National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

EFFECT OF ANTIBIOTICS PRODUCED BY *XENORHABDUS* SPP. ON SOIL BACTERIA

by

GENHUI CHEN

B.Agr., Nanjing Forestry University, 1983

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

March 1992

ISBN 0-315-83658-X

APPROVAL

Name: **CHENHUI CHEN**

Degree: **Master of Science**

Title of Thesis:

EFFECT OF ANTIBIOTICS PRODUCED BY XENORHABDUS SPP. ON SOIL BACTERIA.

Examining Committee:

Chairman: Dr. Z.K. Punja

Dr. J.M. Webster, Professor,
Department of Biological Sciences, SFU

Dr. G.B. Dunphy, Associate Professor,
Department of Entomology, McGill University

Dr. M.M. Moore, Assistant Professor,
Department of Biological Sciences, SFU

Dr. H.R. MacCarthy, Adjunct Professor,
Department of Biological Sciences, SFU

Dr. G.H.N. Towers, Professor,
Department of Botany, UBC
Public Examiner

Date Approved 24 March, 1992

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

EFFECT OF ANTIBIOTICS PRODUCED BY XENORHABDUS SPP. ON SOIL BACTERIA

Author:

(signature)

GENHUI CHEN

(name)

26/03/92

(date)

Abstract

Steinernema spp. and *Heterorhabditis* spp., together with their associated bacterial *Xenorhabdus* spp. are used as biological control agents of insect pests including soil-living insects. This study was done to determine the effect of *Xenorhabdus* spp. on soil bacteria. The research focused on the interaction of *Steinernema* and *Xenorhabdus*, the properties of the antibiotics produced by *Xenorhabdus* spp. and the effect of these antibiotics on soil bacteria. It was shown that more than two forms of bacteria occur in each of *X. nematophilus and X. bovienii*. In *X. bovienii*, these form variants differ not only in antibiotic activity but also in growth and pigmentation. The antibiotic activity of extracts from *Galleria* larvae cadavers was similar regardless of whether the insects were killed by infecting them with nematodes and their symbionts or with *X. nematophilus* alone. The antibiotic activity of *X. nematophilus* cultures was due to several substances which have different molecular weights and are water and organic soluble. *In vitro* experiments showed that the antibiotic activity of *X. nematophilus* was strongly influenced by abiotic factors such as temperature, pH, aeration and nutrition. Antibiotic activity was optimum at 25°C and pH 7.0 and no antibiotic activity was detected when *X. nematophilus* was cultured at 30°C or above, under anaerobic conditions or in 1% peptone water. The antibiotics

from *in vitro* cultures of *X. nematophilus* were stable in heat, in storage and to sunlight UV, and inhibited the growth of various bacterial species in Petri dish cultures. This antibiotic activity was quickly lost when the bacterial solution was applied to sterile soil. There was no antibiotic effect when *X. nematophilus* culture was applied to sterile soil containing exogenous populations of *Bacillus subtilis* and *Rhizobium phaseoli*. Although the insect cadavers killed by *X. nematophilus* retained their antibiotic activity for more than 10 days in natural garden soil there was no evidence that antibiotics contained in the cadavers had an adverse effect on the soil bacterial population, even when bacteria infected cadavers were present in unnaturally large numbers.

Acknowledgments

This work has been the result of encouragement, advice and help received from many people. My deepest gratitude is to Dr. J. M. Webster, my senior supervisor, for his guidance, support, encouragement and help throughout. I would also like to express my gratitude to Dr. G. B. Dunphy for his invaluable ideas and advice. A visit to his laboratory leaves one with great respect for his achievements and contributions to our knowledge and better understanding of insect pathology. Thanks are also due to Dr. H. R. MacCarthy for his numerous suggestions and critical corrections of the manuscripts. I express my gratitude to Dr. M. M. Moore for her helpful suggestions and comments during my research and the revision of the manuscript; to Dr. S. Zervos for her advice and stimuli during the study; to Dr. Z. Mracek for identifying some of the nematode species and teaching me taxonomy of insect nematodes; to Mr. A. Syed for rearing *Galleria mellonella;* to many friends in the Department of Biological Sciences for sharing a lot of enjoyable time with me; to many people mentioned in the text who so generously provided bacterial cultures; to the Department of Biological Sciences for providing financial aid and the facilities; and to the Natural Sciences and Engineering Research Council for financial support through a grant to my senior supervisor. Finally, I express special gratitude to my wife, Xu Xiaofang, for her personal support throughout the course of my study from its first inception.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

It has been almost sixty years since Glaser and Farrel (1935) applied insect nematodes, *Neoaplectana glaseri* (*Steinernema glaseri*), to control Japanese beetle. Great progress has been made in entomopathogenic nematology, including breakthroughs in mass production technology, that have led to the commercialization and large-scale field application of parasitic nematodes against insect pests(Bedding, 1984; Friedman, 1990; Georgis, 1987, 1990). Research into the use of entomopathogenic nematodes as biological control agents has focused mainly on investigations of the families Steinernematidae and Heterorhabditidae. Nematodes of these families are symbiotically associated with bacteria of the genus *Xenorhabdus*. The symbiotic bacteria are carried monoxenically within the intestines of the infective juveniles(IJ) of these nematodes. After penetrating into the insect's haemocoel, the IJs release the bacteria which then kill the insect and enhance the conditions for nematode reproduction(Poinar and Thomas, 1966). It has been reported that these symbiotic *Xenorhabdus* spp. produce antibiotics which inhibit the growth of a wide range of other micro-organisms(Akhurst,1982a).

Although they are adapted to the soil environment, the biotic interactions of the released entomopathogenic nematodes with other soil micro-organisms are virtually unknown. It would be premature to claim that releases of very

large numbers of entomopathogenic nematodes in insect control programs have no impact on soil micro-organisms in view of the production of antibiotics by their symbiotic bacteria. These antibiotics, which are capable of inhibiting the growth of a wide range of micro-organisms, may well have an impact on soil micro-organisms. Consequently, it is appropriate that the impact on soil micro-organisms of these released nematodes and their associated bacteria should be investigated as part of the study of their efficacy as environmentally safe biological control agents. Therefore, the objective of this study was to investigate the properties of the antibiotics produced by *Xenorhabdus* spp. and to determine their effect on soil bacteria.

1-1. The nematode-bacteria complex

The classification of the Steinernematidae is confusing because of inadequate descriptions or loss of type specimens or both. Depending upon the author, there is a single genus, *Steinernema* (Wouts *et al.*, 1982) or two genera, *Steinernema* and *Neoaplectana* (Poinar, 1979, 1984, 1986). However, most recent authors have accepted the prime status and use of *Steinernema*. To minimize confusion and to establish consistency, Poinar (1990) proposed a nine-species nomenclature (Table 1) in which many earlier species are synonymized with *Steinernema*.

Table 1. Recognized species of *Steinernema* and their synonyms in *Neoaplectana**

Recognized species	Synonyms
S.affinis (Bovien,1937)	-
S.anomali (Kozodoi,1984)	?*N.arenaria* Artyukhovsky,1967
S.carpocapsae (Weiser, 1955)	*N.belorussica* Veremchuk,1966
	N.chresima Steiner in Glaser, Mccoy and Girth,1942
	N.dutkyi Turco *et al.*, 1971
	N.dutkyi Jackson, 1965
	N.dutkyi Welch, 1963
	N.elateridicola Veremchuk,1970
	N.semiothisae Veremchuk and Litvinchuk,1971
	N.agriotos Veremchuk, 1969
	N.feltiae Filipjev *sensu* Stanuszek,1974
S.feltiae(Filipjev,1934) *sensu* Filipjev	*N.bibionis* Bovien,1937
	N.bothynoderi Kirjanova and Puchkova,1955
	N.kirjanovae Veremchuk,1969
	N.leucaniae Hoy, 1954
	N.menozzii Travassos, 1932
S.glaseri (Steiner,1929)	-
S.intermedia (Poinar,1985)	-
S.kraussei (Steiner, 1923)	-
S.kushidai Mamiya, 1988	-
S.rara (Doucet,1986)	-
S.scapterisci Nguyen and Smart,1990	*N.carpocapsae*,Uruguay strain Nguyen and Smart,1988

* Based on Poinar's nomenclature(1990), in which *S. kraussei* was omitted. However, Mracek(Pers. comm.)[‡] and Ehlers *et al.* (1991) support the validity of *S. kraussei*.

[‡] Z. Mracek, Research Scientist, Entomology Institute, CSAS, Ceske, Budejovice, Czechoslovakia

The family, Heterorhabditidae, was erected by Poinar (1976). Unlike Steinernematidae, the classification of the Heterorhabditidae is relatively clear. Three species have been described: *Heterorhabditis bacteriophora* Poinar 1976, *H. zealandica* Poinar and *H. megidis* Poinar,Jackson and Klein, 1987. As new isolates were found (Poinar, 1990), new techniques, such as DNA analysis, were used to help define the taxonomy of heterorhabditids (Smits *et al.*, 1991) and more new species may be described in the near future.

Steinernematids and heterorhabditids are groups of obligate entomopathogenic nematodes capable of infecting a wide range of insects. Their life cycles (Fig.1) include the egg, four juvenile stages and the adult. The infective juvenile is the third-stage and it is capable of searching for and entering the body of a host insect. In spite of their similarity there are important differences between *Steinernema* and *Heterorhabditis*. One major difference is in their development subsequent to the infective stage. The IJs of *Steinernema* spp. develop into an amphimictic generation of females and males, whereas the IJs of *Heterorhabditis* spp. develop into hermaphroditic females (Poinar, 1990).

Of the characters that set steinernematids and heterorhaditids apart from other rhabditids it is their association with the symbiotic bacteria, *Xenorhabdus*, that makes them unique. As well it is this character that enables them to be so efficient as insect pathogens.

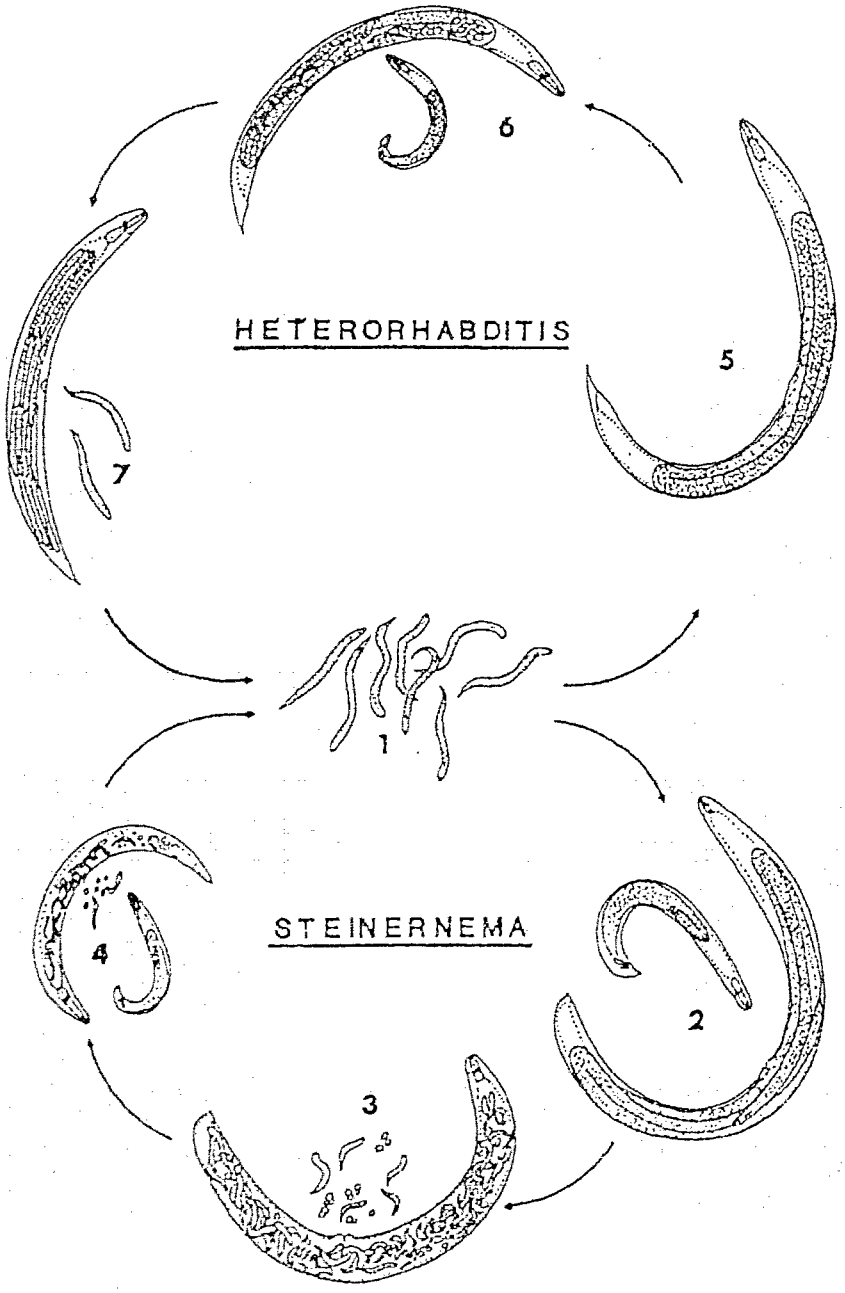

Fig. 1. Generalized life cycle of species *Steinernema* and *Heterorhabditis* (from Poinar, 1990)

1.Infective juveniles
2.Amphimictic, female and male
3.Female, eggs and juveniles
4.Female, male, eggs and juveniles of the second generation
5.Hermaphroditic female
6.Amphimictic, female and male
7.Juveniles inside and outside the female

Poinar and Thomas (1965) described this unusual association of a bacterium with a nematode and named it "*Achromobacter nematophilus*". Subsequently, as more species of bacteria were described with unusual characteristics, the genus *Xenorhabdus* was erected (Thomas and Poinar, 1979) and now there are five species(Table 2). *Xenorhabdus luminescens* is commonly associated with *Heterorhabditis* spp. and the other four with *Steinernema* spp.

1-2. Biology and physiology of *Xenorhabdus*

Xenorhabdus are gram-negative, asporogenous, peritrichous, motile and rod-shaped bacteria varying from 0.8-2 μm x 4-10 μm. In older cultures, crystalline inclusions and intracellular coccoid bodies occur. The bacteria are facultatively anaerobic, having both a respiratory and a fermentative type of metabolism. *Xenorhabdus* do not form spores and do not have an environmentally resistant stage. There is no record of them occurring free in the soil but only in their nematode vectors, in the nematode infected insects and in some wounds of human patients(Thomas and Poinar, 1983; Farmer, *et al.* 1989). Thomas and Poinar(1983) described two species and several subspecies and, based on phenotypic characteristics and phase variations, Akhurst and Boemare (1988) proposed the elevation of these subspecies to species. Five species have been described (Table 2). However, new evidence (Grimont *et al.*, 1984; Colepicolo *et al.*, 1989; Farmer *et al.*, 1989; Akhurst *et al.*,1990; Han *et al.*,1990 and

Xi *et al.*, 1991) indicates that *X. luminescens* is a multispecies taxon and several taxonomic changes may be made in the future. Farmer *et al.*(1989) isolated bacteria from human wounds which were identified as new *Xenorhabdus* species. This occurrence indicates that *Xenorhabdus* spp.may include non-nematode associates. Moreover, based on DNA homology, Akhurst *et al.* (1990) suggested that there are several species of *Xenorhabdus* associated with the *Steinernema* spp. and that the bacterial symbionts of *Heterorhabditis* spp. and *Steinernema* spp. may belong to different genera. The case for *X. luminescens* being placed in a separate genus is further supported by recent work (Akhurst and Boemare, 1990; Leclerc and Boemare, 1991).

In common with all rhabditids, the steinernematids and heterorhaditids are bacterial feeders. The developing nematodes feed on and digest *Xenorhabdus* spp. in the insect haemocoel (Poinar, 1990). It has been reported (Poinar and Thomas, 1966) that nematodes are unable to reproduce in axenic insects without their *Xenorhabdus* symbionts to provide suitable nutrient conditions. However, many of these conditions have not been identified. In fact, rich media without bacteria, such as sterile rabbit kidney and raw liver extracts can sustain axenic cultures of *Steinernema* spp.(Glaser, 1940; Stoll, 1953). Although the bacterium, *Pseudomonas aeruginosa*, is able to provide suitable conditions for the nematode, *S. carpocapsae* strain DD136, to reproduce, the nematode's reproduction is limited. However,

Ehlers *et al.*(1990) found no evidence that *Escherichia coli* had a negative influence on the reproductive potential of *S. carpocapsae* when the nematode was cultured on *E. coli* in very rich media. They observed a delay in the nematode development.

The bacteria, once released from the intestine of the nonfeeding IJ, multiply in the insect haemocoel and provide the nutrients essential for nematode development. *Xenorhabdus* prevents the insect cadaver from being contaminated with other micro-organisms by producing various antimicrobial substances (Paul *et al.*, 1981; Akhurst, 1982a; Richardson *et al.*, 1988 and McInerny *et al.*, 1991a,b). Each species of nematode usually has a specific natural association with a single *Xenorhabdus* species(Akhurst, 1982b). However, a *Xenorhabdus* sp. may be associated with more than one nematode species(Dunphy *et al.*, 1985; Akhurst and Boemare, 1990; Han *et al.*, 1991)(see Table 2).

As symbionts of entomopathogenic nematodes, the *Xenorhabdus* spp. are considered to be a diverse and unusual bacterial group(Nealson *et al*, 1990), because they form variations that differ in their production of various metabolites, antibiotics, extracellular enzymes, intracellular protein crystals and bioluminescence.

Table 2. *Xenorhabdus* species and their nematode associates(Akhurst and Boemare, 1990)

Xenorhabdus species	Associated nematode species
X. beddingii	Undescribed *Steinernema* sp.
X. bovienii	*S. feltiae*
	S. kraussei
	S. affinis
	S. intermedia
X. nematophilus	*Steinernema carpocapsae*
X. poinarii	*S. glaseri*
X. luminescens	All *Heterorhabditis* spp.

Xenorhabdus can be cultured in standard bacterial media. According to the 1984 edition of Bergey's Manual, the optimum temperature for *Xenorhabdus* is about 25°C. In lipid broth *X. nematophilus* has a doubling time of 0.8-1.2 h at 25°C (Dunphy *et al.*, 1985). *X. luminescens* has a doubling time of about 1.5 h in peptone water and 2.5-3 h in larvae of *Galleria mellonella*, the greater wax moth at 25°C (Poinar *et al.*, 1980). The bacteria are usually maintained either by deep-freezing or frequent subculture at room temperature (Woodring and Kaya, 1988). In studies of *Xenorhabdus* several workers (Poinar and Thomas, 1967; Götz *et al.*, 1981 and Xu *et al.*, 1989) reported low plating efficiency, in which various factors could be involved, such as osmotic sensitivity, nutrient requirements and sensitivity to O_2 tension. As well, *Xenorhabdus* spp. that are catalase negative are sensitive to H_2O_2 produced in media exposed to ordinary fluorescent light (Xu and Hurlbert, 1990). Poinar *et al.*(1980) failed to recover viable *X. luminescens* after they inoculated the bacteria into sterile soil.

Akhurst (1980) was the first to report the occurrence of two forms of *Xenorhabdus*, and showed that there were major differences between the forms in their production of antibiotic substances and lecithinase and in their absorption of dyes. He designated them initially as primary and secondary forms. On nutrient agar, colonies of the primary form of *X. nematophilus* are white, opaque, convex and circular with an irregular margin; but colonies of the

secondary form are translucent, flatter and of greater diameter. On nutrient agar supplemented with bromothymol blue(BTB) and triphenytetrazolium chloride(TTC) (NBTA medium, see appendix II), primary form colonies are surrounded by cleared zones in the blue agar because of their absorption of the BTB. Primary form colonies of *Steinernema* symbionts are colored from blue to blue-green and those of *Heterorhabditis* symbionts from greenish to brown. There is no clear zone around the secondary form colonies because they lack the ability to absorb BTB. The color of secondary form colonies are usually from reddish to rusty because of their absorption and reduction of TTC(Woodring and Kaya, 1988). In *X. luminescens*, except for the non-bioluminescent strain (Akhurst and Boemare, 1986), bioluminescence is stronger in the primary than in the secondary form (Boemare and Akhurst, 1988; Nealson *et al.*, 1988), and the primary form is sensitive to a phage particle to which the secondary form is not (Poinar *et al.*, 1989). There is recent evidence of the possibility of more than two forms in *X. luminescens* (Hurlbert *et al.*, 1989; Boemare and Akhurst, 1990; Ehlers *et al.*, 1990).

Primary and secondary forms are considered to be equally pathogenic towards *G. mellonella* larvae, but the primary form creates better conditions for nematode reproduction (Akhurst, 1980). It has been strongly suggested that reversion from the secondary to the primary form is common in *Xenorhabdus* spp.(Akhurst, 1980; Boemare and

Akhurst, 1988), however, reversion in *X. luminescens* has not been detected. Although the IJ is capable of retaining the secondary form of its *Xenorhabdus* symbiont within its intestine, nematodes collected in nature almost invariably contain only primary form cells(Akhurst and Boemare, 1990). There is no evidence for secondary to primary form reversion of *Xenorhabdus* within the IJs of *Steinernema* and *Heterorhabditis*. Poinar *et al.*(1989) proposed that the form change might result from a bacteriophage which lyses the primary but not the secondary form of *X. luminescens*. Akhurst (1982a) was unable to prove this by using mutagens and physical identification methods. Couche *et al.*(1987) ruled out the possibility of plasmid involvement by demonstrating that identical plasmid DNA was present in both forms. Wouts (1990) observed small cells associated with the primary form of *Xenorhabdus*. He assumed that these small cells were responsible for the differences between the forms and suggested that these slow-growing cells were a new and unnamed bacterial species. Recent physiological studies(Boemare and Akhurst, 1990) have shown that the mechanism of form changes is complicated. They suggest that the form shift is enhanced by anaerobic cultural conditions, and that it may be related to differences in nutrient assimilation and the requirements of the primary and secondary forms. However, the mechanisms of the form change remain unclear.

Xenorhabdus luminescens is the only bacterial species in the terrestrial environment known to have this property although there is a non-luminescent strain (Akhurst and Boemare, 1986). When an insect is infected by *X. luminescens*, the cadaver becomes visibly luminescent (Poinar *et al.*, 1980). The bacteria emit light most efficiently at 33°C in a low-salt medium with the emission spectrum peaking at 480nm *in vivo* and 490nm *in vitro*. The luminescence is maximal as the cultures approach the stationary phase. The bioluminescence is believed to be catalyzed by an enzyme similar in substrate requirements and subunit size to other bacterial luciferases (Colepicolo *et al.*, 1989; Nealson *et al.*, 1990).

Many *Xenorhabdus* species produce pigments when cultured in media or in insects, resulting in a color range from yellow to rusty. Some workers use the presence of pigment as a taxonomic character(Grimont *et al.*,1984). One pigment from *X. luminescens* was purified and identified as an anthraquinone derivative. Its color depends on the pH: yellow below and red above pH 9 (Richardson *et al.*, 1988).

Production of crystalline inclusions is another common feature in *Xenorhabdus* species. Couche *et al.* (1987) reported that crystalline inclusions were present in both *X. nematophilus* and *X. luminescens* when they were cultured in liquid media. Crystal formation occurs in primary, but not in secondary forms of *X. nematophilus*, whereas both forms of *X. luminescens* produce inclusions. Ensign *et al.* (1990) obtained

large intracellular crystals from *X. luminescens* cells isolated from insect cadavers. Two morphologically distinct forms of inclusion were purified, namely, large, cigar-shaped and smaller, ovoid forms. The inclusions contained protein almost exclusively. The crystalline protein is not toxic to insect larvae and its function is unknown (Ensign *et al.*, 1990).

In common with many gram-negative bacteria, *Xenorhabdus* species produce insect toxins. Dunphy and Webster (1988b) reported that *X. nematophilus* released lipopolysaccharide into the insect host where itacts as a haemocytotoxin. In another paper these workers showed that *X. luminescens* produced the same toxin in *Galleria* larvae (Dunphy and Webster, 1988a). Ensign *et al.* (1990) detected an insecticidal toxin produced by *X. luminescens* both *in vitro* and *in vivo*. The formation of the toxin in peptone broth begins as the cells enter the stationary phase of growth and reaches a maximum level at 48-60 hours. The purified toxin kills the fifth instar larvae of *Manduca sexta* in 12-24 hours when injected into the larvae but is not toxic when fed to the larvae. Interestingly, McInerney *et al.*(1991a) reported that an antibiotic, xenorhabdin 2, produced by *Xenorhabdus* spp. had *per os* larvicidal activity against *Heliothis punctigera*.

1-3. Antibiotics produced by *Xenorhabdus*

Dutky *et al.* (1964) suggested that the bacterial symbiont of the DD136 strain of *Neoaplectana(Steinernema) feltiae* produced antibiotics. Poinar *et al.* (1980) reported that both *X. nematophilus* and *X. luminescens* inhibited the growth of *Bacillus cereus* var. *mycoides* and *B. thuringiensis*. Paul *et al.*(1981) reported that nine strains of *X. nematophilus* and *X. luminescens* produced antibiotics that inhibited growth of the luminous bacteria, *Vibrio* spp. and *Photobacterium* spp. Moreover, Akhurst (1982a) documented antibiotic activity of *Xenorhabdus* spp. against a variety of micro-organisms, including gram-positive and gram-negative bacteria and yeasts. In this study, Akhurst concluded that: a) all primary forms of *Xenorhabdus*, except the symbiont of *S. glaseri* (presumably *X. poinarii*), produced antibiotics; b) some *Xenorhabdus* strains or species were mutually inhibitory; c) the anaerobic cultures of *Xenorhabdus* produce no antibiotics; d) the antibiotic activity was not affected by the culture media and heat. Noticing the spectrum variation of antibiotic activity within and between *Xenorhabdus* species, both Paul *et al.* (1981) and Akhurst (1982a) indicated that different compounds were produced by different *Xenorhabdus* species e.g., each of these *Xenorhabdus* species or strains produces one or more different antibiotics. In spite of the previous work, little is known of the production rates of these antibiotics. Paul *et al.* (1981) obtained 500mg of semipurified antibiotic extract from 15L cell-free *X.*

nematophilus spent culture medium. They isolated five compounds from *X. nematophilus* and two from *X. luminescens*, all of which have antibiotic activity. Richardson *et al.* (1988) purified and characterized antibiotic compounds from *X. luminescens*, and found that these chemicals belong to two distinct groups, namely indole and hydroxystilbene derivatives. It has been reported (McInerney *et al.*, 1991a,b) that seven antibiotic compounds have been isolated from *Xenorhabdus* spp., and of these, five related compounds named xenorhabdins, are organic soluble. The other two, xenocoumacins 1, 2 are water soluble, and they are produced in 1:1 ratio in insect cadavers infected with *X. nematophilus* (Dunphy*, Pers. comm.). It was found that xenocoumacins have antibacterial activity and that xenocoumacin 2 has antifungal and potent antiulcer activity (McInerney *et al.*, 1991b).

It is well-known that *Xenorhabdus* spp. produce a range of inhibitory substances but their role in the nematode infection of insects has not been determined. The hypothesis that antibiotic(s) prevent(s) the insect cadaver from putrefying and so maintain proper conditions for nematode growth and reproduction, seems logical. The presence of many different antibiotic classes and their production by different strains of *Xenorhabdus* may help to explain strain variation in *Xenorhabdus*. It is unclear, however, if the presence of these antibiotics is sufficient to explain all

---

*Professor, Dept. of Entomology, Macdonald College of McGill University, Montreal, Quebec.

the variation. In view of the number of species and strains already studied for antibiotic production and the discovery of more strains or species of *Xenorhabdus*, it is likely that more antibiotics will be isolated. Moreover, the production and activity of antibiotics *in vivo* may be different from that in a defined medium.

In short, although there has been great interest in and a lot of work done on insect nematodes and their bacterial symbionts, there is an incomplete understanding of this nematode/bacteria association. The objective of this study was to increase our understanding of the antibiotics produced by *Xenorhabdus* spp. with special reference to their properties and their antibiotic activity against other bacteria in the soil.

Chapter 2. Materials and methods

2-1. General materials and methods

A. Sources and preparation of nematodes

All nematode species or strains were from the collection in Dr. J. M. Webster's laboratory at Simon Fraser University. Table 3 shows the source of the nematode species and strains used in this study.

All nematode species were cultured in larvae of the greater wax moth, *Galleria mellonella*(L.), to produce fresh stock material for the study. The culture procedure was a modification of the method described by Dutky *et al.*(1964).

Last-instar larvae of *G. mellonella*, reared according Dutky *et al.*(1962), were provided by the insectary of the Department of Biological Sciences, Simon Fraser University. Upon receipt of the insects, large larvae of 230-260mg were collected and stored at 4°C until needed.

Nematodes kept in a storage sponge(4°C) were allowed to warm for 1h at room temperature before suspensions were made by squeezing the sponge in distilled water. Twenty milliliters of nematode suspension were poured into a 50ml test tube, the nematodes were allowed to settle for 15-20 min., and then were pipetted into another test tube and washed twice with distilled water.

Table 3. The sources of the nematode species and strains used in this study.

Nematode species	Strain	Sources
Steinernema carpocapsae	DD136	G. O. Poinar, University of California, Berkeley, California
S. feltiae	A326	Isolated from British Columbia, Canada
S. feltiae	NCA11	Biosys, Palo Alto, California
S. feltiae	A21-1	Isolated from British Columbia, Canada
S. carpocapsae	BC1* (NC162)	
S. intermedia	BC2* (SC)	
Heterorhabditis sp.	#90	Isolated from British Columbia, Canada

* Cultures of unknown origin due to reidentification of culture after many passages.

Finally, nematodes in aliquot samples were counted and a suspension of estimated concentration was made. To infect the insects, 1ml of the nematode suspension at a concentration of about 500 IJs/ml was evenly distributed on a 9cm Whatman #1 filter paper in a 10cm Petri dish, and 10 *Galleria* larvae were added to each dish. To reduce larval movement, a similar piece of dampened filter paper was placed on top of the insects and lightly pressed to make a paper-insect sandwich. Finally, each dish was covered with its lid, sealed with Parafilm(American National Can, Greenwich, CT.) and incubated in the dark at 24°C. After 24-48h incubation the prepared dishes were opened, the top filter papers removed and the dishes were resealed and incubated again. After 8-10 day's incubation, the nematodes were ready for harvest.

To harvest the nematode IJs, a trap was made by wrapping the lid of a 6cm Petri dish externally with filter paper and placing it open-side down in a larger dish. The larger dish was filled to a depth of 2-3mm with distilled water so that the filter paper came into contact with the water surface. The nematode infected insect cadavers were carefully removed from the Petri dish and about 40 of them were placed on the top of each filter paper-covered lid. After the emergence of the IJs had been observed for 2 days, they were harvested by rinsing the larger dish and collecting the nematode suspension. To remove unwanted insect tissue and noninfective stage nematodes, the collected nematode suspension was poured into a 500-mesh sieve positioned over a collection container.

The container was filled with enough distilled water so that the sieve screen came into contact with the water surface. The active IJ nematodes were left to migrate through the sieve overnight, and the resulting IJ suspension was washed twice with distilled water. Finally, the IJs were pumped into a plastic bag containing a wet sponge and stored at 4°C until future use.

B. Sources and preparation of test bacteria

i). Sources of test bacteria

Bacterial cultures were generously provided by the following individuals: *Bacillus subtilis*, by Dr J. E. Rahe, Department of Biological Sciences, Simon Fraser University; *Rhizobium phaseoli* TAL 182, by Dr. F. B. Holl, Department of Plant Sciences, *Salmonella typhimurium* SL1344 by Dr. B. Findley, Biotechnology laboratory, the University of British Columbia, Vancouver; *Escherichia coli* PA63, PA67 and *Staphylococcus aureus* by Dr. A. Clarke, Department of Microbiology, St. Paul's Hospital, Vancouver, B. C.; *Bacillus thuringiensis* subsp. *kenyae* IP1, *Proteus vulgaris* MAC 774, *Pseudomonas aeruginosa* ATCC9026, *Alcaligenes faecalis* ATCC8750, *Enterobacter aerogenes* MAC438, *Serratia marcescens* MAC21 and *Azotobacter chroococcum* MCD1, by Dr. G. B. Dunphy, Department of Microbiology, Macdonald College, McGill University.

ii) Preparation of test bacteria

Upon receipt of the cultures, all bacteria, except for *R. phaseoli* and *A. chroococcum*, were cultured on nutrient agar(NA, see appendix III). *R. phaseoli* was cultured on yeast extract-mannitol agar(YMA, see appendix VII) and *A. chroococcum* was cultured on Cron's nitrogen-free media (CNFM) (Pawsey, 1974), (see appendix I). *A. chroococcum* and *R. phaseoli* were maintained at room temperature, and the remainder were maintained at 4°C after first being cultured at room temperature.

iii). Preparation of spore suspensions

As one of the most commonly used bacteria for bioassay of antibiotics and very sensitive to antibiotics produced by *Xenorhabdus* spp. *B. subtilis* was chosen as the standard bacterial indicator of antibiotic activity, and a standard spore suspension was made. The procedure was a modification of Hewitt and Vincent (1989) as follows:1) 10ml of bacterial broth was grown overnight at 37°C on tryptic soy broth(TSB), 1ml of the bacterial broth was transferred into a one litre flask with 500ml TSB; 2) the flask was incubated at 37°C, and shaken at 80rpm for 10 days; 3) the bacterial spores and cells were collected by centrifuging at 10,000rpm for 5min and washed three times with sterile water; 4) the spores and cells were resuspended in a sterile 0.9% NaCl solution; 5) the suspension was pasteurized at 80°C in a water bath for 20 min; 6) a concentrated spore suspension of $6x10^9$ colony-forming-units(CFU)/ml was made from the pasteurized saline

suspension and the spore suspension was then transferred into 50ml tubes with screw caps and stored at 4°C until required.

C. Preparation of *Xenorhabdus* spp.

i). To isolate *Xenorhabdus* spp., *Galleria* larvae were infected with *Xenorhabdus* spp. by their respective nematode associates using about 25 IJs/larva, as described in section 2-1.A. After 24-48h of infection the insect cadavers were surface disinfected by dipping them into 95% ethanol and igniting them. Then the cadavers were aseptically opened with sterile forceps and the hemolymph was streaked onto NBTA (see appendix II).

ii). To obtain pure *Xenorhabdus* spp. cultures, bacterial colonies which resembled the primary forms of *Xenorhabdus* were subcultured onto fresh NBTA plates. After 2-3 days of incubation in the dark at 24°C, the bacteria were checked under the microscope for their cell size, motility, and gram stain. Their antibiotic activities against *B. subtilis* were tested on agar plates (see section 2-1D below).

iii). To obtain the secondary form of *Xenorhabdus* spp., the primary form culture was maintained at 24°C. A large proportion of bacterial colonies became secondary after about 20 day's maintenance without subculture, and those secondary colonies were subcultured onto NBTA and tested for antibiotic activity(see section 2-1D).

iv). Two methods were used to maintain *Xenorhabdus* spp. For short-term maintenance, bacterial cultures grown on NBTA

were incubated at 24°C and subcultured on NBTA every 2 weeks. For long-term maintenance, bacteria were grown in TSB for 24h, centrifuged and suspended in TSB containing 15% glycerol and then stored at -20°C.

D. Bioassay of antibiotic activity

Before testing, all test solutions from spent bacterial culture media of *Xenorhabdus* species or insect extracts were neutralized with 6N HCl and centrifuged at 10,000rpm for 20 min. at 4°C. Then they were sterilized by filtration through 0.2μ-pore filter(Millipore, Bedford, MA)

An agar diffusion bioassay (Hewitt and Vincent, 1989) was employed throughout the whole study. Except where otherwise stated, all antibiotic activity tested on agar plates followed the procedure outlined below.

The bacterial agar-based media were autoclaved for 15 min and held in a water bath at 50°C for 1h. Aseptically, 10ml of media were poured into each 10cm Petri dish. After cooling, the plates were stored at room temperature until required.

Test bacteria were cultured in their suitable broths, shaken at 80rpm at 25°C over-night and 100μl of the bacterial culture or *B. subtilis* spore suspension(see 2-1.B) was inoculated onto each assay plate. The inoculum was evenly spread on the plate with a sterile spreader. The plates were dried in a laminar air-flow with their lids open for 20-30 min.

Before test solutions were added to the plates, 2-4 wells, each of 0.5cm diameter, were cut in the inoculated agar with a alcohol-flame sterilized cork borer and the agar plugs were removed with sterile forceps. Then 50μl of test solution were added into each well, the plates were covered with their lids and incubated at 25°C. After 24h incubation, the diameter of the inhibition zone of the test bacteria was measured with hand-held calipers.

2-2. General observations and tests of *Xenorhabdus* spp.

To decide the conditions for short term laboratory maintenance of *Xenorhabdus* spp., the following tests were conducted: a) The primary forms of *X. nematophilus* and *X. luminescens* were cultured separately on NBTA at 24°C for 4 days. Then three plates of each species were moved into a 4°C refrigerator. After 1, 5, 10, 15, 20, 25 and 30 day's treatment at 4°C, the bacterial colonies from 3 parts of each plate were streaked separately on NBTA and incubated again at 24°C. After 4 day's incubation, the viability of the colonies of each species was recorded. b) Each last-instar larva of *Galleria* was injected with 5μl of a 24h-old culture of *X. nematophilus*. One day after larval death, the cadavers were refrigerated at 4°C. After 5, 10, 30 and 60 day's incubation, three cadavers were sampled and the bacteria were isolated as described in 2-1C and incubated in the dark for 3-4 days at 24°C, after which the bacterial form and viability were checked according their growth on the plate.

It was observed that colonies of *Xenorhabdus* isolated from different insect cadavers killed by the same nematode strain had different colony colors on NBTA, therefore, the following observation and experiment were conducted. To observe the form variation, *Xenorhabdus* spp. were isolated from insect cadavers as described in section 2-1C. The bacteria were streaked onto plates containing 10ml NBTA and incubated in the dark at 24°C. After 4 day's incubation, the plates were checked for dye absorption by the bacteria. Those colonies which appeared obviously different in color from others of the same species, were subcultured separately on NBTA again. After 4 day's incubation, the pure cultures of these colonies were cultured in TSB, four replicates for each of the isolates, them the antibiotic activity, pH level and optical absorbance of the cultures were measured 6 days after culture.

The growth curves of both primary and secondary forms of *X. nematophilus* (from DD136) were determined by adding 0.1ml of the bacterial inoculum(see 2-3.1A, below) to 100ml TSB in 250ml sterile flasks, shaking at 80rpm, three replicates for each form. The growth was measured spectrophotometrically at 540nm at different incubation times. To determine the form changes during the culture, a serial dilution was made from each sample, plated on NBTA and after 4 day's incubation, the number and the proportion of primary and secondary colonies(very light blue, translucent) were counted. To record the pH change during the incubation, the pH of each of

the above culture samples was measured, using a Fisher 220 pH meter.

2-3. Antibiotic activity of *Xenorhabdus* spp. *in vitro* and *in vivo*

Except where otherwise stated, all procedures were conducted at 25°C.

2-3.1. *In vitro*

A. Inoculum preparation.

Inocula of *Xenorhabdus* spp. were prepared by adding one loopful of overnight stock culture to 50ml of TSB in 100ml Erlenmeyer flasks. Cultures were shaken at 80rpm on a horizontal, gyratory shaker for 24h.

B. Effect of abiotic factors on antibiotic activity of *Xenorhabdus nematophilus*

The antibiotic activity of *X. nematophilus* at different temperatures, nutrition and pH levels was determined by inoculating 0.1ml of the above *X. nematophilus* inoculum into 100ml of culture media in 250ml flasks, three replicates for each treatment and shaking them at 80rpm on a horizontal shaker. After 6 day's incubation, the cultures were treated and the antibiotic activities were tested by bioassay on agar plates as described in section 2-1D, using *B. subtilis* as the indicator. The conditions of temperature, pH, nutrition and aeration were established as follows: 1). The effect of temperature on antibiotic activity was measured by culturing

X. nematophilus in flasks of TSB and the inoculated flasks were incubated separately at 15, 20, 25, 30 and 35°C. 2). To determine the effect of pH on the antibiotic production the bacteria were cultured in TSB where the pH had been adjusted using 6N HCl and 6N NaOH to 5.8, 6.4, 7.0, 7.6 or 8.0. 3). The effect of nutrition on antibiotic activity was determined by culturing the bacteria separately in TSB or 1% peptone water (four replicates). 4). The effect of aeration on antibiotic activity was determined by inoculating 0.1ml of the *Xenorhabdus* inoculum into 10ml of TSB in 20ml sterile, polypropylene test tubes. The inoculated test tubes were treated in one of three different ways, namely: a) aerobic, incubated on a horizontal, gyratory shaker at 80rpm ; b) incubated statically; c) anaerobic, put in a boiling water bath for 10min before inoculation, allowed to cool to room temperature, inoculated and the surface of the medium was covered with a layer of sterile, melted Vaseline and the tubes were incubated statically.

C. Determination of antibiotic concentration and potency

To determine antibiotic concentration(Nielsen *et al.*, 1990), serial, twofold dilutions were made with filter-sterilized test solutions of spent *X. nematophilus* culture media(see 2-1D) and sterile water. From each dilution, 5µl were placed on the surface of a TSA(TSB with 1.5% agar) plate seeded with a 0.1ml spore suspension of *B. subtilis*. Then the plates were incubated at 25°C for 24h. One arbitrary unit(AU) was defined as 5 µl of the highest dilution of the test

solution causing a definite zone(ø ≈ 4mm) of inhibition on the lawn of the indicator, *B. subtilis*.

To determine minimum inhibitory concentrations and minimum killing concentrations, serial, twofold dilutions were made with the filter-sterilized test solution of spent *X. nematophilus* culture media and sterile TSB. To each 20ml tube containing 10ml of diluted media, 100µl of bacterial culture of *B. subtilis* were added and the tubes were incubated and shaken at 80rpm. The minimum inhibitory concentration was determined as the lowest concentration of test solution which prevented visible turbidity in tubes after 24h. Each of the above tubes showing no turbidity was vortexed, and 0.1 ml of its contents was transferred to 10ml of TSB in a 20ml tube and incubated at 25°C. The minimum killing concentration was defined as the lowest original test concentration allowing no visible turbidity in the tubes after 24h's incubation.

D. Comparison of antibiotic activity of *Xenorhabdus* spp.

Inocula of all *Xenorhabdus* spp. isolated from nematodes listed in Table 3 were prepared as described in 2-3.1A. One milliliter of bacterial inoculum was added to each 2,000ml flask containing 1000ml TSB creating two replicates for each species. The inoculated flasks were incubated at 25°C and shaken at 80rpm on a horizontal shaker. After 6-day's incubation, the cultures were bioassayed on agar plates as described in 2-1D.

2-3.2. *In vivo*

Last-instar larvae of *G. mellonella* were infected by either IJs or pure *Xenorhabdus* culture. The procedure for infecting the insect larvae with IJs was as described in 2-1.1.A. except that the concentration of IJs used was 650 IJs/ml. To infect the insect larvae with pure bacteria, the bacterial cell number of a *Xenorhabdus* culture was determined under a microscope using a hemocytometer. Each insect was aseptically injected in the haemocoel with 5µl of the required *Xenorhabdus* culture, at a concentration of $5x\ 10^6$ cells/ml, using a microinjector. After the injection, the insects were sealed in wet Petri dishes and incubated at 25°C. From each treatment of both nematode- and pure-*Xenorhabdus*-killed insects, 20 insect cadavers were sampled daily for 7 days after the insect's death. The cadavers sampled on each day were divided equally into two groups, crushed and mixed with distilled water at a ratio of one larva/ml. The mixtures were then stirred on a magnetic plate for 20min. To remove insect tissue and fat droplets the mixtures were centrifuged at 7,000rpm for 20min at room temperature. The supernatants were neutralized by 12N HCl and centrifuged again at 10,000rpm for 20min. The antibiotic activity of each supernatant was tested on agar plates as described in section 2-1D.

2-4. Antibiotic properties and separation

The spent culture medium of *X. nematophilus* (from DD136) was used for the following experiments, the culture solutions were prepared and bioassayed on agar plates for their antibiotic activity as described in section 2-3.1D and 2-1D.

A. Effect of dialysis on antibiotic activity.

The culture of *X. nematophilus* was dialysed in running distilled water overnight with two replicates. Three types of dialysis tubings were used with molecular weight cut-off (MWC) ranges of 8-9,000, 12-14,000 and 25,000. Antibiotic activity of the dialysates were tested on agar plates as described in section 2-1D. Since this experiment was designed to test the qualitative effect of dialysis no volume adjustment of the dialysate was conducted.

B. Properties

Three experiments were done on the *X. nematophilus* to test the antibiotic products for their sensitivity to heat, to UV and their stability in storage.

i). Sensitivity to heat

The test solutions were heated either at 20, 40, 60, 80, or 100°C in a water bath for 20min, or autoclaved at 121°C for 20min, with three replicates for each treatment. After heat treatment all the solutions were cooled to room temperature and bioassayed.

ii) Sensitivity to sunlight.

Into each of 12 3ml quartz photometer test tube 2ml of test solution was added and to minimize evaporation each tube was plugged with its lid and sealed with tape. Then the test tubes were exposed to direct sunlight, three tubes were moved into a 4°C refrigerator each time, after treatment of 0.5, 1, 3 and 7 hour. To ensure maximum sunlight absorption the tubes were tilted at an angle of about 45° with their transparent faces towards the sun. The position was adjusted every hour from the beginning of the test at 10:30 A.M. to the end at 5:30 P.M., as the sun moved. After treatment the antibiotic activity of each solution was tested.

iii). Stability in storage.

To test the antibiotic stability, 100 ml of 6-day-old *X. nematophilus* culture was prepared as described in 2-3.1D, of which 50 ml was centrifuged at 10,000 rpm for 20 min. at 4°C, sterilized by a 0.2μ-pore-size filter, and poured equally into four sterile 50ml test tubes. The tubes containing the culture with or without the cells of *X. nematophilus* were closed with their screw caps and stored in the dark at 24°C for 5, 10, 30, 60 and 90 days, after which the antibiotic activity of each culture was neutralized and tested.

C. Separation

To eliminate the interference of the culture media on antibiotic activity the antibiotic was extracted by ethyl acetate. The separation procedure was similar to that used by Richardson *et al.*(1988). Spent bacterial culture media of *X.*

nematophilus were prepared as described in section 2-3.1D and 2-1D but without sterilization. One litre of cell-free bacterial culture was mixed with 2L of ethyl acetate and shaken vigorously for 1h. The ethyl acetate fraction was collected and the aqueous fraction was extracted again with ethyl acetate. After three such extractions, the ethyl acetate fractions were combined, concentrated on a rotary evaporator at 25°C and the dried residue was suspended into 30ml distilled water. The antibiotic activity of the ethyl acetate and aqueous fractions were tested on agar plates as described in section 2-1D.

2-5. Effect of the antibiotics on soil bacteria in Petri dishes.

Agar diffusion bioassay (see 2-1D.) was used to test the antibiotic activity of *X. nematophilus*, from either *in vitro* or *in vivo Galleria* culture, against the soil bacteria listed in section 2-1B. All bacteria were grown in TSB and tested on TSA, except for *A. chroococcum* and *R. phaseoli*. For *A. chroococcum*, CNFM was used as for inoculum preparation and test. The procedure was as follows: a streak-plate of *A. chroococcum* was incubated for 4 days, the bacterial growth was scraped from the agar and transferred into a 10 ml test tube containing 5 ml of sterile 0.9% NaCl solution. The cell suspension was homogenized by manual shaking for about 30sec. and the suspension was spread immediately onto test plates that were incubated for 4 days before the growth was

recorded. For *R. phaseoli*, the inoculum was grown in yeast extract-mannitol broth(see appendix VI) and tested on YMA. Test plates were incubated at 25°C for 2 days before recording.

2-6. Tests in sterile and garden soils

2-6.1 Preparation and test of soil

A. Preparation of soil

Soil was collected from grassland areas on the Simon Fraser University campus. A sample of the top 10cm of soil was taken, manually mixed, spread out and dried at room temperature for about one month. Then plant debris and stones were removed by sieving the soil through a 30-mesh sieve. The sieved soil was stored at room temperature until required.

The soil, which has a composition of clay:4%, silt:18% and sand:78% with 2.72% of organic matter and pH, 5.73, was analyzed in the Department of Renewable Resources, McGill University.

To prepare sterile soil, the soil was autoclaved three times at 121°C for 30 min, with a 3 day interval between each autoclaving. The sterility was checked by adding one gram of the soil to each TSA plate and incubating them at room temperature. If bacterial growth occurred, a fourth autoclaving was done.

B. Culture methods for bacterial population in soil

The methods used to investigate bacterial population of soil in this study were culture methods, modified from Wollum

II (1982) and Herbert (1990). After treatment, the soil bacterial population was investigated as outlined below:

i). Preparation of soil dilutions

For each sample, a dilution bottle containing 95 ml diluent of 0.9% NaCl was made. To make a series of soil dilutions, several 90ml dilution blanks were made with the same diluent. All dilutions were autoclaved at 121°C for 20 min. and allowed to cool to room temperature. Then 10g of the soil sample(see 2-6.2D; 2-6.3B, below) were transferred into a bottle containing the 95ml of diluent. The bottle was shaken vigorously by hand for 30 sec. Immediately thereafter, 10ml of the soil suspension was taken from the center of the suspension and added to a fresh 90ml blank. This bottle was shaken and 10ml of the suspension was removed from it as described above. This sequence was continued until the desired dilution was reached.

ii). Preparation of plates.

On the basis of previous experience with the samples, a range of three of the above dilutions which produced the optimum number of organisms for counting were chosen. Either 1ml or 0.1ml of each selected dilution was transferred into each of three Petri dishes. Before each pipetting, the dilution was drawn up and pushed back into the bottle at least three times to ensure a well mixed dilution. This process was carried out at all dilutions. Tryptic soy agar(See appendix V) (Martin, 1975) was used for test bacteria.

The media were prepared as follows: After being autoclaved, the containers of media were put in a water bath for 1-2h to allow the media to cool to 48°C. For pour plates, 10-15ml of the medium were poured into each Petri dish containing 1ml soil dilution. Immediately after pouring, the plates were carefully and thoroughly swirled to mix their contents. For spread plates, 10 to 15ml of autoclaved media were poured into each Petri dish before adding the soil dilutions. The plates were allowed to solidify and dry on a laminar air-flow bench overnight or alternatively, stored at room temperature for several days before use. Then 0.1ml of soil dilution was transferred into each plate and spread with a sterile spreader on the agar surface.

iii). Incubation and counting

All plates were incubated at 24°C in the dark. Bacterial colonies were counted after incubating for 2 days. The plates from the dilutions which yielded from 20-300 colonies per plate were selected for counting.

2-6.2. Antibiotic production and persistence of *Xenorhabdus* in sterile soil

A. Persistence of *Xenorhabdus* spp. in sterile soil.

To determine the persistence of *Xenorhabdus* spp. in sterile soil, the following experiments were performed. Primary forms of *X. nematophilus*. and *X. luminescens* were cultured separately in 50ml flasks each containing 25ml TSB, six flasks for each species, and were shaken on a horizontal

shaker at 80 rpm for 24h. To decide if washing causes fatal cell damage or not, cultures of three flasks from each species were washed three times by centrifugation(10,000rpm, 5min.) with sterile water(10ml) and after each washing, a 0.1ml sample of the bacterial contents was plated on NBTA. After washing, the bacteria were resuspended into 25ml sterile water. Each flask of washed bacterial culture and the half that was not washed were then added separately to a 50g sample of the sterile soil, mixed and incubated at 24°C in the dark. Bacterial survival was tested by spreading 1g of the inoculated soil on each NBTA plate at intervals of 1, 5, 10, 20, 30 and 60 days. The plates were incubated in the dark at 24°C and examined for viable colonies after 3 days.

B. Antibiotic production of *X. nematophilus* and *X. luminescens* in soil

To test antibiotic production in soil(Dhingra and Sinclair, 1985), *X. nematophilus* and *X. luminescens* were cultured in 25 ml of TSB in 50 ml flasks and shaken at 24°C for 24h. Then the bacterial cultures were poured into 50g of the sterile soil and mixed, three replicates for each species. In the control the sterile soil was mixed with sterile TSB. After being incubated for 5, 10 and 15 days, 5g of the soil was, aseptically, transferred into separate 10cm Petri dishes, then 10ml of 48°C TSA were poured into each Petri dish and mixed thoroughly with the soil. When the agar was solid and cool, 0.1ml of 24h-old *B. subtilis* culture was inoculated into each plate. To clarify the effect of TSB

three plates of TSA without soil were inoculated with the *B. subtilis* culture. After 24hr incubation at 24°C, the growth of *B. subtilis* was observed.

C. Antibiotic persistence in sterile soil.

Seven milliliter spent culture medium of *X. nematophilus*, prepared as in section 2-3.1D and 2-1D, were mixed with each 10g of sterile soil in a 50 ml tube with screw cap, and incubated. To assay the antibiotic activity in the soil, another 3 ml of sterile water were added to each tube and the tubes were vortexed, 0.5h after incubation, spun at 7,000 rpm for 5 min and the supernatants assayed for their antibiotic activity on agar plates. This experiment was repeated three times with three replicates each time.

D. Antibiotic activity against *B. subtilis* and *R. phaseoli* in sterile soil.

As the result of unsuccessful separation of the antibiotics from the the bacterial culture of *X. nematophilus*, all antibiotic solutions used below and in 2-6.3A are spent bacterial cultures of *X. nematophilus*. To prepare the inoculum, *B. subtilis* was cultured on TSB overnight. The bacteria were washed three times with sterile water by centrifugation and a bacterial suspension of washed *B. subtilis* was made. *R. phaseoli* was grown on YMA, and after being incubated for 2 days at 24°C, the bacterial growth was scraped from the agar and transferred into a 25 ml tube containing 10 ml sterile water. The cell suspension was homogenized by manual shaking. Before being inoculated into

sterile soil, the number of bacteria per milliliter were counted under a microscope using a hemocytometer. Bacterial inocula containing $6x10^7$ cells/ml of *B. subtilis* and $5x10^8$ cells/ml of *R. phaseoli* were made. Then 5ml of these inocula were added separately into each 10g of sterile soil in 6cm Petri dishes, four replicates for each species. The inoculated dishes were sealed and incubated at 24°C. The bacteria were allowed to colonize the soil, 1 day for *B. subtilis* and 2 days for *R. phaseoli*. The dishes were then treated by adding 5ml antibiotic solution(see 2-1D) of *X. nematophilus* into each dish, for controls, the same volume of water or TSB was added to each dish, and allowing the dishes to dry in a laminar air-flow with their lids open until the weight of each dish was the same weight as that before the treatment. The dishes were covered with their lids, sealed with Parafilm and incubated at 24°C. In order to show the antibiotic activity in the soil the bacterial populations of these soils were estimated on spread plates as described in section 2-6.1B after incubating for 2 days(*B. subtilis*) or 4 days *(R. phaseoli)*.

2-6.3. Tests in garden soil

A. Effect of *Xenorhabdus* cultures on bacterial population and total microbial activity in nonsterile soil

Tests on the effect of the antibiotic solutions were made by adding 5ml of the *X. nematophilus* antibiotic solution(2-3.1D and 2-1D) into each 10g sample of soil(see section 2-6.1) in 6cm Petri dishes and mixing them thoroughly. As no antibiotics was produced by the secondary form of *X. nematophilus*, dishes treated with spent secondary form culture medium of *X. nematophilus*, TSB and water were used as controls. The dishes with the treated soils were covered, sealed with Parafilm and incubated at 24°C. The bacterial populations were assayed on spread plates after incubating for 1, 2, 3, 4, 6 and 8 days as described in section 2-6.1B, three replicates for each day.

To measure the total microbial activity of the soil, fluorescein diacetate (FDA) hydrolysis was used as an estimator(Schnürer and Rosswall, 1982). The soil was treated the same as described above and the method for the measurement of FDA hydrolysis was as follows: FDA solution was prepared by dissolving 0.1g FDA powder(Sigma Chemical Co. St. Louis, MO) in 50ml acetone(Sigma) and the solution was stored at -20°C until required. For determination of FDA hydrolytic activities, each 10g (dry weight) soil was suspended in 50ml sterile 60mM sodium phosphate buffer, pH

7.6. Immediately, 35µl of the FDA solution was added to the soil suspension, and the suspension was incubated at 24°C on a rotary shaker for 15 min. After incubation, the large soil particles of the suspension were removed by centrifugation at 7,000rpm for 3 min., the supernatants were put on ice and their optical absorbances at 490nm were measured. This experiment was conducted for a period of 6 days with three replicates for each day.

B. Effect of insect cadavers on soil bacterial populations

The effect on the soil bacteria of insects killed by *X. nematophilus*, and the antibiotic persistence was tested. Last-instar larvae of *G. mellonella* were infected with primary and secondary cultures of *X. nematophilus* as described in section 2-3.2. When they died, each insect cadaver was punctured 10 times with a dissecting needle 2 days after death. To prevent the bacteria inside the cadavers from moving into the test soil, either 1 or 5 insect cadavers were placed in a dialysis tubing which had a MWC of 25,000; 0.1ml of sterile water was added to each cadaver inside the tubing and the ends of each tubing were sealed with a tubing closure. This system allowed the antibiotic but not the bacteria to enter into the soil. Each tubing was buried in 10g of soil in a 6cm dish, 7.5ml sterile water was added to each dish, which was covered, sealed and incubated at 24°C, 3 replicates for each treatment. After incubation of 2, 4, 6 and 10 days, the dialysis-tubing package was removed from the

dish. The bacterial population in the soil was assayed on pour plates as described in section 2-6.1B at the same time.

To test the persistence of the antibiotic from *X. nematophilus*-killed insects in the soil, the above dialysis tubings were carefully opened and the insect cadavers inside were removed. These insect cadavers were then extracted in the same way as described in section 2-3.2 and the antibiotic activity of the extracts was tested on agar plates as described in 2-1D.

2-7. Data analysis

All data were analysed on SAS software; Student's *t-test* (P=0.05) was used for differences among means. Data are presented as the mean $\pm$ standard error of the means.

Chapter 3. Results

3-1. General observations and tests of *Xenorhabdus* spp.

A. Isolation and observation of the form variation of *Xenorhabdus*

In 1-2 days after insect death(see 2-1C) *Xenorhabdus* spp. could be isolated from *Galleria* larvae killed by nematodes. After that time it was very difficult to obtain *Xenorhabdus*. By 4 days after the insect's death *X. nematophilus* was never isolated from the insects killed by nematode strain DD136. At or after 4 days the bacteria isolated from the insect cadavers were always catalase positive and red on NBTA which are characteristics of contamination by other bacteria. Noticeably, these insect cadavers from which no *Xenorhabdus* were isolated, but only contaminated bacteria were isolated 4 days after insect death, have the typical yellowish brown color and could produce nematodes normally. Similarly, when *X. luminescens* was isolated from insects killed by heterorhabditids, *X. luminescens* could be obtained only during the 1st and 2nd days after infection

The colonies of *Xenorhabdus* spp. isolated from different insect cadavers killed by the same strain of nematode, varied in color on NBTA(see 2-2). This phenomenon occurred in isolates of *Xenorhabdus* from insects killed by *Steinernema* or *Heterorhabditis*. In isolates of *X. bovienii* from *S. feltiae* strain A21-1, the color of colonies varied

from greenish blue to dark blue. When these colonies were exposed to normal room light, the colony color changed from greenish to blue and eventually all became dark blue after more than 6h exposure. When these colonies were cultured separately in TSB broth, the color of the cultures appeared quite different, from light yellow to dark yellow; they showed small differences in their final cell densities and in the pH of the cultures 6 days after incubation. The antibiotic activities of these cultures were also different(Table 4).

When the primary form of *X. nematophilus* (from DD136) was inoculated and cultured in TSB(see 2-2), only a very small proportion (about 0.5%) of secondary form colonies were obtained after 6 day's incubation at 25°C. Both primary and secondary forms had very low plate efficacy when cultured in TSB. No primary form was obtained from the culture inoculated with the secondary form. When NBTA plates of *Xenorhabdus* spp. were maintained at 24°C in the dark(see 2-1C), the change from primary to secondary form began after 6-16 day's incubation, depending on the strain and isolate. For *X. luminescens* the change began as early as 6 days. For *X. nematophilus*, the change began in about 12 days and after 20 day's incubation without subculture, about 30-40% of the colonies had changed to secondary. In plates of *X. bovienii* from *S. feltiae* strain A21-1, about 20% of isolate #1 , 5% of #2 and 1% of #3 changed into secondary forms.

Table 4. Final absorbance, antibiotic activity and pH level of three isolates of *Xenorhabdus bovienii*, isolated from *Steinernema feltiae* strain A21-1, cultured in TSB, shaken at 80rpm and incubated in the dark at 24°C for 6 days.

Isolates	Absorbance *	Inhibition zone(cm) +	pH @
1	2.16±0.01a #	2.29±0.02a	8.1
2	2.38±0.01b	2.04±0.07b	8.2
3	2.26±0.00c	2.40±0.16a	7.8

*. Final absorbance at 540nm(n=4)

+. Antibiotic activities against plated *Bacillus subtilis*, expressed as the diameter of the inhibition zone(n=12).

@. The final pH of the bacterial cultures.

Expressed as means ± SE., means in a column with the same letter are not significantly different.

In *X. nematophilus*, no secondary to primary form change was observed on NBTA after 20 day's incubation without subculture.

B. Temperature for the maintenance of *Xenorhabdus*

The effect of temperature on maintenance(see 2-2) of *Xenorhabdus* spp. was not consistent. Colonies from some plates of both *X. nematophilus* and *X. luminescens* were not viable after 4-5 day's incubation at 4°C whereas others remained viable. Both species showed slower growth and poorer colony-forming than the untreated ones on NBTA after 15 day's treatment. The bioluminescence of *X. luminescens* was also decreased after 10 day's treatment. No changes of the primary to the secondary form or *vice versa* were observed in either species after 15 day's treatment at 4°C. Eventually, all *Xenorhabdus* lost their viability 25 days after having been stored at 4°C. When insect cadavers, killed by the primary form of *X. nematophilus*, were maintained at 4°C bacterial cells of reduced viability were isolated after one month. The primary form bacteria maintained their viability after two month's storage at 4°C, and no secondary form bacteria were isolated from the insect cadavers even after two months.

C. Growth of *Xenorhabdus nematophilus*(see 2-2)

The growth of both primary and secondary forms of *X. nematophilus* (from DD136) were different from each other. When overnight cultures of both forms were inoculated in TSB the lag phase of the secondary form lasted much longer than that of the primary. The final absorbance at 540nm between

the cultures of primary and secondary forms of *X. nematophilus* had a difference of 0.3 after 132hr's culture(Fig.2 A.). During culture, the pH of the primary form decreased in the first 20 hr from 7.0 to 6.3 and then increased gradually thereafter. However, little change of pH was recorded in the secondary form culture during the first 32 hrs. Thereafter, it had a similar rate increase to that of the primary form but there was a 0.3 difference in the final pH between the primary and secondary forms(Fig.2 B).

3-2. Antibiotic activity/production of *Xenorhabdus* spp. *in vitro* and *in vivo*

3-2.1. *In vitro*

A. Effect of abiotic factors on antibiotic activity(see 2-3.1B)

(i). Temperature.

Antibiotic activity of *X. nematophilus*(from DD136) was greatly influenced by temperature in that it gradually increased with culture temperature from 15°C to reach a maximum at 25°C. No antibiotic activity was detected in cultures grown at 30°C or 35°C (Fig.3 A).

(ii). pH level

When the primary form of *X. nematophilus* was cultured in TSB at a range of pH from 5.8 to 8.0, the antibiotic activity of the bacterial cultures showed significant increases from those at pH 5.8 to those at pH 7.0 after 6 day's culture

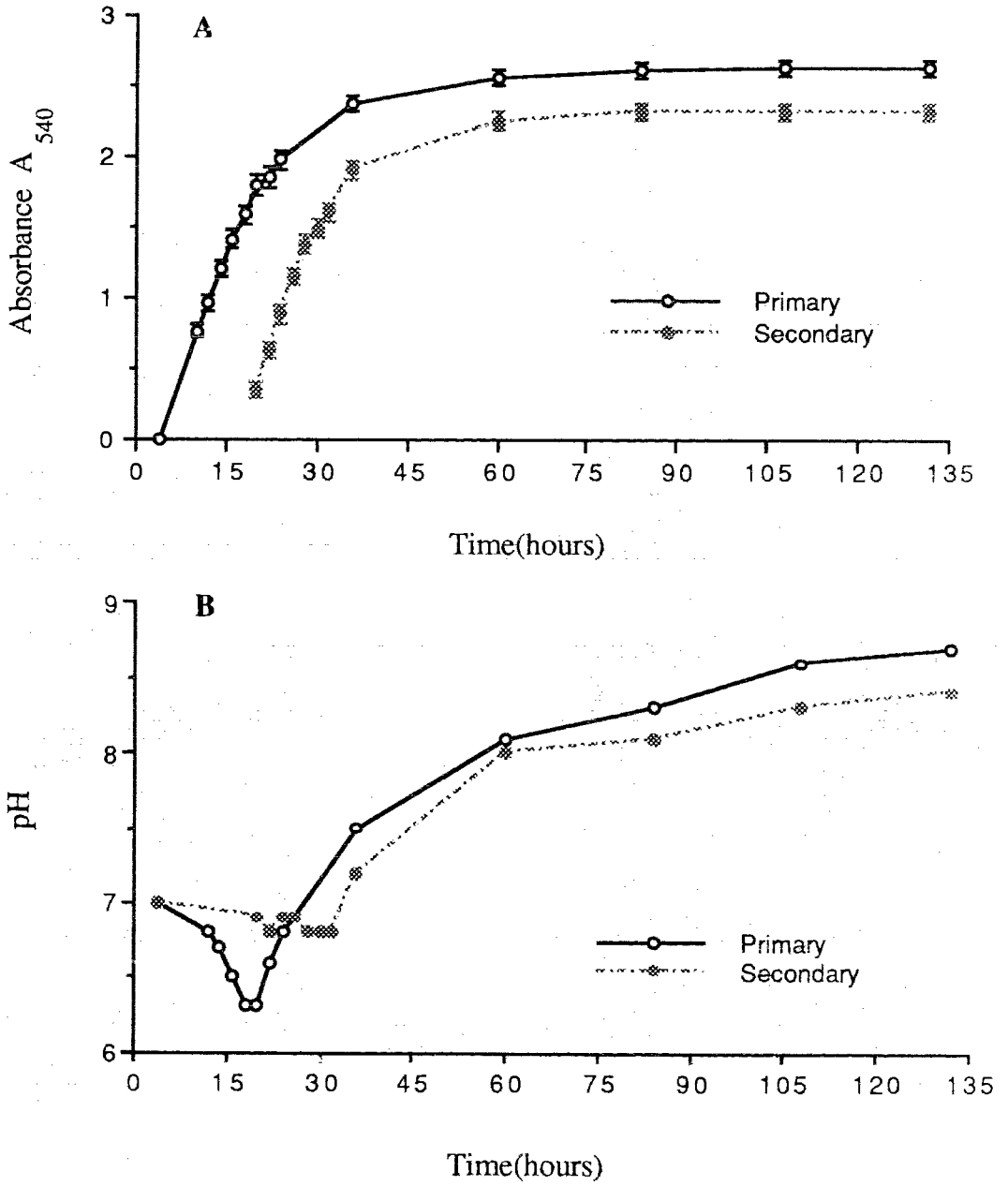

Fig.2.Growth and culture pH of the primary and secondary forms of *Xenorhabdus nematophilus* in TSB. Expressed, as in A the absorbances at 540nm, and in B the pH of the bacterial cultures during 132 hours(n=3).

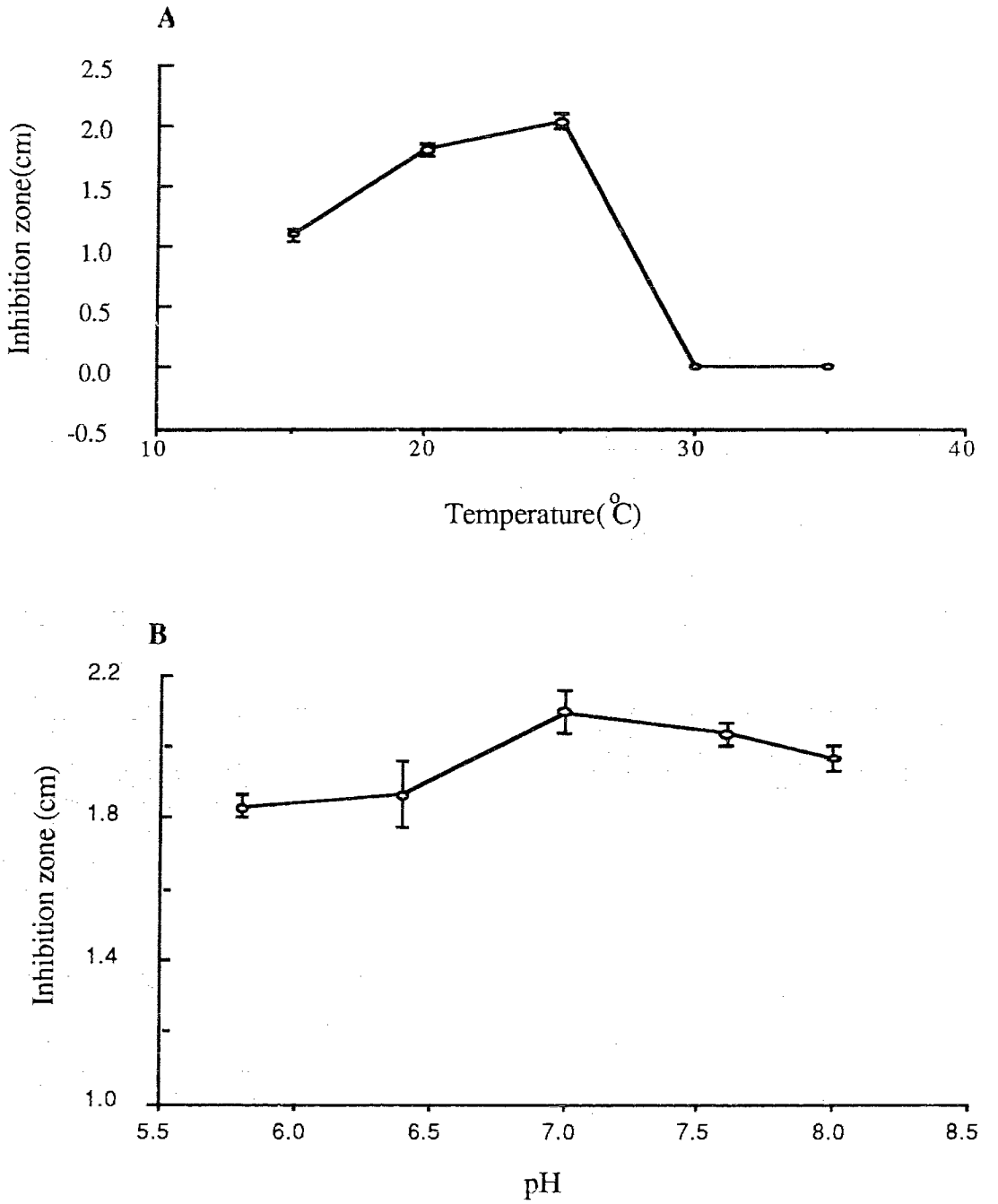

Fig.3. Antibiotic activity of *Xenorhabdus nematophilus* cultured in TSB at different temperatures (A) and pH levels (B) after 6 days. Expressed as the diameter of the inhibition zone on plated *Bacillus subtilis* (n=9).

Thereafter, the antibiotic activity decreased modestly but significantly as the pH increased from 7.0 to 8.0 (Fig.3 B).

(iii). Aeration

When *X. nematophilus* was cultured in TSB under different aerobic conditions, antibiotic activity was detected from the 2nd day in both shaken and static cultures. The activity increased in all aerobic cultures with time and reached a maximum by the 6th day. No antibiotic activity was detected in any of the anaerobic cultures. The antibiotic activity of cultures under static and shaken conditions in test tubes were similar to each other(Table 5).

(iv). Nutrition

When the primary form of *X. nematophilus* was cultured in 1% peptone water, the growth was poor and no antibiotic activity was detected after 6 days of incubation (Table 6).

B. Antibiotic concentration.

The *X. nematophilus* culture had 3200 arbitrary units(AU)/ml against *B. subtilis*. The minimum inhibitory concentration against *B. subtilis* was 50 AU/ml and the minimum killing concentration against *B. subtilis* was 100 AU/ml.

C. Comparison of antibiotic activities of *Xenorhabdus*

The antibiotic activity of six different *Xenorhabdus* spp. isolates when cultured in TSB(see 2-3.1D) were not the same.

Table 5. Antibiotic activity of *Xenorhabdus nematophilus* cultured in TSB at 24°C under different conditions of aeration over time as measured by the diameter of the inhibition zone(cm) using *Bacillus subtilis* as the target organism on TSA

Time (days)	Treatment		
	Aerobic (shaken)	Aerobic (static)	Anaerobic (static)
1	0±00a*	0±00a	0±00a
2	1.40±0.14a	1.33±0.06a	0±00b
3	1.50±0.00a	1.57±0.18a	0±00b
4	1.53±0.06a	1.63±0.06a	0±00b
6	1.67±0.03a	1.67±0.06a	0±00b

*Expressed as the mean(n=9) ± SE, means in a day with the same letter are not significantly different.

Table 6. Antibiotic activity and final absorbances of *Xenorhabdus nematophilus* cultured in different media for 6 days.

Media	Number of replicates	Inhibition zone(cm)*	Final absorbance+
Standard TSB	3	2.06±0.04a#	2.63 ± 0.02a
1% peptone water	4	0.00±0.00b	0.31 ± 0.01b

*Expressed as mean ± SE diameter of inhibition zone(n=9) on plated *Bacillus subtilis*; + the absorbances at 540nm; # means in a column with the same letter are not significantly different.

The cultures from BC1, BC2 and A32-6 showed the strongest activity whereas that from #90 was significantly weaker than the rest, and that of the DD136 was significantly weaker than for all the others except #90 (Fig.4).

3-2.2. Antibiotic activity *in vivo*.

Extracts (see 2-3.2)from insect cadavers killed either by infecting *G. mellonella* larvae with strain DD136 of *S. carpocapsae* and its *Xenorhabdus* symbionts or by injecting them with *X. nematophilus* alone, showed strong antibiotic activity within 2 days of insect death. The antibiotic activity in insects killed by *X. nematophilus* alone increased gradually from the 2nd to the 3rd day after the insect death and thereafter, there was no significant increase. The antibiotic activity in insects killed by nematodes and their bacterial symbionts fluctuated over time but did not increase significantly for the next 6 days. The profile of antibiotic activity was similar in the cadavers killed by the two methods(Fig.5).

3-2.3. Comparison of antibiotic activity *in vitro* and *in vivo*

Comparison of antibiotic activities from the three cultures of two *Xenorhabdus* strains demonstrated different inhibition potencies against *B. subtilis*.

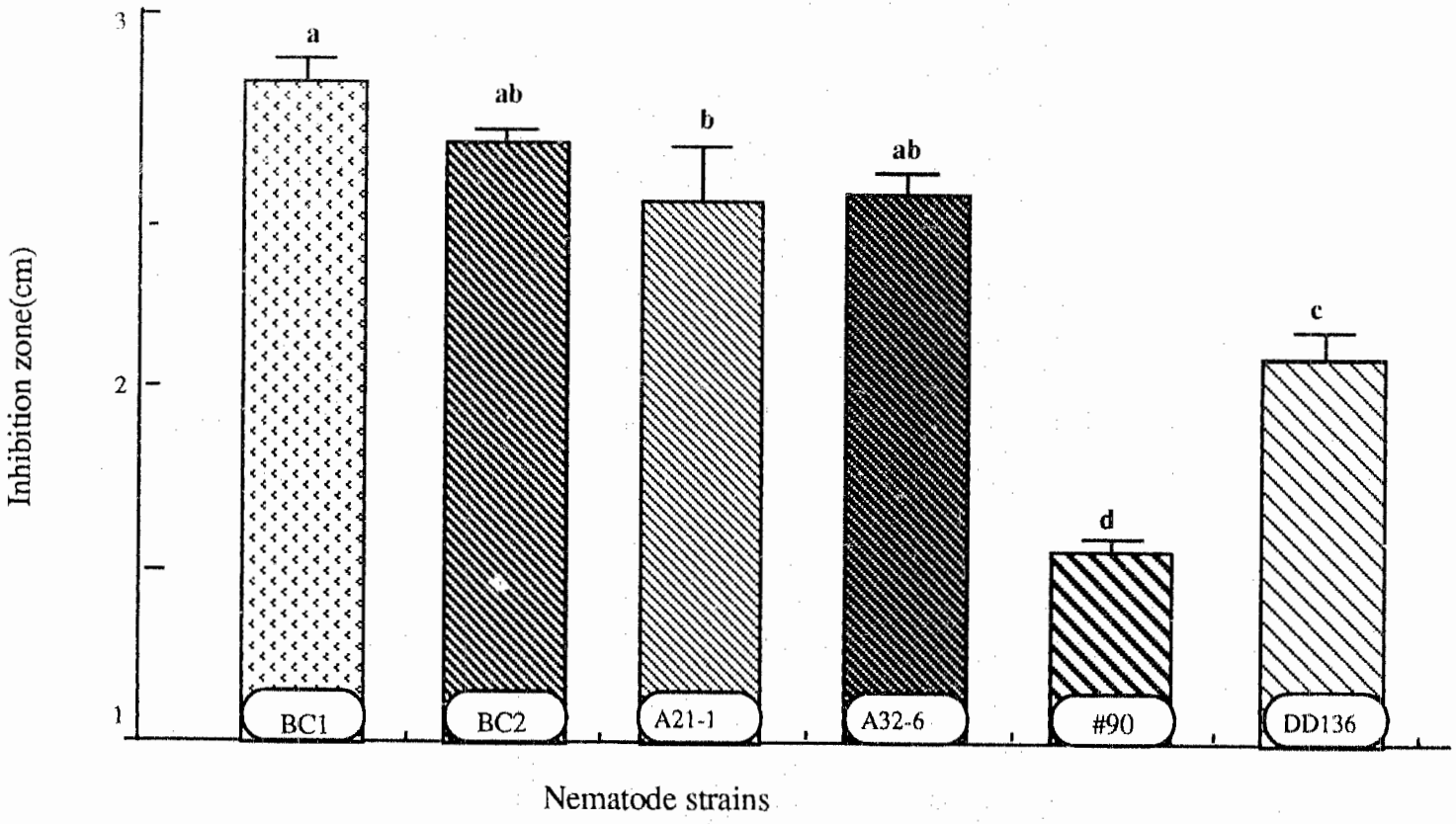

Fig.4. Antibiotic activity of *Xenorhabdus* isolates from different species and strains of *Steinernema* (all except #90) and *Heterorhabditis* (#90), expressed as the diameter of the inhibition zone on plated *Bacillus subtilis*. Means with the same letter (on top of the columns)are not significantly different(n=6).

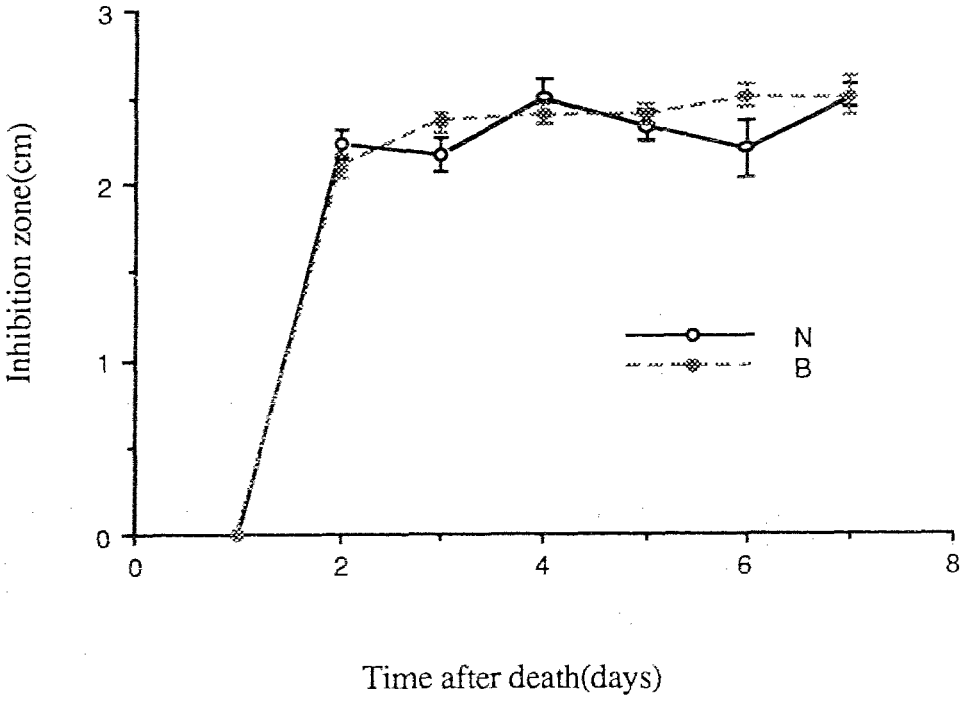

Fig.5. Antibiotic activity of *Xenorhabdus nematophilus*, from extracts of *Galleria mellonella* cadavers infected with either *Steinernema carpocapsae* plus symbiotic *X. nematophilus* (N) or *X. nematophilus* alone(B), as expressed by the inhibition zone on *Bacillus subtilis* plates over time.

For DD136, extracts from insects killed by both bacteria and nematode-bacteria treatments showed significantly stronger antibiotic activity than that from the bacterial culture. By contrast, the antibiotic activity of the bacterial culture of BC1 was significantly stronger than that of an extract of the nematode-killed insects. The antibiotic activity of the bacterial culture of the DD136 symbiont was the weakest whereas that of the bacterial culture of the BC1 symbiont was the strongest(Table 7.).

2-3. Properties and separation of the antibiotics

A. Effect of dialysis on antibiotic activity of *X. nematophilus* culture(see 2-4A).

Dialysis changed the antibiotic activity of the bacterial culture. After overnight dialysis, the dialysates from the tubings of 8-9,000 and 12-14,000 MWC were active against *B. subtilis*. However, the inhibition zones were reduced from 2.0cm diameter in the control to 0.8cm after dialysis. The dialysate from the 25,000 MWC tubing was not active against *B. subtilis*.

B. Properties(see 2-4B)

(i). Sensitivity to heat

When the cell-free solution from a culture of *X. nematophilus* was heated in a water bath, the solution exhibited no significant (P=0.01) reduction in antibiotic activity after having been heated to 80°C for 20 min.

Table 7. Comparison of the antibiotic activity of *Xenorhabdus* spp. from *Steinernema carpocapsae* DD136 and BC1 from three forms of culture. Expressed as the mean ± SE diameter of the inhibition zone(cm) on plated *Bacillus subtilis*

Nematode strains	Culture type+		
	1	2	3
DD136	2.37±0.06bc*	2.43±0.06bc	2.06±0.12d
BC1	2.63±0.26ab	2.17±0.16cd	2.80±0.11a

\+ 1. Extracts from *Galleria mellonella* killed by injection of *Xenorhabdus*.

2. Extracts from *G. mellonella* killed by nematodes and their *Xenorhabdus* symbionts.

3. Bacterial cultures grown in TSB in the dark at 24°C for 6 days.

* Means(n=6) with the same letter are not significantly different.

However, the activity was significantly (P=0.01) reduced when the culture was heated to 100°C or autoclaved at 121°C (Fig.6, A). A large precipitate, probably protein, and loss of the typical yellow color of the culture were observed in all heated cultures except those at 20°C.

(ii). Sensitivity to sunlight

When the cell-free bacterial culture was exposed to sunlight outdoors, the antibiotic activity was very little reduced, even after 7h exposure (Fig.6. B). A large precipitate and loss of the typical yellow color of the culture solution were observed after 2 hr's exposure.

(iii). Storage

When cultures of *X. nematophilus* were stored in the dark at room temperature either with or without (filter sterilized) the bacterial cells, the antibiotic activity decreased slowly with time. The antibiotic activity was significantly decreased after the first 20 day's storage. No significant difference in antibiotic activity was detected between 20 and 60 days and the antibiotic activity was significantly decreased between 30 and 90 day's storage. No protein precipitation or color changes were observed. No significant difference was detected in antibiotic activity between the sterile and non-sterile cultures during 90 day's storage (Fig. 7).

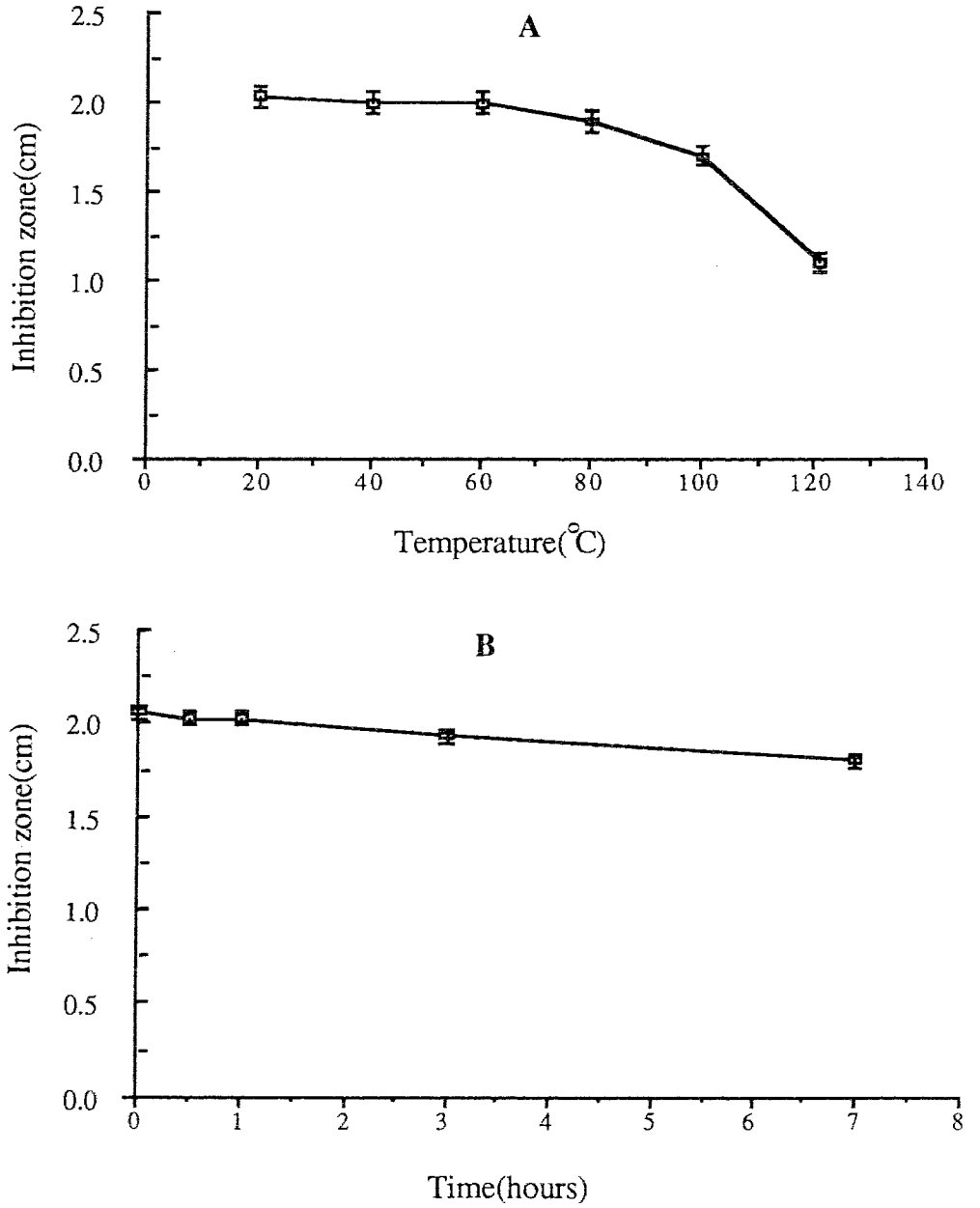

Fig.6. Antibiotic activity of *Xenorhabdus nematophilus* culture after having been heated in a water bath (A) or exposed to sunlight(B), expressed as the diameter of the inhibition zone of *Bacillus subtilis*.

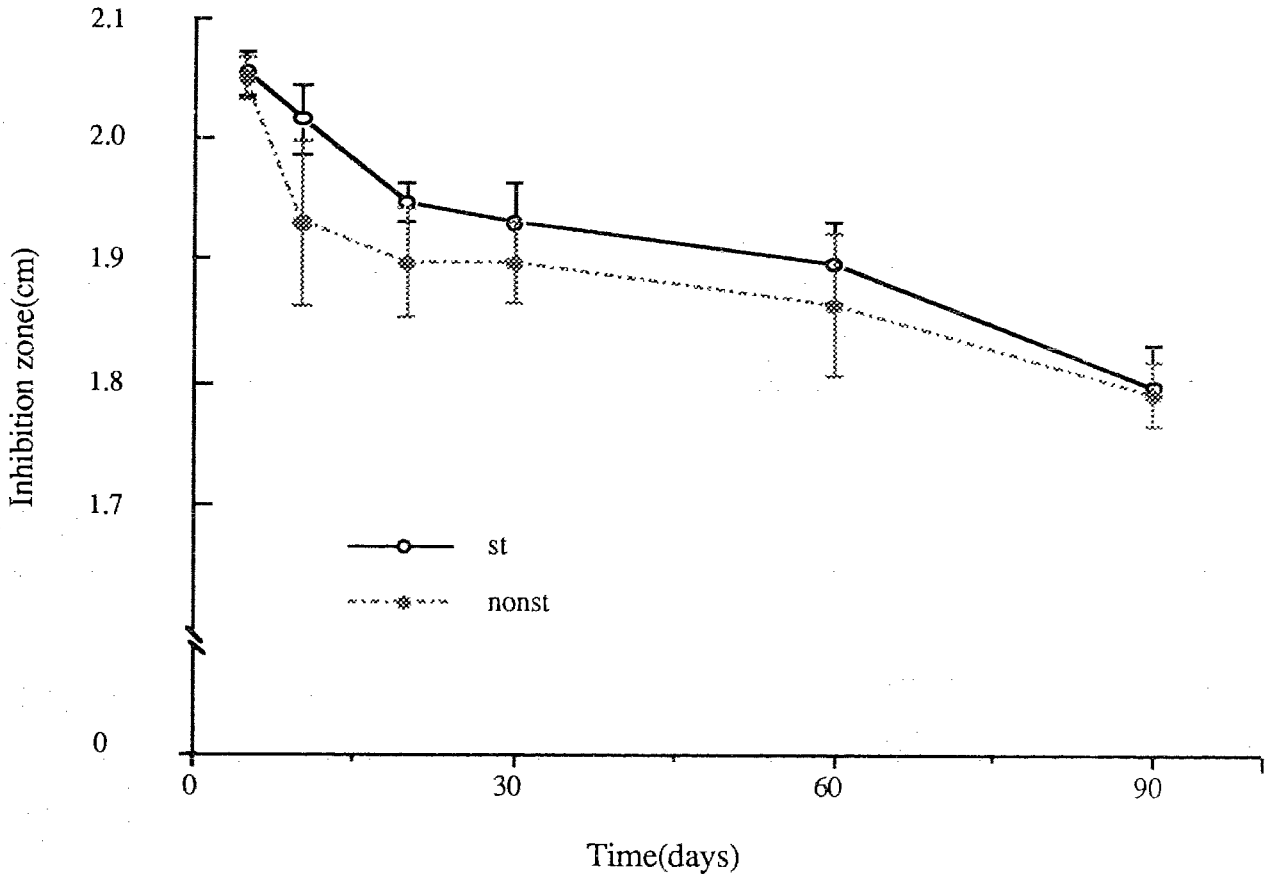

Fig.7. Antibiotic activity of *Xenorhabdus nematophilus* stored at room temperature over 90 days, expressed as diameter of inhibition zone of *Bacillus subtilis*. st= *X. nematophilus* culture sterilized by filtration; nonst= *X. nematophilus* culture.

C. Separation(see 2-4C)

When the spent bacterial medium of *X. nematophilus* was extracted with ethyl acetate, both the ethyl acetate and the aqueous fraction had antibiotic activity against *B. subtilis*. Fifty µl of suspension from the ethyl acetate fraction formed a 3.5 ± 0.09 cm inhibition zone whereas that of the culture of *X. nematophilus* and the aqueous fractions formed 2.0 ± 0.17 and 1.1 ± 0.09 cm zones respectively.

3-4. Antibiotic activity against soil bacteria in Petri dishes.

Both the insect extract(see 2-3.2) and the culture of *X. nematophilus* (see 2-3.1D) inhibited the growth of a range of bacterial cultures on agar media in Petri dishes. These bacteria included gram-positive and negative species. It is worth noting that the growth of the nitrogen-fixing species *A. chroococcum* and *R. phaseoli*, and the human pathogens, *E. coli* PA63,67, *S. aureus* and *S. typhimurium* SL1344 were also inhibited (Table 8).

Table 8. Antibiotic activity *of Xenorhabdus nematophilus*, from either TSB culture or *in vivo Galleria* culture against various bacterial species in Petri dishes. Expressed as whether the bacterial growth was inhibited(+) or was not inhibited(-).

Bacteria	Gram reaction	Bacterial growth	
		TSB	*in vivo*
Alcaligenes faecalis	-	+	+
Azoctobacter chroococcum	-	+	+
Bacillus subtilis	+	+	+
B. thuringiensis	+	+	+
Enterobacter aerogenes	-	+	+
Escherichia coli	-	+	+
Pseudomonas aeruginosa	-	-	-
Proteus vulgaris	-	-	-
Rhizobium phaseoli	-	+	+
Serratia marcescens	-	+	+
Salmonella typhimurium	-	+	+
Staphylococcus aureus	+	+	+

3-5. Tests in sterile soil

3-5.1. Persistence and antibiotic activity of *Xenorhabdus*

A. Persistence of *Xenorhabdus* spp. in sterile soil

Neither washed *X. nematophilus* nor *X. luminescens* were able to survive in sterile soil one day after inoculation. However, when the washed *Xenorhabdus* were plated on NBTA before being inoculated into the soil, they were viable on the plate even after three washes. Viable primary form of *Xenorhabdus* cells were recovered from the soils inoculated with bacterial cultures of *X. nematophilus* and *X. luminescens*. *X. luminescens* were viable 30 days and *X. nematophilus* were viable 60 days after inoculation (Table 9). Decline in colony number of both species over the incubation was observed.

B. Antibiotic production in sterile soil

When the primary forms of both *X. nematophilus* or *X. luminescens* in TSB were released into sterile soil no antibiotic activity was detected 5, 10 and 15 day's after the inoculation(Table 10).

C. Antibiotic persistence in sterile soil

When spent culture medium of *Xenorhabdus nematophilus* was released into the sterile soil, no antibiotic activity was found 30 min after the release.

Table 9. Viability of *Xenorhabdus nematophilus*(N) and *X. luminescens*(L) over time after inoculation into sterile soil with either the culture broth or after being washed 3 times before inoculation, at 24°C.

Treatments		Days 1	5	10	20	30	60
With culture broth	N.	+*	+	+	+	+	+
	L.	+	+	+	+	+	-
washed	N.	-	-	-	-	-	-
	L.	-	-	-	-	-	-

* + Viable; - not viable

Table 10. Antibiotic production during 15 days, of *Xenorhabdus nematophilus* and *X. luminescens* in sterile soil supplemented with TSB.

Species	Time in soil(days)		
	5	10	15
X. nematophilus	-*	-	-
X. luminescens	-	-	-

* - indicates no antibiotic activity was detected.

3-5.2. Effect of the antibiotics on soil bacteria

The population of *B. subtilis* in the soil treated(see 2-6.2D) with TSB was larger than those in soil treated with water and primary (P) cultures of *X. nematophilus;* and the population treated with water was the smallest 2 days after treatment. The means of the populations between those treated with the primary (P) and secondary (S) cultures of *X. nematophilus* were not significantly different (Fig.8 A).

The population of *R. phaseoli* in soil treated with TSB was significantly larger than when treated with water or spent *Xenorhabdus* culture media(cell free). The populations in the treatments of primary and secondary cultures of *X. nematophilus* were not significantly different from each other but were significantly larger than that of the water treatment (Fig.8 B).

3-6. Tests in garden soil

3-6.1. The effect of *X. nematophilus* culture on soil

A. Bacterial populations

The bacterial populations in all treatments(see 2-6.3A) were low after one day, but increased rapidly thereafter (Table.11), with sharp population increases from the 2nd to the 3rd day after treatment. The populations with TSB and water treatments reached their maximums on the 3rd day whereas those of *Xenorhabdus* treatments attained their maximums on the 4th day.

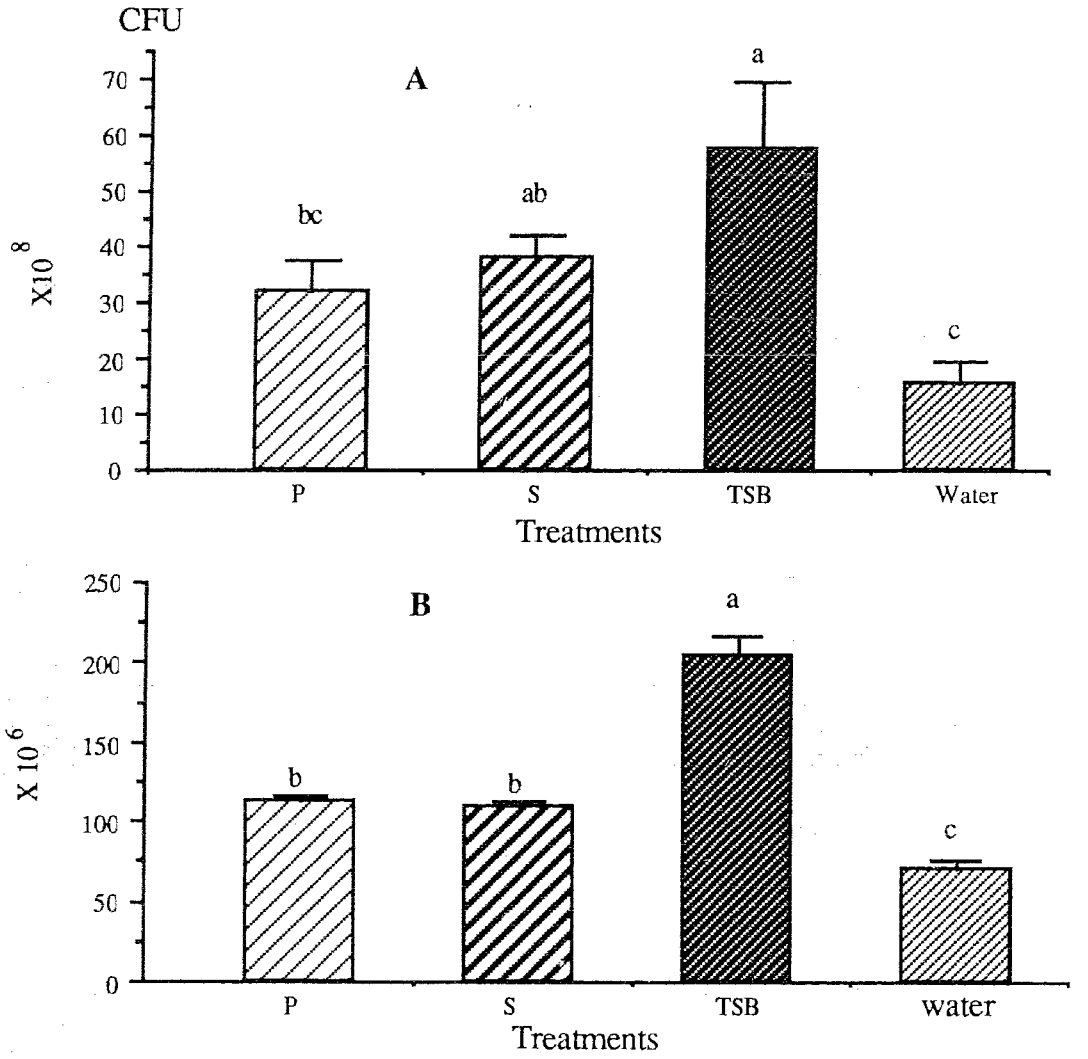

Fig.8. Population sizes of *Bacillus subtilis* (A) and *Rhizobium phaseoli* (B) in sterile soil after having been treated with the 6-day-old primary (P) and secondary (S) cultures of *Xenorhabdus nematophilus*, tryptic soy broth (TSB) and water. Means with the same letter are not significantly different (n=12)

Table 11. Estimated total soil bacterial population, expressed as the mean number(±SE) of colony forming units(x 10^8), in garden soil during 8 days after having been treated with 6-day-old primary and secondary form cultures of *Xenorhabdus nematophilus*, tryptic soy broth(TSB) and water.

Time after treatment (days)	Treatment			
	Primary	Secondary	TSB	Water
1	18.3±2.9a*	16.3±1.9a	18.7±1.9a	13.0±1.2a
2	66.7±27.3a	88.7±35.7a	163.3±63.6a	57.0±36.9a
3	121.0±13.2a	116.0±20.1a	169.7±23.4a	156.3±7.9a
4	123.7±18.9a	148.3±35.9a	127.7±29.2a	133.3±23.8a
6	94.3±26.4a	102.7±27.1a	121.3±26.3a	113.3±27.8a
8	107.0±24.3a	98.3±21.9a	111.0±23.6a	106.3±26.7a

* Means in a day with the same letter are not significantly different.

Variations in bacterial populations were recorded among these treatments but no significant differences were found between each treatment in any day during the test.

B. Total microbial activity.

Of all treatments, the FDA hydrolysis of the soil treated with water was always significantly weaker over the 6 days of test. The hydrolytic activities of TSB were always significantly higher than the others except on the 4th day and were equal to that of the primary form treatment on the 3rd day. As reflected by the FDA hydrolysis, the total microbial activities between the soils treated with primary and secondary form cultures of *X. nematophilus* were not consistently different. The activities with the secondary culture were significantly higher than those with the primary culture on the first and last day. However, the activity with the primary culture was higher than that with the secondary in the 2nd day. There were no significant differences between them on the 3rd and 4th days(Fig.9).

3-6.2. Effect of insect cadavers on soil bacteria

A. When insect cadavers were buried(see 2-6.3B) in garden soil the soil bacterial population in the soil changed during the time. However, no significant differences were recorded between treatments(*e.g.*insects killed by the primary or secondary form of *X. nematophilus*) on any single day during the test(Fig. 10).

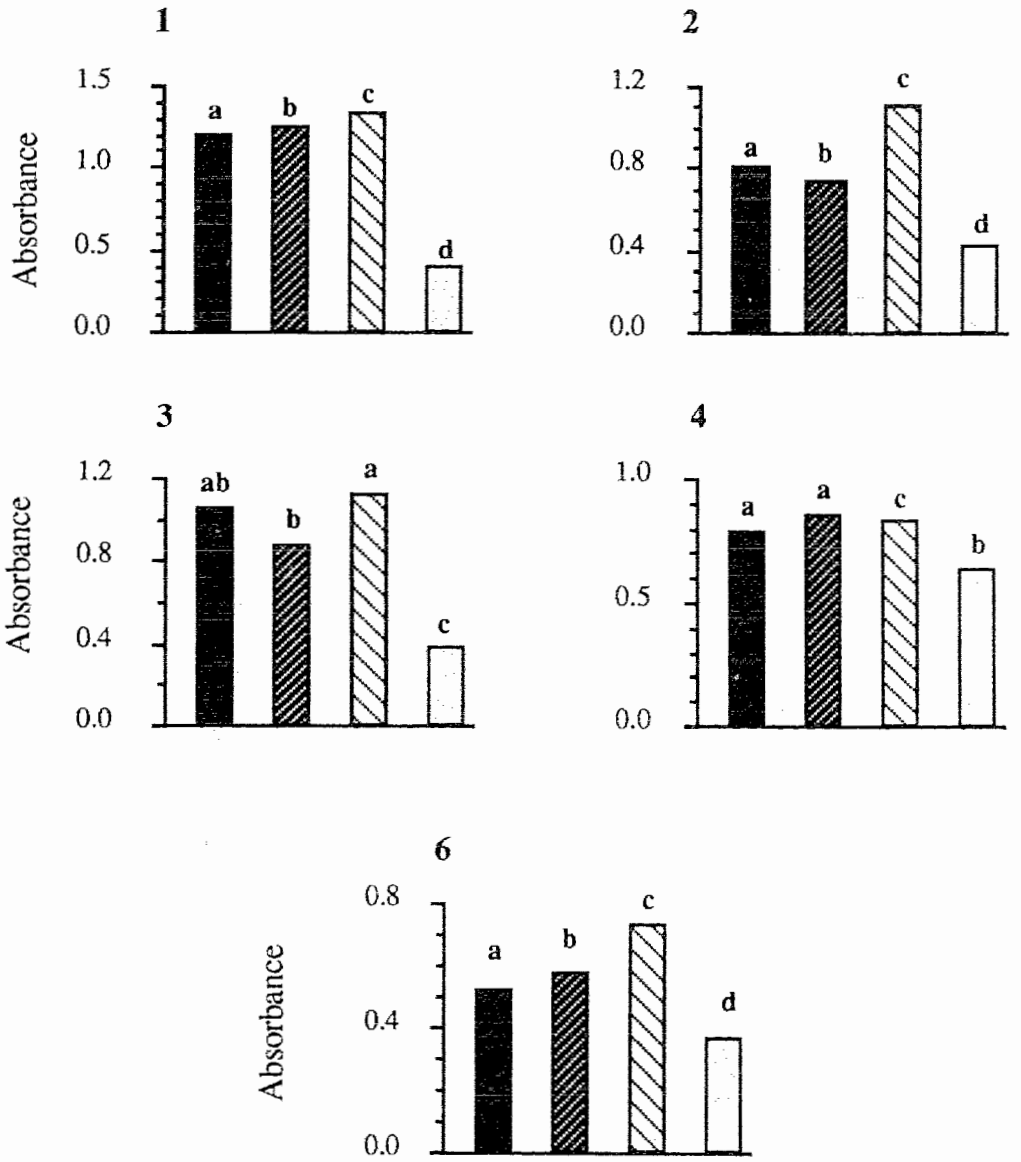

Fig. 9. Total soil microbial activity of garden soil after having been treated with primary (■), and secondary form (▨) cultures of *Xenorhabdus nematophilus*, TSB (▭) and water (▭);1, 2, 3, 4 and 6 days after treatment, determined by spectrophotometry at 490nm, using the hydrolysis of fluorescein diacetate as the indicator. Means (n=3) with the same letter are not significantly different.

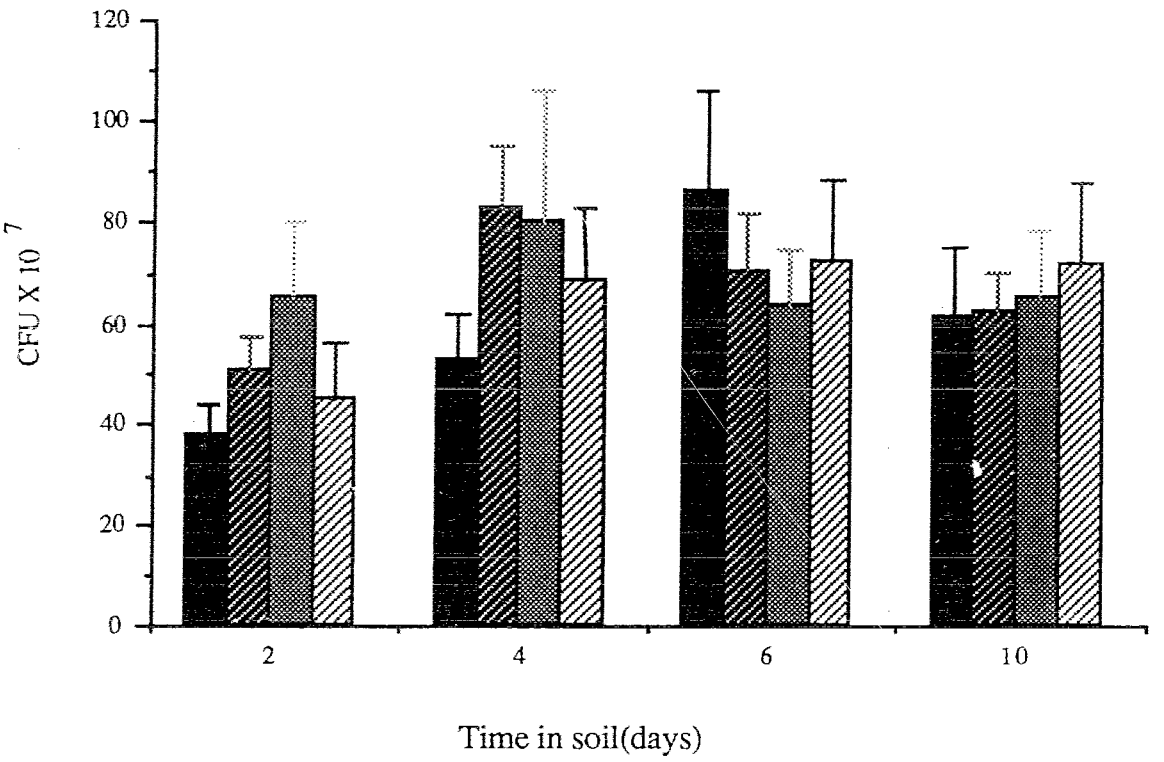

Fig.10. Population of soil bacteria in four batches of garden soil, in each of which was buried cadavers of *Galleria mellonella* killed by *Xenorhabdus nematophilus*.

The treatments and their indicators are as follows:

- **One cadaver killed by primary form bacteria**
- **Five cadavers killed by primary form bacteria**
- **One cadaver killed by secondary form bacteria.**
- **Five cadavers killed by secondary form bacteria.**

B. The antibiotic activity of the contents of insect cadavers killed by *X. nematophilus* significantly (P=0.01) decreased after 6 days of the cadavers being buried in soil. However, extracts from the insect cadavers continued to show antibiotic activity against *B. subtilis* after 10 day's burial in soil(Fig.11).

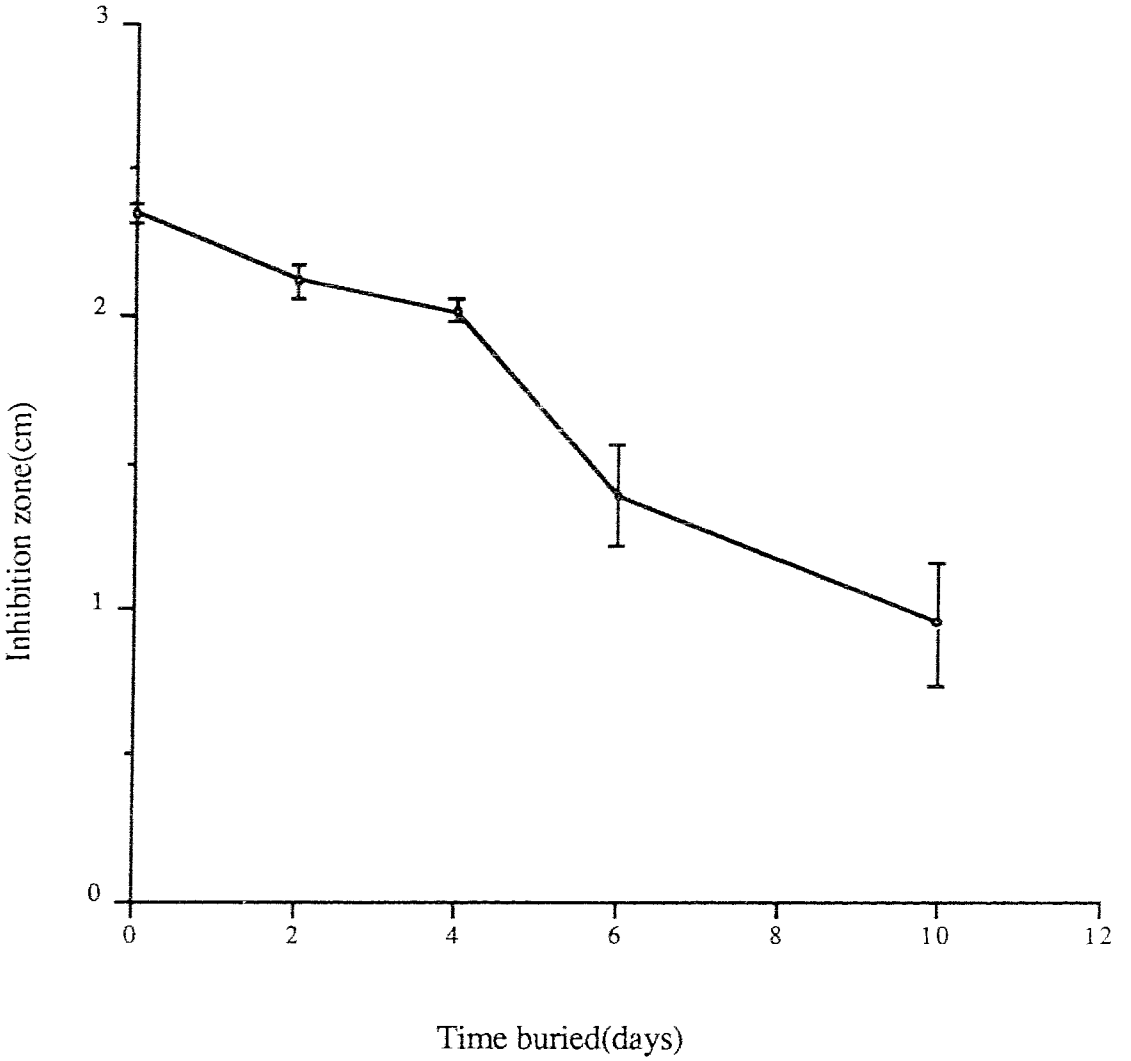

Fig.11. Antibiotic activity of extracts from insect cadavers after being buried in garden soil for different periods of time. Expressed as the average diameters of the inhibition zones(cm) on plates of *Bacillus subtilis*.

Chapter 4. Discussion

As research has proceeded, it has become increasingly apparent that this nematode/bacterial association is very complex. The present study has provided new information on several aspects of this nematode/bacteria association and especially on the biology of *Xenorhabdus* and on the characteristics and activity of the antibiotics.

4-1. Interaction of the nematodes and bacteria, and the biology of *Xenorhabdus* spp.

Insects killed by nematodes were often used as sources from which to isolate *Xenorhabdus* spp.. The isolation usually was carried out within 1-2 days of infection, at which time *X. nematophilus* was easily obtained. However, as the length of infection increased it became harder to isolate *Xenorhabdus*. In this study *X. nematophilus* was never isolated from nematode-killed *Galleria* cadavers later than 4 days after infection. From this time onwards the bacteria isolated were always catalytic positive and red on NBTA, indicating the presence of contamination by other bacteria. A similar phenomenon of cadaver-contamination was observed in the isolation of *X. luminescens* from *Galleria* killed by *Heterorhabditis*. When isolating *Xenorhabdus* spp. from insect cadavers it is advisable that the isolation be carried out as

early as possible after the insect has shown symptoms of infection or soon after death.

Poinar (1966) found various bacteria, rather than only *Xenorhabdus*, along with the nematode DD136 inside the cadavers of *G. mellonella*. It is quite possible that some bacteria from the insect gut invaded the haemocoel because of the breakdown of the insect tissues. It is hard to imagine that an insect cadaver is not contaminated by gut-inhabiting bacteria 4 days after infection, because by that time there is probably no physical barrier between the gut and the haemocoel and not all micro-organisms are sensitive to the antimicrobial substance produced by *Xenorhabdus* (Akhurst, 1982a).

Insects infected by nematodes are subject to secondary invasion by other micro-organisms which may modify conditions within the cadaver to the detriment of the nematode's development and reproduction (Nealson *et al.*, 1990). However, the secondary invasion does not necessarily result in the failure or reduction of nematode reproduction. *S. carpocapsae* can reproduce in culture with some species of bacteria other than *Xenorhabdus* (Akhurst and Boemare, 1990; Ehlers *et al.*, 1990). The normal process of infection in these cadavers, as observed here(see, 3-1.A), is that the presence of some bacterial species other than the nematode's symbionts in the cadavers appears to have no negative effect on the nematode's development and reproduction, and may be normal in nature. However, the strongly selective retention of *Xenorhabdus* in

the intestines of IJ nematodes suggests that the symbionts are essential in the nematode's development subsequent to the infective stage. The importance of *Xenorhabdus* to the nematode is probably mainly in the early stages of the infection. In the early stages of the infection, the insect immune system minimized the competition of other organisms and ensured the development of *Xenorhabdus* (Dunphy and Thurston, 1990). At the same time the development of *Xenorhabdus* in the insect haemocoel facilitates the development of the nematode (Akhurst and Boemare, 1990). The failure of isolating *Xenorhabdus* after a certain stage of infection indicates that once the insect is killed and its tissues destroyed, various other bacteria invade the cadaver and establish the domination of non-*Xenorhabdus* bacteria in the insect cadaver. The domination of the non-*Xenorhabdus* may indicate that *Xenorhabdus* does not play an important part in the development of the nematode at that stage.

The production of antimicrobial substances by *Xenorhabdus* in the insect cadaver, as Nealson *et al.*(1990) hypothesized, plays a part in minimizing the secondary invasion of the insect haemocoel and, in the early stage of infection, ensures the domination of *Xenorhabdus*, which converts the insect's tissue into suitable nutrition for nematode development. However, this antimicrobial prevention may be selective rather than exclusive to some of the organisms which are harmful to the nematodes. The main function of the antimicrobials might be in protecting early

nematode development and keeping the cadaver from collapse rather than from contamination. It is unlikely that the insect cadaver would be completely destroyed by *Xenorhabdus* and other invading bacteria surviving in the cadaver during the nematode's development, because the cuticle of insect larvae is usually very tough. However, the overwhelming growth of other organisms, such as fungi, may destroy the insect cuticle and the cadaver in a short time if their mycelia grow through the cuticle. This part of antifungal activity might be played by *Xenorhabdus* spp's production of antifungal substances, such as xenocoumacin 2 (McInerney *et al.*, 1991b). The rapid increase in pH in the *Xenorhabdus* culture may also play a part in preventing the growth of fungi on the cadaver, since many saprophytic fungi grow poorly at high pH.

The growth of the primary form of *X. nematophilus* differed from that of the secondary form in the length of the lag phase, and in the final cell density and pH of the culture broth(Fig.2). Boemare and Akhurst(1990) reported that primary and secondary forms of *X. nematophilus* had different final cell densities when they were cultured *in vitro* for 2 days. However, they found no differences in total cell count between the two forms and they assumed that the differences in the cell density may be due to the size and morphology of the primary form cells, which are larger, pleomorphic and have cellular inclusions.

The different profile of the growth of primary and secondary *X. nematophilus*, as observed in this study, may reflect fundamental physiological rather than morphological differences between the forms. It is suggested that the two forms may have different metabolic systems as stated by Boemare and Akhurst(1990), and that a much lower rate of increase of the secondary form in the haemolymph, compared with that of the primary form as observed by Dunphy and Webster(1984), may be the result. The other differences between the two forms such as nematode yield *in vivo*, observed by Akhurst(1980), also may be partially attributed to these differences. The different development of the two forms in *Galleria* may affect the conversion of the host into nutrients for the nematodes.

Form variation is common in *Xenorhabdus* spp. Akhurst(1980) reported it in 15 of 17 isolates of *Xenorhabdus* spp. tested. Other variations rather than the primary and secondary forms have been detected in *X. luminescens* (Hurlbert *et al.*, 1989; Ehlers *et al.*, 1990). Those differences among different isolates from the same nematode strain in their growth, pH changes and antibiotic activity, as documented in this present study(Table 4), may reflect new aspects of form variation because each nematode only associates with a single *Xenorhabdus* species in nature(Akhurst and Boemare, 1990). It appears that the primary and the secondary forms represent the extremes of form variation and that there are intermediates between these

two forms. For example, in *X. bovienii* isolates of *S. feltiae* strain A21-1, the differences among their final cell densities, pHs, and antibiotic activities(Table 4) seem to represent a gradient between the forms. The pleomorphic phenomenon of the primary form observed by Boemare and Akhurst(1990), Hurlbert *et al.*(1989) and Ehlers *et al.*(1990) may reflect different types in a gradient between these extremes. It is quite possible that the small, slow-growing bacteria observed by Wouts(1990) is one of the types in the gradient of types between these two forms. The three *X. bovienii* isolates from nematode A21-1 showed differences not only in their cell density, pH, antibiotic activity and their pigment production in TSB but also in their rates of change to the extreme secondary form on NBTA(see 3-1 A). These may represent substantial differences in the way they use the media and in their adaptability to different environments.

Boemare and Akhurst(1988) reported that the secondary form may revert to the primary form. In the present study, however, when a secondary form from DD136 was obtained after several subcultures and maintained for a long time, I could not obtain the primary form after the secondary form of *X. nematophilus* was inoculated in TSB, on NBTA or TSA.

It was reported by Poinar *et al.*,(1980) that the symbionts of *H. bacteriophora* could be stored under refrigeration at 4°C on nutrient agar slants. In the present study, however, *Xenorhabdus* spp. were found to be sensitive to cold. When they were stored at 4°C on NBTA, their

viability was decreased. The eventual loss of all our stored *Xenorhabdus* spp. indicated that they could not be stored at 4°C for long term maintenance. However, the fact that in this study the *Galleria* larvae were killed by the primary form of *X. nematophilus* that had been maintained inside the cadavers at 4°C for about 2 months suggested that this technique might be an alternative for maintenance of specific *Xenorhabdus*.

Xenorhabdus spp. have never been isolated in the past from soil even after having been inoculated into sterile soil(Poinar *et al.*, 1980). The successful recovery of *Xenorhabdus* from sterile soil in this study and the isolation of *X. luminescens* from human wounds reported by Farmer *et al.*(1989), suggest that some *Xenorhabdus* can survive under certain limited conditions in nature outside of insects and nematodes.

It is understandable that *Xenorhabdus* spp. have not been found in and described from soil before, because they grow very slowly compared with most bacteria and perhaps because they need nutrient augmentation or have some special, unknown requirements. That the washed cells did not survive in the sterile soil(Table 9) suggests that *Xenorhabdus* spp. cannot be widespread as free-living species. However, the reasons why they were not recovered from the sterile soil are unclear. Nutrition might not be the only reason because, after 2 months, the originally amended nutrient must have been consumed. Physical damage by washing could be partially responsible. However, washed cells retained their viability

when plated on NBTA. The reason why *X. luminescens* survived for a shorter time than *X. nematophilus* in the sterile soil is also not clear. The short survival time of *X. luminescens* plus its apparent greater sensitivity to cold and readiness to change to the secondary form than *X. nematophilus*, may suggest that *X. luminescens* is vulnerable to such factors. If this vulnerability is confirmed, it might be one of the reasons why *Heterorhabditis* is more difficult to culture than is *Steinernema*.

4-2. Antibiotic activity *in vitro* and *in vivo*

Favorable temperatures and pH for the growth of *X. nematophilus* resulted in the strongest antibiotic activity in the cultures. The failure of antibiotic production in 30 and 35°C cultures could be attributed to the inhibition of bacterial growth(Fig.3). The inability of an anaerobic culture of *X. nematophilus* to inhibit growth of other micro-organisms, as observed by Akhurst(1982a), was confirmed. *X. nematophilus* produces antibiotic only in aerobic conditions(Table 5). Nutrition also affects the antibiotic activity of *X. nematophilus*. Its growth was poor and no antibiotic activity was detected when it was cultured in 1% peptone water 6 days after incubation at 25°C(Table 6). However, it appears that richer nutrient media enhance its antibiotic production *in vitro*. Akhurst(1982a) reported that *Xenorhabdus* isolated from different nematode strains differed

in their antibiotic activities, and this study confirmed Akhurst's observation (Fig. 4).

To my knowledge, the profile of antibiotic activity in insect cadavers has not been previously documented. The timing of the antibiotic activity appearing *in vivo*, is similar to that *in vitro*. Fluctuation in antibiotic activity in nematode-killed *Galleria* cadavers might represent an effect of the nematode's metabolism or development(Fig. 5). However, the overall similarity of the *Galleria* killed by nematodes and their symbionts or by *Xenorhabdus* alone suggests that the direct contribution of the nematode to antibiotic activity is limited.

The stability of antibiotics when exposed to heat, as observed by Akhurst (1982a), was confirmed here, but their stability to UV irradiation(sunlight) had not been previously documented(Fig.6). Precipitation of a large amount of protein and loss of the pigment from the cell cultures were observed when the cultures were heated or exposed to UV light. However, the antibiotic reduction in heat of high temperature and to UV did not necessarily result from the precipitation of protein and loss of pigment, because the precipitation and depigmentation also happened in these treatments where little reduction of antibiotic activity was evident.

When the spent culture media of *X. nematophilus* were stored at room temperature either with or without bacteria no differences in antibiotic activity were detected between them during the storage(Fig.7). These similar changes in

antibiotic activity between the cell-bearing and cell-free cultures during storage of *X. nematophilus*, illustrate that there was apparently no continuous antibiotic production by the bacteria after a certain stage.

The antibiotic activity shown here in dialysed *Xenorhabdus* culture did not agree with that obtained by Akhurst(1982a), *i.e.* the dialysates showed antibiotic activity against *B. subtilis* after having been dialysed in dialysis tubing with a MWC of 12-14,000 overnight. The reason is unknown. However, the antibiotic activity of the dialysates indicates that the antibiotic activity evidently involved substances having different molecular weights and in the present case their molecular weights are less than 25,000. Antibiotic activity in the water fraction after having been extracted by ethyl acetate, indicates that *X. nematophilus* produces water- and organic-soluble antibiotic substances and further separation is needed.

4-3. Effect of the antibiotics produced by *Xenorhabdus* spp. on soil bacteria

The sensitivity of some soil bacteria to antibiotics produced by *X. nematophilus* was demonstrated in Petri dishes(Table 8). However, this antibiotic effect could not be confirmed in sterile soil where exogenous populations of *B. subtilis* and *R. phaseoli* were unaffected by the release of the bacterial cultures of *X. nematophilus* (Fig.8).

Furthermore, the failure to detect antibiotic activity in sterile soil shortly after release of the antibiotics, suggests that they might not be capable of affecting the bacteria in sterile soil. The failure to detect many antibiotics after the antibiotics were inoculated into soil, as described by Williams (1982) and Gottlieb (1976) is common. The reasons are not clear but could be as Williams and Vickers(1986) hypothesized: a) chemical or bio-degradation of the antibiotics; b) adsorption of antibiotics onto clay and humus colloids; or c) insufficient sensitivity in detection of the antibiotics. In this study, reason a) is unlikely because the antibiotics are extremely stable in various situations as described earlier, and there is no bio-activity in sterile soil. Given the basic nature of some antibiotics produced by *X. nematophilus*(McInerney *et al.*, 1991b) reason b) adsorption to clay and humus colloids, could well be a reason, and if so, this might also be a clue to the chemical properties of some other antibiotics. Basic and amphoteric antibiotics are absorbed to soil but not acidic or neutral ones (Williams, 1982). Reason c) lack of sensitivity of detection, could well be one of the reasons. This could be verified by using more sensitive indicator organisms or by chemical means if the antibiotics should be purified and characterized. However, whether it is because of the adsorption or insufficient detection, the apparent absence of antibiotics in sterile soil indicated that besides being inactivated, the antibiotics, if any, in the sterile soil

might be too low in amount to be effective against soil bacteria.

It would be generally accepted that sterile soil is not normal and that the activity of antibiotics in sterile soil would not be the same as in natural soil. When antibiotics were released into natural garden soil they not only could be inactivated by the means that existed in sterile soil but also could be bio-degraded. The data arising from this study in natural soil are difficult to interpret(Table 11, Fig 9). However, there was no evidence that the release of the antibiotics had any effect on the populaticns of naturally occurring soil bacteria. The differences in the total soil microbial activity among different treatments were not correlated with the soil bacterial population, but seemed rather to be affected by the nutrients in the various treatments.

The release of insect cadavers injected with *X. nematophilus* closely represents the real situation in nature, where insects are killed by this nematode-bacterial complex, except for the unnaturally high number of insect cadavers in an area. The effect of antibiotics produced by insects infected by the nematodes could be very well reflected by this model. The antibiotic activity on soil bacteria was not evident even in the extremely high dosage released(Fig.10). The slow release of antibiotics from the insect cadavers suggested the possible presence of antibiotics around the cadaver for more than 10 days(Fig.11). However, it is

doubtful that even if these antibiotics were present in lower concentrations, below the level of detection, they would continue to antagonize soil bacteria.

It has been demonstrated that insect cadavers, killed by insect nematodes and their symbionts, contain antibiotics which are very stable and capable of inhibiting the growth of various bacteria. Although the antibiotics showed an adverse impact on bacteria in Petri dishes, this impact was not evident in soil even when a large amount of the antibiotics and an unnaturally high number of insect cadavers killed by *Xenorhabdus* were present. Based on the results of the experiments it is suggested that the antibiotics produced by *Xenorhabdus* do not have an adverse impact on soil bacteria in soil, and consequently, it is unlikely that inundative release of entomopathogenic nematodes will adversely effect the environment by killing the soil bacteria. However, this conclusion may not necessarily apply to other groups of the soil micro-fauna. It is recommended that the effect of all *Steinernema* and *Heterorhabditis* species and their symbionts on the major groups of the micro-fauna be investigated before a generalized conclusion is made concerning their environmental impact.

References

Akhurst, R. J.(1980). Morphological and functional dimorphism in *Xenorhabdus* spp. Bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* 121:303-309.

Akhurst, R. J. (1982a).Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *Journal of General Microbiology* 128:3061-3065.

Akhurst, R. J. (1982b). A *Xenorhabdus* sp. (Eubacteriales: Enterobacteriaceae) symbiotically associated with *Steinernema kraussei* (Nematoda: Steinernematidae). *Revue de Nematologie* 5:277-280.

Akhurst, R. J. and N. E. Boemare (1986). A non-luminescent strain of *Xenorhabdus luminescens*(Enterobacteriaceae). *Journal of General Microbiology* 132:1917-1922.

Akhurst, R. J. and N. E. Boemare (1988). A numerical taxonomic study of the genus *Xenorhabdus* (Eubacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species. *Journal of General Microbiology* 134:1835-1845.

Akhurst, R. J. and N. E. Boemare (1990). Biology and taxonomy of *Xenorhabdus*. *In* Entomopathogenic Nematodes in Biological Control (R. Gaugler and H. K. Kaya, eds). CRC Press Inc., Boca Raton, FL. 365pp.

Akhurst, R. J., N. E. Boemare and R. G. Mourant(1990). DNA homology groups in the genus of *Xenorhabdus*. *In* Proceedings and abstracts of VI International Colloquium on Invertebrate Pathology and Microbial Control. 1990, Adelaide, Australia.

Bedding, R. A.,(1984). Large scale production, storage and transport of the insect-parasitic nematodes *Neoaplectana* spp. and *Heterorhabditis* spp. *Annals of Applied Biology* 104:117-120.

Boemare, N. E. and R. J. Akhurst (1988). Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). *Journal of General Microbiology* 134:751-761.

Boemare, N. E. and R. J. Akhurst(1990). Physiology of phase variation in *Xenorhabdus* spp. *In* Proceedings and abstracts of VI International Colloquium on Invertebrate Pathology and Microbial Control. 1990, Adelaide, Australia.

Colepicolo, P., K. W. Cho, G. O., Poinar and W. Hastings (1989). Growth and luminescence of the bacterium *X. luminescens* from a human wound. *Applied and Environmental Microbiology* 55(10):2601-2606.

Couche, G. A., P. R. Lehrbach, R. G. Forage, G. C. Cooney, D. R. Smith and R. P. Gregson (1987). Occurrence of intracellular inclusions and plasmids in *Xenorhabdus* spp. *Journal of General Microbiology* 133:967-973.

Dhingra, O. D. and J. B. Sinclair(1985). Basic plant pathology methods. CRC Press Inc., Boca Raton, FL. 355pp.

Dunphy, G. B. and J. M. Webster (1984). Interaction of *Xenorhabdus nematophilus* subsp. *nematophilus* with the haemolymph of *Galleria mellonella*. *Journal of Insect Physiology* 30:883-889.

Dunphy, G. B. and J. M. Webster (1988a). Virulence mechanisms of *Heterorhabditis heliothidis* and its bacterial associate, *Xenorhabdus luminescens*, in non-immune larvae of the greater wax moth, *Galleria mellonella*. *International Journal for Parasitology* 18:729-731.

Dunphy, G. B. and J. M. Webster (1988b). Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galleria mellonella* (Insecta:Lepidoptera) larvae. *Journal of General Microbiology* 134:1017-1028.

Dunphy, G. B and G. S. Thurston (1990). Insect immunity. *In* Entomopathogenic Nematodes in Biological Control (R. Gaugler and H. K. Kaya, eds). CRC Press Inc., Boca Raton, FL. 365pp.

Dunphy, G. B., T. A. Rutherford and J. M. Webster (1985). Growth and virulence of *Steinernema glaseri* influenced by different subspecies of *Xenorhabdus nematophilus*. *Journal of Nematology* 17:476-482.

Dutky, S. R., J. V., Thompson and G. E., Cantwell(1962). A technique for mass rearing the greater wax moth (Lepidoptera:Galleridae). *Proceedings of the Entomological Society of Washington* 64:56-58.

Dutky, S. R., J. V., Thompson and G. E., Cantwell(1964). A technique for the mass propagation of the DD-136 nematode. *Journal of Insect Pathology* 6:417-422.

Ehlers, R. U., S. Stoessel and U. Wyss(1990). The influence of phase variants of *Xenorhabdus* spp. and *Echerichia coli* (Enterobacteriaceae) on the propagation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. *Revue de Nematologie* 13:417-424.

Ehlers, R. V., K. V. Deseo and E. Stackebrant(1991). Identification of *Steinernema* spp.(Nematoda) and their symbiotic bacteria *Xenorhabdus* spp. from Italian and German soils. *Nematologica* 37:360-366.

Ensign, J. C., D. J. Bowen and B. Bintrin (1990). Cystalline inclusion proteins and an insecticidal toxin of *Xenorhabdus luminescens* strain NC19. *In* Proceedings and abstracts of VI International Colloquium on Invertebrate Pathology and Microbial Control. 1990, Adelaide, Australia.

Farmer, J. J. III, J. H. Jorgensen, P. A. D. Grimont, R. J. Akhurst, G. O. Poinar, E. Ageron, G. V. Pierce, J. A. Smith, G. P. Carter, K. L. Wilson and F. W. Hickman-Brenner,(1989). *Xenorhabdus luminescens* (DNA hybridization group 5) from human clinical specimens. *Journal of Clinical Microbiology* 27:1594-1600.

Friedman, M. J.,(1990). Commercial production and development. *In* Entomopathogenic Nematodes in Biological Control (R. Gaugler and H. K. Kaya, eds). CRC Press Inc., Boca Raton, FL. 365pp.

Georgis, R. (1987). Nematodes for biological control of urban insects. Division of Environmental Chemistry of the American Chemical Society meeting 30, New Orleans.

Georgis, R.(1990). Formulation and application technology.*In* Entomopathogenic Nematodes in Biological Control (R. Gaugler and H. K. Kaya, eds). CRC Press Inc., Boca Raton, FL. 365pp.

Glaser, R. W.(1940).The bacteria-free culture of a nematode parasitic in Japanese beetle. *Proceedings of the Society for Experimental Biology and Medicine* 43:512-514.

Glaser, R. W. and C. C. Farell (1935). Field experiments with the Japanese beetle and its nematode parasite, *Journal of the New York Entomological Society* 43:345.

Gottlieb, D. (1976). The production and role of antibiotics in soil. *The Journal of Antibiotics* 29:987-1,000.

Götz, P., A. Boman and H. G. Boman (1981). Interactions between insect immunity and an insect-pathogenic nematode with symbiotic bacteria. *Proceedings of Royal Society of London* (B)212:333-350.

Grimont, P. A. D., A. G. Steigerwalt, N. E. Boemare, F. W. Hickman-brenner, C. Deval, F. Grimont and D. J. Brenner (1984). Dexyribonucleic acid relatedness and phenotypic study of the genus *Xenorhabdus*. *International Journal of Systematic Bacteriology* 34:378-388.

Han, R., W. M. Wouts and L. Li(1990). Development of *Heterorhabditis* spp. strains as characteristics of possible *Xenorhabdus luminescens* subspecies. *Revue de Nematologie* 13:411-415.

Han, R., W. M. Wouts and L. Li(1991). Development and virulence of *Heterorhabditis* spp. strains associated with different *Xenorhabdus luminescens* isolates. *Journal of Invertebrate Pathology* 58:27-32.

Herbert, R. A. (1990). Methods for enumerating micro-organisms and determining Biomass in natural Environments. *In* Methods in Microbiology (Grigorova, R. and J. R. Norris eds) Vol. 22, Academic Press Inc., San Diego. 627pp.

Hewitt, W and S. Vincent (1989). Theory and application of microbiological assay. Academic Press Inc, San Diego. 323pp.

Hurlbert, R. E., J. Xu and C. Small (1989) Colonial and cellular polymorphism in *Xenorhabdus luminescences*. *Applied and Environmental Microbiology* 55:1136-1143.

Leclerc, M. C. and N. E. Boemare(1991). Plasmids and phase variation in *Xenorhabdus* spp. *Applied and Environmental Microbiology* 57:2597-2601.

Martin, J. K. (1975). Comparison of agar media for counts of viable bacteria. *Soil Biology and Biochemistry* 7:401-402.

McInerney, B. V., W. C. Talor, M. J. Lacey, R. J. Akhurst and R. P. Gregson.(1991a). Biologically active metabolites from *Xenorhabdus* spp., Part 1. Dithiolopyrrolone derivatives with antibiotic activity. *Journal of Natural Products* 54:774-784.

McInerney, B. V., W. C. Talor, M. J. Lacey, R. J. Akhurst and R. P. Gregson.(1991b). Biologically active metabolites from *Xenorhabdus* spp., Part 2. Benzopyran-1-one derivatives with gastroprotective activity. *Journal of Natural Products* 54:785-795.

Nealson, K, T. M. Schmidt and B. Bleakley (1988). Luminescent bacteria:symbionts of nematodes and pathogens of insects. *In* Cell to Cell Signals in Plant, Animal and Microbial Symbiosis. NATO ASI Series, Vol. H17, S. Scannerini, ed, Springer-Verlag, Berlin. 414pp.

Nealson, K, T. M. Schmidt and B. Bleakley(1990). Biochemistry and physiology of *Xenorhabdus*. *In* Entomopathogenic Nematodes

in Biological Control (R. Gaugler and H. K. Kaya, eds). CRC Press Inc., Boca Raton, FL. 365pp.

Nielsen, J. W., J. S. Dickson and J. D. Crouse (1990). Use of a bacteriacin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology* 56:2142-2145.

Paul, V. J., S. Frautschy, W. Fenical and K. H. Nealson (1981). Antibiotics in microbial ecology, isolation and structure assignment of several new antibacterial compounds from the insect-symbiotic bacteria *Xenorhabdus* spp. *Journal of Chemical Ecology* 7:589-597.

Pawsey, R. K.(1974). Techniques with bacteria. Hutchinson Educational Ltd, London. 160pp.

Poinar, G. O. (1966). The presence of *Achromobacter nematophilus* in the infective stage of *Neoaplectana* sp. (Steinernematidae:Nematoda). *Nematologica* 12:105-108.

Poinar, G. O. (1976). Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida: Heterorhabditidae N. Fam.). *Nematologica* 21:1463

Poinar, G. O.(1979). Nematodes for biological control of Insects. CRC Press Inc., Boca Raton, FL. 143pp.

Poinar, G. O.(1984) On the nomenclature of the genus *Neoaplectana* Steiner, 1929 (Steinernematidae:Rhabditida) and the species *N. carpocapsae* Weiser, 1955. *Revue de Nematologie* 7:199-200.

Poinar, G. O.(1986). Recognition of *Neoaplectana* species (Steinernematidae: Rhabditida) *Proceeding of Helminthology Society of Washington* 53:121-129.

Poinar, G. O.(1990). Biology and Taxonomy of Steinernematidae and Heterorhabditidae. *In* Entomopathogenic Nematodes in Biological Control (R. Gaugler and H. K. Kaya, eds). CRC Press Inc., Boca Raton, FL. 365pp.

Poinar, G. O. and G. M. Thomas (1965). A new bacterium, *Achromobacter nematophilus* sp. nov. (Achromobacteriaceae: Eubacteriales) associated with a nematode. *In. Bull. Bacteriol. Nomencl. Taxon.* 15:249-252.

Poinar, G. O. and G. M. Thomas (1966). Significance of *Achromobacter nematophilus* Poinar & Thomas (Achromobacteriaceae: Eubacteriales) in the development of the nematode, DD136 (*Neoaplectana* sp. Steinernematidae). *Parasitology* 56: 385-390.

Poinar, G. O. and G. M. Thomas (1967). The nature of *Achromobacter nematophilus* as an insect pathogen. *Journal of Invertebrate Pathology* 9:510-514.

Poinar, G. O., R. T. Hess and G. Thomas (1980). Isolation of defective bacteripophages from *Xenorhabdus* spp. (Enterobacteriaceae). *IRCS Medical Sciences* 8:141.

Poinar, G. O., G. M. Thomas, M. Haygood and K. H. Nealson(1980). Growth and luminescence of the symbiotic bacteria associated with the terrestrial nematode, *Heterorhabditis bacteriophora*. *Soil Biology and Biochemistry* 12:11-14.

Poinar, G. O., R. T. Hess, W. Lanier, S. Kinney and J. H. White (1989). Preliminary observations of a bacteriophage infecting *Xenorhabdus luminescences*(Enterobacteriaceae), *Experientia* 45:191-192.

Richardson, W. H., T. M. Schmidt and K. H. Nealson (1988). Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. *Applied and Environmental Microbiology* 54:1602-1605.

Schnürer, J. and T. Rosswall (1982). Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology* 43:1256-1261.

Smits, P. H., J. J. M. Groenen and G. D. Raay (1991). Characterization of *Heterorhabditis* isolates using DNA restriction fragment length polymorphism. *Revue de Nematologie* 14:445-453.

Stoll, N.(1953). Axenic cultivation of the parasitic nematode, *Neoaplectana glaseri*, in a fluid medium containing raw liver extract, *Journal of Parasitology* 39:422-444.

Thomas, G. M. and G. O. Poinar (1979). *Xenorhabdus* gen nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. *International Journal of Systematic Bacteriology* 29:352-360.

Thomas, G. M. and G. O. Poinar (1983). Amended description of the genus *Xenorhabdus* Thomas and Poinar. *International Journal of Systematic Bacteriology* 33:878-879.

Williams, S. T. and J. C. Vickers (1986). The ecology of antibiotic production. *Microbial Ecology* 12:43-52.

Williams, S. T.(1982). Are antibiotics produced in soil? *Pedobiologia* 23:427-435.

Wollum II, A. G.(1982). Cultural methods for soil micro-organisms. *In* Methods of soil analysis part 2.Chemical and microbiological properties. Agronomy Monograph No.9 (2nd Edition). ASA-SSSA, Madison, WI. USA.

Woodring, J. L. and H. K. Kaya(1988). Steinernematid and Heterorhabditid Nematodes: A handbook of Techniques. Southern Cooperative Series Bulletin 331, Arkansas Agricultural Experiment Station. 29pp.

Wouts, W. M.(1990). The primary form of *Xenorhabdus* species (Enterobacteriaceae:Eubacteriales) may consist of more than one bacterial species. *Nematologica* 36:313-318.

Wouts, M., Z. Mracek, S. Gerdin and R. A. Bedding (1982). *Neoaplectana* Steiner, 1929 a junior synonym of *Steinernema* Travassos, 1927 (Nematoda: Rhabditida). *Systematic Parasitology* 4:147-154.

Xi, L., K. W. Cho and S. C. Tu (1991). Cloning and nucleotide sequences of lux genes and characterization of luciferase of *Xenorhabdus luminescens* from a human wound. *Journal of Bacteriology* 173:1399-1405.

Xu, J. and R. E. Hurlbert (1990). Toxicity of irradiated media for *Xenorhabdus* spp. *Applied and Environmental Microbiology* 56:815-818.

Xu, J., S. Lohrke, I. M. Hurlbert and R. E. Hurlbert (1989). Transformation of *Xenorhabdus nematophilus*. *Applied and Environmental Microbiology* 55:806-812.

APPENDIX

The media used in this study are listed in the following appendices

Except where otherwise stated all media are expressed as the quantity of ingredients required per liter

I. Crone's nitrogen free medium(Pawsey,1974)

KCl	5g	K_2HPO_4	1.25g
$CaSO_4.2H_2O$	1.25g	$MgSO_4 \cdot 7H_2O$	1.25g
$Ca_3(PO_4)_2$	1.35g	$Fe_3(PO_4)_2$	0.01g

Grind up the salts together, add 1.5g of this and 15g agar to 1 liter water and autoclave at 121°C.

II. NBTA (Woodring and Kaya,1988)

1 liter Nutrient Agar(NA) before autoclaving

0.025g Bromothymol Blue(BTB)

0.04g Triphenyltetrazolium chloride(TTC)

Mix NA and BTB. Autoclave at 121°C for 15 min. Add TTC to a few milliliters distilled water, run through a millipore filter(0.2 micron). Cool the media to 48°C, add the TTC solution to the media and mix thoroughly.

III. Nutrient agar

Beef extract	3g	Peptone	5g
Bacto agar	15g		

IV. Tryptic soy broth(TSB)

Bacto tryptone	17g	Bacto soytone	3g
Bacto dextrose	2.5g	NaCl	5g
K_2HPO_4	2.5g		

V. Tryptic soy agar(Martin,1975)

Tryptic soy broth	3g	Agar	15g

VI. Yeast extract-manitol broth

Manitol	10g	K_2HPO_4	0.5g
$MgSO_4 \cdot 7H_2O$	0.2	NaCl	0.1g
Yeast extract	0.5g		

pH was adjusted to 6.8.

VII. Yeast extract-manitol agar(YMA)

Yeast extract-manitol broth plus 15g agar per liter