# ANTIMICROBIAL ACTIVITY OF THE NEMATODE SYMBIONTS, *XENORHABDUS* AND *PHOTORHABDUS* (ENTEROBACTERIACEAE), AND THE DISCOVERY OF TWO GROUPS OF ANTIMICROBIAL SUBSTANCES, NEMATOPHIN AND XENORXIDES

by

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Antimicrobial activity of the nematode symbionts, Xenorhabdus and Photorhabdus (Enterobacteriaceae) and the discovery of two novel groups of antimicrobial substances, nematophin and xenorxides

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ANTIMICROBIAL ACTIVITY OF THE NEMATODE SYMBIONTS, *XENORHABDUS* AND *PHOTORHABDUS* (ENTEROBACTERIACEAE), AND THE DISCOVERY OF TWO GROUPS OF ANTIMICROBIAL SUBSTANCES, NEMATOPHIN AND XENORXIDES

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# ABSTRACT

The antimicrobial activity of *Xenorhabdus* and *Photorhabdus* species (Enterobacteriaceae), bacterial symbionts of the entomopathogenic nematodes *Steinernema* and *Heterorhabditis*, respectively, was studied using *X. nematophilus* (three strains), *X. bovienii* (one strain) and *P. luminescens* (one strain). Metabolites produced by the primary form of *Xenorhabdus* and *Photorhabdus* species were found to be inhibitory *in vitro* against 32 species of fungi from a range of habitats. Many plant pathogenic fungi were inhibited by all primary forms whereas the growth of two mycorrhizal (*Oidiodendron griseum, Suillus pseudobrevipes*) and two entomopathogenic fungi (*Beauveria bassiana, Metarhizium anisopliae*) was inhibited slightly or not at all. The metabolic products of the secondary form of *X. nematophilus* were shown to have antimycotic but not antibacterial activity. The growth and antimicrobial activity among strains and species of these symbiotic bacteria differed under similar culture conditions. All primary forms of *Xenorhabdus*, but not of *Photorhabdus*, produced non-proteinaceous, water-soluble substances with both antimycotic and antibacterial activities. The antimicrobial activity of *X. nematophilus* was greater than that of *X. bovienii* or *P. luminescens*.

A large variation in chemical diversity, in levels of production among different species and among different strains of the same species was found. Isolation and characterization of the active agents revealed that these bacteria produced a wide range of antimicrobial substances. The spectrum of antimicrobials produced by *X. bovienii* A21 strain includes four indole derivatives, four xenorhabdins and two new xenorxides. The later belong to a novel class of chemical compounds. *X. nematophilus* produces xenocoumacins as well as one new compound, nematophin. The C9 strain of *P. luminescens* appears to produce two anthraquinones and a

stilbene. *In vitro* tests showed that some of these substances had potent antibacterial and/or antimycotic activity against many bacterial and fungal species of medical and agricultural importance. The novel compound, nematophin, had strong antibacterial activity against Gram-positive bacteria, in particular, activity against methicillin-resistant *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) as low as 0.75μg/ml. Xenorxides had both antibacterial and antimycotic activity with MICs at a range of 0.75-12 μg/ml against *S. aureus, Micrococcus luteus, Aspergillus fumigatus, Botrytis cinerea* and *Cryptococcus neoformans*. The indoles and the stilbene had a wide spectrum of activity against both bacteria and fungi.

All strains tested showed chitinase activity with both exo- and endo-chitinases being present using p-nitrophenyl-N-acetyl-β-D-glucosaminide and nitrophenyl-β-D-N,N',N"-triacotylchitotriose as substrates. Variation in exo- and endo-chitinase activities between different species and strains of these symbiotic bacteria was detected. The strongest activity was shown by *X. nematophilus* and the weakest by *P. luminescens*. The primary form of *X. bovienii* showed significantly stronger chitinase activity than did the secondary form. The partially purified protein of *X. bovienii* showed significant antimycotic activity against conidial germination and mycelial growth of *B. cinerea*, but neither lysozyme nor β-glucanase activity was detected.

It is established that some of the antimicrobial metabolites of *Xenorhabdus* and *Photorhabdus* include compounds new to science, and that these substances have significant potential in agroforestry and medical application.

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# ABBREVIATIONS

A21: a bacterial strain of *Xenorhabdus bovienii*;

AF: aqueous fraction which is the cell-free supernatant extracted by ethyl acetate;

AFWP: AF from which the protein has been removed by acetone precipitation;

ATC: a bacterial strain of *X. nematophilus*, number 19061, obtained from the American Type Culture Collection;

BB: bromothymol blue;

BC1: a bacterial strain of *X. nematophilus*;

C9: a bacterial strain of *Photorhabdus luminescens*;

CFS: cell-free supernatant;

CFU: colony-forming-units;

D1: a bacterial strain of *X. nematophilus*;

Da: dalton;

DS: the secondary form of the bacterial strain D1;

EAF: ethyl acetate extracts;

HPLC: high performance liquid chromatography;

$IC_{50}$:50% inhibitory concentration;

ID1: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole;

ID2: 3-(2'-acetoxy-4-methyl-3'-oxopentyl)-indole;

ID3: 3-(2'-hydroxy-4'-methyl-3-oxohexyl)-indole;

ID4: 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole;

IJ: infective juvenile;

MIC: minimum inhibitory concentration;

NB: nutrient broth;

NBTA: nutrient agar supplemented with bromothymol blue and triphenyltetrazolium chloride;

NID: nematophin;

NMR: nuclear magnetic resonance;

PAGE: polyacrylamide gel electrophoresis;

PDA: potato dextrose agar;

PMSF: phenylmethylsulfonyl fluoride;

PNP: p-nitrophenol;

ppGpp: guanosine-3',5'-bis-pyrophosphate;

SDS: sodium dodecyl sulfate;

STB: 3,5-dihydroxy-4-isopropylstilbene;

TSA: the agar medium of TSB;

TSB: tryptic soya broth;

XO1:xenorxide 1;

XO2:xenorxide 2;

XR1: N-hexanoyl-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole;

XR2: N-(5'-methylhexanoyl)-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole;

XR3: N-(3-methylbutanoyl)-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole;

XR4: N-butanoyl-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole.

# CHAPTER 1. INTRODUCTION

It has become increasingly apparent in recent years that the problems of pests and diseases of man, domestic animals and crops that were once controlled by the use of synthetic pesticides and antimicrobial agents have re-emerged in many places in the world, due to both social and biological changes. In agroforestry, the unnecessarily heavy and widespread use of synthetic, chemical pesticides has resulted in the development of resistance to pesticides and has been identified as a major source of environmental contamination and ecosystem damage. In general, the chemical pesticides that were once regarded so highly are now seen as a potential health and environmental threat to humans. Consequently, many synthetic pesticides have been either banned from use or voluntarily withdrawn from the market (May, 1993). Similarly, in the medical field, antibiotics that formerly killed bacterial pathogens with ease are becoming ineffective as a result of resistance developed by bacterial and fungal pathogens. The development of multi-drug resistance in many bacteria has made many currently available antimicrobial drugs ineffective and in certain cases the resistance has almost reached crisis level, because what were once manifest as mild infections can now be life-threatening (Neu, 1992; Travis, 1994). Moreover, for diseases like tuberculosis, the ever increasing population of immune compromised patients has resulted in a sharp increase of morbidity and mortality to such an extent that the World Health Organization is urgently appealing to governments to step-up their research and development to prevent its spread (Nowak, 1995).

There is, therefore, an urgent need for new agrochemicals and antimicrobial drugs to control diseases effectively. The diversity of microbial products from soil inhabiting microorganisms has been a traditional source for the discovery of new pharmaceuticals and

agrochemicals (Porter and Fox, 1993). However, the drug industry is looking for antimicrobial substances from sources other than soil (Gabay, 1994) in the hope that these alternative antimicrobials are less likely to have their efficacy overcome so rapidly by existing resistance mechanisms. Various forms of biological control are also being considered as environmentally safe alternatives to the chemical control of pests, especially when integrated into pest management programs.

One of the more recent developments in biological control has been the commercialization of a nematode-bacteria combination (*Steinernema* spp.-*Xenorhabdus* spp.) against insect pests, because it is harmless to non-target animals and to plants (Poinar, 1990). A crucial feature of this biocontrol agent is that the bacterial symbiont (*Xenorhabdus* spp.) of the nematode (*Steinernema* spp.) produces a wide range of bioactive metabolites including antimicrobial substances that inhibit the growth of bacteria, fungi and yeasts (Akhurst and Dunphy, 1993; Chen *et al.*, 1994; Li *et al.*, 1995a,b). The diversity of metabolites produced by these bacteria suggests that these metabolites are potential sources of new agrochemicals and antimicrobial drugs, and they are the subject of this study. This first Chapter of the thesis presents the state of knowledge of the nematode/bacteria association with emphasis on the bacteria and their secondary metabolites.

Fifteen *Steinernema* and three *Heterorhabditis* species, and their bacterial associates, are known (Table 1). Both of these entomopathogenic nematode genera have undescribed strains of some of the species that are designated by code names (Poinar, 1990). As more new strains are found, the molecular techniques are being used to help define their taxonomy (Curran, 1990), and it is anticipated that several new species will be described in the near future.

**Table 1. Entomopathogenic nematode species and their respective symbiotic bacterial species.**

| Nematode species | Bacterial species | References |
|---|---|---|
| *Steinernema affinis* | *Xenorhabdus bovienii* | Akhurst and Boemare, 1990 |
| *S. anomali* | *Xenorhabdus* sp. | Akhurst and Boemare, 1990 |
| *S. carpocapsae* | *X. nematophilus* | Akhurst and Boemare, 1990 |
| *S. cubana* | *Xenorhabdus* sp. | Mracek *et al.*, 1994 |
| *S. feltiae* | *X. bovienii* | Akhurst and Boemare, 1990 |
| *S. glaseri* | *X. poinarii* | Akhurst and Boemare, 1990 |
| *S. intermedia* | *X. bovienii* | Poinar, 1990 |
| *S. kraussei* | *X. bovienii* | Akhurst and Boemare, 1990 |
| *S. kushidai* | *Xenorhabdus japonicus* | Nishimura *et al.*, 1994 |
| *S. longicaudum* | *Xenorhabdus* sp. | Kaya *et al.*, 1993 |
| *S. neocurtillis* | *Xenorhabdus* sp. | Nguyen and Smart, 1992 |
| *S. rara* | *Xenorhabdus* sp. | Akhurst and Boemare, 1990 |
| *S. riobravis* | *Xenorhabdus* sp. | Cabanillas *et al.*, 1994 |
| *S. ritteri* | *Xenorhabdus* sp. | Kaya *et al.*, 1993 |
| *S. scapterisci* | *Xenorhabdus* sp. | Nguyen and Smart, 1990 |
| *Steinernema* sp. | *X. beddingii* | Kaya *et al.*, 1993 |
| *Heterorhabditis bacteriophora* | *Photorhabdus luminescens* | Poinar, 1990 |
| *H. megidis* | *P. luminescens* | Poinar *et al.*, 1987 |
| *H. zealandica* | *P. luminescens* | Poinar, 1990 |

*Xenorhabdus* is the bacterial genus symbiotically associated with *Steinernema* and *Photorhabdus* is similarly associated with *Heterorhabditis*. In nature each nematode species is associated with only one bacterial species, although each *Xenorhabdus* or *Photorhabdus* species may be associated with more than one nematode species (Akhurst and Boemare, 1990). Five *Xenorhabdus* and one *Photorhabdus* species are now recognized, but many strains remain to be described (Table 1). The relationship between the nematodes, both *Steinernema* and *Heterorhabditis*, and their respective symbionts are complex and are not well-understood (Akhurst and Dunphy, 1993).

The non-feeding, infective juveniles (IJ) of *Steinernema*, carry their symbionts in the modified, ventricular portion of the intestine whereas the IJs of *Heterorhabditis* carry their symbionts in this location and also throughout the intestinal lumen (Bird and Akhurst, 1983; Poinar, 1990). The IJs enter their insect hosts through natural openings; *Heterorhabditis* also can penetrate directly through the integument into the hemocoel (Poinar, 1990). Once in the hemocoel of the host although the response of the host defense system to the nematodes varies with insect species, the nematode-bacteria combination is capable of killing many insect species by either overloading the host defense system or by triggering a nonself reaction toxic to the host insect (Welch and Bronskill, 1962; Beresk and Hall, 1977; Dunphy and Thurston, 1990). In nonimmune larvae of *Galleria mellonella*, the greater wax moth, the nematode was not recognized as nonself and subsequently, the nematode was able to evade the immune response of the insect host (Dunphy and Webster, 1986; Dunphy and Thurston, 1990). For about 4-6 h post infection (at room temperature) the symbiotic bacteria are released into the hemocoel (Dunphy and Webster 1988a). Within 2 h of release, the number of bacterial cells doubles and endotoxins are produced.

The insect host is killed within 24-48 h by the multiplying bacteria and associated toxin, depending on the host and nematode species (Dunphy and Webster, 1985, 1988a; Bowen *et al.*, 1988; Ehlers, 1991). This development time for the bacteria and the time of death of the insect host parallels approximately the development time from IJ to egg hatching of the second generation in *Steinernema* (Wouts, 1984) and *Heterorhabditis* (Glazer *et al.*, 1993). In nonimmune insects, such as *G. mellonella*, the lethal dose of bacterial cells required to kill 50% of the insects varied from one to ten, depending on bacterial and insect species (Poinar and Thomas, 1967; Dunphy and Thurston, 1990). The developing nematodes feed on the multiplying bacteria and host tissues, reproduce, and emerge from the cadaver as IJs to search for new hosts (Poinar, 1990). This life cycle is a good example of a mutualistic relationship between nematode and bacterial symbionts in that the nematode acts as a vector for the bacterium and provides protection from environmental factors such as host defense mechanisms (Götz *et al.*, 1981) and soil antagonists. The bacterium helps to kill the host and to provide optimal conditions for nematode development (Akhurst and Dunphy, 1993). In the early stage of an infection, the insect host's phagocytes destroy contaminating organisms that might accidentally be introduced into the hemocoel during the entry of the nematode. Consequently, the nematode is able to establish a virtually monoxenic culture with its bacterial symbionts within the host insect. The nematode benefits because the multiplying bacteria quickly kill the host, convert the host tissue into nutrients for the nematodes and maintain monoxenic culture conditions for the nematodes and bacterium in the cadaver by producing antimicrobial agents that prevent the growth of secondary micro-organisms (Akhurst, 1993; Akhurst and Dunphy, 1993).

Entomopathogenic nematode/bacterium complexes are able to kill a wide range of insect species (Begley, 1990; Klein, 1990) by overcoming and /or evading the defense mechanisms of the insect host, especially under laboratory conditions. Like many other Gram negative bacteria, *Xenorhabdus* and *Photorhabdus* species produce endotoxins (Kamionek 1975; Dunphy and Webster, 1988a, b) and exotoxins (Bowen *et al.*, 1988; Ehlers, 1991). The *X. nematophilus* endotoxins are lipopolysaccharide components of the cell wall that are toxic to the hemocytes of *G. mellonella* (Dunphy and Webster 1988a). Ensign *et al.* (1990) detected an insecticidal toxin produced by *P. luminescens* both *in vitro* and *in vivo*. The purified toxin killed the fifth instar larvae of *Manduca sexta* in 12-24 h when injected into the larvae but was not toxic when fed to the larvae. The nature and mode of action of this toxin have not yet been determined, but the substance is apparently neither a protease nor a phospholipase (Ensign *et al.*, 1990). Interestingly, McInerney *et al.*(1991a) reported that an antibiotic, xenorhabdin 2, produced by *Xenorhabdus* spp. had *per os* larvicidal activity against *Heliothis punctigera*. It is suggested that xenorhabdins might be involved in the mechanism of pathogenic action against the host insect through their insecticidal properties. In complexes such as *S. carpocapsae/X. nematophilus* (Boemare *et al.*, 1983; Burman 1982) and *S. feltiae/X. bovienii* (Akhurst, 1993) both the nematode and bacterium components produce toxins. *S. carpocapsae* produces a proteinase that is able to disorganise gut cells of the insect host (Laumond *et al.*, 1989). Kucera and Mracek (1989) identified a protease in *S. kraussei*. However, not all *Steinernema* spp. produce toxins, *S. glaseri* apparently does not and must rely on its bacterial symbiont to kill the host (Akhurst 1986). There are no reports of *Heterorhabditis* spp. producing a toxin.

An important contribution of the bacterial symbiont to nematode development that is beneficial to the complex, is the nutritional environment induced by the bacterium. Although *Steinernema* spp. can be cultured axenically *in vitro*, they are unable to reproduce in insects without the intervention of microbial activity. When axenic *S. carpocapsae* infected axenic *G. mellonella*, the nematodes matured but did not reproduce (Poinar and Thomas, 1966; Boemare *et al.*, 1983). However, when axenic *S. carpocapsae* were introduced into G. *mellonella* with bacteria such as *Enterobacter agglomerans, Serratia liquefaciens, Pseudomonas fluorescens* or their symbiont, *X. nematophilus*, they were able to reproduce (Boemare *et al.*, 1983; Aguillera and Smart, 1993). Similar results have been obtained for other *Steinernema* spp. Although *Steinernema* species are able to reproduce in rich culture media without their respective symbionts (Ehlers, *et al.*, 1990; Lunau *et al.*, 1993) their natural symbionts are generally much superior to other bacterial species in *vitro* and *in vivo* for facilitating nematode reproduction (Akhurst 1983; Boemare *et al.*, 1983; Dunphy *et al.*, 1985; Han *et al.*, 1990). However, Aguillera and Smart (1993) reported that *S. scapterisci* could be cultured *in vitro* monoxenically with many non-symbiotic bacterial species and that the nematodes produced were equally effective against insects to those produced *in vivo*. This suggests a possible alternative method for producing these entomopathogenic nematodes, using non-symbiotic bacteria.

The nature of the essential nutrients provided by bacteria has not been identified. In axenic culture *Steinernema spp.* have a requirement for sterols (Dutky, 1967; Dutky, 1974) and some essential amino acids (Jackson, 1973). However, since yields from axenic cultures are much lower than those from monoxenic cultures, there are probably other nutritional factors provided by bacteria that are important for nematode development. Among these factors may be the

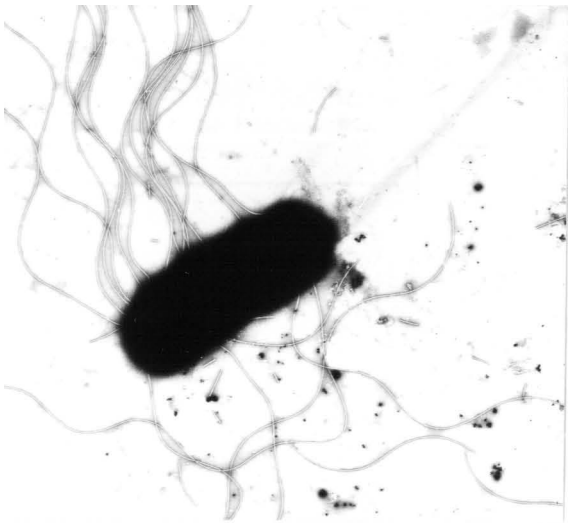
concentration of essential nutrients that improve nematode feeding efficiency. There is no information available on the provision of nutrients by the symbionts of *Heterorhabditis*. Unlike *Steinernema* spp., that are easily cultured axenically (Glaser, 1940; Jackson, 1962; Akhurst, 1983), *Heterorhabditis* cannot be axenically cultured (Strauch *et al.*, 1994).

Although the existence of a bacterial species associated with nematodes was postulated as early as 1937 (Dutky, 1937) such a bacterium was first described by Poinar and Thomas in 1965. Initially, they named the bacterium *Achromobacter nematophilus* (Poinar and Thomas, 1965). As a result of the rejection of the genus *Achromobacter* (Buchanan and Gibbons, 1974; Hendrie *et al.*, 1974) and the discovery of more bacterial strains associated with this group of nematodes, the new genus *Xenorhabdus* was created (Thomas and Poinar, 1979) and later amended (Thomas and Poinar, 1983). Later, Akhurst and Boemare (1988) elevated four subspecies of *X. nematophilus* to species status on the basis of numerical analysis of 45 variable characters of 20 strains. However, *X. beddingii*, *X. bovienii* and *X. poinarii* were not included in "Bergey's Manual of Systematic Bacteriology" because a full description and elevation of them from subspecies to species, had not been published (Holt *et al.*, 1993). As knowledge of these symbiotic bacteria has accumulated their taxonomy has apparently become more complicated. Ehlers *et al.* (1988) analyzed the relative phylogenetic positions of different *Xenorhabdus* and *Photorhabdus* strains by 16S rRNA cataloguing, and demonstrated that *P. luminescens* and *X. nematophilus* were not as closely related as they were originally thought to be. They proposed to treat the bacterial symbionts of entomopathogenic nematodes as a taxonomic unit equivalent to that of the family Enterobacteriaceae into which they are currently placed. Subsequently, Boemare *et al.* (1993a) proposed the new taxon, *Photorhabdus*, to accommodate those bacteria associated with

*Heterorhabditis* because DNA analysis showed these bioluminescent bacteria to differ significantly from *Xenorhabdus* species. Five *Xenorhabdus* species associated with 15 species of *Steinernema* nematodes have been described whereas only one *Photorhabdus* species associated with three species of *Heterorhabditis* nematodes has been described (Boemare *et al.*, 1993a; Nishimura *et al.*, 1994). Recently, however, Rainey *et al.* (1995), following the analysis of 16S rRNA gene sequences of all type strains of both genera, suggested that the taxonomy of these bacteria is much more complex and more taxonomic changes are anticipated.

*Xenorhabdus* and *Photorhabdus* are Gram negative, rod-shaped cells approximately 0.3-2.0 x 2-10 μm in size but occasionally they are filamentous (15-50 μm long). In older bacterial cultures the cells contain crystalline inclusions. Spheroplasts or coccoid bodies, resulting from disintegration of the cell wall, appear in the last third of exponential growth (Akhurst, 1983). Both genera are motile with peritrichous flagella (Figure 1). They are facultatively anaerobic and chemoorganotrophic, having both a respiratory and a fermentative type of metabolism (for characterization see 2.2. and Table 4-6). Although these bacteria are found mostly in the intestinal lumen of entomopathogenic nematodes and the body cavities of insects infected by these nematodes, several strains of *P. luminescens* have been isolated from human wounds and blood (Farmer *et al.*, 1989). These bacteria have never been isolated from soil even after being introduced axenically into autoclaved soil (Poinar *et al.*, 1980b; Chen, 1992). *Xenorhabdus* and *Photorhabdus* can be cultured in standard bacterial media. According to the 1994 edition of Bergey's Manual, the optimum temperature for *X. nematophilus* growth is about 25 °C, and at this temperature in lipid broth it has a doubling time of 0.8-1.2 h (Dunphy *et al.*, 1985). *P. luminescens* has a doubling time of about 1.5 h in peptone water and 2.5-3.0 h in larval *G. mellonella*, at 25°C (Poinar *et al.*, 1980b). It has been suggested (Götz *et al.*, 1981) that these

**Figure 1. Electron microscope photograph showing the peritrichous flagella of a bacterial cell of *Xenorhabdus bovienii*. The bacteria were negatively stained with 2% (w/v) uranyl acetate and examined with a Philips EM-300 transmission electron microscope (× 3,300).**



bacteria are sensitive to various factors such as osmotic variation, nutrient variability and $O_2$ tension. As well, *Xenorhabdus* spp. are sensitive to $H_2O_2$ produced in media exposed to ordinary fluorescent light (Xu and Hurlbert, 1990).

Like many bacteria (Brock and Madigan, 1991), form variation is one of the important features of *Xenorhabdus* and *Photorhabdus* biology. Akhurst (1980) was the first to report the occurrence of form variation in *Xenorhabdus* and *Photorhabdus*, and initially designated the two extreme colony forms as primary and secondary. The primary form, which is naturally associated with the IJs, often changes to the secondary form when these bacteria are cultured *in vitro*. The two forms exhibit distinct cell morphology and physiology (Boemare and Akhurst, 1988; Boemare and Akhurst, 1990; Nealson *et al.*, 1990). In general, the primary form produces protease, lipase, and crystalline protein, is able to bind to specific dyes and may be luminescent (only in *P. luminescens*). The secondary form does not have this range of biochemical characters (Bleakley and Nealson, 1988). In general, the primary form bacteria (except *X. poinarii*) produce antibiotics but the secondary form does not. However, in *X. nematophilus*, although the secondary form does not have antibacterial activity it does have antimycotic activity (Chen *et al.*, 1994). Several culture isolates of these bacteria have been reported that have intermediate properties (Hurlbert *et al.*, 1989; Chen, 1992; Gerritsen *et al.*, 1995). Bleakley and Nealson (1988) and Brehelin *et al.* (1993) found that the cell surface partially differs between the form variants. Fluorescein conjugated, wheat germ agglutinin bound to secondary form variants more than to primary form variants, probably due to different components of the cell surface. Smigielski *et al.* (1994) revealed differences in the respiratory activity between the two form variants of both *X. nematophilus* and *P. luminescens*, and speculated that the primary form cells are better

adapted to conditions in the insect and the nematode, whereas secondary form cells may be better adapted to conditions in soil as free-living organisms. Recent evidence suggests that the primary and secondary forms are different immunologically (Gerritsen *et al.*, 1995) and in cell outer membrane protein (Leisman *et al.*, 1995). The primary and secondary forms of *X. nematophilus* exhibited different surface translocation phenomena in that the primary form swarmed on a solid medium but that the secondary did not. Recent analysis has shown that the secondary form of these bacteria are not flagellated because of a defect in flagellin synthesis (Givaudan *et al.*, 1995), and that the secondary form of *X. nematophilus* do not have fimbrae (Brehelin *et al.*, 1993; Moureaux *et al.*, 1995)

Of practical relevance for nematode reproduction is the ability of the primary form variant to support nematode propagation. The secondary form variant does not support the multiplication of nematodes as effectively as the primary form (Akhurst, 1980; Bedding 1984; Ehlers *et al.*, 1990). Primary and secondary forms are considered to be equally pathogenic to *G. mellonella* larvae and both forms multiply rapidly in the hemocoel (Akhurst, 1980; Dunphy and Webster, 1985). It has been strongly suggested that reversion from the secondary to the primary form is common in both *Xenorhabdus* (Akhurst, 1980; Boemare and Akhurst, 1988) and *Photorhabdus* (Gerritsen *et al.*, 1995). Recent results (Krasomil-Osterfeld, 1995) showed that form shift from primary to secondary in *P. luminescens* is reversible by changes in the osmolarity of the culture medium. This reversion would prevent a total loss of the desirable primary form from the nematodes. 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). Apparently, the nematode has a preference for retaining

the primary form bacteria, partially due to differences in the cell surface (Binnigton and Brooks, 1993).

Form variation in enteric bacteria is normally triggered by environmental factors (Silverman and Simon, 1983). However, the mechanisms of form conversion and maintenance in *Xenorhabdus* and *Photorhabdus* are not known. Poinar *et al.*(1980a) proposed that form change might result from a bacteriophage that lyses the primary but not the secondary form of *P. luminescens*. Akhurst (1982) was unable to prove this by using mutagens and physical identification methods. Akhurst (1980) noticed that form shift from primary to secondary appeared after frequent subcultures, and Bleakley and Nealson (1988) speculated that form shift from primary to secondary is provoked by oxygen limitation and the accumulation of metabolites. Two independent groups (Couche *et al.*, 1987; Leclerc and Boemare, 1991) ruled out the possibility of plasmid involvement by demonstrating that identical plasmid DNA was present in both forms. Leclerc and Boemare (1991) declared that there is no involvement of plasmid loss or gain in form variation. Akhurst *et al.* (1992) were able to prove that form variation is not due to genomic rearrangements. Wang and Dowds (1993) showed that lipase production associated with primary to secondary form shift is regulated at a post-translation level. Recent results (Krasomil-Osterfeld, 1995) showed that among factors like osmolarity, low oxygen levels, light, extreme pH and temperature, osmolarity was the only factor that could reliably trigger form shift in *P. luminescens*.

Physiological studies have shown that the mechanism of form change is complex and unpredictable with respect to extent and timing, but it occurs mostly in the stationary phase (Boemare and Akhurst, 1990). They suggest that the form shift is enhanced by anaerobic culture

conditions, and that it may be related to differences in nutrient requirement and assimilation of the primary and secondary forms. However, Poinar (1993) suggested recently that the *Heterorhabditis-Photorhabdus* complex evolved from rather different ancestors than the *Steinernema-Xenorhabdus* complex and that the typical characteristics common to both have developed by convergent evolution. Form variation in the symbiotic bacteria, therefore, probably has an important function because otherwise it probably would not have evolved independently in *Photorhabdus* and *Xenorhabdus.*

*P. luminescens* is a bioluminescent, soil-living bacterial species, but a non-luminescent strain of *P. luminescens* has been reported (Akhurst and Boemare, 1986). When an insect is infected by *P. luminescens* the whole cadaver shows the characteristic luminescence (Poinar *et al.*, 1980b). The luminescence is maximal as the cultures approach stationary phase. The bacteria emit light most efficiently at 33°C in a low-salt medium with the emission spectrum peaking at 480 nm *in vivo* and 490 nm *in vitro.* 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). Although the role of bioluminescence is not known in the bacteria/nematode system, it has been speculated that it is involved in the attraction of other organisms to the infected insect cadaver (Nealson *et al.*, 1990).

Many *Xenorhabdus* species produce yellow to rusty brown pigments when cultured in media or in insects. Richardson *et al.* (1988) and Sztaricskai *et al.* (1992) purified a pigment from *P. luminescens* and identified it as an anthraquinone derivative. In recent work on identification of pigments produced by *P. luminescen,* Li *et al.* (1995b) isolated two more anthraquinone pigments from the strain C9 of this bacterium. The pigment color depends on pH; yellow below and red

above pH 9. Interestingly, most anthraquinone derivatives occur in higher plants, and also in lichens and *Streptomyces*. Some workers use the presence of pigment as a taxonomic character (Grimont *et al.*, 1984), but as the color varies with pH its value in taxonomy is limited. The function of the pigments are not known. However, the antimicrobial activity expressed by these pigments (Li *et al.*, 1995b) indicates that these pigments probably function as part of the antagonistic activity of the symbiotic bacteria against other microorganisms in the insect cadaver.

The presence of crystalline inclusions is another common feature in *Xenorhabdus* species. It has been reported (Couche *et al.*, 1987; Couche and Gregson, 1987) that crystalline inclusions occur in both *X. nematophilus* and *P. luminescens* when they are cultured in liquid media. Crystal formation occurs in primary, but not in secondary forms of *X. nematophilus*, whereas both forms of *P. luminescens* produce inclusions. Ensign *et al.* (1990) obtained intracellular crystals from *P. luminescens* cells isolated from insect cadavers. The inclusions are composed almost exclusively of protein, and are not toxic to insect larvae (Ensign *et al.*, 1990). The function of the crystalline protein is unknown, but Couche and Gregson (1987) speculated that it might be a storage protein that is metabolized in the presence of the nematode and provides the nematode with nutrients.

Dutky (1974) first suggested that one of these entomopathogenic bacteria, namely, the one from DD136 strain of *Neoaplectana* (*Steinernema*) *feltiae*, produced antibiotics. Poinar *et al.* (1980a, 1989) showed the existence of bacteriophage in *Xenorhabdus* and *Photorhabdus* (Table 2). Subsequently, Boemare *et al.* (1992, 1993b) demonstrated the common occurrence of lysogeny and bacteriocinogeny in both *Xenorhabdus* and *Photorhabdus* after mitomycin or high-temperature treatment. Phage DNA, purified from *X. nematophilus* A24, hybridized to several fragments of A24 chromosomal DNA, also confirmed that the phage genome is incorporated into

**Table 2. Antimicrobial agents known from *Xenorhabdus* and *Photorhabdus* species.**

| Species | Strain | Antimicrobial agents | Properties | Reference |
|---|---|---|---|---|
| *X. nematophilus* | ATCC 19061 | Phage | 1* | Poinar *et al.*, 1980a |
| | ATCC 53200 | Xenocoumacin 1 | 1, 2 and 3 | McInerney *et al.*, 1991b |
| | | Xenocoumacin 2 | 1,3 | McInerney *et al.*, 1991b |
| | A24 | Bacteriocin | 1 | Boemare *et al.*, 1992 |
| | | Phage | 1 | Boemare *et al.*, 1992 |
| | F1 | Xenorhabdicin | 1 | Thaler *et al.*, 1995 |
| | | Phage | | Baghdiguian *et al.*, 1993 |
| *X. bovienii* | R | Indole 1-2 | 1 | Paul *et al.*, 1981 |
| | T319 | Xenorhabdin 1-3 | 1 and 4 | McInerney *et al.*, 1991a |
| | Umea | Xenorhabdin 1,2,4,5 | 1 | McInerney *et al.*, 1991a |
| | A21 | Indole 1-4 | 1 and 2 | Li *et al.*, 1995a |
| | | Xenorhabdin 1,2,6,7 | 1 and 2 | Li *et al.*, 1995a |
| | Dan | Bacteriocin | 1 | Boemare *et al.*, 1992 |
| | | Phage | 1 | Boemare *et al.*, 1992 |
| *X. beddingii* | Q58 | Bacteriocin | 1 | Boemare *et al.*, 1992 |
| | | Phage | | Boemare *et al.*, 1992 |
| *Xenorhabdus* sp. | ATCC 39497 | Xenorhabdin 1-4 | 1, 2 and 4 | McInerney *et al.*, 1991a |
| | | Xenocoumacin 1 | 1, 2 and 3 | McInerney *et al.*, 1991b |

continues on next page

| Species | Strain | Antimicrobial agents | Properties | Reference |
|---|---|---|---|---|
| *P. luminescens* | ATCC29999 | Bacteriocin | 1 | Baghdiguian *et al.*, 1993 |
| | | Phage | 1 | Poinar *et al.*, 1980a |
| | | Stilbenes | 1 | Paul *et al.*, 1981 |
| | C1 | Bacteriocin | 1 | Baghdiguian *et al.*, 1993 |
| | HK | Anthraquinone | | Richardson *et al.*, 1988 |
| | | Stilbene | 1 | Richardson *et al.*, 1988 |
| | H90 | Anthraquinones | 1 | Li *et al.* 1995b |
| | | Stilbene | 1 and 2 | Li *et al.* 1995b |
| | ATCC29304 | Bacteriocin | | Baghdiguian *et al.*, 1993 |
| | X[1] | Anthraquinones | 1 | Sztaricskai *et al.*, 1992 |
| | K80 | Bacteriocin | 1 | Boemare *et al.*, 1992 |
| | Hm | Bacteriocin | 1 | Baghdiguian *et al.*, 1993 |
| *Photorhabdus* sp. | Hi | Bacteriocin | 1 | Baghdiguian *et al.*, 1993 |
| | K18 | Bacteriocin | 1 | Baghdiguian *et al.*, 1993 |

• 1= antibacterial; 2= antimycotic; 3 = antiulcer and 4=insecticidal.

[1] no strain number was provided.

the bacterial chromosome. Subsequently, a bacteriocin, named xenorhabdicin, was purified and partially characterized as protein particles similar in shape to phage tail consisting of two major subunits of 20 and 43 kDa (Thaler *et al.*, 1995). Bacteriocins, by definition, are highly specific antibacterial proteins, produced by certain strains of bacteria and they are active mainly against other strains of the same species (Mayr-Harting *et al.*, 1972). Bacteriocins have been detected in limited quantities in many *Xenorhabdus* and *Photorhabdus* cultures without any induction, and in increased quantity if induced (Baghdiguian *et al.*, 1993). It is believed that the bacteriocins and phage elements from these bacteria are similar to those associated with lysogenic strains of *Pseudomonas* or *Erwinia* spp. Although the culture media were active even after being diluted $10^5$ times these bacteriocins were inactivated by proteinase and high temperature treatment (Boemare *et al.*, 1993b).

Apparently, the antimicrobial systems of *Xenorhabdus* and *Photorhabdus* species act in two broad ways in that the wide-spectrum, chemical antimicrobials inhibit other microbial species, and the bacteriocins inhibit closely related species or strains of the same species. Paul *et al.*(1981) reported that nine strains of *X. nematophilus* and *P. luminescens* produce antibiotics that inhibit growth of the luminous bacteria, *Vibrio* spp. and *Photobacterium* spp. Moreover, Akhurst (1982) documented antibiotic activity of *Xenorhabdus* spp. against a variety of micro-organisms, including the Gram-positive species of *Micrococcus, Staphylococcus, Bacillus*, Gram-negative species of *Escherichia, Shigella, Enterobacter, Serratia, Proteus, Erwinia, Xenorhabdus, Flavobacterium, Pseudomonas* and yeast species of *Candida* and *Saccharomyces*.
In this study, Akhurst showed that all primary forms of *Xenorhabdus*, except *X. poinarii*, produced antibiotics and that some *Xenorhabdus* strains or species were mutually inhibitory.

Maxwell *et al.*(1994) showed that two strains of *X. nematophilus* produced broad-spectrum antibiotics both *in vitro* and *in vivo* and that the antibiotic activity was heat-stable. The spectrum of bacterial species affected varies with the strain of *X. nematophilus*, and the antibiotic released from *Xenorhabdus*-killed insect cadavers temporarily decreases the number of bacteria in the soil. Recently, Chen *et al.*(1994) showed that not only the primary form of *X. nematophilus*, *X. bovienii* and *P. luminescens* but also the secondary form of *X. nematophilus* produce substances that have antimycotic activity against a wide range of fungal species. Noting the variation in spectrum of antibiotic activity within and between *Xenorhabdus* species, these workers suggested that each of these *Xenorhabdus* species or strains produces one or more different antibiotics.

Little is known of the rate of production of these antibiotics. Paul *et al.* (1981) obtained 500 mg of semi-purified antibiotic extract from 15 L of cell-free, spent culture medium of *X. bovienii*. They isolated four indole derivatives from *X. bovienii* and hydroxystilbene derivatives from *P. luminescens*, all of which have antibiotic activity (Table 2). Richardson *et al.* (1988) purified the same hydroxystilbene derivative from *P. luminescens*. Recently, anthraquinone pigments with antibacterial activity have been isolated also from strain C9 of *P. luminescens* (Li *et al.*, 1995b) and from an unspecified strain of *P. luminescens* (Sztaricskai *et al.*, 1992). Naturally occurring anthraquinones are known to be produced by fungal species (Anke *et al.*, 1980b). These compounds were shown to have antimicrobial and antitumor activities and are toxic to animals (Anke *et al.*, 1980a).

Sundar and Chang (1992, 1993) have investigated the activity and mechanism of action of the indole and stilbene derivatives isolated by Paul *et al.* (1981). Both the indole and stilbene derivatives were effective against both Gram-positive and Gram-negative bacteria, causing a

severe inhibition of RNA synthesis by inducing an accumulation of the regulatory nucleotide, guanosine-3',5'-bis-pyrophosphate in susceptible bacteria. It has been reported (McInerney *et al.*, 1991a, b) that seven antibiotic compounds have been isolated from *Xenorhabdus* spp., and of these, five dithiolopyrrolone derivatives, named xenorhabdins, are organic soluble (see Tabie 2). The other two, benzopyran-1-one derivatives, named xenocoumacin 1 and 2, are water soluble.

Dithiolopyrrolone compounds which were initially isolated from *Streptomyces* species have activity against a variety of fungi, amoebae, Gram-positive and Gram-negative bacteria (Celmer and Solomons, 1955). Xenorhabdin 2, a dithiolopyrrolone compound isolated from *X. bovienii* has antibacterial activity against Gram-positive bacteria (McInerney *et al.*, 1991a). The mechanisms of action of dithiolopyrrolone derivatives include membrane stablization in animals (Ninomiya *et al.*, 1980) and inhibition of RNA and protein synthesis in yeast (Jimenez *et al.*, 1973; Tipper, 1973).

Xenocoumacins belong to the same class of compounds, with regard to structure and pharmacological activity, as the amicoumacins which are isolated from *Bacillus pumilus* (Shimojima *et al.*, 1982). Xenocoumacin 1 and 2 are considered to arise from leucine (McInerney *et al.*, 1991b ) and produced in 1:1 ratio in insect cadavers infected with *X. nematophilus* (Maxwell *et al.*, 1994). Xenocoumacins are highly active against Gram-positive bacteria, including *Staphylococcus* and *Streptococcus* species and also against some strains of *Escherichia coli* (McInerney *et al.*, 1991b ). However, most enterobacteria and *Pseudomonas aeruginosa* are resistant to xenocoumacins, as is the drug-resistant strain of *Staphylococcus aureus* (McInerney *et al.*, 1991b). Xenocoumacin 1 exhibits also antimycotic activity against species of *Cryptococcus*, *Aspergillus*, *Trichophyton* and *Candida* (McInerney *et al.*, 1991b ). Both xenocoumacin 1 and 2

displayed potent antiulcer activity against stress-induced ulcers when dosed orally in rats (McInerney *et al.*, 1991b). The pharmacological activity of xenocoumacins has not been tested, but was speculated to be similar to that of amicoumacins ( McInerney *et al.*, 1991b) which have been shown to have high acute toxicity to rats (Shimojima *et al.*, 1982).

It is well-known that *Xenorhabdus* and *Photorhabdus* species produce a range of inhibitory substances. The hypothesis that these antibiotics prevent the insect cadaver from putrefying and so maintaining optimal conditions for nematode growth and reproduction, seems logical. Although a variety of antimicrobial agents have been isolated from two genera of entomopathogenic bacteria, their activity spectrum, production and the relative abundance of the known groups of compounds is not fully known.

The objective of this study was to examine the antimicrobial activity of these bacterial symbionts of entomopathogenic nematodes, namely, *Xenorhabdus* and *Photorhabdus* species. In particular, the study would examine the antimicrobial spectrum, chemical nature, bioactivity, production and potential usefulness of the metabolites produced by selected bacterial strains.

# CHAPTER 2. MATERIALS AND GENERAL METHODS

## 2.1. SOURCE, ISOLATION AND MAINTENANCE OF *XENORHABDUS* AND *PHOTORHABDUS*.

All species and strains of nematode and their associated bacteria that were used in this research project were from the laboratory collection of Dr. J. M. Webster (Department of Biological Sciences, Simon Fraser University), and their sources are as listed in Table 3. The nematode cultures were maintained by periodic passage through larvae of the Greater Wax Moth, *Galleria mellonella* (obtained from the Simon Fraser University Insectary), at 24 °C, and the nematodes were stored in moist sponge at 4 °C (Dukty *et al.*, 1964). Bacterial strain C9 of *P. luminescens* was isolated from IJs, whereas all other strains were isolated from nematode-infected *G. mellonella* larvae. Newly molted, last instar larvae of *G. mellonella* were infected with IJs, carrying the respective *Xenorhabdus* or *Photorhabdus* spp., at a rate of 25 IJs/larva. After 24-48 h the dead larvae were surface disinfected by dipping them into 95% ethanol and igniting them (Woodring and Kaya, 1988). The cadavers were aseptically opened with sterile forceps, hemolymph was streaked onto 9-cm Petri plates of nutrient agar medium (NBTA:beef extract 3.0 g, peptone 5.0 g, agar 15.0 g, bromothymol blue 0.025 g and 2,3,5-triphenyltetrazolium chloride 0.04 g per liter of distilled water) (Woodring and Kaya, 1988) and incubated in the dark at room temperature (approximately 24 °C).

Where isolation from infected insects was difficult (e.g., C9 strain) the bacterial symbiont was isolated from the IJs using a method modified after Dunphy and Webster (1984), in which IJs were surface-sterilized by immersion in 0.2% Thermosol (Sigma) for 2 h, rinsed thoroughly with

**Table 3. Species and sources of *Xenorhabdus* and *Photorhabdus*, and their nematode symbionts, used in this study.**

| Bacterial species | Bacterial strains | Nematode species and strains | Source |
|---|---|---|---|
| *X. nematophilus* Poinar and Thomas | D1 | *Steinernema carpocapsae* (Weiser) (DD136) | G. O. Poinar, University of California, Berkeley, USA. |
| | ATC | *S. carpocapsae* | American Type Culture Collection, Rockville, Maryland, USA. |
| | BC1 | *S. carpocapsae* | Unknown |
| *X. bovienii* Akhurst and Boemare | A21 | *S. feltiae* (Filipjev) (A21) | Isolated from soil in Merrit, British Columbia, Canada. |
| *P. luminescens* Akhurst and Boemare | C9 | *Heterorhabditi megidis* Poinar, Jackson and Klein (H90) | Isolated from soil in Summerland, British Columbia, Canada |

sterile water and homogenized. The homogenous mixture was spread on NBTA and incubated as described above. The resulting primary form, as defined by Boemare and Akhurst (1988), of each bacterial strain was characterized (see below) and subcultured at 14-day intervals on NBTA. To obtain the secondary form of *Xenorhabdus* species, the primary form was maintained without subculture for 20 days at 24°C. During this extended period many bacterial colonies produced secondary forms. The secondary form of each *Xenorhabdus* and *Photorhabdus* strain was confirmed by testing its antibiotic activity against the bacterium *Bacillus subtilis* (see below), and each strain was subsequently maintained on NBTA, as described above, and subcultured biweekly until needed.

## 2.2. CHARACTERIZATION OF *XENORHABDUS* AND *PHOTORHABDUS* STRAINS.

The characterization was done, unless otherwise stated, using methods described by Holding and Collee (1971) and Smibert and Krieg (1981), and was repeated once. All tests were conducted at 25 °C using the primary form of each strain. The cell size of each bacterial species was measured microscopically when examining 24 h cultures in nutrient broth (NB: beef extract 3 g and peptone 5 g in one liter of distilled water). The bacterial motility was observed under the microscope (× 1,000). Flagellum position was examined by observation of the negatively stained bacterial cells from 24-h-old NB cultures with the electron microscope. Briefly, aliquots (10 µl) of bacterial suspension were placed onto carbon-coated copper grids (200 mesh). The bacteria were negatively stained with 2% (w/v) uranyl acetate. After 1 min of staining, excess material was removed, the grids air-dried and the bacteria examined with a Philips EM-300 transmission electron microscope. Colony pigmentation and bioluminescence were recorded from 4-day-old colonies on nutrient agar (NB plus 1.5% agar). Cytochrome oxidase reaction was determined by

cytochrome oxidase testing paper according to the instructions of the manufacturer (Unipath Ltd, Basingstoke, Hampshire, England). All of the following tests were conducted according to methods described by Smibert and Krieg (1981). Catalase activity was tested by flooding 24 h-old nutrient agar culture with 10% hydrogen peroxide and observing the release of oxygen. Lecithinase was tested on egg yolk agar which consisted of peptone 40.0 g, $Na_2HPO_4$ 5.0 g, NaCl 2.0 g, $MgSO_4$ 0.5% solution 0.2 ml, glucose 2.0 g, agar 25.0 g and two egg yolks in one liter distilled water. Urease was tested on Christensen urea agar which consisted of peptone 1.0 g, glucose 1.0 g, NaCl 5.0 g, $KH_2PO_4$ 2.0 g, phenol red 0.012 g, agar 20.0 g in one liter distilled water.

Acid production by the bacterial species from various carbohydrates was determined 7 days after incubation by visual observation of color changes in 1% (w/v) peptone water containing 0.0025% (w/v) bromothymol blue (BB) and 1% (w/v) carbohydrate as described by Smibert and Krieg. (1981). Because of the limitation of BB's sensitivity to pH changes, the pH level of the bacterial culture tubes was measured also using a pH meter to confirm the production of acid. The utilization of carbon sources listed in Table 6 was assessed in minimal medium which consisted of $NH_4NO_3$ 0.3%, $K_2HPO_4$ 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% at pH 7.0 plus 0.2% (w/v) carbon source. Growth was determined by visual observation of turbidity of the culture broth after 6 days of incubation in the dark at 24 °C.

Table 4, Table 5 and Table 6 show the morphological, physiological and biochemical characteristics of the five bacterial strains studied in this project. All primary form strains are motile, Gram-negative and rod-shaped with an average rod length greater than 5.0 μm and having

**Table 4. Morphological and physiological characteristics of *Xenorhabdus* and *Photorhabdus* strains used in this study.**

| Characteristics | Bacterial strains* | | | | |
|---|---|---|---|---|---|
| | A21 | C9 | BC1 | D1 | ATC |
| Gram reaction | –[†] | – | – | – | – |
| Cell size (μm) | 5.3×2.2 | 5.8×1.2 | 5.5×1.6 | 5.1×2.1 | 5.3×1.2 |
| Motility | + | + | + | + | + |
| Peritrichous flagella | + | + | + | + | + |
| Pigmentation | yellow | orange | white | white | white |
| Bioluminescence | – | + | – | – | – |
| Catalase | – | + | – | – | – |
| Oxidase | – | – | – | – | – |
| Urease | – | + | – | – | – |
| Lecithinase | + | + | + | + | + |

[†] + positive; - negative.

*see Table 3 for explanation of the codes of the bacterial strains.

**Table 5. Acid production by *Xenorhabdus* and *Photorhabdus* strains used in this study when tested on carbohydrate sources.**

| Carbohydrates† | Bacterial strains* | | | | |
|---|---|---|---|---|---|
| | A21 | C9 | BC1 | D1 | ATC |
| D-Arabinose | +/- | – | – | – | – |
| Esculin | – | – | – | – | – |
| D-Fructose | + | + | + | +/- | +/- |
| D-Galactose | – | – | – | – | – |
| D-Glucose | + | + | + | + | + |
| Inositol | +/- | + | – | – | – |
| Insulin | – | – | – | – | – |
| D-Lactose | – | – | – | – | – |
| D-Maltose | + | + | + | +/- | + |
| D-Mannitol | – | – | – | – | – |
| D-Mannose | + | + | + | + | + |
| D-Raffinose | – | – | – | – | – |
| D-Sorbitol | +/- | – | – | – | – |
| L-Sorbose | – | – | – | – | – |
| D-Xylose | – | – | – | – | – |

† Acid production was defined by the pH level in the test tube: pH <6.0 (+); pH 6.0-6.5 (+/-); and pH > 6.5 (–).

*see Table 3 for explanation of the codes of the bacterial strains.

**Table 6. Utilization of carbon sources by *Xenorhabdus* and *Photorhabdus* strains used in this study.**

| Carbon sources‡ | Bacterial strains* | | | | |
|---|---|---|---|---|---|
| | A21 | C9 | BC1 | D1 | ATC |
| Asparagine | + | + | + | + | + |
| Cysteine | – | – | – | – | – |
| Glycine | – | – | – | +/- | – |
| Tyrosine | + | + | + | + | + |
| Nicotinic acid | – | – | – | – | – |
| Ethanol | – | – | – | – | – |
| Methanol | – | – | – | – | – |
| Inositol | +/- | + | +/- | +/- | + |
| Mannose | + | + | + | + | + |
| D-Galactose | – | – | – | – | – |
| D-Glucose | + | + | + | + | + |
| D-Lactose | – | – | – | – | – |
| D-Mannitol | – | – | – | – | – |
| D-Sorbitol | – | – | – | + | – |
| D-Ribose | + | + | +/- | + | + |

‡ Utilization of carbon: (+) turbidity within 6 days, (+/-) delayed turbidity within 6 days and (–) no turbidity over 6 days.

*see Table 3 for explanation of the codes of the bacterial strains.

peritrichous flagella (Figure 1). Crystalline inclusions were clearly visible in all tested strains. They were negative for oxidase and positive for lecithinase, absorbed bromothymol blue from NBTA and formed blue to greenish blue colonies. All strains produced acid from D-fructose, D-glucose, D-maltose and D-mannose whereas none of them produced acid from esculin, insulin, D-lactose, D-mannitol, D-raffinose, L-sorbose and D-xylose. The results of their utilization of 15 carbon sources showed that all tested strains utilized asparagine, tyrosine, inositol, mannose, glucose and ribose but not cysteine, nicotinic acid, ethanol, methanol, D-lactose and D-mannitol. The above characteristics distinguish these bacterial strains from other closely related genera. Strain C9 is positive for bioluminescence, catalase and urease, which are typical for *P. luminescens*. These characteristics distinguish C9 from all of the other strains tested. The strain A21 was unique among those tested in being positive for lipase, producing yellow pigment on nutrient agar (NB plus 1.5% agar) and in producing acid from D-arabinose and D-sorbitol. These characteristics collectively are typical of *X. bovienii* (Akhurst and Boemare, 1988). Strains BC1, D1 and ATC produced no pigments on nutrient agar, shared the same nematode origin and were similar in almost all characteristics tested. These three strains have characteristics typical of *X. nematophilus*.

## 2.3. TEST ORGANISMS.

A range of bacterial, yeast and fungal species were used to test the bioactivity of the bacterial metabolites and they were either purchased from commercial sources or generously donated by the individuals listed (see Appendix 1). The bacterial strains were usually maintained on TSA [TSB (Difco) plus 1.5% agar], incubated in the dark at room temperature and subcultured weekly. For long term preservation the bacteria were grown in nutrient broth (25 °C

at 120 rpm) for 24 h, then the bacterial culture was mixed with an equal amount of 24% sucrose, lyophilized and then preserved at -20 °C as a powder.

The fungal and yeast species were maintained and subcultured biweekly on potato dextrose agar (PDA) in the dark at room temperature or were stored in mineral oil at 4 °C.

## 2.4. BIOASSAYS.

### 2.4.1. Bioassay of antibacterial activity.

*Bacillus subtilis* (see Appendix 1) was chosen as the standard bacterial indicator of antibiotic activity, because it is one of the most commonly used bacteria for bioassay of antibiotics, and initial tests showed it to be sensitive to antibiotics produced by *Xenorhabdus* and *Photorhabdus*. A standard spore suspension of *B. subtilis* was made using a modified method of Hewitt and Vincent (1989) as follows: 1) inoculum was prepared by adding a loopful of slant culture of *B. subtilis* into 10 ml of sterile TSB (shaken at 120 rpm, overnight at 37 °C), then 1 ml of the bacterial broth was transferred into a one liter flask containing 500 ml TSB; 2) the flask was incubated at 37 °C, and shaken at 80 rpm for 10 days; 3) the bacterial spores and cells were collected by centrifuging at 11,000 × g for 5 min and washed three times with sterile water; 4) the spores and cells were resuspended in a sterile 0.9% (w/v) 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 transferred into batches of 1.5-ml tubes for storing at -20 °C until required.

An agar diffusion bicassay, based on Hewitt and Vincent (1989), was employed throughout the whole study as the standard initial test of antibacterial activity. Except where

otherwise stated, all antibiotic activity tested on agar plates followed the procedure outlined below.

TSA was autoclaved for 15 min and 10 ml of media were poured aseptically into each 9-cm Petri dish. After cooling, the plates were stored at room temperature until required. One hundred microlitres of the *B. subtilis* spore suspension was pipetted onto each assay plate. The inoculum was evenly spread over the surface of the agar plate with a sterile spreader and then the plates were dried in a laminar air flow with their lids open for 10-20 min.

Before test solutions were added to the *B. subtilis* plates, 2-4 wells, each of 4 mm diameter, were cut in the inoculated agar with an alcohol-flame sterilized cork borer and the agar plugs were removed with sterile forceps. Then 40 µl of test solution were added to each well, the plates were covered with their lids and incubated at 25 °C. After 24 h incubation, inhibition zones (clear zones without bacterial growth) were visualized (qualitative tests) and, as necessary, the radiuses of the inhibition zones were measured with hand-held calipers.

2.4.2. Bioassay of antimycotic activity.

*Botrytis cinerea* was used for the routine, analytical bioassay organism for antimycotic activity because of its ability to produce conidia and the ease of their subsequent germination. *B. cinerea* was grown on PDA for about 7 days at 24 °C under laboratory light conditions to stimulate the production of conidia. The conidia were harvested by flooding the fungal plate with sterile, distilled water, adjusting the number of conidia to $2x10^4$ conidia/ml and then adding potato dextrose broth to the suspension at a final concentration of 10%. The bioassay was performed in dual-well, cavity slides. To each well was added 50 µl of the test solution that was to be bioassayed. The slide was then placed in a laminar air-flow until the solution had dried, then

50 µl of conidial suspension was pipetted into each well, and the cavity slide was incubated in the dark at 24 °C overnight in a moist chamber. Conidial germination was determined microscopically and expressed as a percentage of the first 100 conidia randomly found in each well. This bioassay system was used for the monitor of antimycotic activity in all fractions during the purification process in 4.2.1.

To further quantify the antimycotic activity of *Xenorhabdus* and *Photorhabdus* species, the test solutions were tested also against *B. cinerea* on plates. One milliliter of the conidial suspension ($2\times10^4$ conidia /ml ) was thoroughly mixed with 9 ml water agar (1.4%) at 49 °C in a 9-cm Petri dish, after the agar had set, several wells (4 mm diameter) were made, and 40 µl of the test solution was added into each well. These plates were incubated at 24 °C for 48 h, and the radiuses of inhibition zones were measured with hand-held calipers after incubation.

2.4.3. Determination of the efficacy of purified antimicrobial substances.

To determine minimum inhibitory concentration (MIC) of the purified compounds, the standard procedure for testing antibiotics was followed (The National Committee for Clinical Laboratory Standards, 1983). Briefly, test chemicals were dissolved in dimethyl sulphoxide (DMSO), filter sterilized and diluted into distilled water resulting in a final DMSO concentration < 0.4% (v/v) at a chemical stock concentration of 200 µg/ml. The active compounds were tested in serially twofold dilutions over concentrations ranging from 100 µg/ml to 0.1 µg/ml (i.e. 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1) for the determination of MICs. The testing medium (pH 7.0) for bacteria comprised bacteriological peptone 6.5 g, yeast extract 1.5 g, sodium chloride 3.5 g, dextrose 1.0 g, $K_2HPO_4$ 3.7 g and $KH_2PO_4$ 1.3 g in one liter of distilled water with or without 15 g agar for agar dilution or liquid dilution tests. The testing medium for fungi and

yeasts was PDA. Test bacteria and the two test yeasts (*C. tropicalis* and *C. neoformans*) were grown on nutrient agar (PDA for the yeasts) for 24 h (25°C), then were scraped from the plate by flooding the plate with 0.8% saline and diluted with the saline to make inocula (containing 2.5-2.8x$10^7$ CFU/ml). *Aspergillus* spp. and *B. cinerea* were grown on PDA for 7 days (25 °C) before the conidia were harvested by flooding the plate with sterile, distilled water and diluted to make the final inocula (2.5-3.0x$10^6$ conidia/ml). For the agar dilution test, 5 µl of the inoculum was added to the agar plate containing the test compound which was then incubated at 35 °C (*B. cinerea* 24 °C) and the MICs were visually determined after 24 h incubation (2 days for *B. cinerea*). This system allowed 3-4 organisms to be tested on a single plate. For the liquid dilution test, an amount of the inoculum was added such that the final bacterial or yeast concentration in the test solution was 6x$10^5$ CFU/ml. The inoculated medium was incubated under the same conditions as for agar plates. Medium without chemical compound was prepared the same way and used as control. The MIC was defined as the lowest chemical concentration that prevented visible growth of the test organism at the above conditions. The tests on *Pythium* and *Phytophthora* were done on water agar (1.5%) or rye agar (Caten and Jinks, 1968). Test chemicals were diluted with distilled water, mixed with an equal amount of agar. After the medium had solidified, each plate was inoculated in the center with a mycelium plug (5 mm diam.) and incubated at 24 °C (room temperature for *P. infestans*). MICs were determined after 24 h (4 days for *P. infestans*).

## 2.5. EXPERIMENTAL REPETITION AND STATISTICAL ANALYSIS.

Except where otherwise stated, tests done throughout the study were repeated three times. Data were analyzed on SAS software using Tukey's Studentized Range Test for variables ($P$=0.05); data are expressed in the thesis as means ± standard errors.

# CHAPTER 3. THE ANTIMICROBIAL ACTIVITY

## 3.1. INTRODUCTION.

Although the antimicrobial activity of *Xenorhabdus* and *Photorhabdus* has been known for some time (Dutky, 1974), and several antibiotics have been purified and characterized (Paul *et al.*, 1981; Richardson *et al.*, 1988; McInerney *et al.*, 1991a, b), little is known about the antimicrobial spectrum of these bacteria and, in particular, about their activity against fungi. To help overcome this deficiency in our knowledge a study was done to investigate the antimicrobial activity of *Xenorhabdus* and *Photorhabdus*.

## 3.2. MATERIALS AND METHODS.

Inocula of the primary form *Xenorhabdus* and *Photorhabdus* species and of the secondary form of *X. nematophilus* were grown in 50 ml of TSB in 100 ml Erlenmeyer flasks. Cultures were shaken in darkness on a horizontal, gyratory shaker (80 rpm, 24°C) for 24 h.

### 3.2.1. *In vitro* spectrum of activity against fungi.

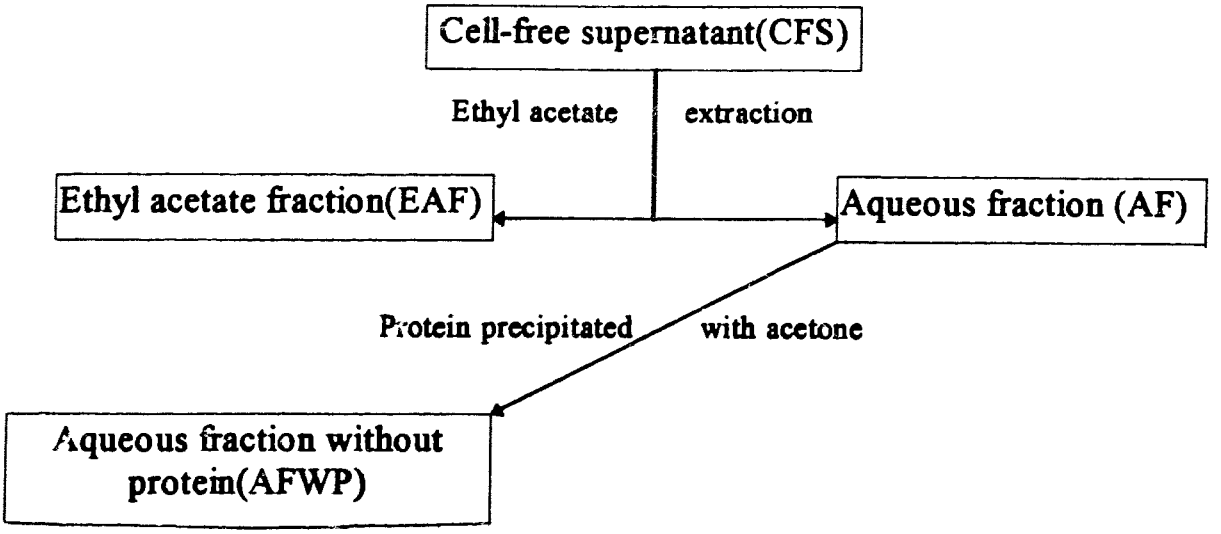
The initial determination of the antimycotic activity spectrum of *Xenorhabdus* and *Photorhabdus* species was done by adding 50µl of the inocula of each *Xenorhabdus* and *Photorhabdus* strain aseptically into the center of separate 9-cm Petri dishes each containing 10 ml of autoclaved TSA which was then incubated in the dark at 24°C for 4 days. Control plates were inoculated with TSB minus *Xenorhabdus* and *Photorhabdus*. Each plate was subsequently inoculated in a triangular fashion with three mycelial plugs (each of 0.5 cm diameter placed about 8 mm from the edge of the plate) of one of the 32 test fungi cut from freshly grown cultures on

PDA plates. Each test was replicated three times using three plates. The growth of the fungal species on each plate was compared with that of the controls, and incubation was terminated when the fungal colony in the control plates reached the center of the plate.

3.2.2. Growth, fractionation and antimicrobial activity of *Xenorhabdus* and *Photorhabdus*.

The antibacterial and antimycotic activity of the metabolites from each strain were tested by adding 1 ml inoculum, which had been adjusted to an absorbance of 1.50 at 600 nm, of each bacterial strain to 30 ml TSB in a 100 ml flask, and culturing in the dark at 25 °C on a shaker (120 rpm). Three replicate flasks were collected each sampling time at day 1, 2, 3, 4 and 5. No sample was taken prior to 24 h because the main interest of this study was antimicrobial activity and, normally, secondary metabolites such as antibiotics of microorganisms are produced in a relatively late stage of growth. TSB minus bacteria was used as control and was incubated and treated in parallel and in the same way as the bacterial cultures, as outlined below. From each sample 1 ml of bacterial culture was drawn, diluted 10 times with TSB and measured at 600 nm for absorbance. The rest of the culture was measured for pH value, neutralized with 6 M HCl, and centrifuged (11,000 × g, 20 min 4°C) to yield a cell-free supernatant (CFS). In order to determine the chemical nature of the secondary metabolites the CFS was processed as described below (see diagram). It is known (Paul *et al.*, 1981; Richardson *et al.*, 1988; McInerney *et al.*, 1991a, b) that all non-proteinaceous, antimicrobial substances isolated from *Xenorhabdus* and *Photorhabdus*, except xenocoumacins, are ethyl acetate extractable. Ten milliliters of the CFS sample were each extracted twice with 10 ml ethyl acetate each time, the extracts were combined and the water in the extracts was removed by passing through an anhydrous sodium sulfate column (40×4 mm).

The ethyl acetate fractions were evaporated in an evaporator to dryness. Preliminary data (not shown) established that the ethyl acetate extracts had low antimicrobial activity when




reconstituted with solvent and bioassayed, which made the quantitative bioassay difficult. Alternatively, the ethyl acetate extracts from 10 ml of CFS were dissolved in 0.5 ml methanol to yield the ethyl acetate fraction (EAF) of the samples, which represents a 20-fold-concentration of the extract, and subsequently bioassayed. The remaining, extracted CFS was frozen in liquid nitrogen, lyophilized to dryness and, when required, the dry powder was reconstituted with distilled water to give the aqueous fraction (AF) of the sample and subsequently bioassayed. To determine the possible role of proteinaceous agents in the overall antimicrobial activity and their possible synergistic activity with the non-proteinaceous antimicrobial substances, half of the above extracted CFS of strain A21 was treated with acetone at a final concentration of acetone of 65% (v/v) to precipitate the protein prior to lyophilization. The precipitate was removed by centrifugation (12,000 x g, 20 min, 4 °C) and the supernatant without protein was lyophilized. The dry powder was then reconstituted with sterile, distilled water to give the aqueous fraction without protein (AFWP). The CFS, EAF, AF and AFWP of the samples and of the TSB control

were bioassayed against *B. subtilis* and *B. cinerea* for their antibacterial and antimycotic activities, respectively on TSA or water agar plates as outlined in 2.4.1 and 2.4.2.

3.2.3. Preparation of cell-free filtrates from cultures of *Xenorhabdus* and *Photorhabdus*.

Cell-free filtrates of the primary form of the two *Xenorhabdus* and of the *Photorhabdus* species were prepared for further tests on the antimicrobial activity of these bacterial strains. One milliliter of inoculum of each strain of *Xenorhabdus* and *Photorhabdus* was added separately, and aseptically to 700 ml of autoclaved TSB in 2,000 ml flask. The flasks were incubated at 24°C on a horizontal gyratory shaker (120 rpm). After 4 day incubation, the pH of the spent media was adjusted to 7.0 with 6 M HCl, the culture was centrifuged (11,000 × g, 20 min, 4 °C) to remove the bacteria, and the supernatant was filter-sterilized (0.22 μm, Millipore) and stored at 4 °C until required. The control consisted of the same volume of uninoculated TSB which was treated similarly.

3.2.4. Effect of the cell-free filtrates on conidial germination of *B. cinerea*.

The bioassay for conidial germination was performed on cavity slides as outlined in 2.4.2. Conidial germination was determined under a compound microscope and was based on counting and observing the status of 100 conidia in each of three replicate slides after 14 h incubation.

3.2.5. Effect of heat on the antimycotic activity of filtrates.

To determine heat stability of the filtrates on conidial germination, the cell-free filtrates prepared as described above (section 3.2.3), of strains of *X. nematophilus*, *X. bovienii* and *P. luminescens* were heated in a water bath at either 60 or 100°C or autoclaved at 121°C for 20 min with autoclaved TSB as control. The heat-treated filtrates were then centrifuged at 11,000 × g, at

room temperature for 10 min to remove precipitates, filter-sterilized (0.22 μm) and bioassayed as described in 2.4.2.

3.2.6. Toxicity of *Xenorhabdus* and *Photorhabdus* filtrates to fungi.

Filtrates of strains *X. nematophilus, X. bovienii* and *P. luminescens,* prepared as in 3.2.3. above were each lyophilized to powder. The powdery yield of each cell-free filtrate varied from 18-26 mg/L, depending on the bacterial strain. The powders were dissolved with sterile, distilled water and a serial twofold dilution was done to produce concentrations for each strain ranging from 0.5-100 mg/ml. Each dilution was then mixed thoroughly with an equal amount of 3.0% water agar (49°C) and poured into 9-cm Petri dishes (10 ml/dish). Plates with TSB and water agar were used as controls. When the plates were cold an agar disc (5 mm diameter) was removed from the periphery of actively growing PDA plates of the desired test fungus and placed in the center of each treatment and control plate. There were six replicates of each filtrate concentration. The plates were incubated in the dark at 24 °C until the fungal hyphae in any of the controls were about 5 mm from the edge of the plate. Growth inhibition was determined by the following equation: % inhibition = $100(1-R^2/r^2)$, where R represents the radius of the fungus colony in the treated plates and r represents the average radius of the fungal colonies in both the TSB and water control plates. Test of the conidial germination was done as described in 2.4.2.

To determine the concentration of the cell-free filtrate that inhibited 50% ($IC_{50}$) growth of the test fungi the percentage inhibition of each individual fungus was plotted against the log concentration of lyophilized filtrates, the linear regression of each test was generated, the $IC_{50}$ calculated and, the $IC_{50}$s from each test were analyzed as described in 2.5 using Tukey's Studentized Range Test.

## 3.3. RESULTS.

### 3.3.1. Spectrum of antimycotic activity.

The inhibition of fungal growth by antimycotic substances varied with the fungal species tested and with the bacterial strain (Table 7). The growth of all of the fungi except for the mycorrhizal fungus *Suillus pseudobrevipes* was inhibited to some extent by one or more of the strains of *Xenorhabdus* and *Photorhabdus*. Seven species of plant pathogenic fungi, *B. cinerea*, *Ceratocystis ulmi*, *C. dryocoetidis*, *Mucor piriformis*, *Pythium coloratum*, *P. ultimum* and *Trichoderma pseudokoningii*, were inhibited by the primary forms of all the *Xenorhabdus* and *Photorhabdus* strains. The strains of *Xenorhabdus* and *Photorhabdus* differed in their spectra of activity against the fungal species tested; the BC1 strain of *X. nematophilus* had the widest activity spectrum and the secondary form variant (DS) of D1 of *X. nematophilus* had a much more restricted range than its primary form variant. Considerable resistance to these antimycotics was demonstrated by *Aspergillus niger*, *Cephaloascus fragrans*, by the insect pathogenic fungi *Beauveria bassiana*, and *Metarrhizium anisopliae*, and by the mycorrhizal fungi *Oidiodendron griseum*, and *S. pseudobrevipes*.

It was observed that the hyphae of some these fungi, which had their growth severely inhibited, were lysed in the presence of *Xenorhabdus* and *Photorhabdus*.

### 3.3.2. Growth of *Xenorhabdus* and *Photorhabdus*, and their antimicrobial activity.

Absorbances of the cultures varied significantly among the strains of *Xenorhabdus* and *Photorhabdus* (Figure 2). The BC1 strain of *X. nematophilus* had the highest absorbance and the A21 strain of *X. bovienii* had the lowest at the end of the 5 day of culture. The maximum

**Table 7. Spectrum of antimycotic activity of species and strains of *Xenorhabdus* and *Photorhabdus* against various fungal species when grown in dual culture on agar plates.**

| Fungal species | *Xenorhabdus* and *Photorhabdus* strains* | | | | |
|---|---|---|---|---|---|
| | D1 | DS' | BC1 | A21 | C9 |
| *Alternaria sp. strain* A | ++** | | ++ | ++ | + |
| *Alternaria sp. strain* B | ++ | | ++ | ++ | + |
| *Aspergillus niger* | - | | + | + | - |
| *Beauveria bassiana* | - | - | + | + | - |
| *Botrytis cinerea* | +++ | ++ | +++ | +++ | +++ |
| *Cephaloascus fragrans* | + | | + | + | - |
| *Ceratocystis ulmi* | +++ | | +++ | +++ | +++ |
| *C. dryocoetidis* | +++ | | +++ | +++ | +++ |
| *Fusarium oxysporum* | ++ | ++ | ++ | ++ | ++ |
| *F. solani* | ++ | ++ | ++ | ++ | ++ |
| *Geotrichum candidum* | ++ | + | ++ | ++ | ++ |
| *Gloeosporium perannans* | ++ | | ++ | ++ | - |
| *Metarrhizium anisopliae* | - | - | + | - | - |
| *Monilinia fructicola* | ++ | | ++ | + | - |
| *Mucor piriformis* | +++ | ++ | +++ | +++ | +++ |
| *Oidiodendron griseum* | - | | + | - | - |
| *Ophiostoma piceae* | ++ | +++ | ++ | ++ | ++ |
| *Pythium coloratum* | +++ | ++ | +++ | +++ | +++ |
| *P. ultimum* | +++ | | +++ | +++ | +++ |
| *Penicillium expansum* | ++ | | ++ | + | + |

continues on next page

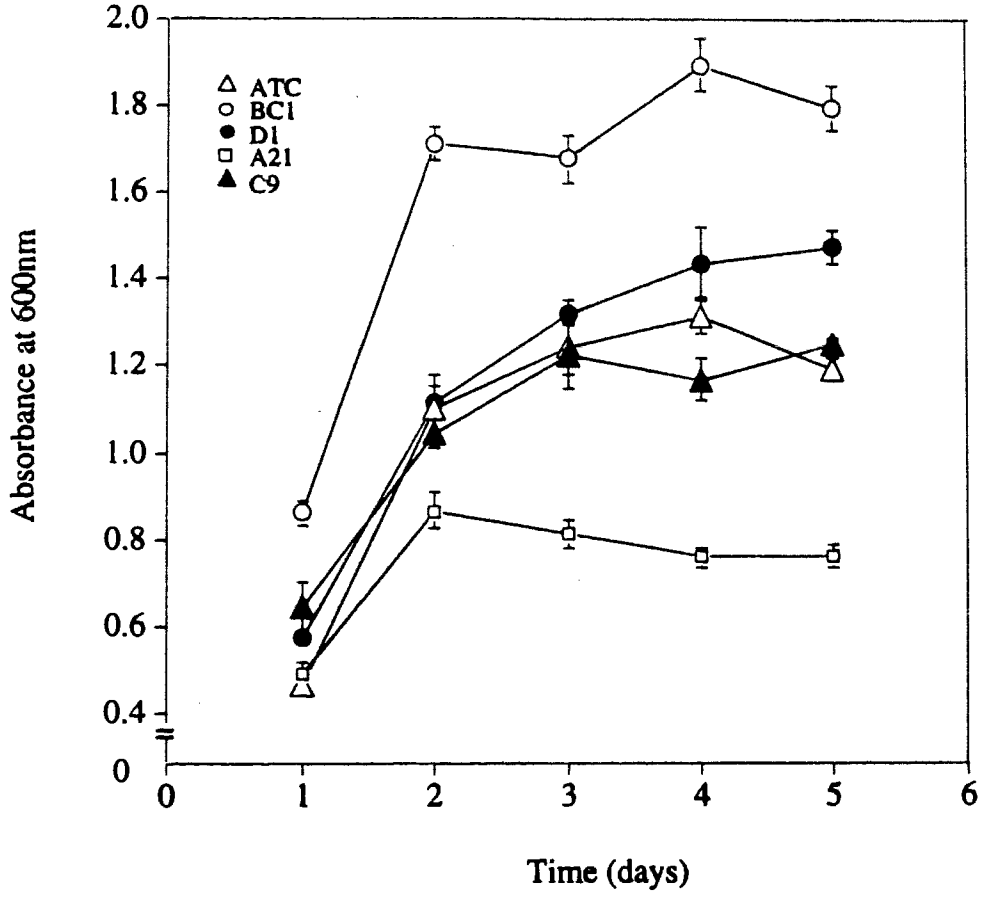
| Fungal species | *Xenorhabdus* and *Photorhabdus* strains* | | | | |
|---|---|---|---|---|---|
| | D1 | DS' | BC1 | A21 | C9 |
| *P. notatum* | **+++ | | +++ | + | ++ |
| *Penicillium sp.* | +++ | | +++ | +++ | + |
| *Rhizoctonia cerealis* | +++ | ++ | +++ | +++ | ++ |
| *R. solani* | +++ | ++ | +++ | +++ | ++ |
| *Rhizopus stolonifer* | ++ | | ++ | ++ | ++ |
| *Sclerotinia minor* | ++ | | +++ | ++ | ++ |
| *Suillus pseudobrevipes* | - | | - | - | - |
| *Thielaviopsis basicola* | ++ | | +++ | +++ | ++ |
| *Trichoderma pseudokingii* | +++ | | +++ | +++ | +++ |
| *Venturia inaequalis* | + | | + | + | + |
| *Verticillium albo-atrum* | ++ | | +++ | ++ | ++ |
| *V. dahliae* | +++ | | +++ | ++ | +++ |

* Full explanation of the codes of the bacterial strains is given in Table 3.

**Four levels of antimycotic activity as measured by the degree of inhibition of fungal growth defined as: -, no inhibition; +++, clear inhibition zone that persisted for at least one week after its formation; ++, clear inhibition that was subsequently colonized by aerial hyphae or small clusters of hyphae after a few days; +, a zone of poor fungal growth surrounding the *Xenorhabdus* and *Photorhabdus* inoculum. Blanks indicated that test was not done.

' secondary form of strain D1.

**Figure 2. Time course of the absorbance (600 nm) of *Xenorhabdus nematophilus* strains ATC, BC1, D1, *X. bovienii* strain A21 and *Photorhabdus luminescens* strain C9 in TSB.**


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0
Absorbance at 600nm
ATC
BC1
D1
A21
C9
0
1
2
3
4
5
6
Time (days)

absorbance of BC 1 strain of *X. nematophilus* was twice as much as for strain A21 of *X. bovienii*. The absorbance of strain A21 reached its highest level after 48 h of incubation, declined slightly thereafter and remained significantly lower than those of the other strains (Figure 2). The absorbances of the strains ATC, D1 of *X. nematophilus* and strain C9 of *P. luminescens* had a similar pattern where the absorbances increased from 24 to 48 h, but did not significantly increase thereafter. The strain BC1 had a significantly higher absorbance than all other tested strains including strains D1 and ATC of the same species. The maximum absorbances of the strains ATC, BC1, and D1 of *X. nematophilus* differed significantly (Figure 2).

During the culture period the pH level of the culture media increased significantly for all strains, from 7.1 at the beginning to between 8.6 (A21) and 9.0 (ATC) after 5 days (Figure 3). The pattern of pH increase was similar for all strains. The pH levels of the culture broth for strains A21 of *X. bovienii* and C9 of *P. luminescens* were similar to each other, but the final level at the end of the culture period were significantly lower than those of the three strains of *X. nematophilus*.

The antibacterial and antimycotic activity of the CFS varied among strains and species. The antibacterial activity of strain A21 of *X. bovienii* was significantly lower than that of all strains of *X. nematophilus* during the culture period except the first day at which time the activity of D1 was the lowest among all strains tested (Figure 4). Among the three strains of *X. nematophilus*, the CFS of BC1 had (except the 4th day) the higher activity against bacteria than those of the other two strains (D1 and ATC). The activity of BC1 on the first day reached a level that was not significantly exceeded during the rest of the culture period. The activity of the strain

**Figure 3. Time course of the pH value of the culture broth (TSB) of *Xenorhabdus nematophilus* strains ATC, BC1 and D1, *X. bovienii* strain A21 and *Photorhabdus luminescens* C9.**

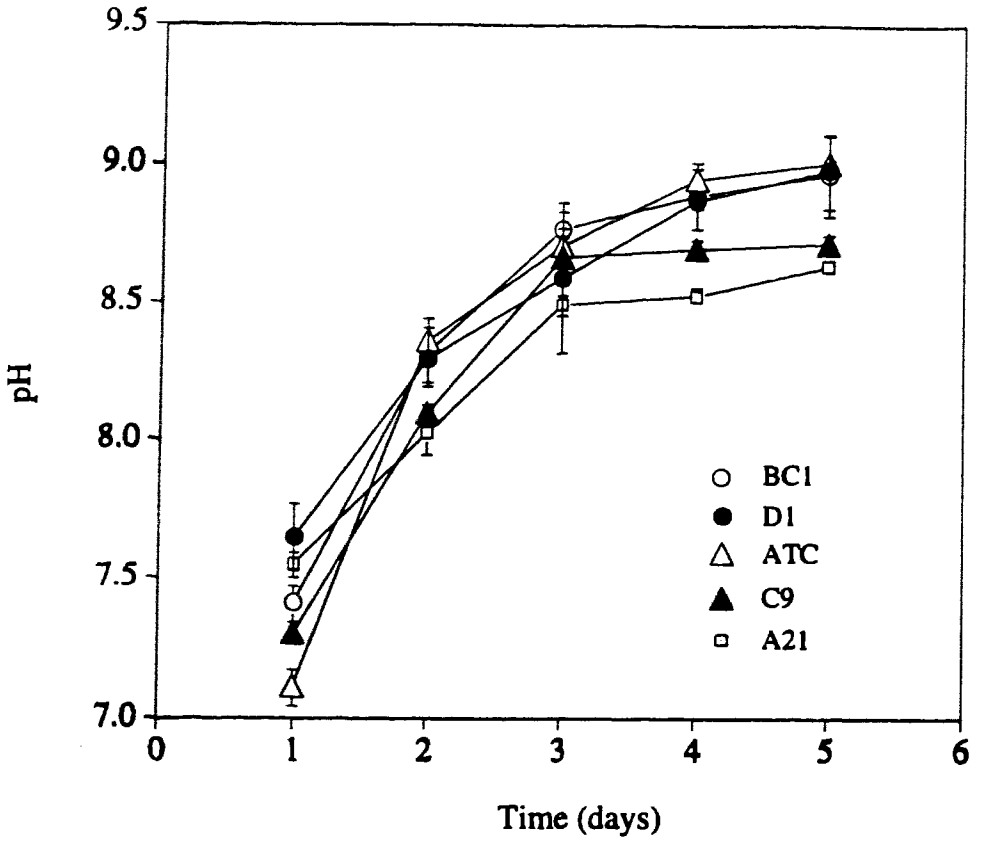
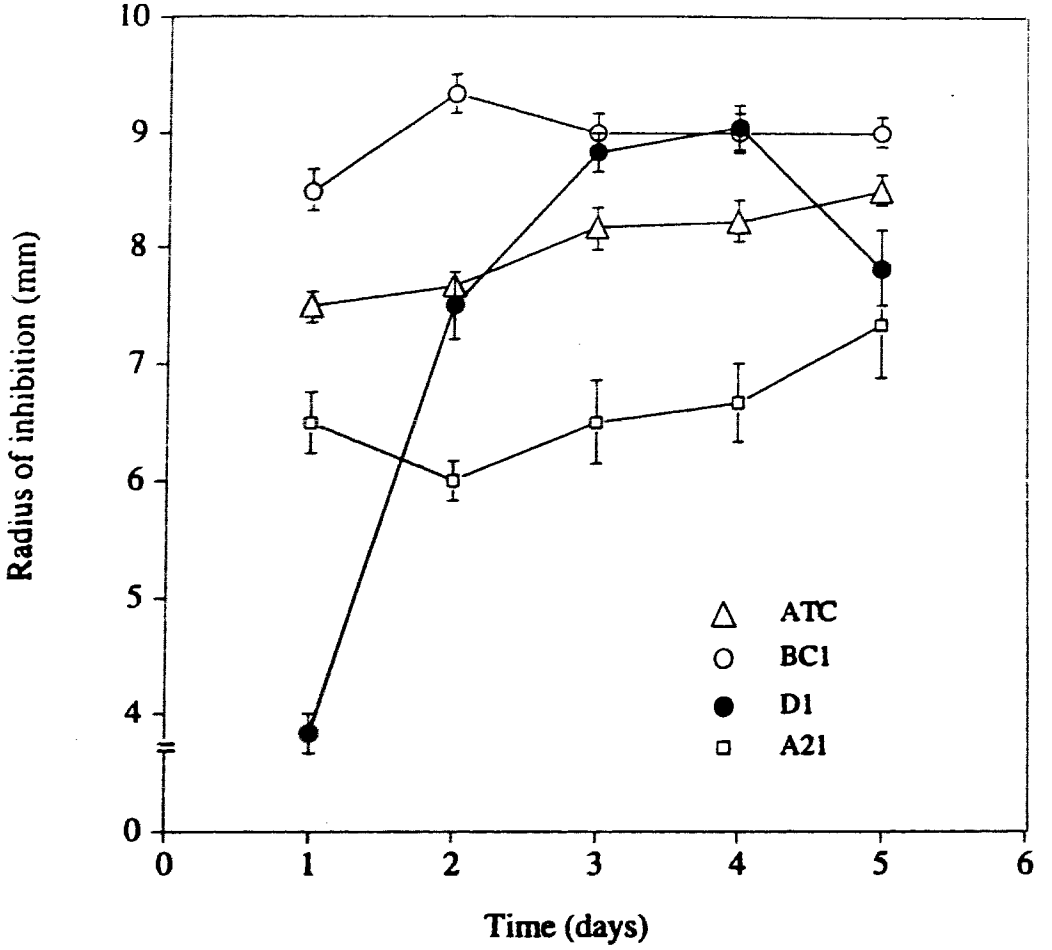

9.5
9.0
8.5
8.0
7.5
7.0
pH
0
1
2
3
4
5
6
Time (days)
BC1
D1
ATC
C9
A21

**Figure 4. Time course of the antibacterial activity of *Xenorhabdus nematophilus* strains ATC, BC1 and D1 and *X. bovienii* strain A21, measured as the radius of inhibition zone of the cell-free supernatant against *Bacillus subtilis*.**


Radius of inhibition (mm)
10
9
8
7
6
5
4
0
0
1
2
3
4
5
6
Time (days)
ATC
BC1
D1
A21

D1 significantly increased from the first day to the third, reached a maximum level on the fourth day and significantly decreased by the fifth day (Figure 4).

The antimycotic activity of the strains of *X. nematophilus* was two to three fold greater than that of the strain A21 of *X. bovienii* (Figure 5). The antimycotic activity of the strain A21 of *X. bovienii* increased gradually from 3.83 and reached a maximum of only 5.0 by the end of the culture period of 5 days. The antimycotic activity of both strains ATC and D1 increased significantly from the 1st to the 2nd day and remained at about the same level thereafter, whereas the activity of BC1 rose rapidly to the 3rd day and did not differ significantly thereafter. Among the three strains (BC1, ATC and D1) of *X. nematophilus* the activity of BC1 was significantly stronger than that of D1 but not ATC over the 5 days except for the 1st and 2nd day at which time there was no significant difference among them (Figure 5).

Unlike strains of *X. nematophilus* and *X. bovienii*, no detectable inhibition was evident when the CFS of the strain C9 of *P. luminescens* was tested against both *B. subtilis* and *B. cinerea*. Neither antibacterial nor antimycotic activity was detected in the TSB controls.

In addition to the activity of the CFS, the EAFs and the AFs of all strains of *Xenorhabdus* examined showed significant antibacterial and antimycotic activities whereas in strain C9 of *P. luminescens* only the EAF showed activity (Table 8, Table 9). In general, the EAFs appeared to have a higher antibacterial and antimycotic activity than did the AFs because of the 20-fold-concentration of the extracts used for the bioassay. Similarly to the activity of the CFS, EAFs and AFs of the strains of *X. nematophilus* had greater antimicrobial activity than did the strain A21 of *X. bovienii*. The AFWPs (see 3.2.2 ) of the strain A21 of *X. bovienii* had a similar level of activity against both *B. subtilis* and *B. cinerea* after the protein was removed as that of the AFs (Table 8).

**Figure 5. Time course of the antimycotic activity of *Xenorhabdus nematophilus*, strains ATC, BC1 and D1, and *X. bovienii*, strain A21, measured as the radius of the inhibition zone of the cell-free supernatant against *Botrytis cinerea*.**

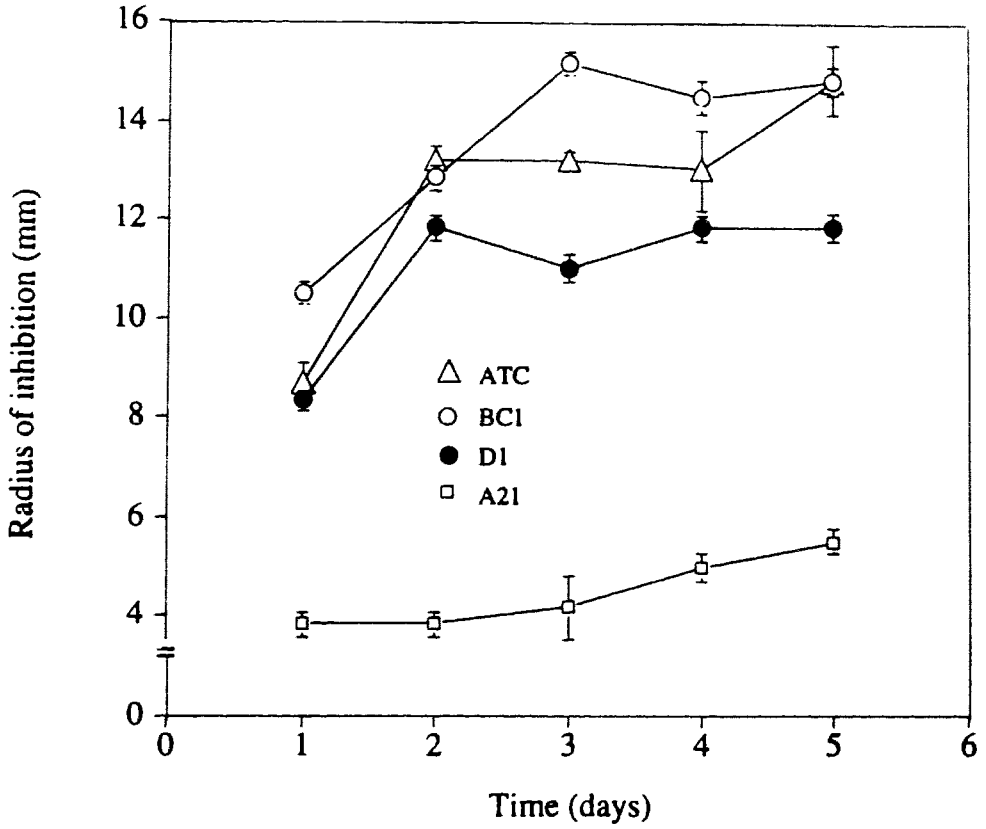

Radius of inhibition (mm)
16
14
12
10
8
6
4
0
0
1
2
3
4
5
6
Time (days)
ATC
BC1
D1
A21

**Table 8. Antibacterial and antimycotic activity of different fractions of the fifth day's culture supernatants of strains ATC, BC1 and D1 of *Xenorhabdus nematophilus*, strain A21 of *X. bovienii* and of the TSB control.**

| | Antibacterial | | | | | Antimycotic | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fraction | A21 | ATC | BC1 | D1 | TSB | A21 | ATC | BC1 | D1 | TSB |
| AF* | †5.0 | 8.5 | 8.7 | 7.2 | 0 | ‡3.2 | 10.3 | 12.5 | 8.7 | 0 |
| EAF** | 9.2 | 12.5 | 13.2 | 13.2 | 0 | 10.5 | 13.2 | 14.3 | 13.3 | 0 |
| AFWP*** | 4.5 | nd§ | nd | nd | nd | 2.7 | nd | nd | nd | nd |

†expressed as the means (n=3) of the radius (mm) of the inhibition zone against *Bacillus subtilis*.

‡ expressed as the means (n=3) of the radius (mm) of the inhibition zone against *Botrytis cinerea*.

§ nd=not determined.

*AF: aqueous fraction.

** EAF:ethyl acetate fraction, 20-fold-concentration extracts were used for the bioassay.

***AFWP: aqueous fraction without protein.

**Table 9. Antibacterial and antimycotic activity of ethyl acetate extract of *Photorhabdus luminescens* C9 strain.**

| Time (day) | Radius (mm) of inhibition | |
|---|---|---|
| | Antibacterial | Antimycotic |
| 1 | †9.0±0.0 | ‡4.5± 0.8 |
| 2 | 8.4± 0.6 | 4.8± 0.4 |
| 3 | 8.2± 0.2 | 4.8± 0.9 |
| 4 | 9.0± 0.6 | 4.7± 0.2 |
| 5 | 8.7± 0.3 | 6.8± 0.9 |

†expressed as the means (n=3) of the radius (mm) of the inhibition zone against *Bacillus subtilis.*

‡ expressed as the means (n=3) of the radius (mm) of the inhibition zone against *Botrytis cinerea.*

For strain C9 of *P. luminescens* only the EAF had activity against both *B. subtilis* and *B. cinerea*, and the activity did not significantly change over time (Table 9). No antibacterial or antimycotic activity was detected from any fraction of the TSB controls (Table 8).

Statistical analysis showed that for the three strains of *X. nematophilus* there was a significant correlation between the antibacterial and antimycotic activity of the EAFs (ATC: r=0.91, BC1:r=0.78, D1:r=0.87). There was no such correlation evident in strain A21 of *X. bovienii* and strain C9 of *P. luminescens*.

3.3.3 Antimycotic efficacy of the culture filtrates.

Conidial germination of *B. cinerea* was completely inhibited by all filtrates of the primary forms of *X. nematophilus*, *X. bovienii* and *P. luminescens* (Table 10). There was no detectable reduction in antimycotic activity of the filtrates of *X. nematophilus* and *X. bovienii* after having been treated up to 121 °C for 20 min. The activity of *P. luminescens* C9 did not show a decrease after the filtrate had been treated at 60 and 100 °C but was significantly decreased by autoclaving (121 °C) (Table 11).

The 50 % inhibitory concentration ($IC_{50}$) values of the filtrates varied with test fungal stage, species and bacterial strains (Table 12). In general, the filtrates had a smaller $IC_{50}$ for the conidial germination than for the mycelium growth of *B. cinerea*. Strains D1 and BC1 of *X. nematophilus* had significantly lower $IC_{50}$s for conidial germination of *B. cinerea* than did strain A21 of *X. bovienii* and strain C9 of *P. luminescens*. *B. cinerea* and *F. solani* showed a lower degree of susceptibility to the filtrates of all bacterial strains tested.

**Table 10. Effect of cell-free filtrates of *Xenorhabdus* and *Photorhabdus* strains on the conidial germination and germ tube growth of *Botrytis cinerea* after 14 h incubation.**

| Strains | % germination | Length of germ tube(μm) |
|---|---|---|
| C9 | *0 | 0 |
| ATC | 0 | 0 |
| BC1 | 0 | 0 |
| D1 | 0 | 0 |
| A21 | 0 | 0 |
| TSB | 91.8±2.2 | 135±7 |
| Water | 94.5±1.4 | 182±10 |

*n=3.

**Table 11. Effect of heat treatment of cell-free filtrates of *Xenorhabdus* and *Photorhabdus* strains at different temperatures for 20 min on their inhibitory activity against conidial germination and germ tube growth of *Botrytis cinerea*.**

| | % of germination | | | Length of germ tube(μm) | | |
|---|---|---|---|---|---|---|
| Strains | 60 °C | 100 °C | 121°C | 60 °C | 100 °C | 121°C |
| C9 | *0 | 0 | 67±4 | 0 | 0 | 16±1 |
| ATC | 0 | 0 | 0 | 0 | 0 | 0 |
| BC1 | 0 | 0 | 0 | 0 | 0 | 0 |
| D1 | 0 | 0 | 0 | 0 | 0 | 0 |
| A21 | 0 | 0 | 0 | 0 | 0 | 0 |
| TSB | 91.8±2.2 | 92.1±3.3 | 89.7±1.9 | 141±20 | 152±14 | 142±7 |
| Water | 94.5±1.4 | 92.1±2.7 | 90.0±2.2 | 149±15 | 142±10 | 146±14 |

*n=3.

**Table 12. $IC_{50}$ of the freeze-dried powder of the cell-free filtrate of strains of *Xenorhabdus nematophilus* (D1 and BC1), *X. bovienii* (A21) and *Photorhabdus luminescens* (C9) on mycelial growth of five fungal species and the conidial germination of *Botrytis cinerea*.**

| Fungal species | $IC_{50}$ (mg/ml) | | | |
|---|---|---|---|---|
| | A21 | D1 | BC1 | C9 |
| *Botrytis cinerea* (conidia) | *1.61±0.01 Bc | 0.41±0.15 Cd | 0.88±0.23 Cc | 10.4±0.2 Ac |
| *B. cinerea* (mycelium) | 4.61±0.36 Ba | 6.88±0.39 Ba | 4.21±0.19 Bb | 24.1±1.4 Aa |
| *Fusarium solani* | 2.33±0.14 Db | 4.02±0.17 Cb | 7.11±0.41 Ba | 13.8±0.1 Abc |
| *Monilinia fructicola* | 1.95±0.19 Bb | 1.98±0.11 Bc | 0.52±0.07 Bc | 15.0±0.7 Ab |
| *Pythium ultimum* | 0.20±0.05 Cc | 1.89±0.21 Bc | 0.28±0.08 Cc | 12.2±0.2 Abc |
| *Rhizoctonia solani* | 0.41±0.23 Dc | 2.18±0.29 Cc | 4.57±0.05 Bb | 11.4±0.6 Abc |

*Means with either the same capital letter in the same row or the same small letter in the same column are not significantly different ($P$=0.05, n=3).

## 3.4. DISCUSSION.

The inhibitory effect of the antimicrobial substance(s) varied with bacterial strains and the target fungal species (Table 7). The growth of all of the fungi except for the mycorrhizal fungus *S. pseudobrevipes* was inhibited to some extent by one or more of the strains of *Xenorhabdus*. The spectrum of bioactivity of the *Xenorhabdus* and *Photorhabdus* strains differed depending on the fungal species tested. The BC1 strain of *X. nematophilus* had the broadest activity in that it had inhibitory activity against 31 of the 32 fungal species tested. The secondary form variant (DS) of *X. nematophilus* had the most restricted range of bioactivity. This suggests that the different activity spectra might be due to quantitative or qualitative differences in the antimycotic substances produced by each species, strain and form variant of *Xenorhabdus* and *Photorhabdus*. Considerable resistance to these antimycotic(s) was demonstrated by *A. niger*, *C. fragrans*, by the insect pathogenic fungi *B. bassiana* and *M. anisopliae*, and by the mycorrhizal fungi *O. griseum* and *S. pseudobrevipes*. Neither the resistance nor susceptibility observed is correlated with a particular taxonomic group of fungi. For instance, both *A. niger* and *B. bassiana* show resistance, and *B. cinerea* and *T. basicola* are susceptible. All are Hyphomycetes. The mechanisms of antagonistic interaction between micro-organisms are normally parasitism, direct competition and antibiosis (Singh and Faull, 1988). The latter two possibilities could explain the results of these initial inhibition studies of many of the fungal species (Table 7). The clear inhibition zones on *B. cinerea* plates extending beyond the well of bacterial culture supernatant of the different strains (Figure 5) showed that the inhibitory effect of *Xenorhabdus* on the fungi was caused by the secretion of antimicrobial substances from the *Xenorhabdus* rather than by direct microbial competition for nutrients and/or space between the bacteria and fungi.

*X. nematophilus* and *P. luminescens* have been shown to be antagonistic to the insect pathogenic fungus *B. bassiana* (Barbercheck and Kaya, 1990). This antimycotic activity against *B. bassiana* was confirmed here, but only weakly so and for one primary form strain of *X. nematophilus* (BC1) but not by another (D1). However, the A21 strain of *X. bovienii* did inhibit growth of this insect fungus. The primary form of *P. luminescens* did not inhibit the growth of *B. bassiana*. In my experiments *B. bassiana* showed more resistance to the antimycotic(s) than did most of the other fungal species tested. This discrepancy may reflect differences in susceptibility between the fungal strains tested. It is also possible that the different media used in the present and previous studies (Barbercheck and Kaya, 1990) and the different culture history (Maxwell *et al.*, 1994) of the strains may contribute to the differing levels of activity expressed in the different experiments.

Many economically damaging plant pathogenic fungi, such as *Fusarium* spp., *Pythium* spp., *Rhizoctonia* spp. and *Verticillium* spp. were inhibited under laboratory conditions by the antimycotics of *Xenorhabdus* species while the mycorrhizal fungi, *O. griseum* and *S. pseudobrevipes* were affected only slightly, or not at all. Interestingly, Olthof *et al.* (1991) found that when *S. feltiae* and *H. heliothidis* were inundatively applied to control a sciarid fly infestation on mushrooms in greenhouses, the mushroom mycelium development was slowed. Although not confirmed, it is possible that the mushroom mycelium may have been temporarily inhibited by antimycotic substances released into the medium from large numbers of sciarid fly larvae killed by *Xenorhabdus* spp. from the high doses of *Steinernema* (Chen *et al.*, 1994).

One of the important features differentiating the primary and secondary forms of *Xenorhabdus* and *Photorhabdus* is that the primary but not the secondary form of the bacteria

produces antibiotics (Akhurst, 1980). Although the secondary form (DS) of strain D1 of *X. nematophilus* did not show any antibacterial activity against *B. subtilis*, strain DS did have significant, albeit diminished, antimycotic activity (Table 7). This antimycotic activity of the secondary form strain clearly provides another distinction between the primary and secondary form bacteria. The secondary form may well produce different levels or types of antimicrobials rather than there being a total loss of ability to produce antimicrobial substances, as previously thought (Akhurst and Dunphy, 1993). The fact that the primary and secondary forms have different levels of extracellular chitinase activity (see Chapter 5.) supports this hypothesis. However, as there are intermediates between the primary and secondary forms, as well as shifting from secondary to primary (Hurlbert *et al.*, 1989; Chen, 1992; Krasomil-Osterfeld, 1995) more work needs to be done to define and determine the differences in antimicrobial activity between the form variants of *Xenorhabdus* and *Photorhabdus*.

The observation of fungal hyphae lysis suggests that there are extracellular enzymes produced by *Xenorhabdus* and *Photorhabdus*, which have the ability to lyse fungi. It is known that chitinases and β-glucanase cause lysis of fungal hyphae (Bielecki and Galas, 1991; Flach *et al.*, 1992) and that chitin is a major component of fungal cell walls as well as insect cuticle. However, early work on *Xenorhabdus* chitinase activity was not positive (Khan and Brooks, 1977). Considering the antimycotic activity of chitinase and β-glucanase, and their possible role in the overall antimicrobial activity of *Xenorhabdus* and *Photorhabdus*, further studies of the activity of these enzymes will be discussed in Chapter 5.

It has been reported that types of culture media strongly influence the growth and consequently, the antimicrobial activity of *X. nematophilus* and *P. luminescens* (Nealson *et al.*, 1990,

Maxwell *et al.*, 1994; Chen *et al.*, 1996). There is some limitation in the use of absorbance values to indicate growth, because absorbance is only a measurement of average cell volume in the culture. However, absorbance reflects the relative growth patterns of these bacterial strains in the culture period measured, and was evident that some of these bacterial strains differed from each other in their growth patterns in a particular medium.

The increase in pH of culture media could be attributed to the metabolism of nutrients into alkaline products by the bacteria. For example, many amino acids which are abundant in TSB medium, are easily deaminized, resulting in the release of ammonia which could change the pH of the culture broth. The discovery of large amounts of ammonia in the culture broth released from *Xenorhabdus* (Li, personal communication[1]) supports this hypothesis. The correlation between the absorbance and pH of the culture broth, suggests that the amount of alkaline substance released into the media is related to bacterial growth. However, this correlation disappeared when *X. nematophilus* was cultured anaerobically (Chen *et al.*, in press), and in a defined medium, when the concentration of amino acids and salts was changed (Nealson *et al.*, 1990). Although strain D1 of *X. nematophilus* raised the pH level in insect cadavers no correlation was evident in insects between the pH level and the antibacterial activity (Maxwell *et al.*, 1994). However, in the present study the pH level was significantly correlated with the antibacterial activity ($r=0.78$) of strain D1 in TSB culture. This difference in pH and antibacterial activity between cultures in insects and TSB is attributed to the influence of nutrients in the hemocoel of the dying or dead insect being different from those nutrients in the TSB, because it is known that different media influence differently the antibiotic activity of *P. luminescens* (Nealson *et al.*, 1990) and of *X. nematophilus* (Maxwell *et al.*, 1994).

[1] J. Li, research associate, Department of Biological Sciences, Simon Fraser University.

The differences in the antimicrobial activity among different strains of *X. nematophilus* and among different species is probably attributable, in part, to the overall physiological differences between individual strains and/or species. The different patterns of antibacterial and antimycotic activity shown by the same bacterial strain indicate that there are qualitative and/or quantitative differences in production of the agents responsible for these activities. Good correlation between antibacterial and antimycotic activity of the EAFs of all the strains of *X. nematophilus* tested indicates that the same agent(s), extractable by ethyl acetate, is responsible for both activities. So far, the only known compound produced by *X. nematophilus* which is both antibacterial and antimycotic is xenocoumacin 1 (McInerney *et al.*, 1991b). However, xenocoumacin is not extractable by ethyl acetate. Therefore, there must be some unknown ethyl acetate extractable compounds which have both antibacterial and antimycotic activity produced by *X. nematophilus*. This question will be examined in Chapter 4. In contrast, the poor correlation between antibacterial and antimycotic activity of the EAFs of *X. bovienii* strain A21 and of *P. luminescens* strain C9 clearly suggests that either the agents responsible for both activities are not the same or that the same agent has different activities against bacteria and fungi. Indoles and xenorhabdins have been reported to be produced by *X. bovienii* and would be present in the EAF of *X. bovienii*. These compounds would be partially responsible for the antimicrobial activity in the EAFs since they are active against bacteria, and xenorhabdins are active against fungi as well (McInerney *et al.*, 1991a). Similarly, stilbenes and anthraquinones have been isolated from *P. luminescens*. The stilbenes have wide-spectrum antimicrobial activity and anthraquinones have activity against bacteria(Sudar and Chang, 1992). The antimicrobial activity of the EAFs of *P. luminescens* could be attributed to the presence of the stilbenes and anthraquinones. The difference between the stilbenes and anthraquinones in activity

against bacteria and fungi might help to explain the poor correlation between antibacterial and antimycotic activity of the EAF of strain C9.

Although several classes of antimicrobial agents have been isolated from the organic extraction of *Xenorhabdus* and *Photorhabdus*, unfortunately, only xenocoumacins have been isolated from aqueous fractions of *X. nematophilus* and from an unidentified *Xenorhabdus* strain (McInerney *et al.*, 1991b). No non-proteinaceous antimicrobial substance has been isolated from the AF of any strain of either *X. bovienii* or *P. luminescens*. Xenorhabdins and indoles produced by strains of *Xenorhabdus* are known compounds which have both antibacterial and antimycotic activity (Paul *et al.*, 1981, McInerney *et al.*, 1991a, Li *et al.*, 1995a). However, these compounds probably are not responsible for these activities in the AFs of *Xenorhabdus* because they would have been removed by the ethyl acetate extraction. Xenocoumacins could be partially responsible for the antibacterial and antimycotic activity of strains of *X. nematophilus*, since xenocoumacin is active against bacteria and fungi (McInerney *et al.*, 1991b). For *X. bovienii*, this study has clearly shown, for the first time, that the AFs of *X. bovienii* have antimicrobial activity. This suggests that there are other unknown antimicrobial agents produced in the AFs of *X. bovienii*. Bacteriophages and bacteriocins (Boemare *et al.*, 1992, 1993b) have been reported from *X. bovienii*, but they have not been reported to show activity against *B. subtilis*. The antimicrobial activity of these AFs could not be attributed entirely to the existence of these agents because bacteriophages and bacteriocins are not likely to have antimycotic activity. Moreover, the fact that the elimination of proteins from the AFs of *X. bovienii* resulted in almost no reduction in antimicrobial activity (Table 8) suggests the existence of unknown, non-proteinaceous, antimicrobial agents and that there is probably no synergistic activity between proteinaceous and non-proteinaceous agents.

The heat stability of the antimycotic activity parallels the report of heat stability for the antibacterial activity reported by Akhurst (1982) and Maxwell *et al.*(1994). Although the activity of the autoclaved filtrate of strain C9 of *P. luminescens* was significantly decreased (Table 11) it still retained antimycotic activity that significantly reduced conidial germination and growth of the germ tube of *B. cinerea.* This suggests that a significant component or all of the agent(s) responsible for the antimicrobial activity is probably not a protein.

The $IC_{50}$ data are a quantitative demonstration of the potency of these bacterial filtrates against fungi. These potency data further reflect the results of the antimycotic spectrum tests in that the *Xenorhabdus* and *Photorhabdus* have broad-spectrum, antimycotic activity which is not selective towards a particular taxonomic group, whether it is an oomycete, hyphomycete or agonomycete. The high $IC_{50}$ value of *P. luminescens* illustrates the weaker antimycotic activity of this bacterium compared with that of *X. nematophilus* and *X. bovienii*. This weakness further reflects the fact that the antimicrobial activity of the CFS or AF of C9 was not detectable in *B. cinerea* plates. This weakness is due partially to the fact that, *in vitro*, the primary form of *P. luminescens* is readily changed into secondary or intermediate forms and that secondary forms are known to have a low antimicrobial activity in culture (Akhurst, 1982; Hurlbert *et al.*, 1989). This was reflected in the fact that when liquid culture of C9 was plated on NBTA a large proportion ( >83% within 5 days) of the bacterial colony was very lightly-colored, little-pigmented and was very low in bioluminescence, indicating a change to the secondary or one of the intermediate forms.

# CHAPTER 4. PURIFICATION AND ANALYSIS OF ANTIMICROBIAL SUBSTANCES

## 4.1. INTRODUCTION.

There are seven known classes of antimicrobial substances including anthraquinones, bacteriocins (xenorhabdincin), indoles, phage, stilbenes, xenocoumacins and xenorhabdins isolated from various strains of *Xenorhabdus* and *Photorhabdus*. Although bacterial phage and proteinaceous bacteriocins exist in all strains of *Xenorhabdus* and *Photorhabdus* investigated, the other substances are species related. Xenocoumacins are produced by *X. nematophilus* and an unidentified *Xenorhabdus* (McInerney *et al.*, 1991b), indoles and xenorhabdins by *X. bovienii*, and stilbenes and anthraquinones by *P. luminescens* (Akhurst and Dunphy, 1993). Little is known about the spectrum of antimicrobial substances produced by any one strain of *Xenorhabdus* or *Photorhabdus*, or about the efficacy and production of these known antimicrobial agents.

These symbiotic bacteria have not only a wide-spectrum of activity but also their activity varies with bacterial growth, strain and species (see Chapter 3). Given the abundance of secondary metabolites produced by these bacteria-nematode associations, the apparent variation of antibiotic activity of these bacteria and of the known properties of some of the substances they produce, it follows that a closer examination of the chemical nature of these antimicrobial substances is needed.

## 4.2. EXPERIMENTS.

To purify antimicrobial agents from *Xenorhabdus* and *Photorhabdus*, 5 liters of culture broth from each of strains A21 of *X. bovienii*, BC1 of *X. nematophilus* and C9 of *P. luminescens*

were prepared by adding 30 ml of the respective inocula, prepared as outlined in 3.2, to each of five 2-liter flasks containing 1 liter of TSB and growing them in the dark (125 rpm, 25 °C) for 4 days. The spent culture media were then centrifuged (11,000 × g, 4 °C, 20 min ) to remove the bacterial cells. The supernatants of these bacterial cultures were subjected to the following separation processes.

4.2.1. Purification and characterization of antimicrobial substances.

The purification work in this section was done jointly with Dr. J. Li and the chemical characterization of the purified compounds was done solely by Dr. Li. During the purification process each step was carefully monitored for bioactivity, using *B. subtilis* and *B. cinerea* bioassays as outlined in 2.4.1. and 2.4.2. The purification was repeated whenever it was necessary to obtain more compounds for chemical characterization and bioassay.

A. Purification of antimicrobial substances from strain A21 of *X. bovienii.*

Each 1 liter of the cell-free supernatant was passed through 150 g of Amberlite XAD-2 (Sigma) resin suspended in distilled water in an 8 cm diam column. The Amberlite column was washed with 500 ml distilled water, followed by 1 liter methanol. This process was carried out separately for all 5 liters of supernatant. The methanol fraction was collected and concentrated in a rotary evaporator at room temperature to about 2.0 g of oily residue, which was then extracted three times, each time with 100 ml of ethyl acetate (analytical grade). The ethyl acetate extracts from each 1 liter of original centrifuged supernatant were combined and approximately 10 g of anhydrous sodium sulfate was added to the 300 ml ethyl acetate extract to remove the water. The extract was evaporated to dryness, and residue was dissolved in 1 ml methanol. The methanol solution was separated by high performance liquid chromatography (HPLC) on a $C_{18}$ reverse

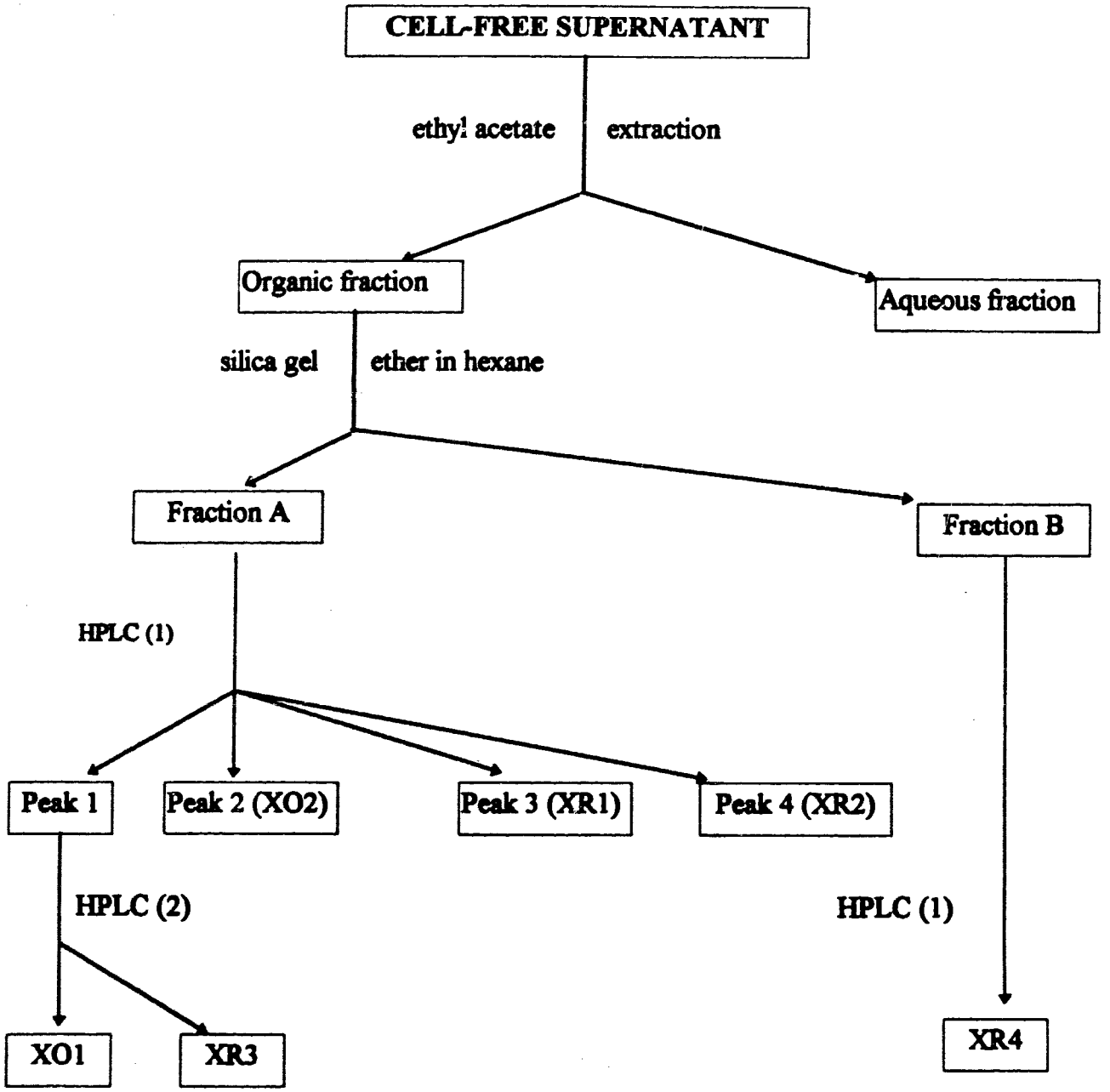
phase preparative column (Sphersorb 10 (ODS(1)), 250 x 10 mm, 10 micron, Phenomenex, Torrance, CA) at a steady flow rate of 2.5 ml/min, using the following mobile phase gradient of $CH_3CN$ in $H_2O$. The elution was monitored at 254 nm.

| Time | Composition | |
|---|---|---|
| (min) | % $H_2O$ | %$CH_3CN$ |
| 0 | 90.0 | 10.0 |
| 5.0 | 90.0 | 10.0 |
| 20.0 | 75.0 | 25.0 |
| 30.0 | 75.0 | 25.0 |
| 40.0 | 40.0 | 60.0 |
| 55.0 | 40.0 | 60.0 |
| 58.0 | 90.0 | 10.0 |
| 60.0 | 90.0 | 10.0 |

From this process four major peaks were observed in the elution profile. These peaks were collected and bioassayed.

In order to isolate other active compounds, the isolation process was modified as outlined in the diagram on the next page. Briefly, each 1000 ml of culture supernatant was extracted four times with 500 ml of ethyl acetate. The combined 2000 ml of extracts were dried with anhydrous sodium sulfate and then filtered through glass-wool. The filtrate was concentrated on a Buchi rotary evaporator at 30°C to 40°C. The above isolation process was repeated 10 times to yield about 2.8 g of the oil organic fraction from 10 liters of culture broth. This oil was dissolved in 2 ml of methanol and processed through a silica gel (200 g silica gel 60, 40 x 5 cm, EM Science, Darmstadt, Germany) chromatographic column. The silica gel column was first equilibrated with 1:1 of ether and hexane. The 2 ml of sample was added, then the column was eluted with 250 ml of 50% ether in hexane as the initial eluent which was collected as 25 ml fractions, then with each 250 ml of 60%, 70%, 80% and 90% ether in hexane and finally with 250 ml of 100% ether. All fractions were collected and bioassayed directly for antibacterial and antimycotic activities, as

## ISOLATION PROCESS[2]




[2] This process was conceived and the chromatographic work was done by Dr. J. Li.

described in 2.4.1 and 2.4.2. Two bioactive fractions, A and B were found in the 70% ether in hexane eluents. Fraction A was separated by HPLC on a $C_{18}$ preparative column (Spherisorb 10 (ODS(1)), 250 x 10 mm, 10 micro, Phenomenex, Torrance, CA), eluting with 10% $CH_3OH$ in $H_2O$ for 5 min and gradually increasing to 90% $CH_3OH$ in $H_2O$ in 25 min, isocratic for 5 min at 2.5 ml/min [HPLC (1)]. The eluent was monitored at 254 nm. The eluted fractions representing 20 peaks were collected and bioassayed for antimicrobial activity. Peak 1 was further separated by HPLC on the same column using an isocratic mobile phase with 50% acetonitrile (HPLC grade) in water [HPLC (2)] as the eluent and two further peaks were obtained. Fraction B was purified by HPLC using the same [HPLC (1)] program as for fraction A, and an active compound was obtained.

B. Purification of antimicrobial substance from strain C9 of *P. luminescens*[3].

The preparation of a cell-free supernatant and the subsequent ethyl acetate extraction process of *P. luminescens* strain C9 was the same as that for *X. bovienii* strain A21 outlined above. This extraction process yielded about 2 g extract residue. The residue was loaded onto a silica gel-60 column (50×8cm) and the column eluted first with 250 ml of 30% ether in hexane, then 250 ml of 60% ether in hexane and finally 250 ml of 100% ether, and the eluents were collected as fractions of 25 ml. Each fraction was bioassayed for antimicrobial activity as described in 2.4.1 and 2.4.2. The most active fraction was eluted with 60% ether in hexane, while another less active, yellow fraction was eluted with 100% ether. The bioactive fractions were collected separately, and further concentrated on a Buchi rotary evaporator to give two active fractions at a yield of about 40 mg/L and 70 mg/L, respectively.

[3] The chromatographic work was done by Dr. J. Li.

C. Purification of antimicrobial substances from strain BC1 of *X. nematophilus*[4].

For the organic soluble compound the separation process was the same as for *P. luminescens* except that the eluent used was 250 ml of 30% ethyl acetate in hexane. Only one bioactive eluent was eluted from the silica gel with 30% ethyl acetate in hexane. This bioactive eluent was collected and concentrated on a Buchi rotary evaporator.

For the isolation of water soluble compounds, 3 liters of the cell-free supernatant of *X. nematophilus* strain BC1 was extracted with 500 ml of dichloromethane three times. The aqueous phase was then run through an Amberlite XAD-2 column (20 cm × 2.5 cm), and eluted first with 500 ml of water, then 500 ml of ethanol, and finally with 500 ml of acetone. The ethanol and acetone eluents were combined and concentrated. This partially purified material was then run through a $C_{18}$ reverse phase chromatographic column (30 cm × 1.5 cm) with a mobile phase of $CH_3OH$ in $H_2O$ gradually changed from 20% to 100%. Fractions were collected for each minute. Each fraction was bioassayed for antimicrobial activity. The bioactive fractions were then combined and concentrated. This concentrated material was further purified by HPLC using a $C_{18}$ preparative column (Spherisorb 10 (ODS(1)), 250 x 10 mm) and a mobile phase of 20% of $H_2O$ containing 0.1% $CF_3COOH$ in $CH_3CN$ for 5 min, then gradually increased to 90% within 20 min, kept at 90% for 10 min, then back to 20% within 3 min. Fractions from 23 min to 27 min were bioactive, and were concentrated to give the final material for analysis.

D. Chemical and biological characterization of the purified compounds.

The chemical structures of the purified compounds were determined using NMR, mass-spectrum and/or other appropriate analyses. The biological activity of each known substance was

[4] The chromatographic work was done by Dr. J. Li.

then assayed and determined as outlined in 2.4.3. The bioassay was repeated at least five times, depending on the test compounds and organisms.

4.2.2. Analysis and concentration of the purified compounds from different strains and species.

All strains listed in Table 3 were cultured and treated as described in section 3.2.2. Then 10 ml of the culture supernatant of each strain culture was extracted twice with 10 ml ethyl acetate, the extracts were combined and water was removed by passing the extracts through an anhydrous sodium sulfate column (40×4 mm). The ethyl acetate fractions were evaporated at room temperature to dryness and the resulting residue of each sample was then dissolved in 0.5 ml methanol, and 20 µl of the methanol solution was injected into the HPLC for analysis of chemical composition and concentration. The HPLC analysis was done on a $C_{18}$ reverse phase analytical column (Lichrosorb 10 RP-18, 250 × 6 mm ) using an elution program of 10% $CH_3CN$ in $H_2O$ for 5 min and gradually increasing to 90% $CH_3CN$ in $H_2O$ in 25 min and then isocratic for 5 min at 1.5 ml/min. The eluent was monitored at 254 nm. Pure compounds isolated from each individual bacterial strain were used as external standards for determination of the chemical concentration by comparing the peak areas. Out of a total of 12 identified compounds, only six were present in the bacterial culture in sufficient concentration to permit quantification. This analytical study was repeated four times.

4.3. RESULTS.

4.3.1. Purification of *X. bovienii* compounds.

From strain A21 of *X. bovienii* four indole derivatives, which had HPLC retention times and approximate yields of 45.43 min, 40 mg/L (ID4), 47.13 min, 100 mg/L (ID3), 48.71 min, 18

mg/L (ID2) and 50.81 min, 45 mg/L (ID1), respectively (Figure 6) were obtained and their chemical structures were assigned (Appendix 2). The four bioactive indoles are: ID1: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole; ID2: 3-(2'-acetoxy-4-methyl-3'-oxopentyl)-indole; ID3: 3-(2'-hydroxy-4'-methyl-3-oxohexyl)-indole; ID4: 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole. Bioassay of the four indoles showed that they have both antibacterial and antimycotic activity (Table 13 ). ID1, ID3 and ID4 had MICs ≥100 μg/ml against the four bacteria tested, whereas ID2 had MIC=12 μg/ml and 6 μg/ml against the two Gram-positive bacteria, *Micrococcus luteus* and *S. aureus*, respectively. The antimycotic activity varied with both test species and chemical. ID1 and ID2 were active against the yeast *Cryptococcus neoformans* at MIC=50 μg/ml and 25 μg/ml, respectively, and against *Phytophthora infestans* at MIC=100 μg/ml whereas ID3 and ID4 were active against *B. cinerea* (MIC=12 μg/ml) and *P. infestans* (MIC= 100μg/ml, 50 μg/ml, respectively). The MIC of these four indoles against the other fungal species was ≥100 μg/ml.

The six additional compounds purified by silica gel from fraction A of strain A21 of *X. bovienii* gave the HPLC elution profile shown in Figure 7. Peak 2 (32.9 min.), peak 3 (33.8 min.) and peak 4 (34.7 min.) represented compounds XO2, XR1 and XR2, respectively. The approximate yields of these compounds were 0.2 mg/L, 1 mg/L and 2 mg/L for XO2, XR1 and XR2, respectively. Further separation from peak 1 (31.7 min) (Figure 7) resulted in the pure compounds XO1 (17 min.) with a concentration of about 0.2 mg/L and compound XR3 (12.9 min, about 0.2 mg/L) (Figure 8.). From fraction B an active compound XR4 (30.5 min.) with a concentration of about 0.5 mg/L (Figure 9) was obtained. These compounds (shown in Appendix 2) are: XR1: N-hexanoyl-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole

**Figure 6. HPLC elution profile of the organic extract of the culture supernatant of *Xenorhabdus bovienii* strain A21, showing the relative retention times of four indoles through a preparative $C_{18}$ column using a gradient mobile phase of $H_2O$ and $CH_3CN$.**

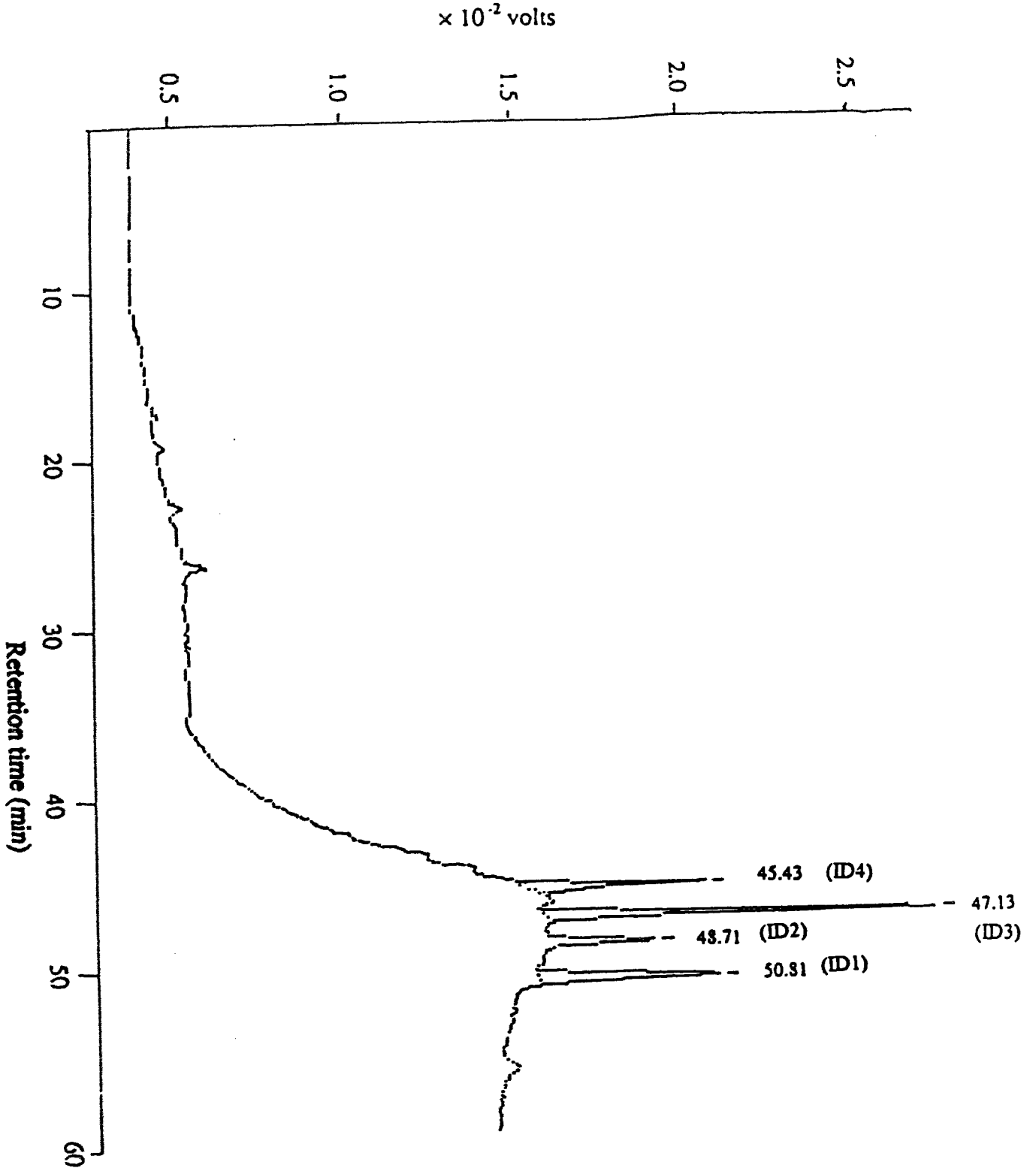

× 10⁻² volts
0.5
1.0
1.5
2.0
2.5
10
20
30
40
50
60
Retention time (min)
45.43 (ID4)
47.13
(ID3)
48.71 (ID2)
50.81 (ID1)

**Table 13. Minimum inhibitory concentrations(MIC) of the indoles isolated from cell-free supernatant of *Xenorhabdus bovienii* strain A21 against bacteria, yeast and fungi.**

| Organisms | MICs(μg/ml) | | | |
|---|---|---|---|---|
| | *ID1 | ID2 | ID3 | ID4 |
| *Bacillus subtilis* | >100** | >100 | >100 | >100 |
| *Escherichia coli* ATCC25922 | >100 | >100 | >100 | >100 |
| *Micrococcus luteus* | 100 | 12 | >100 | >100 |
| *Staphylococcus aureus* ATCC 29213 | 100 | 6 | >100 | >100 |
| *Aspergillus fumigatus* ATCC 13073 | >100 | >100 | >100 | >100 |
| *A. flavus* ATCC 24133 | >100 | >100 | >100 | >100 |
| *Botrytis cinerea* | >100 | >100 | 12 | 12 |
| *Candida tropicales* CBS 94 | >100 | >100 | >100 | >100 |
| *Cryptococcus neoformans* ATCC14117 | 50 | 25 | >100 | >100 |
| *Phytophthora infestans* | 100 | nd*** | 100 | 50 |
| *Pythium ultimum* | >100 | nd | >100 | >100 |

* ID1: 3-(2'-acetoxy-4'-methyl-3'-oxohexyl)-indole; ID2: 3-(2'-acetoxy-4-methyl-3'-oxopentyl)-indole; ID3: 3-(2'-hydroxy-4'-methyl-3-oxohexyl)-indole; ID4: 3-(2'-hydroxy-4-methyl-3'-oxopentyl)-indole.

** n=6.

***nd=not determined.

**Figure 7. HPLC elution profile of fraction A derived from silica gel separation of an organic extract of the culture supernatant of *Xenorhabdus bovienii* strain A21, showing the relative retention time of the bioactive peak 1, XO2, XR1 and XR2 through a preparative $C_{18}$ column using a gradient mobile phase of $H_2O$ and $CH_3OH$[5].**

[5] Chromatographic work was done by Dr. J. Li.

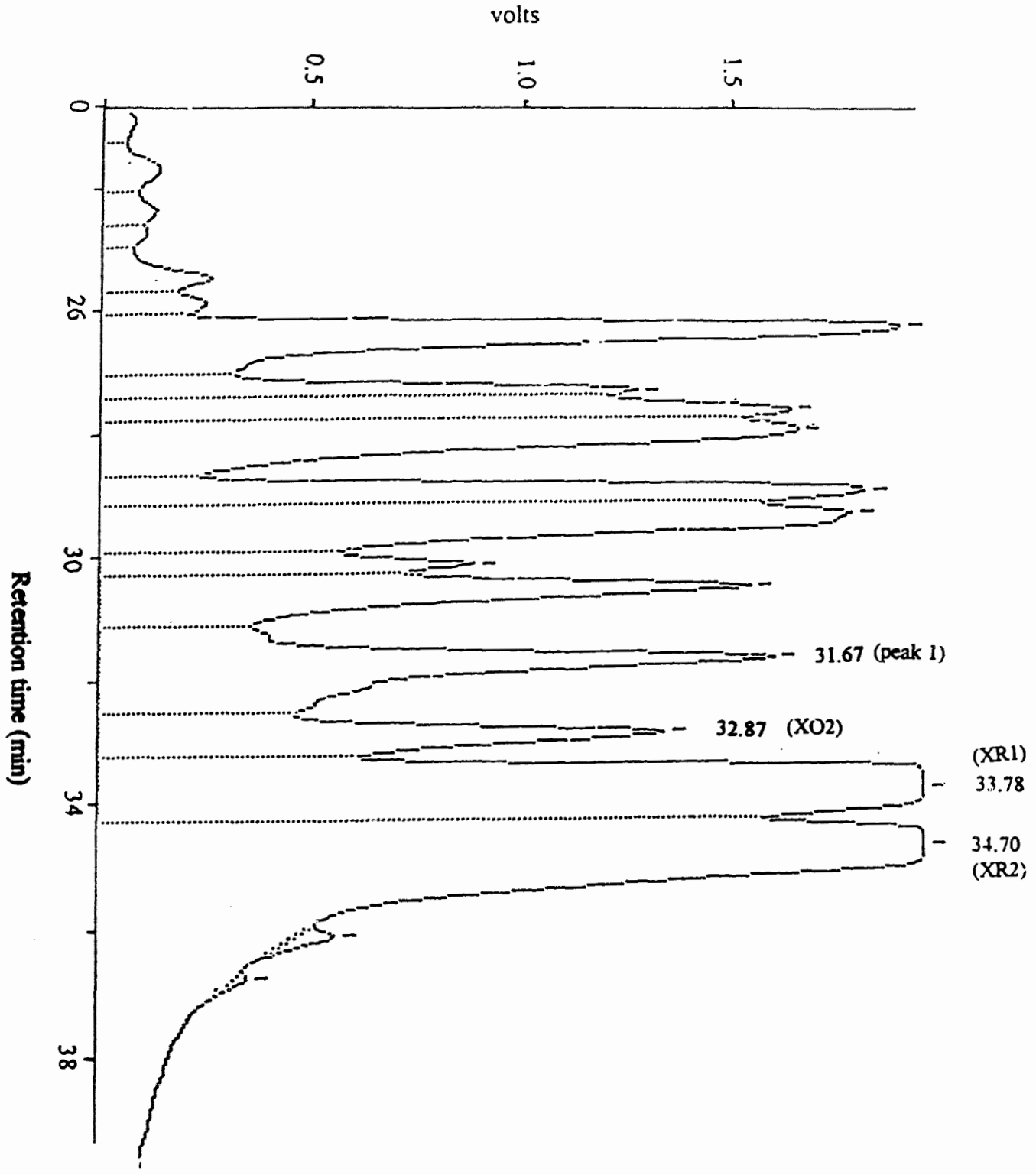

volts
0.5
1.0
1.5
0
26
30
34
38
Retention time (min)
31.67 (peak 1)
32.87 (XO2)
(XR1)
33.78
34.70
(XR2)

**Figure 8. HPLC elution profile of peak 1 (see Figure 7) derived from initial HPLC separation of the organic extract of the culture supernatant of *Xenorhabdus bovienii* strain A21, showing the relative retention times of XO1 and XR3 through a preparative $C_{18}$ column using an isocratic mobile phase with 50% acetonitrile in water[6].**

[6] Chromatographic work was done by Dr. J. Li.

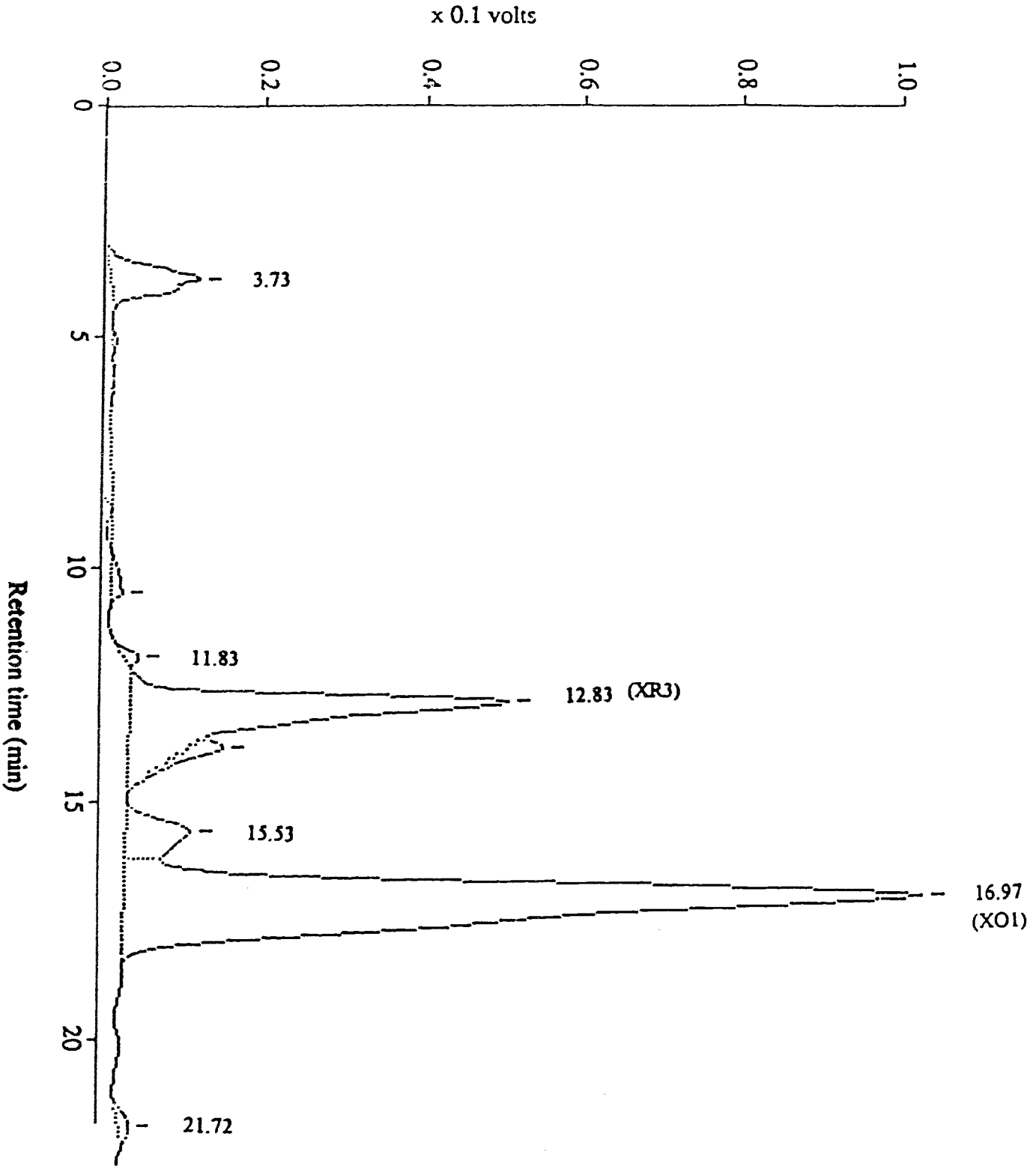
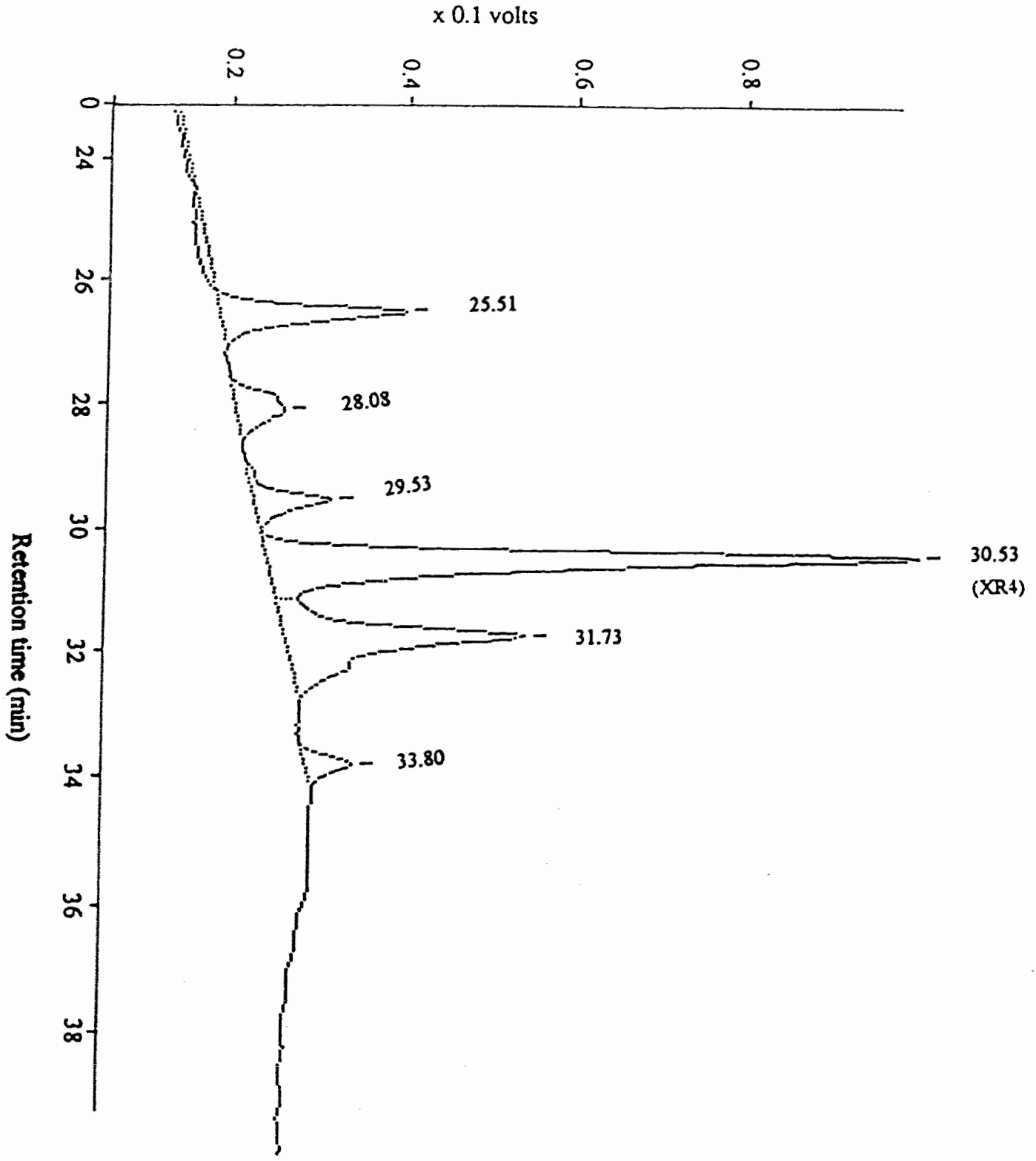

x 0.1 volts
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
20
Retention time (min)
3.73
11.83
12.83 (XR3)
15.53
16.97
(XO1)
21.72

**Figure 9. HPLC elution profile of fraction B derived from the silica gel separation of the organic extract of the culture supernatant of *Xenorhabdus bovienii* strain A21, showing the relative retention time of the active peak XR4 through a preparative $C_{18}$ column using a gradient mobile phase of $H_2O$ and $CH_3OH$[7] .**

[7] Chromatographic work was done by Dr. J. Li.


x 0.1 volts
0.2
0.4
0.6
0.8
0
24
26
28
30
32
34
36
38
Retention time (min)
25.51
28.08
29.53
30.53
(XR4)
31.73
33.80

(xenorhabdin); XR2: N-(5'-methylhexanoyl)-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole (xenorhabdin); XR3: N-(3-methylbutanoyl)-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole; and XR4: N-butanoyl-6-amino-4,5-dihydro-4-methyl-5-oxo-1,2-dithiolo-(4, 3-b) pyrrole. XR1 and XR2 are known xenorhabdins. XR3 and XR4 are xenorhabdins which are reported for the first time from *X. bovienii*. XO1: N-hexanoyl-6-amino-4,5-dihydro-4-methyl-5-oxo-1-sulfano-2-thiolo-(4, 3-b) pyrrole. XO2: N-(5'-methylhexanoyl)-6-amino-4,5-dihydro-4-methyl-5-oxo-1-sulfano-2-thiolo-(4, 3-b) pyrrole. XO1 and XO2 are novel compounds and we name them xenorxide 1 and xenorxide 2, respectively. Xenorxides had strong antibacterial activity against Gram-positive bacteria with MICs ranging from 1.5 to 25 μg/ml. Of particular interest is that these xenorxides showed strong activity against drug-resistant *S. aureus* (Table 14). The xenorxides also showed strong antimycotic activity against fungi and yeast with MIC ranging from 0.75 to 25 μg/ml (Table 15).

4.3.2. Purification of *P. luminescens* and *X. nematophilus* compounds.

From strain C9 of *P. luminescens* the analysis of the fraction eluted by the 60% ether in hexane result in one bioactive compound, namely STB: 3,5-dihydroxy-4-isopropylstilbene. It showed antimycotic activities against all fungi tested (Table 15), but was generally less active than the xenorxides. The other bioactive yellow fraction (about 70 mg/L) was a mixture of two compounds and they were not separable by HPLC with either acetonitrile-water or methanol-water as mobile phase. However, further chemical characterization revealed that they were 3,8-dimethoxyl-1-hydroxyl-9,10-anthraquinone (minor) and 1,3-dimethoxyl-8-hydroxyl-9,10-anthraquinone (major). They were in a mixture of approximately 1:3 ratio. As further

**Table 14. Minimum inhibitory concentrations (MIC) of xenorxides (XO1, XO2) and nematophin (NID) isolated from *Xenorhabdus bovienii* strain A21 and *X. nematophilus* strain BC1, respectively against bacteria.**

| Bacterial species tested | Gram | MICs(μg/ml) | | |
|---|---|---|---|---|
| | | XO1 | XO2 | NID |
| *Bacillus subtilis* | + | *6 | 6 | 12 |
| *Escherichia coli* ATCC 25922 | - | >100 | >100 | >100 |
| *Escherichia coli* UBC1005† | - | >100 | >100 | >100 |
| *Micrococcus luteus* | + | 25 | 6.0 | >100 |
| *Pseudomonas aeruginosa*† | - | >100 | >100 | >100 |
| *Staphylococcus aureus* ATCC 29213 | + | 3.0 | 3.0 | 0.75 |
| *S. aureus* 0012** | + | 0.75 | 0.75 | 1.50 |
| *S. aureus* 0017** | + | 0.75 | 1.50 | 0.75 |

* n=6;

**clinical, multi-drug-resistant strains.

† test was done by S. Farmer, Canadian Bacterial Diseases network, University of British Columbia.

**Table 15. Minimum inhibitory concentrations (MIC) of xenorxides (XO1, XO2), nematophin (NID), and the stilbene derivative (STB) isolated from strain A21 of *Xenorhabdus bovienii*, strain BC1 of *X. nematophilus*, strain C9 of *Photorhabdus luminescens*, respectively against yeast and fungi.**

| Organisms | MICs(μg/ml) | | | |
|---|---|---|---|---|
| | XO1 | XO2 | NID | STB |
| *Aspergillus fumigatus* ATCC 13073 | *0.75 | 1.50 | >100 | 12 |
| *A. flavus* ATCC 24133 | 0.75 | 1.50 | >100 | 25 |
| *Botrytis cinerea* | 12 | 25 | 12 | 12 |
| *Candida tropicales* CBS 94 | >100 | >100 | >100 | 25 |
| *Cryptococcus neoformans* ATCC14117 | 6 | 6 | >100 | 12 |

* n=5.

chromatographic separation was unsuccessful and their low solubility made the evaluation of bioactivity difficult. Their bioactivity was not evaluated further.

Only one active compound, which we named nematophin (NID), was purified from the organic fraction of *X. nematophilus* strain BC1. This compound is a novel chemical (see Appendix 2). It showed strong antibacterial activity against *B. subtilis* (MIC=12 μg/ml), *S. aureus* (MIC=0.75-1.5 μg/ml), and in particular against drug-resistant *S. aureus* (Table 14). Nematophin also showed antimycotic activity (Table 15). Although purification was done for each of the three strains (ATC, BC1 and D1) of *X. nematophilus*, the only bioactive compound found in the ethyl acetate fractions was nematophin. From the aqueous fraction only one active fraction was found. Although the active material was not 100% pure, its $^1$HNMR spectrum showed clearly that it belonged to the xenocoumacin group of the water-soluble benzopyran-1-one derivatives. Thus further work on this was terminated.

4.3.3. Analysis of antimicrobial compounds in culture broth.

HPLC analysis (Figure 10) showed that the concentration of nematophin differed significantly during the period of culture (Table 16). Strain BC1 of *X. nematophilus* had a higher (from 2-5 times) concentration of NID through the whole culture period than did strains D1 and ATC. The average NID concentrations in ATC, BC1 and D1 cultures increased significantly from the 1st to 2nd day, and remained relatively high thereafter. Variations were evident in the average NID concentration in all cultures throughout the test period.

The concentrations of the four individual indoles differed significantly during the period of culture (Figure 11). ID1 and ID2 had their maximum levels of 17.00 μg/ml and 11.56 μg/ml, respectively on the 1st day of culture, but the concentrations decreased thereafter, and reached the

**Figure 10. Representative HPLC elution profile for the nematophin standard (the inset) and the organic extract of the *Xenorhabdus nematophilus* BC1 culture supernatant, showing the relative retention time of the nematophin through an analytical $C_{18}$ column using a gradient mobile phase of $H_2O$ and $CH_3CN$.**

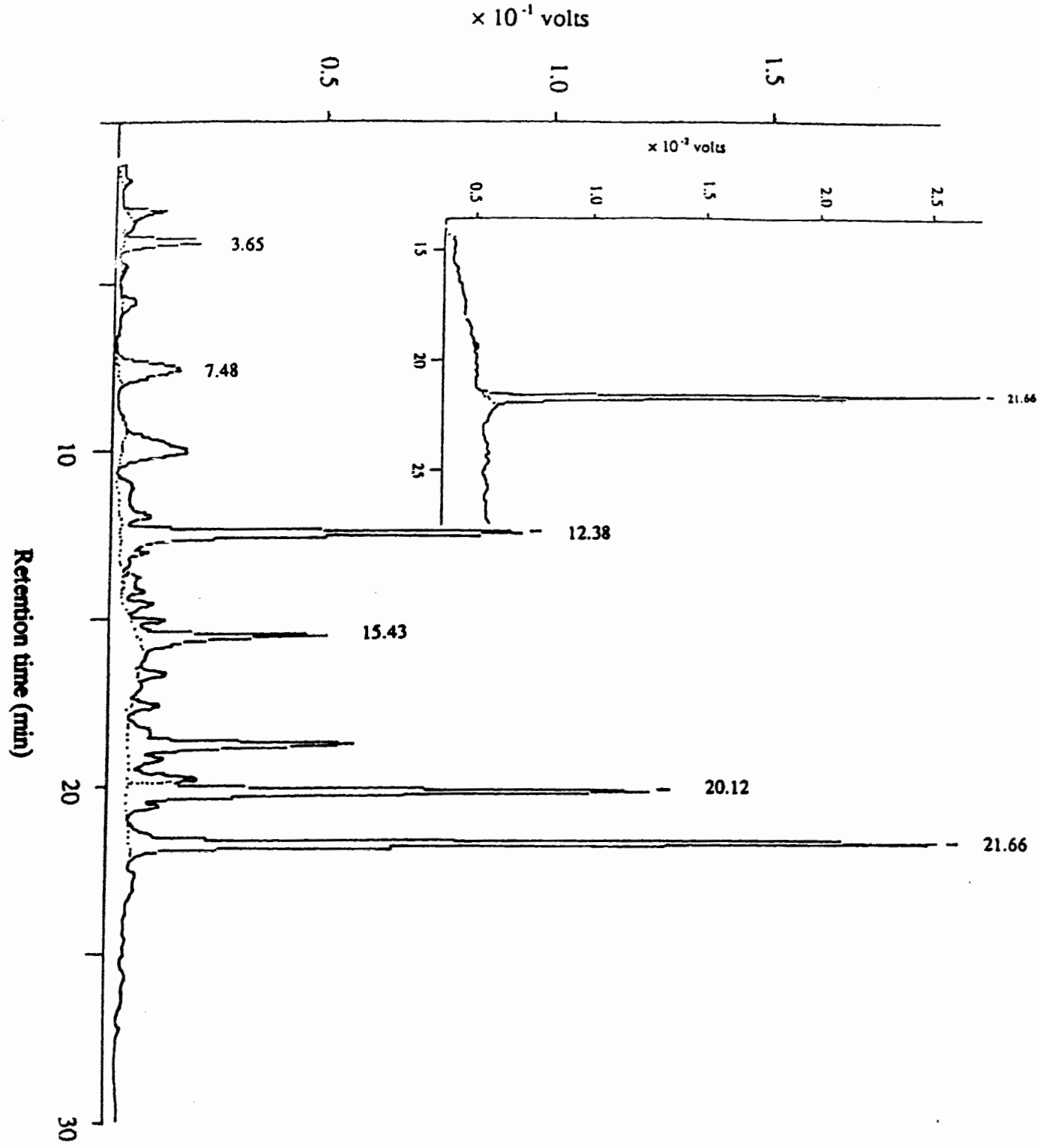
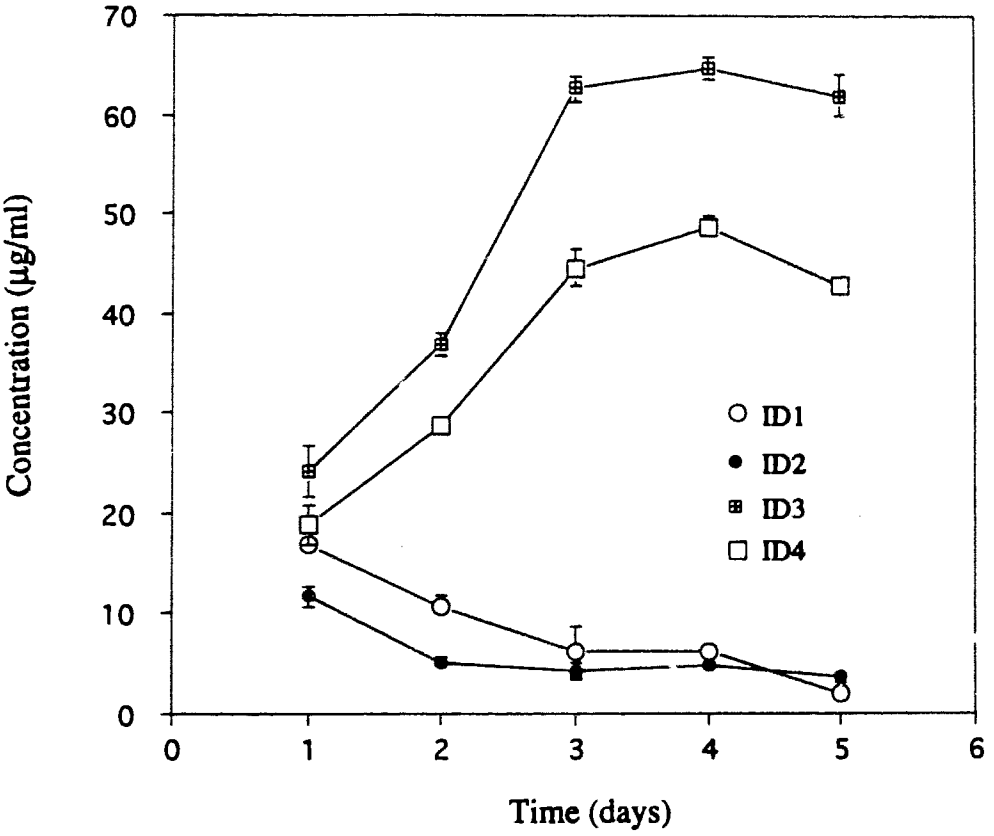

× 10⁻¹ volts
0.5
1.0
1.5
× 10⁻² volts
0.5
1.0
1.5
2.0
2.5
1.5
2.0
2.5
3.65
7.48
21.66
12.38
15.43
20.12
21.66
10
20
30
Retention time (min)

**Table 16. Concentrations of nematophin produced in TSB cultures of *Xenorhabdus nematophilus* strains ATC, BC1 and D1 at 25 °C.**

| | Concentration of nematophin (μg/ml) | | |
|---|---|---|---|
| Time(days) | BC1 | D1 | ATC |
| 1 | *116.83<br>Ac | 54.84<br>Bb | 11.25<br>Cc |
| 2 | 605.34<br>Aa | 200.67<br>Ba | 148.04<br>Ba |
| 3 | 478.48<br>Aab | 39.93<br>Cb | 88.72<br>Bab |
| 4 | 369.28<br>Ab | 36.14<br>Bb | 36.69<br>Bb |
| 5 | 564.20<br>Aab | 108.00<br>Bab | 70.00<br>Bab |

*means in the same column with the same small letter or means in the same row with the same capital letter are not significantly different (n=4, $P$=0.05).

**Figure 11. Time course of the indole concentrations (ID1, ID2, ID3 and ID4) in the TSB culture supernatant of *Xenorhabdus bovienii* A21 strain at 25 °C.**


70
60
50
40
30
20
10
0
Concentration (µg/ml)
0
1
2
3
4
5
6
Time (days)
ID1
ID2
ID3
ID4

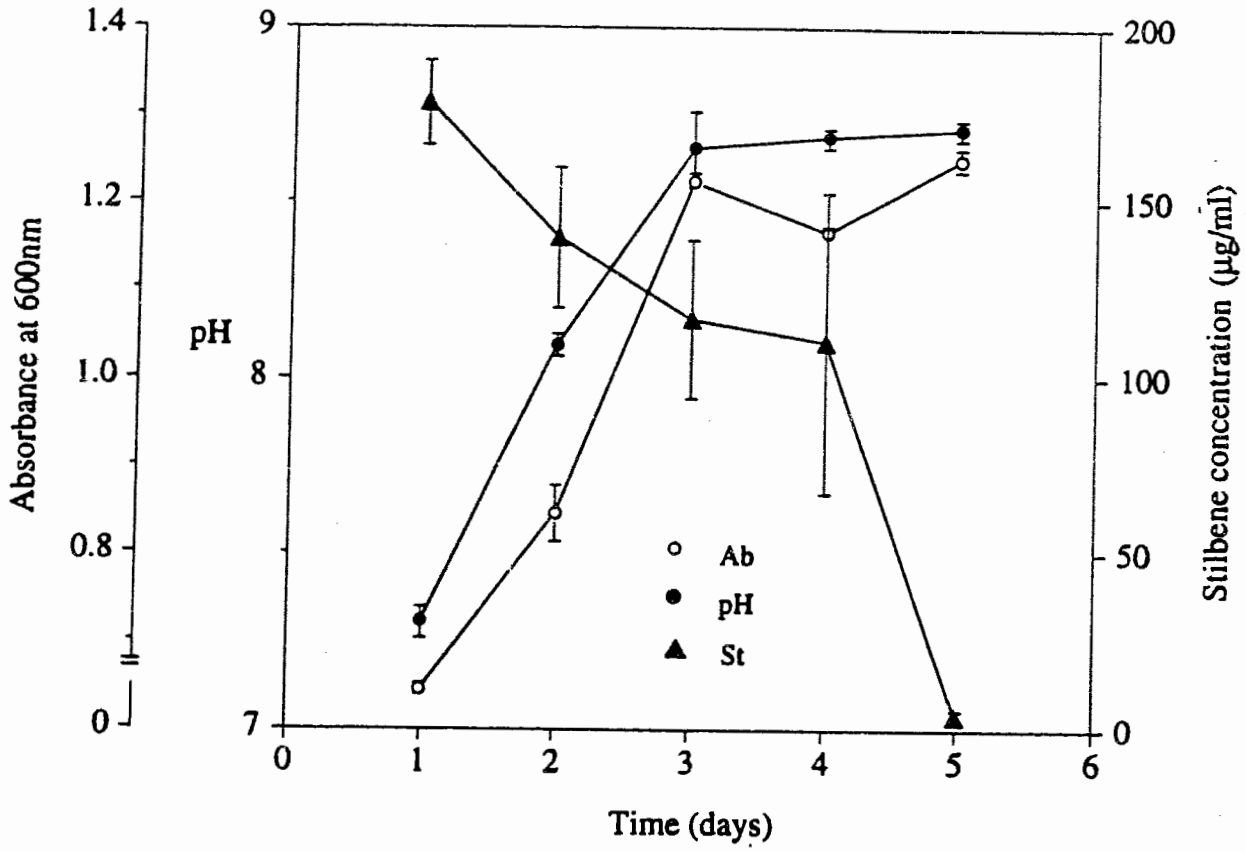
minimum levels of 2.06 μg/ml and 3.46 μg/ml, respectively at the end of the culture period (Figure 11). In contrast, ID3 and ID4 had their lowest levels of 24.42 μg/ml and 18.87 μg/ml, respectively on the 1st day, and the concentrations significantly increased thereafter to their maximum on the fourth day. The total indole concentration of the A21 strain of *X. bovienii* significantly increased in the first two days of culture and reached the maximum by the fourth day. There was a significant positive correlation between total indole concentration and absorbance (r=0.67) of the culture. None of these antimicrobial compounds was detected in any of the TSB control samples.

The concentration of STB in the culture of *P. luminescens* had a maximum (178.53 μg/ml) on the 1st day and tended to decrease thereafter to a significantly lower level (4.3 μg/ml) at the end of the culture period (Figure 12). The anthraquinone concentration in the culture was not analyzed because of technical reasons. However, continuous decline similar to that of the stilbene was noted by observing the color changes of the culture.

## 4.4. DISCUSSION.

All previous reports on the production of antimicrobial substances by *Xenorhabdus* and *Photorhabdus* were limited to the isolation of the most abundant classes of antimicrobial compound (e.g. indoles, stilbenes and xenorhabdins). The present research has, for the first time, isolated several classes of antimicrobial compounds from a single bacterial strain, the compounds have been characterized, several of them are new to science, and their bioactivity and production have been documented (Table 14, 15 and 16). From the organic fraction alone three classes of antimicrobial agents, indole derivatives, xenorhabdins and xenorxides, have been isolated from *X. bovienii* A21 strain. These four indole derivatives, four xenorhabdins and two xenorxides, clearly

**Figure 12. Time course of absorbance (Ab), pH and stilbene concentration (St) in TSB culture of *Photorhabdus luminescens* strain C9 at 25 °C.**


Absorbance at 600nm
1.4
1.2
1.0
0.8
0
pH
9
8
7
Stilbene concentration (µg/ml)
200
150
100
50
0
0
1
2
3
4
5
6
Time (days)
Ab
pH
St

demonstrate the wide-spectrum of antimicrobial substances produced by this one bacterial strain. Indole derivatives have been reported from *X. nematophilus* by Paul *et al.* (1981) and Sundar and Chang (1993). However, the identity of the bacterial strain examined by Paul *et al.*(1981) was questioned by Grimont *et al.* (1984), who suggested, after DNA analysis, that the bacterium was not a strain of *X. nematophilus*. It appeared to be a strain of *X. bovienii* as described by Akhurst and Boemare (1988). Four xenorhabdins have been reported from an *X. bovienii* strain and an unidentified *Xenorhabdus* strain (McInerney *et al.*, 1990a). Out of the four xenorhabdins isolated from A21 in this present study, two are identical with the ones isolated by McInerney *et al.* (1991a) and the other two are isolated for the first time from a natural source. Xenorhabdins are dithiolopyrrolone derivatives, first isolated from *Streptomyces pimprina* about 50 years ago, which have been shown to have a wide-spectrum of antibacterial and antimycotic activity (Celmer and Solomons, 1955). These compounds are believed to inhibit RNA synthesis of microorganisms and to be a membrane stabilizer in rats (Ninomiya *et al.*, 1980).

Paul *et al.* (1981) and Sundar and Chang (1993) reported that the indole derivatives had antibacterial activity. These compounds were found in the present research to have antimycotic activity as well. However, unlike the wide-spectrum of antibacterial activity previously reported (Sundar and Chang, 1993) only ID2 demonstrated strong antibacterial activity and this was against some Gram-positive bacteria, but not Gram-negative bacteria. The reason for this difference in our respective findings is not clear. Sundar and Chang (1993) used a mixture of all the extracted indole derivatives for their bioassay, whereas I tested each indole derivative separately. It does not seem likely that these indoles have synergistic activity because my test, using a mixture of all indoles, did not show any synergism. The present research demonstrates

also that the indoles have antimycotic activity, though not wide-spectrum and the bioactivity appears to vary among different derivatives. Sundar and Chang (1993) reported that the mode of action of the indole derivatives they found increased the intracellular levels of the regulatory nucleotide, guanosine-3',5'-bis-pyrophosphate (ppGpp) that leads to the inhibition of net RNA synthesis. Since ppGpp mediated macro-molecular regulation is not the same in all micro-organism systems (Sundar and Chang 1992) it may be expected that these compounds could exhibit different levels of activity against different micro-organisms by this method.

It has been speculated (McInerney *et al.*, 1990a) that xenorhabdins have similar bioactivity to that of closely-related chemical compounds such as thiolutin which has been isolated from various sources and shown to have antibacterial and antimycotic activity (Celmer and Solomons, 1955). Chemically, xenorxides differ from xenorhabdins in the dithiolopyrrolone ring structure in that the disulfide of xenorhabdins was oxidized. Therefore, xenorxides are oxidized forms of xenorhabdins. Presumably, there will be as many xenorxides as xenorhabdins produced by the same bacteria. However, only two xenorxides were found while four xenorhabdins were found from strain A2 of *X. bovienii*. The reason is probably due to the minute amount of these compounds produced so that the other xenorxides are in concentrations too low to be detected. The antibacterial activity of xenorxides appears to be selective for Gram-positive but not Gram-negative bacteria. The antimycotic activity is wide-spectrum with activity against all fungi tested.

The separation work on the cell-free culture of *X. bovienii* strain A21 confirmed the observation of Chapter 3 that there are non-protein, antimicrobial agent(s) present in the aqueous fraction. The nature of these agents was not examined because of time and the physical limitations of the laboratory.

The data obtained on the production of individual indoles have significant implications for the improvement of fermentation. The pattern of total indole production during the growth course has confirmed that reported by Sundar and Chang (1993). However, the total indole production from the present work is about five times higher than that reported by these workers. There is no clear explanation for the difference. The difference may be partially attributed to the different bacterial strains, culture conditions and media used since these conditions significantly influence bacterial growth and metabolic activity (Nealson *et al.*, 1990; Maxwell *et al.*, 1994; Chen *et al.*, in press). Further, the different efficiency of analytical techniques used between this study and that of Sundar and Chang (1993) may well contribute to some of the difference. Unfortunately, neither detailed specification of the culture media nor variance of the means were presented in the Sundar and Chang (1993) report. The data for the different production pattern of individual indole compounds is of significance for their industrial production in the event that a particular indole is commercialized for its antimicrobial activity. The strong correlation between the total indole concentration and absorbance clearly indicated a continuous production and accumulation of some of these indoles during the culture period.

Unlike *X. bovienii*, only a single antimicrobial agent, nematophin, was isolated from the organic fraction of *X. nematophilus*. Nematophin is a novel indole antimicrobial agent. The fact that all of the three strains of *X. nematophilus* produced nematophin suggests that nematophin might be present in many other strains of the same species. Indole derivatives produced by *X. bovienii* are probably biosynthesized from tryptophan, and their production was enhanced by the addition of tryptophan to the media (Sundar and Chang, 1993).The antibacterial activity of nematophin is probably restricted to Gram-positive bacteria (Table 14), but it also shows

antimycotic activity against the plant pathogen, *B. cinerea* (Table 15). The fact that only xenocoumacins were present in the aqueous fraction of *X. nematophilus* strain BC1 suggests that strains of *X. nematophilus* have a narrower spectrum of antimicrobial substances than that of *X. bovienii*. Based on the successful identification of the antimicrobial substances in both the organic and aqueous fractions, a complete spectrum of antimicrobial substances can be drawn for *X. nematophilus*, that includes nematophin, xenocoumacins, bacteriophage, bacteriocins and other proteinaceous agents (see Chapter 5).

It has been established that the xenorxides and nematophin could have significant potential for use against medically important human pathogens because both chemical groups have MICs of less than 2.0 μg/ml against drug-resistant bacteria. In addition to this strong activity, the simple structure and ease of production of nematophin, by biological or synthetic means, should make this group of chemicals an excellent starting point for the development of new drugs or agrochemicals.

There appear to be two antimicrobial groups isolated from *P. luminescens*, i.e. stilbenes and anthraquinones. Stilbene derivatives were previously reported to have strong activity against both Gram-positive and Gram-negative bacteria (Sundar and Chang, 1992). The production of the stilbene isolated in this study from the bacterial culture is interesting in that it was at maximum level at a relative early stage of the bacterial culture, substantially slowed as the growth continued and eventually disappeared at the end of the culture period. In general, the production of secondary metabolites, such as antibiotics of microorganisms, is triggered by unfavorable environmental factors and commences in a relatively late stage of growth. The reason for the early production and decline of stilbene is not clear. However, its decline in concentration suggests the

possible cessation of stilbene synthesis. The cessation of antibiotic synthesis usually results from the following possible reasons: disappearance of enzymes of the antibiotic synthetic pathway, the inhibitory effect of the accumulated antibiotic, and/or deletion of intermediary precursors of the antibiotic (Martin and Demain, 1980). Although none of these possibilities is ruled out it is not likely that the stilbene has an inhibitory effect on *P. luminescens* itself at the concentration achieved in the medium (178.53 μg/ml) because *P. luminescens* is resistant to stilbene (MIC>600 μg/ml, data not shown). It appeared that the anthraquinone production in the culture had a similar decline during the culture, but again, the reason for the decline is not clear. However, one possible explanation for the decline of both the stilbene and the anthraquinone might be the fast conversion of the primary form of this bacteria into secondary or intermediate forms. As observed in Chapter 3, strain C9 was not stable and it readily changed into its secondary or intermediate form. It would be possible to analyze the production of these antimicrobial substances of *P. luminescens* if a stable primary form was used.

# CHAPTER 5. EXOCELLULAR CHITINASE ACTIVITY

## 5.1. INTRODUCTION.

It has been reported that strains of *Xenorhabdus* and *Photorhabdus* species have antimicrobial activity, such as suppressing soil microflora populations (Maxwell *et al.*, 1994), inhibiting the growth of blastospores of the insect pathogenic fungus *B. bassiana* (Barbercheck and Kaya, 1990), and slowing or inhibiting the mycelial growth and spore germination of many fungal species on agar plates (Chen *et al.*, 1994). The identities of the bioactive substances produced by these bacteria, especially those in the aqueous fraction, are only partially known. In Chapter 3 it was observed that *Xenorhabdus* and *Photorhabdus* lysed fungi. This suggested that these bacteria probably produce chitinase and/or β-glucanase, and it led to an investigation of exo-enzymatic activity of *Xenorhabdus* and *Photorhabdus* species.

Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine, is a common constituent of a wide range of organisms, including fungi (cell walls), insects (cuticle) and nematodes (egg shell) (Flach *et al.*, 1992). Chitinases are those enzymes that cleave the chitin polymer. In general, the enzymes that cleave chitin randomly and release oligosaccharides are referred to as endo-chitinases (EC 3.2.1.14). The enzymes that hydrolyze non-reducing, N-acetyl-glucosamine residues from the terminal non-reducing ends of chitobiose and higher analogues, releasing N-acetylglucosamine from chitin are considered to be exo-chitinases (EC 3.2.1.30) (Gooday, 1991). Organisms that contain chitin also possess chitinase. In addition, organisms such as soil bacteria, and many plant and animal species, that do not contain chitin may produce chitinases in order to obtain carbon by degrading the polymer (Roberts and Selitrennikoff, 1988).

Plant chitinases are often associated with pathogenesis, and are induced by the presence of some pathogens such as fungi. Fungal chitinases are involved in the growth of the fungus itself. Insect chitinases are found principally in the moulting fluid and integument of insects, and are believed to participate directly in cuticular chitin degradation. Bacterial chitinases play a role mainly in chitin mineralization as extracellular enzymes (Flach *et al.*, 1992). In addition, chitinases express antimycotic activity through the destruction of fungal cell walls, and some chitinases have lysozyme activity which hydrolyze the peptidoglycans in bacterial cell walls and may, therefore, be antibacterial (Flach *et al.*, 1992). Among the Enterobacteriaceae, only a few species, such as *Serratia liquefaciens* (Molise and Drake, 1973) and *S. marcescens* (Young *et al.*, 1985) produce chitinases. In an earlier study Khan and Brooks (1977) failed to identify clearly the presence of chitinase activity in *Xenorhabdus* and *Photorhabdus* species.

β-glucan, as either linear or branched polymers of anhydroglucose, linked by glucosidic bonds, is found in higher plants, in fungi and yeasts as structural component of the cell walls. β-glucanases, which hydrolyze β-glucan, are produced by plants, fungi, yeast and bacteria, such as *Bacillus* and *Pseudomonas* species. Like chitinase, β-glucanase has been shown to have antimycotic activity against fungi and yeasts (Bielecki and Galas, 1991).

These properties of chitinase and β-glucanase enzymes probably form part of the collective antimicrobial activity of *Xenorhabdus* and *Photorhabdus*, but little is known of them in these bacteria. Therefore, the chitinase and β-glucanase activity of these bacteria was investigated to determine their possible role in the bacteria-nematode-insect interaction determined.

## 5.2. MATERIALS AND METHODS.

### 5.2.1. Enzyme preparation and detection.

Bacterial inoculum (10 ml) from an overnight culture (25 ml in 100 ml flask shaken at 120 rpm., 25°C) of the primary or secondary forms of *Xenorhabdus* or *Photorhabdus* species was added to 300 ml TSB and cultured separately in 1,000 ml flasks on a gyratory shaker (150 rpm.) in the dark for 60 h. The cell suspension was then centrifuged (11,000 × g, 4 °C, 20 min) to remove bacterial cells, the cell-free culture was treated with $(NH_4)_2SO_4$ to 20% saturation, stirred for 2 h at 4 °C and centrifuged (11,000 × g, 4°C), and the supernatant was decanted from the protein, treated again with $(NH_4)_2SO_4$ to 80% saturation, stirred (4 °C, overnight) and centrifuged (11,000 × g, 30 min, 4 °C). The precipitated protein was pelleted, dissolved in distilled water, dialyzed (MW cut-off: 8-9,000) overnight against 4 liters of distilled water (two changes) and then dialyzed against 4 liters of 10 mM sodium acetate buffer (pH 5.0, 4 °C, overnight). The dialysate was centrifuged to remove any precipitate and the resulting supernatant was concentrated to 5 ml by adding Sephadex G-10 powder to remove water.

Protein concentration in the enzyme preparations and bacterial cultures was determined using the Coomassie brilliant blue G-250 assay (Bradford, 1976) with bovine serum albumin as the standard. Enzyme solutions were kept at 4 °C and used immediately or within 1 week; preliminary tests established that the specific activity did not decline during this period.

The proteinaceous preparations were separated using non-denaturing polyacrylamide gel electrophoresis (PAGE) at 4 °C (Pan *et al.*, 1991). After electrophoresis, the PAGE gel, attached to supporting glass plates, was incubated in 0.1 mM sodium acetate (pH 5.9) for 5 min, covered

with a 0.75 mm thick polyacrylamide overlay gel (7.5%) containing 0.04% glycol chitin in 0.1 M sodium acetate buffer (pH 5.0) and incubated at 40 °C overnight under moist conditions. The overlay gel was then stained by incubating in freshly prepared 0.01% (w/v) fluorescent Brightener 28 (Sigma) in 500 mM Tris-HCl (pH 8.9) at room temperature for 5 min. The Brightener solution was discarded and the overlay gel was incubated in distilled water at room temperature in the dark for 2-3 h. Chitinase isozymes were visualized as clear zones by placing the overlay gel on a UV transilluminator and then photographed.

To detect β-1,3-glucanases, the above non-denaturing PAGE gels (after electrophoresis) were washed with distilled water, incubated with 0.05 M sodium acetate (pH 5.0) for 5 min, and then incubated at 40 °C in 150 ml of 0.025 M sodium acetate buffer (pH 5.0) containing 0.75% laminarin for 30 min. The gel was then incubated in a mixture of methanol:water:acetic acid (5:5:2) for 5 min at room temperature, washed with distilled water, and stained with 0.3 g of 2,3,5-triphenyltetrazolium chloride in 200 ml of 1.0 M NaOH in a boiling, water bath for 15 min.

Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed as described by Zhang and Punja (1994). Briefly, denatured samples were loaded onto an SDS-PAGE gel containing glycol chitin as the substrate for chitinase. After running the gel, SDS was removed from the gel by shaking overnight at 38-40°C in sodium acetate buffer (0.1 M, pH 5.0) containing purified Triton X-100, and the proteins in the gel were renatured. The gels were then stained and destained as described above for the non-denaturing PAGE/overlay gels. The lytic zones were visualized and photographed as described above for the overlay gels under UV. The molecular weight of the chitinase protein was obtained by comparing it with standard marker proteins on the same SDS-PAGE gel (Trudel and Asselin, 1989).

5.2.2. Biochemical analysis of the enzyme activities.

To compare the enzymatic activity of different bacterial species and strains, the five bacterial strains listed in Table 3 were grown separately in 30 ml of TSB in 125 ml flasks (25 °C, shaken at 150 rpm) in the dark for 48 h, and tested for $A_{600}$, protein concentration and enzymatic activity as outlined below. The comparison test between these strains was repeated three times. Preliminary tests revealed strong chitinase enzyme activity in the A21 strain of *X. bovienii*. Therefore, this strain was chosen to monitor the levels of enzymatic activity during the growth of the primary and secondary forms. Experiments were initiated by growing the A21 strain in 100 ml of TSB in each 250 ml flask (25 °C, shaken at 150 rpm). The cultures were sampled at 0.5, 1, 2, 3, 4 and 5 days. For comparison of the primary and secondary forms, these two forms of the A21 strain of *X. bovienii* were grown separately under the same conditions. Bacterial growth was measured as absorbance at 600 nm and then the media were centrifuged (11,000 × g, 20 min, 4 °C) to remove bacterial cells and debris, and the supernatant of each of the cultures was subjected to the following tests.

To quantify exo-chitinase, the release of *p*-nitrophenol (PNP) from the substrate *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) was assayed using the modified methods of Roberts and Selitrennikoff (1988) and Ohtakara (1988). Briefly, 10 µl of the supernatant were added to a 1.5 ml Eppendorf tube. Sixty microliters of the substrate solution, containing 100 µg/ml of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 50 mM potassium phosphate (pH 6.7), was mixed with the test sample and the tubes were incubated at 41 °C in a water bath for 30 min. The reaction was terminated by adding 1.43 ml of 0.4 M $Na_2CO_3$ to each tube and the concentration of PNP in the solution was determined by absorption at 405 nm (Tronsmo and

Harman, 1993). One unit of enzyme activity was defined as the amount of enzyme that released 1μmol of *p*-nitrophenol/min under the above conditions.

Endo-chitinase activity was quantified by using the same assay as described for exo-chitinase, but using nitrophenyl-β-D-N,N',N''-triacetylchitotriose as a substrate (Tronsmo and Harman, 1993). Test samples (10 μl ) of culture supernatant were added to each 1.5 ml Eppendorf tube followed by 50 μl of the substrate solution (250 μg/ml) dissolved in 50 mM potassium phosphate (pH 6.7 ). The tubes were incubated at 41 °C for 30 min and the reactions were stopped by adding 0.95 ml of 0.4 M $Na_2CO_3$.

β-1,3-glucanase activity of the partially purified enzymatic solutions was tested as described by Sock *et al.* (1990) using laminarin as substrate by measuring the amount of reducing sugars.

5.2.3. Partial purification of the chitinase.

To assay the antimycotic activity the chitinase enzymes were partially purified. Half a liter of bacterial culture of *X. bovienii* strain A21 was prepared in 1,500 ml flasks under the above culture conditions. The supernatant, after dialysis and further centrifugation as described in 5.2.1., was frozen in liquid nitrogen and freeze-dried. Finally, the dry, protein powder was dissolved in distilled water and subjected to chromatographic separation using Sephadex G-100 (12×3.5 cm). The column was eluted with water at 6 ml/min, the fractions were collected every minute and their absorbance was monitored at 280 nm. The exo-chitinase, endo-chitinase, lysozyme and proteolytic activities of each fraction were measured as described and the antimycotic activity was bioassayed (see below).

The activity of lysozyme was assayed using a modified lysoplate method as described by Yousif (1993). Briefly, 40µl of the test sample was pipetted into wells (4 mm in diameter) cut into 0.5% agarose (Sigma) in a 9-cm Petri plate containing 10 ml of agarose. The agarose had been amended with 0.6 mg/ml of freeze-dried cells of *Micrococcus lysodeiktiens* (Sigma). Chicken egg-white lysozyme (1,000 µg/ml) was used as control. The lysozyme activity was qualitatively assessed after 20 h incubation at 25 °C.

Assay for proteolytic activity was tested using the method outlined by Holding and Collee (1971). Wells of 4 mm were made in 1.5% water agar plates (10 ml in a 9 cm plate) containing 0.4% of gelatin, and 40µl of test sample was added to each well. The agar plates were incubated at 25 °C for 40 h before the plates were flooded with 1% tannic acid, and then the clear proteolytic zones around the wells were qualitatively assessed.

5.2.4. Bioassay of antimycotic activity.

To quantify the antimycotic activity of *Xenorhabdus* and *Photorhabdus* species, the partially purified protein solutions were tested against *B. cinerea* on cavity slides as described in section 2.4.2.

To detect further the lytic activity of the chitinase enzymes on germ tubes, the conidia of *B. cinerea* were allowed to germinate for 12 h prior to mixing with an equal amount of test solution containing 0.1-10 µg/ml protein. The mixture was incubated in a humid chamber, in the dark at 24 °C for an additional 12 h, examined microscopically and photographed.

Preliminary tests showed proteolytic activity of the freeze-dried protein and fractions after partial purification. Consequently, in order to eliminate the possible effect of the proteolytic activity on germination of the fungal conidia the fractions, after Sephadex separation, were either

heated in a water bath at 41 °C for 30 min or were added together with phenylmethylsulfonyl fluoride (PMSF, 3 mM, final) prior to the test. Both methods resulted in undetectable proteolytic activity in the fractions.

## 5.3. RESULTS.

### 5.3.1. Enzyme activity.

*Xenorhabdus* and *Photorhabdus* species showed enzymatic activity by their ability to hydrolyze glycol chitin after electrophoresis (Figure 13). Two major activity bands were detected for both *X. bovienii* (A21) and *X. nematophilus* (BC1, D1), three bands for ATC of *X. nematophilus*, and one band for *P. luminescens* strain C9 on the overlay gel. SDS-PAGE of the A21 strain showed one band with chitinase activity at a molecular mass of 38.8 kDa. The activity of both enzymes for the A21 strain of *X. bovienii* increased during the first 24 h (Figure 14) with the exo-chitinase activity peaking by 24 h post-inoculation and remaining at a constant level until the fourth day after which it decreased significantly. The endo-chitinase activity increased rapidly throughout the first 48 h and was more or less constant thereafter. The overall level of activity of exo-chitinase was greater than that of endo-chitinase during the five-day period of culture.

### 5.3.2. Enzyme analysis.

The levels of specific endo-chitinase and exo-chitinase activity differed among the strains of *Xenorhabdus* and *Photorhabdus* (Table 17). Among the three strains of *X. nematophilus*, ATC, BC1 and D1, the BC1 culture had the lowest activity for both enzymes whereas the ATC culture had the highest activity for endo-chitinase and D1 had the highest activity for exo-chitinase both enzymes. The strains ATC and D1 but not BC1 of *X. nematophilus* had higher

**Figure 13. Photos showing the isoform banding patterns for the chitinase enzyme of strains of *Xenorhabdus* and *Photorhabdus*: (a) Native PAGE of A21, C9, BC1, D1 and ATC strains followed by overlay gel of glycol chitin; (b) SDS-PAGE of A21 strain with chitinase activity (S) number marker on the right (k Da) and standard protein marker (M) stained with Coomassie blue.**

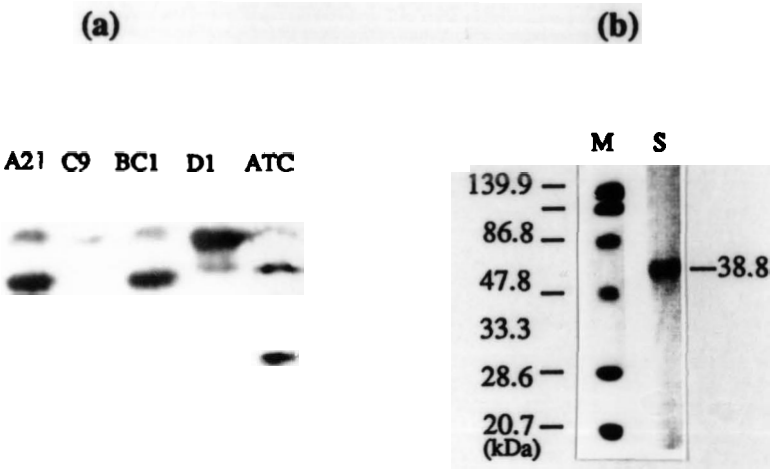

(a)
(b)
A21
C9
BC1
D1
ATC
M
S
139.9
86.8
47.8
33.3
28.6
20.7
(kDa)
38.8

**Figure 14. Time-course of *Xenorhabdus bovienii* A21 strain development showing exo-chitinase (Ex) and endo-chitinase (En) activity in TSB.**

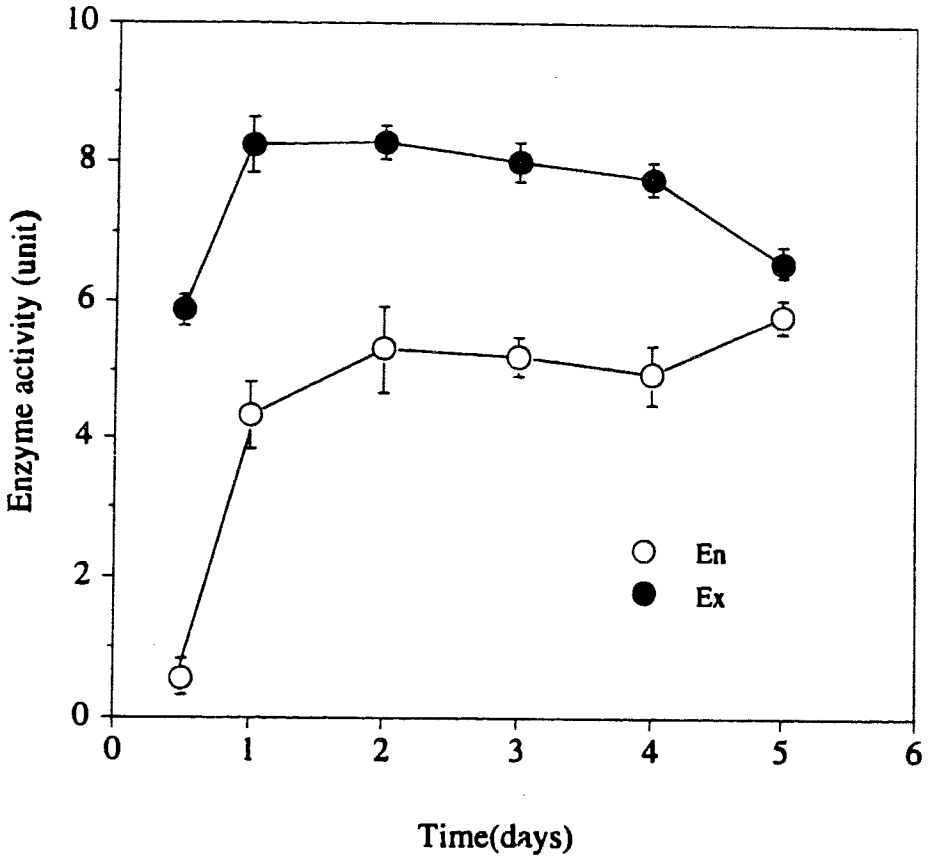

10
8
6
4
2
0
0
1
2
3
4
5
6
Enzyme activity (unit)
Time(days)
En
Ex

**Table 17. Comparison of the final absorbance, total protein concentration and specific exo- and endo-chitinase enzyme activity in the cultures of three strains of *Xenorhabdus nematophilus* (ATC, BC1, D1), and one strain each of *X. bovienii* (A21) and *Photorhabdus luminescens* (C9).**

| | Bacterial strains* | | | | |
|---|---|---|---|---|---|
| Characteristics | ATC | BC1 | D1 | C9 | A21 |
| Final absorbance (600nm) | 2.9±0.1 | 3.3±0.0 | 3.1±0.0 | 2.9±0.0 | 2.7±0.0 |
| Total protein (× 100 μg /ml) | 1.3±0.1 | 9.5±0.3 | 2.4±0.1 | 4.6±0.2 | 6.3±0.3 |
| Exo-chitinase (units/mg) | 29.6±2.2 | 10.3±0.1 | 35.2±1.7 | 8.0±0.5 | 12.5±1.1 |
| Endo-chitinase (units/mg) | 32.1±3.0 | 9.1±0.1 | 17.8±0.7 | 8.8±0.5 | 7.0±0.3 |

*n=3

levels for both enzymes than had *P. luminescens* strain C9 and *X. bovienii* strain A21. The chitinase activity was not enhanced by adding 0.5% chitin to the culture media (data not shown).

The chitinase enzyme activity of the primary form of *X. bovienii* A21 strain was significantly greater than that of the secondary form (Table 18). Exo-chitinase activity was about ten times higher and endo-chitinase about four times higher in the primary form cultures than in the secondary form cultures. There was no significant difference ($P>0.05$) in the final absorbance or the total protein level between the primary and secondary forms (Table 18).

The freeze-dried protein of A21 strain did not show lysozyme activity but had proteolytic activity before and after the chromatographic separation (data not shown). The chitinase enzyme activity of A21 was detected from the 4th to the 14th fraction for exo-chitinase and from the 4th to the 9th for endo-chitinase following Sephadex gel separation. This corresponded with the first small peak of the protein absorbance and the antimycotic activity (Figure 15). There was a major peak of protein absorbance present between fraction 10 and 25, and this peak corresponded with a high level of antimycotic activity. However, this major peak of protein absorbance corresponded with very weak chitinase activity. The antimycotic activity decreased in the fractions between the small and the major peaks. The inhibition of proteolytic activity with PMSF and heat treatment did not change their antimycotic activity (Figure 15, A) but the heat treatment totally eliminated the antimycotic activity correlated with the major peak (Figure 15, B). The partially purified protein solutions from the pooled fractions of the small peak showed antimycotic activity against conidial germination and germ tubes of *B. cinerea* (Figure 16). This was expressed as inhibition of conidial germination and of germ tube elongation on exposure to enzymatic protein at 0.1-1

**Table 18. Comparison of the absorbance, total protein concentration and specific chitinase activity in primary and secondary form cultures of *Xenorhabdus bovienii* A21 strain.**

| Characters | Primary | Secondary |
|---|---|---|
| Final absorbance (600nm) | *2.5±0.0 | 2.5±0.0 |
| Total protein (× 100 μg/ml) | 5.0±0.3 | 5.2 ± 0.3 |
| Exo-chitinase (units/mg) | 12.4±0.7 | 1.2±0.1 |
| Endo-chitinase (units/mg) | 9.0±0.9 | 2.3±0.1 |

* n=6.

**Figure 15. Profile of endo-chitinase (En) and exo-chitinase (Ex) activity in the different fractions following separation of protein from *Xenorhabdus bovienii* strain A21 culture through Sephadex-G100-120. Protein concentration (Ab), as expressed by absorbance at 280 nm, and antimycotic activity (%), expressed as the percentage inhibition of *Botrytis cinerea* conidial germination in these fractions, are shown. In (B) but not in (A) the fractions were heated at 41 °C for 30 min prior to test for antimycotic activity.**

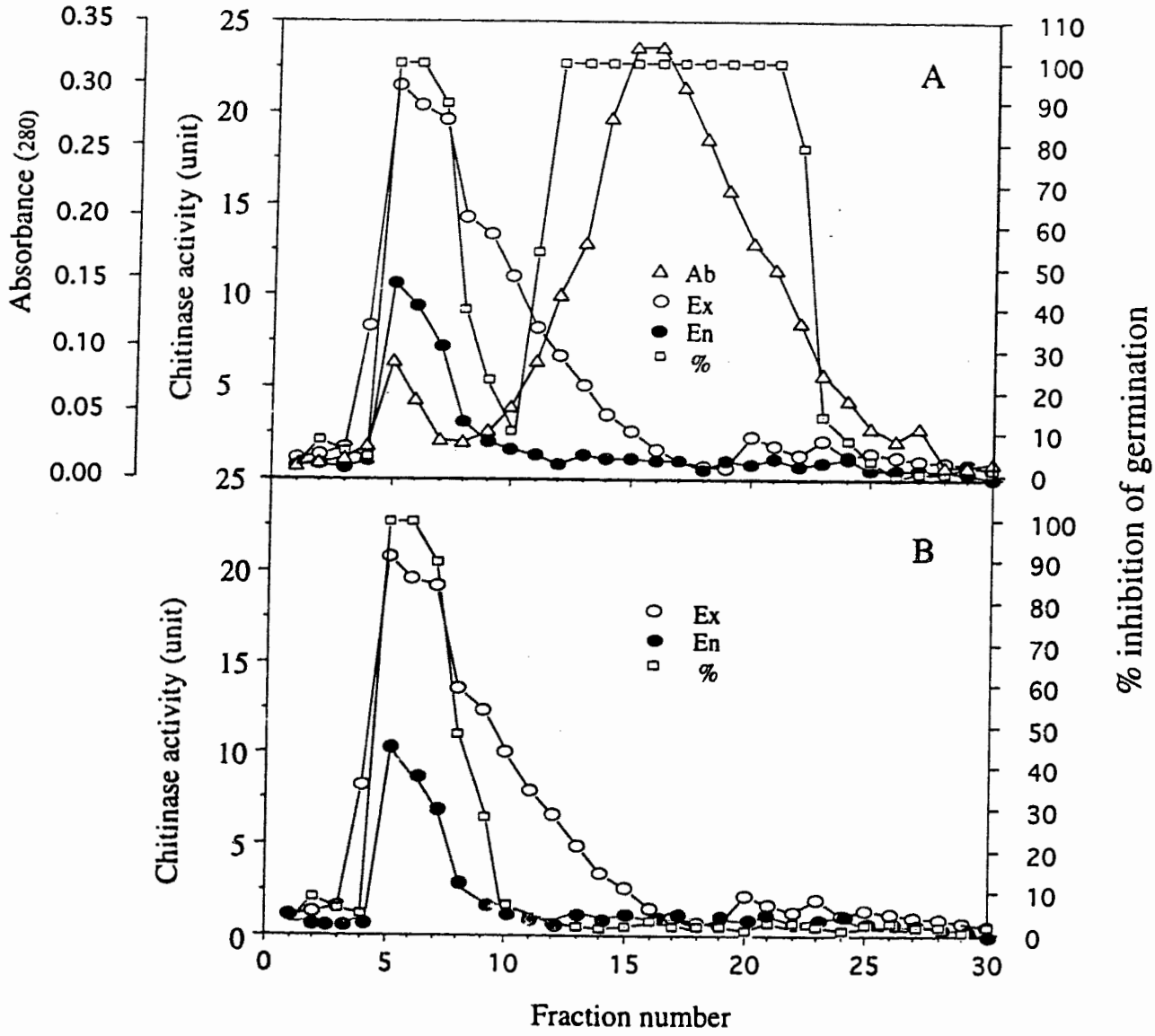
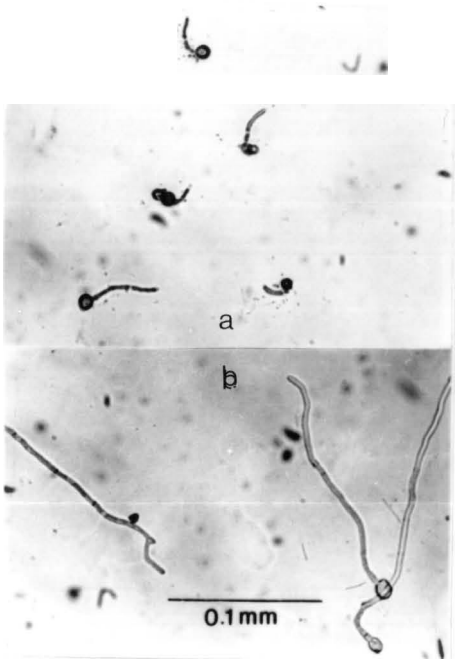

Absorbance (280)
Chitinase activity (unit)
% inhibition of germination
A
B
Ab
Ex
En
%
Fraction number

**Figure 16. Photos showing lysis of the germ tube of *Botrytis cinerea* (a) caused by partially purified chitinase of *Xenorhabdus bovienii* A21 in comparison with an untreated control (b).**


a
b
0.1mm

μg/ml, and as severe cell wall lysis and distortion of the germ tube on exposure to enzymatic protein at 5 μg/ml (Figure 16).

Neither the electrophoresis nor biochemical assay detected any β-glucanase activity in any of the strains of *Xenorhabdus* and *Photorhabdus* examined.

## 5.4. DISCUSSION.

The chitinase activity of *Xenorhabdus* and *Photorhabdus* has been clearly demonstrated by the fact that the proteinaceous preparations of their respective cultures hydrolyzed glycol chitin after gel electrophoresis. The different isoform binding patterns for the chitinase of strains of these bacteria (Figure 13) suggests that the chitinase activity varied qualitatively and quantitatively between the species and strains tested.

The antimicrobial activity of species of *Xenorhabdus* and *Pnotorhabdus* is due to the production of antibacterial and antimycotic substances and these include chitinases. These enzymes have particularly strong antimycotic activity as shown by their effects on conidial germination, and on the growth and morphology of the germ tube of *B. cinerea* at a concentration as low as 0.1 μg/ml. The result is consistent with the idea that the chitinase of these bacteria contributes to the overall antimicrobial activity, based on the correlation of the antimycotic activity and chitinase activity in the absence of lysozyme and proteolytic activity in the partially purified fractions. Proof will require purification of the protein fractions. The mechanism of action of these enzymes produced by *Xenorhabdus* and *Photorhabdus* species is not clear. However, the rapid increase (Figure 14) in enzymatic activity in the first 24-48 h of bacterial culture suggests that these enzymes may play an important role in the initial antimycotic mechanisms of the dying insect against microbial invasion, since by then the protection from the immune system of the insects is fading and the antibiotic production of these

bacteria is not maximized. The relatively stable enzymatic level suggests further possible roles, including the facilitation of juvenile hatching by softening the nematode egg shell, and juvenile emergence from the insect cadaver at a later stage of nematode development by softening the insect cuticle. The former is probably a similar mode of action to that of chitinase activity during egg hatch of the animal parasitic nematode, *Ascaris suum* (Fuhrman, 1995). The softening of the insect cuticle is probably in a manner similar to the eclosion of the adult sugar beet root maggot, *Tetanops myopaeformis*, from the pupa with the aid of chitinase from *S. marcescens* and *S. liquefaciens* (Iverson *et al.*, 1984).

The proteolytic activity of the crude protein preparation confirmed two earlier reports on protease of *Xenorhabdus* (Boemare and Akhurst, 1988) and of *Photorhabdus* (Schmidt *et al.*, 1988). Although it is not confirmed, the proteolytic activity may play a part, not only in the inactivation of insects' defense systems (Schmidt *et al.*, 1988; Dunphy and Thurston, 1990) but also in nutrient utilization by the nematode through conversion of some insect proteins to amino acids. The large protein absorbance peak after chromatographic separation indicates the presence of large amounts of protein in these fractions (Figure 15a). The fact that these fractions did not have chitinase activity but did have antimycotic activity, suggests that some other enzyme or protein plays a role in the collective antimicrobial activity of *Xenorhabdus* in the symbiotic system. The clearly diminished antimycotic activity in fractions between the small chitinase peak and the major protein peak indicates that the antimycotic activity in the fractions corresponding to the major peak is very unlikely to be derived from the tailing effect of the chitinase fraction or from something common to both peaks. The inhibition of the proteolytic activity in all fractions and the inhibition of antimycotic activity corresponding to the major protein peak by heat

treatment (Figure 15b) suggests that the agents responsible for these activities are not heat stable. The nature of the large amount of protein shown by the major peak has yet to be determined, since these proteins have neither chitinase, lysozyme nor β-glucanase activity.

The primary and secondary forms differ from each other in many physiological properties (Boemare and Akhurst, 1988; Nealson *et al.*, 1990), and this study shows that one strain, A21, differs also in chitinase enzyme activity (Table 18). The chitinase enzyme activity of the bacterial culture of the primary form of strain A21 was several times stronger than that of the secondary form of the same strain, but their overall absorbance and total protein concentration were similar. This may be attributed to the nature of the overall differences in their antimicrobial activity. Since the $LD_{50}$s of the primary and secondary forms of *X. nematophilus* and *X. bovienii* are the same in *G. mellonella* (Dunphy and Webster, 1985), the chitinases may not be part of the virulence mechanisms of the bacteria.

*Xenorhabdus* and *Photorhabdus* species have not been tested routinely for chitinase enzyme activity. In an earlier study, Khan and Brooks (1977) observed a doubtful positive for chitinase activity using the bacteria associated with the DD136 isolate of *S. carpocapsae* (presumably, the strain ATCC 19061 deposited by Poinar and Thomas). However, in this study, this strain of bacteria (ATC) associated with DD136 was positive for chitin hydrolysis activity, but its overall activity on glycol chitin and chitin residues were not the strongest among the strains tested (Figure 13, Table 17). It is quite possible that its weak chitinase activity was not detected previously, either because the methods used to detect this activity were not sufficiently sensitive or the bacteria did not grow well on the medium used by Khan and Brooks (1977). It is also possible that they used the secondary form, which is probably weak in chitinase activity, but were unaware of it

because it was not recognized at that time. Chitinase is not a character which has been associated with many species of the Enterobacteriaceae. However, chitinase enzyme activity was common to all of the five strains tested in this study. Therefore, chitinase should be routinely tested for in the isolation and identification of *Xenorhabdus* and *Photorhabdus* species. This test will help to differentiate these bacteria from many other species in the Enterobacteriaceae.

# CHAPTER 6. GENERAL DISCUSSION

It has been reported since the early seventies (Dutky, 1974) that the symbiotic bacteria of this nematode/bacteria (*Steinernema* spp.) complex produce antimicrobial substances that protect the insect cadaver from secondary invasion by micro-organisms. Although some aspects of the antimicrobial activity of these bacteria has been examined in recent years and several classes of compounds have been identified (Akhurst and Dunphy, 1993), the overall spectrum or the bioactivity of antimicrobial substances produced by these bacteria is not fully known.

The present research has established clearly that there are at least three antimicrobial systems employed by different nematode-bacteria complexes. In the *X. nematophilus/S. carpocapsae* complex, I have shown that *X. nematophilus* has an antimicrobial effect in the production of nematophin in addition to the xenocoumacins (McInerney *et al.*, 1991b) and the proteinaceous chitinases and bacteriocins (Boemare *et al.*, 1992). In the *X. bovienii/S. feltiae* complex, the antimicrobial substances produced by bacteria are xenorxides as well as the indoles, xenorhabdins, the unknown water-soluble-non-proteinaceous substance(s), the proteinaceous chitinases, bacteriocin (Boemare *et al.*, 1992) and an unknown protein. The bacteria of the *P. luminescens/Heterorhabditis* spp. complex produce the stilbene, anthraquinones and the proteinaceous chitinases and bacteriocin (Boemare *et al.*, 1993b). It appears that the *X. bovienii/S. feltiae* complex produces the most diverse range of compounds, producing at least seven classes of antimicrobial agents, whereas the simplest system of *P. luminescens/Heterorhabditis* produces only four classes. The presence of chitinases and bacteriocins are common to all three complexes which suggests the essential role played by these agents in the biology of the nematode/bacteria complex.

Chitinase hydrolyzes chitin in many organisms including fungi, nematode egg shell and insect cuticle. One role of chitinases is apparently part of the collective antimycotic activity of the symbiotic bacteria as shown in this present study. However, unlike some other bacterial chitinases that have both antimycotic and lysozyme activities (Flach *et al.*, 1992) the chitinases of *Xenorhabdus* and *Photorhabdus* are unlikely to contribute to the antibacterial activity because they do not have lysozyme activity. In contrast to the large variation in species-specific, non-proteinaceous substances as antimycotic agents employed by the different *Xenorhabdus* and *Photorhabdus* species, chitinases are the proteinaceous, antimycotic agents that are common to all species tested. This suggests that the chitinases may play additional roles in the nematode/bacteria system. Although not yet confirmed, one of the possible roles may be in inducing nematode egg hatch at a particular time and, subsequently, in assisting the nematodes to emerge from the insect cadaver, as has been demonstrated for other nematode and parasitic systems (Fuhrman, 1995). The occurance of nematode egg hatch at a particular time may well reflect the timely availability of chitinase activity in the insect cadaver.

Compared with the large variation in species-specific, non-proteinaceous substances as antibacterial agents produced by the different species of bacteria, the ubiquitous production of bacteriocins (Table 2) by both *Xenorhabdus* and *Photorhabdus* spp. suggests that there may be a significant function for the bacteriocins. Production of bacteriocins is widespread among both Gram-negative (Dykes, 1995) and Gram-positive bacteria (Jack *et al.*, 1995). Basically, bacteriocins are effective against closely related bacterial strains or species (Boemare *et al.*, 1993b), but their exact role in the nematode/bacteria complex is not clear. Given their high degree of specificity, bacteriocins would only help in competition among related strains of *Xenorhabdus*

and *Photorhabdus*, to establish population predominance in a manner similar to *Lactobacillus plantarum* (Ruiz-Barba *et al.*, 1994) and *Myxococcus* spp. (Smith and Dworkin, 1994).

When two different entomopathogenic nematodes co-infected an insect, only one species was able to reproduce (Alatorre-Rosas and Kaya, 1991). Boemare *et al.* (1993b) suggested that the first symbiont to establish in the host may initiate the spontaneous production of bacteriocins which may kill related symbionts and, consequently, may inhibit development and reproduction of the second nematode. This suggestion is rather controversial. Elimination of the symbiotic bacteria *in vivo* does not necessarily result in failure of nematode reproduction because these nematodes are able to reproduce on other bacteria. The fact that non-symbiotic bacteria are dominant in the insect cadaver at a certain stage of nematode development (Maxwell *et al.*, 1994: Akhurst, 1982) further illustrates the flexibility of nematode development on the bacterial species *in vivo*. Moreover, the relatively small proportion of antibacterial activity that is derived from the proteinaceous component of the bacterial metabolites (Chapter 3.) clearly suggests that the elimination of a closely related bacterium is not a major target of the overall antimicrobial activity. Several workers (Lysenko and Weiser, 1974; Poinar, 1966) noted that fresh, infective juveniles from insect cadavers contain bacterial contaminants, and for them to become monoxenic a "starving time" is required, during which the contaminants are eliminated. The effect of bacteriocins in eliminating the closely related species or populations probably occurs at a stage when the juveniles are acquiring their symbionts and/or after emergence from the insect cadaver when inter-specific competition organisms in the cadaver. This is because the closely related bacterial symbionts and/or populations have very similar affinities for their nematode symbionts (Binnigton

and Brooks, 1993; Moureaux *et al.*, 1995). Both bacterial genera have evolved very similar antimycotic and antibacterial strategies for producing chitinases and bacteriocins. Poinar (1993) suggests that their similarities are a result of convergent evolution arising from their mutualistic association with the nematodes. This evolutionary convergence further supports the relative importance of the chitinases and bacteriocins in the nematode/ bacteria complex.

The form variants of *Xenorhabdus* and *Photorhabdus* differ physiologically (Boemare and Akhurst, 1988), but these differences are ill-defined and the shifting between forms is unpredictable (Smigielski *et al.*, 1994; Krasomil-Osterfeld, 1995). The difference in the antibiotic activity between primary and secondary forms is one of the features that differentiates them. However, the present study shows that this feature is also highly variable with regard to the qualitative nature of the antimycotic activities. The presence of antimycotic activity in the secondary form in the absence of antibacterial activity suggests that these activities probably would be only a part of the overall antimicrobial arsenal that differs between forms. Apparently, the secondary forms do not produce the small molecular antimicrobial agents, such as indoles and xenorhabdins, in *X. bovienii* or nematophin and xenocoumacins, in *X. nematophilus* (Chapter 3. 4.). However, the secondary forms do produce some of the proteinaceous agents, such as chitinases and bacteriocins (Chapter 5; Boemare *et al.*, 1993b). Variation in antimicrobial agents and their activities between forms is complicated by the reciprocal shifting of these forms. Precise characterization of the differences in antimicrobial activity between forms is possible only when stable primary and secondary forms are found.

In the spectrum of antimicrobial substances produced by these symbiotic bacteria this study has shown an extensive list of non-proteinaceous substances, especially, from *X.*

*nematophilus*. These include the organic-soluble nematophin and water-soluble xenocoumacins. It is noted that non-proteinaceous antimicrobial substances from all the strains of *X. nematophilus* examined in the present study, are constant qualitatively in the same culture conditions. It is known also that there is a variable spectrum of non-proteinaceous antimicrobial substances in the strains of *X. bovienii* (McInerney *et al.*, 1991a; Chapter 4; Li *et al.*, unpublished data). This difference may reflect that a higher degree of variation exists between strains of *X. bovienii* than between strains of *X. nematophilus* with regard to the spectrum of antimicrobial substances.

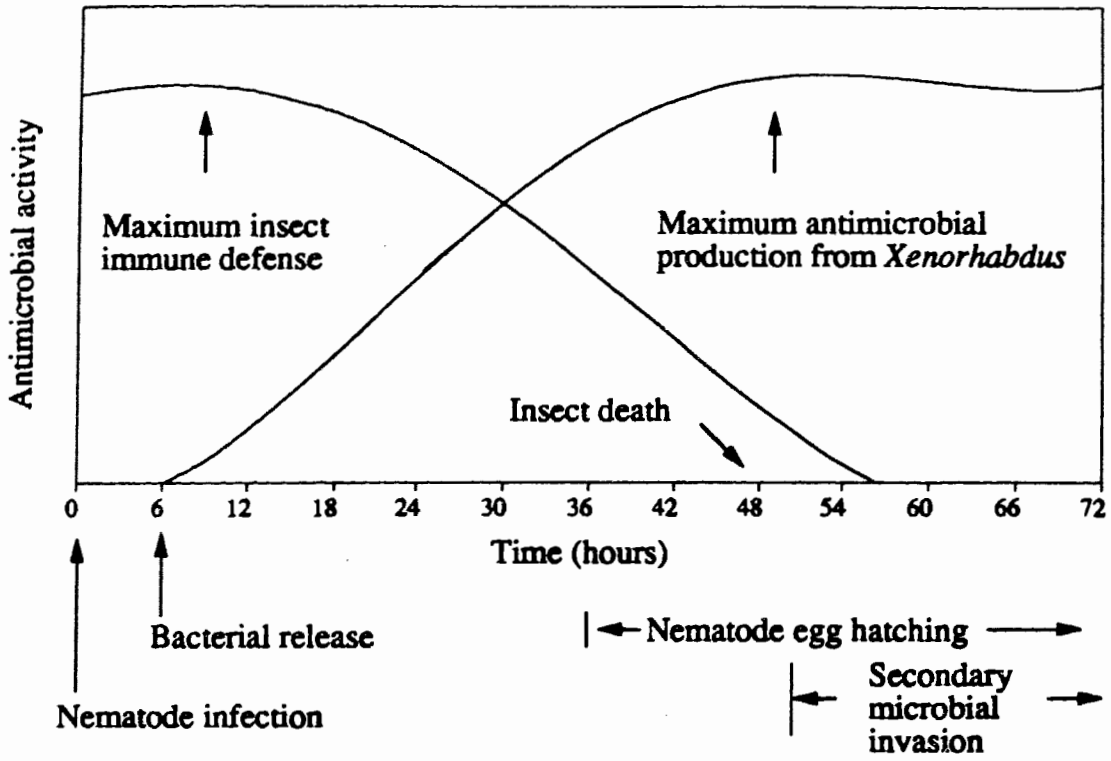
Although little is known about the efficacy of many of these antimicrobial substances in the symbiotic development of the nematodes and bacteria within an insect cadaver in nature, the *in vitro* efficacy of these compounds have led to speculation that they could be effective antibacterial and antimycotic agents in medicine, in agriculture, forestry or aquaculture. The fact that *Xenorhabdus* and *Photorhabdus* species have been found in only nematodes, nematode-infected insects and, in rare cases, from human wounds suggests that these bacteria have evolved in relative isolation from other micro-organisms. Consequently, the bioactive substances produced by these bacteria are less likely to be overcome by existing mechanisms of resistance. The novel nematophin and the xenorxides are of particular significance. The notable antibacterial activity of nematophin, especially its activity against drug-resistant human pathogens, such as *S. aureus*, suggests that it could be an exceptional candidate as an antibiotic and be a lead compound for potential chemotherapy agents. The activity of this compound is rather unusual in that it is active against *S. aureus*, but not active against the closely related *M. luteus* although it also has antimycotic activity. This specific activity against both prokaryotes and eukaryotes is unusual. While this selective activity may limit its range of application, it also provides a clue to its

probable novel mode of action. Further exploration of this compound in terms of its potential usefulness and mode of action deserve immediate consideration. The antimicrobial activity of xenorxides is probably similar to that of the structurally related compounds which have been shown to have high mammalian toxicity. The toxicity of xenorxides needs to be examined as soon as possible, and should they prove to be non-toxic, this group of compounds would offer themselves as exceptional antibiotic candidates and as lead compounds for medicinal and agroforestry applications.

Although the antimicrobial activity of these bacteria has been studied in recent years and several classes of compounds have been identified (Akhurst and Dunphy, 1993, Chapter 4), little is known of the protective mechanism of these antimicrobial substances or of their roles in the nematode reproduction. Nevertheless, this present study has provided additional information for a better understanding of the protective mechanism of the antimicrobial activity in the nematode/bacteria/insect complex. Based on information from the present study and the few earlier studies (Paul *et al.*, 1981; Richardson *et al.*, 1988; McInerney *et al.*, 1991 a, b) a model of the protective mechanisms is presented in Figure 17.

During the first several hours post-infection by the nematodes, the nematodes trigger the insect's immune system to eliminate all microorganisms introduced by the nematode at the time of insect penetration (Dunphy and Thurston, 1990). In nonimmune insects such as *G. mellonella*, about 6 h post-infection, the symbiotic bacteria are released from the nematode into the host hemocoel, and they multiply rapidly (Dunphy and Webster, 1985, 1988a). These symbionts apparently are somewhat resistant to the insect's immune system or are not recognized as nonself (Dunphy and Thurston, 1990). The insect host dies due to the rapid growth of the bacteria and nematode and to the toxins produced by them (Akhurst and Dunphy, 1993). Meanwhile

**Figure 17. Model summarizing the biological processes that occur when *Steinernema* spp. and their associated *Xenorhabdus* spp. infect a larval *Galleria mellonella*; in particular it shows the antimicrobial activity, nematode development, insect's immune system activity and other activities within the insect in the first 72 h post-infection.**


Antimicrobial activity
Maximum insect immune defense
Maximum antimicrobial production from *Xenorhabdus*
Insect death
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (hours)
Bacterial release
Nematode egg hatching
Secondary microbial invasion
Nematode infection

antimicrobial substances are produced by the bacteria, and these substances gradually compensate for the diminishing effectiveness of the insect defense system. The antimicrobial activity of these substances of bacterial origin gradually increases, and approaches a maximum, replacing the host's immune defenses against microorganisms between 30 to 48 h post-infection. The overall antimicrobial activity remains at about the same level thereafter. There is, over a period of about 48 h, a fundamental change in the antimicrobial arsenal from a mainly host immune system to the antimicrobial substances produced by the symbiotic bacteria parallels the development of the nematode from the infective juvenile to the second stage juvenile of the next generation of nematodes.

It appears that the antimicrobial protection in the insect host is needed for only a selected period of nematode development, namely, for approximately 72 h following the initial infection and, thereafter, for defense against certain organisms such as fungi. It has been observed in previous studies (Akhurst, 1982; Maxwell *et al.*, 1994) that the monoxenic conditions in the cadaver are usually maintained only temporarily, for about 48-72 h post-infection, and also that the cadaver is subsequently dominated by non-symbiotic bacteria as a result of the breakdown of the mid-gut of the insect. The antimicrobial substances maintain a modest infection level and so ensure a slow disruption of the integrity of the cadaver rather than totally eliminating the non-symbiotic organisms, because these nematodes are known to reproduce on non-symbiotic organisms (Poinar, 1990; Akhurst and Dunphy, 1993; Aguillera and Smart, 1993). For example, rapid destruction of the integrity of the cadaver by fungal penetration of the cadaver cuticle would destroy the environment within the cadaver and thereby would stop nematode development. This hypothesis is supported by the fact that a wide array of antimycotic compounds are produced by

all the bacterial symbionts of these nematodes to prevent the cadaver from fungal invasion. The overall antimycotic activity is achieved in different ways by different nematode species, depending on the species of bacterial symbiont. For example, in *X. bovienii* this antimycotic effect is achieved by producing several classes of agents including indoles, xenorhabdins, xenorxides, chitinase and the unknown protein. In *P. luminescens* , the antimycotic activity is apparently achieved by the stilbenes and the chitinases (Chapter 4 and 5). On the other hand, in *X. nematophilus* three classes of agents, namely, nematophin, xenocoumacins and the chitinases provide the antimycotic activity.

It appears that a simple antimycotic system such as that in *P. luminescens* is as effective as a complex system, such as that in *X. bovienii*. However, a complex system may offer more stable and reliable antimycotic protection to the insect cadaver than a simple one. The greater range of antimycotic substances may explain why it is generally easier to culture *Steinernema* spp. than *Heterorhabditis* spp. over long periods. The symbionts of *Steinernema* produce a more diverse spectrum of antimycotics than do those of *Heterorhabditis*. Consequently, the former would appear to offer more complete antimycotic protection for nematode reproduction. For example, a failure in the production of the stilbenes of *P. luminescens* would result in the loss of a major portion of the antimycotic arsenal whereas the absence of the indoles would remove only a small proportion of the overall antimycotic activity contributed by several classes of antimycotics. Destruction of the cadaver by microbial organisms would cause failure of the nematode reproduction.

In contrast to the antimycotic activity, the antibacterial activity required for nematode reproduction is a selective inhibition of certain bacterial species, probably, the Gram-positive

ones, rather than total elimination of non-symbiotic bacteria. This hypothesis is based on the fact that (1) all the antibacterial agents produced by *Xenorhabdus* spp. are active against Gram-positive but not Gram-negative bacteria; (2) these nematode entomopathogens can be successfully cultured on many non-symbiotic, Gram-negative bacteria (Ehlers, *et al.*, 1990; Poinar, 1990; Aguillera and Smart, 1993); and (3) the dominance of non-symbiotic bacteria in the insect cadaver 48-72 h post-infection (Maxwell, *et al.*, 1994; Akhurst, 1982). It appears that Gram-positive bacteria are not suitable for nematode reproduction, whereas Gram-negative bacteria (including *Xenorhabdus* and *Photorhabdus*) are generally satisfactory for the nematode reproduction. This is reflected in the fact that many Gram-negative bacteria have been successfully used for nematode reproduction, whereas no Gram-positive bacteria have been reported to be successfully used. It will be of interest to compare the monoxenic reproduction of these nematodes on Gram-positive and Gram-negative bacteria to determine what impact Gram-positive bacteria have on nematode reproduction.

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# APPENDIXES

**Appendix 1. Habitat and sources of test organisms**

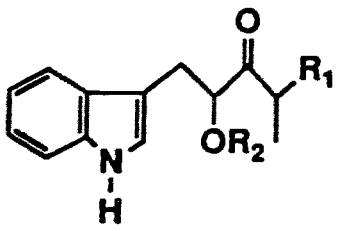
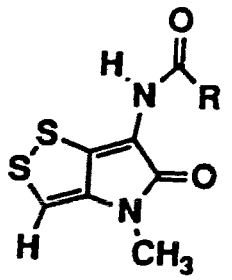
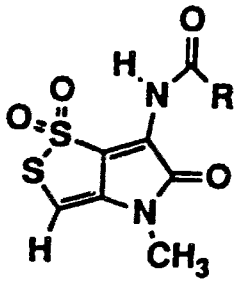
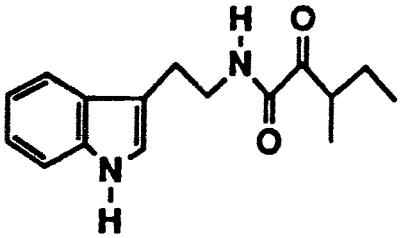
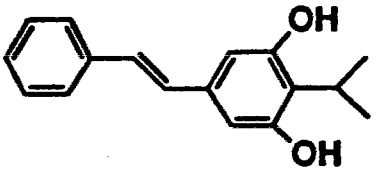
| Species and habitats | Organism | Sources* |
|---|---|---|
| ANIMAL PATHOGENS | | |
| *Aspergillus fumigatus* ATCC 13073 | fungus | MM |
| *A. flavus* ATCC 24133 | fungus | MM |
| *Candida tropicales* CBS94 | yeast | MM |
| *Cryptococcus neoformans* ATCC14117 | yeast | MM |
| *Escherichia coli* ATCC25922 | bacterium | ATCC |
| *Staphylococcus aureus* ATCC 29213 | bacterium | ATCC |
| *S. aureus* 0012 | bacterium | SM |
| *S. aureus* 0017 | bacterium | SM |
| FOREST | | |
| *Cephaloascus fragrans* | fungus | FT |
| *Ceratocystis dryocoetidis* | fungus | JB |
| *Ceratocystis ulmi* | fungus | MH |
| *Ophiostoma piceae* | fungus | FT |
| *Penicillium sp* | fungus | FT |
| *Trichoderma pseudokingii* | fungus | FT |
| INSECT PATHOGENS | | |
| *Beauveria bassiana* | fungus | GD |
| *Metarhizium anisopliae* | fungus | GD |
| MYCORRHIZAE | | |
| *Oidiodendron griseum* | fungus | SB |
| *Suillus pseudobrevipes* | fungus | SB |
| PLANT PATHOGENS | | |
| Foliage | | |

continues on next page

| Species and habitats | Organism | Sources* |
|---|---|---|
| *Alternaria* sp. isolate A | fungus | GD |
| *Alternaria* sp. isolate B | fungus | ZP |
| Postharvest | | |
| *Aspergillus niger* | fungus | GD |
| *Botrytis cinerea* | fungus | PS |
| *Geotrichum candidum* | fungus | GD |
| *Gloeosporium perannans* | fungus | PS |
| *Monilinia fructicola* | fungus | ATCC |
| *Mucor piriformis* | fungus | PS |
| *Penicillium expansum* | fungus | PS |
| *P. notatum* | fungus | PS |
| *Rhizopus stolonifer* | fungus | GD |
| *Venturia inaequalis* | fungus | PS |
| Soil born | | |
| *Fusarium oxysporum* | fungus | JR |
| *F. solani* | fungus | JR |
| *Phytophthora infestans* | fungus | ZP |
| *Pythium coloratum* | fungus | JR |
| *P. ultimum* | fungus | JR |
| *Rhizoctonia cerealis* | fungus | GD |
| *R. solani* | fungus | GD |
| *Sclerotinia minor* | fungus | ZP |
| *Thielaviopsis basicola* | fungus | ZP |
| *Verticillium albo-atrum* | fungus | JR |
| *V. dahliae* | fungus | JR |
| SOIL | | |
| *Bacillus subtilis* | bacterium | JR |

*ATC: American type culture collection (ATCC), Rockville Maryland, USA; FT: Forintek Canada Corp. Ottawa, Canada; GD: Dr. G. B. Dunphy, Department of Natural Resource Sciences, McGill University, Montreal; Jʙ: Dr. J. H. Borden, Department of Biological Sciences, Simon Fraser University, Burnaby; JR: Dr. J. E. Rahe, Department of Biological Sciences, Simon Fraser University, Burnaby; MH: Dr. M. Hubbes, Department of Forestry, University of Toronto, Toronto.; MM: Dr. M. M. Moore, Department of Biological Sciences, Simon Fraser University, Burnaby; PS: Dr. P. Shelberg, Agriculture Canada Summerland; SB: Dr. S. Birch, Department of Soil Science, University of British Columbia, Vancouver; SM: S. Farmer, Canadian Bacterial Diseases network, Vancouver; ZP: Dr. Z. Punja, Department of Biological Sciences, Simon Fraser University, Burnaby, BC.

**Appendix 2.** The chemical structure and the bioactivity of antimicrobial substances isolated from *Xenorhabdus* and *Photorhabdus* species.

| Bacterial sources | Chemicals produced | Code | Activity* | |
|---|---|---|---|---|
| | | | 1 | 2 |
| *X. bovienii* strain A2 |  ID1:$R_1$=CH3; $R_2$=Ac | ID1 | + | + |
| | ID3:$R_1$=CH3; $R_2$=H | ID2 | + | + |
| | ID2:$R_1$=CH2CH3; $R_2$=Ac | ID3 | + | + |
| | ID4: $R_1$=CH2CH3; $R_2$=H | ID4 | + | + |
| |  XR1: R=$(CH_2)_3CH_2CH_3$ | XR1 | nt | nt |
| | XR2:R=$(CH_2)_3CH(CH_3)_2$ | XR2 | nt | nt |
| | XR3:R= $CH_2CH(CH_3)_2$ | XR3 | nt | nt |
| | XR4:R=$CH_2CH_2CH_3$ | XR4 | nt | nt |
| |  XO1: R=$(CH_2)_3CH_2CH_3$ | XO1 | + | + |
| | XO2:R=$(CH_2)_3CH(CH_3)_2$ | XO2 | + | + |
| *X. nematophilus* strain BC1 |  | NID | + | + |
| *P. luminescens* strain C9 |  | STB | + | + |

* 1= antibacterial; 2 = antimycotic, nt= not tested.