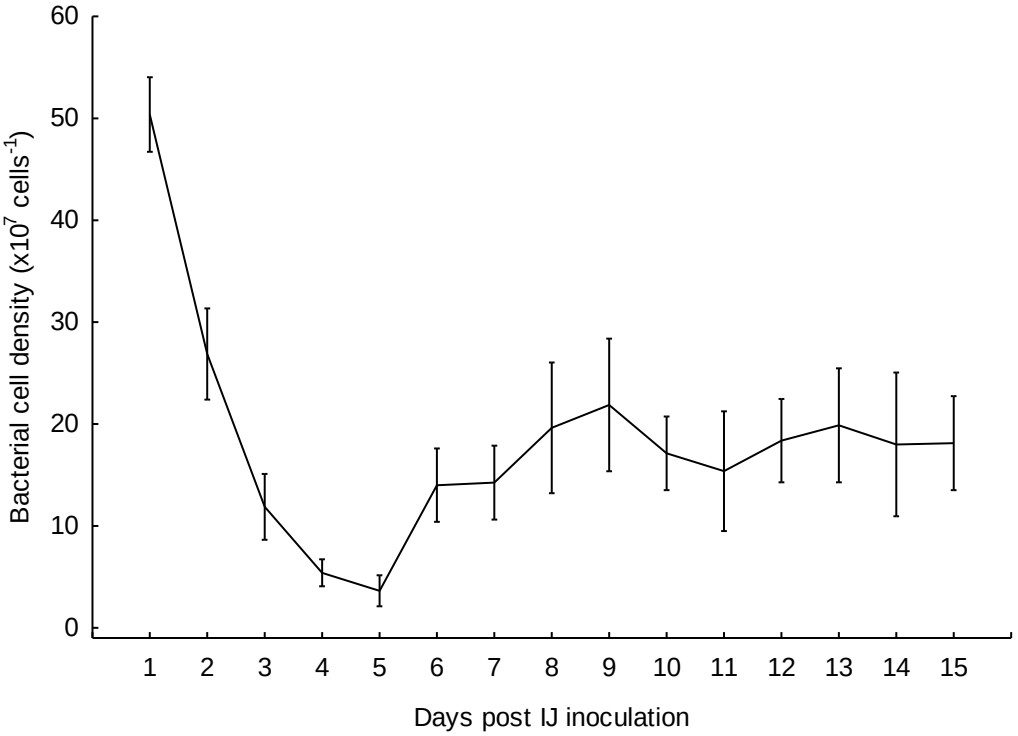


**Fig. 6.6.** The colony forming units (95% confidence interval) $ml^{-1}$ of *Xenorhabdus* sp. in liquid culture over a period of 15 days, at 25˚C (one-way ANOVA; $F_{(14, 98)}$ = 29.7).

*Virulence studies*

A high percentage mortality rate of *G. mellonella* larvae was obtained for both *in vitro*- and *in vivo*-produced IJs of S. *yirgalemense* (Fig. 6.7). However, the *in vivo*-produced nematodes were found to cause a higher percentage mortality of *G. mellonella* larvae. The results were analysed by means of a one-way ANOVA ($F_{(1, 68)}$ = 41.783; $\rho$ = < 0.0001), indicating the percentage mortality to differ significantly between the *in vitro*- and *in vivo*-produced nematodes.

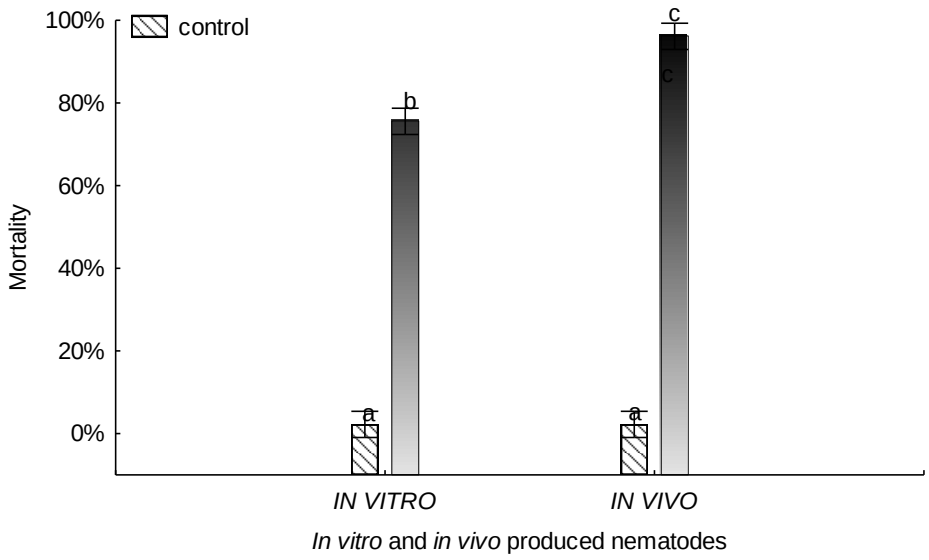


**Fig. 6.7**. The mean percentage mortality (95% confidence interval) of *Galleria mellonella* larvae two days after inoculation with 200 IJs/insect of *Steinernema yirgalemense* (one-way ANOVA: $F_{(1,68)}$ = 41.783; $\rho$ = < 0.0001). ). Black bars depict mortality. Different letters indicate significant difference.

## Discussion

For nematode population development in liquid culture to be successful, IJ recovery is the primary factor determining the number of adults in the parental generation, as it affects the size of the F1 generation (Hirao & Ehlers, 2010). The recovery of IJs varies unpredictably among cultures, making it difficult to adjust the number of nematodes feeding on the bacteria. As a consequence, food depletion and subsequent IJ formation does not always take place after a single generation. Likewise, a non-synchronous recovery also exposes early offspring to conditions of sufficient food, for the starting of another generation. Hence, maximum population density is sometimes reached only after a prolonged process time (Ehlers, 2001).

Results obtained by Hirao and Ehlers (2010) showed IJ recovery of > 90% for *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982. In the current study, the recovery rate of IJs was recorded as being 67%. Such a low percentage recovery rate for the IJs could be ascribed to the low bacterial cell density in the medium. In order to assist nematode development in liquid culture, the symbiotic bacteria were inoculated into the liquid medium at least one day prior to IJ inoculation. The volume of the bacterial culture inoculated was between 0.5 and 1% of the culture volume (Ehlers, 2001). The pre-cultured symbiotic bacteria triggered the recovery of the IJs by providing appropriate food signals (Aumann & Ehlers, 2001; Hirao & Ehlers, 2009a). Doing so was

essential, as artificial media do not tend to produce sufficient food signals to allow for IJ recovery (Strauch & Ehlers, 1998).

When *S. yirgalemense* IJs was inoculated, the cell density of *Xenorhabdus* was $5 \times 10^8$ cfu $\cdot ml^{-1}$. When *S. feltiae* IJs were inoculated, the cell density of *X. bovienii* (Akhurst, 1983) Akhurst & Boemare, 1993 was $10 \times 10^9 \cdot ml^{-1}$ (Hirao & Ehlers, 2010), which was substantially higher than that of the bacterial symbiont in the current study. Results from the research conducted by Hirao and Ehlers (2010) indicate that bacterial cell density is the key factor for IJ recovery, and not IJ inoculum density, as was previously thought.

The method used for determining cell density in the present study differed from the method used by Hirao and Ehlers (2010), who assessed bacterial cell density by counting cells in a Thoma chamber. In the current study, dilution series were made of each sample, and colony-forming units (cfu) were determined by streaking out onto NBTA plates (Atlas, 1988; L. Dicks, personal communication, 2012). Bacteria were initially grown in TSB to determine general growth characteristics, including their growth rate and optimum growth temperature, as no work with this specific *Xenorhabdus* symbiont of *S. yirgalemense* had yet been done. Thereafter, the bacteria were grown in the complex medium before it was inoculated with *S. yirgalemense* IJs. During both growth experiments, the cfu were determined, and no difference was found between the bacteria grown in either the TSB or the complex medium. The different methods used could explain the difference in bacterial cell density found between the results of the current study and that which was found in Hirao and Ehlers' (2010) study. Direct cell counts would probably, it was concluded, have been a better option for determining cell density, and should rather be used in future.

When asynchronous (when all IJs do not start developing simultaneously) recovery occurs in heterorhabditid cultures, the subsequent development of a second generation can cause a complete loss of the liquid culture process, due to the inability of F1 amphimicitic adults to copulate in liquid cultures (Strauch *et al.*, 1994). In comparison, *Steinernema* are generally less vulnerable to the low recovery rate of IJs, because the adults are able to copulate in liquid (Strauch *et al.*, 1994). In this study, IJ recovery continued to increase after newly hatched juveniles were observed, and no new generation IJs were observed four days after IJ inoculation. This indicates an asynchronous development in the first generation. Hirao and Ehlers' (2010) research showed that the optimum inoculum density for *S. carpocapsae* (Weiser, 1955) Wouts, Mracek, Gerdin & Bedding, 1982 was

between 3-6 × $10^3$ IJs·$ml^{-1}$, which would result in 1-3 × $10^3$·$ml^{-1}$ parental females being required for successful production. For *S. feltiae*, the optimum inoculum density is > 5 × $10^3$ IJs·$ml^{-1}$, which would result in > 2 × $10^3$ parental females per ml (Hirao & Ehlers, 2010). In the current study, the inoculum density for *S. yirgalemense* was 4 × $10^3$ IJs·$ml^{-1}$, which resulted in a parental female density of only 1 × $10^3$·$ml^{-1}$ on day 9, and an increased density of 4 × $10^3$·$ml^{-1}$ on day 15. The increase in adult density after day 8 indicates that the progeny of the late recovered IJs, as well as that of the second and third generations, were induced to develop into adults, instead of into IJs. The cell density of *Xenorhabdus* reached the lowest density on day 5, after which the cell density increased again. The increase prevented the starvation conditions necessary for inducing development into IJs, rather than into adults. The cell density reached two peaks, one of which was on day 1 and one on day 9, during the 15-day developmental period. Control of the bacterial cell density is critical at the moment at which the progeny of the F1 generation hatches, as achieving high numbers of IJs within a short process time is required.

In the current study, the sex ratio was not constantly biased towards the females, as was recorded in previous research done on *Steinernema* (Selvan *et al*., 1993; Alsaiyah *et al*., 2009; Hirao & Ehlers, 2010). The ratio concerned varied between the different days of the study, and whether this was caused by the low initial recovery of the IJs, the low bacterial cell density, or both, still requires investigation. Inoculum density has a strong effect on the fertility of the parental females (Hirao & Ehlers, 2010), and, in the present study, the bacterial concentration was relatively constant from day nine onwards. Therefore, fewer nutritional resources were available for the females, with increasing densities (Hirao & Ehlers, 2010). This was shown to have the same effect on *S. carpocapsae* as it did on *S. feltiae* parental females in the research that was undertaken by Hirao and Ehlers (2010). Reproduction of *Steinernema* is amphimictic, with male and female copulation occurring, and with the females producing eggs. When food is abundant in the liquid culture, the offspring develop further into reproductive adults. In comparison, the response to depleting food resources tends to encourage the development of IJs (Strauch *et al.*, 1994).

The development of a large number of offspring into adult males and females might cause problems when downstreaming of the process is required. As all non-IJs rapidly died off during formulation, the amount of contaminants increased, resulting in the nematode product being spoiled. Therefore, these stages should be removed from the IJ suspension before formulation. Second- and

third- generation adults should also be avoided, as the increase concerned is caused by an increase in the bacterial density. This results in switching to adult development, instead of IJ formation, as the attainment of a one-generation process is the objective, as was achieved for *S. carpocapsae* in the past (Hirao & Ehlers, 2010). Results from this study suggest that the occurrence of second- and third-generation adults could be the rule for *S. yirgalemense*, and that the density of the IJ inoculum would then not be able to influence it.

Basic knowledge, such as that of the body length of various life stages of *S. yirgalemense*, was monitored to improve culture conditions for the successful production of *S. yirgalemense* in liquid. The data could be used to estimate the population development and yields in commercial production, and future improvements in culture conditions. Results from the current study showed, that in terms of body length, most of the stages were significantly different from one another. A few of the nematode life stages were, however, similar in size to one another, such as the J4 larval stage of the males and the J2 larval stage of the IJs. The J4 larval stage is the final larval stage before the nematodes concerned develop into males or females, and, therefore, at said stage of larval development, it is mainly the reproductive organs that are still developing. The J4 larval stages and the males were distinguished by the presence or absence of male genitals. In regard to the J2 larval stage and the IJs, the latter are a specialised J3 larval stage, which could explain the body length overlapping. When comparing measurements of body lengths of the nematodes in the current study with the measurements taken of nematodes by Nguyen *et al*. (2005), a difference is discernible in the body length of the first- generation females and males. This difference in body length can be ascribed to the different method of culturing used, namely *in vivo* compared to *in vitro*, and is similar to what Ehlers and Shapiro-Ilan (2005) found when observing the decreasing body length of hermaphrodites in flask cultures.

The virulence of IJs produced from employing *in vivo* methods was more effective compared to that of IJs produced from *in vitro* methods, with a significant difference being observed between the two. Similar results were obtained from research done by Gaugler and Georgis (1991), in which *H. bacteriophora* Poinar, 1976 produced by liquid culture achieved significantly lower mortality against the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeoidea), than those did that were reared *in vivo* (Gaugler and Georgis, 1991). A concentration of 200 IJ·50µl$^{-1}$ was used in this study, which was high for *G. mellonella* larvae, as they were already highly susceptible to

entomopathogenic nematodes. Even with the relatively high concentration, there was still a significant difference between the *in vivo*- and *in vitro*-produced IJs. Such differences could have been the result of the *in vivo*-produced IJs containing higher amounts of lipids (Molyneux, 1985). The nematodes that have been cultured *in vitro* appear to be visibly more transparent when compared to their *in vivo*-produced counterparts. This condition is generally associated with the reduced presence of lipids, and is related to a reduction in pathogenicity against *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae, according to Vanninen (1990). As the amount of time between production and application increases, the reduced pathogenicity also increases (Vanninen, 1990). This has important consequences for *S. yirgalemense,* as the desired end result is commercial production and application in the field.

The successful culturing of *S. yirgalemense in vitro* led to valuable information being gained in the current study. However, the liquid culture production of *S. yirgalemense* requires further investigation, especially regarding the production of IJs within a single generation. The bacterial growth should be further analysed, as the nutrients provided by the medium might not be fully exploited by the bacteria used. Improvement of the culture medium, as well as increasing the IJ inoculum, might result in an improved final number of IJs of *S. yirgalemense*, in a shorter process time.

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# CHAPTER 7

## Investigating the growth characteristics of *Xenorhabdus*, a bacterial symbiont associated with the entomopathogenic nematode *Steinernema yirgalemense*


### Abstract

In South Africa, *Steinernema yirgalemense* has shown potential as a biological control agent of insect pests in agriculture. For the commercial use of these nematodes for the key pests of fruit crops, they have to be cultured and formulated in massive numbers. When this species of nematodes is cultured in industrial fermenters, monoxenic liquid cultures have to be pre-incubated with its symbiont, *Xenorhabdus*, before infective juveniles (IJs) of *S. yirgalemense* are to be inoculated. This study is the first to investigate the growth characteristics of this unknown *Xenorhabdus* sp., associated with *S. yirgalemense*, in a 20-L fermenter. Parameters that can be used to determine accurately when stationary growth phase conditions occur are bacterial cell density and dissolved oxygen ($DO_2$). Bacterial cell density of $50 \times 10^7$ cells·ml$^{-1}$ was reached after 36 h, and the $DO_2$ rate started to increase after 24 h, reaching 60% after 36 h. The results showed the stationary phase of *Xenorhabdus* was reached after 36 h at 30˚C in the 20-L fermenter, which took 6 h less than did the same procedures followed with the *Xenorhabdus* sp. cultured in Erlenmeyer flasks on orbital shakers. This is the first step in the future liquid mass culture of *S. yirgalemense* in industrial-size fermenters, as data from this study can indicate the optimum time required before adding IJ to the bacterial culture in the fermenter. This will ensure the optimum recovery of IJs, and a subsequent high yield of nematodes within a minimum amount of processing time.


### Introduction

*Xenorhabdus* are motile gram-negative bacteria that belong to the family Enterobacteriaceae (Akhurst, 1980; Boemare & Akhurst, 1988; Boemare *et al*., 1993). *Xenorhabdus* bacteria are associated with EPNs from the family Steinernematidae, and are carried in the intestine of the infective juvenile (IJ) stage of the nematode (Poinar, 1990; Akhurst, 1993; Akhurst & Dunphy, 1993; Forst & Nealson, 1996). A diversity of aerial and soil insects can be controlled by EPNs, but the soil

stage of insects is naturally accessible to the free living IJ. In the soil, the IJs can locate the insect by secreted cues such as $CO_2$ left behind by the insect and, when the digestive track of the host is entered, they penetrate through the lining to the haemocoel (Poinar, 1990; Akhurst & Dunphy, 1993). Access to the haemocoel of the insect can also be gained through the spiracles (Poinar, 1990). The IJ release their symbiotic bacteria as soon as they enter the haemocoel of the insect host. The insect is rapidly killed off by the bacteria and nematode combination, although the bacteria are usually more virulent (Akhurst & Dunphy, 1993). The bacteria grow to stationary phase inside the haemocoel of the insect host, while, simultaneously, the IJ recover to start feeding, developing and reproducing sexually. EPN reproduction is most favourable when the natural symbiont dominates the microbial flora inside of the host. When the food supply in the insect is depleted, the bacteria and nematode reassociate, with the latter developing into the IJ stage, which is a nonfeeding, specially adapted, third larval stage. The bacteria are carried in the intestinal tract of the IJ, and subsequently emerge in the intestine of the IJ from the depleted insect carcass into the soil, in search of a new host (Akhurst, 1993; Kaya & Gaugler, 1993).

*Xenorhabdus* can be grown under standard laboratory conditions as a free-living organism, although they have never been reported as occurring freely in nature (Akhurst, 1993; Kaya & Gaugler, 1993). Numerous extracellular products, such as lipase(s), phospholipase(s), protease(s) and several different broad-spectrum antibiotics, are secreted as the bacteria enter the stationary phase of their growth cycle (Akhurst, 1982; Boemare & Akhurst, 1988; Gaugler & Kaya, 1990).

Another important characteristic of *Xenorhabdus* is the formation of phenotypic variant forms (Akhurst, 1980; Bleakley & Nealson, 1988; Boemare & Akhurst, 1988; Hurlbert *et al*., 1989; Bermudes *et al*., 1993). During prolonged incubation under stationary phase conditions, such phenotypic variant forms can be isolated. The variant forms, also called phase II cells, are different from one another in terms of various properties, and are not naturally associated in the nematode. Phase I cells represent the form occurring in the IJ (Boemare & Akhurst, 1988). This phase variance should always being aware of during *in vitro* culture of EPNs as these phase II bacteria cause the IJ to lose its virulence.

The optimum growth temperature of a bacterial symbiont should always be defined before mass culturing of the nematode is attempted (Ehlers *et al*., 2000). Deviation from the optimum temperature can potentially induce a switch to phase II cells, which will impede nematode reproduction (Ehlers *et al*., 2000). When the bacterial culture medium is started, the pH should ideally

be between 5.5 and 7, and the oxygen saturation rate should be kept above 30%, as doing so will prevent the bacteria from switching to phase II cells (Ehlers & Shapiro-Ilan, 2005). The aeration rate is very important, and Strauch and Ehlers (2000) compared yields of *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, with one culture being aerated at 0.3 vvm and another at 0.7 vvm (Strauch & Ehlers, 2000). The researchers concerned found a significantly higher number of adults 8 days after IJ inoculation, and a higher final yield in the culture that was aerated at a higher rate.

Increasing the aeration rate in a fermenter often leads to increased foaming which affects the nematodes negatively. Silicon oil can be used to prevent foaming, but should be used cautiously, as high concentrations can affect the nematodes negatively. Long-chain fatty acids used to control foaming have been found to affect *H. bacteriophora* negatively (Ehlers & Shapiro-Ilan, 2005).

The pathogenic potential of the bacteria/nematode complex can be very useful as a biological pest control agent (Klein, 1990). For nematodes to be used as a commercial biocontrol agent, they are produced in industry-scale fermenters (Ehlers, 1996; 2001). In these ferementers, prior to the inoculation of the IJs, the liquid medium is incubated with the bacterial symbiont (Ehlers *et al*., 1998), with the recovery of the IJs tending to vary quite noticeably in the liquid culture. The above is very different to IJ recovery in the haemocoel of the insect, where almost 100% recovery takes place within a day after entry of the haemocoel (Strauch & Ehlers, 1998). Therefore, *in vitro* production is possible due to the preculturing of the symbiotic bacteria, which excrete the food signals used by the IJs to induce their feeding, into the medium (Aumann & Ehlers, 2001), for recovery of the IJs to take place. Research undertaken by Strauch and Ehlers (1998) found that the highest recovery of IJs was obtained when bacteria were in the stationary growth phase.

In this study, the aim was to determine when *Xenorhabdus* reaches the stationary phase when grown in a 20-L fermenter, as this would be the optimum time to add the IJs of *Steinernema yirgalemense*. The effect of stationary phase conditions on the bacterial cell density and the dissolved rate ($DO_2$) in the fermenter was investigated.

## Materials and methods

*Isolation of symbiotic bacteria*

The primary form of the *Xenorhabdus* was isolated from last larval instars of *Galleria mellonella* (L.) (Coleoptera: Pylaridae) infected with *S. yirgalemense* (Chapter 6). After 24 h post inoculation with IJs of *S. yirgalemense*, the *G. mellonella* larva was surface sterilised by dipping them briefly into 95% (v/v) ethanol, igniting them and immediately thereafter plunging the insect into sterile water. The cadaver was dissected, using a sterile syringe and, with a sterile inoculating loop, the haemolymph was transferred and spread onto an NBTA (supplemented with 0.004 % (w/v) triphenyltetrazolium chloride and 0.025 % (w/v) bromothymol blue) plate. Plates were incubated at 25-28˚C for 48 h (Akhurst, 1980; Kaya & Stock, 1997), propagated in trypticase soy broth (TSB) and stored in 15% glycerol at -80˚C. When required, the glycerol stocks were melted at room temperature, propagated in 10 ml TSB in 25 ml test tubes for 2 days at 30˚C, and subsequently used.

*Culture conditions*

The *Xenorhabdus* culture was grown in liquid media (complex medium) that consisted of 15.0 $g \cdot l^{-1}$ yeast extract (Merck), 20.0 $g \cdot l^{-1}$ soy powder (Nature's Choice, Meyerton, South Africa), 4 $g \cdot l^{-1}$ NaCl, 0.35 $g \cdot l^{-1}$ KCl, 0.15 $g \cdot l^{-1}$ $CaCl_2$, 0.1 $g \cdot l^{-1}$ $MgSO_4$ (Merck), and 46 $ml \cdot l^{-1}$ vegetable oil (Ehlers *et al.*, 1998). The culture was grown in a fermenter (New Brumswick Scientific Bioflo IV, 20L reactor) (www.harlowscientific.com ) filled with 8 L of the above medium and supplemented with 0.03% (v/v) anti-foam (Antifoam 204 Sigma-Aldrich). The process temperature was set at 30˚C, and the pH was controlled at 7, using KOH (4N) and citric acid as a base and acid, respectively. The $DO_2$ concentration was set to remain above 30% by adjusting the agitation speed. The airflow rate (filtered) was set at 4 L/min and the velocity of the turbine was set between 200 and 1000 revolutions per minute (rpm).

The fermenter filled with the liquid medium was steam sterilised *in situ* for 15 minutes at 121˚C, and then cooled down to 30˚C. The steam originated from a boiler that was connected to the fermenter. The medium in the vessel was inoculated with 80 ml of a 24 h phase I bacterial culture grown in the same liquid medium that was in the fermenter. Bacterial cell samples of 10 ml were taken aseptically from the fermenter every 3 hours over a period of 60 hours. Steam was used to

sterilse the sample port each time before and after samples were taken. The same experiment was conducted on two different test dates.

*Assessment of bacterial growth*

Bacteria cell samples were washed with 0.8% (w/v) sterile saline by centrifuging and washing three times. Cells were counted using a Petroff-Hausser Counter (Hausser Scientific). Counting was done using a Leica DM2000 Leica (Microsystems, Wetzlar, Germany) research microscope at a 1000 × magnification. Of each sample that was taken, 4 ml was divided into 1 ml samples, from which the dilution series was made up. The experiment was repeated on two different test dates.

*Statistical analysis*

The results were analysed using STATISTICA 10 (Statsoft Inc., 2011). Analysis of the data was done using descriptive statistics.

## Results

The stationary growth phase of *Xenorhabdus* in the fermenter was reached after 36 h at a bacterial cell density of 50 × $10^7$ cells·$ml^{-1}$. A lag phase occurred from 0 to 3 h, and the exponential phase lasted from 3 to 33 h, with the bacterial cell density increasing from 0.05 × $10^7$ cells·$ml^{-1}$ to 49 × $10^7$ cells·$ml^{-1}$ (Fig. 7.1.). A drastic decrease occurred in the $DO_2$ level from 0 to 3 h (Fig. 7.2.), as *Xenorhabdus* started to grow. From 3 to 27 h, the $DO_2$ rate was kept just above 30%, but, as a result of the delayed reaction of the reactor, the $DO_2$ level declined to 29%. Said period coincides with the exponential phase (Fig. 7.1.) of *Xenorhabdus* growth (Fig. 7.3.). The $DO_2$ level increased at 30 h, as *Xenorhabdus* developed into the stationary phase, which required less oxygen. From 42 to 60 h, the $DO_2$ level rose steadily, except for a decrease at 45 h and 54 h, which coincided with the plateau that was reached with the bacterial cell density, at around 50 x $10^7$ cells·$ml^{-1}$ (Fig. 7.3.). The effect of the bacterial cell density on the $DO_2$ levels can clearly be seen in Figure 7.3. After 60 h, the $DO_2$ level was 95% (Fig. 7.2.).

The percentage $DO_2$ was kept above 30% by increasing the speed of the agitation (rpm) provided by the turbine (Table 7.1.), which was set at a minimum of 200 rpm. After 24 h, the percentage $DO_2$ started to increase above 30%, as *Xenorhabdus* was reaching the end of the

exponential phase of its growth (Fig. 7.1.). At 36 h, the rpm declined outomatically to a minimum of 200 rpm, as the stationary phase started. The rpm was automatically controlled by the fermenter. As soon as the $DO_2$ level went below 30 % the rpm increased. At 60 h, very little oxygen was needed in the reactor (Table 7.1.).

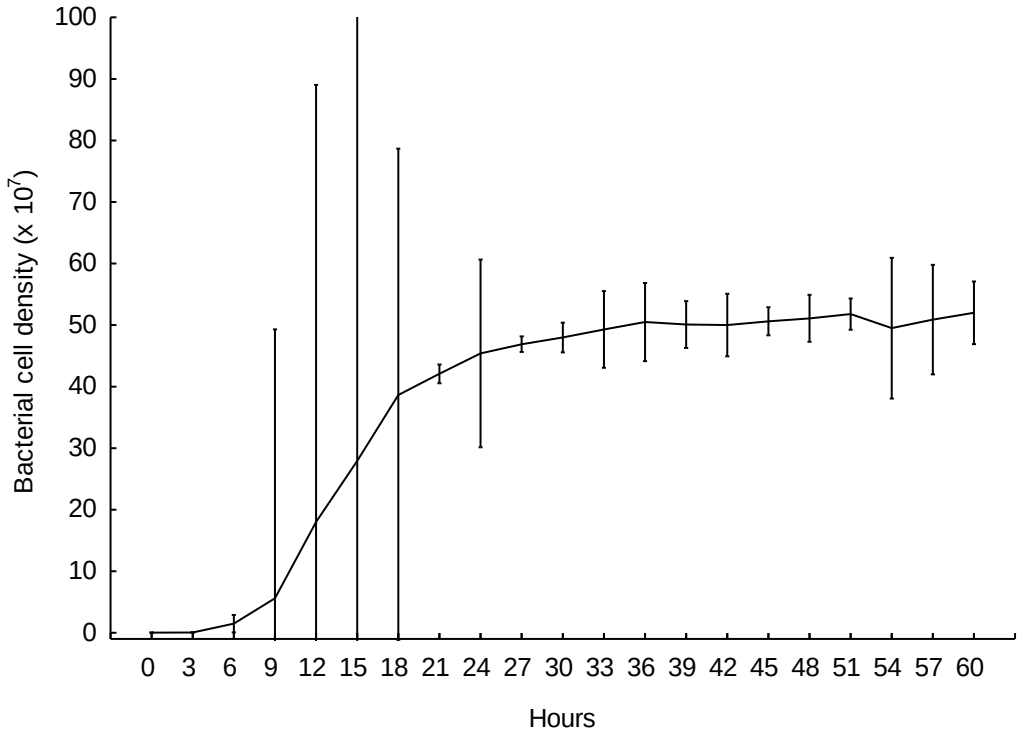


**Fig. 7.1**. The cell growth, measured as bacterial density·ml$^{-1}$ of *Xenorhabdus* spp. in liquid medium, in three hour periods over a period of 60 h in a 20-L fermenter at 30˚C.

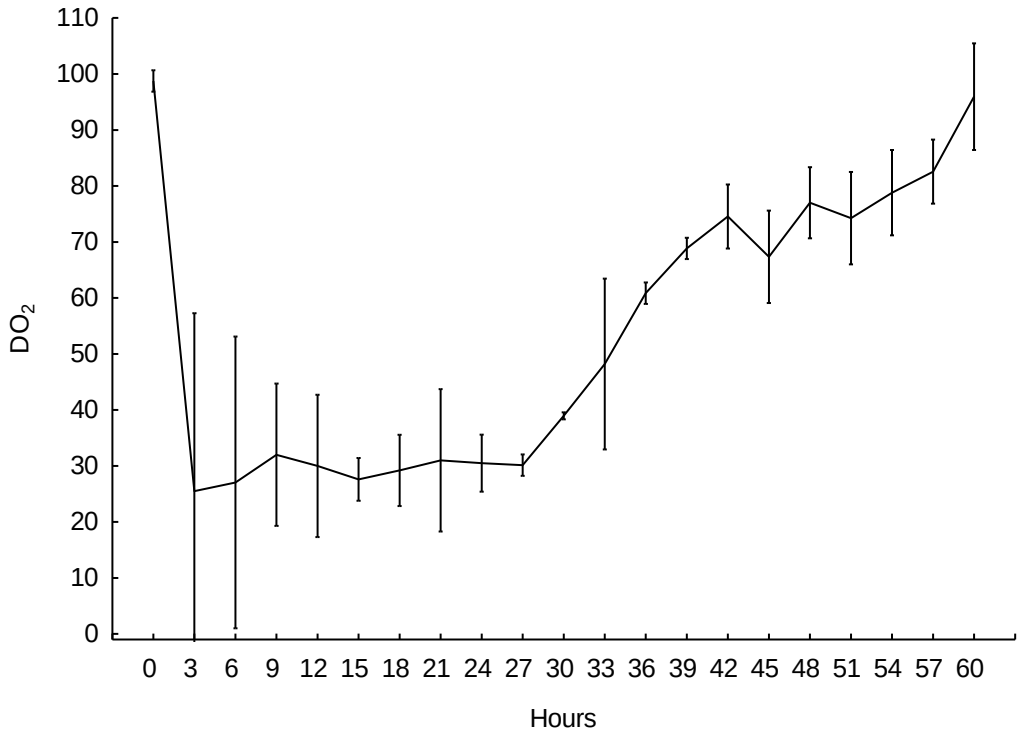


**Fig. 7.2.** The dissolved oxygen ($DO_2$) readings taken every 3 h, over a period of 60 h, in a 20-L fermenter, in which *Xenorhabdus* was cultured in the liquid medium at 30˚C.

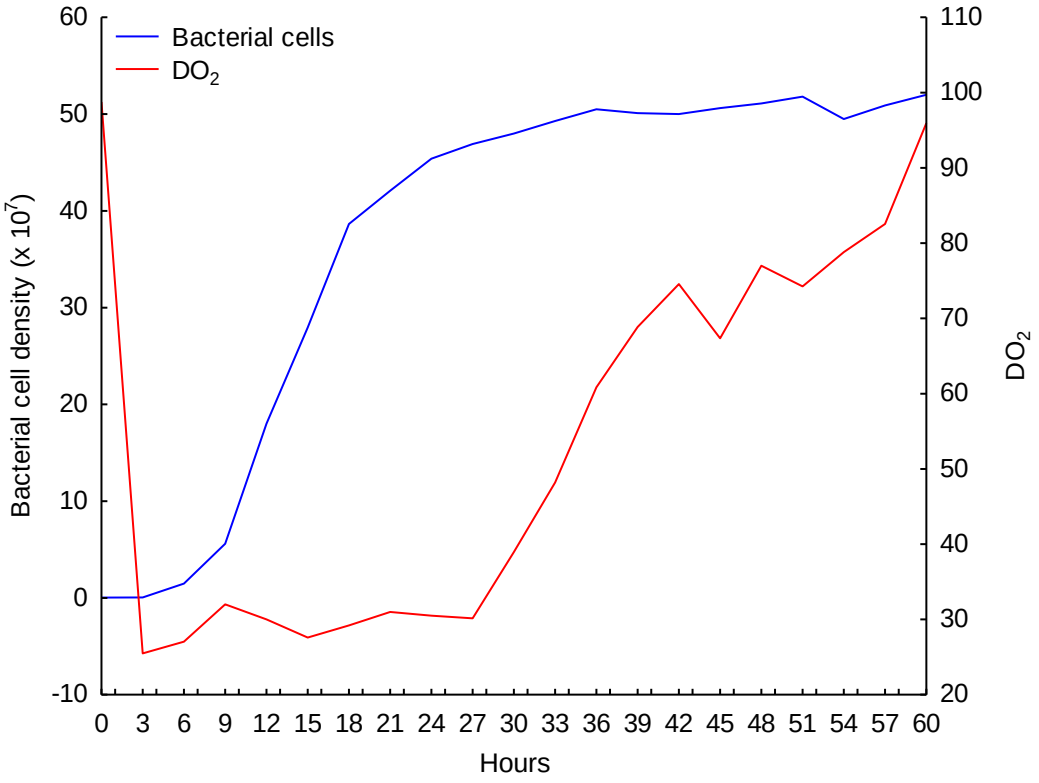


**Fig. 7.3.** The variation in bacterial cell density and dissolved oxygen levels ($DO_2$) every three hours for a period of 60 h in a 20-L fermenter in which *Xenorhabdus* spp. was cultured in the liquid medium at 30˚C.

**Table 7.1.** Dissolved oxygen ($DO_2$) and medium agitation measured in revolutions per minute (rpm) recorded every 6 h during the growth phase of *Xenorhabdus* in a 20-L industrial fermenter.

| h | 0 | 6 | 12 | 18 | 24 | 30 | 36 | 42 | 48 | 54 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $DO_2$ (%) | 98 | 27 | 30 | 29 | 30 | 38 | 60 | 74 | 77 | 78 | 95 |
| rpm | 200 | 273 | 359 | 240 | 211 | 204 | 200 | 200 | 200 | 200 | 200 |

## Discussion

*Steinernema yirgalemense* has been successfully cultured using Erlenmeyer flasks on orbital shakers (Chapter 6). The next step for commercial production would be to upscale the culturing of the nematodes and their associated bacterial symbionts to industrial size fermenters. In such fermenters,

the bacteria and the nematodes would be exposed to different environmental conditions, as with each step in the upscaling process. Results of this study provide the first guidelines as to the successful future mass culture of *Xenorhabdus* from Erlenmeyer flasks to a 20-L fermenter.

For the nematodes to recover from the arrested state and to resume feeding and reproduction, the bacteria must be in a stationary phase within the fermenter, as this would provide sufficient food-induced cues (Johnigk *et al*., 2004). The optimum time concerned can be determined by means of such process parameters as $DO_2$. Data from the cell counts were used to distinguish between the different growth phases of the bacteria (Johnigk *et al*., 2004). Calculating cell density, using colony-forming units (cfu), as was done in Chapter 6 to determine when *Xenorhabdus* reached stationary phase, gave similar results to the direct cell counts. Cell counts were lower than expected, which could possibly be ascribed to washing the cells prior to counting. High variation was observed in the bacterial cell density between the two test dates from 12 h to 18 h (Fig. 7.1). This could be ascribed to the exponential phase being reached earlier during the first experiment, compared to the experiment repeated on the second test date. This experiment was conducted twice and the data combined, and it would be advisable to repeat it further for fine tuning and reproducibility before future mass culturing in high-volume fermenters.

Optical density (OD) was not used to measure growth, as a complex medium was used, including such components as oil which would result in inaccurate OD readings of the bacteria. Research undertaken by Johnigk *et al*. (2004) found that, when working with a complex medium, OD cannot be used to determine the growth phase of bacteria, and that using other parameters tends to provide more reliable information such as the $DO_2$ level . Changes in $DO_2$ levels can be interpreted as changes in bacterial metabolism (Johnigk *et al*., 2004), as the bacteria require less oxygen when they enter the stationary phase. In the current study, the $DO_2$ was found to be efficiently kept around 30% by increasing the agitation speed concerned of the medium concerned. As soon as the stationary phase of the bacteria was reached at 36 h, the $DO_2$ rate increased to 60%, and the agitation speed of the propeller decreased to its minimum of 200 rpm (Table 7.1.). This could possibly indicate the reduced oxygen needs of the bacteria when their stationary phase is reached. The stationary phase of *Xenorhabdus* in the 20-L fermenter was reached after 36 h, which was 6 h earlier than when *Xenorhabdus* was grown in Erlenmeyer culture flasks (Chapter 6). It could be ascribed to the different environment concerned when scaling up from 250-ml volume Erlenmeyer flasks to a 20-L fermenter.

More oxygen would have been available for the bacteria in the 20-L fermenter, as air was actively being pumped in at a rate of 4 L/min, combined with the agitation speed of the propeller for improved circulation and oxygen transfer.

Although fermenter design is an engineering issue, it is necessary to understand the factors that influence the needs of both the symbiotic bacteria, as well as the nematodes, which include correct temperature and enough oxygen (Gaugler & Han, 2002). In the future, such data from the study can be used to determine when it would be the optimum time to inoculate the IJs into the fermenter.

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# CHAPTER 8

## Conclusion

A critical aspect to advancing research within the field of nematology is conducting surveys to search for new EPN species. Many surveys have been conducted worldwide in search of new isolates. In South Africa, however, knowledge on the distribution is still limited, and therefore surveying for new EPNs should always be a priority. Discovering novel EPNs can potentially lead to discovering novel bacterial species associated with such nematodes. Consequently, the field of research is very exciting at present, as the surface has only, so far, been scratched with regards to the development in the knowledge regarding EPNs and their bacterial symbionts on the African continent. Collecting endemic isolates and investing time and funds in producing a local biological control product is more ideal than is importing an exotic EPN species, and avoiding biological pollution.

The South African deciduous fruit and citrus industries regard the use of entomopathogenic nematodes (EPNs) as important in an integrated pest management system for the production of pest- and residue-free fruit for the export market. For this goal to be realised, however, research has to be focused on *in vitro* liquid mass production of nematodes for commercial application. To fast track the use of EPNs in orchards, importation of a formulated nematode product from Germany was agreed upon by the two industries. However, research into production and supply of indigenous nematode species should continue to remain a priority.

The first part of this study was aimed at isolating the symbiotic bacteria-associated *Heterorhabditis noenieputensis*, *Steinernema khoisanae* and *Heterorhabditis zealandica*, respectively. The former two species are new nematode species that were described from South Africa, while the occurrence of the latter was recorded for the first time in South Africa. No research on the associated bacteria of EPNs occurring in South Africa had previously been completed, and it was found that all three of the nematode species were associated with novel symbiotic bacterial species and/or subspecies, which were subsequently characterised and described in this thesis.

The bacteria of *Xenorhabdus* and *Photorhabdus* generally associated with EPNs belong to the family Enterobacteriaceae, and are irreversibly locked together with their nematode partner. Together, they form a formidable mutual alliance that is capable of killing a wide range of insect pests. During

the current study, the importance of the bacterial partner in the nematode insect pathogenicity was realised. Most research undertaken during the last 30 years has mainly been aimed at the nematode and bacteria in combination, while research on the bacteria alone, has been lagging behind. The success of nematodes as biological control agents is highly dependent on our knowledge and understanding of the symbiotic bacteria that is associated with each nematode species as they work very closely together and are most effective against pest insects when in combination As each nematode-bacteria complex is unique, each complex requires separate investigation in terms of the process of mass culture, in order to gain more knowledge about their characteristics with regard to optimum liquid medium required, optimum temperature and duration of life cycle. Fortunately, the bacterial symbionts can easily be cultivated in artificial media and studied under laboratory conditions.

The first bacterial symbiont described in this study was isolated from a *Heterorhabditis* species that was isolated from citrus orchards in the Mpumalanga province of South Africa. This was also the first work to be published on the characterisation of nematode symbiotic bacteria for South Africa. The associated bacterium, *Photorhabdus luminescens* subsp. *noenieputensis* n. sp., was named after the nematode from which it was isolated, *H. noenieputensis*. By injecting *Photorhabdus noenieputensis* directly into the haemocoel of *G. mellonella* larvae, they were found to be highly pathogenic, without the nematode, which essentially acts as the vector.

Interest in the symbiotic bacteria is increasing rapidly, as more knowledge is gained about this unique genus. The bacteria associated with *S. khoisanae* and *H. zealandica* were also isolated and characterised. A new *Xenorhabdus* species was found and named *X. khoisanae* n. sp., after the nematode from which it was isolated. In addition, a new *Photorhabdus* species was isolated from *H. zealandica*, which is quite unique, as it is not just another subspecies, but another species entirely. This is the first study worldwide to show that *H. zealandica* associates with a different *Photorhabdus* species, and not with the known *P. temperata*. As shown by the results in this study, SF41 share a DNA homology of 50.8% with *P. asymbiotica* ssp*. australis,* even though four genes (recA, *gyrB*. *dnaN, gltX*) show the strains are closely related to *P. asymbiotica* ssp. *asymbiotica.* This raises the question of how significant house keeping genes and concatenated sequences is. In comparison to *Steinernema* the number of *Heterorhabditis* species is limited, as well as the associated bacteria, and more reseach is needed in species identification and their relatedness.

The second part of the study was aimed at investigating the mass production of two endemic EPN species, *H. zealandica* and *Steinernema yirgalemense*, using *in vitro* liquid culture technology. This was done in order to investigate the potential commercialization of one or both of these species for the use as a biopesticide. Only one isolate of *S. yirgalemense* has been found in South African surveys, and it has only been reported from Ethiopia. The two nematodes concerned have not previously been cultured in liquid, making this study the first attempt to accomplish such a feat.

EPNs as commercial biological control agents can fill the gaps left after the banning or restriction of numerous organophosphate and carbamate insecticdes. In the present study, the attempt was first made to mass culture *H. zealandica* in liquid medium, as the species had shown, in previous research, to be highly virulent against such pest insects *Phlyctinus callosus*, *Cydia pomonella*, *Thaumatotibia leucotreta*, *Planococcus citri* and *Pseudococcus viburni*. When working with a *Heterorhabditis* species in liquid culture, one very important aspect that needs to be taken into consideration is that the male and female cannot copulate in liquid, as a result of their mating behaviour. *Heterorhabditis* has a ‘y’ or lambda copulation behaviour, which means that the male cannot attach itself to the female. Only the self-fertilising hermaphrodites can produce offspring and, subsequently, the final yield is predicted by the density of the hermaphrodites. In this study, two generations occurred in liquid, with *H. zealandica* and its *Photorhabdus* symbiont being mass cultured. More research is required to optimise the process, in order for the more desirable one-generation process to take place.

IJ recovery is another critical factor that should be improved upon in the liquid culture of *H. zealandica*. The recovery that occurred is not nearly sufficient for full-scale commercial production to be successful and economically viable. The symbiotic bacteria are a key factor when it comes to adequate recovery aimed at obtaining high infective juvenile (IJ) yields. The bacteria are responsible for producing food signals that induce IJ recovery. Said bacterial food signal is much less efficient when compared to the food signal in an insect host, which immediately induces the recovery of the IJs.

Future research should be aimed at optimising the *Photorhabdus* growth in the medium. Different mediums could be investigated, as well as methods for enhancing stronger food signals. *Heterorhabditis zealandica* is already commercially available in Australia, and is used for the control of a number of turf and pasture pests. The product, which is produced by Ecogrow Australia Pty Ltd

(www.ecogrow.com.au), is known as 'Weevilnem™'. It is not, however, mass cultured using *in vitro* liquid culture technology, but instead by means of three-dimensional monoxenic culture, using sponge as the medium.

During the current study, *S. yirgalemense* was isolated during research that was conducted into the occurrence of EPNs in citrus orchards, and was shown, in different studies, to be more potent against codling moth, false codling moth and mealybug than was *H. zealandica*. Since *Steinernema* in general is known to be less difficult to culture in liquid, it was decided to investigate the liquid mass culture of this species. It was found that *S. yirgalemense* produced higher yields of IJs than did *H. zealandica.* Although the initial inoculation density of *H. zealandica* was less than that of *S. yirgalemense*, a general trend was previously seen in the preliminary trials, with *S. yirgalemense* producing higher yields than did *H. zealandica*. The higher yield from *S. yirgalemense* could be ascribed to the different copulation behaviour of the *Steinernema* species. *Steinernema* males and females are able to copulate in liquid culture, because of the curling mating behaviour of the male. As a result of the occurrence of male and female copulation, the females can produce fertilised eggs. The initial percentage recovery of *S. yirgalemense* was also higher than was that of *H. zealandica*. This could possibly be ascribed to a stronger food signal being emitted from *Xenorhabdus*. This study indicated that *S. yirgalemense* has more potential to be mass cultured in a liquid medium from a commercial point of view, than does *H. zealandica*. Future research should be aimed at: increasing the percentage recovery, shortening the processing time; and optimising the overall process, in order to achieve a higher yield of IJs. A key to success with the process is to reduce the cost of culturing the nematode in liquid medium, which can be achieved by further improving the population dynamics involved.

The Faculty of Engineering at Stellenbosch University is equipped with state-of-the-art fermenters that are used for the culturing of bacteria, mostly for pharmaceutical purposes. One of their 20-L fermenters was used to upscale the culture of the *Xenorhabdus* associated with *S. yirgalemense*. The results from the study looked promising for the successful culture of the bacteria, with the next step being to add the nematodes. Upscaling, which is an important factor in the commercialisation of *S. yirgalemense*, is important to achieve in a cost-effective way. Even though the cost of nematode products has substantially decreased since liquid culture technology was first introduced, such products still cost more than do chemicals. A possible solution for the problem is to

focus on such high-cash crops as blueberries, as it is more economically feasible for these growers to invest in a biological control management tool.

When importing nematodes from other countries, which is currently occurring, two factors act as obstacles. The first is the amount of time that it takes for the nematodes to reach South Africa, combined with the temperature at which they have to be kept while in transit (keeping them at cooler temperatures than usual, is unfortunately, not always possible). The second problem is the cost that is associated with importing nematodes from another country, which, due to such importing not being economically feasible in the long run, is, therefore, only a short-term solution. Consequently, our own endemic species need to be mass cultured and produced as a biopesticide in South Africa. South Africans can also serve as the leaders for other African countries to use nematodes as biocontrol agents.