THERMAL ADAPTATION IN Xenorhabdus SPP., BACTERIAL SYMBIONTS OF ENTOMOPATHOGENIC NEMATODES, Steinernema SPP.

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Thermal Adaptation in *Xenorhabdus* spp., Bacterial Symbionts of Entomopathogenic Nematodes, *Steinernema* spp.

By

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Abstract

Physiological mechanisms of adaptation to temperature were investigated in four strains of *Xenorhabdus* spp. that originated from various geographical areas: *Xenorhabdus bovienii* NF strain and *Xenorhabdus bovienii* Umeå strain (boreal origin), *Xenorhabdus nematophilus* All strain (temperate origin) and *Xenorhabdus* sp. TX strain (subtropical origin). The criteria included the effect of temperature on growth, capacity to synthesize isozymes of metabolic enzymes and modify fatty acids. In addition, the TX strain (undescribed) and the NF strain (newly isolated) were characterized through physiological and biochemical tests and cellulose acetate electrophoresis was evaluated for use in the taxonomy of this category of bacteria.

The isozymes of nine enzymes were separated by cellulose acetate electrophoresis and compared among the four bacterial strains. The results indicated that these strains could be distinguished from one another on the basis of isozyme patterns at 25^{0}C. Four enzymes [fumarate hydratase (FUM), malate dehydrogenase *(NAD)* (MDH), malate dehydrogenase *($NADP^+$)* (ME), and phosphoglucomutase (PGM)] displayed species-specific isozyme patterns, and the isozyme patterns of arginine phosphokinase (APK) distinguished between the NF strain and the Umeå strain. Additionally, the isozyme patterns in the NF and Umeå strains were temporally stable for all enzymes, except ME (Umeå strain) and IDH (NF strain). These findings suggested that cellulose acetate electrophoresis could be an important tool for the identification of *Xenorhabdus* species or even strains.

Xenorhabdus sp. TX strain was physiologically and biochemically distinguishable

from the five described *Xenorhabdus* species and from the related bacterium, *Photorhabdus luminescens*. The TX strain differed from any of the five described *Xenorhabdus* species or *P. luminescens* in at least one of the following characteristics: growth at 10^0C (-), growth at 37^0C (+), catalase (-), bioluminescence (-), absorption of bromothymol blue dye (+), lipase (-), urease (-), phosphatase (-), alkaline phosphatase (w: weak), ribose acidification (-), glycerol acidification (+), salicin acidification (-), cefalothin resistance (-), amoxilline & calvulanic acid resistance (-), and esculin hydrolysis (-). The NF and Umeå strains displayed identical reactions for all the tests in this study, indicating that they were inseparable on the basis of common physiological and biochemical tests.

Growth of the four *Xenorhabdus* strains was examined over a wide range of temperatures. The boreal strains (NF, Umeå) grew at culture temperatures from 0^0C to 32^0C, the All strain from 10^0C to 32^0C, and the TX strain from 15^0C to 38.5^0C. The optimal temperature, based on growth rates, was 25^0C for the two boreal strains, 30^0C for the All strain and the TX strain. The boreal strains (NF, Umeå) were categorized as psychrotrophs, and the All and TX strains as mesophiles.

The effect of culture temperature on the isozymes of seven enzymes was studied in the four *Xenorhabdus* strains. All four strains displayed temperature-related variations in isozyme patterns. Five enzymes displayed temperature related modifications in isozyme banding patterns in the Umeå strain, four in the NF strain, three in the All strain and two in the TX strain. These results indicated that these bacteria may physiologically adapt to temperature by altering the synthesis of isozymes.

All four strains responded to low temperatures by increasing monounsaturated fatty acids (16:1$_{\omega}$7 and 18:1$_{\omega}$9) with concomitant decreases in the prominent saturated fatty acid (16:0), indicating that these bacteria could adapt to temperature by modifying the degree of fatty acid unsaturation. Other fatty acids (14:0, 17:0, 17:0$_c$, 20:0), present in lower amounts, were affected by temperature in three strains (All, NF, Umeå) while they did not significantly vary from 20°C to 35°C in the TX strain. This study suggested that cold adaptation in *X. bovienii* may involve shifts in fatty acids composition induced by temperatures well above freezing.

This thesis is dedicated to the memory of my father who was the continuous source of inspiration for me in every walk of life.

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Chapter 1

General introduction

1.1. Entomopathogenic nematodes as biological control agents

Widespread applications of chemicals in insect pest management has resulted in the acquisition of resistance to chemical pesticides among pest populations with consequent resurgence of pests, pesticide residues in the food-chain, and environmental pollution. Biological control is one of the most attractive alternatives for chemical control in pest management (Ehlers and Hokkanen, 1996; Metcalf and Luckmann, 1994). Insect-parasitic nematodes are among the most important biological control agents (Ehlers, 1996; Kaya *et al.*, 1993). Nematodes from three families (Mermithidae, Steinernematidae and Heterorhabditidae) have been viewed as potentially effective biocontrol agents (Bedding *et al.*, 1993; Gaugler and Kaya, 1990). However, because of the difficulties in their artificial culture and storage, mermithids are no longer viewed as potential biocontrol agents in the foreseeable future (Kaya *et al.*, 1993). Current research is on steinernematids and heterorhabditids, both of which are entomopathogenic nematodes because they carry symbiotic bacteria. These nematodes can be artificially mass-produced, easily stored and transported (Bathon, 1996; Bedding *et al.*, 1993, Gaugler and Kaya, 1990). Some of these nematodes are being commercially produced or developed as bioinsecticides (Smart, 1995).

1.2. Entomopathogenic nematodes and their mutualistic bacteria

Non-feeding infective juveniles (J_3, dauer larvae) of steinernematids and heterorhabditids carry, in their intestines, species-specific bacterial symbionts belonging to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Akhurst and Boemare, 1990). These infective juveniles enter the insects' hemocoel via the mouth, anus, the respiratory spiracles or by directly penetrating the insects' cuticle (Poinar, 1990; Bedding and Molyneux, 1982). Once the nematodes are in the hemocoel of the insect hosts, they release their symbiotic bacteria. These bacteria grow and reproduce in the hemocoel, then produce toxins to kill the host, and antibiotics to prevent other microbes from growing in the hemocoel. This creates a non-competitive environment for the growth and reproduction of the bacteria and their nematode hosts (Poinar, 1990). After proliferating for 2-3 generations, infective nematode juveniles carrying bacterial symbionts in their intestines emerge from the insect cadavers, and enter the soil in search of new insect hosts (Kaya and Gaugler, 1993).

In these mutualistic associations, the nematodes transport their bacterial symbionts between insect hosts, protect bacteria from the soil environment in which these bacteria are not competitive with other microbes, and carry bacteria into the hemocoel of insect hosts (Akhurst, 1993). On the other hand, the bacteria produce toxins to kill the insects, release antibiotics to prevent insect cadavers from putrefaction, and modify the cadavers to provide suitable nutrients for the reproduction of nematodes (Akhurst, 1982, 1993; Forst and Nealson, 1996).

1.3. Bacterial associates

1.3.1. Taxonomy of *Xenorhabdus* and *Photorhabdus*

The genus *Xenorhabdus*, formerly *Achromobacter* (Poinar and Thomas, 1965), was initially proposed by Thomas and Poinar (1979), with two species described: *Xenorhabdus nematophilus* from nematodes of the genus *Steinernema* (syn. *Neoaplectana*) and *Xenorhabdus luminescens* from nematodes of the genus *Heterorhabditis* (syn. *Chromonema*). Akhurst(1983) suggested three sub-species (subsp. *nematophilus*, subsp. *bovienii*, and subsp. *poinarii*) for the species *X. nematophilus*. Several years later, another sub-species (subsp. *beddingii*) was proposed for the same species (Akhurst, 1986a). Based on the results of numerical taxonomic studies of 240 characters in 21 strains, the four sub-species (*X. nematophilus* subsp. *nematophilus*, subsp. *bovienii*, subsp. *poinarii*, and subsp. *beddingii*) were suggested as four species (*X. nematophilus*, *X. bovienii*, *X. poinarii* and *X. beddingii*, respectively) (Akhurst and Boemare, 1988). Using DNA-DNA hybridization, *Xenorhabdus* spp. were separated from each other and a new genus, *Photorhabdus*, was proposed, with *X. luminescens* renamed as *Photorhabdus luminescens* (Boemare *et al.* 1993). Nishimura *et al.* (1994) suggested a new *Xenorhabdus* species (*Xenorhabdus japonicus*). To date, five species of bacteria have been described in the genus *Xenorhabdus* (*Xenorhabdus nematophilus*, *Xenorhabdus bovienii*, *Xenorhabdus poinarii*, *Xenorhabdus beddingii*, and *Xenorhabdus japonicus*) and one species for *Photorhabdus* (*Photorhabdus luminescens*).

Morphological, physiological and biochemical characteristics are widely used in the taxonomy of the bacterial associates of entomopathogenic nematodes (Akhurst, 1983, 1986a; Boemare and Akhurst, 1988; Nishimura *et al.*, 1994). Modern molecular approaches have also been utilized to classify these bacteria. DNA-DNA hybridization was used to analyze the genomic differences among these bacterial associates; the four *Xenorhabdus* species (i.e. not including *X. japonicus*) were found to be different from one another, and *X. luminescens* was transferred into a new genus, *Photorhabdus* (Boemare *et al.*, 1993). 16S rRNA genes were found to be useful in analyzing the phylogeny of *Photorhabdus* and *Xenorhabdus* species, and also in identifying these bacteria. By comparing the 16S rRNA gene sequences of 16 strains of *Xenorhabdus* or *Photorhabdus*, Liu *et al.* (1997) confirmed the validity of the species *X. bovienii*, *X. nematophilus*, and *X. poinarii*. These authors also found at least two distinct groups in the *Photorhabdus* isolates that they studied. These groups may represent distinct species, a finding that is at odds with the current view that only one species has been reported for this genus. Szállás *et al.* (1997) sequenced the 16S rRNA genes of 47 strains (40 from *Photorhabdus*, and 7 from *Xenorhabdus*). In this study, several subclusters were also found in *Photorhabdus* strains, indicating the existence of several species within *P. luminescens*. By analyzing 16S rRNA restriction patterns of 27 strains (14 *Photorhabdus* strains and 13 *Xenorhabdus* strains), Brunel *et al.* (1997) proved that amplified 16S rRNA restriction analysis was a simple tool for the fast and accurate identification of the bacterial associates of entomopathogenic nematodes.

Fatty acid profiles were also utilized in the taxonomy of *Xenorhabdus* and *Photorhabdus* species. Janse and Smits (1990) analyzed the fatty acid profiles of 33 strains (26 *Photorhabdus* strains and 7 *Xenorhabdus* strains), found that there were three subgroups in *X.* (*P.*) *luminescens*, and suggested that it is questionable to place *X.* (*P.*) *luminescens* in the Family Enterobacteriaceae because of the existence of branched and hydroxy fatty acids. Suzuki *et al.* (1990) also used fatty acid compositions to classify these bacterial associates.

Protein electrophoresis has proved to be a useful taxonomic tool for nematode species and even strains, and also for the bacterial associates of entomopathogenic nematodes. Starch gel (Akhurst, 1987), polyacrylamide gel (Sha, 1985; Kozodoi *et al.*, 1986) and cellulose acetate gel (Jagdale *et al.*, 1996) electrophoreses were applied in nematode taxonomy. Hotchkin and Kaya (1984) used acrylamide gel to examine the total protein profiles and some isozyme patterns in *X. nematophilus* and *X.* (*P.*) *luminescens*, and found that this technique could be used to separate the two species from each other, and also to distinguish among the subspecies of *X. nematophilus*, which were later elevated as species by Boemare and Akhurst (1988).

In my research, *Xenorhabdus* sp. TX strain and *Xenorhabdus bovienii* NF strain, which were isolated, respectively, from the nematodes *Steinernema riobravis* TX strain and *Steinernema feltiae* NF strain, were characterized with traditional physiological and biochemical tests. In addition, cellulose acetate electrophoresis was evaluated for its

possible use in the taxonomy of bacterial species associated with entomopathogenic nematodes by examining isozyme patterns.

1.3.2. Biology

1.3.2.1. Pathogenicity

Usually, either bacteria or nematodes alone are pathogenic to *Galleria mellonella*, whose larvae (last instar) have been widely used to determine the pathogenicity of the bacterial symbionts and their nematode hosts (Forst *et al.*, 1997). However, neither *X. poinarii* nor *S. glaseri* alone displays pathogenicity to *Galleria mellonella* larvae, while these larvae are very sensitive to this bacterium/nematode complex (Akhurst, 1986b). *Xenorhabdus japonicus* alone also shows no pathogenicity to *Spodoptera litura* larvae (Yamanaka *et al.*, 1992). The virulence of the bacteria has been largely ascribed to their endotoxins and exotoxins (Akhurst and Dunphy, 1993).

The endotoxin, lipopolysaccharide (LPS), of *X. nematophilus* is involved in bacterial evasion from the insect immune system and is also considered to be a virulence factor. The lipopolysaccharide is able to prevent phenoloxidase from activation (Dunphy and Webster, 1988). Activated phenoloxidase converts tyrosine to dihydroxyphenylalanine, which binds to the bacterial cell surfaces and probably improves the bacterial adherence to insect hemocytes. Therefore, *Xenorhabdus* species may tolerate or evade humoral defensive response by inhibiting the activation of insect phenoloxidase (Forst *et al.*, 1997). Lipopolysaccharide is also viewed as a virulence factor in *X. nematophilus*, because injecting the LPS purified from this bacterium results in the death of *G.*

mellonella larvae. Actually, the lipid A moiety of LPS in *X. nematophilus* was thought to stimulate hemocyte lysis (Forst *et al.*, 1997). However, the roles of LPS from *P. luminescens* in pathogenicity are different from those in *X. nematophilus*. Despite the fact that the LPS of *P. luminescens* was shown to damage the hemocyte of *G. mellonella* (Dunphy and Webster, 1988), either its purified LPS or dead bacterial cells were shown to be toxic to the same insect species (Clarke and Dowds, 1995).

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* produce exotoxins such as protease, lipase, lecithinase and extracellular enzymes, although not all of these toxins have been shown to be toxic to insects. Two insecticidal exotoxins, a 40-kDa protein from *X.(P.) luminescens* strain NC-19 (Ensign *et al.*,1990) and a 31 kDa protein from *X. nematophilus* (Boemare *et al.*, 1997), have been isolated. Clarke and Dowds (1995) showed that the sterile extracellular culture media of *E. coli* containing the lipase gene of *P. luminescens* strain K122 were toxic to *G. mellonella* while the preparations of *E. coli* containing no K122 lipase gene were safe to the same insects. These authors suggested that the lipase activity in the K122 strain was a virulence factor towards *G. mellonella*.

1.3.2.2. Specificity

Specificity exists in associations between entomopathogenic nematodes and their bacterial associates. A given nematode species is naturally associated with only one bacterial species, although a bacterial species may act as the symbiont in several nematode species (Akhurst, 1993; Akhurst and Dunphy, 1993). The specificity of association between nematode and bacterium has been proposed to operate at three levels: nutrient provision by

bacteria for nematodes, bacterial retention within the intestines of infective juveniles of nematodes (Akhurst and Boemare, 1990), and development of infective juveniles (the third stage, also 'dauer' stage) into the fourth stage (Grewal *et al.*, 1997).

Some degree of specificity exists with respect to nutrient provision by symbiotic bacteria. Some nematodes were found to be capable of growing and reproducing with the aid of bacterial species (including non-*Xenorhabdus* bacteria) other than their natural symbionts. However, natural symbionts are usually the best nutrient providers for the growth and reproduction of their nematode hosts (Akhurst and Boemare, 1990; Poinar, 1990).

A higher degree of specificity was proposed with respect to the capacities of infective juveniles of entomopathogenic nematodes for retaining bacteria in their intestines (Akhurst and Boemare, 1990). No species of *Steinernema* has been shown to retain any bacterial species other than *Xenorhabdus* bacteria, and some nematode species such as *S. carpocapsae* can only retain its natural symbiont. Although the infective juveniles of *S. feltiae* may be experimentally induced to carry the symbionts of other *Steinernema* species, the efficiency is lower than that of retaining its natural symbiont (Akhurst and Boemare, 1990).

Symbiont-specific dauer recovery was recently proposed as a new mechanism for the specificity of associations between nematodes and their bacterial symbionts (Grewal *et al.*, 1997). Dauer recovery involves the ability of the nematodes to develop from the dauer stage to the fourth juvenile stage and includes the exsheathment of the nematode and subsequent

release of the bacteria into the host's hemolymph. *Steinernema scapterisci* was found to have only a weak specificity of association with its symbiotic bacterium at the levels of nutrient provision and of bacterial retention. However, this nematode species displayed a strong dependence on its natural bacterial symbiont for efficient dauer recovery. The dauer recovery of this nematode species was found to be significantly delayed and reduced in the monoxenic cultures of *Xenorhabdus nematophilus* and *Xenorhabdus* sp., symbionts of *Steinernema carpocapsae* and *Steinernema riobravis*, respectively. Cell-free filtrates of the cultures of the natural symbiont improved the dauer recovery of *S. scapterisci* in the monoxenic cultures of *Xenorhabdus nematophilus* and *Xenorhabdus* sp. TX strain, indicating that infective juveniles use chemical signals produced by their natural symbionts for dauer recovery. Grewal *et al.* (1997) suggested that it was unlikely for infective juveniles to start feeding in the hosts previously infected by other *Steinernema* nematodes, mainly because of the existence of improper bacteria.

1.3.3.3. Phase variation

In order to evade host immune responses or bacteriophage infection, bacteria commonly undergo a reversible change in a major antigen such as flagellar or fimbrial protein. This common phenomenon is defined as phase variation. The phase variation occurring in *Xenorhabdus* and *Photorhabdus* is unusual in that it involves alterations in multiple characters, which are not related to each other and also not involved in evading host immune defense and bacteriophage infection. *Xenorhabdus* and *Photorhabdus* usually display two phases. Phase one was isolated from nematode infective juveniles while phase

two was obtained from *in vitro* cultures of phase one. Phase one cells absorb dyes (bromothymol blue and Congo red), produce antibiotics, pigmentation or luminescence (in the case of *Photorhabdus*) while phase two cells lack these characteristics or show much weaker expressions for some of these characters (Akhurst, 1993).

Phase one usually produces a wide range of antibiotics, and provides better nutrient factors for the reproduction of its nematode associates (Akhurst and Boemare, 1990). Smigielski *et al.* (1994) reported that phase two cells of *X. nematophilus* and *P. luminescens* restarted growth within 2 to 4 hours after starvation while phase one cells required 14 hours. Phase two cells also displayed much higher activity of major respiratory enzymes in stationary cultures (short of nutrients). These authors proposed that phase two cells were better adapted to soil environments. Krasomil-Osterfeld (1997) found that low osmolarity induced the trasformation of phase one to phase two, and noted that high osmolarity (400 mOsmol) exists in insect cadavers while low osmolarity (60 mOsmol) is displayed in the intestines of dauer larvae and in the soil environment. It was proposed that phase two is better adapted to the starvation conditions in the intestines of non-feeding infective nematode juveniles, while phase one is more suitable to the nutrient ambience of the insect hemocoel (Krasomil-Osterfeld, 1997; Forst *et al.*, 1997).

The mechanisms for phase variation in *Xenorhabdus* and *Photorhabdus* are not understood. It was shown that the phase variation was not mediated by plasmids (Couche *et al.*, 1987; Smigielski and Akhurst, 1994). Major genomic vatriations are also unlikely to be involved in the phase variation (Akhurst *et al.*, 1992).

1.3.3. Physiology of thermal growth and adaptation to temperature

Temperature has been found to be a factor limiting the successful applications of entomopathogenic nematodes in pest management (Griffin, 1993). This limitation has led to many investigations into the effect of temperature on various aspects of the biology (e.g. infectivity, reproduction, temperature tolerance, enzymatic activity, fatty acid profiles, and isozyme profiles) of these nematodes (Grewal *et al.*, 1994; Jagdale and Gordon, 1997a, 1997b, 1997c, 1998a, 1998b; Mason and Hominick, 1995). Given that bacterial symbionts are the key contributors to the pathogenicity of these nematodes, the effect of temperature on bacterial growth and the nature of the thermal adaptation mechanisms of these bacteria have to be taken into consideration in developing these nematodes as biocontrol agents.

Very limited studies have been done on the temperature conditions pertaining to growth of these bacteria. From 24°C to 30°C, the generation times for *X. nematophilus* and *P. luminescens* were 1-2 hours in complex media 2.5-3.0 hours in defined media (Nealson *et al.*, 1990). Gwynn and Richardson (1994) examined the growth of five *Xenorhabdus* isolates at low temperatures (2, 6 & 10°C) and found that two isolates of *X. bovienii* could grow well at 2°C. This research was done only from 2°C to 10°C. In my research, a wide range of temperatures were examined for their influence on the growth of four *Xenorhabdus* isolates with the geographical origins ranging from boreal, temperate and subtropical areas.

The mechanisms for thermal adaptation of *Xenorhabdus* and *Photorhbadus* are not well understood. Several previous studies examined temperature related changes in fatty acids in several isolates of these bacteria. *Xenorhabdus nematophilus*, under identical

culture conditions, had a less ordered (i. e. more fluid) membrane structure than *P. luminescens* due to the differences in their fatty acid profiles (Fodor *et al.*, 1997). This may explain why bacteria of the genus *Xenorhabdus* are more cold-adapted than those of the genus *Photorhabdus* (Clarke and Dowds, 1994). *Xenorhabdus* sp. TX strain, initially isolated from *S. riobravis*, was shown to increase saturated fatty acids and decrease in unsaturated fatty acids as the temperature increase from 15°C to 30°C (Abu Hatab and Gaugler, 1997a). The existence of relatively high levels of branched fatty acids in *Photorhabdus* bacteria was considered to be the result of their adaptation to low temperature (Clarke and Dowds, 1994). To understand the membrane adaptation of these bacteria, wider ranges of temperatures and of bacterial isolates should be studied with respect to fatty acid profiles. Other aspects of thermal adaptation in these bacteria should also be examined.

According to current publications, thermal adaptation in other bacteria has been shown to involve fatty acid profiles (Suutari and Laakso, 1994), enzymes (Feller *et al.*, 1996), special proteins (e.g. cold shock proteins) (Gumley and Inniss, 1996), and phosphorylation of lipopolysaccharides(Ray *et al.*, 1994).

1.4. Research objectives

To develop entomopathogenic nematodes for effective pest management, it is important to obtain a thorough understanding of the capacities of both the nematodes and their bacterial associates for temperature adaptation and the nature of the physiological

mechanisms deployed. For this reason, I examined the effect of temperature on the growth of four strains of *Xenorhabdus*, which associate with nematodes originating from climatically diverse geographic regions. Additionally, physiological compensatory changes were examined with respect to changing temperature. The four strains of bacteria were: (i) *X. bovienii* NF and Umeå strains, boreally adapted, originating from *S. feltiae* populations in Newfoundland, Canada (Jagdale *et al.*, 1996), and Umeå, Sweden (Pye & Pye, 1985), respectively; (ii) *X. nematophilus* All strain, temperate, isolated from commercially mass cultured *S. carpocapsae*, initially from Georgia, USA (Poinar, 1979); (iii) an unidentified strain of *Xenorhabdus*, subtropical, isolated from *S. riobravis*, a nematode endemic to southern Texas (Cabanillas *et al.*, 1994).

The NF strain of *S. feltiae* has a similar, but not identical isozyme profile to a strain (L1C) that was isolated from Newfoundland on an earlier occasion (Finney-Crawley, 1985). The NF and L1C strains are legitimately considered to be separate strains of *S. feltiae* (Jagdale *et al.*, 1996). The bacterial associates of *S. feltiae* NF strain and the subtropical *S. riobravis* have not been described at all. Therefore, it seems important to include taxonomic and descriptive components to my temperature studies. Accordingly, opportunity was taken to evaluate the use of cellulose acetate electrophoresis for the taxonomic separation of the bacteria. Also, bacterial isolates were characterized using traditional physiological and biochemical tests.

Specifically, the research consisted of:

A. Taxonomy

(a) evaluation of the use of cellulose acetate electrophoresis in the taxonomy of *Xenorhabdus* bacteria

(b) characterization of *Xenorhabdus* sp. TX strain and *X. bovienii* NF strain

B. Physiology

(a) study of the effect of temperature on the growth of four *Xenorhabdus* strains, using a wider range of temperatures than previously employed.

(b) examination of the effect of temperature on isozyme patterns of several metabolic enzymes in *Xenorhabdus* bacteria

(c) investigation of the influence of temperature on fatty acid profiles in *Xenorhabdus* bacteria

Chapter 2

Utilization of cellulose acetate electrophoresis in the taxonomy of *Xenorhabdus* spp.

2.1. Abstract

Cellulose acetate electrophoresis was used to separate the isozymes of nine enzymes in three *Xenorhabdus* species, representing four strains: *Xenorhabdus bovienii* NF strain, *Xenorhabdus bovienii* Umeå strain, *Xenorhabdus nematophilus* All strain, and *Xenorhabdus* sp. TX strain. On the basis of isozyme patterns, the four strains could be distinguished from one another. The isozyme patterns of four enzymes [fumarate hydratase, malate dehydrogenase (NAD^-), malate dehydrogenase ($NADP^-$), phosphoglucomutase] were species-specific. The NF and Umeå strain of *X. bovienii* were separable on the basis of their isozyme patterns of arginine phosphokinase. In addition, the isozyme patterns were found to be temporally stable for all enzymes in the NF and Umeå strains, except ME (Umeå strain) and IDH (NF strain). These findings indicated that cellulose acetate electrophoresis could be a useful tool for the identification of *Xenorhabdus* species or even strains.

2.2. Introduction

Xenorhabdus spp. are mutualistic bacteria which are carried in the intestine of entomopathogenic nematodes, *Steinernema* spp. (Bird and Akhurst, 1983). Since the

early 1980's, these bacterium-nematode complexes have been under development as insect biocontrol agents (Kaya *et al.*, 1993; Akhurst and Boemare, 1990; Boemare *et al.*, 1997; Poinar, 1990). Bacteria play a vital role in the pathogenicity and reproduction of these complexes. They produce toxins to kill insect hosts (Akhurst, 1982, 1993; Kaya and Gaugler 1993) and provide nutrients for nematode reproduction (Akhurst and Dunphy, 1993; Poinar, 1990; Forst and Nealson, 1996; Forst *et al.*, 1997).

Biochemical approaches have been successfully utilized for the taxonomy of *Xenorhabdus* spp. and *Photorhabdus* spp., bacterial symbionts of *Steinernema* and *Heterorhabditis* nematodes, respectively. By restriction analysis of PCR-amplified 16S rRNA genes, 27 strains of *Xenorhabdus* and *Photorhabdus* from a wide range of geographical and nematode host sources were found to be comprised of 17 genotypes. The 27 strains were divisible into two heterogeneous clusters, one including all the 13 *Xenorhabdus* strains and the other including all the 14 *Photorhabdus* strains (Brunel *et al.*, 1997). Comparison of partial 16S rRNA gene sequences was used to determine the phylogeny of *Xenorhabdus* and *Photorhabdus* species (Rainey *et al.*, 1995; Liu *et al.*, 1997). By means of DNA-DNA hybridization, DNA relatedness between *Xenorhabdus* species was studied, and consequently a new genus, *Photorhabdus*, was proposed for bacterial associates of *Heterorhabditis* nematodes (Boemare *et al.*, 1993).

It is possible that physiological criteria other than DNA profiles could also be used in the taxonomy of these bacteria. For example, Janse and Smits (1990), after examining the fatty acid patterns of *X. nematophilus* and *X. (P.) luminescens*, concluded that the two

groups are significantly different from each other, and proposed that they might comprise different genera.

Protein electrophoresis has been widely used for the separation of nematode species and even their strains. Akhurst (1987) used starch gel electrophoresis to separate isozymes of *Heterorhabditis* isolates, and considered it as a useful taxonomic tool. Polyacylamide gel electrophoresis was also used in the taxonomy of entomopathogenic nematodes (Sha, 1985; Kozodoi *et al.*, 1986). Cellulose acetate electrophoresis was recently advanced as a potentially useful procedure for the taxonomy of steinernematid nematodes (Jagdale *et al.*, 1996). This research is intended to evaluate the determinative significance of cellulose acetate electrophoresis in the taxonomy of bacterial associates of steinernematids by the analysis of isozyme patterns of nine enzymes.

2.3. Materials and Methods

2.3.1. Sources of bacteria and nematodes: *Xenorhabdus nematophilus* All strain was isolated from *Steinernema carpocapsae* All strain, which was provided originally by Plant Products Ltd., Brampton, Ontario, Canada. *Xenorhabdus bovienii* NF strain was isolated from *S. feltiae* NF strain, which was isolated from soil in an organic garden near St. John's, Newfoundland, Canada (Jagdale *et al.* 1996). *Xenorhabdus bovienii* Umeå was isolated from *S. feltiae* Umeå strain, which was provided originally by Dr. R. West, Canadian Forest Service (CFS), St. John's, Newfoundland, Canada, from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA.

Xenorhabdus sp. TX strain was isolated from *S. riobravis* TX strain, originally provided by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, Texas. All the nematode isolates were recycled through *Galleria mellonella* larvae at 20°C (Woodring and Kaya, 1988) for 26 months before being used for bacterial isolation.

2.3.2. Bacterial isolation, identification, and subculture: Bacteria were isolated from *G. mellonella* larvae that had been infected by nematodes, based on the procedure described by Woodring and Kaya (1988) . Hemolymph of insect larvae that had been infected 24 hours previously was streaked onto NBTA plates (nutrient agar with bromothymol blue dye and triphenoltetrazolium chloride). Bacteria were purified by subculturing three consecutive times on NBTA plates at 25°C. The purified bacteria were cultured on plates of BUGM medium (Cat.# 70001, Biolog, Inc., Hayward, CA) twice at 25°C, then suspended in sterile saline and transferred into each well of Biolog GN (Gram negative) microplates. After 24-48 hours' incubation at 25°C, a Biolog Microstation System™ was utilized to identify the bacteria (Biolog, Hayward, CA). Wells were read using an automated plate reader.

Following isolation and identification of the bacteria, *X. bovienii* NF and Umeå strains were maintained on NBTA plates at 5°C, *X. nematophilus* All strain at 10°C, and *X.* sp. TX strain at 15°C with a subculture interval of approximately one month. They had been maintained for about one year by the time that they were cultured for isozyme extraction. Each of the four strains was checked periodically for purity by using the Biolog Microstation System™ and just before sample preparation for enzyme analysis.

2.3.3. Preparation of bacterial culture and enzyme extraction:

The bacteria in their primary phase on NBTA plates (primary phase: blue colony; secondary phase: red or pink colony) were used to inoculate 250-ml tryptic soy broth (TSB) (DIFCO, Detroit, MI) in a 500-ml Erlenmeyer flask with a cap. They were cultured at 25°C with shaking at 100 rpm. The bacteria were harvested at the log phase (on the basis of optical density of the culture) of their growth by centrifugation at 14,740 g at 4°C, and washed three times with 0.15M NaCl. Bacterial culture from two flasks was combined into one sample, and for each strain there were three samples (n=3) from six flasks. In total, 24 flasks were utilized in this study. Harvested bacteria were kept at -20°C for 1~2 days. Each sample was resuspended in 3-4 ml buffer (0.09M Tris HCl, pH 9.00). To avoid enzyme degradation, the test tube containing the bacterial suspension was put into ice while the bacterial cell walls were broken using a Braun-Sonic 2000 sonicator. The resultant suspension was centrifuged at 24,790 g at 2°C for 20 minutes. The enzyme extracts (supernatant) were transferred into 1.5-ml polypropylene microcentrifuge tubes and immediately frozen at -70°C until electrophoresis was carried out.

2.3.4. Cellulose acetate electrophoresis: Cellulose acetate plates (Cat.# 3033, Helena Laboratories, Beaumont, TX) were pre-soaked for at least 20 minutes with Tris-Glycine buffer (3.0g Tris, 14.4g glycine, 1L distilled water, pH 8.5) before samples were loaded onto them. A 10-µl aliquot from each sample was placed in a well in a sample holder and maintained on ice. Then, 0.2~0.4 µl of the sample was spotted, based on the enzyme

sensitivity to staining, onto Titan III Zip Zone Cellulose Acetate Plates with a Super Z Applicator (Helena Laboratories, Beaumont, TX). Next, the plates were laid in a horizontal electrophoresis chamber containing Tris-Glycine buffer. Electrophoresis lasted for 14~20 minutes at room temperature (20~25^{0}C) with 1.5 mA current per plate.

2.3.5. Enzyme staining: The procedure of Hebert & Beaton (1989) was used for the staining of following enzymes: arginine phosphokinase (APK), fumarate hydratase (FUM), glycerol-3-phosphate dehydrogenase (GPDH), isocitrate dehydrogenase (IDH), malate dehydrogenase (*NAD*$^{-}$) (MDH), malate dehydrogenase *(NADP*$^{-}$*)* (ME), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGDH) and phosphoglucose isomerase (PGI). The fixative (acetic acid:methanol:distilled water—1:4:10) was used to fix the isozyme banding patterns for 10~15 minutes. After staining and fixing, the plates were left to dry overnight in the dark and photographed. The migration distances of isozyme bands were measured, and their relative electrophoretic mobilities (μ:cm^{2}/sec/v) were calculated (Lehninger, 1979). Based on the criterion recommended by Jagdale *et al.* (1996), isozyme bands among isolates were considered to be the same band if that their mobility varied only within 10% of one another.

2.3.6. Examinations of stability of isozyme patterns: Five months later, after preparing the samples for the above examinations, all the above procedures were repeated for the NF strain and Umeå strains of *X. bovienii* in order to examine the isozyme stability relative to time and bacterial maintenance.

2.4. Results:

2.4.1. Identification of bacteria: After 48 hours' incubation on Biolog plates, the bacterium isolated from *S. feltiae* NF strain and that from *S. feltiae* Umeå strain were identified as *X. bovienii* with SIM (similarity) values of 0.800 and 0.705, respectively. The bacterium from *S. carpocapsae* All strain was identified as *X. nematophilus* with SIM value of 0.553. The bacterium from *S. riobravis* TX strain was not identified as belonging to either *X. nematophilus* or *X. bovienii*, but was an equal match to these two species based on the Biolog SIM value (Table 1).

2.4.2. Electrophoresis of bacterial enzymes

(1) *Arginine phosphokinase* (APK): The isozyme banding pattern for this enzyme distinguished *X. bovienii* from both *X. nematophilus* All strain and *X.* sp. TX strain, which were indistinguishable from each other. The banding pattern of the NF strain of *X. bovienii* contained one more cathodal band than that of the Umeå strain of this species. The Umeå strain of *X. bovienii* displayed a banding pattern consisting of three isozymes, while those of the other three isolates comprised four isozymes (Fig. 1, Table 2).

(2) *6-phosphogluconate dehydrogenase* (6PGDH): *X. bovienii* was distinguishable from *X. nematophilus* All strain and *X.* sp. TX strain on the basis of the isozyme banding patterns of this enzyme while *X. nematophilus* All strain and *X.* sp. TX strain were indistinguishable from each other. The two strains of *X. bovienii* had identical banding patterns. All of the four isolates showed two isozyme bands (one anodal, one cathodal) (Fig. 1, Table 2).

(3) *Malate dehydrogenase (NAD)* (MDH): This enzyme showed species-specific isozyme patterns. *Xenorhabdus bovienii* NF and Umeå strains had an anodal band and a cathodal band while *X.* sp. TX strain expressed one extra cathodal band and *X. nematophilus* All strain had five cathodal bands (Fig. 2, Table 2).

(4) *Fumarate hydratase* (FUM): The isozyme pattern for this enzyme was also species-specific. *Xenorhabdus nematophilus* All strain was the only isolate that lacked an anodal band. *X.* sp. TX strain differed from *X. nematophilus* All strain by having an extra cathodal band. The two strains of *X. bovienii* contained the same two isozymes (one cathodal, one anodal) (Fig. 1, Table2).

(5) *Glycerol-3-phosphate dehydrogenase* (GPDH): On the basis of the isozyme pattern of this enzyme, *X. bovienii* NF and Umeå strains were indistinguishable from each other, but distinguishable from the two other species, whose isozyme patterns were the same as each other. All four strains showed one anodal band, but the *X. bovienii* NF and Umeå strains expressed an extra cathodal band not present in the other two species (Fig.1, Table 2).

(6) *Isocitrate dehydrogenase* (IDH): All of the four isolates showed the same isozyme pattern. The pattern comprised an anodal band and a cathodal band (Table 2).

(7) *Phosphoglucomutase* (PGM): This enzyme gave species-specific isozyme patterns. *X. bovienii* NF and Umeå strains had an anodal band and a cathodal band. However, *X. nematophilus* All strain showed another cathodal band (i.e. three isozymes total). *Xenorhabdus* sp. TX only had an anodal band (Fig. 2, Table 2).

(8) *Phosphoglucose isomerase* (PGI): According to the isozyme patterns of this enzyme, *X. nematophilus* All strain was distinguishable from the other three strains. The other three strains had the same isozyme pattern (one anodal, one cathodal band). *X. nematophilus* All strain only had a cathodal band (Fig. 2, Table 2).

(9) *Malate dehydrogenase (NADP$^+$)* (ME): Like malate dehydrogenase (NAD^+), this enzyme also showed species-specific isozyme patterns. *X. bovienii* NF and Umeå strains had an anodal band and a cathodal band. However, *X.* sp. TX strain showed another cathodal band, and *X. nematophilus* All strain expressed the anodal band and three cathodal bands (Fig. 2, Table 2).

2.4.3. Isozyme stability: Compared to the former isozyme patterns, the Umeå strain displayed an additional cathodal isozyme band for ME, and the NF strain showed an extra cathodal band for IDH. All the other isozyme patterns tested in these two strains were not changed within five months.

2.5. Discussion

Using the Biolog system, three of the four bacterial strains were identified as belonging to currently described species. The bacterium isolated from the TX strain of *S. riobravis* was an equal match for either *X. nematophilus* (SIM value: 0.263) or *X. bovienii* (SIM value: 0.263). These matches were closer than for any other species of Gram negative bacteria included in the Biolog database. This corresponds with the generally-accepted viewpoint that the bacterium associated with nematodes of Steinernematidae is a

Xenorhabdus species (Smart, 1995; Cabanillas *et al.*, 1994). Further studies are needed to assign the bacterial strain isolated from *S. riobravis* TX strain to a currently described species of *Xenorhabdus* other than these that were scanned, or to designate it as a new species.

This study indicated that cellulose acetate electrophoresis was effective in separating the three species of *Xenorhabdus* used in this study from one another on the basis of the isozyme banding patterns of their enzymes. Four (FUM, MDH, ME and PGM) of the nine enzymes studied produced species-specific isozyme banding patterns. APK discriminated between *X. bovienii* NF and Umeå strains. All of the enzymes displayed temporal stability (five months), except for ME (Umeå strain) and IDH (NF strain). The latter enzyme proved unimportant for taxonomic purposes in any event, since it could not distinguish between species. It would further appear that ME should not be used for taxonomic purposes, since electropherograms, spaced five month apart, were not replicable. However, a combination of the APK isozyme pattern and the patterns of FUM, MDH, PGM would be sufficient to discriminate between each of the four strains.

Using the same technology, Jagdale *et al.* (1996) studied eight enzymes for the taxonomy of *Steinernema* nematodes, hosts of *Xenorhabdus* bacteria. Their research showed that a combination of isozyme banding patterns could be used to distinguish five isolates from one another. Using acrylamide gel electrophoresis, Hotchkin and Kaya (1984) separated the isozymes of four enzymes [glucose-6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), MDH and 6PGDH] in three *Xenorhabdus*

species, which were formerly classified as subspecies (subsp. *nematophilus*, subsp. *bovienii*, and subsp. *poinarii*) of *X. nematophilus*. These authors showed that isozyme patterns could be used to separate these species from one another. In the same study, the isozymes of four enzymes (G6PDH, LDH, MDH and PGM) were examined for the isolates of *P.(X.) luminescences*. This investigation indicated that this species may contain at least two subspecies. Examinations of 16S rRNA (Liu *et al.*, 1997; Szállás *et al.*, 1997) and fatty acid profiles (Janse and Smits, 1990) also showed that there may be several distinct groups in *P.(X.) luminescens*.

Cellulose acetate electrophoresis is simpler, more rapid, and more sensitive than starch or polyacrylamide gel electrophoresis (Easteal and Boussy, 1987). Its running time for nematodes' enzymes is only 20-30 minutes (Jagdale *et al.*, 1996). In the case of *Xenorhabdus* bacterial enzymes, only 14-20 minutes was needed for good electrophoretic separation of their isozyme bands.

While this study shows that cellulose acetate electrophoresis could be a useful determinative tool to discriminate bacterial associates of entomopathogenic nematodes, further studies are needed before it can be advocated with confidence. The scope of the inquiry should be broadened to include a greater number of bacterial isolates and enzymes.

This study showed that the isozyme patterns were relatively stable over time. However, the possible effect that maintenance and culture temperatures may have on isozyme profiles should be taken into consideration. In a recent study, Jagdale and

Gordon (1998b) have also shown that certain isozymes in steinernematids are associated with the temperatures at which the nematodes had been maintained or cultured and that the isozyme banding patterns of certain enzymes at a fixed culture temperature were not constant over time. Thus, it is not known with certainty that the isozyme profiles reported herein are exactly the same as those of the field population from which the laboratory stock colony originated. The thermal and temporal stability of the isozyme banding patterns of the bacteria need to be evaluated further.

Fig. 1: Electropherograms of four enzymes in four strains belonging to three species of *Xenorhabdus* at 25°C. All: *Xenorhabdus nematophilus* All strain; NF: *Xenorhabdus bovienii* NF strain; TX: *Xenorhabdus* sp. TX strain; Umeå: *Xenorhabdus bovienii* Umeå strain. Arginine phosphokinase (APK), 6-phosphogluconate dehydrogenase (6PGDH), fumarate hydratase (FUM), glycerol-3-phosphate dehydrogenase (GPDH). Arrow heads indicate the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.

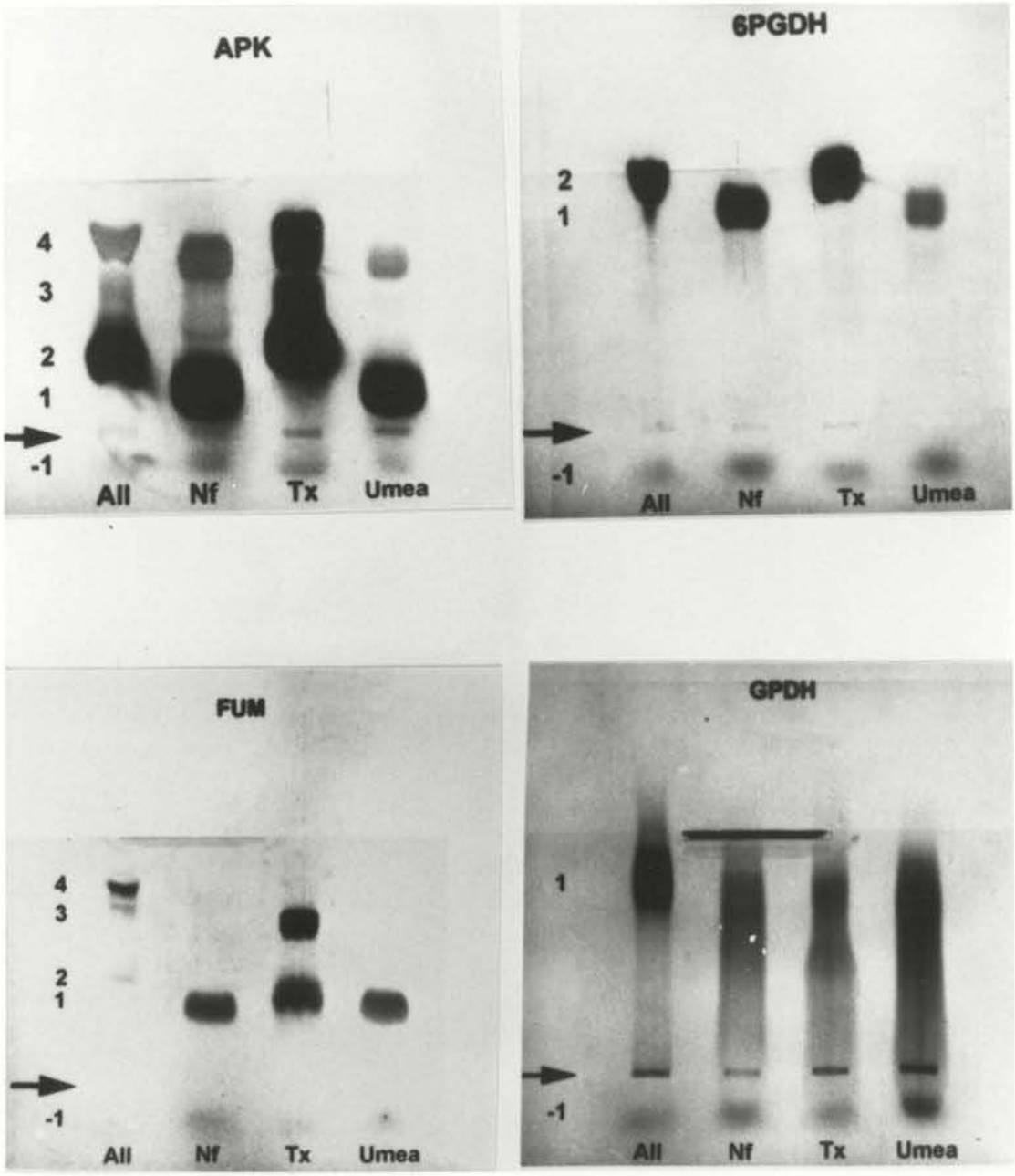
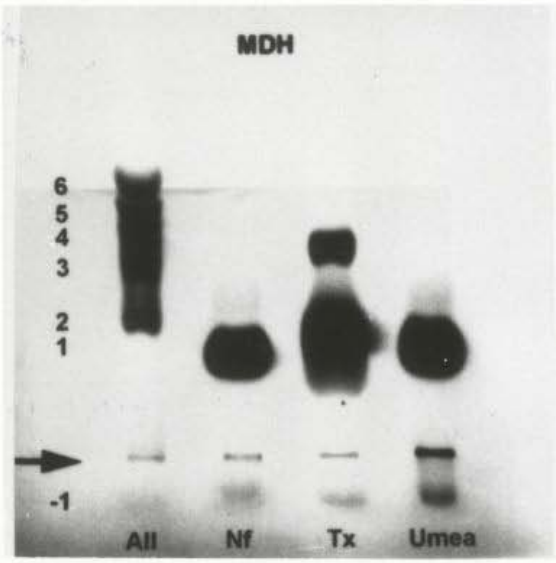
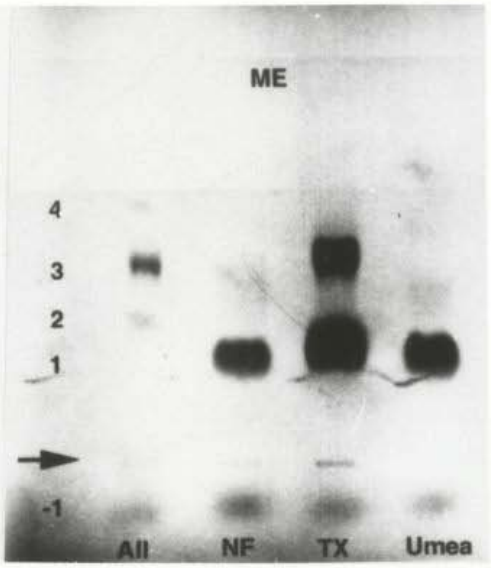
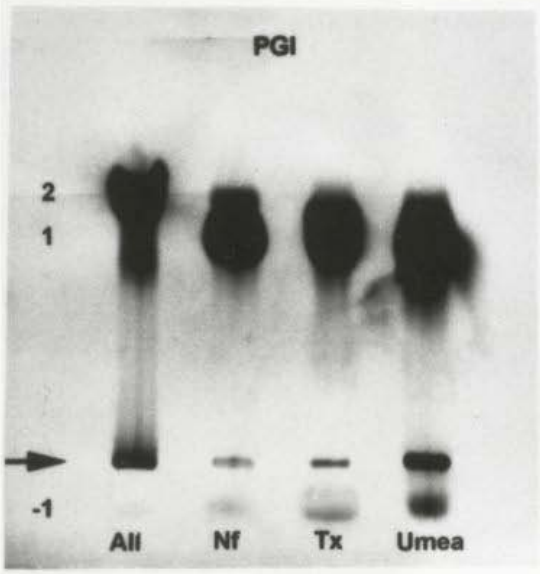

APK
4
3
2
1
-1
All
Nf
Tx
Umea
6PGDH
2
1
-1
All
Nf
Tx
Umea
FUM
4
3
2
1
-1
All
Nf
Tx
Umea
GPDH
1
-1
All
Nf
Tx
Umea

Fig. 2: Electropherograms of four enzymes in four strains belonging to three species of *Xenorhabdus* at 25°C. All: *Xenorhabdus nematophilus* All strain; NF: *Xenorhabdus bovienii* NF strain; TX: *Xenorhabdus* sp. TX strain; Umeå: *Xenorhabdus bovienii* Umeå strain. Malate dehydrogenase (NAD^-) (MDH), malate dehydrogenase ($NADP^-$) (ME), phosphoglucose isomerase (PGI), Phosphoglucomutase (PGM). Arrow heads indicate the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.


MDH
6
5
4
3
2
1
-1
All
Nf
Tx
Umea


ME
4
3
2
1
-1
All
NF
TX
Umea


PGI
2
1
-1
All
Nf
Tx
Umea

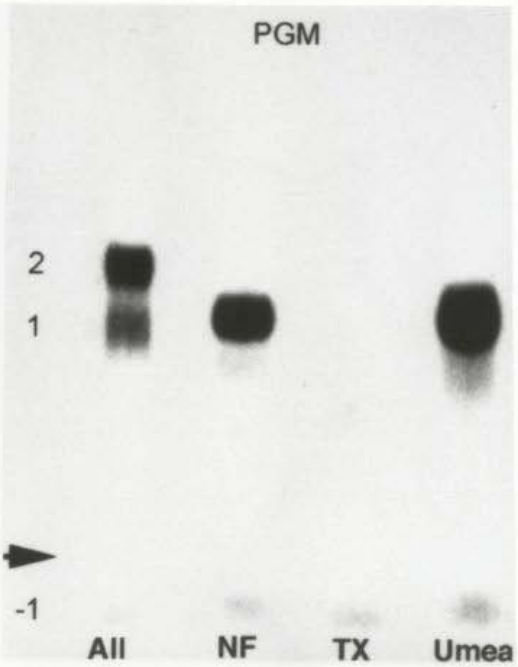

PGM
2
1
-1
All
NF
TX
Umea

Table 1: Similarity values of bacteria, their mutualistic nematodes and geographic origins

Bacterium	SIM value	Nematode	Geographic origin
X. bovienii (NF strain)	0.800	*S. feltiae* (NF strain)	Newfoundland, Canada
X. bovienii (Umeå strain)	0.705	*S. feltiae* (Umeå strain)	Northern Sweden
X. nematophilus (All strain)	0.553	*S. carpocapsae* (All strain)	Georgia, U. S. A.
Xenorhabdus sp. (TX strain)	0.263	*S. riobravis* (TX strain)	Southern Texas, USA

Notes: SIM values were the readings after 48 hours' incubation on Biolog plates at 25°C. The value of *X.* sp. TX strain was an equal match for either *X. nematophilus* or *X. bovienii*.

Table 2: Mean electrophoretic mobility ($cm^2/sec/v$) of isozymes of several enzymes in four isolates of *Xenorhabdus spp.*

Number of bands	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
		Arginine Phosphokinase (APK)		
-1	0.509	0.509	0.509	0.549
1	-	0.862	0.823	-
2	1.411	1.528	-	1.450
3	2.194	-	-	2.312
4	3.174	2.939	3.056	3.213
		6-Phosphogluconate Dehydrogenase (6PGDH)		
-1	0.583	0.583	0.583	0.617
1	-	2.948	2.948	-
2	3.360	-	-	3.463
		Malate Dehydrogenase (NAD^-) (MDH)		
-1	0.514	0.549	0.549	0.514
1	-	1.371	1.371	1.474
2	1.851	-	-	-
3	2.571	-	-	2.640
4	2.983	-	-	-
5	3.326	-	-	-
6	3.737	-	-	-

Table 2: Mean electrophoretic mobility (cm^2/sec/v) of isozymes of several enzymes in four isolates of *Xenorhabdus spp.* (continued)

Number of bands	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
		Fumarate Hydratase (FUM)		
-1	-	0.470	0.470	0.470
1	-	1.371	1.332	1.450
2	1.959	-	-	-
3	3.056	-	-	2.821
4	3.409	-	-	-
		Glycerol-3-Phosphate Dehydrogenase (GPDH)		
-1	0.514	0.514	0.566	0.547
1	2.537	2.503	2.331	2.537
2	-	3.188	2.931	-
		Isocitrate Dehydrogenase (IDH)		
-1	0.617	0.617	0.617	0.669
1	2.880	2.983	2.983	2.777
		Phosphoglucomutase (PGM)		
-1	0.514	0.514	0.514	0.514
1	2.331	2.503	2.537	-
2	2.983	-	-	-

Table 2: Mean electrophoretic mobility ($cm^2/sec/v$) of isozymes of several enzymes in four isolates of *Xenorhabdus spp.* (continued)

Number of bands	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
		Phosphoglucose Isomerase (PGI)		
-1	-	0.514	0.514	0.514
1	-	2.909	2.988	3.066
2	3.394	-	-	-
		Malate Dehydrogenase ($NADP^+$) (ME)		
-1	0.480	0.514	0.514	0.514
1	-	1.371	1.440	1.474
2	2.640	-	-	2.777
3	3.017	-	-	-
4	3.428	-	-	-

1. All the bacterial samples were prepared at 25^0C with shaking at 100 rpm. Three samples (n=3) were obtained for each isolate. Each sample consisted of the combined contents of two flasks. In total, there were twelve samples (24 flasks) for the four isolates.
2. Values ($\times 10^{-1}$) are the means of three samples. For each enzyme of each strain, isozyme bands were numbered in increasing numerical order relative to the distance that they migrated cathodally from the origin. Negative bands (numbered as -1) means that enzymes electrophoretically move towards the anode while positive bands towards the cathode. Dashes mean the absence of bands. Bands were considered the same if their electrophoretic motility values were within 10% of one another.

Chapter 3

Physiological characterization of *Xenorhabdus* sp. TX strain and *Xenorhabdus bovienii* NF strain

3.1. Abstract

Xenorhabdus sp. TX strain (undescribed) and *Xenorhabdus bovienii* NF strain (newly-isolated), bacterial symbionts of entomopathogenic nematodes, were characterized physiologically and biochemically. The TX strain differed from any of the five species of the genus *Xenorhabdus* or *P.(X.) luminescens*, a related nematode symbiont, in at least one of its following characteristics: growth at 10°C (-), growth at 37°C (+), catalase (-), bioluminescence (-), absorption of bromothymol blue dye (+), lipase (-), urease (-), phophatase (-), alkaline phophatase (w: weak), ribose acidification (-), glycerol acidification (+), salicin acidification (-), cefalothin resistance (-), the mixture of amoxilline and clavulanic acid resistance (-), and esculin hydrolysis (-). On the other hand, all the tests used in this study resulted in identical reactions for both the NF strain and the Umeå strain, suggesting that the NF strain could not be distinguished from the Umeå strain through common physiological and biochemical tests.

3.2. Introduction

Entomopathogenic nematodes carry bacterial symbionts that are responsible for killing the insect hosts (Akhurst, 1983; Dunphy, 1994, 1995; Clarke and Dowds, 1995). Two bacterial genera, *Xenorhabdus* and *Photorhabdus*, are associated with two nematode genera, *Steinernema* and *Heterorhabditis*, respectively (Boemare *et al.*, 1993; Ehlers *et al.*,

1988). A given nematode species only naturally carries a specific bacterial species, although one specific bacterial species may be associated with several nematode species (Akhurst, 1983, 1993; Akhurst and Boemare, 1990). To date, only five species of *Xenorhabdus* and one species of *Photorhabdus* have been described and physiologically characterized (Akhurst and Boemare, 1988; Forst *et al.*, 1997; Holt *et al.*, 1994; Nishimura *et al.*, 1994).

Steinernema riobravis is an effective biological control agent against lepidopteran pests at subtropical temperatures. It showed high pathogenicity to the corn earworm, *Helicoverpa zea* (Boddie), which attacks a wide variety of cultivated crops such as corn, cotton, tomato, and soybean (Cabanillas and Raulston, 1994, 1996a, 1996b). As a consequence, this nematode has been commercially produced for pest control. However, its bacterial symbiont has not been characterized and identified. At the other temperature extreme, a strain of *Steinernema feltiae*, isolated from soil in Newfoundland, Canada (Jagdale *et al.*, 1996), is a candidate for development as a biocontrol agent against insect pests in cold climates. Studies have been done to evaluate the efficacy of this nematode relative to other species at a variety of laboratory temperatures (Jagdale and Gordon, 1997a). However, the bacteria associated with *S. feltiae* NF strain has not been studied. The purpose of this study was to characterize these two bacterial strains physiologically.

3.3. Materials and Methods

3.3.1. Bacterial sources and maintenance: *Xenorhabdus nematophilus* All strain was isolated from *Steinernema carpocapsae* All strain, which was provided originally by

Plant Products Ltd. Brampton, Ontario, Canada. *Xenorhabdus bovienii* NF strain was isolated from *Steinernema feltiae* NF strain, which was isolated from soil in an organic garden near St. John's, Newfoundland, Canada (Jagdale *et al.* 1996). *Xenorhabdus bovienii* Umeå strain was isolated from *S. feltiae* Umeå strain, which was provided originally by Dr. R. West, Canadian Forest Service (CFS), St. John's, Newfoundland, Canada from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA. *Xenorhabdus* sp. TX strain was isolated from *Steinernema riobravis* TX strain, originally provided by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, Texas. All the nematode isolates were recycled through *Galleria mellonella* larvae (Woodring and Kaya, 1988) at 20°C for 26 months (Jagdale *et al.*, 1996) before being used for bacterial isolation (see Chapter 2 for the isolation method). After isolation and identification, *X. bovienii* NF and Umeå strains were maintained on NBTA plates at 5°C, *X. nematophilus* All strain at 10°C, and *X.* sp. TX strain at 15°C with a subculture interval of approximately one month. They had been maintained for about one year by the time that this study was carried out. All of the four isolates were identified by using a Biolog Microstation System™ and checked periodically, and just before this study, for culture purity.

Bacteria at their primary phase (blue colonies on NBTA plates) were streaked onto plates of nutrient agar (NA). Colonies that had grown at 25°C for 24 hours on NA plates were used to make the bacterial suspensions for all the tests in this study.

3.3.2. API 50 CH test: An API 50 CH strip (50300, bioMérieux Vitek, Inc., Missouri, USA) is composed of 50 microtubes, each containing a single carbohydrate or its derivative, except one microtube which contains no substrate and is used as the negative control. In this study, the strip is used to examine the bacteria's ability to produce acids from 49 carbon sources under anaerobic conditions. The carbon sources were carbohydrates or their derivatives.

Using a sterile swab, bacterial colonies from an NA plate were suspended in 3-ml sterile distilled water until this suspension had a turbidity equal to that of a 4.0 McFarland standard. One millilitre of this suspension was transferred into an ampoule of API 50 CHE medium (50400, bioMéreux Vitek, Inc., Missouri, USA). Using a sterile pipette, the consequent suspension was stirred and distributed into each of the 50 microtubes of the API 50 CH strip. Then, all of the microtubes were overlayed with sterilized mineral oil to create anaerobic conditions. Finally, the strip was placed in a incubation tray containing water to avoid drying of the bacterial culture. It was incubated at 25^0C with periodic reading. The development of yellow color in a microtube indicated a positive reaction (acid production), and red color as was shown in the control microtube indicated a negative reaction.

3.3.3. ID 32 E test: The ID 32 E strip (bioMérieux Vitek, Inc., Missouri, USA) consists of 32 test cupules, each containing a dehydrated reactive medium. It may be used to examine bacterial enzymatic activities, the abilility to utilize carbohydrates and to produce indole.

Bacterial colonies on an NA plate were suspended in 0.85% sterile saline (0.85 g sodium chloride in 100 ml distilled water) until this suspension had a turbidity equal to that

of a 0.5 McFarland standard. Using a Gilson pipetteman. the bacterial suspension was distributed into each of the 32 test cupules. Then, the strip was placed in an incubation tray containing water to avoid drying of the bacterial culture. and incubated at 25^{0}C for 24 hours. Test reactions were read according to the instructions included with the ID 32 E test panels.

3.3.4. API ZYM test: API ZYM strip (25200. bioMérieux Vitek. Inc.. Missouri. USA) consists of 20 microtubes. among which one control microtube contains no substrate and the other 19 microtubes allowed enzymatic reactions. Bacterial colonies on NA plates were suspended in 0.85% NaCl solution until this suspension had a turbidity between McFarland No. 5.0 and No. 6.0 standard. Using a Gilson pipetteman. 65 μl of this suspension was distributed into each of the 20 microtubes. Then. the strip was placed into an incubation tray containing water to avoid drying the bacterial culture. After 8 hours' incubation at 25^{0}C. one drop of ZYM A reagent and a drop of ZYM B reagent were added to each of the microtubes. Colours developed for five minutes. Colours (violet. orange. blue or brown) indicated positive tests while pale yellow meant a negative test. A value ranging from 0 to 5 (based on colour intensity) was assigned to each reaction corresponding to the colours on a colour chart.

3.3.5. ATB Antibiogram test: The ATB strip is composed of 16 pairs of cupules. 14 of which contain antibiotics at one or two concentrations. allowing examination of bacterial resistance to 19~21 antibiotics. Bacterial colonies on NA plates were suspended in 0.85% NaCl solution until this suspension has a turbidity equal to that of a 0.5 McFarland standard. Ten microliters of the suspension was transferred into an ampoule of ATB

medium and homogenized. Using a Gilson pipetteman, 135 μl of the resulting suspension was distributed into each of the cupules. Then the strip was placed in an incubation tray containing water to prevent the bacterial culture from drying. After 24 hours' incubation at 25°C, visible cloudiness in a cupule indicated a positive resistance test.

3.3.6. Other tests: (1) catalase test: A bacterial colony from a nutrient agar plate was emulsified into one drop of 3% hydrogen peroxide on a glass slide. Immediate bubbling indicated a positive test. (2) Phosphatase test: Bacteria were streaked onto phosphatase medium plates. After five days' incubation, one drop of ammonia solution (40%) was placed in the lid of the inverted plate, and the culture plate was put over it (upside-down). A positive test was indicated by the colonies turning red. (3) Oxidase test: A piece of filter paper was placed on a glass slide and moistened with sterile distilled water. Using a plastic applicator, several colonies were rubbed onto the moistened filter paper. Then, a drop of OX reagent (tetramethyl-p-phenylenediamine : isoamyl alcohol = 1g : 100 ml) was added to the filter paper. A violet colour development within two minutes indicated a positive test. (4) Esculin hydrolysis: Esculin broth was inoculated with one drop of bacterial suspension, and incubated at 25°C for 48 hours. A brownish black appearance and the absence of fluorescence indicated a positive test. (5) Absorption of bromothymol blue (BTB): primary-form bacteria were streaked onto a NBTA plate [nutrient agar containing 0.0025% (wt/vol) BTB and 0.004% (wt/vol) triphenyltetrazolium chloride]. The formation of blue colonies and red colonies after 3-5 days' incubation indicated a positive test and a negative test, respectively.

3.4. Results

All of the four bacterial strains showed negative reactions for indole production and esculin hydrolysis (not included in the Tables). API 50 CH and/or ID32 E tests showed that *Xenorhabdus* sp. TX strain, the bacterium isolated from the nematode *Steinernema riobravis* TX strain, like the other three strains tested, produced acid from glycerol, glucose, fructose, mannose, inositol, N-acetyl-glucosamine, maltose, trehalose, and 5-keto-gluconate. However, it could not acidify ribose, while the other three strains could. In addition, *X. bovienii* NF strain showed the same pattern of acid production as that by the Umeå strain of the same species (Table 1).

On the basis of API ZYM and ID 32 E tests, the TX strain of *Xenorhabdus* sp., like the other three strains tested, produced leucine arylamidase, N-acetyl-β-glucosaminidase and α-glucosidase, and showed weak reactions for esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase. Like *X. nematophilus* All strain and unlike the two strains of *X. bovienii*, it showed a weak reaction for alkaline phosphatase. Additionally, The NF strain and Umeå strain of *X. bovienii* had the same enzyme patterns in this study (Table 2).

Xenorhabdus sp. TX strain displayed resistance only to amoxicilline in the nineteen antibiotics tested. Importantly, it was sensitive to cefalothin while the other three strains were resistant to this antibiotic (Table 3). Like the Umeå strain of *X. bovienii*, the NF strain of the same species was resistant to amoxilline and cefalothin, and partially resistant to the antibiotic mixture of amoxilline and clavulanic acid.

The characteristics that seem of importance in distinguishing among species and strains of *Xenorhabdus* and *Photorhabdus* are summarized (Table 4). Results for *Xenorhabdus poinarii, Xenorhabdus beddingii, and Photorhabdus luminescens* are taken from the literature. The TX strain of *Xenorhabdus* sp. shares the inability to acidify ribose with *X. poinarii* and *Xenorhabdus japonicus*. The TX strain is distinguishable from the others by its inability to grow at 10^0C and its susceptibility to cefalothin. The NF strain of *X. bovienii* is identical to the Umeå strain of the same species with regards to all the features listed in Table 4. *Xenorhabdus bovienii* appears morphologically inseparable from the other *Xenorhabdus* isolates.

3.5. Discussion

In this study, the TX strain of *Xenorhabdus* sp. and the NF strain of *Xenorhabdus bovienii* were physiologically and biochemically characterized. With the exception of resistance to cefalothin, no single test allowed unequivocal separation of either of these isolates from the others previously examined or those that were included in this study. However, differences between these isolates and other individual species were apparent and the data suggested that at least at the species level, a combination of physiological features may be useful in separating the bacteria. The TX strain differed from *Xenorhabdus nematophilus* (Holt *et al.*, 1994) in its temperature range for growth, its inability to produce acid from ribose, and its sensitivity to cefalothin, and additionally from *Xenorhabdus bovienii* (Boemare and Akhurst, 1988; Holt *et al.*, 1994) in its weak reaction for alkaline

phosphatase and its sensitivity to the antibiotic mixture (amoxilline & clavulanic acid). The TX strain of *Xenorhabdus* sp. was distinguishable from *X. beddingii* (Akhurst, 1986a; Holt *et al.*, 1994) in its inability to produce acid from salicin and its negative reactions for lipase and phosphatase. The TX strain differed from *Xenorhabdus poinarii* (Akhurst, 1986b) in its ability to absorb bromothymol blue dye, and its negative reaction for lipase. It was also distinct from *Photorhabdus luminescens* (Holt *et al.*, 1994) in its inability to produce catalase and bioluminescence, and its negative reaction for phosphatase. Finally, *Xenorhabdus* sp. TX strain differed from *X. japonicus* (Nishimura *et al.*, 1994) in its ability to grow at 37°C and to produce acid from glycerol and insitol, and its inability to produce acid from rhamnose.

The NF strain of *X. bovienii* displayed the same characteristics as those of the Umeå strain of the same species in all the tests used in this study. However, the NF strain differed from the TX strain of *Xenorhabdus* sp. in its ability to grow at 10°C and produce acid from ribose, its inability to grow at 34°C, its resistance to cefalothin, and its partial resistance to the antibiotic mixture (amoxilline & clavulanic acid), and additionally from the All strain of *X. nematophilus* in its inability to grow at 34°C, its negative response for alkaline phosphatase and its partial resistance to the antibiotic mixture (amoxilline+clavulanic acid). The NF strain was distinguishable from *X. beddingii* in its inability to grow at 34°C, to hydrolyse esculin, and to produce acid from salicin, its ability to produce acid from ribose, and its negative reaction for phosphatase. The NF strain of *X. bovienii* was distinct from *X. poinarii* in its inability to grow at 34°C, and its capacity of absorbing bromothymol blue dye

and of producing acid from ribose and glycerol, and additionally from *Photorhabdus luminescens* in its negative catalase response, its absence of bioluminescence, and its ability to produce acid from glycerol. The NF strain of *X. bovienii* was different from *X. japonicus* (Nishimura *et al.*, 1994) in its ability to produce acid from glycerol, ribose and insitol, and its inability to produce acid from rhamnose (Table 4).

Acidification of carbohydrates by the Umeå strain of *X. bovienii* gave similar results to those of previous study by Boemare and Akhurst (1988) for this strain. Slight differences in results may be caused by the different incubation times used in the two studies. For example, a positive reaction for ribose was found after ten days' incubation in my study while only a weak response was reported after six days' incubation by Boemare and Akhurst (1988). Similarly, no capacity of acid production from glycerol was recorded for *X. bovienii* after two days' incubation (Holt *et al.*, 1994), whereas a positive test was reported for the Umeå strain of *X. bovienii* after six days' incubation (Boemare and Akhurst, 1988) and after ten days' incubation in this study. However, a negative reaction for gluconate from my work is at variance with the weak reaction for the same carbohydrate reported in the study by Boemare and Akhurst (1988).

The results of acid production from some carbohydrates by the All strain were consistent with what has been previously reported for *X. nematophilus*. Several differences may be attributed to different incubation times between my study and those of other researchers. For example, acid productions from glycerol, trehalose and maltose were

negative for *X. nematophilus* after two days' incubation (Holt *et al.*, 1994), and positive for the All strain of *X. nematophilus* after ten days' incubation in our study.

In this study, the NF and Umeå strains of *X. bovienii* and the All strain of *X. nematophilus* showed negative reactions for oxidase, catalase, phosphatase and urease, as was also found to be the case for *X. bovienii* and *X. nematophilus* (Holt *et al.*, 1994; Akhurst, 1986a; Forst et al, 1997). However, the negative responses for lipase by the NF strain and Umeå strain of *X. bovienii* do not accord with what has been reported for *X. bovienii* (Holt *et al.*, 1994). This may be due to differences in sensitivities among the various procedures used to identify the enzyme. Even within the current study, *X. nematophilus* All strain showed a positive reaction for N-acetyl-β-glucosaminidase using the API ZYM test, but a negative reaction using the ID 32 E test (Table 2).

The patterns of resistance to antibiotics were different among *Xenorhabdus* species, indicating that resistance patterns may be also useful in characterizing these bacteria.

Table 1: Capacity of four strains of *Xenorhabdus* to produce acids from carbohydrates and their derivatives

test	*X. n.* All strain 24 48 96 240h	*X. b.* NF strain 24 48 96 240h	*X. b.* Umeå strain 24 48 96 240h	*X.* sp. TX strain 24 48 96 240h
glycerol	- - w/+ +	- - w +	- w + +	- + + +
erythritol	- - - -	- - - -	- - - -	- - - -
D-arabinose	- - - -	- - - -	- - - -	- - - -
L-arabinose	- - - -	- - - -	- - - -	- - - -
ribose	- - - +	- - w +	- - - +	- - - -
D-xylose	- - - -	- - - -	- - - -	- - - -
L-xylose	- - - -	- - - -	- - - -	- - - -
adonitol	- - - -	- - - -	- - - -	- - - -
β-methyl-D-xyloside	- - - -	- - - -	- - - -	- - - -
galactose	- - - -	- - - -	- - - -	- - - -
glucose	w + + +	w + + +	w + + +	+ + + +
fructose	- + + +	- w + +	- - + +	- + + +
mannose	w/+ + + +	w + + +	w + + +	+ + + +
sorbose	- - - -	- - - -	- - - -	- - - -
rhamnose	- - - -	- - - -	- - - -	- - - -
dulcitol	- - - -	- - - -	- - - -	- - - -
inositol	- - w +	- - w +	- - - w/+	- - + +
mannitol	- - - -	- - - -	- - - -	- - - -
sorbitol	- - - -	- - - -	- - - -	- - - -
α-methyl-D-mannoside	- - - -	- - - -	- - - -	- - - -
α-methyl-D-glucose	- - - -	- - - -	- - - -	- - - -
N-acetyl-glucosamine	+ + + +	+ + + +	+ + + +	+ + + +
amygdalin	- - - -	- - - -	- - - -	- - - -

Table 1: Capacity of four strains of *Xenorhabdus* to produce acids from carbohydrates and their derivatives (continued)

test	*X. n.* All strain 24 48 96 240h	*X. b.* NF strain 24 48 96 240h	*X. b.* Umeå strain 24 48 96 240h	*X.* sp. TX strain 24 48 96 240h
arbutin	- - - -	- - - -	- - - -	- - - -
esculin	- - - -	- - - -	- - - -	- - - -
salicin	- - - -	- - - -	- - - -	- - - -
celiobiose	- - - -	- - - -	- - - -	- - - -
maltose	- w + +	- w + +	- w + +	- + + +
lactose	- - - -	- - - -	- - - -	- - - -
melibiose	- - - -	- - - -	- - - -	- - - -
sucrose	- - - -	- - - -	- - - -	- - - -
trehalose	- - w +	- - w +	- - - +	- - - +
inulin	- - - -	- - - -	- - - -	- - - -
melezitose	- - - -	- - - -	- - - -	- - - -
raffinose	- - - -	- - - -	- - - -	- - - -
starch	- - - -	- - - -	- - - -	- - - -
glycogen	- - - -	- - - -	- - - -	- - - -
xylitol	- - - -	- - - -	- - - -	- - - -
gentiobiose	- - - -	- - - -	- - - -	- - - -
D-turanose	- - - -	- - - -	- - - -	- - - -
D-lyxose	- - - -	- - - -	- - - -	- - - -
D-tagatose	- - - -	- - - -	- - - -	- - - -
D-fucose	- - - -	- - - -	- - - -	- - - -
L-fucose	- - - -	- - - -	- - - -	- - - -
D-arabitol	- - - -	- - - -	- - - -	- - - -
L-arabitol	- - - -	- - - -	- - - -	- - - -
gluconate	- - - -	- - - -	- - - -	- - - -
2-keto-gluconate	- - - -	- - - -	- - - -	- - - -

Table 1: Capacity of four strains of *Xenorhabdus* of producing acids from carbohydrates and their derivatives (continued)

test	*X. n.* All strain 24 48 96 240h	*X. b.* NF strain 24 48 96 240h	*X. b.* Umeå strain 24 48 96 240h	*X.* sp. TX strain 24 48 96 240h
5-keto-gluconate	- w w w	- - - +	- w w/+ +	- - - +
galacturonate	-	-	-	-
palatinose	-	-	-	-
malonate	-	-	-	-
saccharose	-	-	-	-

Notes: 1. The results for galacturonate, palatinose, malonate and saccharose are from ID 32 E test, with all the others from API 50 CH test.

2. *X. n.: Xenorhabdus nematophilus; X. b.: Xenorhabdus bovienii*; *X.* sp.: *Xenorhabdus* sp.

Table 2: Enzymatic activities of four strains of *Xenorhabdus* spp.

Enzymes	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
I. API ZYM (8 hour's incubation)				
alkaline phosphatase	w	-	-	w
esterase (C4)	w	w	w	w
esterase lipase (C8)	w	w	w	w
lipase (C14)	-	-	-	-
leucine arylamidase	+	+	+	+
valine arylamidase	-	-	-	-
cystine aryamidase	-	-	-	-
trypsin	-	-	-	-
chymotrypsin	-	-	-	-
acid phosphatase	w	w	w	w
naphthol-AS-BI-phosphohydrolase	w	w	w	w
α-glucosidase	w	w	w	w
N-acetyl-β-glucosaminidase	+	+	+	+
α-mannosidase	-	-	-	-
α-fucosidase	-	-	-	-
II. ID 32 E (24 hour's incubation)				
ornithin decarboxylase	-	-	-	-
arginine dihydrolase	-	-	-	-
lysine decarboxylase	-	-	-	-
urease	-	-	-	-
β-glucosidase	-	-	-	-
β-giucuronidase	-	-	-	-
N-acetyl-β-glucosaminidase	-	+	+	+

Table 2: Enzymatic activities of four strains of *Xenorhabdus* spp.

Enzymes	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
β-galactosidase	-	-	-	-
α-glucosidase	+	+	+	+
α-galactosidase	-	-	-	-
α-maltosidase	-	-	-	-
L-aspartic acid arylamidase	-	-	-	-
III. Others				
oxidase	-	-	-	-
catalase	-	-	-	-
phosphatase	-	-	-	-

Notes:

1. The results are from API ZYM test or ID 32 E test. The enzymes examined in both tests are reported only once if they showed the identical reaction.
2. For the original readings of API ZYM tests, a value ranging from 0 to 5 was assigned to each test according to the intensity of reaction. Values ranging from 0-0.9, 1-2.9, and 3-5 were qualitatively considered as negative (-), weak (w), and positive (+), respectively.

Table 3: Characteristic resistance of *Xenorhabdus* spp. to antibiotics

antibiotic	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
amoxilline	R	R	R	R
amoxilline & clavulanic acid	S	I	I	S
piperacilline	S	S	S	S
piperacilline & tazobactam	S	S	S	S
ticarcilline	S	S	S	S
cefalothin	R	R	R	S
cefotaxime	S	S	S	S
cerftriaxone	S	S	S	S
ceftazidime	S	S	S	S
aztreonam	S	S	S	S
imipeneme	S	S	S	S
ceftazidime	S	S	S	S
trimethoprim & sulfamethoxazole	S	S	S	S
tobramycine	S	S	S	S
amikacine	S	S	S	S
gentamicine	S	S	S	S
netilmicine	S	S	S	S
pefloxacine	S	S	S	S
ciprofloxacine	S	S	S	S

notes: R: resistance. Growth exists at both low and high concentration of antibiotic; I: intermediate. Growth exists at low concentration, but not at high concentration of antibiotic; S: sensitive. Growth does not exist at both low and high concentration of antibiotic.

Table 4: Physiological characteristics differentiating *Xenorhabdus sp.* TX strain from the known species in *Xenorhabdus* and *Photorhabdus*, and characteristics of *Xenorhabdus bovienii* NF strain*

test	*X.*sp. TX	*X. n.* All	*X. bo.* NF	*X. bo.* Umeå	*X. be.* (**)	*X. p.* (**)	*X. j.* (**)	*P. l.* (**)
growth at 10°C†	-	+	+	+	+	?	?	?
growth at 37°C†	+	-	-	-	+	+	-	+
bioluminescence	-	-	-	-	-	-	-	+
absorption of BBD	+	+	+	+	+	-	+	+
catalase	-	-	-	-	-	-	-	+
lipase	-	-	-	-	+	+	-	?
urease	-	-	-	-	-	-	-	d
phosphatase	-	-	-	-	+	-	-	+
alkaline phosphatase	w	w	-	-	?	?	?	?
acid production from:								
glycerol	+	+	+	+	d	-	-	-
ribose	-	+	+	+	-/w	-	-	?
insitol	+	+	+	+	-	-	-	+
rhamnose	-	-	-	-	?	?	+	?
salicin	-	-	-	-	+	-	?	?
cefalothin (resistance)	-	+	+	+	?	?	?	?
amoxilline & clavulanic acid (resistance)	-	-	I	I	?	?	?	?
esculin hydrolysis	-	-	-	-	+	-	-	?

Notes: 1. *X. n.*: *Xenorhabdus nematophilus*. *X. bo.*: *Xenorhabdus bovienii*. *X. be.*: *Xenorhabdus beddingii*. *P. l.*: *Photorhabdus luminescens*. *X. p.*: *Xenorhabdus poinarii*. *X. j.*: *Xenorhabdus japonicus*.

2. *: All the results are based on primary phase.

3. (**): The results for these species are from Bergey's Manual of Determinative Bacteriology (9th edition) or other reference. All the other results were from my own tests.

4. +: positive; -: negative; w: weak reaction; ?: no data; d: 15 to 85% of the isolates positive. I: resistant at low concentration, and sensitive at high concentration of antibiotic.

5. †: These data for TX, All, NF and Umeå strains are from the study on the effect of temperature on the growth of *Xenorhabdus* spp. (see Chapter 4).

Chapter 4

Effect of temperature on the growth of *Xenorhabdus* spp.

4.1. Abstract

The effect of temperature on the growth of three *Xenorhabdus* species (= four strains) isolated from nematodes that originated from climatically diverse geographical origins (boreal, temperate, and subtropical) was examined over a wide range of temperatures. The two boreal strains (NF, Umeå) of *Xenorhabdus bovienii* grew in tryptic soy broth at culture temperatures from 0°C to 32°C, *Xenorhabdus nematophilus* All strain (temperate origin) from 10°C to 35°C, and *Xenorhabdus* sp. TX strain (subtropical origin) from 15°C to 38.5°C. The NF and Umeå strains displayed their highest growth rates at 25°C, the All and TX strains at 30°C. The NF strain and Umeå strain were categorized as psychrotrophs, and the All strain (temperate origin) and the TX strain (subtropical origin) as mesophiles. The relevance of these data to the feasibility of using nematode-bacterium complexes for pest management in cold climates is discussed.

4.2. Introduction

Bacteria of the genus *Xenorhabdus* are mutualistic symbionts of entomopathogenic nematodes *Steinernema* spp.. These bacteria use their nematode hosts to gain access into the hemocoel of susceptible insect species. In return, the bacteria play a vital role in the nematode life cycle by producing toxins to kill the insect and antibiotics to protect the insect

cadaver from putrefaction, thereby insuring a balanced nutritional environment for nematode proliferation (Akhurst, 1982; Akhurst and Boemare, 1990; Forst and Nealson, 1996; Poinar, 1990).

Environmental temperature is one of the vital factors that influence the survival, dispersal, establishment, reproduction, development and infectivity of entomopathogenic nematodes (Blackshaw and Newell, 1987; Dunphy and Webster, 1986; Grewal *et al.*, 1994; Griffin, 1993; Mason and Hominick, 1995; Molyneux, 1986). The temperatures at which nematodes survived and were able to infect susceptible insect hosts were found to be correlated with the temperature features of the region from which the various nematode strains were initially isolated (Grewal *et al.* 1994; Molyneux, 1986). Temperature tolerance, infectivity and reproduction of entomopathogenic nematodes could be affected by the temperature of recycling systems (Jagdale and Gordon, 1997a, 1998a; Griffin and Downes, 1991), and improved in *in vitro* recycling systems (Glazer *et al.*, 1990; Grewal *et al.*, 1996). Given the critical importance of the bacteria to the efficacy of entomopathogenic nematodes as biological control agents, it is important to establish the degree to which bacterial growth is temperature-related. Therefore, I carried out the present study to compare the effects of temperature on the growth of bacteria that were isolated from four strains of *Steinernema.*

4.3. Materials and Methods

4.3.1. Bacterial sources and maintenance: *Xenorhabdus nematophilus* All strain was isolated from *Steinernema carpocapsae* All strain, which was provided originally by

Plant Products Ltd. Brampton, Ontario, Canada. *Xenorhabdus bovienii* NF strain was isolated from *Steinernema feltiae* NF strain, which was isolated from soil in an organic garden near St. John's, Newfoundland, Canada (Jagdale *et al.* 1996). *Xenorhabdus bovienii* Umeå strain was isolated from *S. feltiae* Umeå strain, which was provided originally by Dr. R. West, Canadian Forest Service (CFS), St. John's, Newfoundland, Canada from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA. *Xenorhabdus* sp. TX strain was isolated from *Steinernema riobravis* TX strain, originally provided by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, Texas. All the nematode isolates were recycled through *Galleria mellonella* larvae (Wooding and Kaya, 1988) at 20°C for 26 months (Jagdale *et al.*, 1996) before being used for bacterial isolation (see Chapter 2 for the isolation method). After isolation and identification, *X. bovienii* NF and Umeå strains were maintained on NBTA (nutrient agar with bromothymol blue dye and triphenoltetrazolium chloride) plates at 5°C, *X. nematophilus* All strain at 10°C, and *X.* sp. TX strain at 15°C with a subculture interval of approximately one month. They had been maintained for about one year by the time that this study was carried out. All of the four isolates were re-identified by using the Biolog Microstation System™ periodically and just before this study.

4.3.2. Cardinal temperatures and bacterial growth

Seven millilitres of TSB (tryptic soy broth, DIFCO) in a 15-ml capped tube was inoculated with blue colonies (primary phase) of the separate bacterial strains on NBTA

plates, and incubated at 25°C with shaking at 100 rpm for 24 hours. For the determination of ultimate temperatures, 2 ml of the above bacterial culture was used to inoculate 250-ml TSB in a 500-ml capped flask. The lowest temperature at which the bacterial cultures showed visible growth within 25 days at 100 rpm was considered as their minimum temperatures for growth. Similarly, the highest temperature at which the bacterial cultures displayed visible growth within 8 days was considered as their maximum temperature for growth. The time periods used for determining whether the bacteria had been killed were selected based on a pilot experiment that I conducted, in which I determined that 8 days at high temperatures (>30°C for the NF and Umeå strains; >35°C for the All and TX strains), 25 days at low temperatures (<5°C for the NF and Umeå strains; ≤15°C for the All and TX starins) were sufficient for growth to be displayed.

Using the bacterial sample harvested after 24 hours' incubation in a 500-ml flask at 25°C with shaking at 100 rpm, a standard curve showing the relationship between dry weight and optical density of bacterial culture was prepared for each of the four strains.

Growth rates were examined at 5°C, 10°C, 15°C, 20°C, 25°C, 27°C and 30°C for the NF and Umeå strains of *Xenorhabdus bovienii*, at 10°C, 15°C, 20°C, 25°C, 27°C, 30°C and 35°C for the All strain of *Xenorhabdus nematophilus*, and at 20°C, 25°C, 30°C and 35°C for the TX strain of *Xenorhabdus* sp., which was isolated from *Steinernema riobravis* TX strain. For this purpose, 250-ml TSB in a 500-ml capped flask was inoculated with 1-ml bacterial culture that was prepared with the same procedure as that used for the determination of the extreme temperature. It was incubated with shaking at 100 rpm in a Psycrotherm incubator

(New Brunswick Scientific, USA) or in Freas 815 incubator inside which a small shaker (Gyrotory® Shaker-Model G2) was placed. Spectrophotometry was used to determine the optical density of the bacterial culture with 3-24 hour intervals (on the basis of temperatures and growth stages). At each time, 1-ml bacterial culture was withdrawn from each flask using a sterile pipette, and diluted up to 5 times with 0.15 M NaCl. Then, 3-4 ml of this diluted culture was transferred into a 4.5-ml cuvette (10 mm thick) (Fisherbrand®:14-385-996). Using a Spectronic Genesys 5 spectrophotometer, optical density was recorded at a wavelength of 600 nm. Three replicates (i.e. three separate flasks) were measured for each of the four strains at each of the culture temperatures, with the exceptions of the NF and Umeå strains of *Xenorhabdus bovienii* at 5°C (one replicate) because of the limited space on the small shaker.

4.3.3. Statistical analysis

In this study, observed optical densities were transformed into true optical densities according to the method of Lawrence and Maier (1977). The conceptual temperatures (T_0) [temperatures that are of no metabolic significance, the X-axis intercept of a plot of the square root of the growth rate against temperature (≤optimal temperature) ($\sqrt{r} = b(T-T_0)$)] were obtained through the method suggested by Ratkowsky *et al.* (1982). Growth rates and generation times were calculated with the BASIC computer program (Gerhardt *et al.*, 1994). These data were further analysed using a one-way ANOVA, t-test (Sokal and Rohlf, 1995) at $P<0.05$.

4.4. Results

Using tryptic soy broth as culture medium. *X. bovienii* Umeå and NF strains were able to grow at temperatures from 0°C to 32°C, but did not grow at -2°C and 34°C. *X. nematophilus* All strain grew at temperatures from 10°C to 35°C, but not at 8°C and 37°C. *Xenorhabdus* sp. TX strain grew at temperatures from 15°C to 38.5°C, but no growth occurred at 12°C and 40°C (Table 1).

The growth curves of bacterial dry weight plotted against time for different culture temperatures are shown in Fig. 1. Growth rates and generation times were calculated from these curves. At temperatures ≤20°C, the two strains of *X. bovienii* showed higher growth rates (Fig. 2, Table 2) or shorter generation times (Table 3) than the All strain of *X nematophilus* and TX strain of *Xenorhabdus* sp.. The All strain of *X. nematophilus* and the TX strain of *Xenorhabdus* sp. showed the highest growth rates at 30°C, and the NF and Umeå strains of *X. bovienii* displayed the highest growth rates at 25°C (Fig. 2). The generation times, at optimal temperatures for growth, were about two hours for all of the four strains studied (Table 3).

The conceptual temperatures of no metabolic significance were 276.16°K (3.16°C) for *X. nematophilus* All strain, 282.52°K (9.52°C) for *X.* sp. TX strain, 270.25°K (-2.75°C) for *X. bovienii* NF strain, and 269.78°K (-3.22°C) for *X. bovienii* Umeå strain (Table 1, Fig. 3).

4.5. Discussion

In this study, cardinal temperatures and temperature (T_0) of no metabolic significance corresponded to the geographic origins of *Xenorhabdus* species. Compared with *X.*

nematophilus All strain (temperate origin: Georgia, USA) and *Xenorhabdus* sp. TX strain (subtropical origin: Texas, USA), the NF and Umeå strains (boreal origin: Newfoundland, Canada and Umeå, northern Sweden, respectively) of *X. bovienii* displayed lower values for optimal, minimum and maximum temperatures, and the temperature of no metabolic significance. In addition, *X. nematophilus* All strain also had lower values for minimum and maximum temperatures, and T_0 than *Xenorhabdus* sp. TX strain.

Morita (1975) defined psychrophiles as those bacteria which could grow at 0^0C and had an upper limit at about 20^0C, with the optimal temperature at about 15^0C or lower. He also defined psychrotrophs as those growing at 0^0C and at temperatures $>25^0$C, with the optimal temperature higher than 15^0C. Based on this definition, the NF and Umeå strains of *X. bovienii* satisfied these requirements and could be considered as psychrotrophic bacteria. Mesophiles are defined as those that may stop growing from 0-10^0C, with an optimal temperature of 30-40^0C (Suutari and Laakso, 1994). Therefore, the All strain of *X. nematophilus* and the TX strain of *Xenorhabdus* sp. could be described as mesophiles. The above grouping also coincided with the classification which was based on the temperature (T_0) of no metabolic significance. Ratkowsky *et al.* (1982) found that T_0 values were 248-263^0K for psychrophiles, 261-269^0K for psychrotrophs, 270-280^0K for mesophiles, 290-296^0K for thermophiles. Therefore, the closest group to the NF strain (T_0=270.25^0 K) and Umeå strain (T_0=269.78^0 K) of *X. bovienii* is psychrotrophs. Similarly, the All strain (T_0=276.16^0 K) of *X. nematophilus* and the TX strain (T_0=282.52^0 K) of *Xenorhabdus* sp. could be considered as mesophiles. *Photorhabdus* sp. K122 strain,

a bacterial associate of heterorhabditid nematodes, was also classified as a psychrotrophic organism on the basis of its cardinal temperatures (Clarke, 1993) and its T_0 value (Clarke and Dowds, 1994).

The minimum temperatures for bacterial growth corresponded with the low limiting temperature for the recycling of the corresponding nematode host. For example, the minimum temperatures for the growth of *Xenorhabdus* sp. TX strain and *X. nematophilus* All strain were 15°C and 10°C, respectively. Their corresponding nematode hosts, *Steinernema riobravis* TX strain and *Steinernema carpocapsae* All strain, could only be recycled at >15°C and >10°C, respectively (Jagdale and Gordon, 1997a). Presumably, at the nematodes' minimum temperatures, the bacteria grew too slowly to produce sufficient antibiotics and/or growth factors needed by the nematodes for growth and development. Thus, the minimum temperature for bacterial growth would become a limiting factor for nematode reproduction, resulting in the failure to recycle these nematodes. Similarly, Gwynn and Richardson (1994) suggested that the nematode's capability of killing insects at low temperatures may depend on their bacterial associates being able to grow.

Gwynn and Richardson (1994) studied the growth of five isolates of bacterial associates of entomopathogenic nematodes, and found that *Xenorhabdus bovienii* L/128 and L/179 isolates were the only two isolates capable of growing at 2°C, and that Nemasys isolate of the same species could grow at 6°C. However, another two isolates, *X. nematophilus* Biosys 252 and *Photorhabdus luminescens* Nemasys H, could grow at 10°C, but not at 6°C. In addition, the three isolates of *X. bovienii* grew more rapidly than the other

two isolates at low temperatures such as 2^0, 6^0, and 10^0C. The current study also showed that two other strains (NF and Umeå) of *X. bovienii* could grow at 0^0C, and had higher growth rates at low temperatures (5^0, 10^0, 15^0 and 20^0C) than the temperate strain (All) and subtropical strain (TX). *S. feltiae* NF and Umeå strains, the nematode hosts of *X. bovienii* NF and Umeå strains respectively, have been shown to be capable of killing *Galleria mellomella* at 5^0C (Jagdale and Gordon, 1997a). These results support the proposition that *X. bovienii* may be a cold-adapted bacterial species, and its nematode host could be used to control sensitive insects in cold areas.

Table 1: Cardinal temperatures and conceptual temperatures in *Xenorhabdus* species

bacterial strain	t^0C for Growth min	opt	max	t^0C without growth low	high	conceptual tem -perature (^0K)
X. bovienii (NF strain)	0	25	32	-2	34	270.25
X. bovienii (Umeå strain)	0	25	32	-2	34	269.78
X. nematophilus (All strain)	10	30	35	8	37	276.16
Xenorhabdus sp. (TX strain)	15	30	38.5	12	40	282.52

Fig. 1: Growth curves of *Xenorhabdus* spp. at different temperatures. A: *Xenorhabdus nematophilus* All strain; B: *Xenorhabdus* sp. TX strain; C: *Xenorhabdus bovienii* NF strain; D: *Xenorhabdus bovienii* Umeå strain.

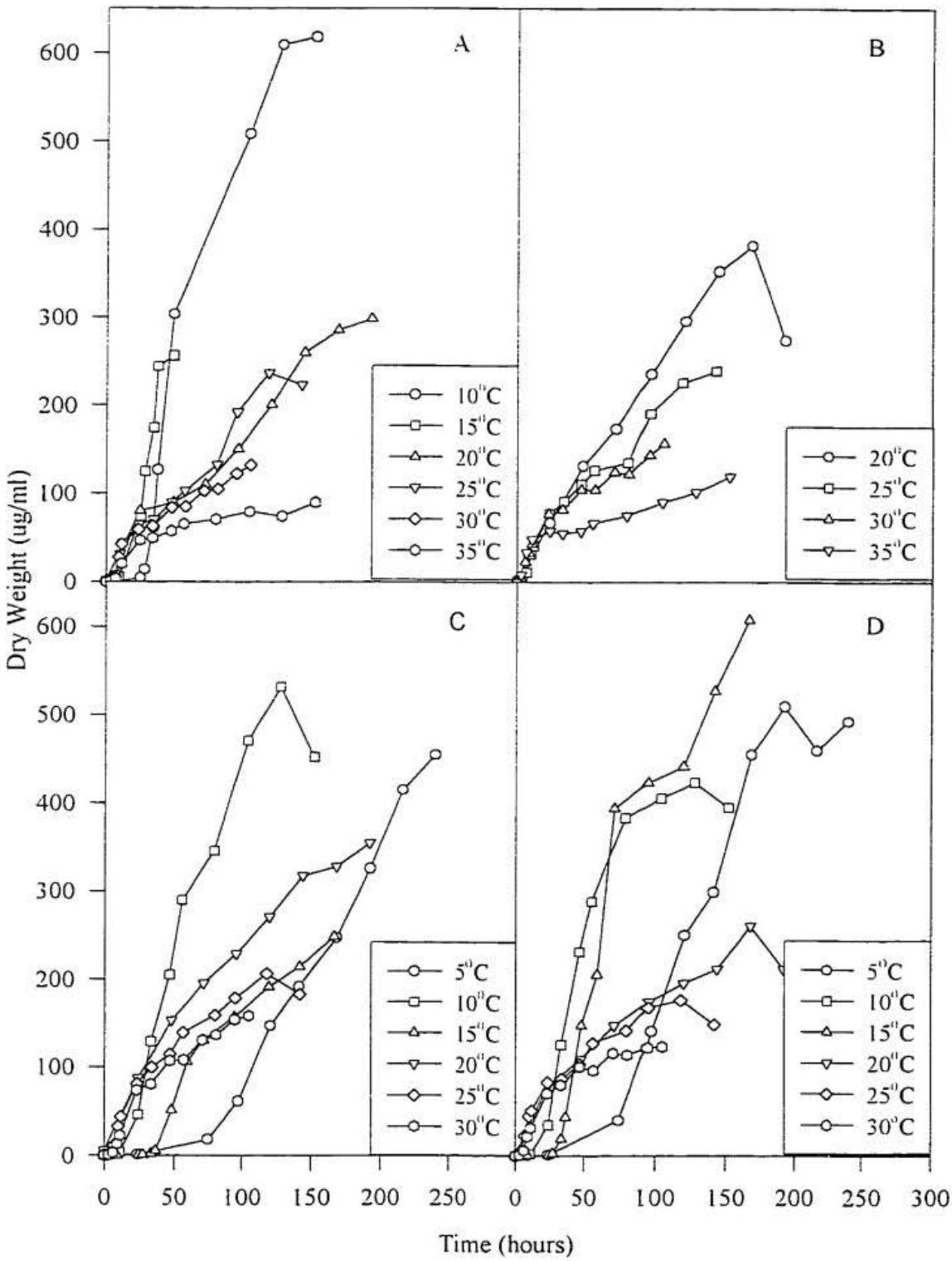

A
B
C
D
Dry Weight (ug/ml)
Time (hours)
0
100
200
300
400
500
600
0
50
100
150
200
250
300
10°C
15°C
20°C
25°C
30°C
35°C
5°C

Table 2: Growth rates of *Xenorhabdus* spp. at different temperatures

t°C	Replicate	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
5	1		0.036	0.030	
	1	0.035	0.051	0.052	
10	2	0.030	0.059	0.050	
	3	0.030	0.048	0.045	
	mean	$0.032 \pm 0.002^{d*}$	0.053 ± 0.003^{d}	0.049 ± 0.002^{e}	
	1	0.098	0.146	0.123	
15	2	0.091	0.171	0.158	
	3	0.084	0.165	0.150	
	mean	0.091 ± 0.004^{c}	0.160 ± 0.008^{c}	0.144 ± 0.011^{d}	
	1	0.158	0.253	0.176	0.149
20	2	0.141	0.180	0.190	0.141
	3	0.160	0.175	0.187	0.152
	mean	0.153 ± 0.006^{b}	0.203 ± 0.025^{c}	0.185 ± 0.004^{c}	0.147 ± 0.004^{c}
	1	0.414	0.367	0.284	0.321
25	2	0.341	0.373	0.315	0.305
	3	0.352	0.365	0.305	0.336
	mean	0.369 ± 0.023^{a}	0.368 ± 0.002^{a}	0.301 ± 0.009^{a}	0.320 ± 0.009^{a}
	1	0.462	0.316	0.197	
27	2	0.371	0.267	0.248	
	3	0.364	0.257	0.238	
	mean	0.399 ± 0.032^{a}	0.280 ± 0.018^{b}	0.227 ± 0.016^{b}	
	1	0.432	0.163	0.122	0.319
30	2	0.395	0.135	0.138	0.310
	3	0.432	0.154	0.119	0.351
	mean	0.419 ± 0.012^{a}	0.151 ± 0.008^{c}	0.126 ± 0.006^{d}	0.327 ± 0.012^{a}
	1	0.163			0.228
35	2	0.180			0.238
	3	0.183			0.230
	mean	0.175 ± 0.006^{b}			0.232 ± 0.003^{b}

*Values are the means ± SE (standard error). Means with the same superscript letter (down the single column) are not significantly different at $P<0.05$ by t-test (Sokal and Rohlf, 1995).

Fig. 2: Effect of temperature on growth rates of *Xenorhabdus* spp.. A: *Xenorhabdus nematophilus* All strain; B: *Xenorhabdus* sp. TX strain; C: *Xenorhabdus bovienii* NF strain; D: *Xenorhbadus bovienii* Umeå strain. Values with the same superscript letter in each of four graphs are not significantly different from temperature to temperature at $P<0.05$ by t-test (Sokal and Rohlf, 1995). The bars represent standard errors.

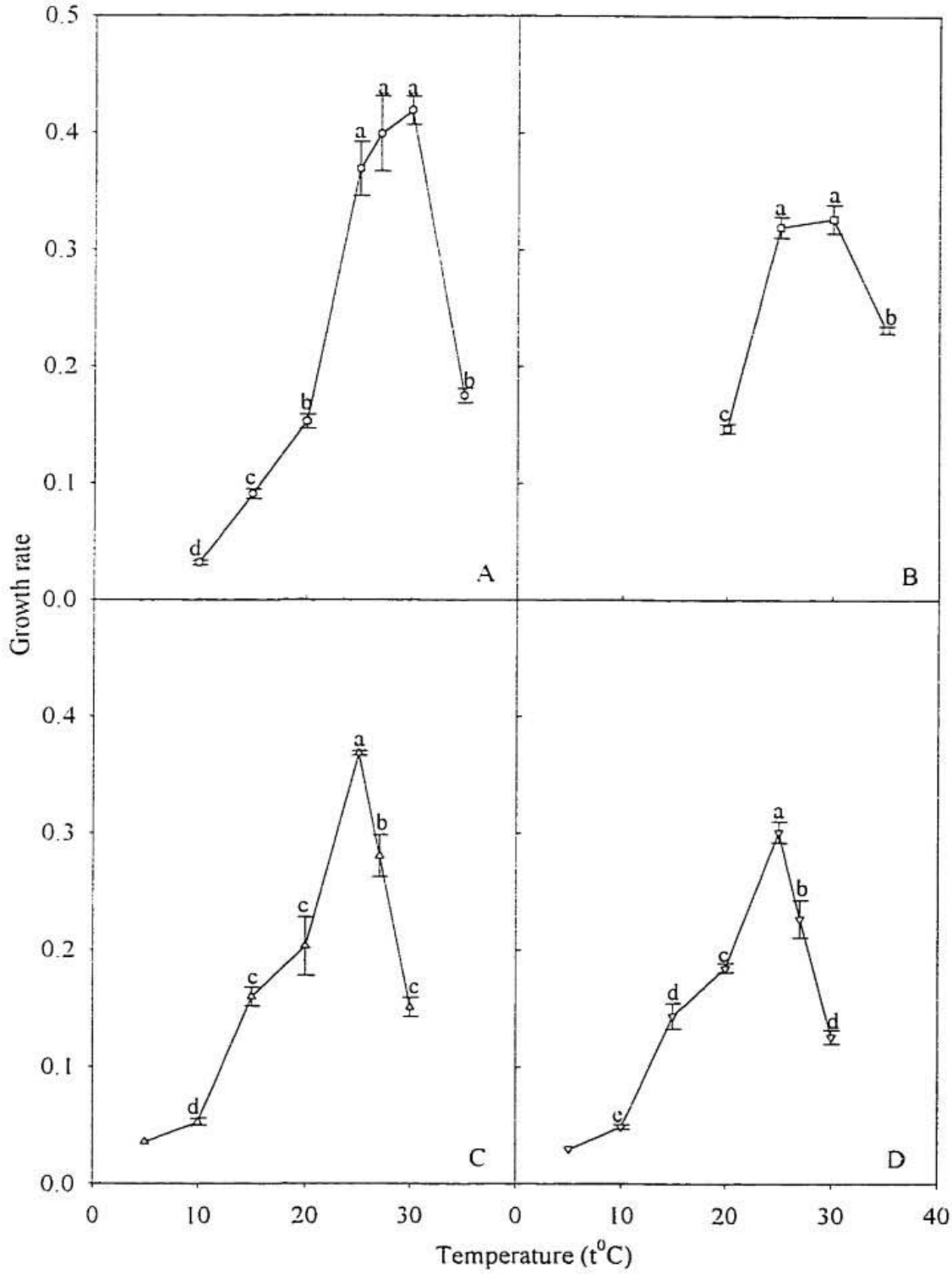
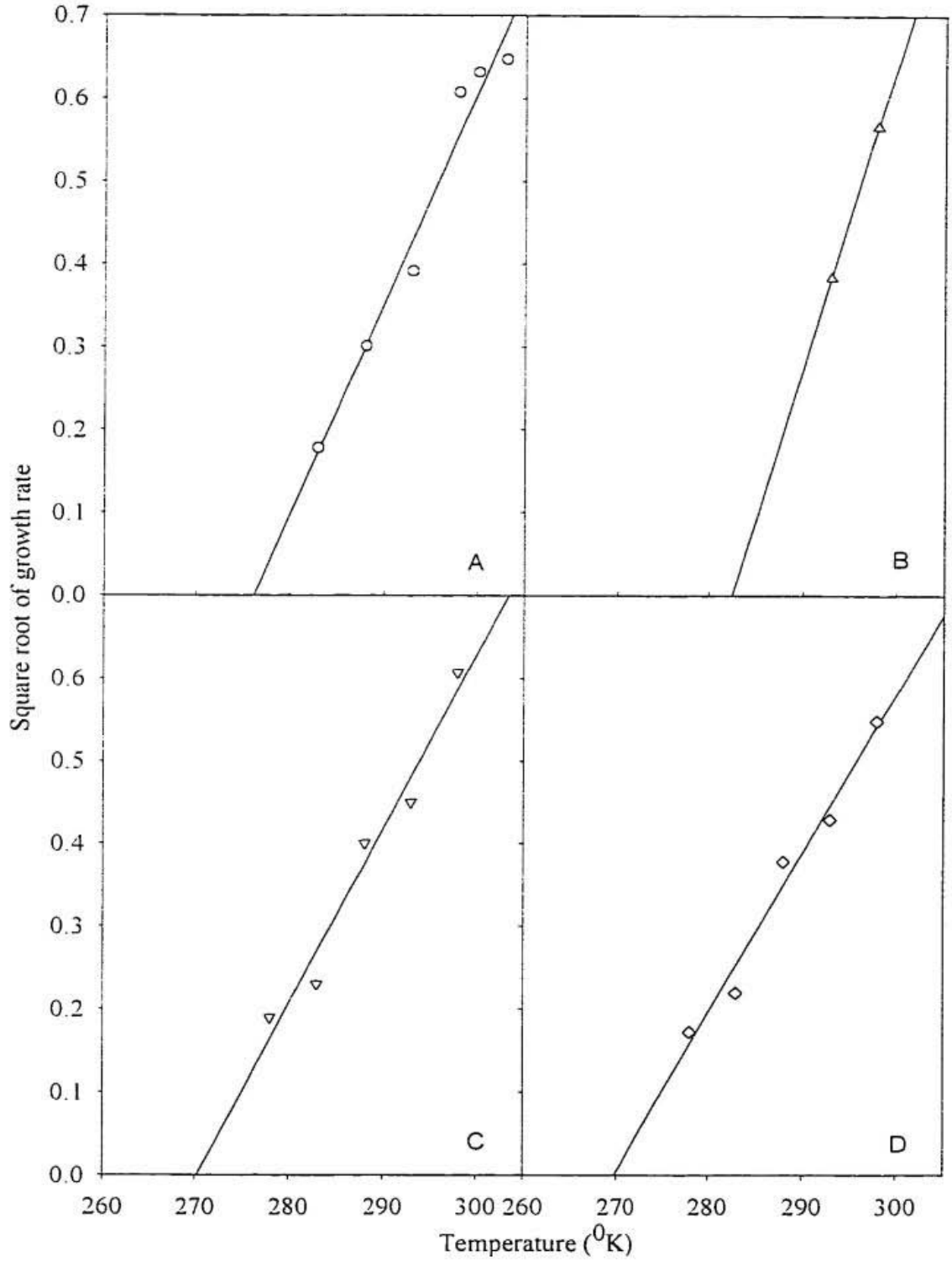

Growth rate
Temperature (t°C)
A
B
C
D
0.0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40

Table3: Generation time (hours) of *Xenorabdus* spp. at different temperatures

t°C	Replicate	*X. nematophilus* All strain	*X. bovienii* NF strain	*X. bovienii* Umeå strain	*X.* sp. TX strain
5	1		19.26	23.17	
	1	19.70	13.55	13.42	
10	2	23.06	11.82	14.00	
	3	22.98	14.31	15.50	
	mean	21.91±1.11[a*]	13.23±0.74[a]	14.31±0.62[a]	
	1	7.09	4.77	5.64	
15	2	7.64	4.05	4.38	
	3	8.27	4.21	4.62	
	mean	7.67±0.34[b]	4.34±0.22[b]	4.88±0.39[b]	
	1	4.38	2.74	3.94	4.65
20	2	4.91	3.85	3.64	4.93
	3	4.34	3.96	3.70	4.55
	mean	4.54±0.18[c]	3.52±0.39[bc]	3.76±0.09[c]	4.71±0.11[a]
	1	1.67	1.89	2.44	2.16
25	2	2.03	1.86	2.20	2.28
	3	1.97	1.90	2.27	2.06
	mean	1.89±0.11[d]	1.88±0.01[d]	2.30±0.07[d]	2.17±0.06[c]
	1	1.50	2.20	3.52	
27	2	1.87	2.60	2.80	
	3	1.90	2.69	2.92	
	mean	1.76±0.13[d]	2.50±0.15[cd]	3.08±0.22[cd]	
	1	1.61	4.26	5.68	2.17
30	2	1.76	5.15	5.03	2.24
	3	1.60	4.49	5.84	1.98
	mean	1.66±0.05[d]	4.63±0.27[b]	5.52±0.25[b]	2.13±0.08[c]
	1	4.26			3.04
35	2	3.85			2.91
	3	3.78			3.02
mean		3.96±0.15[c]			2.99±0.04[b]

*Values are the means ± SE (standard error). Means with the same small letter (down the single column) are not significantly different at P<0.05 by t-test (Sokal and Rohlf, 1995).

Fig. 3: Relationship between square root of growth rate and temperature in *Xenorhabdus* spp. A: *Xenorhabdus nematophilus* All strain; B: *Xenorhabdus* sp. TX strain; C: *Xenorhabdus bovienii* NF strain; D: *Xenorhbadus bovienii* Umeå strain. T_0 value is the intercept on the X-axis of the plot of the square roots of the growth rates against temperatures (≤optimal temperature) [$\sqrt{r} = b(T-T_0)$].


Square root of growth rate
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
B
C
D
260
270
280
290
300
Temperature (^{0}K)

Chapter 5

Effect of temperature on the distribution of isozymes in *Xenorhabdus* spp.

5.1. Abstract

The effect of temperature on the distribution of isozymes of seven enzymes was examined in four *Xenorhabdus* strains using cellulose acetate electrophoresis. *Xenorhabdus* sp. TX strain displayed temperature-related alterations in isozyme patterns for two enzymes [MDH: malate dehydrogenase (NAD^+); ME: malate dehydrogenase ($NADP^+$)], and *Xenorhabdus nematophilus* All strain for four enzymes [FUM (fumarate hydratase), MDH, PGI (phosphoglucose isomerase), PGM (phosphoglucomutase)]. The NF strain of *Xenorhabdus bovienii* expressed temperature-related changes in isozyme patterns for three enzymes [APK (arginine phosphokinase), MDH, PGI], and the Umeå strain of the same species for five enzymes (APK, FUM, ME, PGI, PGM). These results indicated that *Xenorhabdus* bacteria may adapt to temperature changes in their environment by modifying the synthesis of isozymes.

5.2. Introduction

Nematodes of the family Steinernematidae are being commercially produced as biological control agents against soil-dwelling or cryptic insect pests (Dunphy and Thurston, 1990; Smart, 1995). Bacteria of the genus *Xenorhabdus* are symbiotically associated with these nematodes, and carried in the specialized gut vesicles of the non-

feeding infective nematode juveniles (Akhurst and Boemare, 1990; Bird and Akhurst, 1983; Poinar, 1990). After infecting the insect hosts, the nematodes release the bacteria into the insect's haemocoel. Then, the bacteria multiply and release antibacterial and antifungal compounds into the insects' haemolymph, creating a non-competitive ambience for nematode and bacterium growth and reproduction (Akhurst, 1982; Nealson *et al.* , 1990; Poinar and Thomas 1966).

Despite the high pathogenicity of entomopathogenic nematodes to sensitive insect pests under ideal environmental conditions, their applications in pest management are limited by environmental factors (Kaya, 1990; Jagdale and Gordon, 1998b). Temperature influences the nematodes' survival, infection, growth and reproduction, and is considered as one of the most important factors limiting the practical uses of the nematodes (Griffin, 1993; Mason and Hominick, 1995; Grewal *et al.*, 1994; Jagdale and Gordon, 1998a). Therefore, knowledge of the temperature adaptation mechanisms of these nematodes and their bacterial associates is important in the research and the development of this category of nematodes.

It has been established that the nematodes are able to physiologically adapt to the temperatures at which they are recycled by changes in isozyme profiles (Jagdale and Gordon, 1998b), enzyme kinetics (Jagdale and Gordon, 1997b) and fatty acid composition (Jagdale and Gordon, 1997c). However, no such investigations have been made with respect to the bacterial associates, key contributors to overall pest management efficiency. In poikilothermic animals, long-term adjustment to differing temperature

regimes is often accomplished by synthesis of isozymes that are geared to function optimally at the ambient temperatures concerned (Lin *et al.*, 1995, Marcus, 1977). This possible strategy for temperature compensation has not been investigated for bacteria, however. The current study was done to investigate whether or not bacterial associates of three species of steinernematid nematodes synthesize isozymes in response to the temperatures at which they are cultured.

5.3. Materials and Methods:

5.3.1. Sources of bacteria and nematodes: *Xenorhabdus nematophilus* All strain was isolated from *Steinernema carpocapsae* All strain, which was provided by Plant Products Ltd. Brampton, Ontario, Canada. *Xenorhabdus bovienii* NF strain was isolated from *Steinernema feltiae* NF strain, which was isolated from the soil in St. John's, Newfoundland, Canada (Jagdale *et al.* 1996). *Xenorhabdus bovienii* Umeå was isolated from *S. feltiae* Umeå strain, which was provided by Dr. R. West, Canadian Forest Service (CFS), St. John's, Newfoundland, Canada from a stock colony that had been initially obtained from Biological Biocontrol Products, Willow Hill, PA. *Xenorhabdus* sp. TX strain was isolated from *Steinernema riobravis* TX strain, which was provided by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, Texas. All the nematode isolates had been recycled through *Galleria mellonella* larvae (Woodring and Kaya, 1988) at 20°C for 26 months before being used for bacterial isolation.

These bacteria were isolated and identified in August 1996. After that time, *X. bovienii* NF and Umeå strains were maintained on NBTA (nutrient agar with bromothymol blue dye and triphenoltetrazolium chloride) plates at 5°C, *X. nematophilus* All strain at 10°C, and *X.* sp. TX strain at 15°C with a subculture interval of around one month. They were maintained in this manner for about one year, then cultured for isozyme extraction. All of the four isolates were re-identified by means of a Biolog Microstation System™ periodically and just before the samples were prepared for enzyme analysis.

5.3.2. Preparation of bacterial culture and enzyme extraction:

The temperatures at which bacteria were cultured to make samples for isozyme analyses were 0°C, 5°C, 10°C, 20°C, 30°C for *X. bovienii* NF and Umeå strains, 10°C, 20°C, 30°C, and 35°C for *X. nematophilus* All strain, 20°C, 30°C and 35°C for *Xenorhabdus* sp. TX strain.

The bacteria in their primary phase on NBTA plates (primary phase: blue colonies; secondary phase: red or pink colonies) were used to inoculate 250-ml tryptic soy broth (TSB) (DIFCO, 0370-07-05) in 500-ml capped flasks (Kimax, Cat. No. 26505). They were cultured with shaking at 100 rpm, then harvested at the log phase (on the basis of optical density) of their growth by centrifugation at 14740 g at 4°C, followed by washing three times with 0.15M NaCl. Bacterial culture from each flask was considered to be one sample. Three replicates were prepared for each bacterial isolate at each specified temperature. Harvested bacteria were stored at -20°C for 1~2 days. The sample for each

replicate was resuspended in a small glass test tube with 2-3 ml buffer (0.09M Tris HCl, pH 9.00). To avoid enzyme degradation, the small test tube with bacterial suspension was put into ice while cells were ruptured with a Braun-Sonic 2000 sonicator. The suspension produced was centrifuged at 24,790 g at 2°C for 20minutes. The enzyme extracts (supernatant) were transferred into 1.5-ml polypropylene microcentrifuge tubes and immediately frozen at -70°C until used for electrophoresis.

5.3.3. Cellulose acetate electrophoresis: Cellulose acetate plates (Cat.# 3033, Helena Laboratories, Beaumont, TX) were pre-soaked for at least 20 minutes with Tris-Glycine buffer (3.0g Tris, 14.4g glycine, 1L distilled water, pH 8.5) before samples were loaded onto them. Ten millilitres aliquots from each sample were placed in each well in a sample holder and maintained on ice. Then, a 0.2-0.4 µl aliquot of each sample was loaded, based on the enzyme sensitivity to staining, onto a Titan III Zip Zone Cellulose Acetate Plate with a Super Z Applicator (Helena Laboratories, Beaumont, TX), and the plates laid in a horizontal electrophoresis chamber containing Tris-Glycine buffer. Electrophoresis lasted for 14~20 minutes at room temperature(20~25°C) with 1.5 mA current per plate.

5.3.4. Enzyme staining: The procedure recommended by Hebert & Beaton (1989) was used for the staining of the following enzymes: arginine phosphokinase (APK), fumarate hydratase (FUM), malate dehydrogenase*(NAD⁻)*(MDH), malate dehydrogenase*(NADP⁻)* (ME), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGDH) and phosphoglucose isomerase (PGI). After electrophoresis, the plates were removed from the chamber and laid on a horizontal glass surface. The appropriate staining mixture

containing liquified agar was poured over the plates. The plates were kept in the dark for about 10~15 minutes for the development of isozyme banding patterns, then rinsed with tap water, soaked in distilled water for 10 minutes, and fixed (acetic acid:methanol:distilled water—1:4:10) for 10~15 minutes. The plates were left to dry overnight in the dark and photographed, and the bands were measured for the migration rates of isozyme bands. The relative electrophoretic motility (μ: $cm^2/s/v$) for each enzyme was used to compare the migration rates (Lehninger, 1979). Based on the criterion recommended by Jagdale *et al.* (1996), isozyme bands among isolates were considered to be the same if their electrophoretic motility (μ) values varied within 10% of one another.

5.4. Results:

1. Arginine phosphokinase (APK): Its isozyme banding patterns were not temperature-related for *X. nematophilus* All strain and *X.* sp. TX strain. However, they were influenced by temperature for the NF and Umeå strain of *X. bovienii* (Table 1). At temperatures $\leq 5^0$C, the NF strain displayed an additional anodal band, and the Umeå strain expressed an extra cathodal band (Band 3) .

2. Fumarate hydratase (FUM): The isozyme banding patterns of this enzyme were not affected by temperatures for *X. bovienii* NF strain and *X.* sp. TX strain while they were temperature-related for *X. bovienii* Umeå strain and *X. nematophilus* All strain (Table 1). At the highest temperature examined (30^{0}C), the Umeå strain of *X. bovienii* displayed an additional cathodal band. The All strain of *X. nematophilus* had the same

isozyme profile at 30°C and 35°C. At culture temperatures ≤20°C, an additional cathodal band (Band 3) was present, which supplanted the slowest moving cathodal band (Band 1) at the lowest temperature (10°C) (Fig. 1, Table 1).

3. Malate dehydrogenase *(NAD)*(MDH): The isozyme patterns were influenced by temperature for *X. bovienii* NF strain, *X. nematophilus* All strain and *X.* sp. TX strain. However, temperature did not affect the patterns for *X. bovienii* Umeå strain, which showed an anodal band and a cathodal band at all five temperatures. *Xenorhabdus bovienii* NF strain displayed an additional cathodal band (Band 2) at the highest temperature 30°C at which it was cultured. The All strain of *X. nematophilus* displayed one fewer cathodal band at 10°C than at the higher culture temperatures. The TX strain of the unidentified *Xenorhabdus* speices displayed one extra cathodal band at the highest temperature (35°C) and one fewer anodal band at the lowest temperature (20°C) compared with the intermediate culture temperature (30°C) (Fig. 3, Table 1).

4. Malate dehydrogenase *($NADP^+$)*(ME): The isozyme patterns of this enzyme were not affected by temperature for *X. nematophilus* All strain and *X. bovienii* NF strain, but were temperature-related for *X.* sp. TX strain and *X. bovienii* Umeå strain. The strains that showed such temperature sensitivity synthesized one fewer isozyme at the lower end of the temperature range at which they were cultured. *Xenorhabdus* sp. TX strain lacked an anodal band at 20°C, which was present at 30°C and 35°C. *Xenorhabdus bovienii* Umeå strain, cultured at ≤5°C, lacked one of the cathodal bands that bacteria cultured at higher temperatures contained (Fig. 4, Table1).

5. Phosphoglucomutase (PGM): The banding patterns were temperature-independent for *X. bovienii* NF strain and *X.* sp. TX strain, but were temperature-dependent for *X. nematophilus* All strain and *X. bovienii* Umeå strain. The All strain of *X. nematophilus* displayed one fewer cathodal band at the lowest temperature (10°C) than at higher ones. The Umeå strain of *X. bovienii* synthesized an additional (cathodal) isozyme when cultured at temperature ≤5°C (Fig. 5, Table 1).

6. 6-phosphogluconate dehydrogenase (6PGDH): The isozyme patterns were not influenced by temperatures for all the four strains, all of which showed two isozyme bands (Fig. 2, Table 1).

7. Phosphoglucose isomerase (PGI): The patterns of this enzyme were unaffected by culture temperature in *X.* sp. TX strain, but temperature-dependent for *X. bovienii* NF and Umeå strains and *X. nematophilus* All strain. At 30°C, the NF strain of *X. bovienii* failed to synthesize the isozyme represented by an anodal band, which was produced at lower temperatures. The Umeå strain of this species synthesized one additional isozyme (cathodal) at ≤5°C. The All strain of *X. nematophilus* displayed temperature-related differences in isozyme profiles at both ends of the temperature range at which it was cultured. At the highest temperature (35°C), one fewer cathodal band was present, while at the lowest temperature (10°C), this same isozyme, together with another cathodal one, was absent (Fig. 6, Table 1).

5.5. Discussion

The four strains of *Xenorhabdus* bacteria responded to differing culture temperatures by modifying the synthesis of isozymes. The Umeå strain of *X. bovienii* showed temperature-related changes in isozyme patterns for five enzymes (APK, FUM, ME, PGI, PGM), the All strain of *X. nematophilus* for four enzymes (FUM, MDH, PGI, PGM), the NF strain of *X. bovienii* for three enzymes (APK, MDH, PGI), and the TX strain of the unidentified *Xenorhabdus* sp. for two enzymes (MDH, ME). The enzyme MDH displayed temperature-related difference in isozyme patterns for three strains (All, NF, and TX), PGI for three strains (All, NF and Umeå), both FUM and PGM for two strains (All and Umeå), APK for two strains (NF and Umeå), and ME for two strains (TX and Umeå). None of the four strains showed temperature-related isozyme patterns for 6PGDH.

It is generally accepted that poikilothermic animals, in response to their environmental temperature, may produce different isozyme patterns as a temperature adaptation mechanism (Lin *et al.*, 1995; Yamawaki and Tsukuda, 1979; Marcus, 1977). In a recent study, Jagdale and Gordon (1998b) showed that steinernematid nematodes, recycled at different temperatures, displayed differences in the isozyme profiles of several metabolic enzymes. These authors concluded that while some of the changes in isozyme complement were environmentally-induced, genetic drift may have been at least partially responsible for the differences among nematode cultures. In the current study, genetic

effects can be effectively ruled out because all of the replications among all of the temperatures emanated from the same stock colonies on the NBTA plates.

In some instances, recycling temperatures affected isozyme banding patterns of steinernematid enzymes in an apparently adaptive fashion, while in other instances, no discernable trend was apparent and the effects were likely the results of inadvertent genetic mutations (Jagdale and Gordon, 1998b). In the current study, isozymes were gained or lost at either extreme of the temperature range at which a given isolate was cultured. Although it is possible that some of these effects may be the result of thermal stress, they are more readily explicable as being adaptive in nature. Thus, the presence of an additional isozyme of FUM by the Umeå strain of *X. bovienii* (30^0C) and of an additional isozyme of MDH by the NF strain of *X. bovienii* (30^0C), the All strain of *X. nematophilus* ($\geq 20^0$C) and the TX strain of *Xenorhabdus* sp. (35^0C) may be considered as adaptive response to the warmer temperatures. Conversely, the additional isozymes of APK in the NF and Umeå strains of *X. bovienii* ($\leq 5^0$C), and the additional isozymes of PGI and PGM in *X. bovienii* Umeå strain ($\leq 5^0$C), as well as the replacement of the isozymes of FUM in *X. nematophilus* All strain (10^0C) may be interpreted as adaptations to cold temperatures, involving the synthesis of cold adapted isozymes. In some instances, fewer isozymes were synthesized at colder temperatures (eg. ME and MDH in *X.* sp. TX strain, *X. bovienii* Umeå strain PGM and PGI in *X. nematophilus* All strain). This may suggest that the isozymes no longer synthesized may be ill-suited to cold

temperatures, so that it would be advantageous to redirect synthesis toward other enzymes.

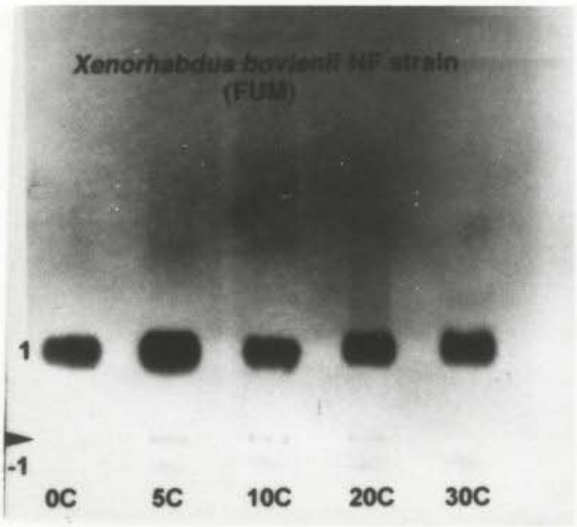
Given that all four strains of bacteria displayed temperature related changes in isozyme profiles and that the presence or absence of an isozyme could be interpreted as being adaptive, it is not possible to evaluate these physiological responses among strains in terms of their *relative* capacities for heat or cold adaptation. Further studies would be required, including isolation and characterization of the isozymes concerned, in order to make such comparisons. Superficially, the data suggest that all four isolates, with geographic origins ranging from boreal (*X. bovienii*), temperate (*X. nematophilus)* to subtropical (*Xenorhabdus* sp.) may display thermal adapation at the physiological level.

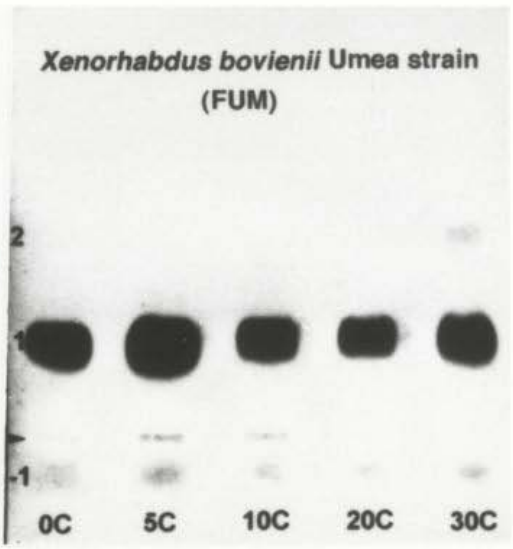
Therefore, this study suggests that entomopathogenic bacteria may physiologically adjust to temperature by altering the synthesis of isozymes. Such a mechanism for temperature compensation would complement other strategies which bacteria are known to use. For example, *Pseudomonas fluorescens* increases the synthesis of its enzymes to compensate for the reduced enzyme activities at low temperature (Margesin *et al.*, 1992). To adapt to their cold environment, bacteria may alter the molecular structure of their enzymes in order to lower the activation energy (Davail *et al.*, 1994; Feller *et al.*, 1996; Narinx *et al.*, 1992). Also, in response to temperature, bacteria may adjust their fatty acid composition (Clarke and Dowds, 1994; Fulco, 1970; Margesin *et al.* 1994, Suutari *et al.*,1994), and synthesize special proteins such as cold shock proteins and cold acclimation proteins (Araki, 1991; Gumley *et al.*, 1996; Jones *et al.*, 1987). *Pseudomonas*

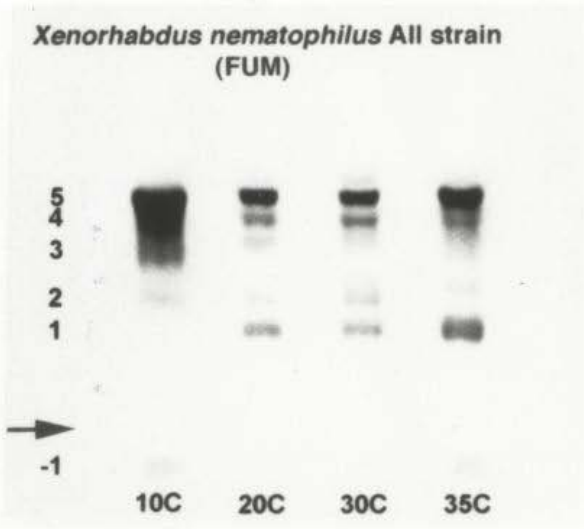
syringae may adjust the permeability of its outer membrane in response to low temperature by reducing the phosphoration of its lipopolysacchride (Ray *et al.*,1994).

Regardless of the biological significance of the temperature-induced variations in isozyme profiles, the fact that they did occur suggests that isozyme banding patterns would have to be used cautiously for the purpose of taxonomy of this group of bacteria. Enzymes (eg. 6PGDH), in which isozyme banding patterns were independent of temperature, would have to be used or in the case of enzymes that are temperature sensitive, would have to be carefully controlled and reported.

Fig 1: Electropherograms of fumarate hydratase (FUM) in four *Xenorhabdus* strains belonging to three species at different temperatures. Arrow heads indicate the the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.


Xenorhabdus bovienii NF strain
(FUM)
1
-1
0C
5C
10C
20C
30C


Xenorhabdus bovienii Umea strain
(FUM)
2
1
-1
0C
5C
10C
20C
30C


Xenorhabdus nematophilus AII strain
(FUM)
5
4
3
2
1
-1
10C
20C
30C
35C

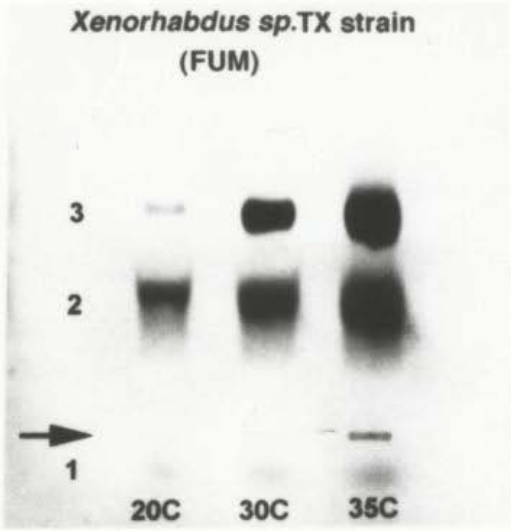
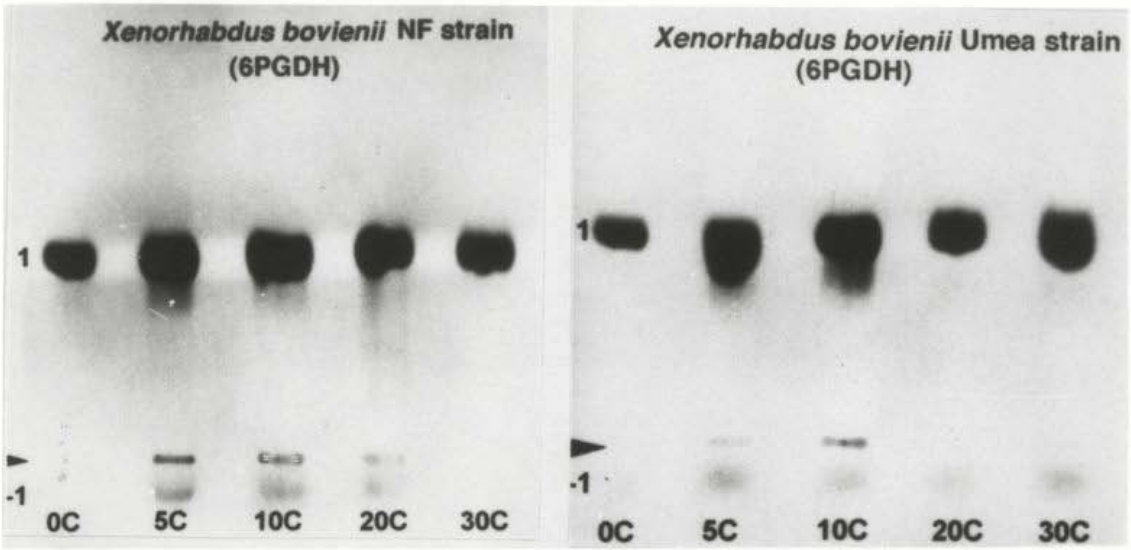

Xenorhabdus sp.TX strain
(FUM)
3
2
1
20C
30C
35C

Fig 2: Electropherograms of 6-phosphogluconate dehydrogenase (6PGDH) in four *Xenorhabdus* strains belonging to three species at different temperatures. Arrow heads indicate the the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.


Xenorhabdus bovienii NF strain
(6PGDH)
1
-1
0C
5C
10C
20C
30C
Xenorhabdus bovienii Umea strain
(6PGDH)
1
-1
0C
5C
10C
20C
30C

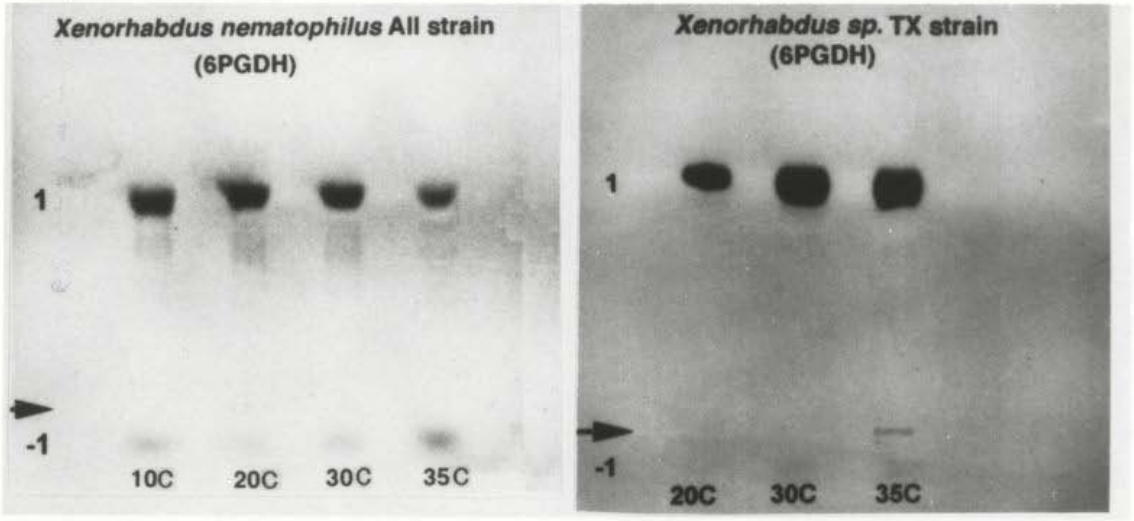
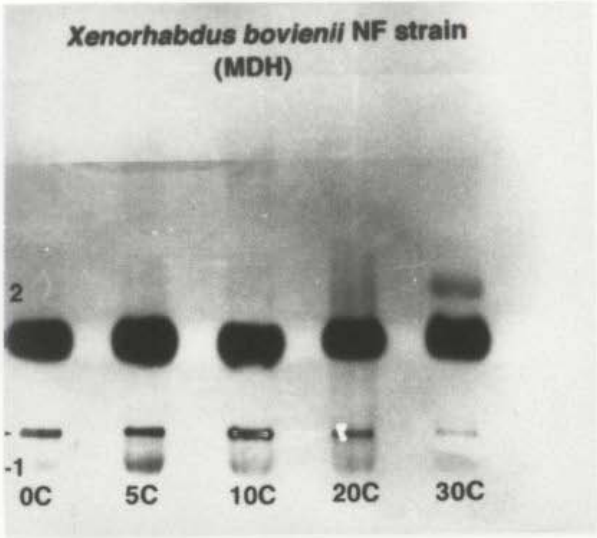
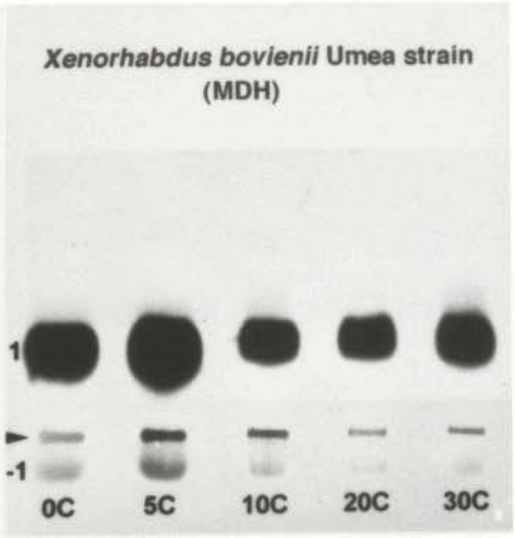
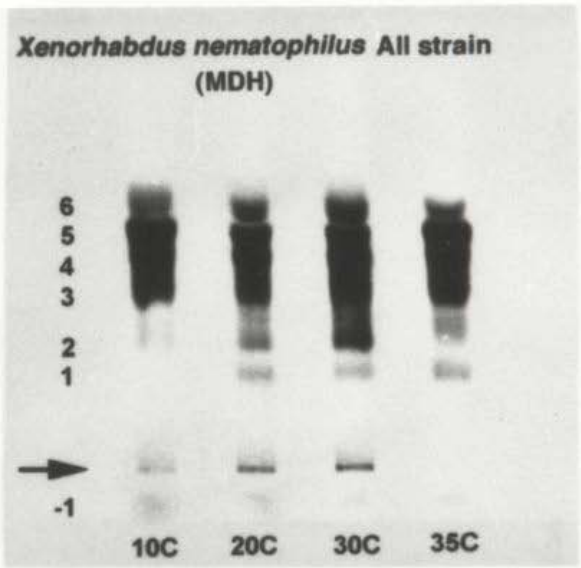

Xenorhabdus nematophilus AII strain
(6PGDH)
1
-1
10C
20C
30C
35C
Xenorhabdus sp. TX strain
(6PGDH)
1
-1
20C
30C
35C

Fig 3: Electropherograms of malate dehydrogenase (NAD^+) (MDH) in four *Xenorhabdus* strains belonging to three species at different temperatures. Arrow heads indicate the the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.


Xenorhabdus bovienii NF strain
(MDH)
2
-1
0C
5C
10C
20C
30C


Xenorhabdus bovienii Umea strain
(MDH)
1
-1
0C
5C
10C
20C
30C


Xenorhabdus nematophilus All strain
(MDH)
6
5
4
3
2
1
-1
10C
20C
30C
35C

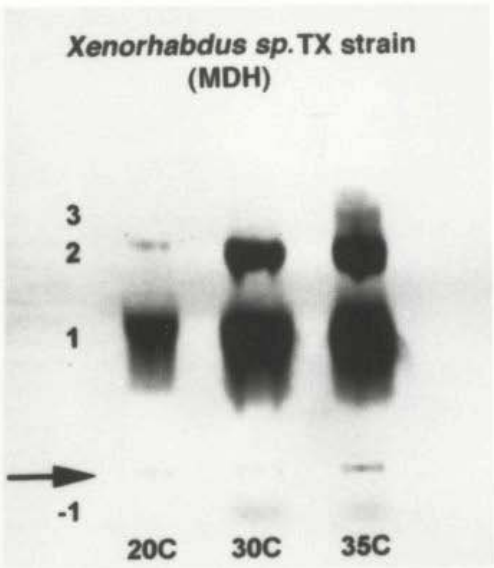
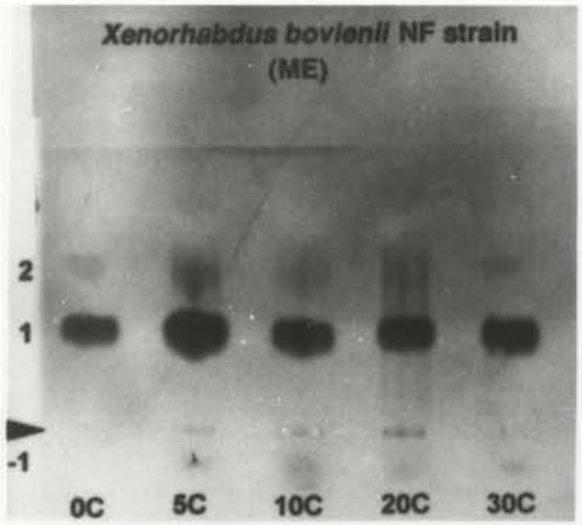
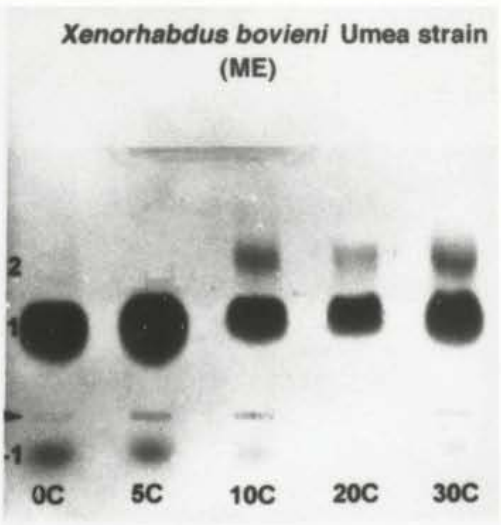
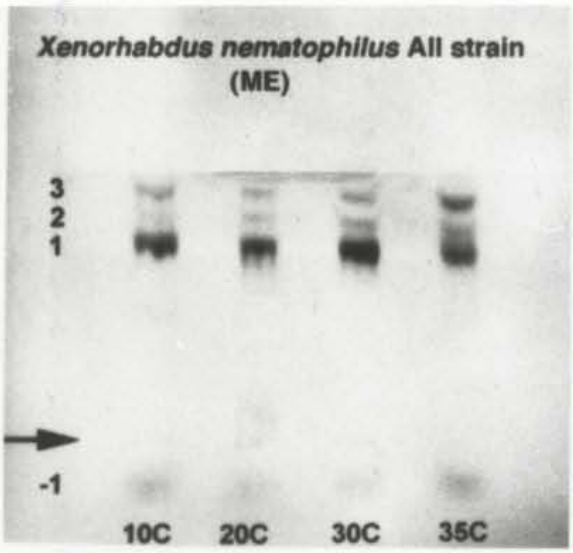

Xenorhabdus sp. TX strain
(MDH)
3
2
1
-1
20C
30C
35C

Fig 4: Electropherograms of malate dehydrogenase ($NADP^+$) (ME) in four *Xenorhabdus* strains belonging to three species at different temperatures. Arrow heads indicate the the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.


Xenorhabdus bovienii NF strain
(ME)
2
1
-1
0C
5C
10C
20C
30C


Xenorhabdus bovieni Umea strain
(ME)
2
1
-1
0C
5C
10C
20C
30C


Xenorhabdus nematophilus All strain
(ME)
3
2
1
-1
10C
20C
30C
35C

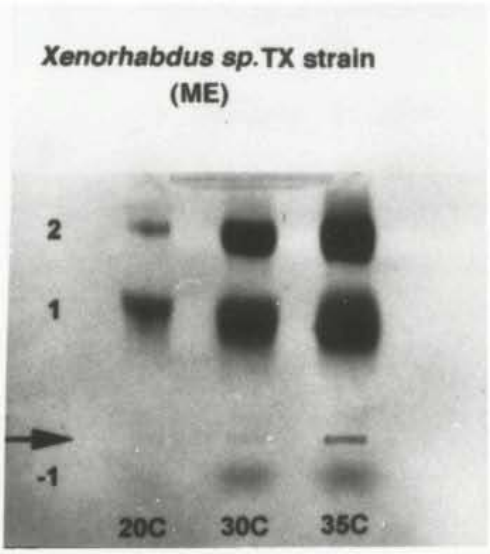
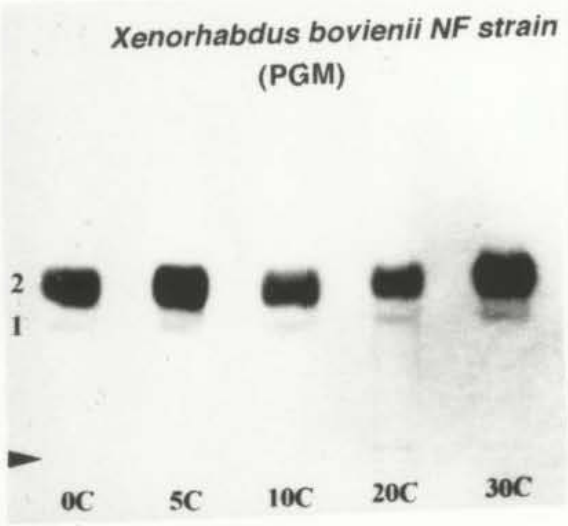
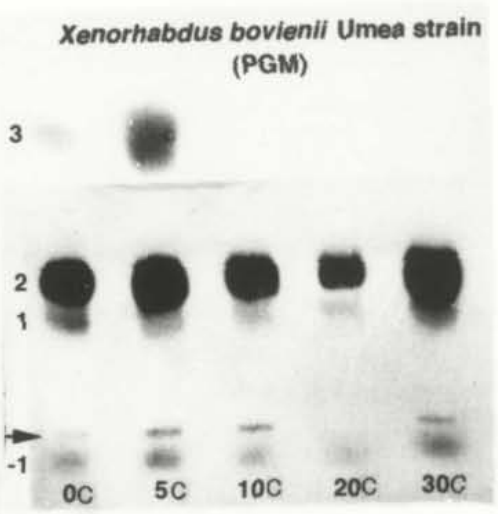
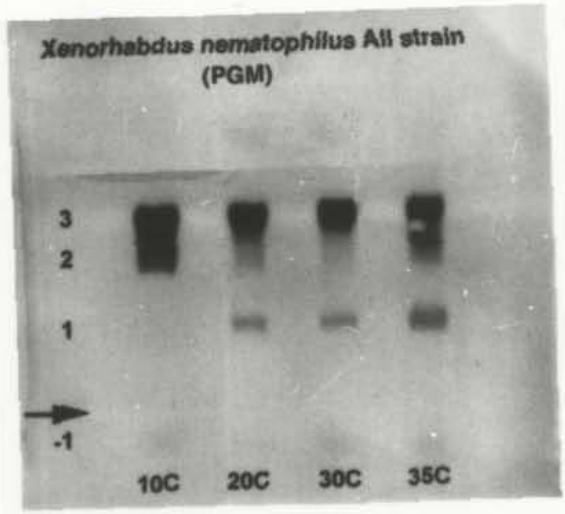

*Xenorhabdus sp.*TX strain
(ME)
2
1
-1
20C
30C
35C

Fig. 5: Electropherograms of phosphoglucomutase (PGM) in four *Xenorhabdus* strains belonging to three species at different temperatures. Arrow heads indicate the the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.


Xenorhabdus bovienii NF strain
(PGM)
2
1
0C
5C
10C
20C
30C


Xenorhabdus bovienii Umea strain
(PGM)
3
2
1
-1
0C
5C
10C
20C
30C


Xenorhabdus nematophilus All strain
(PGM)
3
2
1
-1
10C
20C
30C
35C

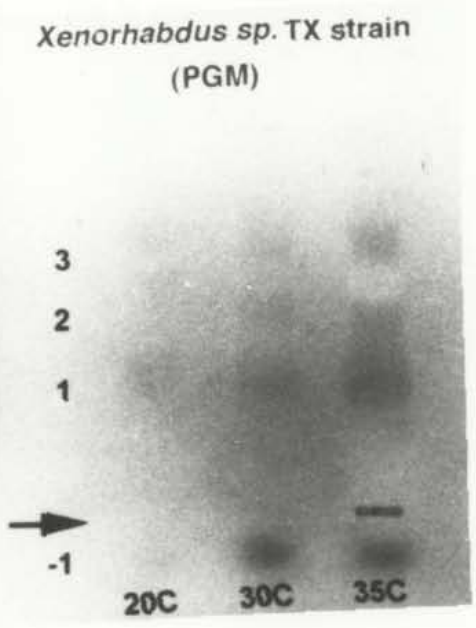

Xenorhabdus sp. TX strain
(PGM)
3
2
1
-1
20C
30C
35C

Fig. 6: Electropherograms of phosphoglucose isomerase (PGI) in four *Xenorhabdus* strains belonging to three species at different temperatures. Arrow heads indicate the the line of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated from the original line of sample application.

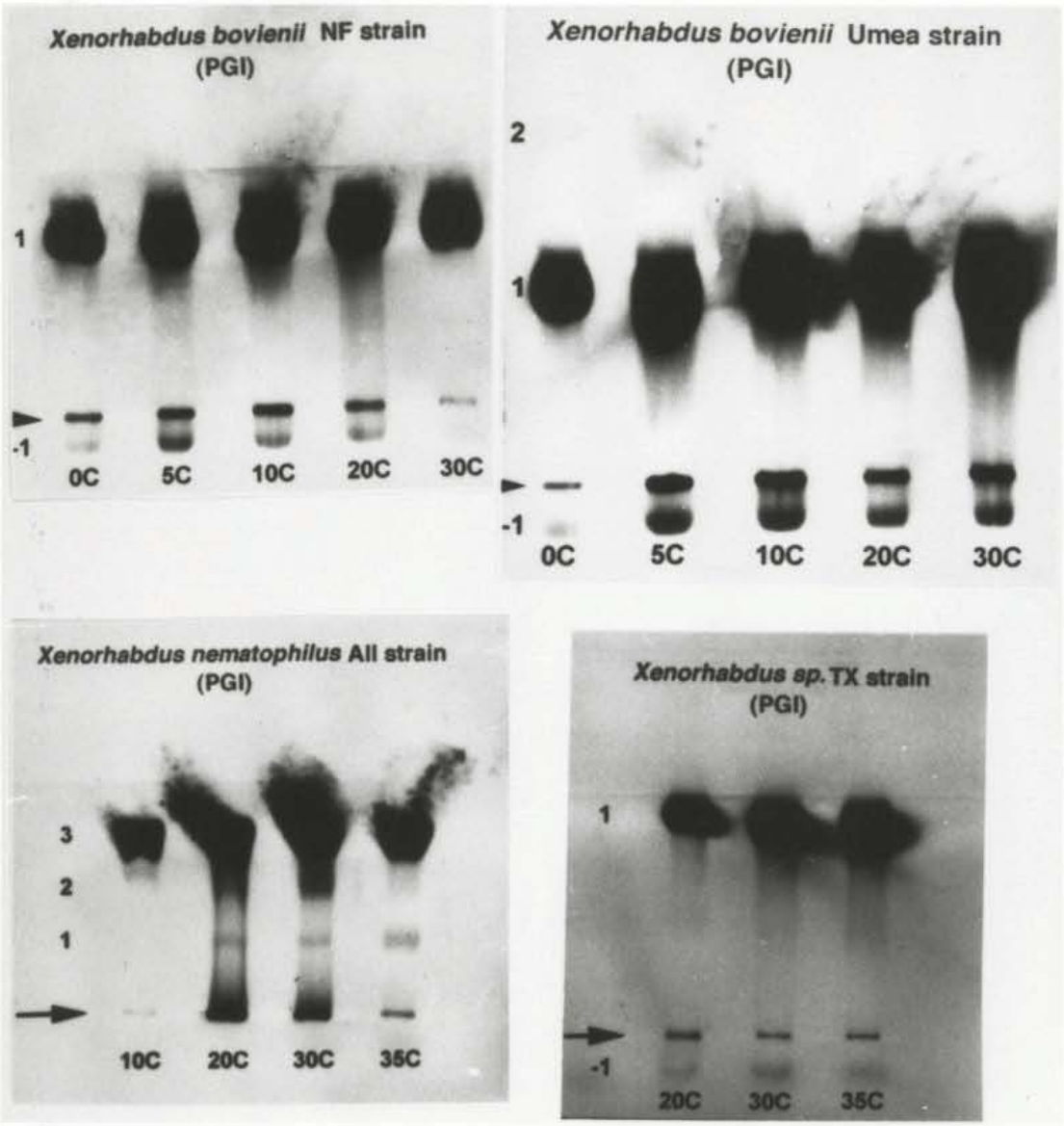

Xenorhabdus bovienii NF strain
(PGI)
1
-1
0C
5C
10C
20C
30C
Xenorhabdus bovienii Umea strain
(PGI)
2
1
-1
0C
5C
10C
20C
30C
Xenorhabdus nematophilus AII strain
(PGI)
3
2
1
10C
20C
30C
35C
Xenorhabdus sp. TX strain
(PGI)
1
-1
20C
30C
35C

Table 1: Effect of temperature on the isozyme patterns of seven enzymes in *Xenorhabdus* spp.*

bands	10°C	20°C	30°C	35°C
		***Xenorhabdus nematophilus* All strain**		
		Arginine phosphokinase (APK)		
1	1.543†	1.543	1.543	1.543
2	1.886	1.851	1.851	1.851
3	2.194	2.160	2.160	2.160
4	2.503	2.468	2.468	2.503
5	4.148	4.114	4.148	4.114
		Fumarate hydratase (FUM)		
-1	0.514	0.514	0.514	0.514
1	-	1.234	1.234	1.131
2	1.543	1.611	1.543	1.543
3	2.160	2.228	-	-
4	2.537	2.606	2.571	2.571
5	2.880	2.914	2.880	2.880
		Malate dehydrogenase *(NAD⁺)*(MDH)		
-1	0.514	0.514	0.514	0.514
1	-	1.166	1.131	1.166
2	1.474	1.543	1.577	1.577
3	2.126	2.126	2.126	2.194
4	2.606	2.606	2.606	2.640
5	3.017	2.983	3.017	2.983
6	3.428	3.394	3.394	3.394

Table 1: Effect of temperature on the isozyme patterns of seven enzymes in *Xenorhabdus* spp.*(continued)

bands	10°C	20°C	30°C	35°C
		***Xenorhabdus nematophilus* All strain**		
		Malate dehydrogenase *(NADP⁻)*(ME)		
-1	0.446†	0.446	0.411	0.411
1	2.331	2.366	2.468	2.468
2	2.777	2.708	2.708	2.708
3	3.188	3.257	3.120	3.154
		Phosphoglucomutase (PGM)		
-1	0.446	0.411	0.411	0.411
1	-	1.509	1.440	1.440
2	2.468	2.468	2.434	2.468
3	2.983	2.983	2.983	2.983
		6-phosphogluconate dehydrogenase (6PGDH)		
-1	0.480	0.480	0.480	0.480
1	2.880	2.880	2.880	2.880
		Phosphoglucose isomerase (PGI)		
1	-	1.097	1.166	1.131
2	-	2.023	1.989	-
3	2.743	2.777	2.777	2.811

*:Bands were considered the same if their electrophoretic motility values were within 10% of one another. Negative bands (numbered as -1) means that enzymes electrophoretically move towards anode while positive bands towards cathode. Dashes means the absence of bands.

†: Motility values ($cm^2/sec/v$) are expressed as 1×10^{-4} times, and are the mean of three samples, each from a separate culture flask.

Table 1: Effect of temperature on the isozyme patterns of seven enzymes in *Xenorhabdus* spp.* (Continued)

bands	0°C	5°C	10°C	20°C	30°C
		***Xenorhabdus bovienii* NF strain**			
		Arginine phosphokinase (APK)			
-1	0.617†	0.617	-	-	-
1	1.029	1.029	0.960	0.960	0.994
2	3.634	3.668	3.600	3.600	3.600
		Fumarate hydratase (FUM)			
-1	0.309	0.309	0.309	0.309	0.309
1	1.234	1.234	1.234	1.234	1.234
		Malate dehydrogenase *(NAD)*(MDH)			
-1	0.411	0.446	0.446	0.446	0.446
1	1.234	1.131	1.131	1.131	1.200
2	-	-	-	-	1.817
		Malate dehydrogenase *($NADP^-$)*(ME)			
-1	0.480	0.480	0.514	0.514	0.480
1	1.097	1.063	1.097	1.131	1.166
2	1.886	1.920	1.886	1.920	1.954

Table 1: Effect of temperature on the isozyme patterns of seven enzymes in *Xenorhabdus* spp.* (Continued)

bands	0°C	5°C	10°C	20°C	30°C
		***Xenorhabdus bovienii* NF strain**			
		Phosphoglucomutase (PGM)			
1	1.714†	1.680	1.680	1.680	1.680
2	2.194	2.194	2.160	2.194	2.194
		6-phosphogluconate dehydrogenase (6PGDH)			
-1	0.480	0.480	0.480	0.480	4.800
1	2.503	2.503	2.503	2.503	2.503
		Phosphoglucose isomerase (PGI)			
-1	0.411	0.411	0.411	0.411	-
1	2.571	2.468	2.468	2.537	2.571

*:Bands were considered the same if their electrophoretic motility values were within 10% of one another. Negative bands (numbered as -1) means that enzymes electrophoretically move towards anode while positive bands towards cathode. Dashes means the absence of bands.

†: Motility values ($cm^2/sec/v$) are expressed as 1×10^{-4} times, and are the mean of three samples, each from a separate culture flask.

Table 1: Effect of temperature on the isozyme patterns of seven enzymes in *Xenorhabdus* spp.* (Continued)

bands	0°C	5°C	10°C	20°C	30°C
		***Xenorhabdus bovienii* Umeå strain**			
		Arginine phosphokinase (APK)			
-1	0.617†	0.617	0.617	0.617	0.617
1	0.857	0.857	0.857	0.857	0.857
2	3.326	3.360	3.394	3.394	3.394
3	5.554	5.520	-	-	-
		Fumarate hydratase (FUM)			
-1	0.514	0.514	0.514	0.514	0.514
1	1.131	1.166	1.166	1.200	1.200
2	-	-	-	-	2.400
		Malate dehydrogenase *(NAD⁻)*(MDH)			
-1	0.480	0.480	0.480	0.480	0.514
1	1.097	1.131	1.097	1.166	1.166
		Malate dehydrogenase *(NADP⁻)*(ME)			
-1	0.514	0.514	0.514	0.514	0.514
1	1.131	1.131	1.131	1.166	1.234
2	-	-	1.954	1.954	1.989

Table 1: Effect of temperature on the isozyme patterns of seven enzymes in *Xenorhabdus* spp.*(Continued)

bands	0°C	5°C	10°C	20°C	30°C
		***Xenorhabdus bovienii* Umeå strain**			
		Phosphoglucomutase (PGM)			
-1	0.446†	0.480	0.480	0.480	0.480
1	1.543	1.509	1.509	1.543	1.509
2	2.160	2.091	2.091	2.160	2.057
3	4.251	4.251	-	-	-
		6-phosphogluconate dehydrogenase (6PGDH)			
-1	0.480	0.446	0.446	0.480	0.480
1	2.297	2.263	2.297	2.400	2.297
		Phosphoglucose isomerase (PGI)			
-1	0.514	0.514	0.514	0.514	0.514
1	2.297	2.263	2.297	2.434	2.400
2	4.217	4.217	-	-	-

*:Bands were considered the same if their electrophoretic motility values were within 10% of one another. Negative bands (numbered as -1) means that enzymes electrophoretically move towards anode while positive bands towards cathode. Dashes means the absence of bands.

†: Motility values ($cm^2/sec/v$) are expressed as 1×10^{-4} times, and are the mean of three samples, each from a separate culture flask.

Table 1: Effect of temperature on the isozyme patterns of six enzymes in *Xenorhabdus* spp.(Continued)

bands	20°C	30°C	35°C
		***Xenorhabdus.* sp. TX strain**	
		Arginine phosphokinase (APK)	
1	2.023[+]	2.023	1.954
2	2.331	2.331	2.263
3	3.051	2.948	2.948
4	4.183	4.148	4.114
		Fumarate hydratase (FUM)	
-1	0.411	0.411	0.411
1	1.749	1.680	1.680
2	2.777	2.708	2.708
		Malate dehydrogenase *(NAD)*(MDH)	
-1	-	0.411	0.411
1	1.749	1.714	1.680
2	2.846	2.777	2.708
3	-	-	3.257
		Malate dehydrogenase *($NADP^+$)*(ME)	
-1	-	0.5143	0.514
1	1.509	1.440	1.440
2	2.640	2.503	2.537

Table 1: Effect of temperature on the isozyme patterns of six enzymes in *Xenorhabdus* spp.* (Continued)

bands	20°C	30°C	35°C
		***Xenorhabdus.* sp. TX strain**	
		Phosphoglucomutase (PGM)	
-1	0.411†	0.411	0.411
1	1.440	1.440	1.440
2	2.160	2.160	2.160
3	2.914	2.914	2.983
		6-phosphogluconate dehydrogenase (6PGDH)	
-1	0.480	0.480	0.514
1	3.463	3.463	3.463
		Phosphoglucose isomerase (PGI)	
-1	0.514	0.514	0.514
1	2.983	3.086	3.154

*:Bands were considered the same if their electrophoretic motility values were within 10% of one another. Negative bands (numbered as -1) means that enzymes electrophoretically move towards anode while positive bands towards cathode. Dashes means the absence of bands.

†: Motility values ($cm^2/sec/v$) are expressed as 1×10^{-4} times, and are the mean of three samples, each from a separate culture flask.

Chapter 6

Effect of temperature on the composition of fatty acids in total lipids of four bacterial strains of the genus *Xenorhabdus*

6.1. Abstract

The impact of temperature on the fatty acid compositions of total lipids was studied for four strains of *Xenorhbadus*, isolated from nematodes that were initially recovered from various geographical areas: *Xenorhabdus bovienii* NF strain (boreal origin), *X. bovienii* Umeå strain (boreal origin), *Xenorhabdus nematophilus* All strain (temperate origin), and *Xenorhabdus* sp. TX strain (subtropical origin). The most prominent fatty acids were palmitic (16:0), palmitoleic ($16:1_{\omega}7$) and oleic ($18:1_{\omega}9$) acids in all four strains. As temperature declined, all of the strains significantly increased the proportions of the two major unsaturated fatty acids ($16:1_{\omega}7$, $18:1_{\omega}9$), with concomitant decreases in the prevalent saturated fatty acid (16:0). Additionally, four other saturated fatty acids (14:0, 17:0, $17:0_{c}$, 20:0) significantly declined in three strains (All, NF, Umeå) as temperature decreased, while they did not significantly vary in the TX strain from 35°C to 20°C. These results indicated that *Xenorhabdus* bacteria may respond to environmental temperature variations by modifying the degree of fatty acid unsaturation.

6.2. Introduction

Xenorhabdus bacteria are mutualistically associated with nematodes of the genus *Steinernema*. The bacteria are vectored into the insects' hemocoel by infective juveniles

of the nematodes. The insects are killed by the combined pathogenicity of the bacteria and the nematodes, although the bacteria are considered to be the major killers (Akhurst and Dunphy, 1993). Then, the bacteria and the nematodes continue to reproduce on the insect cadavers. After proliferation for about three generations, new combined bacteria-nematode complexes leave the cadavers and search for new hosts (Poinar, 1990).

Effective use of the nematode depends on the capacity of both the nematode and the bacterium to perpetuate their life cycles in the temperature regimes into which they are administered (Grewal et al., 1994; Grewal et al., 1996; Gwynn and Richardson, 1994; Molyneux, 1986; Clarke and Dowds, 1994). Temperature optima of various nematode species and strains are correlated with their geographic origins, indicating that they are adapted to the conditions in their natural environment. The nematodes usually function best at the temperatures that typify the habitats from which they originated (Griffin, 1993; Molyneaux, 1986).

The nematodes have been shown to possess physiological mechanisms to adjust to changing temperature. One mechanism is to alter the degree of fatty acid saturation (Jagdale and Gordon, 1997c; Abu Hatab and Gaugler, 1997b). However, the capacity and mechanisms of bacteria to adapt to varying temperature are poorly understood. This study was done to compare the physiological capacities of four strain of bacteria to adjust to temperature by changes in fatty acids.

6.3. Materials and Methods

6.3.1. Bacterial sources and maintenance: *Xenorhabdus nematophilus* All strain was isolated from *Steinernema carpocapsae* All strain, which was provided originally by Plant Products Ltd. Brampton, Ontario, Canada. *Xenorhabdus bovienii* NF strain was isolated from *Steinernema feltiae* NF strain, which was isolated from soil in an organic garden near St. John's, Newfoundland, Canada (Jagdale et al.1996). *Xenorhabdus bovienii* Umeå strain was isolated from *S. feltiae* Umeå strain, which was provided originally by Dr. R. West, Canadian Forest Service (CFS), St. John's, Newfoundland, Canada from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA. *Xenorhabdus* sp. TX strain was isolated from *Steinernema riobravis* TX strain, originally provided by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, Texas. All the nematode isolates were recycled through *Galleria mellonella* larvae (Woodring and Kaya, 1988) at 20°C for 26 months (Jagdale et al., 1996) before being used for bacterial isolation. After isolation (see Chapter 2) and identification, *X. bovienii* NF and Umeå strains were maintained on NBTA plates at 5°C, *X. nematophilus* All strain at 10°C, and *X.* sp. TX strain at 15°C with a subculture interval of approximately one month. They had been maintained for about one year by the time that this study was carried out. The purity of all the four isolates was identified using a Biolog Microstation System™ periodically and just before this study.

6.3.2. Culture temperatures and preparation of bacterial culture:

Culture temperatures were 10^0, 20^0, 30^0 and 35^0C for *X. nematophilus* All strain, 20^0, 30^0 and 35^0C for *Xenorhabdus* sp. TX strain, and 0^0, 5^0, 10^0, 20^0 and 30^0C for the NF strain and Umeå strain of *X. bovienii*. Blue bacterial colonies (primary phase) on NBTA (nutrient agar with bromothymol blue dye and triphenoltetrazolium chloride) plates were used to inoculate 250-ml tryptic soy broth (Difco) in a 500-ml capped flask. It was cultured with shaking at 100 rpm at a given temperature, harvested at the mid-log phase (on the basis of optical density of the culture) of their growth by centrifugation at 14,740 g at 4^0C, then washed three times by repeated centrifugation, decanting and replenishing with 0.15M NaCl. The bacterial pellet was transferred into a plastic cap, which was then covered with filter paper, and frozen at -20^0C for 24 hours. The sample was freeze-dried for 48 hours using a Labconco® freeze dry system, Lypho-lock 6 (Labconco Corp., Kansas City, MO, USA). The dried sample was transferred into a 6.0 ml capped vial, and kept at -20^0C until it was used for lipid extraction. Three samples from three separate flasks were prepared for each bacterial strain at each of the given temperatures.

6.3.3. Extraction of lipids and analysis of fatty acids in total lipids

Fatty acid components in total lipids were determined by means of gas liquid chromatography (GLC). Lipids were extracted from bacteria using the method of Bligh and Dyer (1959), as modified by Jagdale and Gordon (1997c). Thirty milligrams of each of the freeze dried samples was placed into separate polypropylene microcentrifuge tubes (1.5 ml) to which 750 µl methanol, 250 µl distilled water, 250 µl chloroform and a few crystals of

hydroquinone (antioxidant agent) were then added. Each sample was vortexed, left at room temperature (about 25°C) for 3-4 hours with intermittent shaking, then centrifuged at 13,150 rpm for 2 minutes at room temperature. The supernatant was transferred into a microcentrifuge tube to which 300 µl distilled water and 300 µl chloroform were added. This mixture was vortexed and incubated at room temperature for 30 minutes. The lower lipid-containing layer (chloroform + lipids) was transferred into a 6.0-ml transmethylation vial, and evaporated to dryness under a stream of nitrogen. Then, 2.0-ml transmethylating agent (methanol:sulfuric acid=94:6) and a few crystals of hydroquinone were added to this vial, vortexed, and incubated for 4 hours at 70°C. Then 1.0 ml distilled water and 1.5 ml hexane were added and shaken. After 10 minutes at room temperature, the upper layer [hexane + fatty acid methyl esters (FAMEs)] was transferred into a small capped vial (4.0 ml) and blown to dryness under a stream of nitrogen. Finally, 100 µl carbon disulphide (CS_2) was utilized to dissolve the extracted FAMEs. Then, 0.25 µl of this solution was injected into the GLC apparatus, a Hewlett Packard 5890 series II gas liquid chromatograph equipped with a flame ionization detector. The column was a 30 m Supelcowax 10/0.53 mm (Supelco, Supelco Park, Bellfonte, PA, USA). Helium was used as the inert carrier at a flow rate of 1.00 ml per minute. Temperatures were set at 205°C, 240°C and 240°C for the oven, the injector and the detector, respectively. The fatty acid components were identified by referring to the retention times of fatty acid standards in bacterial acid methyl esters CP™ mix (Catalog No. 1114, Matreya, Inc., Pleasant Gap, PA), and of some standards obtained from Supelco and Sigma Chemical Co.

6.3.4. Statistical analysis

Data were transformed with $Log_{10}(1+X)$, and analyzed using one-way ANOVA. Significant differences were determined by t-test (Sokal and Rohlf, 1995) at $P<0.05$.

6.4. Results

The most prominent fatty acids in all four strains were palmitic acid (16:0) and its monounsaturated form, palmitoleic acid ($16:1_{\omega}7$). Collectively, these 16C fatty acids accounted for 47-65 percent of total fatty acids. Oleic acid ($18:1_{\omega}9$) was the next most abundant fatty acid, accounting for 8-35% of the total. Each of the other fatty acids, including myristic (14:0), margaric (17:0), cyclopropane ($17:0_c$), stearic (18:0), arachidic (20:0), and *cis*-vaccenic ($18:1_{\omega}7$) acids, accounted for <10% of the total fatty acids, regardless of strain or temperature.

Fatty acid compositions were influenced by culture temperatures. When the temperature decreased, two major unsaturated fatty acids ($16:1_{\omega}7$ and $18:1_{\omega}9$) significantly increased while five saturated fatty acids (14:0, 16:0, 17:0, 17:0c, 20:0) decreased in three strains (NF, Umeå, All). In the case of the TX strain, temperature decreases also caused increases in palmitoleic ($16:1_{\omega}7$) and oleic ($18:1_{\omega}9$) acids, with concomitant significant decreases in only one saturated fatty acid (16:0). Stearic acid (18:0) was not significantly affected by temperature in all four strains. Additionally, the minor unsaturated fatty acid ($18:1_{\omega}7$) decreased when temperature was lowered (Table 1).

At a culture temperature of 0°C, the proportions of 16:1$_{\omega}$7 and 18:1$_{\omega}$9 in the fatty acid moiety of *X. bovienii* NF were approximately double those of bacteria which had been cultured at 30°C. Conversely, 14:0 and 16:0 accounted for approximately half the proportion of fatty acids at 0°C as was the case at 30°C. The same trend was displayed for 14:0 and 16:0 in the Umeå strain of *X. bovienii*, the proportions in 0°C cultured bacteria being approximately half those of 30°C cultured ones. The proportion of 18:1$_{\omega}$9 was tripled, and the proportion of 16:1$_{\omega}$7 elevated about five fold in the fatty acids of bacteria cultured at 0°C (cf. 30°C). For the two strains of *X. bovienii*, the proportions of the most abundant fatty acids C16:0 and C16:1 changed at temperatures ≤ 10°C in a fashion that was consistent with the general trends over the entire temperature range, i.e. C16:0 decreased, while C16:1 increased as a proprtion of the total fatty acids as temperature declined from 10° to 0°C. The concentrations of all other identified fatty acids remained stable over this low temperature range (Figs. 1, 2). However, at the upper end of the temperature range (≥20°C), all four strains displayed the same general trend, involving a continual increase in the proportion of C16:0, with concomitant decreases in 16:1 and 18:1$_{\omega}$9, as the temperature increased (Figs. 3, 4). Other fatty acids, present in lower amounts, remained stable and showed small scale changes in concentration over this higher temperature range.

6.5. Discussion

It has been established that 90% of the fatty acid methyl esters in *E. coli* are acylated to phospholipids, and that almost all of the phospholipids exist in the cytoplasmic membrane

and the inner leaflet of the outer membrane. Based on this fact, fatty acid profiles in total lipids were directly used to discuss the temperature adaptation of *Photorhabdus* spp. (Clarke and Dowds, 1994). Like *E. coli* and *Photorhabdus* spp., bacteria of the genus *Xenorhabdus* are also Gram negative, and have the same basic cell structures as that in *E. coli*. Therefore, the fatty acid profiles in this study can also be utilized to analyze the thermal adaptation of *Xenorhabdus* bacteria.

The four strains of *Xenorhabdus* bacteria involved in this study responded to low temperature by increasing monounsaturated fatty acids ($16:1_{\omega}7$ and $18:1_{\omega}9$) with concomitant decreases in saturated fatty acids (TX: 16:0; other strains: 14:0, 16:0, 17:0, 17:0c, 20:0). Adaptation to low temperature (10^{0}C) involved compensatory changes in the relative proportions of the dominant fatty acids 16:0 and 16:1. At higher temperatures, changes in the relative proportions of 16:0, 16:1 and $18:1_{\omega}9$ characterized the temperature compensatory mechanism.

It is a common mechanism for poikilotherms to adapt to low temperature by increasing unsaturated fatty acids with the accompanying reductions of saturated fatty acids (Jagdale and Gordon, 1997c; Hazel, 1995; Suutari and Laakso, 1994). Many bacterial species such as *E. coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Xenorhabdus* TX strain were found to increase the degree of fatty acid unsaturation at low temperature (Suutari and Laakso, 1994; Abu Hatab and Gaugler, 1997a). Unsaturated fatty acids with different positions and different numbers of double bonds have different conformations. These different conformations prevent hydrocarbon chains of fatty acids

from becoming stacked together at low temperature, and the temperature at which the membrane crystalizes is consequently lowered. Therefore, increases of fatty acid unsaturation at low temperature increase the membrane fluidity, and aid in maintaining normal membrane functions (Suutari and Laakso, 1994). In addition, unsaturated fatty acids also increase membrane permeability (Hakomori, 1986; Abu Hatab and Gaugler, 1997a), to compensate for the lowering of membrane permeability that would otherwise occur at low temperature.

From the data reported in this study, it would appear that all four strains of bacteria modified the level of saturation of their fatty acids in an adaptive fashion at high temperatures. At cold temperatures, the two strains of *X. bovienii* displayed adaptive changes; studies on the All and TX strains were precluded by their inability to grow at low temperatures. Such broad capacities for physiological adaptation to warm temperature by the bacteria were not recorded for their steinernematid vectors. The unsaturation indices of *S. feltiae* NF strain, *S. feltiae* Umeå strain, and *S. carpocapsae* All strain changed adaptively with temperature over the range 5-25^{0}C. However, *S. riobravis* TX strain displayed no changes in the unsaturation index over the temperature range, leading Jagdale and Gordon (1997c) to conclude that it had a lower degree of physiological adaptation to changing temperatures than the other strains.

The first three predominant fatty acids in the four strains of *Xenorhabdus* bacteria were found to be palmitic (16:0), palmitoleic (16:1$_{\omega}$7) and oleic (18:1$_{\omega}$9) acids, which was in agreement with the study on *Xenorhabdus* TX (Abu Hatab and Gaugler, 1997a). Much less

cyclopropane (17:0c) was found in this study than in a previous report (Janse and Smits, 1990), in which these authors determined the fatty acid profiles of 32 *Xenorhabdus* strains at 28°C, and used these profiles to classify these bacteria. This may be due to the differences in culture media and the harvesting phases of the bacterial samples. The bacteria were cultured in tryptic soy broth (Difco) and harvested at mid-log phase in my research, while bacterial samples were harvested at late-log growth phase from trypicase soy broth agar (Difco) in the other studies (Abu Hatab and Gaugler, 1997a; Janse and Smits, 1990). In fact, cyclopropane has been found to be significantly influenced by culture media, temperature and growth phase in *Xenorhabdus* sp. TX strain (Abu Hatab and Gaugler, 1997a). For example, this strain displayed only 1.7% cyclopropane when it was cultured at 15°C with *Galleria mellonella* crude lipid (GmCL) as the primary carbon source in culture media, and harvested at log phase. However, it showed 30.6% cyclopropane [very close to the 27.7% cyclopropane in the study by Janse and Smits (1990)] when cultured at 30°C with glucose as the main carbon source and harvested at stationary phase.

In the study by Abu Hatab and Gaugler (1997a), fatty acid compositions in total lipids were examined only for *Xenorhabdus* sp. TX strain cultured at 15, 20, 25, and 30°C on media with glucose or GmCL as the primary carbon source. In this study, saturated fatty acids increased and unsaturated fatty acids decreased as temperature increased, regardless of the main carbon source in culture media. Compared with this study, my research involved three species (four strains) which originated from various geographical regions, which made

it possible to compare the capacities, with respect to fatty acids, of *Xenorhabdus* bacteria from different areas for thermal adaptation.

Fatty acid patterns have been used for the taxonomy of the bacteria of the genera *Xenorhabdus* and *Photorhabdus* (Janse and Smits, 1990; Suzuki et al., 1990). This study shows the importance of specifying culture temperature if fatty acids are to be used in this manner. In addition, fatty acid profiles can be affected by culture media, temperature and growth phases in *Xenorhabdus* bacteria (Abu Hatab and Gaugler, 1997a). These conditions also have to be carefully specified in taxonomic studies.

Table 1: Effect of temperature on fatty acids in total lipids of three *Xenorhabdus* species including four strains*

fatty acids	t°C	Bacterial strains NF	Umeå	All	TX
	0	$^{b}1.97\pm0.12^{cd}$	$^{a}3.42\pm0.27^{b}$	-	-
	5	$^{a}2.41\pm0.13^{c}$	$^{a}2.64\pm0.17^{c}$	-	-
	10	$^{b}1.84\pm0.16^{d}$	$^{a}3.49\pm0.40^{b}$	$^{b}1.59\pm0.34^{b}$	-
14:0	20	$^{b}3.82\pm0.30^{b}$	$^{b}3.89\pm0.10^{b}$	$^{b}4.13\pm0.41^{a}$	$^{a}6.87\pm0.89^{a}$
	30	$^{bc}5.41\pm0.11^{a}$	$^{b}5.58\pm0.26^{a}$	$^{c}4.80\pm0.24^{a}$	$^{a}7.73\pm0.21^{a}$
	35	-	-	$^{b}4.24\pm0.12^{a}$	$^{a}8.61\pm0.56^{a}$
	0	$^{a}16.00\pm0.63^{c}$	$^{a}17.52\pm0.27^{d}$	-	-
	5	$^{a}18.37\pm0.80^{d}$	$^{a}17.53\pm0.32^{d}$	-	-
	10	$^{a}22.37\pm1.12^{c}$	$^{a}21.06\pm0.16^{c}$	$^{a}21.58\pm2.21^{c}$	-
16:0	20	$^{a}30.45\pm1.34^{b}$	$^{a}28.86\pm0.39^{b}$	$^{a}31.17\pm0.66^{b}$	$^{a}33.70\pm1.59^{c}$
	30	$^{ab}40.23\pm1.04^{a}$	$^{b}38.42\pm0.77^{a}$	$^{a}41.71\pm0.65^{a}$	$^{ab}39.55\pm0.74^{b}$
	35	-	-	$^{a}47.70\pm0.71^{a}$	$^{b}44.29\pm0.48^{a}$
	0	$^{a}47.24\pm2.30^{a}$	$^{a}47.47\pm1.19^{a}$	-	-
	5	$^{a}47.17\pm3.32^{a}$	$^{a}44.17\pm1.35^{a}$	-	-
	10	$^{a}45.08\pm2.95^{a}$	$^{a}43.11\pm2.55^{a}$	$^{a}33.07\pm3.43^{a}$	-
16:1$_{\omega}$7	20	$^{a}26.16\pm2.74^{b}$	$^{a}24.12\pm0.36^{b}$	$^{a}23.46\pm2.17^{b}$	$^{a}20.49\pm2.04^{a}$
	30	$^{a}18.26\pm1.64^{c}$	$^{c}8.95\pm0.13^{c}$	$^{ab}16.59\pm0.54^{c}$	$^{b}15.50\pm0.21^{b}$
	35	-	-	$^{b}8.28\pm0.54^{d}$	$^{a}13.32\pm0.14^{c}$

*Note: Values are expressed as the percentages in total fatty acids. Each of the values is the mean ± SE (standard error) of three independent replicates. The values with the same superscript letter (across columns) on the left of the mean are not significantly different at $P<0.05$ (among strains). The values with the same superscript letter (down the columns) on the right of the SE are not significantly different at $P<0.05$ (among temperatures).

Table 1: Effect of temperature on fatty acids in total lipids of three *Xenorhabdus* species including four strains* (continued)

fatty		Bacterial strains			
acids	t°C	NF	Umeå	All	TX
	0	$^{a}0.05\pm0.05^{b}$	$^{a}0.08\pm0.08^{c}$	-	-
	5	$^{a}0.09\pm0.09^{b}$	$^{a}0.38\pm0.25^{c}$	-	-
	10	$^{a}0.00\pm0.00^{b}$	$^{a}0.31\pm0.16^{c}$	$^{a}0.45\pm0.22^{b}$	-
17:0	20	$^{a}1.00\pm0.54^{a}$	$^{a}0.97\pm0.07^{b}$	$^{a}0.79\pm0.10^{ab}$	$^{a}0.37\pm0.37^{a}$
	30	$^{b}1.62\pm0.25^{a}$	$^{a}2.69\pm0.12^{a}$	$^{bc}1.28\pm0.18^{a}$	$^{c}0.96\pm0.06^{a}$
	35	-	-	$^{a}1.39\pm0.05^{a}$	$^{a}0.97\pm0.15^{a}$
	0	$^{a}1.19\pm0.05^{b}$	$^{b}0.21\pm0.21^{b}$	-	-
	5	$^{a}1.13\pm0.06^{b}$	$^{a}0.60\pm0.30^{b}$	-	-
	10	$^{a}0.59\pm0.59^{b}$	$^{a}0.90\pm0.51^{b}$	$^{a}0.61\pm0.30^{c}$	-
$17:0_c$	20	$^{a}2.85\pm0.38^{a}$	$^{a}3.98\pm0.19^{a}$	$^{a}2.83\pm0.17^{b}$	$^{a}3.44\pm0.54^{a}$
	30	$^{ab}4.88\pm0.56^{a}$	$^{a}5.85\pm0.50^{a}$	$^{bc}3.58\pm0.43^{b}$	$^{c}2.72\pm0.26^{a}$
	35	-	-	$^{a}7.87\pm0.51^{a}$	$^{b}2.51\pm0.15^{a}$
	0	$^{a}5.80\pm1.02^{a}$	$^{a}5.21\pm0.26^{a}$	-	-
	5	$^{b}3.85\pm0.50^{a}$	$^{a}5.88\pm0.31^{a}$	-	-
	10	$^{a}4.20\pm0.53^{a}$	$^{a}5.59\pm0.54^{a}$	$^{a}4.99\pm0.65^{a}$	-
18:0	20	$^{a}7.87\pm2.90^{a}$	$^{a}6.37\pm0.49^{a}$	$^{a}3.81\pm0.11^{a}$	$^{a}5.17\pm0.44^{a}$
	30	$^{a}6.22\pm0.28^{a}$	$^{a}6.23\pm0.18^{a}$	$^{b}4.75\pm0.23^{a}$	$^{b}4.55\pm0.17^{a}$
	35	-	-	$^{b}4.41\pm0.19^{a}$	$^{a}5.60\pm0.09^{a}$

*Note: Values are expressed as the percentages in total fatty acids. Each of the values is the mean ± SE (standard error) of three independent replicates. The values with the same superscript letter (across columns) on the left of the mean are not significantly different at $P<0.05$ (among strains). The values with the same superscript letter (down the columns) on the right of the SE are not significantly different at $P<0.05$ (among temperatures).

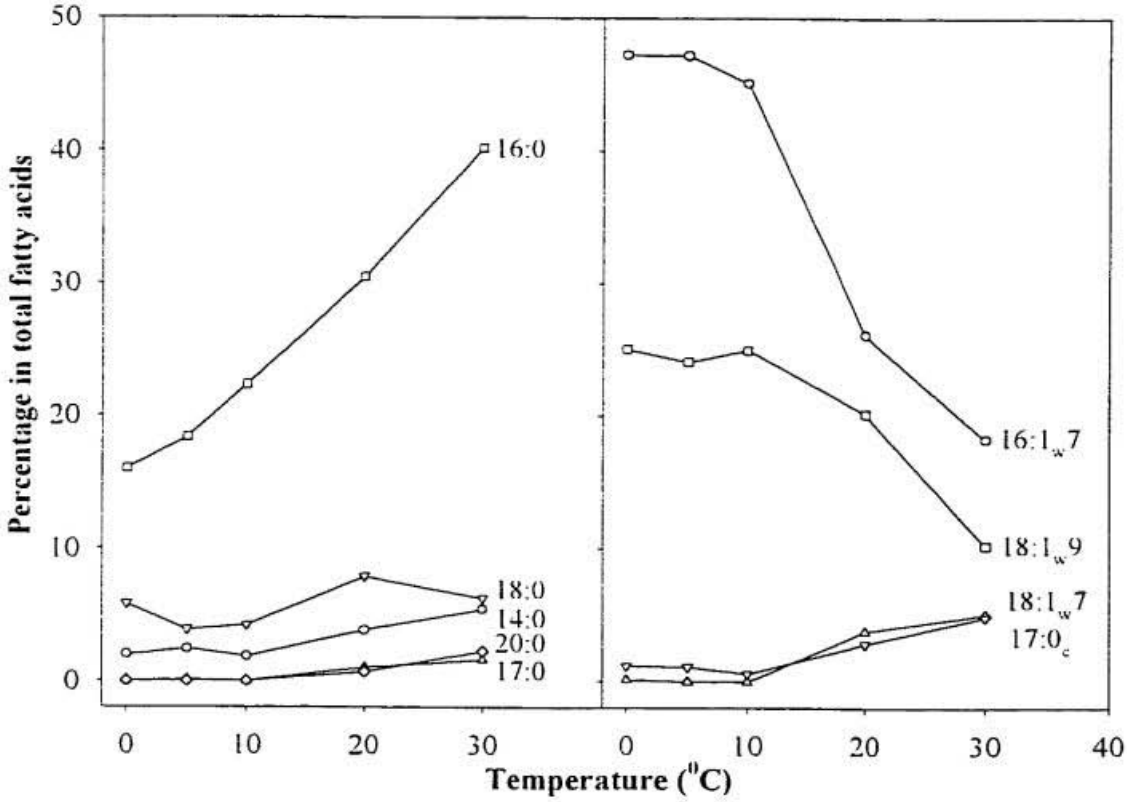
Table 1: Effect of temperature on fatty acids in total lipids of three *Xenorhabdus* species including four strains* (continued)

fatty acids	t^{0}C	Bacterial strains NF	Umeå	All	TX
	0	^{a}25.07±1.40^{a}	^{a}24.20±0.76^{ab}	-	-
	5	^{a}24.12±0.95^{a}	^{a}26.27±1.23^{a}	-	-
	10	^{a}24.99±1.73^{a}	^{a}22.93±0.78^{bc}	^{a}35.49±6.89^{a}	-
18:1$_\omega$9	20	^{b}20.17±0.98^{b}	^{b}21.25±0.73^{c}	^{a}26.44±1.85^{a}	^{b}21.36±1.11^{a}
	30	^{c}10.25±0.29^{c}	^{d}8.35±0.13^{d}	^{b}18.52±0.26^{b}	^{a}21.02±0.73^{a}
	35	-	-	^{b}11.38±0.52^{c}	^{a}17.56±0.30^{b}
	0	^{a}0.17±0.17^{b}	^{a}0.17±0.17^{c}	-	-
	5	^{a}0.00±0.00^{b}	^{a}0.57±0.29^{c}	-	-
	10	^{a}0.00±0.00^{b}	^{a}0.66±0.34^{c}	^{a}0.56±0.28^{b}	-
18:1$_\omega$7	20	^{ab}3.79±0.34^{a}	^{a}4.03±0.33^{b}	^{b}3.08±0.05^{a}	^{a}4.30±0.20^{a}
	30	^{b}5.06±0.57^{a}	^{a}7.93±0.19^{a}	^{c}3.65±0.36^{a}	^{c}3.66±0.17^{a}
	35	-	-	^{a}4.86±0.44^{a}	^{b}3.04±0.16^{b}
	0	^{a}0.00±0.00^{b}	^{a}0.00±0.00^{c}	-	-
	5	^{a}0.00±0.00^{b}	^{a}0.00±0.00^{c}	-	-
	10	^{a}0.00±0.00^{b}	^{a}0.15±0.15^{c}	^{a}0.00±0.00^{c}	-
20:0	20	^{a}0.69±0.69^{b}	^{a}1.91±0.18^{b}	^{a}1.44±0.17^{b}	^{a}0.30±0.30^{a}
	30	^{b}2.23±0.40^{a}	^{a}4.61±0.23^{a}	^{bc}1.34±0.12^{b}	^{c}0.62±0.36^{a}
	35	-	-	^{a}2.86±0.20^{a}	^{b}0.49±0.25^{a}

Note: Values are expressed as the percentages in total fatty acids. Each of the values is the mean ± SE (standard error) of three independent replicates. The values with the same superscript letter (across columns) on the left of the mean are not significantly different at $P<0.05$ (among strains). The values with the same superscript letter (down the columns) on the right of the SE are not significantly different at $P<0.05$ (among temperatures).

Fig. 1: Effect of temperature on fatty acids in total lipids of *Xenorhabdus bovienii* NF strain

Fig. 2: Effect of temperature on fatty acids in total lipids of *Xenorhabdus bovienii* Umeå strain


Percentage in total fatty acids
50
40
30
20
10
0
16:0
18:0
14:0
20:0
17:0
16:1w7
18:1w9
18:1w7
17:0c
0
10
20
30
0
10
20
30
40
Temperature (0C)

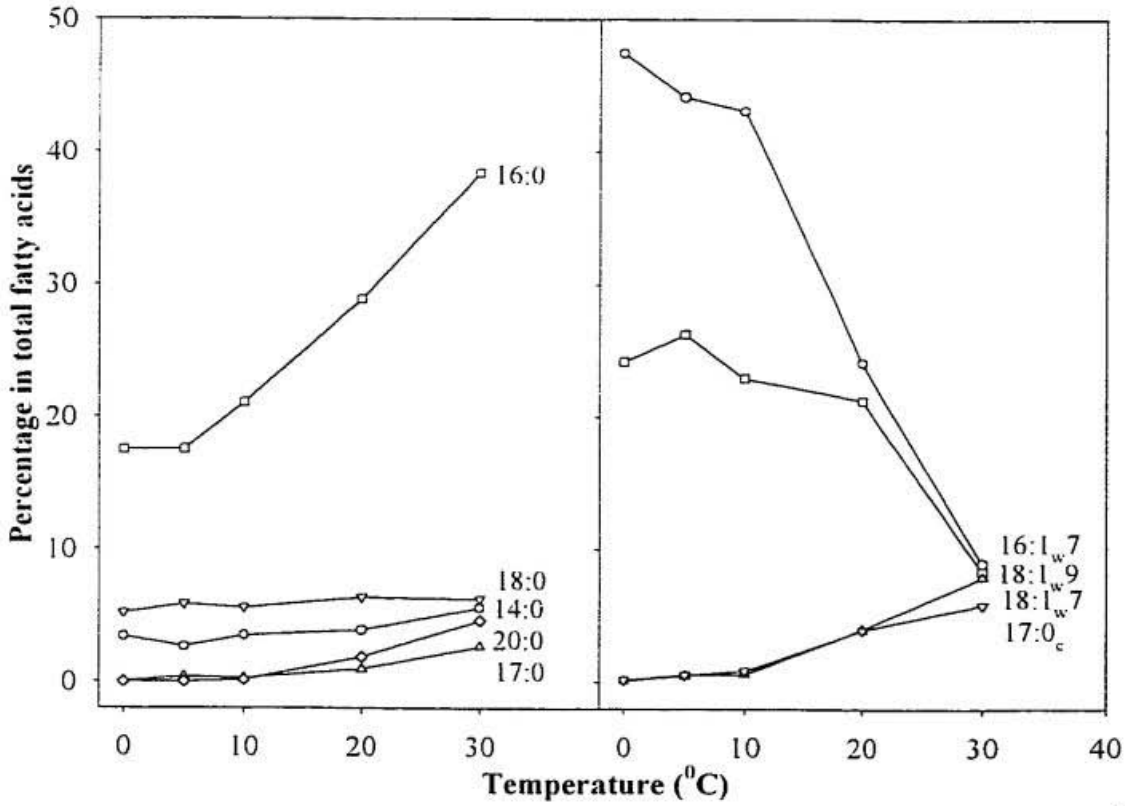
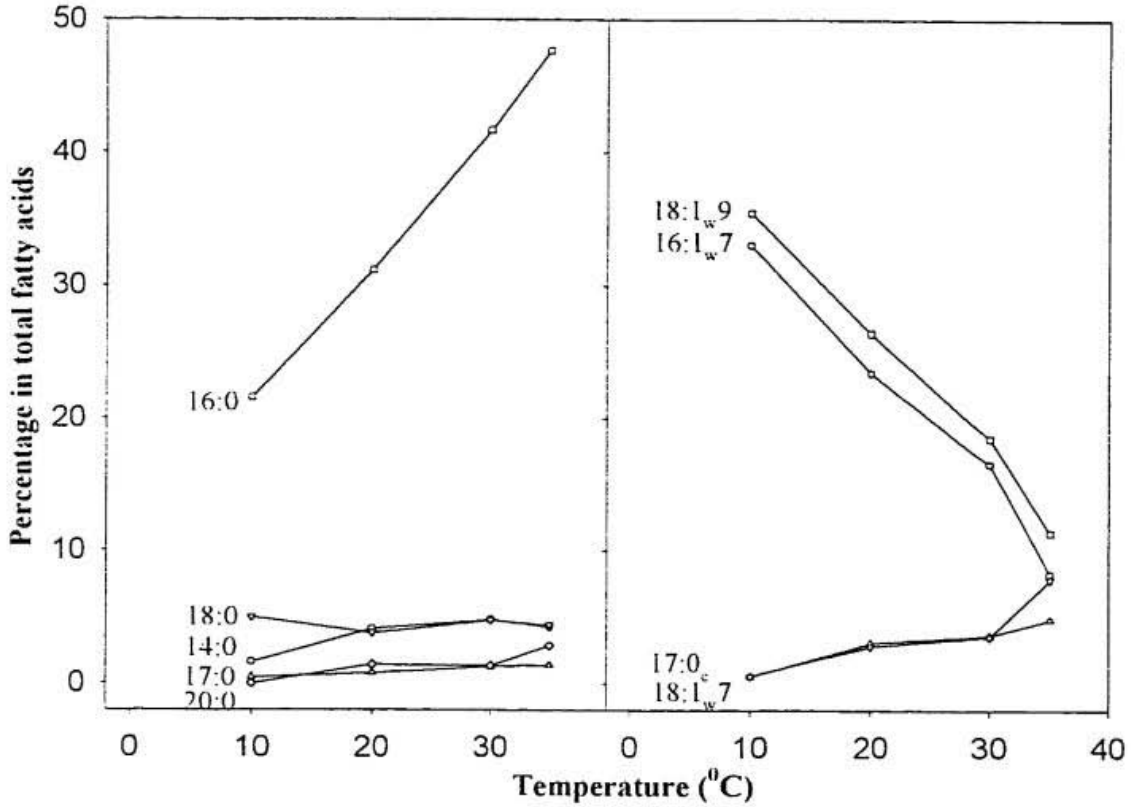

Percentage in total fatty acids
50
40
30
20
10
0
16:0
18:0
14:0
20:0
17:0
16:1w7
18:1w9
18:1w7
17:0c
0
10
20
30
0
10
20
30
40
Temperature (0C)

Fig. 3: Effect of temperature on fatty acids in total lipids of *Xenorhabdus nematophilus* All strain

Fig. 4: Effect of temperature on fatty acids in total lipids of *Xenorhabdus* sp. TX strain


Percentage in total fatty acids
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40
30
20
10
0
16:0
18:0
14:0
17:0
20:0
18:1w9
16:1w7
17:0c
18:1w7
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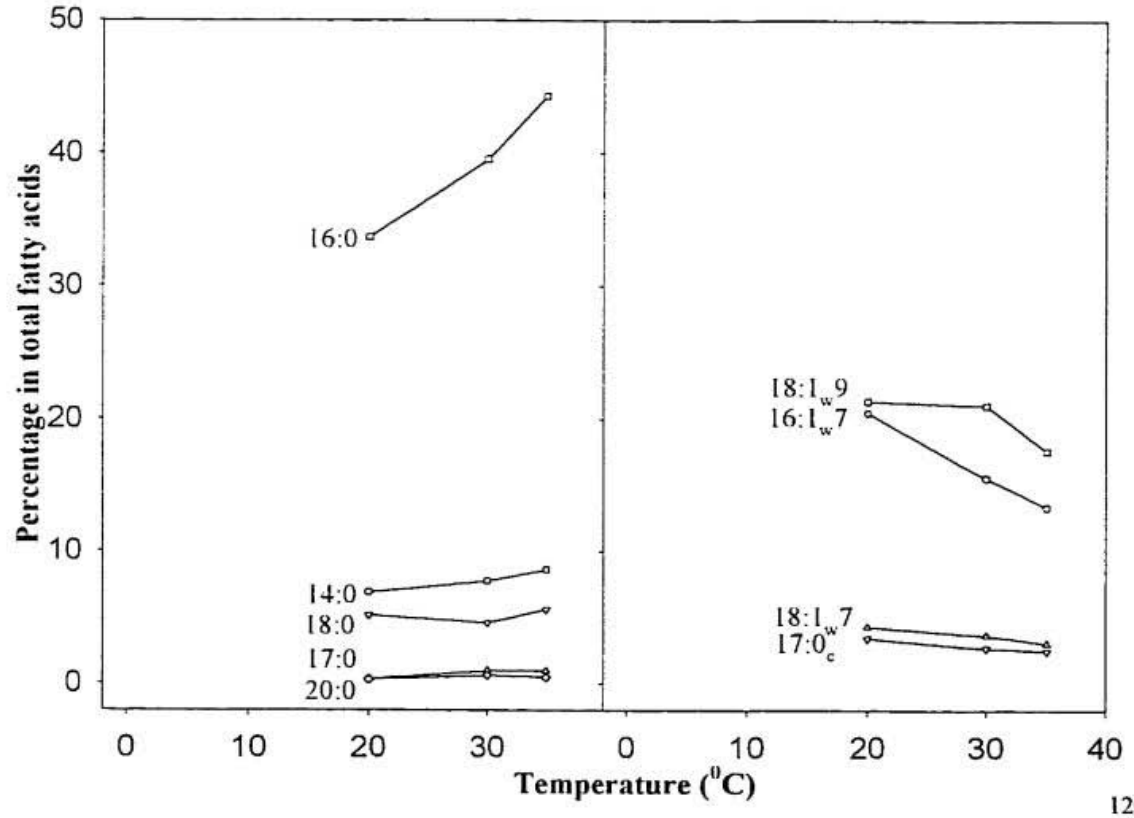
10
20
30
0
10
20
30
40
Temperature (0C)


Percentage in total fatty acids
50
40
30
20
10
0
16:0
14:0
18:0
17:0
20:0
18:1w9
16:1w7
18:1w7
17:0c
0
10
20
30
0
10
20
30
40
Temperature (0C)

Chapter 7

General discussion

Xenorhabdus sp. TX strain, the bacterial symbiont of the nematode *Steinernema riobravis*, was physiologically and biochemically different from all the described bacterial species that were natually associated with entomopathogenic nematodes. Moreover, its similarity value, obtained using the Biolog system, showed that it was neither *X. bovienii* nor *X. nematophilus*. These findings suggest that this bacterium is probably a new species. On the other hand, *Xenorhabdus bovienii* NF strain could not be distinguished from the Umeå strain of the same species by common physiological and biochemical tests and it had a similarity value coincident with *X. bovienii*. These two strains were separable from each other using the isozyme patterns of arginine phosphokinase (APK). However, although this enzyme was temporally stable, its pattern of isozymes was affected by temperature; so, its value for taxonomic purpose is questionable.

The present study has shown that, like their nematode hosts (Jagdale and Gordon, 1998b), the bacterial associates of entomopathogenic nematodes contain enzymes that are temperature sensitive with respect to isozyme synthesis. Only one (6PGDH) of the seven enzymes studied had an isozyme pattern that was independent of temperature and this enzyme failed to discriminate among species. Thus, if techniques such as cellulose acetate electrophoresis or acrylamide gel electrophoresis (Hotchkin and Kaya, 1984) are to be advocated for use in the taxonomy of bacterial associates of entomopathogenic nematodes, a broader survey of enzymes is needed to determine those that are both diagnostic and

temperature independent. Alternatively, the temperature at which the bacteria are grown for taxonomic studies should be carefully controlled and specified.

Moreover, although the results on temporal stability of the enzymes are encouraging, revealing only two out of a possible fourteen [2 isolates (NF and Umeå), 7 enzymes] situations where isozyme patterns changed over time, the long-term stability of isozyme patterns should be examined for more enzymes over periods of time in excess of the five months used in this study, and for a wider range of bacterial species and strains. Only the isozyme patterns with good stability over time could be used for taxonomic purposes.

In response to the changes in culture temperatures, all four bacterial strains modified the synthesis of the isozymes of their metabolic enzymes and the degree of fatty acid unsaturation in total lipids. These findings suggest possible physiological mechanisms used by *Xenorhabdus* bacteria for temperature adaptation. The strategy of synthesizing isozymes, geared to function optimally at either end of the organism's temperature range, has been documented for poikilothermic animals (Jagdale and Gordon, 1998b; Marcus, 1977; Smith and Hubbes, 1986). Thus far, It has not been reported to occur in bacteria. However, the fact that differences in enzyme banding patterns occurred at either end of the temperature range, rather than randomly, relative to temperature is suggestive of an adaptive response. By contrast, the nematode hosts displayed temperature related changes in isozyme profiles of metabolic enzymes that in many instances, were suggestive of random, non-adaptive genetic mutations (Jagdale and Gordon, 1998b). It should be noted, however, that the term of exposure to the culture temperature (ca. 2 years) was considerably greater in the

nematode studies than in the current investigations on their bacteria. So, the two situations may not be directly comparable.

The changes in the saturation of fatty acids that occurred in all bacterial strains in response to temperature is consistent with what has been reported to occur for other types of bacteria (Suutari and Laakso, 1994), as well as for *Xenorhabdus* sp. TX strain (Abu Hatab and Gaugler, 1997a). As bacteria use lipids mainly for structural purposes (Clarke and Dowds, 1994), it seems reasonable to conclude that the temperature related changes in saturation levels are adaptive, maintaining membrane fluidity and permeability over a broad temperature range.

From this study, it appears that temperature adaptive mechanisms such as changes in isozyme synthesis and fatty acid saturation levels are common to all the bacterial strains, so the observed differences in temperature/growth must be attributed to other strain specific properties.

The climatic profiles of the geographic origins of the bacteria corresponded to the temperature ranges for their growth and limited their physiological capacity of adapting to temperature. In this study, the two boreal bacterial strains (NF, Umeå) could grow under much lower temperatures than the temperate strain (All) and the subtropical strain (TX). A similar tendency was also found in the temperature requirements for recycling the corresponding nematode hosts of these bacteria, so that the lower temperature limit for recycling *S. feltiae* < *S. carpocapsae* < *S. riobravis* (Jagdale and Gordon, 1997a).

Whether bacterial associates grow well at a given temperature has relevance to the successful recycling of their corresponding nematode host species (Gwynn and Richardson 1994; Jagdale and Gordon, 1997a). The reasons are due to the roles that bacteria play in the bacterium-nematode complexes. First, bacteria provide their nematode hosts with nutrients for efficient nematode reproduction (Akhurst and Dunphy, 1993; Forst and Nealson, 1996). Bacterial failure to grow would result in inadequate nutrition for nematode reproduction. Additionally, in most nematode-insect interactions, bacteria are the key contributors to the pathogenicity of their corresponding bacterium-nematode complexes (Akhurst and Dunphy, 1993; Forst and Nealson, 1996), so if bacteria are unable to grow well, this could affect the efficiency of the corresponding bacterium-nematode complexes in controlling the pests.

The strains of *Xenorhabdus bovienii* are well adapted to low temperatures, capable of growing at a temperature as low as 0°C, with maximum growth occurring at 25°C. This finding agrees with that of Gwynn and Richardson (1994), who showed that two other strains (L/128 and L/179) of *Xenorhabdus bovienii* could grow at 2°C. The nematode host species (*Steinernema feltiae*) of the bacterial strains used in this study were shown to have superior tolerance than the nematode hosts of the other bacterial isolates, and could kill *Galleria mellonella* larvae at low (5°C) temperatures (Jagdale and Gordon, 1997a, 1998a; Grewal et al., 1994). Thus, it may be concluded that *X. bovienii* and its nematode host (*S. feltiae*) may be utilized for pest management in cold regions. On the other hand, the bacterial TX strain displayed good growth only at warm temperatures (>15°C) and its nematode host (*S. riobravis*) infected insect hosts at 10-39°C (Grewal et al., 1994, Jagdale

and Gordon, 1997a). So, the bacterial TX strain should be viewed as a warm-adapted species, and *S. riobravis* could be used to control sensitive insects in warm areas. Finally, *S. carpocapsae* All strain, which has undergone extensive laboratory colonization, showed similar temperature-growth relations as *S. riobravis* TX strain (Jagdale and Gordon, 1997a,1998b). Its bacterial associate (growing at $\geq 10^0$C), *X. nematophilus* All strain, is a little more cold-adapted than the TX strain of *Xenorhabdus* (growing at 15^0C). So, it seems that *S. carpocapsae* All strain would function well for insect pest management in temperate and warm regions.

Chapter 8

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