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THE INTERACTION OF SURFACE COMPONENTS OF *XENORHABDUS NEMATOPHILUS* (*ENTEROBACTERIACEAE*) WITH THE HEMOLYMPH OF NONIMMUNE LARVAE OF THE GREATER WAX MOTH, *GALLERIA MELLONELLA* (LEPIDOPTERA: GALLERIDAE)

by

Philip W. Maxwell

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Department of Natural Resource Sciences
Macdonald Campus of McGill University
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Abstract

The following studies were done to identify factors that influence the virulence of *Xenorhabdus nematophilus* and the interactions of the bacterium with the nonself defence systems of nonimmune *Galleria mellonella*. Isolates of *X. nematophilus* that are qualitatively similar in biochemical properties differed significantly in virulence (LT_{50} values, the to kill 50 % of the insects injected with a given bacterial dosage) for *G. mellonella* larvae. The production of enzymes such as proteases could not account for differences in the virulence of the isolates.

Growth conditions, influenced the growth rate and the interactions of the bacterium with nonimmune *G. mellonella* larvae. In general, *X. nematophilus* cells grown under aerobic conditions were more susceptible to the nonself defences of *G. mellonella* larvae than those grown under less than ideal conditions, resulting in increased clearance of the bacteria from the hemolymph (blood) of the insects. Clearance of the bacteria from the hemolymph of the insect was positively correlated with culture condition, culture age, and attachment to insect hemocytes *in vitro*. However, clearance of the bacteria from the hemolymph of the insect was not correlated with cell surface hydrophobicity, electrostatic charge or cell viability of the bacteria.

Isolates of *X. nematophilus* produced flagella and fimbriae when grown under microaerobic and aerobic conditions. The type of fimbriae produced was influenced by culture conditions. *X. nematophilus* fimbriae consist of 3 major proteins (67, 40, and 32 kDa), and *X. nematophilus* flagellar consist of 4 major flagella proteins (67, 40, 35, 32 kDa). The injection of both flagella and fimbriae in picogram quantities into nonimmune *G. mellonella* caused an increase in total hemocyte counts within these insect larvae. The injection of fimbrial and flagellar antigens into *G. mellonella* larvae caused changes in the hemocyte types found in circulation in the insects' hemolymph.

Co-injection of picogram amounts of *X. nematophilus* fimbrial and flagellar antigens with *X. nematophilus* cells did not impact upon bacterial clearance from the hemolymph. When *Proteus mirabilis* was co-injected into nonimmune *G. mellonella* larvae with these antigens, flagella reduced the clearance of *P. mirabilis* and the fimbriae had no effect. The surface appendages of *X. nematophilus* may prevent cellular recognition of bacteria by blocking hemocyte receptors for certain surface antigens of bacteria.

Résumé

Les études suivantes ont été menées afin d'établir les facteurs qui ont une incidence sur la virulence de *Xenorhabdus nematophilus* et les interactions de la bactérie avec kes systèmes de défense non-soi de *Galleria mellonella*. Les isolats de *X. nematophilus* qui ont des propriétés biochimiques qualitativement semblables varient considérablement du point de vue de la virulence (valeurs de LT 50) sur les larves de *G. mellonella*. Les facteurs enzymatiques (notamment les protéases) n'étaient pas responsables des écarts dans le taux de virulence des isolats.

Les conditions de croissance, notamment l'oxygénation, influent sur le taux de croissance de la culture et les interactions de la bactérie avec les larves de *G. mellonella* non immunes. En général, les cellules de *X. nematophilus* produites sous condition aérobie se prêtent plus facilement a l'action des défenses non-soi des larves de *G. mellonella*, entraînant une clairance accrue de l'hémolymphe (du sang) des insectes. La clairance était positivement reliée a la condition de culture, a l'âge de la culture et a la fixation aux hémocytes de l'insecte *in vitro*. La clairance n'était pas reliée a l'hydrophobicité ou a la charge électrostatique de la surface de la cellule, ou a la viabilité de celle-ci.

Les isolats de *X. nematophilus* produisent les flagelles et des fimbriae lorsqu'ils sont cultivés sous condition microaérobie ou aérobie. Les conditions de culture avaient une incidence sur le genre de fimbriae produit. Les fimbriae de *X. nematophilus* comprennent 3 protéines principales (67, 40 et 32 KDa) et les flagelles de *X. nematophilus* comptent 4 protéines principales (67, 40, 35 et 32 KDa). L'injection dans l'hémocèle de *G. mellonella* de picogrammes de flagelles et de fimbriae peut causer une hausse du taux d'hémocytes total dans les larves. Des changements dans les genres d'hémocytes circulant dans l'hemolymphe ont été provoqués par injection d'antigènes de fimbriae et de flagelles dans les larves de *G. mellonella*.

L'injection combinée de mg d'antigènes de cellules de *X. nematophilus* et de fimbriae et flagelles de *X. nematophilus* n'a pas eu d'incidence sur la clairance bactérienne de l'hemolymphe. Lorsqu'on a remplacé *X. nematophilus* par *Proteus mirabilis* dans les antigènes injectés, les flagelles réduisaient la clairance de *Pr. mirabilis* et les fimbriae n'avaient aucun effet. Par conséquent, les appendices de surface de *X. nematophilus* jouent peut-être un rôle dans les défenses cellulaires de l'insecte en recouvrant la bactérie d'une surface non réactive.

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Table of Abbreviations

AGS	-	artifical *Galleria* serum
CFU	-	colony forming unit
EIC	-	electrostatic interaction chromatography
HIC	-	hydrophobic interaction chromatography
KDO	-	3 deoxy - D - manno - octulosonic acid
IJ	-	infective juvenile
LPS	-	lipopolysaccharide
LT50	-	lethal time to kill 50 % of a test population
NSS	-	nine salts solution
OD	-	optical density
OM	-	outer membrane
OMP	-	outer membrane protein
PBS	-	phosphate buffered saline
PDT	-	population doubling time
PO	-	phenoloxidase
PPO	-	prophenoloxidase
THC	-	total hemocyte count
TSB	-	tryptic soy broth
TSA	-	tryptic soy agar
TTC	-	tripheyltetrazolium chloride
YSB	-	yeast salts broth

Introduction

Pest insects cause billions of dollars in crop losses yearly. The use of chemical insecticides is becoming less appropriate due to increased resistance of pest insects to these insecticides and the impact of the chemical insecticides on the environment as a whole. This has resulted in the greater emphasis on the development of biological control agents for the regulation of pest populations *in lieu* of chemical pesticides to reduce negative enviromental impact. One biological control agent for use against pest insects is the nematode *Steinernema carpocapsae* (F. Steinernematidae). At present a number of insect pests have been successfully controlled with this and other steinernematids and with the closely related heterorhabditid nematodes [31, 57, 72].

The steinernematids are symbiotically associated with bacteria of the genus *Xenorhabdus* (F. *Enterobacteriaceae*)[60]. The nonfeeding, infective stage of the nematode (infective juvenile (IJ)), which carries the bacteria within the intestinal tract, generally enters an insect host via the mouth, anus, and spiracles [55] and subsequently releases the virulent bacteria shortly after entering the insect hemocoel [59, 60]. The bacteria multiply making the host suitable for nematode reproduction [60] through the release of antimicrobial compounds which either kill or slow the growth of adventitious bacteria [1, 8]. The level of virulence of the bacterial-nematode complex of steinernematids has been linked to (i) the degree of control of pest insects [25, 36] (ii) ecological and behavioral parameters of the nematodes in nonimmune insects [35, 48] and (iii) the strains of *X. nematophilus* carried by the nematode [2, 36].

Nonimmune insects that are susceptible to the nematode-bacterium complex have systems that respond to nonself material but, by virtue of dosage (1-10 cells) *X. nematophilus* required to kill 50 % of a population of nonimmune test insects, the antimicrobial defences of the insect blood (hemolymph) are ineffective [27]. The interactions of *X. nematophilus* with the cellular (hemocytes) and humoral (enzymes) antibacterial systems of nonimmune insects have been described for *Galleria mellonella* and *Lymantria dispar* [24, 27]. Dunphy [23], using chemically mutagenized *X. nematophilus*, established that bacterial attachment to insect hemocytes was correlated with the susceptibility of the bacteria to the humoral factor lysozyme which digests peptidoglycan. Results with closely related *Photorhabdus luminescens* (*X. luminescens*)[9] have led to the proposal that fimbriae may be important to the ability of the bacteria to colonize host tissue or resist host defences [13]. However, little is known about the relationship of bacterial metabolites, fimbriae and flagella of *X. nematophilus* to bacterial

virulence or their contribution to the interaction of the organism with host defences of insects.

The objectives of the thesis are as follows;

(i) to determine the relationship of the biochemical properties, (such as secreted enzymes and utilized substrates) culture conditions and physicochemical surface properties of selected varieties of *X. nematophilus* that differ in virulence for nonimmune *G. mellonella* larvae.

(ii) to determine the relationship of culture conditions and physicochemical surface properties of varieties of *X. nematophilus* to bacterial interaction with selected antibacterial systems of nonimmune *G. mellonella* larvae.

and (iii) to determine the contribution of the flagella and fimbriae of *X. nematophilus* to the interaction of *X. nematophilus* with selected antibacterial systems of nonimmune *G. mellonella* larvae.

The antibacterial responses examined included bacterial attachment to insect hemocytes *in vitro*, the patterns of removel of the bacteria from the hemolymph *in vivo* and the activation of the melanizing system, the prophenoloxidase system.

Chapter I: Literature Review

I. Cellular Antibacterial Systems: Nonself Responses of Nonimmune Insects

Insects exist in a myriad of environments, due in part to their ability to resist bacterial infections [26] with two types of antimicrobial actions; (i) nonimmune responses to nonself materials (antigens) and (ii) bactericidal immune proteins induced by prior exposure to an antigen. The nonself responses of nonimmune insects consist of two interactive components. The first of these operates at the cellular level involving the hemocytes and, depending on the insect species and nature of the antigen, may involve phagocytosis, nodule formation and encapsulation [64, 66]. The second component includes humoral factors such as the prophenoloxidase cascade, constitutive lectins, lysozyme and certain biogenic amines [6, 11, 15]. In immunized insects resistance is based on induced antibacterial proteins and lectins, and elevated levels of lysozyme. These aspects will be discussed generally for insects with emphasis placed on *Galleria mellonella* in the following text.

1.0 Phagocytosis of Foreign Particles

Phagocytosis is an important process in vertebrate cellular immunity. In insects, little is known about this process although studies have shown phagocytosis to play an important role in defence reactions of insects against pathogens [66]. Five types of hemocytes (blood cells) are present in the hemolymph of *G. mellonella* including the prohemocytes, plasmatocytes, granulocytes, spherule cells, and oenocytoids. The plasmatocytes are considered to be the main phagocytic blood cells in most insects including *G. mellonella* although the granulocytes may exhibit phagocytosis in other insect species [14, 67].

Electron microscopy has established that the characteristic attachment and ingestion phases of the phagocytic process in vertebrates also occurs in *G. mellonella* and other insects and involves filopodial and lobopodial engulfment of the bacteria by the plasmatocytes. This process culminates in the formation of bactericidal vacuoles (phagocytic vacuoles) and cell wall degradation [66, 67]. Phagocytosis is driven by ATP generated by glycolysis and requires the functioning of the cytoskeletal system of the hemocytes [39]. Although lysosomes are known to occur in insect hemocytes, they are not apparent in the plasmatocytes of *G. mellonella* and the origin of the bacteriolytic enzymes involved in the degradation processes in *G. mellonella* is unknown [66]. The efficiency of phagocytosis varies with the nature of the bacteria, virulent pathogens surviving the process [34]. Phagocytosis may occur concertedly with nodule formation [78].

2.0 Nodulation

Nodules are melanized aggregates consisting of usually two types of hemocytes with foreign particles (*e.g. Bacillus cereus*) that have either exceeded a critical level of virulence and/or concentration [15, 64, 68]. Initiation of the process occurs with degranulation of fragile granulocytes during contact with the foreign agent. Granulocyte lysis is a prerequisite for the attachment of other granulocytes and bacteria prior to the prenodule being surrounded by successive layers of plasmatocytes [38, 74]. In *G. mellonella*, the center of the nodule is composed of both necrotic and intact granulocytes whereas the periphery consists of layers of plasmatocytes [67, 74].

The release of a plasmatocyte depletion factor from the granulocytes as a response to wounding or injection of foreign materials causes the plasmatocytes to drop from the plasma onto the insect's tissue [17]. These plasmatocytes are attracted to the prenodule as it drops from suspension in the hemolymph onto the tissues and they then effectively wall off the bacteria [17]. Foreign particles (bacteria) initiate the formation of large compact nodules with the hemocytes, while soluble molecules (peptidoglycan) initiate small to medium nodules [38, 67]. The efficiency of the response varies with the insect species due to variation in the number of hemocytes [34] and the nature of the antigen(s) but in general the bulk of removal of the bacteria occurs within 5 minutes postinjection [37, 63, 68]. Despite this hemocyte response pathogenic Gram-positive bacteria overcome the larvae of *G. mellonella* [78].

Nodulation of bacteria could result in (1) bacterial death due to active killing by antimicrobial mechanisms, (2) bacterial death due to physiological stress, (3) bacterial survival with entrapment in multicellular nodules, or (4) bacterial survival, multiplication, and escape from the nodule [78].

3.0 Cellular Encapsulation

Cellular encapsulation is a response to parasites too large to be phagocytosed and involves the aggregation of host hemocytes around the parasite with the resultant formation of a multi-layered cellular sheath of plasmatocytes surrounding a necrotic mass of granulocytes [74]. Encapsulation in *G. mellonella* is a biphasic process similar to nodule formation where phase one is modified clot formation resulting from granulocyte lysis, and phase two results in capsule formation, consisting of plasmatocyte attachment to the nonself material [74].

II. Humoral Antibacterial Systems of Nonimmune Insects

Humoral immunity, by tradition, is regarded as consisting of lectins (agglutinins), the prophenoloxidase system and lysozyme, recognizing that many of these factors may have a cellular (including hemocyte) origin [5].

1.0 The Prophenoloxidase System of Insects

A layer of melanin is frequently observed deposited on parasites of insects; thus, melanization is often associated with an insect's cellular response [63]. In insects with large numbers of hemocytes, components of the melanization pathway are believed to be derived from the hemocytes [63, 75]. Melanization can also be a strictly humoral response in insects with few hemocytes such as some species of Diptera [63]. The enzyme phenoloxidase (PO) produces quinones through the oxidation of tyrosine to 3,4, dihydroxyphenylalanine and subsequently to bactericidal derivatives. The bactericidal activity is due to the ability of the carboxyl groups of the products to react with (1) the free amino groups of proteins and other amines and (2) the thiol groups of other compounds [7, 22, 63]. PO is activated by limited proteolysis of the zymogen form (described later in the text). When quinones are present in excess, the black pigment melanin is formed.

Melanization requires the activation of the zymogenic form of phenoloxidase, prophenoloxidase (PPO) to phenoloxidase by a cascade of serine proteases [7]. Calcium-dependant PPO [21] is found in either the plasma and/or hemocytes (eg. granulocytes, oenocytoids) depending on the species of insect [75] and is activated by peptidoglycan from bacterial cell walls, capsular polysaccharides [15] and by ß -1,3-glucans from fungal cell walls [21, 52]. However, teichoic acid, a component of the cell walls of Gram-positive bacteria, does not activate the PPO system. Lipopolysaccharide (LPS) associated with the outer membrane of Gram-negative bacteria varies in its effect on the prophenoloxidase system ranging from activation to no effect depending on the insect species [15, 80]. In the locusts *Schistocerca gregaria* and *Locusta migratoria*, LPS from several bacterial species was found to activate the PPO system in whole hemolymph homogenates but not in hemocyte lysate implying a different method of activation of the PPO system by LPS in locusts. The PO levels generated by LPS *in vitro* were correlated with nodules formed *in vivo* [65]. LPS from *Pseudomonas aeruginosa*, *Shigella flexneri*, *Klebsiella pneumoniae* triggered nodule formation while LPS from *Escherichia coli* 0111 and *E. coli* 055 did not [65].

Brehélin *et al.* [12] purified a 14 kDa protein which inhibits PPO conversion to PO from the plasma and hemocytes of *Locusta migratoria*. The protein strongly inhibited chymotrypsin but not trypsin suggesting that chymotrypsin-catalysed activation of PPO in hemocyte extracts takes place under different controls or at an earlier stage of the cascade [12]. The result is a sticky coating of opsonic protein composed of PO or other factors of the PPO system which mediates the insect's humoral response to foreign particles [7, 52].

2.0 Lysozyme Activity in Insect Hemolymph

Lysozyme of *G. mellonella* is a constitutive, cationic protein, that is similar to avian lysozyme in terms of amino acid composition, molecular mass (14.7 kDa) and ultraviolet absorbance spectrum [61]. However, lysozyme of *Galleria* species differs in both substrate binding affinity and specificity when compared to other lysozymes of the class [61, 62]. The lysozyme of *G. mellonella* releases *N* - acetylmuramic acid from bacterial cell walls, permitting the insect protein to be classified as a mucopeptide *N*-acetylmuramyl-hydrolase ([61, 62]. The lysozyme is heat stable and is found in hemocytes and plasma of insects [61].

A putative role of insect lysozyme is to hydrolyse the cell walls of invading bacteria particularly Gram-positive bacteria [22]. Factors such as crowding, high humidity, and starvation cause *G. mellonella* larvae to increase blood lysozyme levels [37]; however, its presence does not ensure immunity [16].

3.0 The Role of Lectins and Antibacterial Proteins in Nonimmune Insects

Agglutinins (or lectins) are capable of agglutinating vertebrate blood cells and are considered to be lectin-like due to their specificities for carbohydrate groups [40, 69]. In insects, agglutinin activity has been found in the hemolymph of several orders (Orthoptera, Lepidoptera and Diptera).

Lackie [49] proposed that agglutinins in the immune response of insects could function as (1) membrane-bound receptors, (2) humoral opsonic factors, or (3) act independently of the hemocytes agglutinating bacteria. Studies with *Sarcophaga peregrina* and *Spodoptera exigua* larvae showed agglutinins to be produced in response to injury or injection of solutions into the hemocoel suggesting that some agglutinins are inducible and may be involved in wound repair or be a form of protective immunity [47, 58].

Agglutinins have been found in the hemocytes and fat body of *S. peregrina* [47, 56] and the grasshoppers *Melanoplus differentialis* and *M. sanguinipes* [11]. *In vitro* studies with hemocytes of *M. differentialis* and *M. sanguinipes* showed that neither grasshopper serum nor purified agglutinin stimulate the adhesion of hemocytes to particles or phagocytosis, indicating that the agglutinin is not generally involved as either an opsonin or recognition molecule though a limited number of granulocytes have agglutinin binding sites [11]. Lectins of stick insects (phasmids) act as opsonins bridging bacteria to hemocytes [71].

In *S. peregrina* larvae, the hemocytes from normal larvae have a lower affinity for the agglutinin compared with injured larvae. The binding to the hemocytes is specific to the galactose residue on the cell surface, which may be either masked or absent on the surface of normal hemocytes [47]. The association of agglutinins with the hemocyte surface was confirmed when hemocytes of injured insects were exposed to galactose or lactose, and lectin was released from the cell surface without causing cell lysis [47].

In *Periplaneta americana* and *Schistocerca gregaria,* agglutinin activity of whole serum cannot be totally inhibited and shows no obvious specificity for any one type of sugar. This implies that more than one type of agglutinin may be present [49]. However, other studies involving hemolymph of *Glossina* species have shown the presence of agglutinins which exhibit wide specificities for carbohydrate residues on the surface of human erythrocytes [44]. The different types of agglutinin differ in their sugar specificities and are directed mainly towards sorbose, trehalose, glucose, 2-deoxygalactose and to a lesser extent the deoxy, [1-4]- and/or [1-6]-linked derivatives of glucose [44]. The metal ion requirements of lectins for hemagglutinating activity depend on the type of lectin and insect species [20, 45, 58, 70].

4.0 Nonself Recognition

The cellular responses are initiated by the hemocytes recognizing foreign organisms invading the body. The constitutive lectins present in the plasma and the hemocytes have an opsonic role in several insect species and may be part of the recognition system [71] although the evidence is not unequivocal for all insect species [73]. The lectins enhancing bacterial attachment to hemocytes may act as bridging molecules between antigen and hemocyte or promote the activation of the prophenoloxidase system.

Recognition of nonself material(s) by the hemocytes may occur independently of plasma factors. Morton *et al.* [54] established the existence of carbohydrate receptors on the plasmatocytes and granulocytes of *G. mellonella*. The physicochemical properties of

antigens also trigger nonself responses in insects. The adhesion of antigens to hemocytes is influenced by antigen hydrophobicity and degree of electrostatic charge [50]. A two tier system of nonself recognition has been proposed by Lackie [51], one being nonspecific and based on physicochemical properties and the other on specific recognition systems.

III. Antibacterial Proteins in Acquired Immunity

Antibacterial proteins have been found in diverse orders of insects (Table 1); however, lepidopterous larvae have received the most attention due to their greater size and ease of rearing. Antibacterial proteins are produced *de novo* by insects in response to bacterial challenge [10, 46]. Of the antibacterial proteins which have been discovered and studied to date, the cecropins have received the most in-depth analysis. Cecropins from different insects are similar in that they have a strongly basic *N*-terminal region and a long hydrophobic section in the *C*-terminal part of the molecule [10]. The high degree of homology between the various groups of cecropins of *Antheraea pernyi* and *Hyalophora cecropia* indicates a similar evolution through gene duplication [10].

Two different genes exist, responsible for encoding the basic and neutral or acidic forms of the attacin molecules [10]. The attacins are larger in molecular weight than the cecropins and interfere with cell division [43]. The activity spectra of the different subclasses of cecropins and attacins vary [10]. In *G. mellonella,* both live and dead Gram-positive and Gram-negative bacteria act as immunizing agents for the induction of antibacterial proteins. All of these inducing agents give the same type or level of antibacterial activity [41]. The factors found in the hemolymph of *G. mellonella* larvae were similar in properties (heat stability and sensitivity) to the InA factor (an immune inhibitor) produced by *B. thuringiensis* [41]. Hemocytes from immune pupae stimulate the production of cecropins, lysozyme and other antibacterial proteins by *H. cecropia* fat body [77], and in *G. mellonella*, cecropin and antibacterial activity [19].

IV. The Genus *Xenorhabdus*

1.0 The Biochemical and Physiological Properties of *Xenorhabdus*

The genus *Xenorhabdus* (*Enterobacteriaceae*) Thomas and Poinar is comprised of bacteria that are Gram-negative chemoorganotrophic [76], and are peritrichously flagellated asporogenous rods of variable size (0.3 x 2.0 μm to 2.0 x 10 μm) with occasional filaments (15 - 50 μm in length)[3]. The bacteria are symbiotically associated with the entomopathogenic nematodes of the genus *Steinernema* and released into the insect hemolymph, effectively killing the insect [59, 60]. Stationary phase bacteria produce

Table 1. Overview of insect antibacterial proteins.[1]

Protein	Size (kDa)	Source	Activity Spectrum
Cecropins	4	Lepidoptera	Bactericidal, Gram -,+
Andropin	4	Diptera	Bactericidal, Gram -,+
Defensins	4	Diptera, Hymenoptera, Coleoptera, Odonata	Bactericidal, Gram +
Attacins (includes sacrotoxin I & diptericin	9 - 28	Lepidoptera, Diptera	Bactericidal, Gram -
M 13	72	Lepidoptera	Triggers coagulation of hemolymph
Hemolin	48	Lepidoptera	Recognition of nonself materials

[1] Table adapted from Faye and Hultmark [32].

two types of birefringent (non-polybetahydroxybutyrate) phase bright inclusions (type 1 and type 2)[3, 18] which contain traces of DNA, RNA and carbohydrates and are restricted to particular strains. The function(s) of these protein inclusions may be related to the host nematode since *Xenorhabdus* inclusions are metabolized *in vivo* in the presence of larval nematodes [18]. Members of the genus are facultatively anaerobic and exhibits respiratory and fermentative metabolism [3].

The genus currently consists of four species; *X. nematophilus*, *X. bovienii*, *X. poinarii*, and *X. beddingii* each associated with a specific nematode species [4]. Bacteria from the genus exist in two forms, phase I and phase II [8]. The phase I form (1) is carried by infective stage nematodes, (2) absorbs bromothymol blue from agar media, (3) absorbs neutral red from MacConkey's agar forming either red or brown colonies, (4) produces antimicrobial substances, and (5) produces lipase and protease, whereas, phase II form has none of these properties [8]. Phase I and phase II forms are also distinguishable by their soluble proteins and isoenzyme patterns [42]. Boemare and Akhurst [3] studied the biochemical and physical characteristics of colony form variants in *Xenorhabdus* spp. and found phase I variants were sticky and gummy on agar and difficult to disperse in liquids, whereas the phase II variants could be easily dispersed.

Xenorhabdus spp. produce glutamic acid decarboxylase [76] and metabolize *N*-acetylglucosamine, fructose, fumarate, glucose, L-glutamate, DL-glycerate, glycerol, L-malate, maltose, mannose, L-proline, pyruvate, succinate, L-alanine in API LRA medium, and most strains produce acid from utilizing glucose (no gas), mannose, maltose, N-acetylglucosamine, fructose, ribose, trehalose, and glycerol in API CHE medium [3]. Based on the API strips, five sugars were utilized and 13 carbon sources utilized out of 147 compounds tested, by all strains of *Xenorhabdus* tested [3]. All strains utilized citrate, acetate, and DL-lactate when tested in Pye's (1968) medium; some strains did not utilize these compounds in the API tests. All strains gave a negative result for acidification of melezitose solution when tested on API CH strips with API CHE medium [3]. Although these bacteria do not reduce nitrates to nitrites and are generally catalase negative and anaerogenic, they possess the remaining characteristics of the *Enterobacteriaceae* [76].

The bacteria produce cytochrome oxidase, urease, phenylalanine deaminase, catalase, ß-D-galactopyranoside, arginine dihydrolase, ornithine decarboxylase, and lysine decarboxylase, and are methyl red positive and Voges Proskauer positive differentiating them from the *Vibrionaceae* [3]. Indole is not produced and certain strains do not hydrolyze glycogen and starch but do hydrolyze casein, liquify gelatin, and produce

DNAase and lipase on Tween 40 and 60 [3]. Growth is not inhibited by KCN. Hydrogen sulfide is not produced [76].

2.0. The Interaction of *Xenorhabdus nematophilus* with the Hemolymph of Nonimmune *Galleria mellonella*

A pathogenic bacterium which has penetrated into the hemocoel of an insect must have (1) the ability of resisting the humoral defence factors of the hemolymph, and (2) the ability to defend against the attack of phagocytes or evade recognition if infection is to result in lethal septicemia [53]. The nematodes of the *S. carpocapsae-X. nematophilus* complex do not influence the fate of the symbiotic bacteria, *X. nematophilus* [28].

When injected into the hemocoel, the phase I form of *X. nematophilus* var *dutki* is not initially cleared from the hemolymph of *G. mellonella* during the first hour after injection, possibly due to bacterial surface changes induced by humoral enzymes [27]. However, the majority of the bacteria are cleared by 6 h by nodulation followed by bacterial emergence into the hemolymph as represented by an increase in bacterial numbers. The increase in number of *X. nematophilus* cells in the hemolymph is due to bacterial multiplication as evident by an increase in muramic acid (a unique bacterial cell wall compound) [27]. Transient hemocytopenia occurs within 1 h after injection of bacteria and this is followed by an increase in hemocyte counts. The hemocytes are damaged in that they are vacuolated, fail to adhere to coverslips or to exclude trypan blue, and are incapable of binding *X. nematophilus* or *B. cereus* [27].

The removal of phase II *X. nematophilus* var *dutki* from the hemolymph of *G. mellonella* occurred within 30 minutes of injection and the majority of the bacteria were removed from the hemolymph by 4 h. By 8 h, the bacterial numbers increased but by a significantly lower level than when the phase I form of the bacterium was used. Hemocytopenia occurred within 30 min after injection of phase II *X. nematophilus* and the hemocyte counts remained low for 2 h followed by an increase at 4 h [27]. These differences may be attributed to differences in the outer membranes (OMs) of phase I and phase II bacteria.

During the initial period of bacterial incubation in the hemolymph, hemocyte counts were neither elevated nor decreased during the first 30 min in the hemolymph. The absence of a hemocyte decrease during the early stages of infection would be advantageous for *X. nematophilus* and the nematode vector by ensuring the repair of host tissue damaged during nematode invasion and/or enabling the host to contain adventitiously introduced bacteria present on the cuticle of the nematode. This would

allow time for *X. nematophilus* to reach a level which can support the growth and reproduction of the nematode [27].

The increase in the levels of damaged hemocytes and bacteria was shown to be independent of ongoing bacterial metabolism, suggesting that the outer membranes of the bacteria were responsible for damaging the hemocytes and the emergence of *X. nematophilus* from the nodules and for hemocyte damage [27]. As the bacterial infection proceeded, a rapid increase in vacuolated hemocytes occurred followed by a lytic decline of hemocytes thereafter. Therefore a hemocytotoxin exists, which damages hemocytes [29].

The hemocytotoxin in *G. mellonella* infected with *X. nematophilus* was identified as lipopolysaccharide (LPS) because (1) the toxin isolated from infected larval serum by phenol/ water extraction, like LPS from the bacterial cell envelope, induced *Limulus* amoebocyte lysate coagulation, (2) injection of LPS resulted in fat body dissociation and hemocyte damage, and (3) the electrophoretic profiles of the LPS extracts were identical. The correlation of LPS release from varieties of *X. nematophilus* with hemocyte damage and emergence of *X. nematophilus* into the hemolymph established LPS as a virulence determinant of *X. nematophilus* [29]. LPS was released from both living and dead *X. nematophilus* but only when the bacteria were exposed to larval serum. Although serum is believed to modify the envelope of the bacterium, it is not known how this relates to LPS release.

The observations that (1) the LPS gel profiles of the *breton* and *dutky* varieties of *X. nematophilus* differed, (2) injecting LPS samples containing the same concentration of 3-deoxy-D-manno-octulosonic acid (KDO) damaged the hemocytes to the same extent, and (3) the binding of polymyxin B sulphate to lipid A neutralized LPS toxicity suggest that lipid A was the toxic moiety [29]. The LPS is believed to bind to lectin-like molecules on the granulocytes by means of the glucosaminyl group of lipid A. The fatty acids of *X. nematophilus* lipid A are responsible for endotoxic expression [29].

Injecting LPS and the corresponding amounts of lipid A and total oligosaccharide from the LPS showed that only the carbohydrate fraction lowered hemocyte counts below the control levels. Nodules were detected in the hemocoel in close proximity to the injection site in larvae receiving the oligosaccharide. Thus, LPS may bind to hemocytes by both lipid A and the oligosaccharide moieties of LPS [29].

The quantity and release of LPS from the outer membrane of *X. nematophilus* varies with bacterial isolate; wild type *X. nematophilus* contains less KDO than did antibiotic-resistant mutants. Differential rates of LPS release occurs between different

isolates *Xenorhabdus spp.*, but has not been correlated with bacterial virulence [23]. Mutant strains of *X. nematophilus* have reduced outer membrane protein (OMP) content compared with the wild type which is negatively correlated with KDO content [23]. Bacterial interaction with hemocytes is influenced by bacterial hydrophobicity, LPS content, cationic surface charge and PO activity in the test insect [23].

3.0 The Antihemocyte Surface Components of *Xenorhabdus*

The LPS of *X. nematophilus* reduces PO activity in the hemolymph of Lepidoptera *in vivo* [24, 30, 79]. However, incubating either *X. nematophilus* or its LPS in larval serum containing PO previously activated with laminarin did not result in reduced PO activity; thus, the toxin prevents PPO activation as opposed to inhibiting PO activity [30].

Although artificially-activated PO augments cellular responses towards *X. nematophilus* in *Lymantria dispar* [24], the absence of a correlation between PO activity and *X. nematophilus* levels in *L. dispar* and *G. mellonella* implies that PO is not an opsonin during the early stages of septicemia by *X. nematophilus* [24, 30]. Thus, other factors in the hemolymph may be involved. Incubating *X. nematophilus* with larval serum (with inhibited PO activity) altered the bacterial surface resulting in rapid reduction in bacterial levels. Deproteinizing the serum abrogated the effect establishing the opsonic factor(s) as proteinaceous [30].

Larval serum contains α-mannosidase-like, α- and ß-galactosidase-like and ß-N-acetyl-D-glucosaminidase-like (α-N-acetyl-D-glucosaminidase-like activity was not found) enzymes which have specific activities that vary with the enzyme; ß-galactosidase activity was the greatest and ß-N-acetyl-D-glucosaminidase the least [30]. Removal of carbohydrates from the OM of *X. nematophilus* enhanced bacterial attachment to the plasmatocytes of *G. mellonella* without the production of PO and accelerated bacterial removal from the hemolymph. Changes in the physicochemical surface properties alone of the bacteria may not be responsible for these nonself responses as the addition of enzyme substrates to suspensions of modified bacteria suppressed or reduced the responses. This suggests bacterial attachment to hemocytes may be lectin-mediated [30].

The importance of the OM of *X. nematophilus* in the interactions of the bacterium with *G. mellonella* hemocytes was demonstrated by accelerated removal of the bacteria from the hemolymph after the bacterial surface was modified by agents that dissociate protein and extract LPS. Such treatments may have (1) altered the bacterial surface charge and/ or hydrophobicity, (2) removed antihemocyte components, (3) exposed

antigens, or (4) increased bacterial sensitivity to humoral antibacterial factors [30]. The latter was demonstrated when EDTA-treated bacteria were exposed to purified insect lysozyme and insect serum, resulting in an increase in spheroplast formation.

OMPs and envelope appendages may contribute to the antihemocytic nature of *X. nematophilus* since proteolytic digestion of the bacteria elevates the nonself responses. Fimbriae of *X. nematophilus*, have an unknown function in terms of the pathogenicity of *X. nematophilus*, but for the closely allied bacterium, *Photorhabdus luminecens*, fimbriae may facilitate bacterial adhesion to insect hemocytes [13]. Growth conditions of the bacteria may also influence bacterial hemocyte interactions [13].

The OM and surface appendages of *X. nematophilus*, like those of other insect pathogens [33], may contribute to host antibacterial-pathogen interactions. Therefore, the objective of this study was to investigate the interaction of the surface components of *X. nematophilus* var *dutki* with the hemolymph environment inside the hemocel of nonimmune *G. mellonella* larvae. The objective was addressed by: (1) investigating physicochemical surface properties and how they relate to pathogen-host interactions, (2) determining the occurrence of bacterial surface components (fimbriae and flagella), and (3) relating surface properties and components to interactions with the host and determining their role in virulence.

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Chapter II

The Influence of Biochemical Properties, Cultural Conditions, and Physicochemical Surface Properties on the Interaction of *Xenorhabdus nematophilus* with Nonimmune *Galleria mellonella* Larvae

Abstract

Isolates E4 and E7 of *Xenorhabdus nematophilus* were qualitatively similar with respect to their biochemical properties but differed significantly in their LT_{50} (time to kill 50 % of the insects injected with a given bacterial dosage) values for *Galleria mellonella* larvae. Protease production by this bacterium may not be a virulence factor since enzyme activity against asocasein was detected *in vitro* only when the bacteria entered stationary phase. Artificial *Galleria* serum which aproximates *G. mellonella* hemolymph did not enhance the level of protease activity produced by the isolates of *X. nematophilus* when used as a growth medium over the levels of activity produced in YSB medium. The protease was not similar to subtilin because it did not use azocall as a substrate.

Differences in the LT_{50} values were not correlated with growth rates under aerobic or microaerobic conditions in yeast salts broth (YSB) medium. The growth of the bacterial isolates was significantly reduced in synthetic *Galleria* serum and in this medium supplemented with insect hemolymph. Isolates grown under the same cultural conditions to the same physiological age interacted differently with the antimicrobial defences of *G. mellonella* larvae. Cells grown aerobically were removed from the hemolymph more rapidly and for an extended period of time compared with microaerobically grown bacteria. The extent of bacterial removal increased with cultural age when *X. nematophilus* was grown microaerobically; however, this was not the case for aerobically cultured cells.

The clearance of these isolates from the hemocoel of *G. mellonella* was correlated with attachment to hemocytes *in vitro*. Clearance was not correlated with bacterial hydrophobicity, electrostatic charge or cell viability. Clearance of the bacteria from the hemocoel was negatively correlated with the surface area of the isolates.

Introduction

Xenorhabdus nematophilus is a virulent insect pathogen carried into the hemolymph of the host within the intestine of the insect parasitic nematode *Steinernema carpocapsae* [5].

The insect *Galleria mellonella*, a pest of beehives, responds to the bacteria with a combination of cellular and humoral mechanisms, the extent and efficiency of which varies with the bacterial species and strain [16]. The bacteria adhere to the insect hemocytes, initiate bacterial-hemocyte aggregations called nodules, and are thus removed from the hemolymph. These reactions were attributed to enzymes in the hemolymph modifying the carbohydrates and proteins of the bacterial outer membrane facilitating hemocyte contact [28]. Phenoloxidase (PO), a hemolymph enzyme with putative opsonic properties, is usually activated by bacterial cell wall and envelope components [9]. However, PO does not normally act as an opsonin during *X. nematophilus* infections because their lipopolysaccharides (LPS) prevent the activation of the prophenoloxidase (PPO) system [18, 28].

X. nematophilus proliferates within the nodules and subsequently emerges into the hemolymph [28]. Emergence is due to the lysis of the hemocytes caused by the release of LPS into the hemolymph and does not represent the multiplication of a selected subpopulation of bacteria that evaded the hemocytes [16].

Surface components and extracellular enzymes of insect pathogens contribute to pathogenic interaction with the host's antibacterial systems and to virulence [19, 32]. Although LPS and fimbriae may be involved in the adhesion of *X. nematophilus* and *Photorhabdus luminescens* to hemocytes [8, 28], the relationship of the extracellular enzymes and physicochemical properties of the outer membrane components of *X. nematophilus* to the interaction of the bacteria with the antibacterial systems of insects is unknown. Similarly, the effect of culture conditions, known to influence physicochemical properties of vertebrate pathogens [46], on *X. nematophilus* and its relationship to virulence and hemocyte responses is unknown. The present study will determine the contribution of biochemical factors such as secreted enzymes and utilized substrates, culture conditions including flask and tube cultures that were shaken or not shaken and the physicochemical surface parameters of hydrophobicity and type of charge of two strains of *X. nematophilus* that differ in virulence to bacterial interaction with the antibacterial systems of nonimmune *G. mellonella*.

Materials and Methods

Insect Culture

Galleria mellonella larvae were reared to the sixth instar according to Dutky *et al.* [29]. Unless stated otherwise insects weighing 200 mg were used in all experiments.

Isolation of *Xenorhabdus nematophilus* from Nematodes

Ten *G. mellonella* larvae were placed in a glass petri dish containing moistened filter paper (Whatman No. 1). The insects were parasitized with the addition of the infective stage nematodes (10/larva) of *Steinernema capocarpsae* strain DD 136 (supplied by Dr. J.Webster, Simon Fraser University) to the filter paper. The petri dish was incubated at 25 °C in the dark until the insects died. Dead insects were individually surface-disinfected with 95% (v/v) ethanol and passed through a flame. A thoracic proleg was severed with sterile microscisssors; the hemolymph was collected from individual insects with sterile pasteur pipets and was spread on the surface of tergitol-7-agar (Difco) supplemented with 0.04 mg/l of 2,3,5 - triphenyltetrazolium chloride (Sigma)(TTC medium)[1, 57]. Blue colonies were subcultured on TTC medium five times to ensure the purity of the bacterial isolates. Bacteria were considered to be *X. nematophilus* based upon the biochemical characteristics described by Boemare and Akhurst [7]. The six pure cultures so obtained were maintained at room temperature, in the dark, and were subcultured every 2 weeks on TTC medium. The phase I state of the isolates was confirmed based upon bromothymol blue uptake on TTC medium, protease activity and antibiotic production [1].

Bacterial Culture

Isolates of *X. nematophilus,* unless stated otherwise, were grown in 5 ml of yeast salts broth (YSB) media in 20 ml test tubes on a gyrotary shaker at 150 rpm at 27 °C for 18 h. The same conditions were used to grow *Bacillus subtilus* in tryptic soy broth (TSB).

Biochemical Properties of *X. nematophilus* Isolates

(1) Catalase Activity

Catalase activity was tested using a modification of Thomas and Poinar [57] in which the isolates were individually mixed in a drop of 3% (v/v) hydrogen peroxide. A positive test is indicated by the production of bubbles in the hydrogen peroxide.

(2) Nitrate Reduction

The six isolates of *X. nematophilus* were grown in Bacto-nitrate broth (Difco) in test tubes shaken at 150 rpm and 27 °C on a gyrotary shaker. The cultures were sampled at 24 and 48 hours and tested for their ability to reduce nitrate by the addition of sulfanilic acid and α-naphthylamine to each sample. A positive test resulted in the production of a dark red color [15].

(3) Lipase Activity

Lipase activity was determined on bacto-agar (1.2% (v/v)) supplemented with Tween 20 (1.0 % (v/v)) and Tween 80 (1.0% (v/v)) [6]. The plates were incubated at 27 °C for 10 days and lipase activity confirmed by cloudiness in the agar around bacterial colonies.

(4) Lecithinase Activity

Lecithinase activity was determined using egg yolk agar [7]. Bacteria were streaked on the surface of the agar and the cultures incubated at 27 °C for 10 days. A positive result is indicated by a clearing in the yolk agar and the occurrence of a white precipitate.

(5) Protease Activity

Two assays were used to discern protease activity: (1) gelatin hydrolysis at pH 7.2 was based upon the absence of precipitation of the protein induced by acidic $HgCl_2$ around colonies on plates previously incubated for 10 days at 27 °C [6] and (2) quantitative evaluation of proteolytic activity was based upon the release of the azo dye from azocasein (0.6% w/v) in phosphate buffered saline (PBS) [28]. *X. nematophilus* were grown in 2 ml of three types of media, yeast salts broth, tryptic soy broth (Scott), and artificial *Galleria* serum (AGS) [26], in 10 ml culture tubes on a horizontal gyrotary shaker (48 h, 27 °C, 150 rpm). Bacteria-free supernatant (1 ml) was produced by centrifugation (11 000 xg, 3 min., 25 °C) and added to the azocasein solution. The solution was incubated on a horizontal gyrotary shaker (2 h, 27 °C, 150 rpm). Controls consisted of azocasein in buffer with designated medium that was not inoculated with bacteria. The reaction was stopped by the addition of 200 µl of 5 N HCl, the precipitate removed by centrifugation (11 000 xg, 3 min, 25 °C) and the level of liberated azo dye in the supernatant determined spectrophotometerically based on a change in absorbance at a wavelength of 420 nm on a Beckman DU-70 spectrophotometer.

(6) Antibiotic Production *in vitro*

Isolates E4 and E7 of *X. nematophilus* were chosen for subsequent study because they differed in lipase and protease activity, aspects important to virulence [25]. The bacteria were grown in 2 ml of YSB on a horizontal gyratory shaker under conditions (48h, 27 °C, 150 rpm) that will be henceforth referred to a standard conditions. Following centrifugation (12,000 x g, 5 min, 25 °C), 25 µl of supernatant were aseptically added to sterile antibiotic disks and air dried. The disks were subsequently added to tryptic soy agar (TSA) that had been freshly inoculated with *B. subtilis* on a cotton swab to produce a lawn. *B. subtilis* is sensitive to antibiotics produced by *X. nematophilus* [44]. Cultures were incubated for 24 h and the zones of clearing around the disks were measured.

(7) Antibiotic Production *in vivo*

Bacterial isolates E4 and E7 grown in YSB under standard conditions were washed by centrifugation in PBS and 10 µl of the bacterial suspension (2.1×10^7 cells/ ml) injected into insects. The control insects were injected with 10 µl of PBS. Insects were incubated in darkness at 27 °C and at designated times 5 insects were homogenized in 1 ml of deionized water in a 20 ml tissue homogenizer. The homogenate was centrifuged in a 1.5 ml microcentrifuge tube (11 000 xg, 3 min., 25 °C) and the supernatant assayed for antibacterial activity as described by Maxwell *et al.* [44]. The level of bacteria per insect was also determined at the designated times by serially diluting the initial insect extracts. The serial dilutions of the insect extracts were spread on TSA containing bromothymol blue and TTC and incubated in the dark (3 days, 27 °C). Results are reported as colony forming units (CFU) per gram (wet weight) of insect tissue.

(8) Biochemical Characterization using API 20E and API ZYM

The API 20E and API ZYM systems were used to examine biochemical characteristics semiquantitatively. The API system was inoculated with an 18 h old culture grown in YSB under standard conditions and incubated (96 h, 27 °C) in the dark. The reactions were read every 24 h.

Lethal Time to Kill 50% of the Host Population (LT_{50})

Both isolates E4 and E7 of *X. nematophilus* were grown under standard conditions, washed three times by centrifugation in PBS, and the final bacterial level adjusted to 2.1×10^7 cells/ ml with PBS. Ten groups of *G. mellonella* larvae in each of 4 replicates were injected with 10 µl of bacterial suspension per insect per isolate. Control

groups consisted of insects injected with 10 µl of PBS. The insects were incubated at 27 °C in darkness without food. Insects were inspected at regular intervals until signs of dysfunction were evident, and thereafter by inspection at 10 minute intervals until all the insects died. An insect was considered dead when no response could be observed after prodding the facial plates with a pair of forceps.

Growth of Selected Isolates of *X. nematophilus* in Liquid Medium

The study of the growth of the bacterial strains in test tubes and Erlenmeyer flasks was done to determine the effect of different culture conditions on the growth rate of strains E4 and E7. This is important because different culture conditions is known to influence the virulence of bacterial pathogens of plants and mammals [4, 31, 37]. Bacteria previously grown under standard conditions were washed three times with PBS by centrifugation, adjusted to 2.1 x 10^7 cells/ ml after which 100 µl volumes were added to five 500 ml flasks containing 100 ml of YSB and 5 µl volumes were added to 10 ml volume test tubes containing 5 ml of YSB. The bacteria were grown under standard conditions and the cell level determined every 3 h (0.1 ml per sampling) using a Petroff Hausser counter and phase contrast microscopy.

To simulate growth of the bacteria *in vivo*, bacteria were grown under standard conditions, washed as before, and 100 µl of a 2.1 x 10^7 cells/ ml suspension was added to 600 µl of the following media in 1.5 ml microcentrifuge tubes: (1) artificial *Galleria* serum (AGS); (2) AGS with 18% (V/V) larval serum hemolymph; and (3) PBS with 18% (V/V) larval serum. The cultures were grown in the dark without shaking, at 27 °C and the cell level determined at 2 h intervals (10 µl per sampling) using a Pertroff Hausser counter and phase contrast microscopy.

Clearance of Bacteria at Different Physiological Ages from *G. mellonella*

Bacterial isolates (E4 and E7) were grown individually under standard conditions and used as inoculum. Experimental cultures were grown to the early exponential growth, late exponential growth, and stationary phases in test tubes and flasks. Bacteria in flasks were grown also to a physiological age similar to cells in stationary phase in test tubes. The bacteria were washed three times in PBS by centrifugation (11 000 xg, 3 min., 25 °C), adjusted to 2.1 x 10^7 cells/ ml and 10 µl were injected into each insect. Insects were incubated in two groups of five insects for 5, 30, 60, 120, and 240 minutes post-injection prior to bleeding. Hemolymph was collected with a chilled (4 °C) Pasteur pipet following

severance of a prothoracic leg. The pipets containing hemolymph were placed on ice to minimize coagulation and melanization. The hemolymph samples were placed on a hemocytometer and the bacteria and total hemocyte counts determined for each insect at the designated times using phase contrast microscopy.

Viable Plate Counts

To determine if the viability of bacteria in the previous experiment was correlated with the removal of the bacteria from the hemolymph samples of each bactrial group in PBS were plated on TSA amended with bromthymol blue and 2,3,5-triphenyl tetrazolium chloride. The plate counts were performed for isolates E4 and E7 grown in YSB for 6, 9, and 17 h of growth in test tubes and 6, 15 and 23 h of growth in flasks. The bacterial suspensions were plated following the standard wash procedure used to prepare bacteria for injection into the insects.

Bacterial Surface Area

Surface area, which may change as bacteria age, is known to influence the removal of Gram positive bacteria from the hemolymph of insects *in vivo* [51]. To determine if surface area influenced the clearance of isolates E4 and E7 from insects, bacteria were grown in YSB in tube and flask cultures at 27 °C as previously stated, and the bacteria sampled at the appropriate physiological ages. The bacteria were placed on slides, heat fixed, covered with glycerol and a cover slip, and the dimensions of the bacteria were measured with a standardized ocular micrometer. The surface area was calculated using the formula for the surface area of a cylinder ($A = 2\pi rh$, r=radius,h height) and adding it to the surface area of a sphere ($A = 4\pi r^2$) which represent the two ends of the bacteria.

Hemocyte Monolayers

Monolayers of insect hemocytes were used to determine if the differences in the removal of bacteria from the hemolymph observed in earlier experiments was due to differences in bacterial adhesion to the antibacterial hemocytes, the plasmatocytes and granulocytes. Five *G. mellonella* larvae were bled by cutting a prothoracic leg with sterile microsissors. Twenty microliters of hemolymph were collected from each of the insects and combined in an ice-chilled microcentrifuge tube producing a total volume of 100 µl. The 100 µl volume was added to a microscope slide and the hemocytes allowed to attach to the slides for 10 minutes. The slides were rinsed with three 1 ml volumes of

PBS to remove plasma and non adhering hemocytes. Bacteria grown in tubes and flasks to selected physiological ages, as previously described, were washed by centrifugation (11 000 x g, 3 min., 27 °C), resuspended in PBS, and adjusted to 2.1 x 10^7 cells/ ml. One hundred microliters of the bacterial suspension were added to the slides resulting in a bacteria:hemocyte ratio of 50:1. The suspensions were incubated at 27 °C in darkness on a gyrotary shaker (25 rpm) for 10 min. after which an additional 100 µl of PBS was added and the material incubated for an additional 10 min. The slides were rinsed with three 1 ml volumes of PBS to remove non adhering bacteria, and the bacterial-hemocyte complex fixed with 100 µl of 2.5% glutaraldehyde in PBS for an additional 10 minutes. The glutaraldehyde solution was removed using three 1 ml volumes of PBS. The slides were then coated with 100 µl of 5% (v/ v) glycerol in PBS and the monolayers covered with a coverslip. The slides were examined under phase contrast microscopy and the numbers of bacteria per plasmatocyte and granulocyte determined. The number of hemocytes of each type with adhering bacteria was also determined.

Hydrophobic and Electrostatic Interaction Chromatography

Bacteria were grown under standard conditions in test tubes and used as inocula. Media in the test tubes and flasks for each bacterial isolate were inoculated and grown under standard conditions to the appropriate physiological ages. The bacteria were washed once in PBS and suspended to an optical density between 0.1 - 0.4 (OD_{420nm}, using a Bechman DU70) in 1ml of nine salts solution (NSS) [14]. The hydrophobic interaction chromatography (HIC) resin, Octyl-Sepharose CL 4B (Pharmacia), and the electrostatic interaction chromatography (EIC) resins, Dowex 50x8-200 for cationic, and Dowex 1x8-200 for anionic exchange, were washed in NSS and resuspended in NSS (1g of resin/ ml of NSS). Following the adjustment of the bacterial suspension 25 µl of the resin suspension were added and the suspension incubated for 20 minutes. The OD of the samples were determined after the resins had settled to the bottom of the sample tubes. Control groups consisted of NSS without bacteria plus resin suspension and bacteria plus NSS without resin. The level of hydrophobic and electrostatic charge was determined using the following formula: Hydrophobicity/ electrostatic charge = |(initial sample OD - initial control OD) - (final sample (with resin) OD - final control (with resin) OD)| + | initial sample OD (without resin) - initial control OD (without resin)| x 100 %. The final values represent the percentage of cells which are hydrophobic or the percentage with a specific charge.

Statistical Analysis

Separation of means was performed at a significance level of $\alpha = 0.05$ using the Lsd test. All means are expressed as the mean $\pm$ the standard error of that mean.

Results

Isolation and Characterization of *X. nematophilus* Isolates

The six isolates of *X. nematophilus* obtained from the DD136 strain of *S. carpocapsae* were positive for protease, lipase, lecithinase and antibiotic activity and negative for catalase and nitrate reductase confirming the identity of the isolates as *X. nematophilus*.

Protease activity, based on the substrate azocasein, varied with the bacterial isolate and medium used (Table 1). Generally isolates produced the greatest proteolytic activity when grown in yeast salts broth medium and the least activity in tryptic soy broth. In all cases activity was detected when the bacteria were in the stationary phase in culture vessels which may represent nutrient limitation in the culture medium. The proteolytic enzyme(s) showed no activity for the substrate azocoll.

Additional biochemical characterization of isolates E4 and E7 with the API 20E (Table 2) and API ZYM (Table 3) strip systems established semiquantitative differences between the two selected isolates but no qualitative differences. Differences in activity for cysteine aminopeptidase, acid phosphatase, phosphohydrolase and α - glucosidase activity were most evident (Table 3) with isolate E4 producing higher levels than did isolate E7 except for cystine aminopeptidase activity which was more pronounced in cultures of E7 than in E4 cultures. Neither isolate produced either trypsin-like or chymotrypsin-like enzymes. The isolates also did not differ in their antibiotic production profiles within insect cadavers, both isolates producing antibiotics after the death of the insects (Figure 1). Isolate E7 was more virulent for *G. mellonella* larvae than E4 (Table 4).

Growth of *X. nematophilus* under Different Physiological Conditions

To determine if bacterial growth rate was linked to virulence, isolates E4 and E7 were grown under two conditions: (1) in oxygen rich media in shake flasks, and (2) in test tubes which eventually become microaerobic (which more closely approximates the insect hemocoel). The population doubling time (PDT) for isolates grown in YSB medium in test tubes were 1.02 $\pm$ 0.06 h and 1.08 $\pm$ 0.02 h for E7 and E4, respectively, and the PDT for cells grown in YSB medium in flasks were 1.01 $\pm$ 0.02 h and 1.28 $\pm$ 0.10 h for E7 and E4, respectively. The cultural pH for isolate E7 grown in test tubes and flask cultures in YSB medium were 6.67 $\pm$ 0.02 and 7.98 $\pm$ 0.02 at stationary phase (18 h). Individual random samples from test tubes and flasks containing YSB medium and isolate E4 during growth showed that isolate E4 altered the media pH to the same extent as isolate E7 uder similar growth conditions.

Table 1. Protease activity produced per cell of *Xenorhabdus nematophilus* in three types of liquid media using azocasein as a protease substrate.

Isolate	E1[1]	E3	E4	E6	E7	E8
Medium						
TSB	0.046 ± 0.007	0.056 ± 0.007	0.077 ± 0.009	0.046 ± 0.004	0.058 ± 0.002	0.215 ± 0.035
YSB	0.172 ± 0.018	0.156 ± 0.021	0.131 ± 0.004	0.161 ± 0.012	0.161 ± 0.014	0.149 ± 0.014
AGS	0.088 ± 0.002	0.107 ± 0.004	0.100± 0.002	0.096 ± 0.023	0.070 ± 0.002	0.103 ± 0.023

1 Values (mean ± SE) are expressed as the change in absorbance per minute per 2.8 x 10^{8} bacteria (n = 3). TSB - Tryptic soy broth, YSB - Yeast salts broth, AGS - Artificial *Galleria* serum.

Table 2. Biochemical Characterization of two isolates of *Xenorhabdus nematophilus* at designated times on API 20E test strips (API Laboratory Products Ltd.).

	Isolate E4				Isolate E7			
Time (h)	24	48	72	96	24	48	72	96
Enzyme activity or Reaction assayed								
Enzymes								
Oxidase	-	-	-	-	-	-	-	-
ß-galactosidase	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-
Arginine-dihydrolase	-	-	-	-	-	-	-	-
Lysine-decarboxylase	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Ornthine-decarboxylase	-	-	-	-	-	-	-	-
Trytophane-deaminase	ND[a]	ND	ND	-	ND	ND	ND	-
Substrates used								
Citrate	-	-	-	-	-	-	-	-
Indole	ND	ND	ND	-	ND	ND	ND	-
Gelatin-liquefaction	+	++	++	++	+w	++	++	++
Glucose	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-
Inositol	-	-	+	+	-	-	++	++
Sorbitol	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-
Products formed								
Acetoin	ND	ND	ND	-	ND	ND	ND	-
Hydrogen sulfide	-	-	-	-	-	-	-	-
NO_2	ND	ND	ND	-	ND	ND	ND	-
N_2 Gas	-	-	-	-	-	-	-	-

[a] not determined, - = no activity detectable, +w = activity weak but detectable, + = activity easily detectable, ++ = strong activity

Table 3. Characterization of two isolates of *Xenorhabdus nematophilus* using the API ZYM System.

Isolate	E4[1]	E7
Enzyme assayed	Concentration	Concentration
Alkaline phosphatase	0 - 5	0 - 5
Esterase (C4)	10	10
Esterase (C8)	5	5
Lipase (C14)	0	0
Leucine aminopeptidase	≥ 40	≥ 40
Valine aminopeptidase	5	5
Cystine aminopeptidase	0	5
Trypsin	0	0
Chymotrypsin	0	0
Acid phosphatase	20	5
Phosphohydrolase	≥ 40	10
α - galactosidase	0	0
ß - galactosidase	0	0
ß - glucuronidase	0	0
α - glucosidase	≥ 40	5
ß - glucosidase	0	0
N - acetyl- ß -glucosaminidase	30	20
α - mannosidase	0	0
α - fucosidase	0	0

[1] values represent the concentration of enzyme in nanomoles as per the manufacture's procedures.

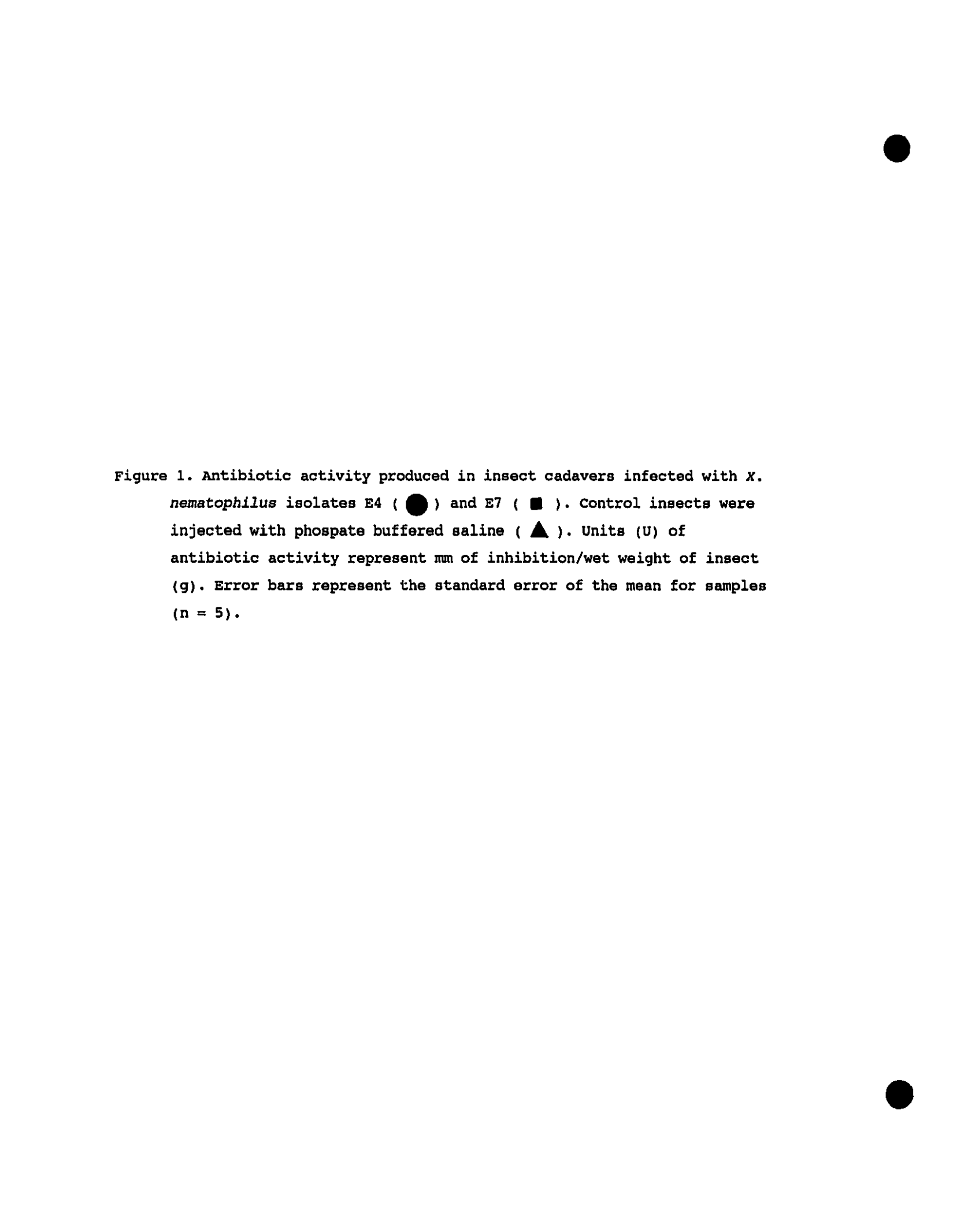


Figure 1. Antibiotic activity produced in insect cadavers infected with *X. nematophilus* isolates E4 (●) and E7 (■). Control insects were injected with phospate buffered saline (▲). Units (U) of antibiotic activity represent mm of inhibition/wet weight of insect (g). Error bars represent the standard error of the mean for samples (n = 5).

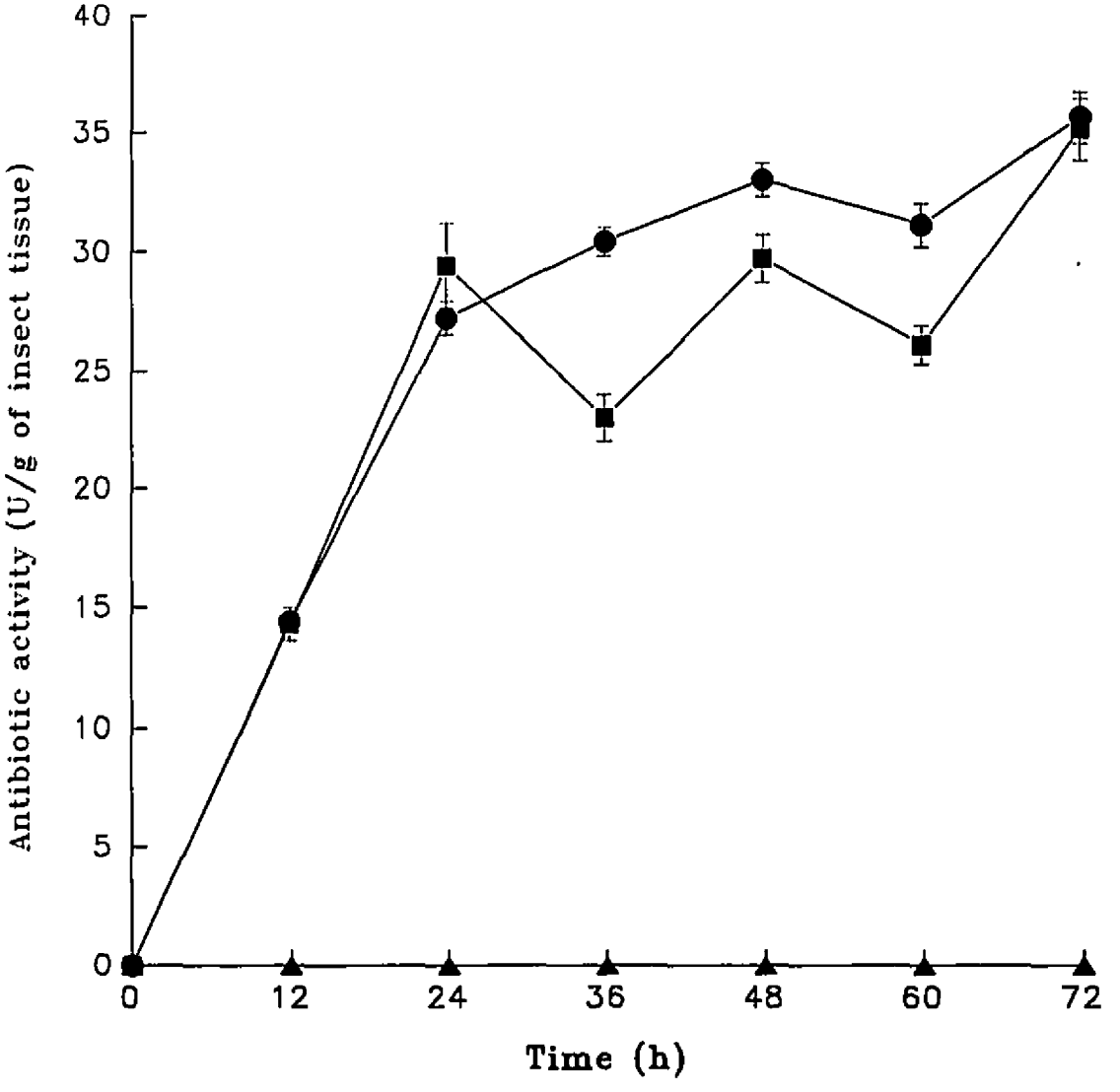

Antibiotic activity (U/g of insect tissue)
40
35
30
25
20
15
10
5
0
0
12
24
36
48
60
72
Time (h)

Table 4. Lethal Time for *Xenorhabdus nematophilus* isolates E4 and E7 to kill fifty percent nonimmune *Galleria mellonella* larvae.

Isolate	Probit line slope x -intercept	LT_{50}[1] (h)	95 % Confidence Intervals
E4	Y = 78.1X + - 82.6	13.2 ± 1.0	13.1 , 13.3
E7	Y = 42.4X + - 41.1	12.2 ± 1.0	12.1 , 12.3

[1] The lethal time to kill 50 % of insects injected with *Xenorhabdus nematophilus* (2.1 x 10^7 cells/ml) is expressed as the mean ± standard error of the mean (n = 40). Y = the probit of the percent cumulative mortality; X = the log of time.

The growth patterns *in vitro* were similar for the two isolates grown under the same conditions (Figure 2 and 3). Thus differences in isolate growth rates may not be responsible for differences in virulence. Although the growth rate *in vitro* may not relect the growth rate *in vivo* differences between isolates biochemical and physiological properties may be enhanced and may yield clues to differences in isolate behavor *in vivo*.

Recognizing that YSB is not similar to the insect hemolymph, PDTs were calculated for bacteria grown in AGS, AGS supplemented with 18% (v/v) larval serum, and phosphate buffered saline (PBS) supplemented with 18% larval serum. No significant difference in PDT occurred for a given isolate in AGS supplemented with larval serum (Figure 4 and 5). It was observed that cultures of E7 in AGS supplemented with larval serum showed less melanization than cultures of E4. The increased amount of melanization may have reduced reduced the growth of isolate E7 or the particles of melanin may have obscured the bacterial cells during on the counting chamber. However, in AGS the PDT was longer for E7 isolate (1.54 ± 0.05 h) of *X. nematophilus* than isolate E4 (1.36 ± 0.04 h). Cultures of isolate E7 also failed to reach stationary phase by 12 h. Isolate E4 of *X. nematophilus* also grew faster in PBS supplemented with larval serum than isolate E7. The PDTs for isolates E4 and E7 were 1.41 ± 0.04 h and 1.70 ± 0.04 h respectively. No relationship between virulence and growth was observed.

In vivo Response of *Galleria mellonella* to *X. nematophilus*

Isolates E4 and E7 grown in tubes to the same physiological age were not similar *in vivo* in their interactions with the insect's antibacterial system. In general, as the physiological age of E4 increased, the extent of bacterial removal from the hemolymph increased (Figure 6). This effect was not seen for isolate E7, the bacteria being cleared to the same level at all ages except for those from the 6 h tube these bacteria were not removed from the hemolymph but rather increased in concentration (Figure 7). Both isolates grown in tubes emerged into the hemolymph at similar rates. Reemergance into the hemolymph was independent of inoculum age for both isolates. Bacteria from flask cultures at the same physiological age as bacteria grown in tubes (17 h) had a clearance pattern from the hemolymph of the insect that differed from those grown in test tubes. The cells of isolate E4 grown in flasks floated in the blood for 30 min and were then gradually removed from circulation to the lowest level by 2 h followed by gradual emergence of the bacteria back into the hemolymph whereas E7 exhibited an immediate and continuous decline for 4 h. With the exception of E7 23 h flask culture, the bacteria of both isolates emerged into the hemolymph. The rates of emergence were similar,

Figure 2. Growth of isolate E4 of *X. nematophilus* in yeast salts broth. Bacteria were grown in tubes (●) and flasks (■) at 27 °C, 150 rpm in darkness. Error bars represent the standard error of the mean of each point (n = 5).

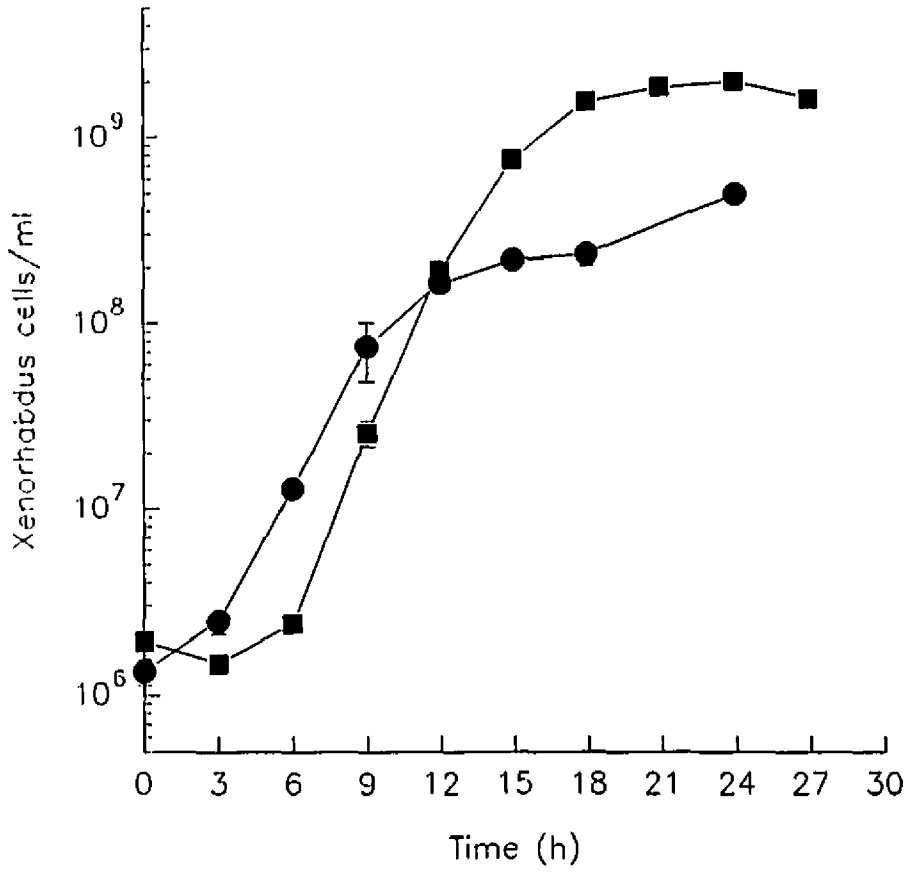

Xenorhabdus cells/ml
10^9
10^8
10^7
10^6
0
3
6
9
12
15
18
21
24
27
30
Time (h)

Figure 3. Growth of isolate E7 in yeast salts broth. Bacteria were grown in tubes (●) and flasks (■) at 27 °C, 150 rpm in darkness. Error bars represent the standard error of the mean of each point (n = 5).

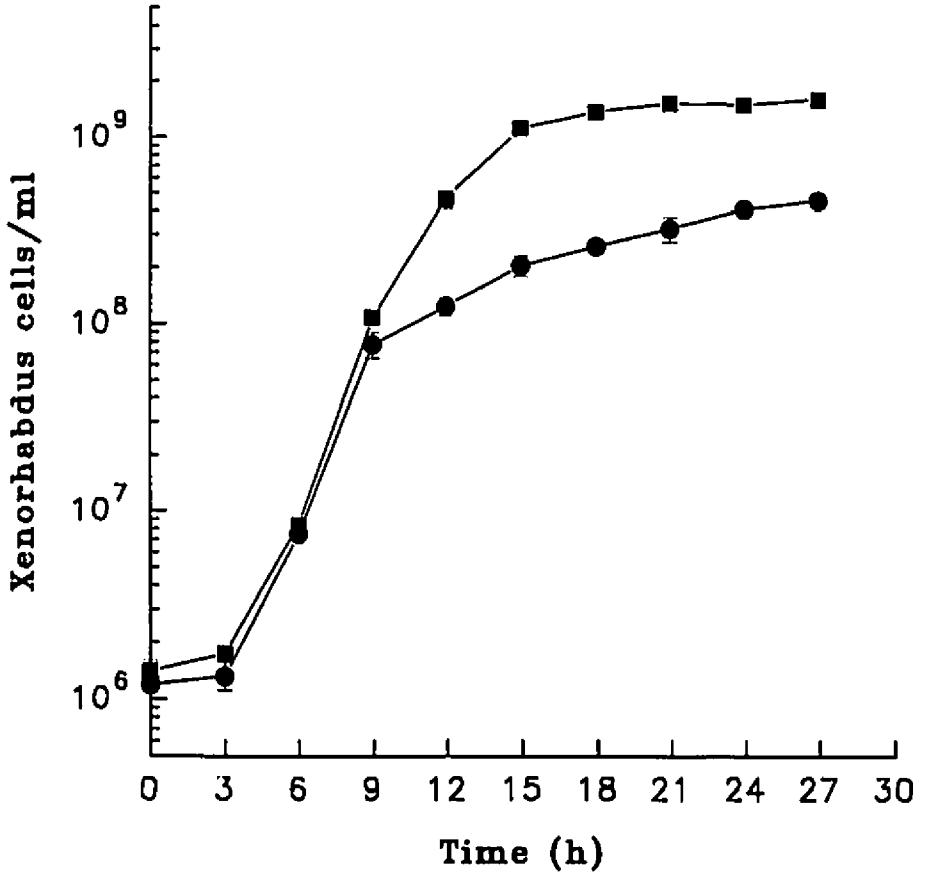

Xenorhabdus cells/ml
10^9
10^8
10^7
10^6
0
3
6
9
12
15
18
21
24
27
30
Time (h)

Figure 4. Growth of isolate E4 in growth medium simulating hemolymph. Bacteria were grown in artificial *Galleria* serum (AGS) (●), AGS + 18% larval serum (v/v) (■), and phosphate buffered saline + 18% larval serum (v/v) (▲) at 27 °C, unshaken in darkness. Error bars represent the standard error of the mean of each point (n = 5).

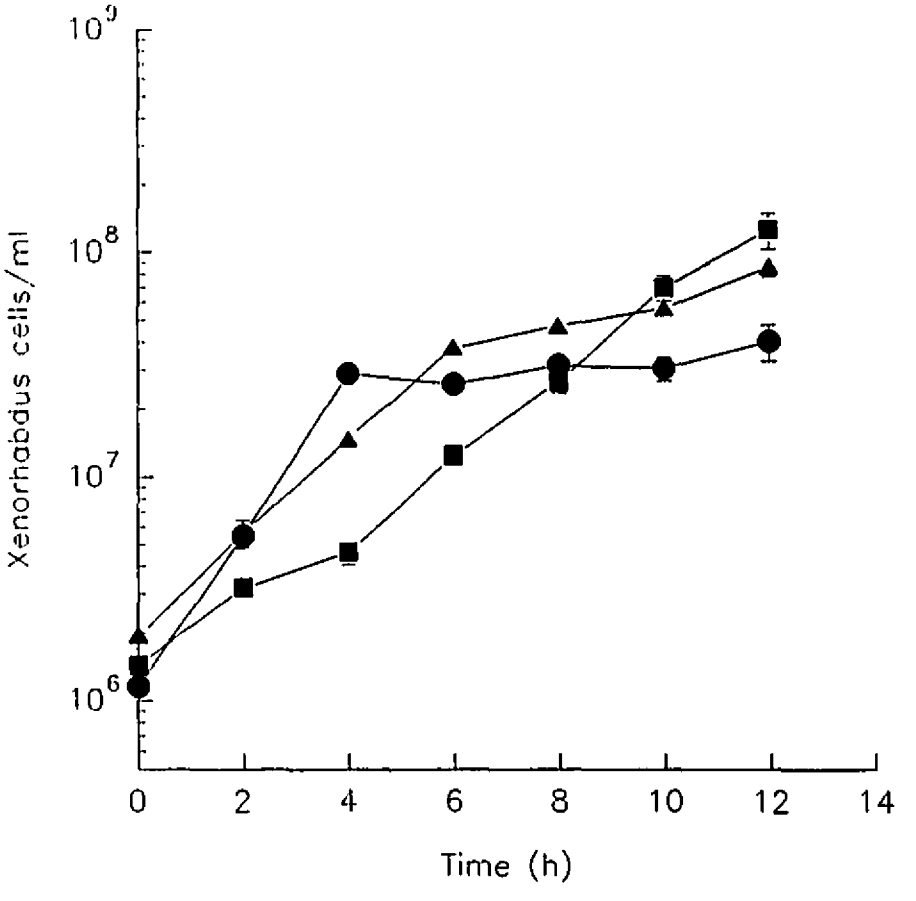

10^9
10^8
10^7
10^6
Xenorhabdus cells/ml
0
2
4
6
8
10
12
14
Time (h)

Figure 5. Growth of isolate E7 in growth medium simulating hemolymph.
Bacteria were grown in artificial *Galleria* serum (AGS) (●),
AGS + 18% larval serum (v/v) (■), and phosphate buffered saline
+ 18% larval serum (v/v) (▲) at 27 °C, unshaken in darkness.
Error bars represent the standard error of the mean of each point (n = 5).

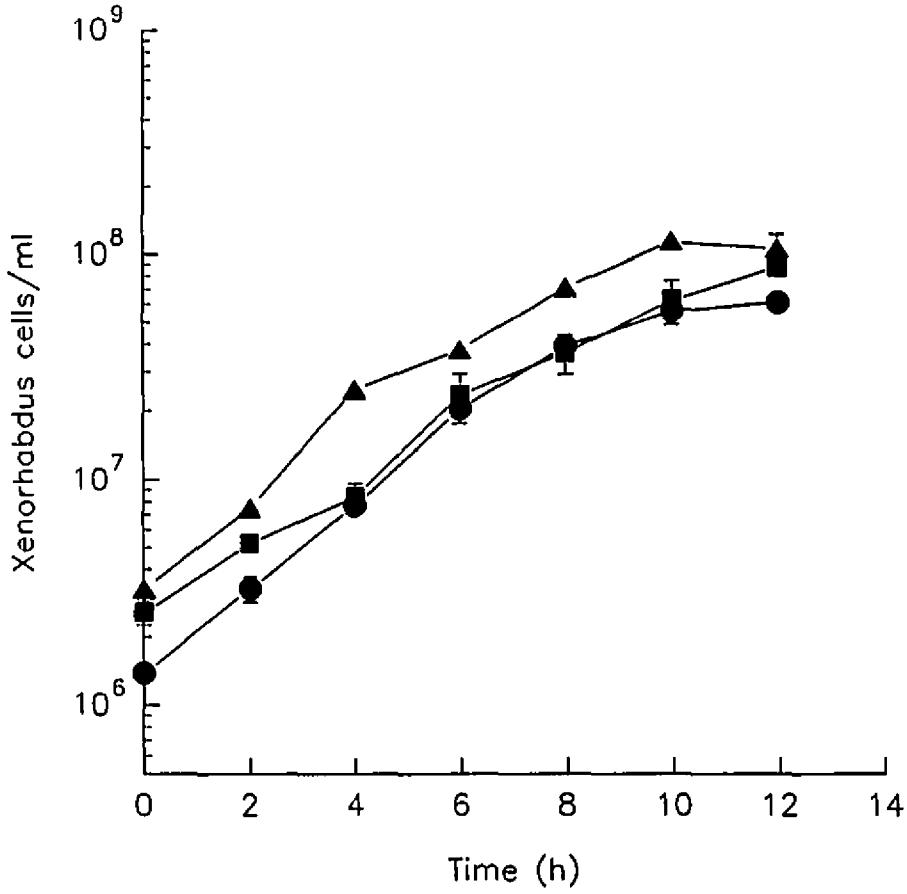
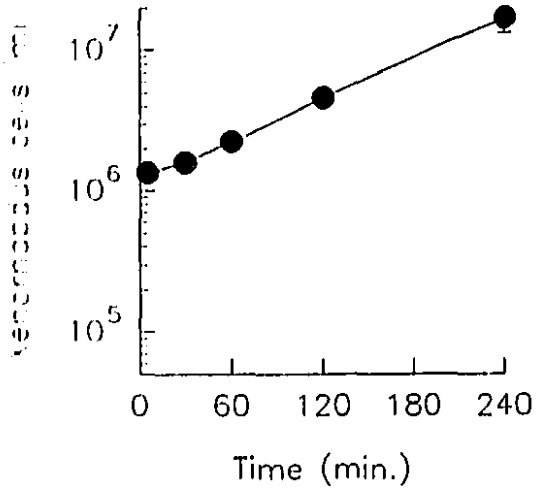
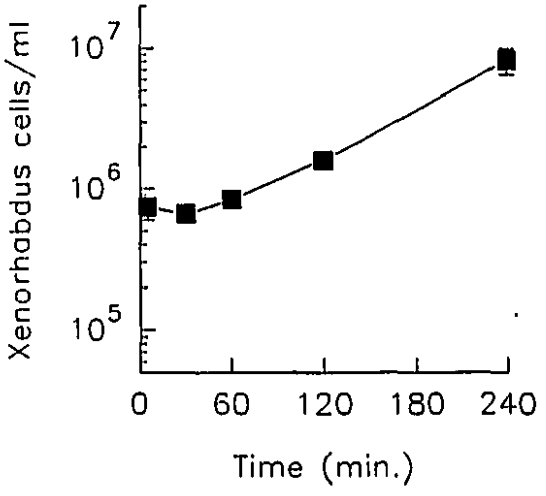
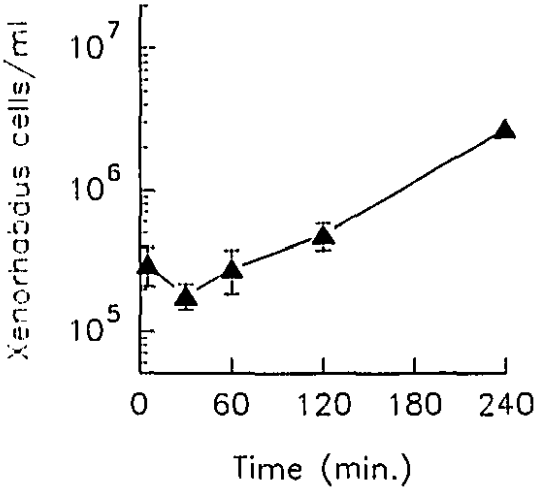

10^9
10^8
10^7
10^6
Xenorhabdus cells/ml
0
2
4
6
8
10
12
14
Time (h)

Figure 6. Clearance of *Xenorhabdus nematophilus* isolate E4 at different physiological ages and from different culture conditions from the hemolymph of nonimmune *Galleria mellonella* larvae *in vivo*; 6 h tube (exponential growth) (●), 9 h tube (late exponential growth) (■), 17 h tube (stationary phase) (▲), and 23 h flask (same physiological age as 17 h tube) (▼). Error bars represent the standard error of the mean and the means represent the number of free floating bacteria (n = 10).


10^7
10^6
10^5
0 60 120 180 240
Time (min.)


Xenorhabdus cells/ml
10^7
10^6
10^5
0 60 120 180 240
Time (min.)


Xenorhabdus cells/ml
10^7
10^6
10^5
0 60 120 180 240
Time (min.)

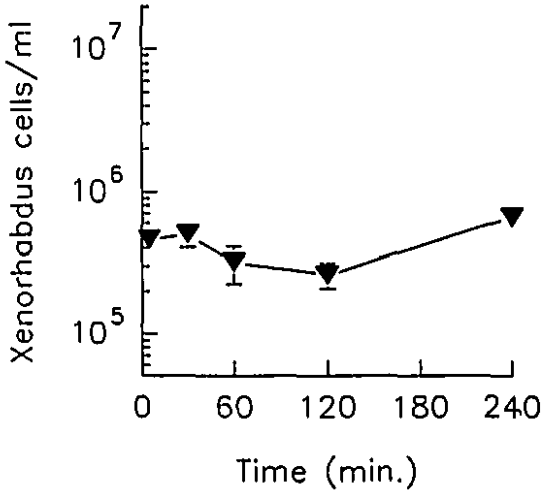
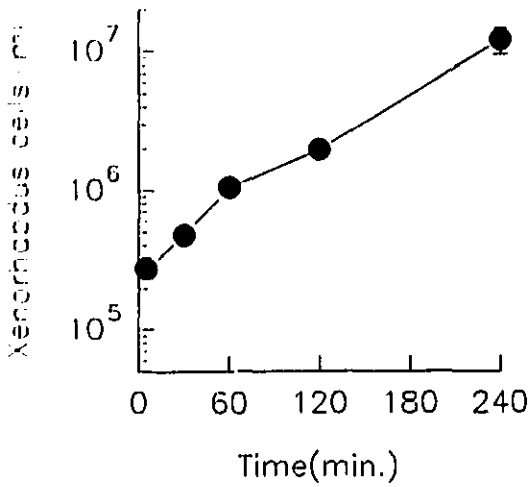
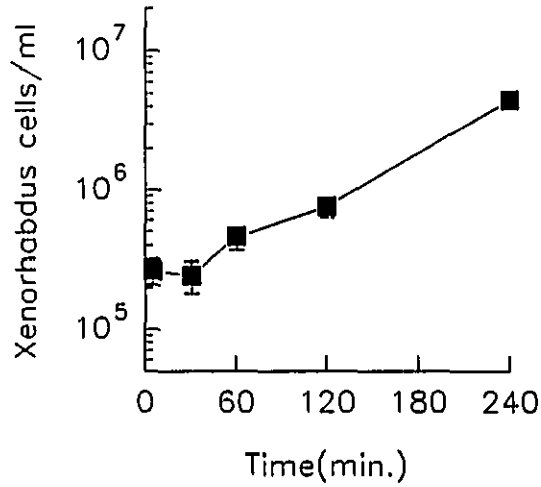
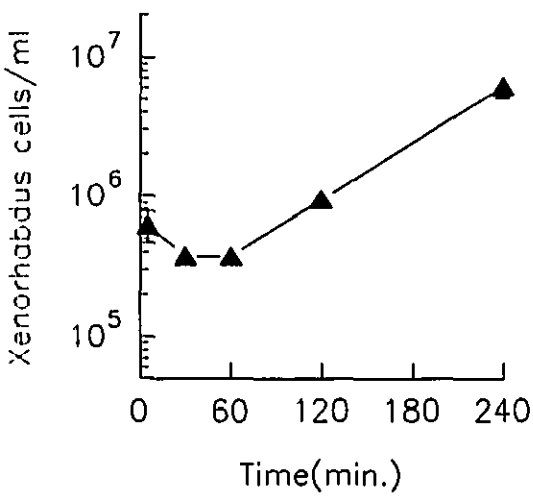
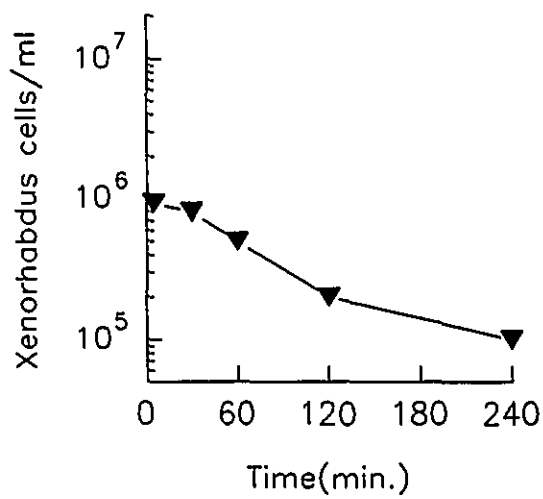

Xenorhabdus cells/ml
10^7
10^6
10^5
0 60 120 180 240
Time (min.)

Figure 7. Clearance of *Xenorhabdus nematophilus* isolate E7 at different physiological ages and from culture conditions from the hemolymph of nonimmune *Galleria mellonella* larvae *in vivo*; 6 h tube (exponential growth) (●), 9 h tube (late exponential growth) (■), 17 h tube (stationary phase) (▲), and 23 h flask (same physiological age as 17 h tube) (▼). Error bars represent the standard error of the mean and the means represent the number of free floating bacteria (n = 10).


10^7
10^6
10^5
0 60 120 180 240
Time(min.)


Xenorhabdus cells/ml
10^7
10^6
10^5
0 60 120 180 240
Time(min.)


Xenorhabdus cells/ml
10^7
10^6
10^5
0 60 120 180 240
Time(min.)


Xenorhabdus cells/ml
10^7
10^6
10^5
0 60 120 180 240
Time(min.)

except for the slower rate of E4 from the 17 h flask culture (Figure 6). Emergence occurred as the hemocyte counts increased. The hemocytes were highly vacuolated at this time indicating damage. Total hemocyte counts increased for both E7 from 23 h flask cultures and E4 from 17 h tube cultures, even though bacteria in the former did not emerge and bacteria from the latter emerged slowly. Bacterial emergence into the hemolymph was not related to virulence. There was no relationship between the rate of removal of the bacteria from the hemolymph and virulence.

To determine if inoculum viability played a role in these results, viable plate counts were done for the two bacterial isolates from the aforementioned growth conditions. The viability of isolate E4 and to a lesser extent the viability of isolate E7 declined in the tubes with increasing culture age (Table 5). However, there was no discernible difference in the viability of isolate E4 and isolate E7 in 6 h tubes or 23 h flasks. A correlation ($r=0.826, P<0.05$) between the viability of bacteria in tubes at different physiological ages and the number of hemocytes with attached bacteria (Table 6). Due to cultural differences no comparisons were possiable with cells grown in tubes to cells grown in flasks.

There was no correlation ($r=0.05, P>0.05$) between the surface area of the bacteria of either isolate and bacterial removal from the hemolymph. Increasing the dose of bacteria injected into the insects to correct for differences in surface area between isolates or age groups was not feasible as it resulted in an increase in contact of the bacteria with the hemocytes. In general, older bacteria were cleared more extensively from the hemolymph than bacteria from younger cultures of a given isolate although the surface area was reduced in the older bacteria.

Hemocyte levels in larvae injected with the bacteria at different physiological ages were lower than in the control insects during the initial 1 h post-injection, indicating recognition of the bacteria by the hemocytes (Figure 8 and 9). With the exception of larvae injected with isolate E4 from a 17 h tube culture and isolate E7 cultured in flasks, hemocyte levels during this time did not differ for either isolate or physiological age. Both isolate E4 and isolate E7 grown in test tubes for 17 h induced the greatest hemocytopenia which was not related to bacterial viability. Although differences in the surface of the bacteria of different ages may have altered the stimulation of the hemocytes, no differences were found between the surface area of bacterial isolates at comparable physiological ages, but significant differences occurred between different physiological ages of isolate E7 as opposed to isolae E4 (Table 7). Thus there was no discernible relationship between bacterial surface area and changes in THC.

Table 5. Viability of *Xenorhabdus nematophilus* at different physiological ages in yeast salts broth.

Isolate	Culture Age (h)	Culture Condition	Viable Count (cells/ml)
E4	6	tube	$1.03 \times 10^{6} \pm 1.39 \times 10^{5}$
E4	9	tube	$8.23 \times 10^{5} \pm 8.19 \times 10^{4}$
E4	17	tube	$2.28 \times 10^{5} \pm 1.61 \times 10^{4}$
E4	23	flask	$1.17 \times 10^{6} \pm 1.38 \times 10^{5}$
E7	6	tube	$1.32 \times 10^{6} \pm 5.37 \times 10^{4}$
E7	9	tube	$7.31 \times 10^{5} \pm 1.18 \times 10^{5}$
E7	17	tube	$6.04 \times 10^{5} \pm 5.77 \times 10^{4}$
E7	23	flask	$1.26 \times 10^{6} \pm 4.08 \times 10^{4}$

The bacteria were grown in yeast salts broth, washed in phosphate buffered saline and adjusted to a total bacterial count of 2.1×10^{7} cells/ml using a Petroff Hausser counter prior to plating on tergitol-7 agar supplemented with 2, 3, 5 - triphenyltetrazolium chloride. Values represent the mean ± the standard error of the mean (n = 12).

Table 6. Adhesion of *Xenorhabdus nematophilus* at designated physiological and cultural conditions to monolayers of hemocytes of *Galleria mellonella* larvae.

Isolate	Physiological Age (h)	Hemocytes with Bacteria (%)		Bacteria per Hemocyte	
		Gr	Pl	Gr	Pl
E4	6	12.0 ± 2.5	5.6 ± 1.8	1.0	1.0
E4	9	24.0 ± 3.0	10.4 ± 2.1	1.0	1.0
E4	17	21.6 ± 2.7	11.2 ± 4.1	1.1	1.4
E4	23[1]	13.6 ± 3.9	24.0 ± 3.8	1.1	1.2
E7	6	12.0 ± 3.6	3.2 ± 1.8	1.0	1.0
E7	9	12.8 ± 1.8	7.2 ± 2.6	1.0	1.0
E7	17	24.0 ± 3.6	18.4 ± 3.5	1.0	1.1
E7	23	17.6 ± 5.7	7.2 ± 0.7	1.1	1.1

1 same physiological age as 17 h in a tube culture. Gr- granulocyte, Pl- plasmatocyte. Ages 6 - 17 h cells grown in tubes in darkness, 150 rpm, 27 ° C. Age 23 h cells grown in flasks in darkness, 150 rpm, 27 ° C. Mean ± standard error of the mean (n = 125).

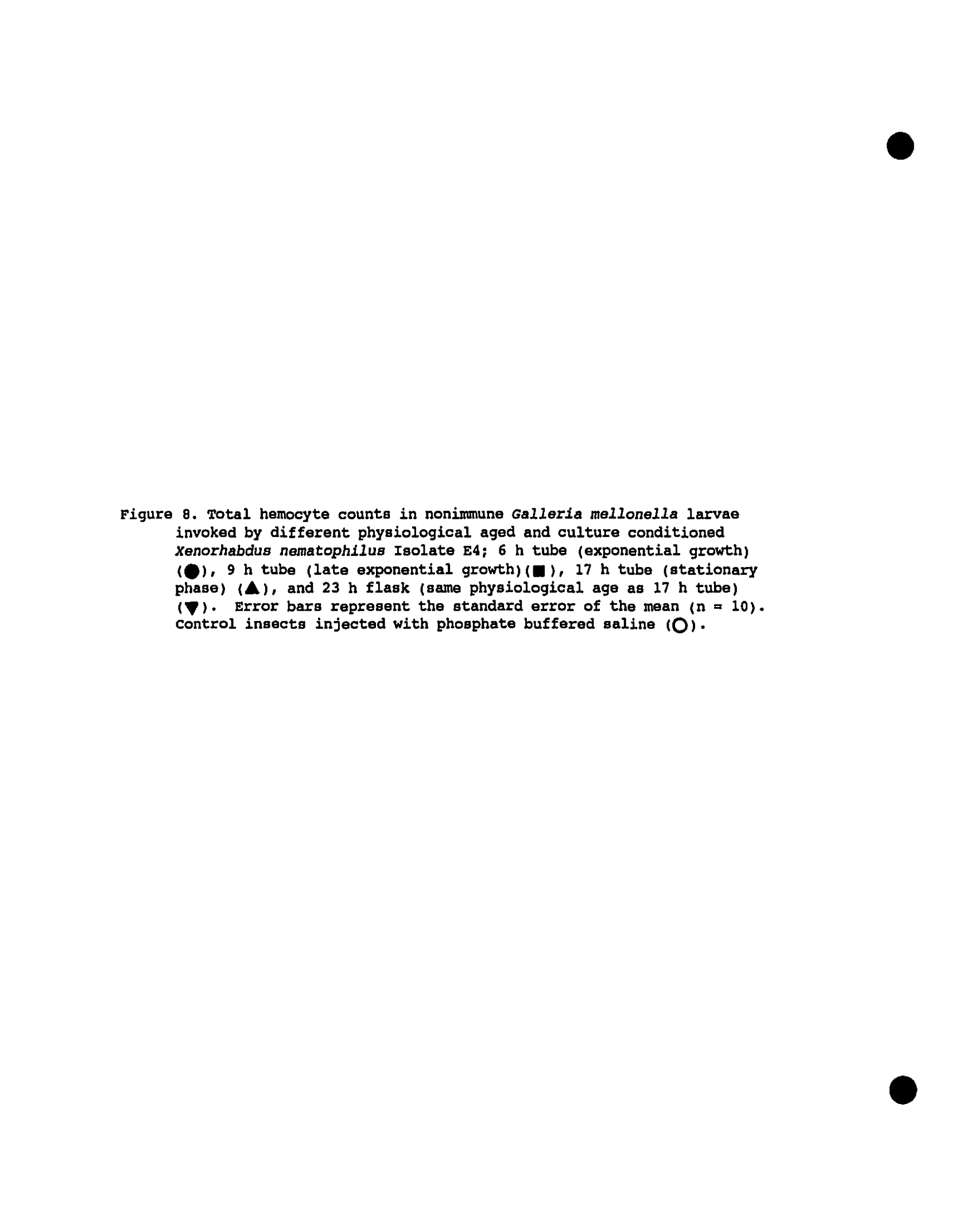
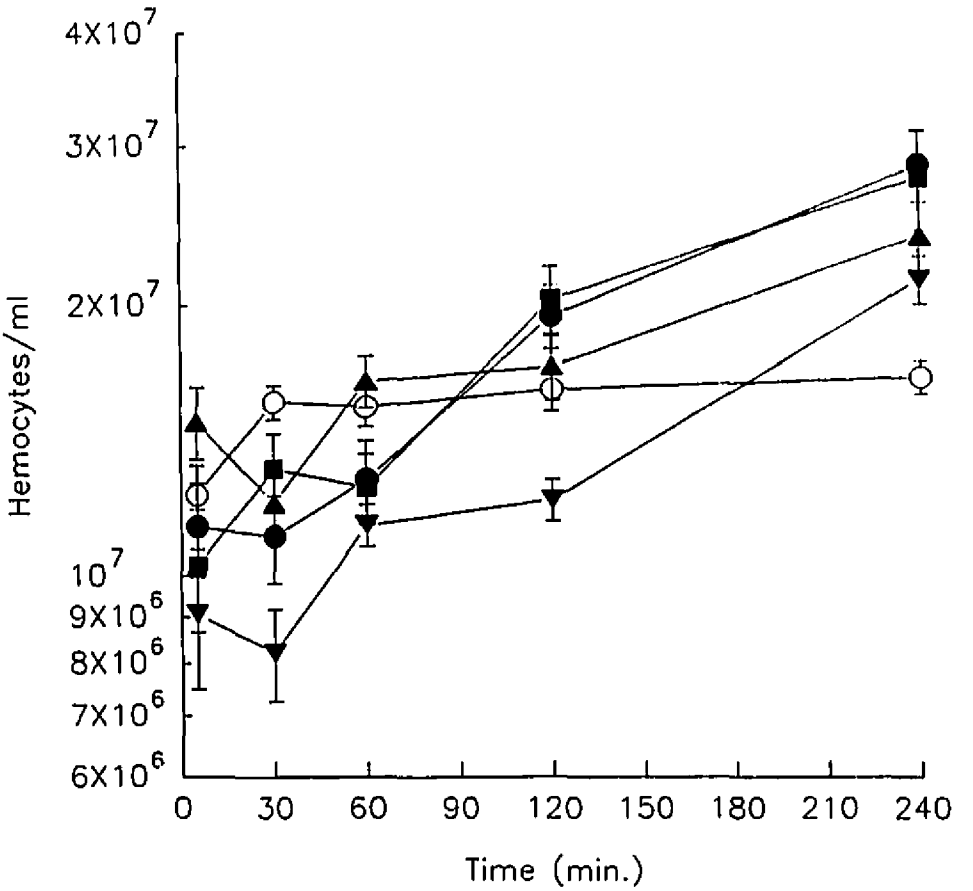


Figure 8. Total hemocyte counts in nonimmune *Galleria mellonella* larvae invoked by different physiological aged and culture conditioned *Xenorhabdus nematophilus* Isolate E4; 6 h tube (exponential growth) (●), 9 h tube (late exponential growth)(■), 17 h tube (stationary phase) (▲), and 23 h flask (same physiological age as 17 h tube) (▼). Error bars represent the standard error of the mean (n = 10). Control insects injected with phosphate buffered saline (○).


4X10^7
3X10^7
2X10^7
10^7
9X10^6
8X10^6
7X10^6
6X10^6
Hemocytes/ml
0
30
60
90
120
150
180
210
240
Time (min.)

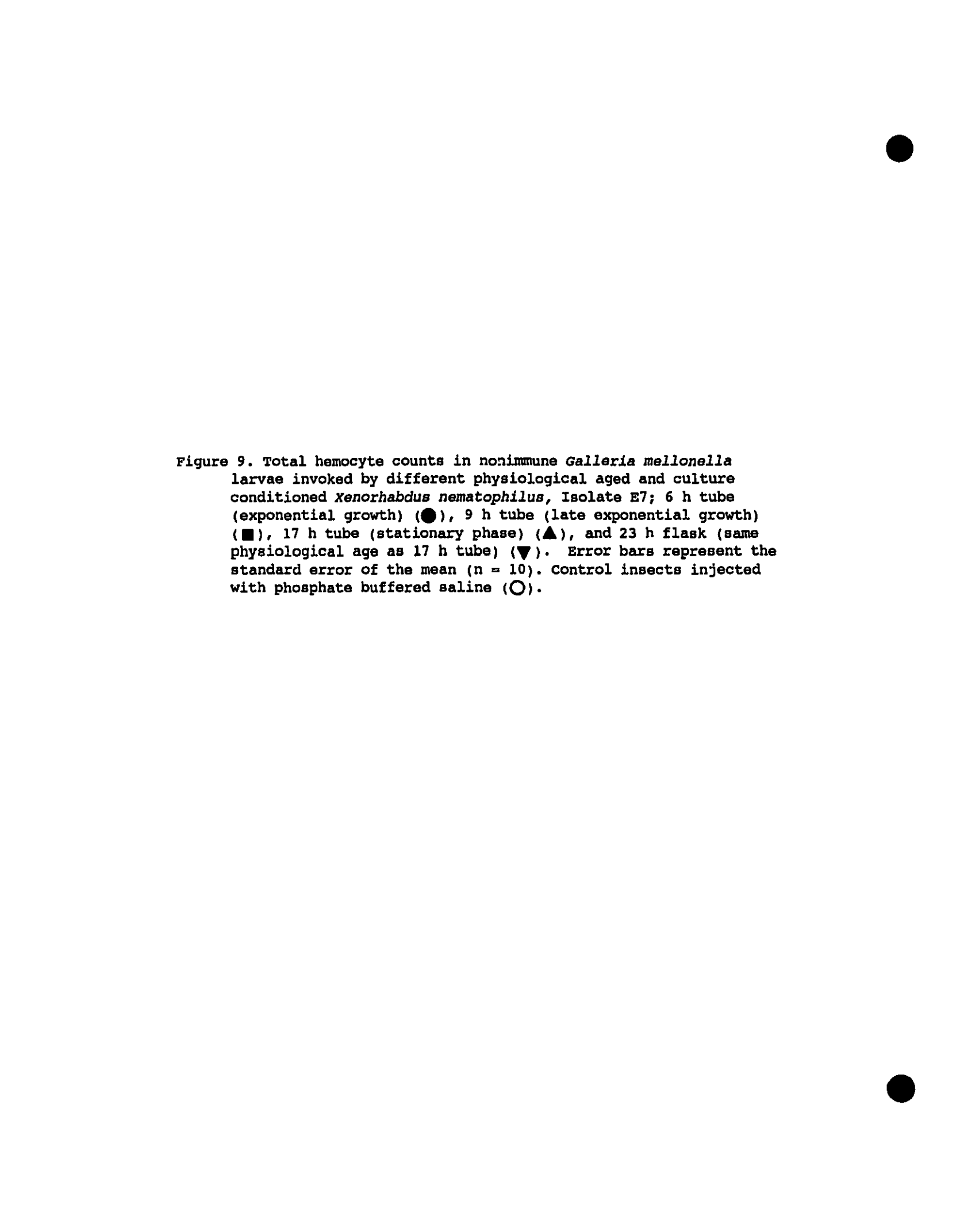


Figure 9. Total hemocyte counts in nonimmune *Galleria mellonella* larvae invoked by different physiological aged and culture conditioned *Xenorhabdus nematophilus*, Isolate E7; 6 h tube (exponential growth) (●), 9 h tube (late exponential growth) (■), 17 h tube (stationary phase) (▲), and 23 h flask (same physiological age as 17 h tube) (▼). Error bars represent the standard error of the mean (n = 10). Control insects injected with phosphate buffered saline (○).

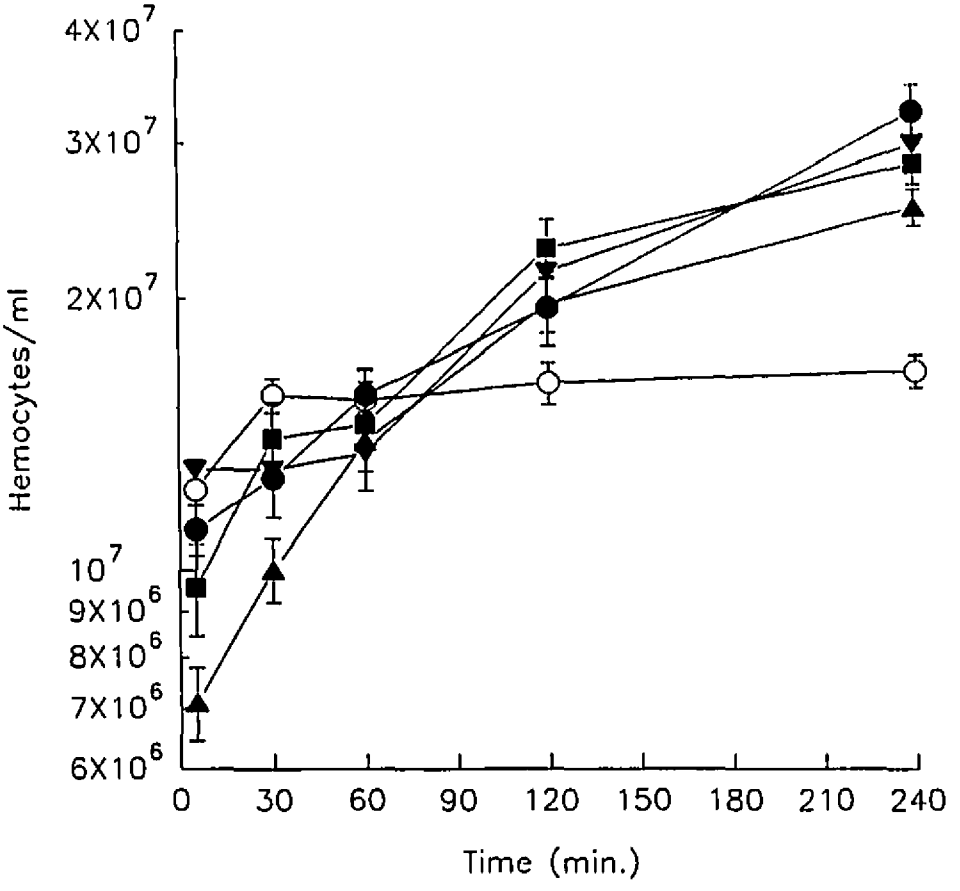

4X10^7
3X10^7
2X10^7
10^7
9X10^6
8X10^6
7X10^6
6X10^6
Hemocytes/ml
0
30
60
90
120
150
180
210
240
Time (min.)

Table 7. Bacterial surface area of *Xenorhabdus nematophilus* isolates E4 and E7 in yeast salts medium at selected physiological ages from two methods of culture.

Isolate	Culture Type	Age (hours)	Measurements (μm) Width	Length	Surface Area per Bacterial Cell (μm^2)
E4	tube	6	1.01±0.12	4.20±1.18	39.47±0.38a
E4	tube	9	1.08±0.11	3.32±1.02	37.18±0.33b
E4	tube	17	1.04±0.12	3.79±1.59	38.36±0.86a
E4	flask	23	1.09±0.11	2.42±0.56	31.50±0.34c
E7	tube	6	1.086±0.0346	4.14±0.18	43.07±0.31a
E7	tube	9	ND	ND	ND
E7	tube	17	0.998±0.017	3.86±0.20	36.72±0.15b
E7	flask	23	1.0975±0.0174	2.77±0.08	34.25±0.05c

The surface area of the bacteria are expressed in μm^2 and ages of the bacteria correspond to different growth phases of the bacteria. Bacteria grown in flasks represent the same physiological age of bacteria grown in tubes (stationary phase). Least significant difference test (LSD) $\alpha = 0.05$ values followed by the same letter in the same column are not significantly different (n = 41). Means are present ± their standard error.

Bacterial adhesion to hemocytes

To determine if the increased removal of bacteria with increasing age from the hemolymph was related to bacterial attachment to hemocytes, hemocyte monolayers were used. The number of bacteria per granulocyte and plasmatocyte was not statistically different (Table 6); however, the level of these hemocytes with bacteria varied with the bacterial isolate and its physiological age. In general, the results showed an increased number of both types of hemocytes with adhering bacteria as the physiological age of the bacteria increased with maximum adhesion occurring for 9 h and 17 h old tube cultures of isolates E4 and E7. More granulocytes were found with adhering bacteria than plasmatocytes, indicating differential recognition of *X. nematophilus* by the hemocytes. Flask grown E7 cells, which were less extensively removed than the 17 h tube grown cells, attached to fewer of either type of hemocyte than did the 17 h old cells grown in tubes. The adhesion patterns of bacteria to the plasmatocytes and granulocytes were correlated with the clearance patterns of the bacteria from the insect hemolymph ($r = 0.777, P = 0.05$) but not with virulence ($r = 0.321$, $P > 0.05$).

Hydrophobic interaction chromatography (HIC) of E4 showed a trend towards a decrease in hydrophobicity with increasing physiological ages (Table 8). Except for isolate E4 grown for 6 h (61 %) in test tubes hydrophobicity of the cells under all conditions (flasks and tubes) and ages was less then 10 % of the cells. Cells rown in flasks also showed a low level of hydrophobicity. Electrostatic interaction chromatography (EIC) studies have shown there to be no relationship between the type and magnitude of the surface charge of the bacteria at different ages or growth conditions and their interaction with the insect's hemocytes (Table 9). the level of positive or negative charge was less than 5%.

Table 8. Level of hydrophobicity of *Xenorhabdus nematophilus* at different cultural ages and from different incubation conditions based on hydrophobic interaction chromatography.

Isolate	Age (h)	Culture conditions	Percentage of cells adhering to resin[1]
E4	6	Tube	61.65 ± 8.71
E4	9	Tube	ND[2]
E4	17	Tube	4.54 ± 0.73
E4	23	Flask	7.65 ± 0.95
E7	6	Tube	7.82 ± 1.24
E7	9	Tube	6.56 ± 0.41
E7	17	Tube	6.57 ± 0.37
E7	23	Flask	3.52 ± 0.25

[1] The hydrophobicity values (mean ± SE) represent the percentage of bacteria removed from suspension by the hydrophobic resin (Octyl-Sepharose CL-4B). The bacteria grown in flasks are the same physiological age as bacteria grown for 17 h in tubes.

[2] ND - not determined

Table 9. Level of surface charge of *Xenorhabdus nematophilus* at different cultural ages and conditions based on electrostatic interaction chromatography.

Isolate	Age (h)	Culture	Percentage of Bacteria with Charge adhering to resin	
			Negative charge	Positive charge
E4	6	Tube	1.74 ± 1.56[1]	2.33 ± 1.30
E4	9	Tube	0.00 ± 0.00	4.10 ± 0.97
E4	17	Tube	3.69 ± 1.03	0.82 ± 0.42
E4	23	Flask	3.71 ± 0.37	0.00 ± 0.00
E7	6	Tube	2.69 ± 0.19	2.43 ± 1.50
E7	9	Tube	1.58 ± 0.39	3.53 ± 3.16
E7	17	Tube	5.33 ± 1.04	0.00 ± 0.00
E7	23	Flask	3.68 ± 0.90	0.00 ± 0.00

[1] The electrostatic values (mean ± SE) represent the bacteria removed from suspension by the electrostatic resins (anion exchanger- Dowex 1x8 - 200, cation exchanger- Dowex 50x8 -200, Aldrich Chemical Company Inc). The bacteria grown in flasks are the same physiological age as bacteria grown for 17 h in tubes.

Discussion

Isolation and Characterization of *Xenorhabdus nematophilus*

The parallel slopes of the probit mortality regressions indicate that both bacterial isolates kill their hosts by the same mode of action [10]. The magnitude of the slope of the lines being greater than one may indicate that a toxin was involved in the death of the insects [10]. Although not tested, the probable toxin may be LPS which was reported by Dunphy and Webster [25] to cause an increase in the total hemocyte counts and the eventual lysis of the insects' hemocytes. The probit mortality regression equations estabished the regressions to have parallel slopes implying the existence of toxins. No discernible relationship between virulence and the measured biochemical parameters was evident. Isolate E4 showed a greater production of acid phosphatase, phosphohydrase, α-glucosidase, and *N*-acetyl-ß-glucosaminidase than isolate E7 although E4 was less virulent than isolate E7 which may preclude these enzymes as virulence factors. Although, these enzymes were not measured *in vivo* due to the enzymatic nature of insect hemolymph. However, it does not preclude the injection into insects of these enzymes in a more detailed study to determine their effect on the insect antibacterial system. It is possible that the slight production of the enzyme cystine aminopeptidase, produced by E7 resulted in increased virulence of this isolate in an as yet unknown manner.

Since *Ph. luminescens* produces an alkaline metalloprotease toxic to *G. mellonella* [53] and the insect pathogen *Serratia marcescens* produces proteases which act as virulence factors [36], it is possible that *in vivo, X. nematophilus* protease plays a role in the bacterium's virulence mechanism (s). Isolates of *X. nematophilus* in this study produced the most proteolytic activity in YSB and slightly less activity in AGS, which may indicate that in the insect little protease would be produced during the early stages of infection until the amino acids in the hemolymph are depleted and the bacteria reached the stationary phase. Protease activity was not measured *in vivo* due to the inherent proteolytic nature of insect hemolymph [28]. Kucera and Mracek [39] have identified two Ca-dependent Cys-SH proteases (41 and 57 KDa) from *X. nematophilus* which are inhibited by a factor in *G. mellonella* hemolymph which implies that should proteases be produced in the early stages of infection they may not contribute to virulence. Both isolates E4 and E7 produce two types of esterases (C4 and C8) in comparable amounts to one another as indicated by API ZYM analysis. The presence of these esterases may explain the inability of these bacteria to utilize the substrate azocoll and its weak use of azocasein as a substrate as some esterases have been shown to have protease like activity.

Proteases of *Ps. aeruginosa* damage the hemocytes of *G. mellonella* and cause the formation of large vacuoles in the hemocyte cytoplasm [42]. However, since the same effect has been observed for LPS from *X. nematophilus* [25], it is unlikely the protease of the latter bacterial species plays the same role as the protease of *Ps. aeruginosa*.

Since the *X. nematophilus* isolates, which were obtained from one nematode strain, differed in semiquantitative API ZYM profiles and LT_{50} values, the bacteria found in the nematode gut may all be of equal benefit to the nematode [3, 30], one bacterium may contribute to the killing power of the nematode/ bacteria complex, while, other bacteria bmay be more important in other aspects of the nematodes life cycle [47, 49].

Bacterial growth

The growth of *Xenorhabdus* isolates in different culture conditions (tubes vs. flasks) resulted in no differences in PDTs. However, the population level was significantly higher and the bacteria entered the stationary phase sooner in flask cultures than those in tube cultures which may reflect oxygen restriction and/ or waste build up in the latter culture system. The PDTs for these isolates were in agreement with those found by Poinar *et al.* [50] and Goetz *et al.* [35] who found that rich media supported a PDT of 1.5 h and 0.9 - 1.3 h *in vitro*, respectively, and a PDT in hemolymph of 2.5 - 3.0 h *in vivo* [2]. However, from the point of view of pH, cells grown in flasks produced a higher medium pH than did cells grown in test tubes, and this higher pH has been shown to control phase variation of *X. nematophilus* [43]. The presence of a lower pH in test tubes than in flasks appears to represent a more accurate portrayal of *in vivo* conditions as reported by Maxwell *et al.* [44].

The PDT in AGS and AGS supplemented with larval serum or PBS with larval serum increased significantly for both isolates compared with those grown in YSB. The increase in PDT may be due to higher osmotic pressure in AGS and/ or factors in the hemolymph which antagonize bacterial growth. Dunphy and Webster [26] has shown that hemolymph enzymes such as lysozyme and proteases influence the PDT of *X. nematophilus* isolates. Isolate E7 grew more slowly in AGS with larval serum than did isolate E4. This was unlikely due to differences in serum composition because pool sera was used. Rather, it may reflect differences in the isolate sensitivity to or interaction with serum factors. Isolates E4 was more effective than isolate E7 at blocking the conversion of prophenoloxidase (PPO) to phenoloxidase (PO). The reduced bacterial growth in insect hemolymph has been reported for other insect pathogens *Ps. aeruginosa*, *P. mirabilis* and *P. vulgaris*, as well as non insect pathogenic bacteria [12].

In vivo response of *G. mellonella* to *X. nematophilus*

Bacteria of increasing physiological age injected into the hemocoel insects resulted in the increased removal of the bacteria from the hemolymph through adhesion to hemocytes. Although differences in removal of *X. nematophilus* and *Ph. luminescens* are known to vary with isolate, strain and insect species [18, 24], this study is the first to report the effects of physiological age and culture conditions on *X. nematophilus* and the bacterium's interaction with the antibacterial system of insects. Physiological age and growth conditions are known to influence the nonself responses of mammals to Gram-negative pathogens [37]. Factors influencing bacterial attachment to antibacterial cellular defences of invertebrates include bacterial age, viability, physiological properties (such as hydrophobicity and surface charge), outer membrane composition and surface appendages [37]. The latter three parameters are influenced by culture conditions the bacteria are grown under [4, 54]. Hemocyte levels generally decreased in response to bacteria of increasing physiological age reflecting increasing levels of nodulation. The surface of the bacteria may have become more antigenic as the bacteria aged.

Bacterial removal from the hemolymph is a complex event as evident by the viability of E4 being correlated with bacterial removal from the blood whereas the removal of E7 was independent of bacterial viability. The results with E4 isolate suggests ongoing bacterial metabolism reduced the removal of the pathogen from the hemolymph, whereas the E7 data suggests that other factors may be involved such as surface structures. Dunphy and Webster [25], using antibiotic killed *X. nematophilus*, reported that bacterial surface structures facilitated the removal of the bacteria by hemocytes in *G. mellonella*. Changes in the OMP/proteinaceous appendages may have facilitated the response [28].

It was observed that as physiological age of the bacteria increased the size of the bacteria decreased, resulting in less surface area, which could result in reduced physical contact with the hemocytes of the insect. Since circulation in the hemocoel is so rapid (2-5 minutes)[33] it can be concluded that bacteria injected into insects have little chance of avoiding circulating insect hemocytes or contact with host tissue However, the bacteria with less surface area were more readily removed from circulation in the insect than cells with greater surface area. This negative correlation may be due to greater interaction with the hemocytes as a result of altered physicochemical properties and/or structural appendages. Ratcliffe and Walters [51] also addressed the clearance of bacteria with different surface areas and concluded that surface area was important with larger Gram-positive bacteria eliciting a weaker cellular response than smaller bacteria. This

relationship was not apparent for the Gram-negative bacteria used by these authors which may reflect their use of different species of bacteria with different antigenic surfaces.

The clearance pattern of *P. luminescens* reported by Dunphy and Webster [27] resembled the findings in this study for *X. nematophilus* grown in tubes except the reactions in this study occurred over a much shorter period of time. The injection of *G. mellonella* with 12 h old *X. nematophilus* var. *mexicanus* [26] produced a clearance and THC response that was similar to *X. nematophilus* isolates grown in test tubes in this study. This suggests similar surface attributes between the two strains.

Bacteria of incresing physiological ages adhered to increasing numbers of hemocytes suggesting a surface change(s) on the bacteria allowing a higher proportion of the bacteria to be recognized by the hemocytes. It is possible that either a specific adhesion antigen(s) becomes more prevalent on the bacterial surface as it ages or immunorepressive factors become less prevelant. The adhesion of rough walled *Ps. aeruginosa* to hemocytes was influenced by the LPS chemotype [20] and LPS of *X. nematophilus* is known to adhere to insect hemocytes [25]. Since bacterial age and culture conditions are known to influence LPS content [38], it is possible that the different adhesion patterns of *X. nematophilus* in the present study may reflect such changes in LPS content and/or chemotype. The level of plasmatocytes with adhering bacteria was lower than the granulocytes; however, since granulocytes are the initial cells responding to bacteria by nodulation, this was expected and supports the concept of receptor heterogeneity proposed for different types and subtypes of hemocytes [34, 45]. However, the magnitude of increased bacterial adhesion from early exponential growth was similar (2 fold). After the bacteria reached late exponential growth, the percentage of hemocytes with adhering bacteria of a given isolate became constant. However, between the E4 and E7 isolates a minor difference in the level of bacteria adhering to specific hemocyte types was noted. Both of these adherence patterns were correlated with bacterial clearance establishing the validity of hemocyte-mediated removal of the bacteria from the hemolymph.

When the adherence of flask grown cells to hemocytes was investigated, the number of plasmatocytes with adhering bacteria exceeded the level of granulocytes with adhering bacteria by almost 2 fold with isolate E4, whereas the opposite occurred for isolate E7. Specific recognition by the hemocytes based upon carbohydrate receptors on the hemocytes with possible lectin mediation has been proposed by several authors [19, 27, 52]. Chadwick *et al.* [13] found that isolates of *Serratia marcescens* with low LT_{50}s for *G. mellonella* adhered to more insect hemocytes than did isolates with high LD_{50}s and

suggested that adherence may be a component of virulence. This was not the situation with mutant *X. nematophilus* [16] and the present results. The differential adherance of cells grown in flasks to a specific blood cell type implies a difference in hemocyte receptor types and the bacterial cell surface.

Physical factors contributing to adhesion of isolate E4 and isolate E7 are complex in that the hydrophobicity of isolate E4 was negatively correlated with contact with the hemocytes and the hydrophobicity of isolate E7 did not change with culture age and did not affect the adhesion of isolate E7 to the hemocytes. Stenström [55] found pH to have only a marginal effect on surface hydrophobicity of *Salmonella typhimuriuna* and bacterial surface characteristics to be the most important factors affecting cell hydrophobicity. The hydrophobicity of bacterial cells is influenced by bacteria capsules, surface appendages, and the amount of LPS and or type found in the outer membrane of the bacteria [11]. Since the hydrophobicity of *X. nematophilus* was low, it is possible that the older cells of isolates E4 and E7 lacked O antigen and/or capsular polysaccharide. The relationship of hydrophobicity to the culture age of both isolates was unexpected since Krekeler *et al.* [38] reported increasing hydrophobicity for bacteria in general as the bacteria aged. The role of hydrophobicity in the responses of hemocytes to nonself material is equivocal. Lackie [40, 41] found plasmatocytes from insects from the order Orthoptera to adhere more readily to hydrophobic surfaces. Pendland and Boucias [48] altered the hydrophobicity of spores of the insect pathogenic fungus *Nomuraea rileyi* and found attachment of the altered spores to hemocytes of *Spodoptera exigua* to be independent of hydrophobicity.

Overall, the surface charge of isolates E4 and E7 appear to be insignificant in terms of the interaction of the isolates with the cellular defences of *G. mellonella*. However, charge localized on surface appendages may contribute to bacterial-hemocyte adhesion as reported by Stenström [55] for bacterial adhesion to soil particles. The increased pH of cultures of *X. nematophilus* tested in this study probably did not affect the surface charge of the bacteria. The pH of bacterial media did not affect the surface charge of bacteria in general at different pH values [38]. Surface charge may be influenced by many outer membrane characteristics such as LPS chemotype, OM phopholipid composition, surface appendages and capsules [11, 21, 55]. Beads differing in the type of surface charge are attacked by insect hemocytes but in a manner that varies with the insect species: negatively charged beads were encapsulated *in vivo* by *Periplaneta americana*, and neutral Sephadex and weakly positive beads were encapsulated by *Schistocerca gregaria* [41]. However, in *Choristoneura fumiferana* and

Lambdina fiscellaria fiscellaria, hemocytes did not attach to negatively charged beads but adhered to postively charged beads [22, 23].

In summary the virulence of isolates E4 and E7 of *X. nematophilus* was not related to biochemical and physiological properties of the bacteria, or their interaction with the cellular defences of *G. mellonella*. Culture age, but not bacterial hydrophobicity or charge was related to the adhesion of the bacteria to the hemocytes *in vitro* and to the removal of the bacteria from the hemolymph. Therefore, other surface attributes such as flagella, fimbriae and outer membrane proteins may be responsible for these binding of the bacteria interactions to the insect's hemocytes.

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Chapter III

Interaction of the Surface Appendages of *Xenorhabdus nematophilus* with the Nonself Defences of Nonimmune *Galleria mellonella* larvae

Abstract

Isolates of *Xenorhabdus nematophilus* produced both flagella and fimbriae when cultured in test tubes and flasks when their growth reached stationary phase. Cells grown under aerobic shaken conditions produced mainly rigid fimbiae, while stationary microaerobic cells produced mainly fimbriae of flacid morphology. The presence of capsular material (indicated on cells grown in shaken flasks by intense electron density) increased in intensity with increasing physiological age of the cells. The amount of flagella protein was 3.15×10^{-10} μg/bacterium and 1.0651×10^{-9} μg of fimbrial protein per bacterium. Electrophoresis revealed fimbrial extracts to consist of 3 major proteins (67, 40, and 32 KDa) and flagella extract to have 4 major proteins (67, 40, 35, and 32 KDa) of similar molecular weights of the fimbriae. These structures may have proteins associated with them that act as virulence factors.

Injections of flagella in doses of 6.6, 66.2, and 661.5 pg/ml into *Galleria mellonella* larvae caused an increase in total hemocyte counts up to 4 hours postinjection. Similar results were achieved with fimbriae at 22.4, 223.7 and 2236.8 pg/ml but to a level greater than the flagella extracts. Similar results were also obtained when the two antigens were injected into *G. mellonella* larvae at the same protein concentrations, indicating that differences in the cellular response found between the two antigens was not due to the different protein concentrations used. Combination of the antigens resulted in an increase in total hemocyte counts that was less than fimbriae and flagella individually but greater than the cellular responses of the control insects. Injections of flagella and fimbriae facilitated an increase in circulating plasmatocytes and a decrease in the number of circulating granulocytes, suggesting that cellular mobilization may be occurring in response to these bacterial surface antigens. Only at 2.2 ng/ml of fimbrial protein did a depletion of circulating plasmatocytes occur, suggesting that a threshold exists between hemocytes phagocytosing foreign particles and removing particles from circulation and becoming associated with host tissue.

Both fimbriae and flagella of *X. nematophilus* when co-injected into *Galleria* larvae did not stimulate cellular interaction with the bacteria. Therefore, fimbriae and flagella did not stimulate bacterial recognition at pg quantities. However, at ng quantities, fimbriae increased bacterial removal slightly and flagella reduced removal slightly of bacteria when they were coinjected into the insects. When *Proteus mirabilis* was substituted for *X. nematophilus*, only flagella prevented or interfered with removal of *Proteus mirabilis* from the hemolymph of *G. mellonella*. Therefore, the surface appendages of *X. nematophilus* may need to be associated with other surface proteins in order to protect the bacteria from recognition by the insect's nonself recognition system at the cellular level.

Introduction

The insect pathogenic bacterium, *Xenorhabdus nematophilus*, is carried into the hemolymph of insects in the gut of the entomoparasitic nematode *Steinernema carpocapsae*. The bacteria encounter the antibacterial systems of the insect immediately upon their discharge from the vector.

The antibacterial systems of the insects consist of interactive humoral and cellular factors that effectively contain nonpathogenic bacteria [38]. The type of response to bacteria and its effectiveness varies with the insect species and bacterial species. Cellular phagocytosis and hemocyte-particle aggregations (nodules) are invoked by bacterial peptidoglycan fragments, lipopolysaccharides (LPS), and fungal ß-1-3 glucans [3]. Peptidoglycan fragments exceeding a minimum size and, for some insect species, some LPS chemotypes activate the melanizing prophenoloxidase system (PPO)[3]. Activation of this system may augment nodulation under specific conditions and for specific foreign bodies [15].

The adhesion of *X. nematophilus* to hemocytes of nonimmune larvae of *Galleria mellonella* was mediated by larval serum enzymes, independent of the prophenoloxidase system and enhanced by a decrease in overall bacterial cationic charge and an increase in bacterial hydrophobicity [14, 18]. However, for isolates E4 and E7 of *X. nematophilus* adhesion to the hemocytes of *G. mellonella* was independent of bacterial surface charge and hydrophobicity (Chapter 2) suggesting that surface structures might be involved.

Bacterial pathogens have a number of surface structures which confer protection from the defences of vertebrate hosts. These structures include capsules, fimbriae, flagella and, in some cases, LPS and outer membrane proteins [34]. Flagella and fimbriae have also been linked to bacterial virulence and colonization of host tissues in vertebrates [34] and for *Pseudomonas aeruginosa* in insects [27]. Fimbriae and outer membrane proteins of the phase one form of *X. nematophilus* (the form normally infecting insects) have been implicated in the removal of the bacteria from the hemolymph of insects [1, 18]. These structures are influenced by the culture conditions of the bacteria [7]. The effect of culture age and method on flagella, fimbriae, and outer membrane proteins of *X. nematophilus* is unknown. The present study will address this aspect and elucidate the contribution of the surface structure and OMPs to bacterial adhesion to the hemocytes of nonimmune *G. mellonella* larvae.

Materials and Methods

Insect Culture

Galleria mellonella larvae were reared to the sixth instar [19]. Unless stated otherwise, insects weighing 200 mg were used in all experiments.

Bacterial Culture

Isolates E4 and E7 of *X. nematophilus,* unless stated otherwise, were grown in 5 ml of yeast salts broth (YSB) in 20 ml test tubes on a gyrotary shaker at 150 rpm, in darkness at 27 °C for 18 h. Hereafter, these parameters will be referred to as standard conditions. *Proteus mirabilis* was grown under the same incubation conditions except the culture media was tryptic soy broth (TSB).

Electron Microscopy

Bacteria were grown to different physiological ages in test tubes and flasks under standard conditions. Bacteria were removed from cultures in 5 µl volumes, added to carbon coated copper grids (200 mesh) and negatively stained with 2% uranyl acetate or 2% phosphtungstic acid [23] to reveal *X. nematophilus* surface appendages. Excess liquid was removed after 1 minute of staining and the grids air dried. The grids were observed with a Zeiss transmission electron microscope.

Isolation of Surface Appendages

Fimbriae of *X. nematophilus* (isolate E7 was chosen as the example strain when possible due to its greater virulence) were induced by growing the bacteria in stationary 500 ml flasks containing 100 ml of YSB (27 °C) in darkness for 8 days. The bacterial concentration was determined with a Petroff Hausser counter, the bacteria pelleted by centrifugation (10 000 xg, 5 °C, 30 min) and resuspended in 150 ml of phosphate buffered saline (PBS) (pH 7.2). The suspension was then blended in a Warring blender for 2 min at room temperature and centrifuged twice (10 000 xg, 5 °C, 30 min) to remove the bacteria. The resulting supernatant containing fimbriae and flagella was retained for subsequent purification. Flagella were pelleted by centrifugation (40 000 xg, 5 °C, 1 h) and fimbriae collected from this supernatant by ultracentrifugation (100 000 xg, 5 °C, 1.5 h). The pellets of both appendages were resuspended in 1.5 ml of PBS (pH 6.5) and stored frozen (-20 °C) in vials until required [22].

Purification of Fimbriae and Flagella

Fimbriae suspended in PBS were dissociated by incubation in 0.15 M ethanolamine-HCl buffer (pH 10.2, 12.5 ml/ g wet wt of bacteria pellet) for 18 h at 25 °C [4]. The fimbriae subunits were precipitated by adding ammonium sulphate to 10 % saturation and stirring for 1 h at room temperature [22]. Fimbriae were collected by centrifugation (100 000 xg, 1.5 h) and resuspended in PBS. Contaminating ethanolamine-HCl buffer was removed by dializing the fimbriae suspension against two 1 L changes of PBS overnight. The fimbriae were centrifuged (100 000 xg) and resuspended in 2 ml of PBS and stored at -20 °C until required.

Flagella suspensions were added to distilled water (10 ml), the pH adjusted to 2.0 with 1 M HCl, and the suspension incubated at 0 °C for 1 h to dissociate the flagella. The solution was centrifuged (100 000 xg, 1 h) to remove insoluble contaminants; the supernatant was adjusted to pH 7.0 with 1 M NaOH and incubated for 1 h to reassociate the flagella which were then reclaimed by centrifugation (100 000 xg, 1 h) and resuspended in PBS [22]. The second method [12] used a cesium chloride gradients of in 6 ml cellulose nitrate tubes 0.832 g/ ml (2.5 ml) overlayed with 0.289 g/ ml solution of cesium chloride (2.5 ml). To this gradient 0.5 ml of flagella suspension was added and the tubes topped up with paraffin oil. The tubes were centrifuged for 18 h at 100 000 xg with an SW 41 rotor. Tubes were punctured with a needle where bands were located and the material collected.

Purity was assessed by electron microscopy after negative staining with 2 % phosphotunsic acid (PTA). The protein concentrations were determined by the Peterson [35] method. The different fractions of fimbriae and flagella were further assessed for purity by SDS-PAGE [31](8 μg of total protein/ well) on a mini protein II gel system (Biorad) and the proteins detected using the silver stain method of Harlow and Lang [21].

Lipopolysaccharide Quantification in Surface Appendage Samples using *Limulus* amoebocyte lysate.

To test for LPS contamination of purified fimbriae and flagella, the quantitative chromogenic Limulus amoebocyte lysate (LAL) kit (QCL-1000, Whittaker Bioproducts) was used. Bacterial endotoxin activates a serine protease in the lysate thus initiating hydrolysis of the substrate acetyl-isoleucyl-glutamyl-arginyl-p-nitroaniline releasing p-nitroaniline. The level of p-nitroaniline was linearly related to the endotoxin level. Endotoxin standards were prepared as recommended by the manufacturer for a range of

0.1 - 1.0 endotoxin unit/ ml. LPS-free distilled water was used throughout the study. Samples to be tested were serially diluted in distilled water in 1.5 ml centrifuge tubes to yield antigen concentrations ranging from 10^{-1} to 10^{0} of the original level. The solutions were assayed as follows: (1) a 50 µl of sample, standard, or distilled water (blank) was added to 1.5 ml centrifuge tubes; (2) 50 µl of LAL were added immediately, the samples vortexed and incubated at 37 °C for 10 min; (3) 100 µl of substrate were added and all tubes were incubated for an additional 6 min at 37 °C; (4) the reaction was stopped with 100 µl of acetic acid, and (5) the amount of released p-nitroanaline was determined spectrophometrically (OD 410 $_{nm}$).

Total and Differential Hemocyte Responses to Surface Appendages

To assess the influence of fimbriae and flagella on the insect hemograms, the fimbriae and flagella antigens (purified by centrifugation) were prepared in three dilutions (fimbriae: 22.4, 223.6, and 2236.8 pg/ ml, flagella: 6.6, 66.2, and 661.5 pg/ ml) in PBS where 66.2 and 223.6 pg/ ml represent the protein concentration of flagella and fimbriae respectively associated with 2.1 x 10^{7} bacteria/ ml (concentration of bacteria used in the bacterial clearence and adhesion studies). To test the influence of fimbriae and flagella together, a series of solutions with pooled antigens were also prepared at 289.8, 2898.2, and 28982 pg/ ml of total protein (combined concentration of fimbriae and flagella on 2.1 x 10^{7} cells/ ml , protein ratio = fimbriae:flagella {3.4 : 1}). However, because the total protein of the pooled antigens was greater than the aforementioned solutions of fimbriae and flagella, control antigen suspensions consisted of fimbriae only and flagella only at the same total protein level as the mixed suspension.

The antigens were injected into 6th instar *G. mellonella* larvae in 10 µl volumes. Groups of 10 larvae were bled individually at 5, 30, 60, 120 and 240 min. post-injection. Hemolymph was collected with, ice-chilled pasteur pipets and placed on ice prior to enumeration of the total hemocyte counts on a hemocytometer.

Particles injected into the hemocoel are known to induce the depletion of plasmatocytes in *G. mellonella* [10]. To determine if fimbriae and flagella influence different hemocyte types, *G. mellonella* larvae were injected with the antigens as before, bled (20 µl) at the designated times and the hemolymph placed on a slide containing 35 µl of 0.1% trypan blue [37] (0.2 %) in PBS. Slides were covered with a 22 x 40 mm cover slip for observation by phase contrast microscopy. One hundred hemocytes were counted per slide for each of five insects per time period and the differential hemocyte counts recorded. Hemocytes were identified according to Price and Ratcliffe [36].

Hemocyte viability was based on the number of cells excluding trypan blue per 100 cells of each type per slide. Both differential counts and viability were expressed as percentages with 95 % confidence limits of arc sin P^{-2} transformed data [46]. Hemocyte viability served as an indicator of LPS contamination since LPS is known to rapidly damage insects hemocytes [17].

The Effect of Surface Appendages on Clearance of Bacteria

To assess the ability of *X. nematophilus* surface components to influence clearance of bacteria from the hemolymph, *X. nematophilus* at a stage (isolate E7) lacking fimbriae and *P. mirabilis* (Department of Natural Resource Sciences, Macdonald College of McGill University) were grown for 24 h in YSB in flasks at 27 °C on a gyrotary shaker at 150 rpm. The bacteria were washed twice by centrifugation (12 000 xg, 3 min) and resuspended (2×10^9 cell/ ml) in PBS containing surface components of *Xenorhabdus*. Three concentrations of fimbriae (22.4, 223.6, and 2236.8 pg/ ml), flagella (6.6, 66.2, 661.5 pg/ ml), and a fimbriae and flagella mixture (3.4 : 1) (289.8, 2898.2, and 28982 pg/ ml) were used with *X. nematophilus*, whereas, 223.6 pg/ ml of fimbriae, 66.2 pg of flagella, and 2898.2 pg/ ml of a 3.4:1 mix of fimbriae and flagella were used with *P. mirabilis*. Groups of ten sixth instar *G. mellonella* larvae were injected with 10 µl of the suspensions of antigens. The insects were incubated for 30 min at room temperature and individually bled by cutting a prothoracic leg. The hemolymph was collected with individual, ice chilled Pasteur pipets and placed on ice. Hemolymph samples were placed on hemocytometers and the number of free floating bacteria and hemocytes enumerated using phase contrast microscopy.

***In vivo* Activation of the Phenoloxidase Cascade by *Xenorhabdus nematophilus* Fimbriae**

Ten *G. mellonella* larvae were injected with one of the following solutions: (1) phosphate buffered saline; (2) a suspension of *X. nematophilus* fimbriae at 22.4 pg/ ml (1x); (3) a suspension of *X. nematophilus* fimbriae at 223.6,pg/ ml; (4) *X. nematophilus* fimbriae at 2236.8 pg/ ml, where 223.6 pg/ ml of fimbriae represents the protein found on the surface of 2.1×10^7 *Xenorhabus* cells/ ml (our standard dose). The treatments consisted of ten injected *G. mellonella* larvae. The injected insects were incubated at 27°C for 5, 30, and 60 min postinjection. The insects were bled at the designated times and the hemolymph added to test tubes containing 3.5 ml of 1/ 10 strength PBS containing 2 mg/ ml DL-dihydroxyphenylalanine and incubated for 1.5 h at room

temperature. The optical density of the reaction mix was measured at 490 $_{nm}$ and the phenoloxidase activity expressed as the change in optical density per minute per mg total protein [5, 13].

Statistics

Means for data were separated using the Lsd test at $\alpha = 0.05$ unless stated otherwise, and all percentage data coded $\sqrt{2}$ p [46] before statistical analysis.

Results

Surface Appendages

Both bacterial isolates possessed peritrichous flagellation (flagella diameter 7.1 ± 0.2 nm (n = 9)) under the different cultural conditions and phases of development (Plate 1, Figure 1). Flagellation was not related to the antibacterial responses described in Chapter 2, as all ages of bacteria were flagellated..

Rigid fimbriae (Plate 1, Figure 2) (diameter 4.6 ± 0.20 nm (n = 10) were detected on the surface of bacteria in the stationary phase of both isolates in both tube and flask cultures (Plate 1, Figure 3,4). Stationary cells from tube cultures also appeared to possess flacid fimbriae (Plate 1, Figure 3). The number of fimbriae on the surface of the stationary tube and flask grown cells was approximately 15 - 20 (n =10)per cell and approximately 30 - 40 (n= 10) per cell, respectively for both isolates (micrographs of isolate E4 representative of cells examined by EM and the estimation of fimbriae is in one plane assuming uniform surface localities). Since there was an increase in both the adhesion of the bacteria to hemocytes and bacterial removal from the hemolymph at this stage of growth, the presence of fimbriae may be responsible for adhesion of the bacteria to the hemocytes and the subsequent removal from the hemolymph by the nonself system(s) of *G. mellonella*.

The isolate E4 grown in test tubes did not stain with phosphotungic acid but readily stained with uranyl acetate. Bacteria from flask cultures stained with the latter stain revealing a diffuse layer around the cells that increased with increasing physiological age (Plate 1, Figure 4). With the advent of fimbriaeation, the diffuse layer became extremely electron dense (Plate 1, Figure 5, 6). The increase in layer density occurred as the bacterial hydrophobicity level declined and the bacterial interaction with the hemocytes increased.

Flagella (previously collected by differential cetrifugation at 40 000 xg and visual estimated to be 90 -95 % pure from EM analysis) were purified by two methods, cesium chloride (CsCl) centrifugation and solubilization at low pH (pH 2.0) in HCl followed by reaggregation at pH 7.0. There were no significant differences in flagellar morphology from semipurified and ethanolamine -HCl insoluble material observed by transmission electromicroscopy (Plate 2, Figure 1) resembling those in the insoluble fraction (Plate 2, Figure 5). The aggregated flagella formed rigid, thickened rods that did not resemble the morphology of the starting structures. The insoluble fraction contained a larger amount of protein band with a molecular weight (30 KDa) of approximately the same (Plate 2, Figure 2).

Plate 1: Investigation of *Xenorhabdus nematophilus* surface structures with transmission electronmicroscopy

Plate 1:

Figure 1. Isolate E4 of *Xenorhabdus nematophilus* grown for six hours in test tubes (early exponential growth). 1 cm bar = 0.336 μm.

Figure 2. Isolate E4 of *Xenorhabdus nematophilus* grown for nine hours in test tubes (late exponential growth). 1 cm bar = 0.328 μm.

Figure 3. Isolate E4 of *Xenorhabdus nematophilus* grown for 17 hours in test tubes (early stationary growth). 1 cm = 0.336 μm.

Figure 4. Isolate E4 of *Xenorhabdus nematophilus* grown for nine hours in flasks (early exponential growth and similar physiological age as Figure 1). 1 cm bar = 0.334 μm.

Figure 5. Isolate E4 of *Xenorhabdus nematophilus* grown for 15 hours in flasks (early exponential growth and similar physiological age as Figure 2). 1 cm bar = 0.334 μm

Figure 6. Isolate E4 of *Xenorhabdus nematophilus* grown for 23 hours in flasks (early exponential growth and similar physiological age as Figure 3). 1 cm bar = 0.334 μm.

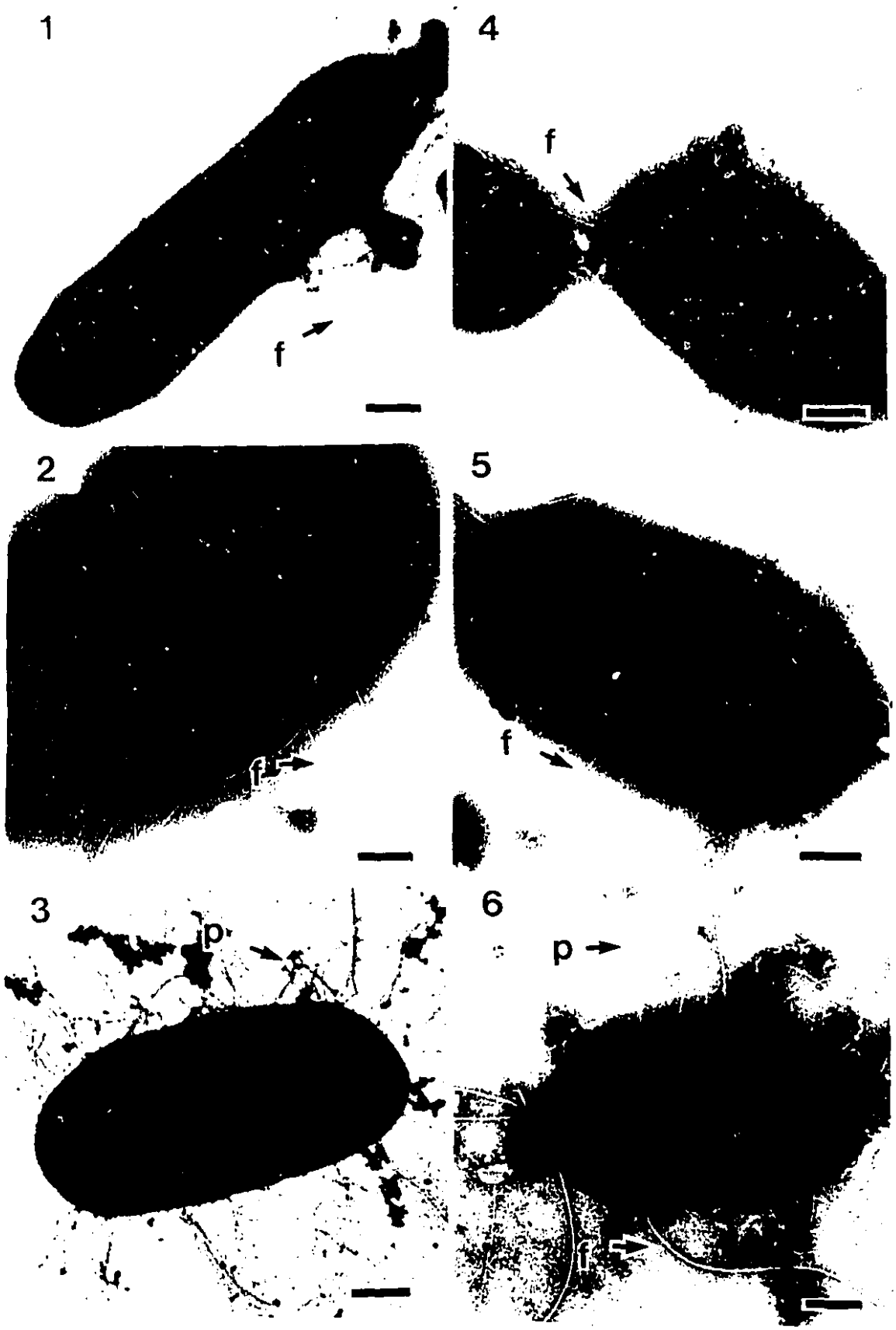

1
f
4
f
2
f
5
f
3
p
6
p
f

Plate 2 : Electronmicroscopy analysis of the purification of *Xenorhabdus nematophilus* fimbriae and flagella by selective solublization and centrifugation

Figure 1: Crude flagella purified by centrifugation at 40 000 xg. 1 cm bar = 0.134 μm.

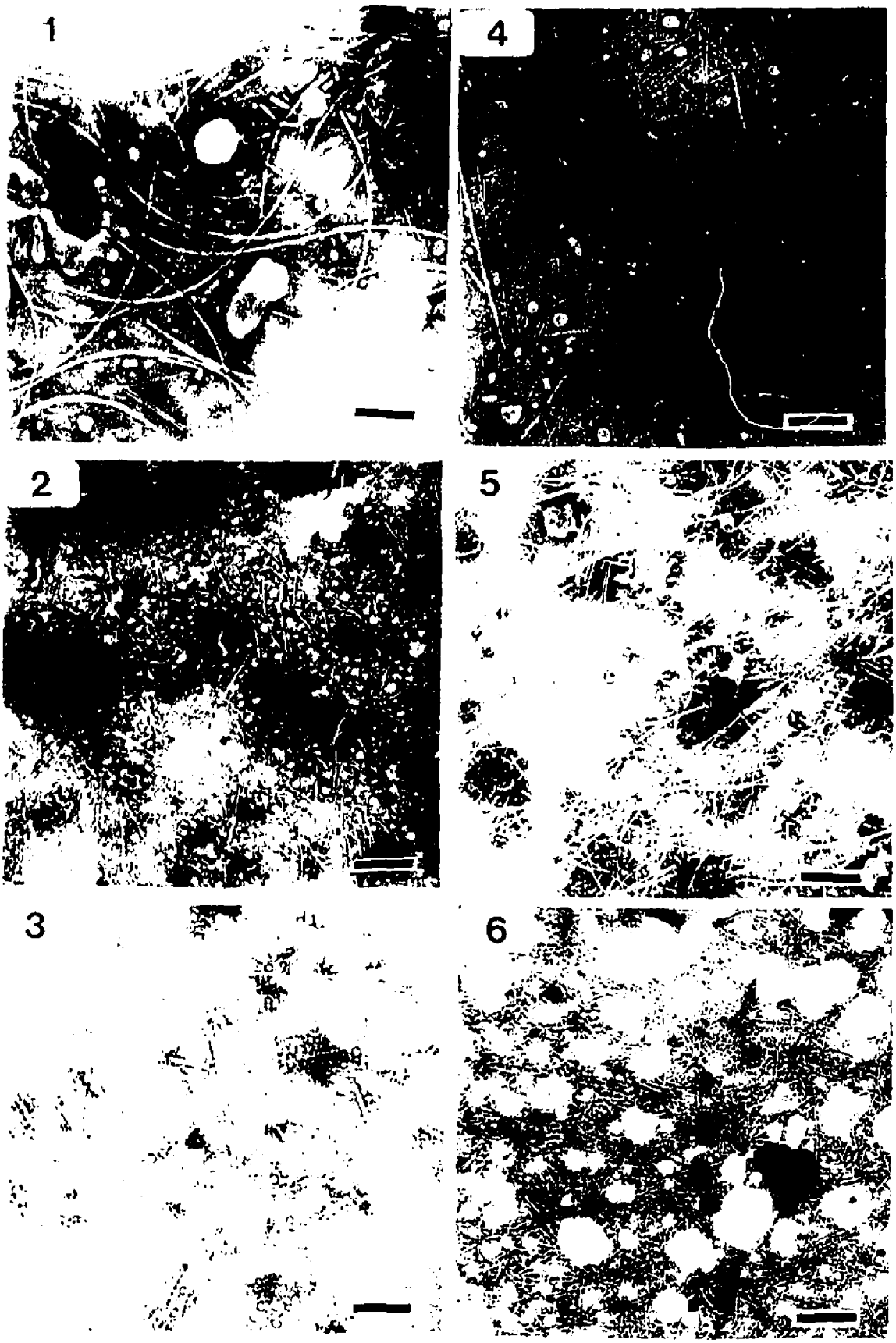
Figure 2: Flagella material that was insoluble at pH 2 and collected by centrifugation at 100 000 xg. 1 cm bar = 0.342 μm.

Figure 3: Flagella extract precipitated at pH 7 and centrifuged at 100 000 xg after removal of insoluble material. 1 cm bar = 0.134 μm.

Figure 4: Crude fimbriae purified by centrifugation at 100 000 xg following the removal of flagella at 40 000 xg. 1 cm bar = 0.134 μm.

Figure 5: Insoluble material in ethanolamine-HCl removed at 40 000 xg. 1 cm bar = 0.134 μm.

Figure 6: Soluble fimbriae material in ethanolamine-HCl precipitated with amonium sulphate and centrifuged at 100 000 xg. 1 cm bar = 0.134 μm.


1
4
2
5
3
6

Solubilization of the partially purified fimbriae (estimated visually to be 85 -90 % pure from EM analysis) in ethanolalamine - HCL, followed by precipitation, produced fimbriae that were similar in morphology to the partially purified fimbriae (Plate 2: Figure 1 and 2) whereas the insoluble fractions produced diffuse irregular aggregations (Plate 2: Figure 5). Similarly, the solubilized and crystallized fraction produced an electropherogram in which there were fewer protein bands but, unexpectedly, no increase in any major bands was observed. It is possible that the treatments may have affected the stainability of the proteins, thus casting doubt as to the actual purification of the proteins. Similar results were detected for flagella (Figure 1: lane 6).

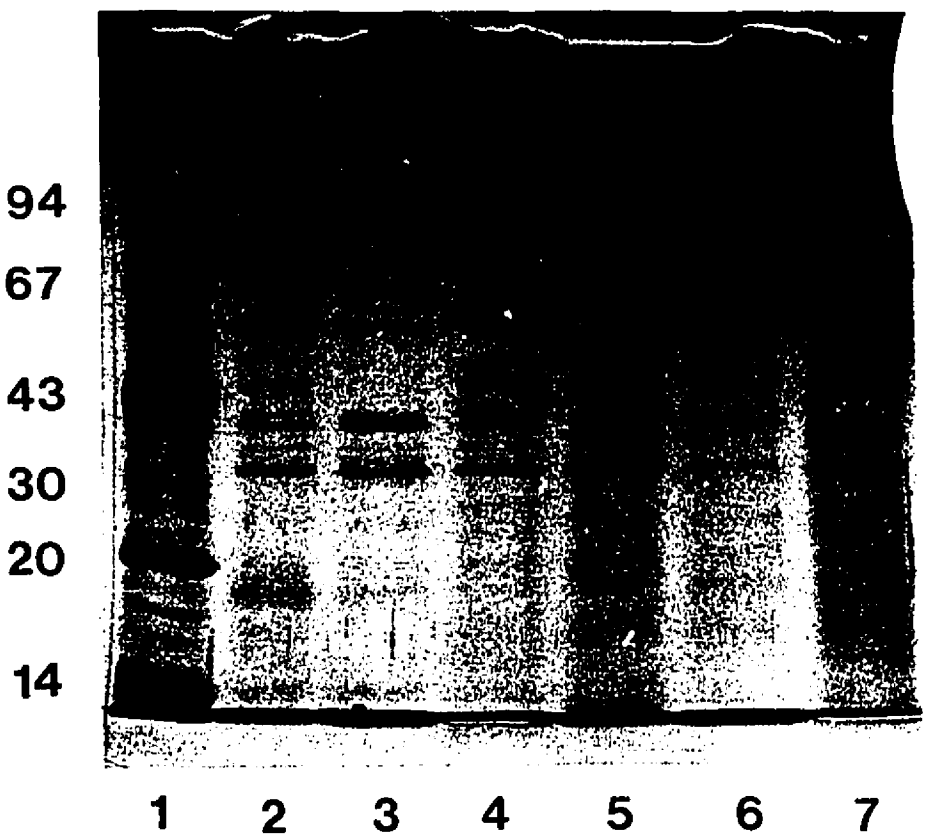
Electrophoretic analysis of the fimbriae and flagella purified by differential centrifugation revealed that the two surface structures have protein bands of similar apparent molecular weight. Major proteins found in flagella consisted of a 32, 35, 40 and 67 kDa, while major proteins composing the fimbriae consisted of 32, 40 and 67 kDa (Figure 1).

Impact of Purified Flagella and Fimbriae on the Total Hemocyte Counts of *Galleria mellonella*

The total flagella and fimbriae protein per bacterium was estimated to be 3.15 x 10^{-10} μg of protein/ cell and 1.065 x 10^{-9} μg of protein/ cell, respectively, for isolate E7. Suspensions of these antigens representing the concentration of fimbriae or flagella found in the bacterial suspension (2.1 x 10^{7} bacteria/ ml) used to define bacterial interactions with insect defences (chapter II). Flagella injected into the larvae at 6.6, 66.2 and 662.5 pg of protein/ ml were compared with the PBS control insects did not cause a decline in hemocyte counts at 5 min postinjection but thereafter there was a rapid increase in THC to a maximum level by 1 h, followed thereafter by a decline in hemocyte counts (Figure 2). The 662.5 pg/ ml suspension induced an increase in the THC to a maximum by 2 h followed by a decline in THC by 4 h to levels comparable to those produced by the other two concentratins of antigens. Injection of fimbriae into larvae also elevated the hemocyte counts (Figure 3). Fimbriae also elicited an increase in THC that reached an essentually constant level by 2 h postinjection (Figure 3). The increase in THC's was greater with fimbrial antigens than with flagellar antigens.

When fimbriae and flagella were mixed in proportions comparable to those on the bacterial surface, the mixture caused an increase in hemocyte levels (2.1 x 10^{7} cells/ ml) similar to flagella alone (2.2 x 10^{7} cells/ ml). To determine if the increased level of the total protein in a flagella-fimbriae suspension influenced the THC, flagella and fimbriae at

Figure 1 Electrophoretic profiles of crude fimbriae and flagella of *Xenorhabdus nematophilus* collected by differential centrifugation. Sample lanes were loaded with 8 μg of protein. Lane 1 - low molecular weight standards; lane 2 - fimbriae: insoluble material from ethanolamine-HCl induced precipitation (40, 000 xg); lane 3 - fimbriae: soluble in ethanolamine-HCl and precipitated with ammonium sulphate; lane 4 - unpurified fimbriae collected by differential centrifugation; lane 5 - flagella extract: insoluble material at pH 2.0 (100,000 xg); lane 6 - flagella precipitated at pH 7.0 (100,000 xg); lane 7 - crude flagella collected by differential centrifugation (40,000 xg).


94
67
43
30
20
14
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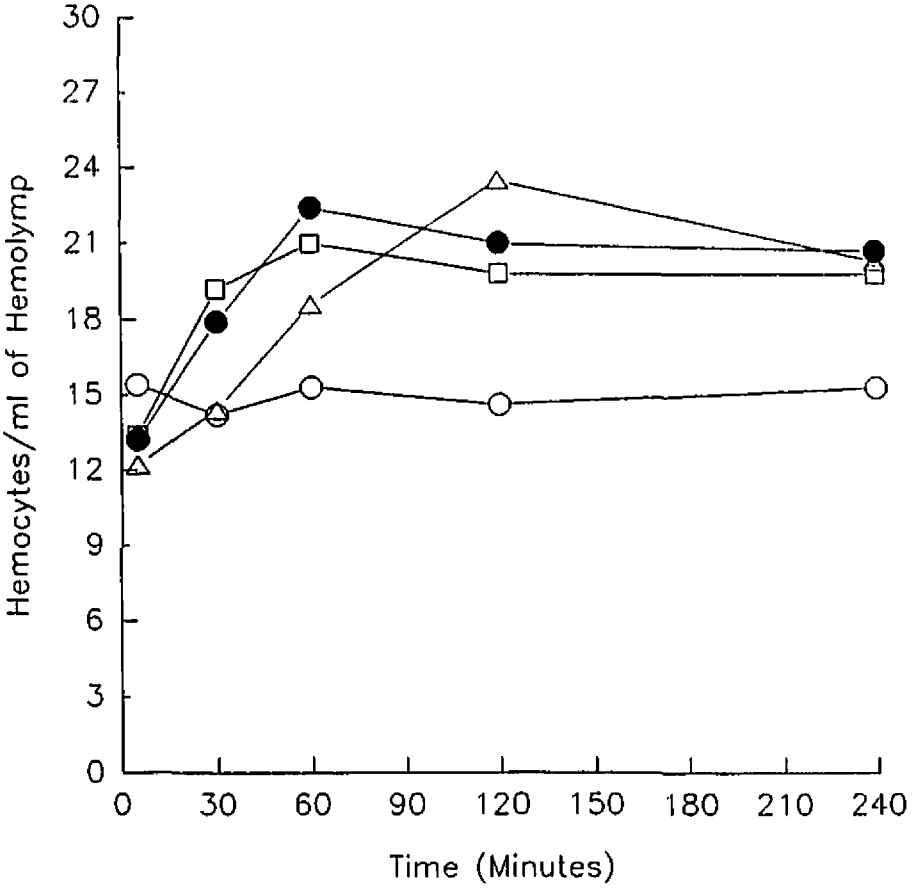
7

Figure 2: Total hemocyte response of nonimmune *Galleria mellonella* to different concentrations of *Xenorhabdus nematophilus* flagella. The antigen in phosphate buffered saline (PBS) was injected into larvae at three concentrations (●) 6.6 pg/ ml, (□) 66.2 pg/ ml, and (△) 661.5 pg/ ml in a 10 µl volume. The control injection consisted of PBS (○). Bars represent the standard error of the mean.


Hemocytes/ml of Hemolymp
30
27
24
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15
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9
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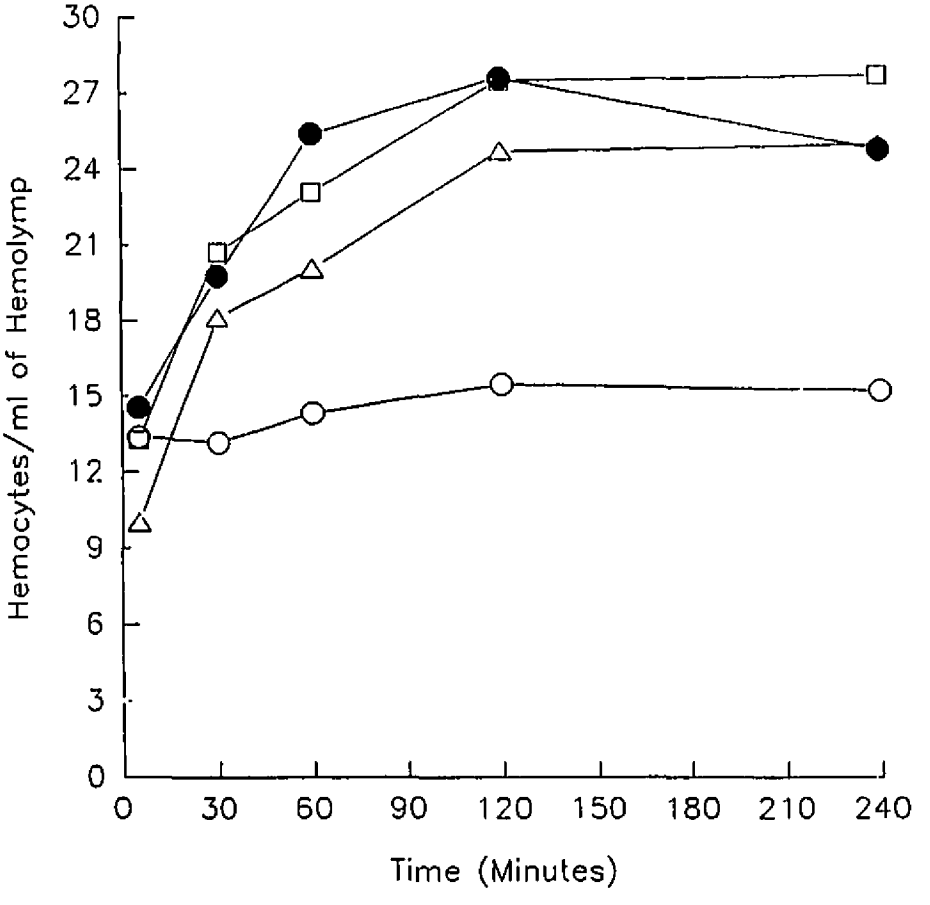
90
120
150
180
210
240
Time (Minutes)

Figure 3: Total hemocyte response of nonimmune *Galleria mellonella* to different concentrations of *Xenorhabdus nematophilus* fimbriae. The antigen in phosphate buffered saline (PBS) was injected into larvae at three concentrations (●) 22.4 pg/ml, (□) 223.6 pg/ml, and (△) 2236.8 pg/ml in a 10 µl volume. The control injection consisted of PBS (○). Bars represent the standard error of the mean.


Hemocytes/ml of Hemolymp
30
27
24
21
18
15
12
9
6
3
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90
120
150
180
210
240
Time (Minutes)

concentrations equal to the total protein of the flagella-fimbriae suspension were injected into larvae as before. The results showed that higher protein concentration did not cause a different results (Figure 4), indicating that the hemocyte response is not a dose dependent event for these antigens within the concentration range tested.

Lipopolysaccharide Quantification in Surface Appendage Samples

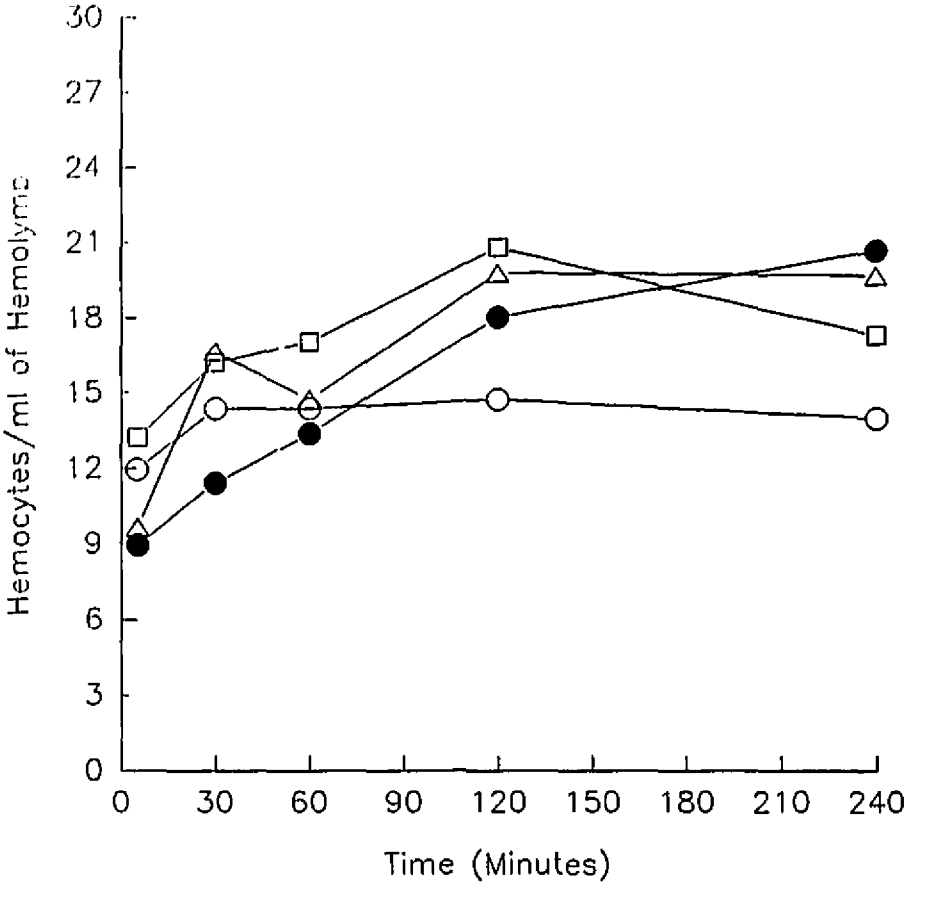
Both fimbriae and flagella samples were found to contain extremely low levels of LPS contamination. Stock suspensions of fimbriae (920 μg/ ml protein) and flagella (900 μg/ ml protein) contained 0.67 EU/ ml (7.39 x 10^{-5} ng of LPS/ μg of protein/ ml) and 0.275 EU/ ml (1.05 x 10^{-4} ng of LPS/ μg of protein/ ml), respectively. Allowing for dilution, this would result in 6.7 x 10^{-6} (6.7 x 10^{-7} ng of LPS) and 2.75 x 10^{-6} EU (2.75 x 10^{-7} ng of LPS) of LPS being injected into the larvae with the flagella and fimbriae respectively. Injecting *X. nematophilus* LPS at the 1 x 10^{-5} EU (1 x 10^{-6} ng of LPS)/ ml did not elevate the hemocyte counts; thus, it is unlikely that the elevated hemocyte counts in the present study were due to LPS contamination of the fimbriae and flagella antigens.

Differential Hemocyte Response to Surface Appendages

The increase in THC response to the injection of the antigens may have consisted of changes in the types of hemocytes thus, differential hemocyte counts were done at 5, 30, and 60 min postinjection. Injections of flagella at 6.6 pg/ ml and 66.2 pg/ ml caused an increase in the number of plasmatocytes in circulation in the hemolymph and a proportionate decrease in circulating granulocytes by 30 min. postinjection when compared with PBS (Table 1). The 661.5 pg/ ml flagellar suspension caused a decline in plasmatocytes and an increase in granulocytes within 5 min of injection and these values remained constant until 60 min. In all flagellar treatments, the proportion of oenocytoids and spherulocytes did not differ from those of the control larvae.

When insects were injected with fimbriae at 22.4 pg, and 223.6 pg/ ml the proportion of plasmatocytes 5 min postinjection was significantly than the levels observed in control larvae injected with PBS. The proportion of plasmatocytes remained at this elevated level for at least 60 minutes while the level of granulocytes remained at the reduced level during this period. Fimbriae at a concentration of 2236.8 pg/ ml caused a lowering of the level of plasmatocytes at 5 min postinjection which was maintained for less than 30 min. The proportion of oenocytoids and spherulocytes was not influenced by the antigen conentration.

Figure 4: Total hemocyte response of nonimmune *Galleria mellonella* to different concentrations of a *Xenorhabdus nematophilus* fimbriae-flagella mix. The mixture of the antigens in phosphate buffered saline (PBS) was injected into larvae at three concentrations (●) 289.82 pg/ml, (□) 2898.2 pg/ml, and (△) 28982 pg/ml in a 10 μl volume. The control injection consisted PBS (○). Bars represent the standard error of the mean.


30
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3
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Hemocytes/ml of Hemolymp
0
30
60
90
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150
180
210
240
Time (Minutes)

Table 1. Differential Hemocyte Counts of Nonimmune Sixth Instar Galleria mellonella larvae in Response to Injection of Xenorhabdus nematophilus Surface Appendages.

Time postinjection/Hemocyte type		5 min				30 min				60 min			
Antigen	Concentration	Pl[b]	Gr	Oe	Sph	Pl	Gr	Oe	Sph	Pl	Gr	Oe	Sph
Flagella	6.6pg/ml	54.4 ± 2.4[a]	39.8 ± 2.1	1.6 ± 0.6	4.2 ± 0.8	65.1 ± 2.0	30.7 ± 2.1	1.7 ± 0.5	2.4 ± 0.6	67.4 ± 1.6	29.1 ± 1.4	1.1 ± 0.2	2.3 ± 0.5
Flagella	66.2pg/ml	55.8 ± 2.1	41.6 ± 3.0	2.1 ± 0.4	3.4 ± 0.8	71.4 ± 2.1	25.4 ± 1.7	1.3 ± 0.4	2.4 ± 0.4	72.0 ± 2.8	25.1 ± 2.6	1.0 ± 0.2	1.9 ± 0.4
Flagella	661.5pg/ml	35.4 ± 1.6	57.8 ± 2.0	1.0 ± 0.4	5.8 ± 1.2	39.2 ± 3.0	52.8 ± 3.4	2.9 ± 0.4	5.0 ± 1.2	62.4 ± 2.7	33.4 ± 1.8	0.6 ± 0.4	3.3 ± 1.0
PBS	-	53.2 ± 2.9	42.2 ± 2.8	1.3 ± 0.4	3.1 ± 0.8	47.7 ± 5.4	50.8 ± 5.2	0.6 ± 0.2	0.8 ± 0.2	55.6 ± 3.6	42.3 ± 3.8	1.0 ± 0.3	1.2 ± 0.7
Fimbriae	22.4pg/ml	73.8 ± 1.3	22.6 ± 1.7	1.6 ± 0.4	2.1 ± 0.4	77.2 ± 1.8	20.9 ± 2.0	0.6 ± 0.1	1.2 ± 0.4	80.8 ± 1.8	17.4 ± 1.8	1.0 ± 0.4	0.8 ± 0.2
Fimbriae	223.8pg/ml	64.4 ± 2.6	30.1 ± 2.4	1.3 ± 0.4	4.2 ± 0.7	68.4 ± 1.5	28.8 ± 1.1	0.8 ± 0.2	2.0 ± 0.6	72.2 ± 1.6	24.6 ± 1.6	0.8 ± 0.2	2.4 ± 0.6
Fimbriae	2238.6pg/ml	34.6 ± 2.7	57.7 ± 2.5	1.9 ± 0.8	5.8 ± 0.9	71.7 ± 1.6	26.0 ± 1.8	0.8 ± 0.2	1.4 ± 0.4	75.4 ± 2.1	22.9 ± 2.1	0.8 ± 0.2	0.9 ± 0.2
PBS	-	55.3 ± 1.8	41.4 ± 1.9	0.8 ± 0.3	2.4 ± 0.9	65.9 ± 2.1	32.4 ± 1.8	0.4 ± 0.2	1.2 ± 0.4	66.3 ± 1.8	31.4 ± 1.6	0.8 ± 0.3	1.4 ± 0.4
Fim & Flag	289.8pg/ml	60.2 ± 2.0	36.5 ± 1.8	0.8 ± 0.3	2.4 ± 0.5	72.3 ± 2.8	25.8 ± 2.8	0.6 ± 0.2	1.3 ± 0.4	68.6 ± 3.1	29.0 ± 3.0	0.4 ± 0.2	2.0 ± 0.6
Flagella	289.8pg/ml	51.6 ± 1.9	45.6 ± 2.0	0.9 ± 0.3	1.9 ± 0.5	67.0 ± 2.5	30.2 ± 2.2	0.7 ± 0.3	2.1 ± 0.8	69.9 ± 2.9	27.3 ± 3.0	1.0 ± 0.2	1.8 ± 0.5
pili	289.8pg/ml	48.1 ± 1.7	47.7 ± 1.7	0.6 ± 0.2	3.5 ± 0.9	60.1 ± 2.7	38.0 ± 3.0	0.2 ± 0.2	1.6 ± 0.4	65.9 ± 1.7	32.6 ± 1.7	0.7 ± 0.1	0.9 ± 0.2
PBS	-	54.2 ± 1.8	42.1 ± 1.4	1.2 ± 0.2	2.4 ± 0.4	65.6 ± 2.4	31.8 ± 2.6	0.8 ± 0.2	1.7 ± 0.5	67.9 ± 1.9	28.1 ± 1.4	0.9 ± 0.4	3.4 ± 1.0

[a] Percentage of a hemocyte type based upon the examination of 100 hemocytes/sample (n=10).

[b] Pl-plasmatocyte, Gr-granulocyte, Oe-oenocytoids, Sph-spherulocytes

In general, insects injected with fimbriae exhibited a higher proportion of circulating plasmatocytes than did insects injected with flagellar fragments.

To assess the contribution of the flagella and fimbriae together, the two antigens were co-injected into insects at 289.8 pg of protein/ ml as no difference in total hemocyte counts were observed between concentrations of 28.9, 289.8, and 2898 pg of protein/ ml of the suspension containing both fimbriae and flagella. To compensate for the increased level of injected protein in the dual antigen suspension, flagella and fimbriae were separately injected into insects at 289.8 pg of protein/ ml, a level of protein that was equivalent to 289.8 pg of protein/ ml of the fimbriae-flagella mixture. There was no significant difference in the hemocyte types for larvae receiving the antigens (Table 1).

The Effect of Surface Appendages on Clearance of Bacteria

Injecting *P. mirabilis* in PBS into the insect hemocoel elevated the THCs above the hemocyte levels in larvae injected with PBS only (Table 2). Although increases in hemocyte counts above the PBS-injected insects were also observed for larvae coinjected with the bacteria and antigens of *X. nematophilus*, the effect was less than with *P. mirabilis* in PBS. The increase in hemocyte counts was not correlated with the removal of *P. mirabilis* from the hemolymph ($r = 0.00, P < 0.05$). Flagella impaired the ability of the hemocytes to remove *P. mirabilis* as evident by the higher bacterial levels compared with *P. mirabilis* levels in the PBS group (Table 2). However, neither fimbriae nor fimbriae plus flagella altered the removal of *P. mirabilis* from the hemolymph.

The antigens at the test concentrations did not influence the total hemocyte counts when compared with PBS containing *X. nematophilus* (Table 3). Similarly, fimbriae and flagella did not alter bacterial removal from the hemolymph. However, the fimbriae-flagella mix did accelerate bacterial removal by the same extent independent of the antigen concentrations compared with those in the control insects.

***In vivo* Activation of the Phenoloxidase Cascade by *Xenorhabdus nematophilus* Fimbriae**

PO activity in insects injected with buffer and the different concentrations of fimbriae were not statistically different at 5 min postinjection whereas the activity in these groups was less than the PO activity in noninjected larvae (Table 4). However, PO levels of larvae injected with fimbriae at 223.8 and 2238.6 pg of protein/ ml were less then those with PBS or 22.4 pg/ ml fimbriae by 30 minutes postinjection. Injections of fimbriae produced PO levels below those of noninjected and PBS injected larvae and was independent of the concentration of fimbriae. PO levels increased in all test groups by 60 min postinjection, although levels in larvae with fimbriae were still less than the PBS and noninjected insects.

Table 2. Effect of partially purified fimbriae and flagella of *Xenorhabdus nematophilus* coinjected with *Proteus mirabilis* on the removal of the bacteria from the hemolymph of nonimmune *Galleria mellonella* sixth instar larvae 30 min postinjection.

Treatment/ Concentration	Bacterial level ($\times 10^6$ cells/ml)	Total Hemocyte Count ($\times 10^5$/ml)
PBS with bacteria	15.0 ± 1.1	6.7 ± 0.4
1 x Fimbriae (223.8pg/ml)	12.8 ± 1.5	3.8 ± 0.3
1 x Flagella (66.2pg/ml)	27.0 ± 4.2	4.6 ± 0.4
1 x Flagella + Fimbriae (289.8pg/ml)	14.1 ± 2.1	4.7 ± 0.5
PBS only	-	114.0 ± 15.1

Values represent mean ± the standard error of the mean (n = 20).

Table 3. Influence of partially purified surface appendages of *Xenorhabdus nematophilus* coinjected with fimbriae minus *Xenorhabdus nematophilus* on total hemocyte counts and the removal of the bacterium from the hemolymph of nonimmune sixth instar *Galleria mellonella* larvae 30 min postinjection.

Treatment	Concentration of *X. nematophilus* (x 10^6/ml)					
	28.9 pg/ml		289 pg/ml		2892 pg/ml	
	Bacterial[1] level	THC[2]	Bacterial level	THC	Bacterial level	THC
PBS with bacteria	17.7 ± 0.7	3.7 ± 0.2	16.6 ± 2.1	3.7 ± 0.3	15.6 ± 1.0	3.1 ± 0.3
Fimbriae	16.5 ± 0.7	3.5 ± 0.2	17.6 ± 1.3	3.6 ± 0.2	13.8 ± 1.2	4.1 ± 0.4
Flagella	14.8 ± 1.1	4.7 ± 0.3	13.6 ± 0.9	4.6 ± 0.4	16.1 ± 1.0	4.5 ± 0.3
Fimbriae + Flagella	11.0 ± 1.5	3.0 ± 0.4	10.3 ± 1.6	2.4 ± 0.3	11.0 ± 1.3	2.9 ± 0.5
PBS only	-	11.1 ± 1.1	-	14.7 ± 0.7	-	13.4 ± 1.4

Values represent the mean ± standard error of the mean (n= 10) for both bacterial levels and total hemocyte counts.
[1] concentration of bacteria 1 x 10 ^{6}/ml
[2] concentration of total hemocytes 1 x 10 ^{6}/ml

Table 4. The Influence of partially purified *Xenorhabdus nematophilus* fimbriae on phenoloxidase activity in nonimmune *Galleria mellonella*.

Treatment	Time Postinjection (Min.)		
	5	30	60
Noninjected larvae	33.1 ± 13.4	58.0 ± 18.6	91.2 ± 12.8
PBS	16.6 ± 2.4	16.0 ± 2.1	118.8 ± 24.2
Fimbriae (22.4pg/ml)	10.2 ± 1.6	11.2 ± 1.2	42.0 ± 4.7
Fimbriae (223.8pg/ml)	14.7 ± 1.2	7.8 ± 0.3	55.1 ± 6.1
Fimbriae (2238.6pg/ml)	12.2 ± 2.2	8.4 ± 1.4	46.1 ± 6.4

[1] Values represent the mean ± the standard error (n=10) in units of activity (Δ OD $_{490}$ / min./ mg of protein).

Discussion

Isolates E4 and E7 of the DD136 strain of *X. nematophilus* both possessed peritrichous flagellation at all physiological ages examined. Peritrichous flagellation has been reported previously for *X. nematophilus* [47], but this is the first time that their presence has been related to a diversity of culture ages and growth conditions. The observations are similar to the limited study of Brehélin *et al.* [1] with other strains of *X. nematophilus*.

This is the first report of fimbriae on the surface of *X. nematophilus* strain DD136 strain in liquid culture. The presence of fimbriae on *X. nematophilus* strain DD136 was found to be correlated with increased removal of stationary phase *X. nematophilus* cells from the insect hemocoel, thus indicating fimbriae as one factor responsible for increased clearance of *X. nematophilus* from *G. mellonella* as culture age increased. The number of fimbriae on the surface of the two isolates was the same when the appendages were present on both isolates, indicating appendage number did not play a role in their different patterns of bacterial removal from the hemolymph. The same isolate grown under aerobic conditions was cleared more readily than those at the same physiological age under conditions of lower oxygen and this may have resulted from the aerobic cultures having more fimbriation. It has been shown that strains of *P. mirabilis* with heavy fimbriation are cleared more readily than lightly fimbriated strains [45]. There appears to be a structural difference between fimbriae produced on cells grown microaerobically and those grown aerobically. The fimbriae produced under microaerobic conditions appear to be a flacid form, while those produced by cells grown under aerobic conditions appear to be of the rigid type. This dimorphism in fimbriae structure has been found in other bacteria and may be related to function such as colonization of host tissue [12]. The true role of *X. nematophilus* fimbriae is not known, but in other bacterial species they play a role in resisting host defences and colonization of host tissue thus contributing to virulence [25, 34]. Some strains of *X. nematophilus* have been found to disappear completely from the hemolymph after injection into *G. mellonella* only to reappear after the insect's death suggesting that the bacteria may be colonizing the insect tissues or have been removed by the insect hemocytes. Fimbriae have also been shown to influence the hydrophobicity of bacteria [8, 48], but this does not appear to be the case in view of the results from Chapter II where cell surface hydrophobicity remained either constant or decreased in the presence of fimbriae. It is possible that isolate E4 may produce a capsule of considerable size as evident by areas of intense stain surrounding the bacterial cells. A capsule has been shown to exist in other strains of *X. nematophilus* in different types of medium [1] but does not seem to have the extensive magnitude which has been observed

in *X. nematophilus* strain DD136.

In order to further study the effects of fimbriae and flagella on insect cellular defence, the appendages were purified. Sampling of fimbriae and flagella by electron microscopy during stages of purification showed strutures that were structurally homogeneous in nature. However, SDS-PAGE of different fractions showed the fimbriae to be 85-90% pure and the flagella to be 90-95% pure, these values were determined visual from micograph. SDS-PAGE of crude fimbriae and flagella collected by differential centrifugation also showed almost identical profiles. No substantial purification was obtained with selective solublization methods as evidenced by the lack of increase in band intensity for protein bands. Cesium chloride centrifugation did cause separation of fimbriae into 3-5 bands. The two major bands were collected and found to contain structures identifible as fimbriae. The lower band had five structures resembling fimbriae whereas the diffuse upper band contained structures that did not resemble either fimbriae or flagella. When these bands were subjected to SDS-PAGE, they produced identical banding patterns suggesting that they were the same structure. Contamination of fimbriae with flagellar protein was expected but at low levels which should have produced bands of low intensity for the same protein load; however, this was not observed using any of the purification procedures. In the literature, fimbriae consists of a single protein [11, 43] and the flagella are composed of flagellin [42]. Fimbriae may also have a minor protein associated with it, *e.g.* adhesion protein at its tip [41]. The only cases of more than one protein associated with flagella is in bacteria which have sheathed flagella [44] or periplasmic flagella [26] and usually comprise the major protein of the structure. It has not been reported in the literature that fimbriae and flagella have proteins with similar molecular weight. Therefore, it may be possible that the isolated fimbriae herein may be an incomplete form of flagella.

When flagella were injected into *G. mellonella* larvae, the total hemocyte levels for all three doses did not significantly drop below the hemoytes levels of the control larvae injected with PBS. This is not like the more traditional response to injected material in which particles trigger nodulation and a decline in THC [20]. However, the flagella induced an increase in THCs that was independent of antigen. Although an increase in THC has been observed in muscid species infected by parasitic nematodes [16] or to bacteria damaging the hemocytes of lepidoptera (*G. mellonella*) [39], the present increase could represent hemocyte damage induced by the LPS of *X. nematophilus*.

Injection of fimbriae into *G. mellonella* at different doses caused an increase in total hemocyte counts that were significantly greater than controls, although the greater the dose, the slower the rate of increase of the hemocyte numbers. The higher dose may be caused

by microscopic nodulation slowing down the rate of increase in the THC. However, nodules were not readily observed. LPS contamination of the antigens or mobilization of the hemocyte community may explain the increase in THC. It is unlikely that this response is a nonspecific response to proteins in general because the flagella at lower concentrations than fimbriae produced a faster increase in THC. The mixture of flagella and fimbriae injected into insects cause an increase in THC that was less then that found for the structures allone. This reduced responce may reflect a conformational change in the protein structure or their interaction. It is unlikely that LPS is the cause as the amount of LPS that each insect received with the fimbriae was 3.0×10^{-4} ng/ insect, well below the level known to incite hemocyte damage [9]. The fact that the larger doses of fimbriae caused a slower increase in hemocyte counts also supports the conclusion that this is not an LPS effect because the rate of increase in THC is related to the concentrations of LPS [17]. The failure of fimbriae to trigger nodulation was unexpected due to the correlation of fimbriae and *X. nematophilus* adhesion. It may represent the effect of particulate size with the antigen suspension not inducing sufficient stress on the granulocytes to cause nodulation. When fimbriae and flagella were coinjected into insects at the concentration found on 2.1×10^{7} bacteria, a similar change in the types of hemocyte was obtained as those for fimbriae and flagella separately. This implies a difference in antigenicity, thus resulting in a different cellular response when introduced to the hemocoel of an insect.

The both antigens showed differences in their ability to alter the levels of the two main types of insect hemocytes in circulation. Flagella at the lower concentrations caused an increase in the circulating number of plasmatocytes and / or a decrease in granulocytes over 60 minutes following injection. However a dose of 661.5 pg/ ml of flagella antigen produced a decline in the number of circulating plasmatocytes (or an increase in granulocytes) compared with control larvae until 60 minutes post injection by which time the pattern reversed. The increasing number of plasmatocytes in *G. mellonella* in response to low levels of antigen may represent activation of the plasmatocytes as opposed to a nodulation effect (which would deplete the granulocytes and favoring an increase in plasmatocytes) since micronodulation was not event. However, when these antigens were co-injected into insects, the effect was abrogated. This may have been due to overloading the system, one antigen nullifying the effect of the other, and/ or it may represent fimbriae and flagella interacting to produce a conformation different from that of either antigen alone.

All doses were in the picogram range of protein, thus indicating the highly sensitive cellular immune system present and the strong antigenicity of these proteins. This result indicates that bacterial surface appendages can invoke a cellular immune response to nonself

material.

The effect of surface appendages on clearance of bacteria

The effect on the clearance of bacteria appears to be dependent on the type of bacteria as, the removal of *X. nematophilus* from the hemolymph differed from the removal of *Pr. mirabilis*. In the case of *X. nematophilus*, the antigens had no effect on bacterial removal whereas for *Pr. mirabilis* the flagella antigen alone had the greatest effect, reducing the removal of the bacteria from the hemolymph. The release of LPS from *X. nematophilus* may have altered the hemocytes too extensively to allow them to remove *X. nematophilus* from the hemolymph.

The ability of flagella from *X. nematophilus* to impair cellular defences of *G. mellonella* against *P. mirabilis* implies that flagella of *X. nematophilus* may preclude bacterial adhesion to hemocytes and thus contribute to the growth of the pathogen in insects. This follows the usual cases where the presence of flagella and motility affects virulence of bacterial pathogens of vertebrates [33, 34, 40] and *Ps. aeurginosa* on *G. mellonella* [27]. The flagella may also be acting as a masking agent, preventing the detection of *P. mirabilis*, as opposed to contributing to molecular mimicry has been reported for bacterial pathogens of animals and eucaryotic pathogens of insects [29, 30].

The reduced PO activity in *Galleria* larvae injected with *X. nematophilus* fimbriae may be due to LPS preventing the activation of the PO system. However, the level of LPS was 2.8×10^{-6} EU (2.8×10^{-7} ng)/ insect, which is considerably lower than the level found to cause a hemocyte response in *Galleria* (0.2 μg) [17]. Since LPS suppression is unlikely, it may be possible that the fimbriae protein may be binding activated PO on the surface of the fimbriae causing a diluted PO response. Alternatively, the fimbraie may be binding to the granulocyte surface blocking receptors that activate the PO system. If this is the case, then fimbriae may act either as a mechanism that dilutes host defences or instills tolerance of host defences which is similar to the role that fimbriae play in serum resistance of *E. coli* in mammals [32].

In summary, *X. nematophilus* surface appendages (fimbriae and flagella) are capable of stimulating a cellular response in *G. mellonella* larvae. These antigens were very effective at stimulating the insect's cellular system stimulating an increase in THC and altering the DHC in insects receiving the antigen. The injection of insects with these antigens did not stimulate the PO system or nodule formation. Flagella also affected the recognition of *Proteus mirabilis*. When *Xenorhabdus* was coinjected with its own surface appendages, clearance was unaffected. Thus, these structures may play a role in host-resistance.

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Thesis Summary

In summary, *Xenorhabdus nematophilus* isolates which are biochemically similar can interact with the nonself system of insects in different ways. The bacterium's interaction with *Galleria mellonella* was influenced by culture conditions. The differences in host response to these conditioned bacteria in general, was not related to physicochemical properties of the bacterial surface. Bacterial adhesion to hemocytes increased with the physiological age of the bacteria as did clearence from the hemocoel of one of the two isolates examined in this study. The importance of cell surface hydrophobicity and electrostatic charge is isolate dependant. The more virulent (by LT_{50}, isolate E7) isolate was removed from the hemocoel more extensively than the less virulent isolate. Isolate E7 also adhered to more hemocytes *in vitro*. Therefore, other factors such as surface appendages were implicated. Investigation of the appendages revealed cells which were removed from the hemolymph were readily covered with fimbriae and flagella. These surface structures were produced in test tubes and flasks in yeast salts broth when the culture reached the stationary phase of growth. Both flagella and fimbriae elicit cellular responses in insects but no detectable humoral changes. Therefore, fimbriae and flagella may play a role in resistance to host antibacterial defences. Fimbriae were found to surpress PO activation when insects were injected with picogram amounts of the structure. This indicates the sensitive nature of the insect antibacteial system. Therefore, it can be concluded that the interaction of isolates of *X. nematophilus* is isolate dependant and consists of multiple events/ host-pathogen interactions.

Further research involving the interaction of *X. nematophilus* with *Galleria mellonella* should investigate the role that outermembrane proteins play in interactions with the nonself defences. The outer membrane proteins responsible for attachment and/ or evasion of host defences should be investigated for *X. nematophilus* under different growth conditions. It is clear that the interaction of *X. nematophilus* is a multi-interactive system where a number of components may play a small part. The differences in the interactions of the two isloates of *X. nematophilus* in these study strongly suggest a role for OMPs.