Secreted proteolytic enzymes of the entomopathogenic bacterium *Xenorhabdus*

MUSTAFA KHALEFA A. O. MASSAOUD

Eötvös Loránd University, Doctorate School of Biology

Head: Dr. Anna Erdei

Structural Biochemistry PhD Program

Head: Dr. László Gráf

Supervisor:

Dr. István Venekei, associate professor

Eötvös Loránd University, Department of Biochemistry

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Abbreviations

AMC	Aminomethyl coumarine;
BSA	bovine serum albumine
CAPS	3-cyclohexylamino-1-propanesulfonic acid;
Dabcyl	4-(4-dimethylaminophenylazo) benzoic acid;
DTT	1, 4-dithiothreitol;
Edans	5-[(2-aminoethyl) amino] naphtalene-1 sulfonic acid;
EDTA	ethylenediaminetetraacetic acid;
Fua	2-furylacryloyl;
Fua-LGPA	Fua-Leu-Gly-Pro-Ala
Fua-ALVY	Fua-Ala-Leu-Val-Tyr
HEPES	4-(2-hydroxyethyl) piperazine-1- ethanesulfonic acid;
IFE	inner filter effect;
IJ	infective juvenile;
LB	Luria-Bertani medium
MES	2-morpholinoethanesulphonic acid;
MOPS	3-(N-morpholino) propanesulfonic acid;
NBTA	nutrient bromothymol blue, triphenyl-2, 3, 5-tetrazolium chloride agar
PAGE	polyacrylamide gel electrophoresis;
PBS	phosphate- buffered saline;
PMSF	phenylmethylsufonyl fluoride;
Ptu	phenylthiourea;
SBzl	thiobenzyl;
SDS	sodium dodecyl sulfate;
Succ	succinyl;
TRIS	2-amino-2-(hydroxymethyl)-1,3-propanediol;

3. INTRODUCTION

3. 1 Insect pathogen bacterium-nematode symbiont pairs

3.1.1 The symbiotic complexes

Perhaps the first studies of nematode-originated diseases were recorded by the physician Aldrovandi about 400 years ago [64]. Aldrovandi also reported on luminescence in insects, but it wasn't until the mid-1970s that Poinar provided the first documentation of a parasitic nematode that employed luminescent bacteria, *Xenorhabdus luminescence*, to invade insects as part of its life cycle [99]. All the species of the entomopathogenic nematodes in the families, *Steinernematidae* and *Heterorhabditidiae*, are symbiotically associated with bacteria of the genera, *Xenorhabdus* and *Photorhabdus*, respectively [1, 2, 3, 4, 26, 92, 93, 94, and 113]. The increasing interest in these bacteria reflects growing awareness that - beyond mass production of the nematode vector - the bacterial partner plays a significant role in nematode/bacterium associations.

Each of the established bacterium species of *Xenorhabdus* and *Photorhabdus* colonize a specific *Steinernematid* and *Heterorhabditid* nematode species, respectively [21]. These symbiotic nematode-bacterium pairs have been used as biological control agents against several agricultural pests [5]. The general features of the life cycles of these bacteria are quite similar, and tightly associated with the life cycle of the partner nematode, as shown in **Fig. 1**. The similarities include habitation in the gut of the nematode, growth in the hemolymph of the host insect with high pathogenic potential. A substantial difference is the association with different families of nematodes. The form of the bacterium that is normally isolated from the symbiotic nematode is referred to as phase I. During the stationary phase, the phase I cells intensively produce proteases, phospholipases, antibiotics, and protoplasmic paracrystaline inclusions composed of crystal proteins [21, 22, 36 and 37].

3.1.2. The infection process of the symbiotic complexes

The bacteria are carried into the susceptible insect larvae by the nematode. Infection by both *Steinernema* and *Heterorhabditis* is initiated by a third-stage, infective juvenile. The infective stage is the only survival stage in the life cycle of these nematodes. Morphological adaptations for this survival period include a compaction or collapse of certain body tissues. For example, the alimentary tract is essentially non-functional because the walls of the

intestine and pharynx have closed together. The mouth and anus are also closed. Symbiotic bacteria (e.g. *Xenorhabdus* spp.), which play an important nutritional role inside the host, are

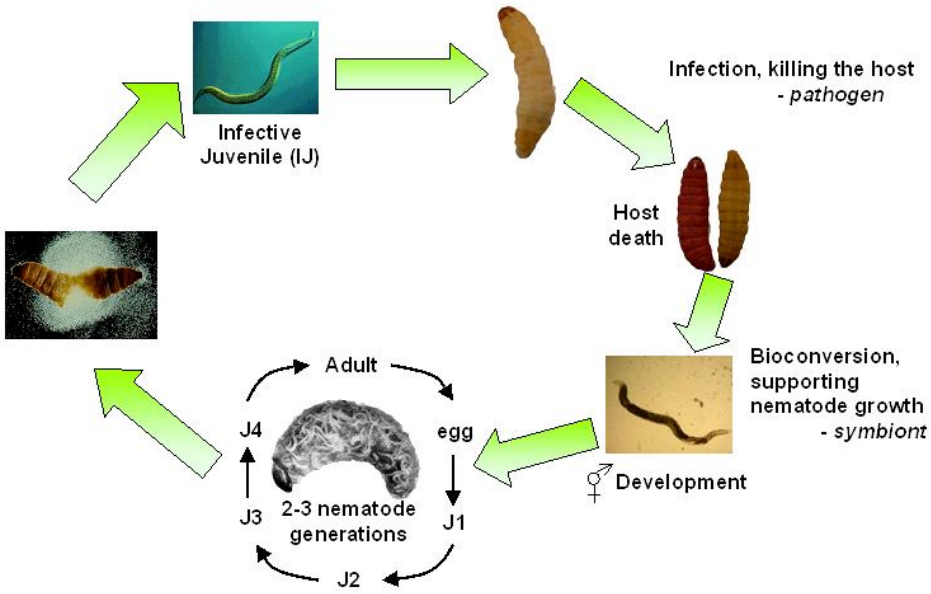


Figure. 1 **The life cycle of the bacterium-nematode symbiotic complex.** (IJ, infective juvenile).

found in the alimentary tract of the infective stage. In *Steinernema*, the great majority of the bacteria are bound in the modified ventricular portion of the intestine, while in *Heterorhabditis*, *Photorhabdus* is found in this location but can also occur throughout the intestinal lumen and even in the pharyngeal lumen.

After a penetration into the hemocoel, the alimentary tract of the nematode becomes functional and cells of the symbiotic *Xenorhabdus* and *Photorhabdus* bacteria are released through the mouth and anus within five hours of nematode invasion. The bacteria start to multiply in the insect's hemocoel, and they participate in the killing of the insect host, which occurs within 48 h [6, 23, 43, 44, 52 and 68]. Their proliferation eventually reaches a stationary phase at a very high cell density, also referred to as reproductive stage of the nematode. During this the bacteria secrete several broad-spectrum antibiotics antimicrobial and nematicidal compounds to protect the insect carcass from invasion by soil organisms including other nematodes. They also produce exoenzymes that degrade insect tissues and macromolecules (a bioconversion process), which contribute to the nutrient base that supports bacterial and nematode reproduction. The insect as food source exhausts after several rounds of nematode reproduction, when the infective juvenile form of the nematode develops, which possesses the gut vesicle that is colonized by the bacterium [19, 80]. The colonized infective

juveniles leave the cadaver and enter the soil environment, initiating a new life cycle with the invasion of a new insect host.

The interaction between nematode and bacterium has been shown to have many facets. The bacterium does not survive well in soil or water [95] and are not pathogenic for insects when ingested [84, 96] *Xenorhabdus* and *Photorhabdus* have not yet been shown to exist as a free-living organism in the soil environment (except for an asymbiotic strain, *P. asymbiotica*). The nematodes provide protection for the bacteria outside the insect host and a means of transmission from cadaver to the hemocoel of a new host. In addition to transporting the bacterium to a new host, the nematodes provide protection from some host defense mechanisms. The bacteria, in turn, are essential for effective killing of the insect larvae and are required for the nematode to efficiently complete its life cycle, which finally develops into an infective juvenile stage. The nematode reproduction is optimal when the natural symbiont (*Xenorhabdus* or *Photorhabdus* spp.) dominate the microbial flora, suggesting that the bacteria can serve as a food source. Indeed, the bacterial contribution is the provision of nutrients for the nematodes. Axenic (bacterium free) nematodes are unable to reproduce in axenic insects and require bacterial activity to produce suitable nutrient conditions [24, 97] Insects infected by nematodes are also subject to secondary invasion by other microorganisms. This contamination of the insect cadaver is minimized initially by the phagocytic activity of the insect hemolymph [29, 45], then by the protection provided by the various antimicrobial agents produced by the bacteria.

3.2. The biology and biochemistry of the symbiotic bacteria

Xenorhabdus and *Photorhabdus* spp. are motile gram-negative bacteria, facultative anaerobes [6, 7, 21, 25, 52, 53, 112, and 120], of which *Photorhabdus* the only terrestrial among three genera of luminescent bacteria is (the others are marine). *Xenorhabdus* and *Photorhabdus* are highly virulent, deadly pathogens of insects. They are phylogenetically sister species in the genus Enterobacteriaceae. That is, at the phenetic level the traits seem similar, but at the genetic level they are quite distinctive. Although there are differences in their gnotobiological (symbiotic partner or host –free) and bacteriological properties (**Table 1**), *Xenorhabdus* and *Photorhabdus* strains share several common properties due to their life strategy e.g. their way of penetration into the hemocoel, which is absolutely dependent on the help of their symbiotic nematode partners in the *Steinernematidae* and *Heterorhabditidae* genera, respectively.

	Heterorhabditis spp.[1]	*Steinernema spp.*[1]	Bioluminesence	Pigmentation	Catalase	BTB binding	Antibiotics	Crystal proteins	Swarming	Lipase	Lecithinase	Gelatinase	DNase
<u>*Xenorhabdus spp.*</u>													
X. nematophilus	+	-	-	-	-	+	+	+	+	+	+	+	+
X. beddingii	+	-	-	+	-	+	+	+	+	-	+	+	+
X. bovienii	+	-	-	+	-	+	+	+	+	+	(+)	+	+
X. poinarii	+	-	-	+	-	-	+	+	-	+	-	+	+
<u>*Photorhabdus spp.*</u>													
P. luminescens (nematode symbiont)	-	+	+	+	+	+	+	+	+	+	+	+	+
P. luminescens (clinical isolate)	-	+	+	+	+	-	-	-	NT	+	-	+	-

Table 1 Phenotypic characteristics of *Xenorhabdus* and *Photorhabdus* species.
+, 90-100% positive; (+), 26-75% positive; -, 0-10% positive; NT, not tested. (Table is after A. Völgyi)

[1] Nematode symbiont.

3.2.1. Phase variation

Xenorhabdus and *Photorhabdus*, isolated from the infective-stage nematode produce dye adsorbing colonies. However, during *in vitro* culturing some non-adsorbing colonies can be detected. The adsorbing and non-adsorbing variants were initially designated as primary and secondary or phase I and phase II, respectively [12]. More precisely they are phenotypic variant forms. Reversion from secondary to primary has not yet been detected in any of the *Xenorhabdus* or *Photorhabdus* strains. Phase variation has been know for many years in *Salmonella* spp., *Neisseria*, and other organisms [107] and is thought to occur in several different mechanisms [103]. Akhurst described first form variation in *Xenorhabdus* and *Photorhabdus* spp. As a type of variation it includes several factors [8, 20, 21 and 69] but it could be confidently delineated by changes in just two biochemical properties, namely, the absorption of the dye bromothymol blue and the reduction of triphenyltetrazolium chloride. The genes involved are apparently intact (not altered or lost) but, by one mechanism or another, their expression is changing. Neither the formation nor the reversion of phase variants is yet understood. The phase II forms are perhaps better suited to survival outside the

symbiotic niche, being active with respect to cellular metabolism and respiration, but they are unable to live under the conditions required by the symbiosis with nematodes.

Xenorhabdus and *Photorhabdus* bacteria are consumed and digested by the developing nematodes during the reproductive stage of insect infection. The phase I variant is the most ideal for nematode development, probably because it furnishes a good source of nourishment and produces an assortment of antibiotics which prohibit the establishment of other microorganisms. For no obvious reason, phase I will convert to phase II variant at low and variable frequencies during prolonged incubation *in vivo* under stationary phase conditions, which neither supplies as much nutritional value nor the types or amount of antibiotics as phase I. Thus they are not found as natural symbionts in the nematodes, as they are unable to it. In defined media, the production of secondary metabolites was deficient in the secondary variants although they grew substantially faster than phase I for any given defined medium [20].

3.2.2. Cell surface properties

Bacterial cell surface adhesions such as fimbria mediate the attachment to host tissues. Fimbria (pilli) rod-like structures are thought to be involved in the establishment of the specific association between the bacterium and the nematode gut. The phase II cells do not produce fimbriae at detectable levels. All strains of phase I of *Xenorhabdus* spp. are motile and exhibit swarming motility on semisolid agar. In contrast, phase II cells of the same strains lack both swimming and swarming ability and do not produce flagella. The capsular material on the surface (glycocalyx) of phase I and phase II cells of *Xenorhabdus* and *Photorhabdus* spp. is of different thickness [30]. This and the possible chemical differences of the glycocalyx of phase I cells relative to phase II cells could contribute to the ability of the former cell type to adhere to a greater extent to the intestinal cells of the nematode.

3.2.3. Antibiotics

The bacteria associated with insect-pathogenic nematodes produce antibacterial activity that keep the insect carcass from putrefying and is probably important in the successful completion of the life cycle by the nematodes [48]. The compounds produced as antibiotics are of quite diverse types [58, 81, 82, 102, 108 and 110]. In general, phase II cells are low or lacking of antibiotic activity [21].

3.2.4. Pigments

Another common characteristic of the *Photorhabdus* spp. and some *Xenorhabdus* spp. is the propensity to produce pigments that accumulate in the growth medium as secondary metabolites. The pigmentation of colonies can be quite variable, depending on the growth medium used and the age of the culture, so that colonies may vary from cream collared to brick red. Some isolates those in the *X. nematophilus* group are apparently non-pigmented. Others show weak or variable pigmentation, which may due to strain variability or to differences in pH of the medium. That pigmentation (like antibiotic activity) develops strongly in late stationary phase, although this has not been quantitatively studied. In general, phase II cells can be identified by their lack of pigmentation [5, 8, 21]. The possible role of pigmentation might be a deterrence of scavenging animals, e.g. birds from eating the insect carcass.

3.2.5. Secreted enzymes

Both *Xenorhabdus* and *Photorhabdus* spp. secrete an array of enzymes [21]. The enzymatic activities that have been identified so far include triglyceride lipases, phospholipases, protease, and DN-ases. In general, the production of the extracellular enzymes appears to increase during the late logarithmic and early stationary phase of the bacterial growth cycle. The production of numerous enzymes is consistent with the idea that these enzymes are involved in the bioconversion of insect tissues for providing a nutrient base for the developing nematode within the hemolymph. It is of interest that the secretion of most of the enzymes from the phase II cells is markedly reduced for both *Xenorhabdus* and *Photorhabdus* spp.[21, 116]. Proteases have been purified from several *Photorhabdus* and *Xenorhabdus* species. Only some of them (51-61 kDa enzymes) were identified and partially but not enzymatically characterized [21, 26, 88], thus their precise function remained unknown. An analysis of *Photorhabdus* genome, which had been sequenced, revealed a large number of proteases and other enzymes that have signal sequence for secretion mechanisms. The extracellular appearance of most of these is still to be confirmed.

3.2.6. Crystalline proteins

A characteristic that distinguishes most *Xenorhabdus* and *Photorhabdus* strains from other members of the Enterobacteriaceae is the production of crystalline inclusion bodies that present in stationary-phase cultures of phase I cells but are not produced in exponentially growing cells [36, 37]. These major crystalline proteins may account for more than 50 % of

the total SDS-solubilized cellular protein. Their function is unknown: there is no evidence to support the idea that the *Xenorhabdus* inclusion body crystalline proteins can function as insect toxins, but they may contribute to the feeding of the nematode symbiont. The amino acid composition and molecular mass of the crystal proteins of *Xenorhabdus* have been determined [36]. The closely related symbiotic bacterium *Photorhabdus* also produces crystal proteins [18]. However, the molecular properties of the crystal proteins of the two bacteria are distinctly different, suggesting that the genes encoding these proteins were laterally acquired from disparate genetic origins.

3.2.7. Bioluminescence

Luminous bacteria in the genus *Photorhabdus* were first noted by military doctors, who occasionally reported luminous wounds [91]. As discussed by Harvey [64, 65], it was taken as a good sign, and it was generally believed that such wounds were likely to heal. The enzyme catalyzing light emission was identified as a typical bacterial luciferase [98], that it uses molecular oxygen to oxidize two substrates (a long-chain aliphatic aldehyde and $FMNH_2$), yielding a blue-green (490 nm) light. The *lux* genes are similar to the *lux* genes of other luminous bacteria. Although the purpose of this function, either for the nematode symbionts or the human wound isolates, is not proven, but it is supposed that luminescence is also a part of the deterrence of scavenging animals [105].

3.2.8 Isolation, growth and maintenance of *Xenorhabdus*

Xenorhabdus can be isolated in the field using methods in which highly susceptible "trap" insects (e.g. *Galleria mellonella*) are exposed to the soil. [9] Insects dying from entomopathogenic nematode infections are then used as sources for the isolation of *Xenorhabdus*, either from the hemocoel of the infected insect, or from infective-juvenile nematodes. [9, 46, 70] The identification include: 1) colony color on NBTA agar plates (the colony should be blue due to the uptake of bromothymol blue, with an area of clearing around them) [10, 20, 21]; 2) the production of antibacterial activity (nearly all isolates produce antibiotics) [89, 102]; 3) the production of pigments ranging from buff to brown to red [63, 70, and 102]; 4) the presence of intracellular protein crystals; and 5) light production or lack of it.

3.3 Genes described to date in *Xenorhabdus* and *Photorhabdus*

3.3.1 *Xenorhabdus* genes

The genome of *Xenorhabdus* (*X. bovienii*) has been sequenced [61]. The outer membrane proteins, Opns, are involved in responding to changes in the environment. In *Xenorhabdus nematophilus* a gene encoding OpnP was cloned. The OpnP protein shows 55 % amino acid identity to the *E. coli* protein OpmF that is one of the main porins in the organism [54, 59]. OpnP is also major component of the membrane. OpnP does not respond to osmolarity changes like OpmF, but is regulated by the bacterial growth conditions and temperature [54]. OmpR in *E. coli* regulates *Opm*F by binding to its promoter. EnvZ and OmpR are part of a two-component regulatory system. EnvZ is activated by environmental changes and phosphorylates OmpR. [111], OmpR in *E. coli* is a central regulatory protein controlling numerous different genes like pathogenicity, production of flagella, fimbriae and osmosensing. It appears that the same system exists in *Xenorhabdus nematophilus*, the amino acid identity with *E. coli* is 57 % for EnvZ and 78 % for OmpR. It is not certain yet if OmpR has the same pleiotropic effect in *X. nematophilus*. The significance of the protein is currently under investigation.

The genes coding for the flagellin protein synthesis were cloned by functional complementation of a *fli*CD *E. coli* strain [59]. Sequence analysis of the DNA fragments and the proteins revealed that the flagellin protein synthesis appears in a similar manner. Both *fli*C and *fli*D genes have σ^{28} promoter region, which was only one base pair different from the equivalent *E. coli* region. The *X. nematophilus* strain (F1) used in this study have a secondary variant form, which lacks motility on semisolid agar surfaces in this and in liquid culture. In previous studies the motility was considered to be a primary specific character. Most secondary strains are non-motile although the reversion from secondary to primary can occur at a low level. An important question is whether the functional *fli*CD operon from the primary *X. nematophilus* strain is able to complement the variant form motility. The secondary strain could not be complemented with *fli*CD although it could restore the motility of *E. coli*. This finding supports the previously favored idea of a master switch controlling a regulatory system resulting in the secondary phenotype in *X. nematophilus*. It has been reported, however, that some secondary strains are motile and the motility in liquid culture is not a primary specific character [115]. It is possible that motility in certain strains fall under a coordinated regulatory network with other primary specific characters like antibiotic and crystal protein production. In other strains motility may not be controlled the same way.

3.3.2 *Photorhabdus* genes

The genome of *Photorhabdus* (*P. luminescens* TT01) has been sequenced and analyzed [33]. The activity of some of genes has been studied in detail. At lower temperature ($9^{\circ}C$) two genes have been shown to turn on in one operon as the bacteria adapt to cold [34]. The operon coding for polynucleotide phosphorylase (Pnp) which degrades mRNA and a ribosomal protein RpsO have bee cloned from *Photorhabdus* sp. strain K122. The cold shock turns on the *nus*A gene, which also encodes for a transcription antiterminator. In *E. coli* both Pnp and NusA are cold shock proteins and share 86 % amino acid identity with the *Photorhabdus luminescence* genes. The upstream regions of the genes are also conserved and primer extension studies revealed two possible σ^{70} promoters and a cold inducible promoter for Pnp between the two genes. A putative binding site for a cold inducible transcriptional regulatory protein (CS7.4) was found for the Pnp gene in *Photorhabdus* but not in *E. coli*.

A fragment of DNA carrying the *mal*B gene was cloned from *Photorhabdus* [34, 38]. The clone also includes part of *mal*E and *mal*Kgenes the regulatory region between the two genes. The maltose operon in *E.coli* is responsible for utilizing maltose from medium. The coding regions of the clone have 65-75 % amino acid identity with *E. coli*. The control region of the *mal*K-*lam*B operon has a very similar organization to *E. coli.* The region has four *Mal*T binding sites like *E. coli* and two sites for the cAMP receptor protein (CRP) while *E. coli* has two sites at the same position and two additional sites.

The primary forms of *Photorhabdus* and *Xenorhabdus* produce pigments [52]. The genes those code for the *Photorhabdus* pigment have been cloned. The red molecule belongs to the anthraquinones and is produced during stationary phase of the primary strain of the *Photorhabdus luminescence*. The fragment cloned carried an 8.2-kb with open reading frames, which in *E. coli* produced pigment. The pigment itself does not have antibiotic activity; but it is related to the polyketide antibiotic synthesis of *Streptomyces* spp.

The genes required for bioluminescence were cloned and analyzed by for different laboratories [56, 109, 117 and 119]. Five genes are necessary for light production, *lux*C, D and E genes are necessary for a fatty acid reduction complex that is producing aldehyde substrate for the luciferase and *lux*A and B which code for the subunits of the luciferase enzyme. The radiolabeled *lux* genes did not hybridize to the *Xenorhabdus* genomic library, implying that the genes responsible for luminescence are not part of this bacterium. The sequence analysis of the *lux* gene revealed homology with the luminous marine bacteria

Vibrio harveyi. The α subunits of the luciferase displayed 85 % and the β subunits had 60 % amino acid identity with the *lux* genes from *Vibrio harveyi*. The *lux* genes are believed to have been incorporated into the *Photorhabdus* genome through lateral gene transfer because *Vibrio* and *Photorhabdus* are distant from each other on the phylogenetic tree. However, the non-nematode symbiont *Photorhabdus* isolate (**Table 1**) has lost the ability to express luminescence. Implying that the lateral transfer occurred more than once or the part of the genome where the *lux* genes are located is not a stable part of the chromosome. Bacterial bioluminescence results from a luciferase catalyzed reaction where the oxidation of a long chain aldehyde and a $FMNH_2$ occurs simultaneously.

3.4 Pathogenicity and virulence mechanism of *Xenorhabdus* and *Photorhabdus*

The efficiency with which the bacterium-nematode complex kills the larvae depends on the insect species infected, the immunological and physiological state of the insect, and on the particular bacterial species [6]. The bacteria must be able to tolerate the host defensive response, evade recognition as nonself, or suppress the host nonself response. The combined effect of varieties of symbiotic pairs on the insect host can be investigated by coinjecting various axenic nematodes with bacterium strains into the hemocoel where they can recombine [4].

3.4.1 The pathogenicity of *Xenorhabdus* and *Photorhabdus*

The pathogenicity of *Xenorhabdus* and *Photorhabdus* spp. depends on their entry into the host hemocoel, the ability to multiply in the hemolymph in spite of the host's defense response, and also on the interactions between the symbiotic partners. The nematodes are supposed to produce some kind of factors [56, 66] that destroy the inducible enzymatic defense response of the insect [11].

Xenorhabdus and *Photorhabdus* spp. and their associated nematodes form host nonspecific entomopathogenic complexes. To function non-specifically they must be able to avoid or overcome the variety of defense mechanisms of a wide range of insects. They must also have several toxin strategies to ensure the kill of insects. The initial cellular defensive response of hosts to bacterial infection is phagocytosis [41]. When a large number of bacteria are present in the hemolymph, phagocytosis is augmented by nodule formation. In this process hemocytes elongate, form filopodia and become more adhesive. The bacteria adhere to the hemocytes (opsonisation) which in turn aggregate with extrcellular matrix to form nodules [41]. Ultimately, the resulting nodules leave the general circulation by adhering to

fatty tissues. The biochemical and cellular processes are very similar during encapsulation, which is for elimination of infection with immobilization and entrapment of pathogens of larger size (e.g. nematodes). As a result of the initial immune response, bacteria are efficiently cleared from the haemolymph within several hours. This, however, is only temporary in the case of *Xenorhabdus* and *Photorhabdus*. In most of the cases they survive somehow the cellular and the later humoral immune response and re-emerge into the 16-20 hours post-infection so that cell number in the hemolymph starts increasing [42]. After the insect dies the bacterium concentration increases dramatically to high levels. Clearly, the pathogenic phase of the bacterial life cycle may in fact be separate from the rapid growth phase, and the septicemia is not essential for virulence.

3.4.2 The virulence mechanisms of *Xenorhabdus* and *Photorhabdus*

In the fight against the immune response of host, *Xenorhabdus* and *Photorhabdus* must have a number of mechanisms and properties for survival. Avirulent mutant strains have pleiotropic phenotypes including a defect in cell motility, an inability to hemolyze sheep erythrocytes, an absence of a 32-kDa protein which binds to insect hemocytes and an altered outer membrane protein profile relative to the wild-type cells [68]. A mechanism that *Xenorhabdus* and *Photorhabdus* use to tolerate or evade the humoral defensive response is the inhibition of the activation of the insect pro-phenoloxidase [47]. The processing of pro-phenoloxidase to the active enzyme results in the conversion of tyrosine (also in proteins) to dihydroxyphenylalanine. The modified phenylalanine binds to the bacterial cell surface, and in itself is toxic to bacteria by initiating the formation of covalent crosslinks between proteins during the process of melanization. It has been suggested to function as an opsonization process promoting adherence to the hemocytes and participating this way in nodule formation and encapsulation also. *X. nematophilus* possesses anti-hemocytic properties that protect the bacterium from being phagocytosed or adhered effectively. LPS is supposed to be a virulence factor in *X. nematophilus* by preventing the processing of pro-phenoloxidase into phenoloxidase [43, 45].

Other participants of the humoral immune response of insects are several bactericidal proteins (attacins) and peptides (cecropins). Attacins were found to alter the permeability of the outer membrane of *E. coli* and to inhibit the production of the major outer membrane proteins OmpF, OmpC, and OmpA [32, 49]. *Xenorhabdus* and *Photorhabdus* may be relatively insensitive to the action of the bactericidal protein so they may be able to inhibit either the induction or the function of these polypeptides [41]. The participation of toxins as

secreted virulence factors in the pathogenicity of *X. nematophilus* spp. has not been carefully assessed yet. However, the closely related species, *Photorhabdus*, was found to produce – among many other toxins – a high molar mass (more than 700 kDa) protein complex, which is toxic for the epithelial cells in the digestive tract of insects. [27] Thus it can be expected that *Xenorhabdus* strains also secrete toxins of similar type.

The high pathogenicity makes *Xenorhabdus* and *Photorhabdus* bacteria good model organisms of pathogens, which can be used for studying the virulence factors and mechanisms and also as a tool for the investigation of the immune system of insects. They also provide an opportunity to study the question of how similar are – at molecular level – the infection mechanisms of two such closely related pathogens. As for model hosts *G. mellonella* (wax moth, Lepidoptera) larvae are highly susceptible to bacterial infection in general while the tobacco horn worm, *Manduca sexta* (Lepidoptera), is more resistant probably due to a faster phagocytosis and nodule formation [42]. However, both larvae are equally sensitive to the highly virulent *Xenorhabdus* and *Photorhabdus* strains they die in 30-42 h post-infection.

3.4.3 Proteolytic enzymes as virulence factors

Recent results of Clarke and Dowds [35] proposed that the lipase activity of *Photorhabdu*s spp. strain K122 was a virluence factor in *G. mellonella*. Such a role for proteolytic enzymes has not been firmly established yet, but it is plausible to suppose. Among the virulence factors secreted proteases can have fundamental roles during the first stage of infection also, for example in the penetration of tissues and in the suppression of the immune response [39] or by exerting toxic effects [114]. The secretion and biochemistry of these enzymes are better studied in *Photorhabdus*, where four secreted proteases could be detected on a screen of 20 strains with the combination of four methods. These are PrtA, a metzincin in the family of Serralysins, PhpC (Photorhabdus protease C). which belongs to the M4 metallopeptidase family of thermolysin-like proteases, OpdA, a collagen peptidase in the family of thimet- oligopeptidases and PhpD, a Furyalcryloy-Leu-Ala-Val-Tyr cleaving, non-matallo enzyme, the identity of which is still unknown. In contrast, although a number of *Xenorhabdus* strains were tested for protease secretion with simple bacteriological plate assays, only one (*X. nematophilus*) was investigated with biochemical detection method. Two activities could be separated with zymography and only one of them has been characterized partially.

All the aboves well document a similarity of the two sister bacterium species at cellular level and also in their life strategy. Therefore, it might be expected at molecular level also

including the types of interactions with potential hosts. But, at the same time, there may be substantial differences too, which manifests in e.g. the choice of their not-exchangeable symbiotic partner nematode.

To investigate how far the similarity can go between them in the pathomechanism, an approach is the comparison the type and role of proteolytic enzymes. As an approach to compare *Xenorhabdus* and *Photorhabdus* in the pathtomechanism regarding the type and role of proteolytic enzymes, I set to investigate 17 *Xenorhabdus* strains for the secretion of proteases with employing the same detection methods what had been used for *Photorhabdus* strains [77]. I wanted to focus on early secreted protease(s), for a comparison with the earliest secreted, potential virulence factor of *Photorhabdus*, PrtA.

4. Goals of the thesis work

The large variety of available of peptidase genes in the genome raises numerous questions: How many of these genes are expressed during infection and what is their role in virulence? Do they help the penetration of host tissues by hydrolyzing the extracellular matrix? Do they cleave components of the defense system, or do they merely have a role in bioconversion? Obviously, it cannot be expected, that a pathogen produces only one type of proteolytic activity.

During the work for my PhD thesis, I tried to draw under the spotlight one small segment of the *Xenorhabdus* proteome, proteolytic enzyme, using the following, biochemical approach:

1) Detection of proteolytic activities with various biochemical methods (SDS and native PAGE coupled zymography, chromogenic substrates); and determining the order their production during culture growth and during the infection of a model insect (*Galleria mellonella*).
2) Identification of the earliest detectable protease(s) via development of a purification procedure, determination of the N-terminal sequence(s) and comparison with that of those enzyme(s) secreted by *Photorhabdus* (PrtA).
3) Biochemical characterization of enzyme(s) produced the earliest by the analysis of the cleavage site and natural substrate protein preference, and the determination of kinetic parameters and inhibitor sensitivity.
4) In this work, the investigation of the biochemical properties of the proteolytic enzymes that are produced during infection will be the first step towards the understanding their biological function, and is a part of a large project to explore the highly virulent nature of *Xenorhabdus.*

5. Materials and methods

5.1 Materials

5.1.1 Bacterium strains and cell culturing conditions*:*

The strains were obtained from the entomopathogenic nematode-bacterium strain collection maintained at the Department of genetics, Eötvös Loránd University, Budapest, Hungary [73]. The identities, taxonomic positions, and origins of strains used in this study are summarized in **(Table-2**). Single colonies were used as starting material, which were grown on Luria-Bertani (LB) medium plates for 48h at 28^{o}C and replica tested on NBTA plates (nutrient agar supplemented with 25 mg of bromothymol blue per litter and 40 mg of triphenyl-2, 3, 5-tetrazolium chloride per litter) to confirm the phase variation status. Liquid cultures were grown in LB medium without antibiotics at 30^{o}C in a rotary shaker.

5.1.2 Insects

Galleria mellonella (Greater wax moth, Lepidoptera) larvae were bred in our laboratory. They were reared at 25 ºC on bee wax sheets supplemented with granulated pollen. Fifth instar larvae were used in all experiments.

5.1.3 Substrates

The substrates were purchased from Sigma-Aldrich (St. Louis, USA) (His-Ser-4-methoxy-naphtylamide, DL-Val-Leu-Arg-pNA, Succ-Ala-Ala-Pro-Phe-SBzl, L-Ser-AMC, Boc-Val-Pro-Arg-AMC, D-Ala-Leu-Lys-AMC, Boc-Leu-Ser-Thr-Arg-AMC, Z-Gly-Gly-Arg-AMC, His-Ser-4-methoxy-β-napthylamide) and from Bachem (Bubendorf, Switzerland) (Fua-Leu-Gly-Pro-Ala, Boc-Val-Leu-Lys-AMC, Boc-Gln-Ala-Arg-AMC, hepatitisA virus 3 C protease substrate), or synthesized earlier as described in the Department of Biochemistry ELTE, (Succ.-Ala-Ala-Pro-Xaa-AMC) [62], or at the ELTE-MTA Research Group of peptide Chemistry (Fua-Ala-Leu-Val-Tyr) [78], and Dabcyl-Glu-Val-Ile-Ala-Val-Glu-Ser-Edans [80]. For the preparation of stock solutions, the substrates were dissolved in dimethylformamide.

Table 2 Designation, source, hosts, origins and accession numbers of *Xenorhabdus* strains used

bacterium: *Xenorhabdus*		nematode symbiont: *Steinernema*	source of			
species	strain/isolate[b]		nematode	bacterium	nematode origin	16S rDNA acc. no.
X. nematophila	DSM 3370[T]	*S. carpocapsae*	Z. Mracek	E. Stackebrandt	Czech Rep.	X82251
	AN6/1		R. Akhurst	S. A. Forst	USA	AY278674
	AN6/2	No host		S. A. Forst	In the lab.	-
X. cabanillassii	RIO -HU /1	*S. riobrave*	B. Adams	A. Fodor	Texas	Z7638
	RIO /2	No host	-	A. Fodor	In the lab.	-
X. bovienii	DSM 4766 [T]	*S. feltiae*	I.N. Filipjev	E. Stackebrandt	Russia	X82252
	"Krausseii"	*S. kraussei*	J. Gunter	J. Gunter	Switzerland	
X. kozodoii	"Anomali AZ"	*S. arenarium*	N. Simoes	A. Fodor	Russia	
	"Intermedium BIO"	*S. intermedium*	N. Simoes	A. Fodor	S. Carolina	
	"Morocco"	*S. sp.*	R.-U. Ehlers	A. Fodor	Morocco	
X. poinarii	DSM 4768 [T]	*S. glaserii*	G. Steiner	E. Stackebrandt	N. Carolina	
	"Cubanum"	*S. cubanum*	Z. Mracek	A. Lucskai	Cuba	
X. beddingii	DSM 4764 [T]	*S. longicaudatum*	Sheng & Wang	E. Stackebrandt	China	-
X. budapestensis	DSM 16342 [T]	*S. bicornutum*	B. Tallosi	A. Fodor	Vojvodina	AJB10293
X. szentirmaii	DSM 16338[T]	*S. rarum*	B. Adams	A. Fodor	Cordoba	AJB10295
X. ehlersii	DSM 16337[T]	*S. longicaudatum*	B. Adams	A. Fodor	China	
X. innexi	DSM 16336[T]	*S. scapterisci*	B.Adams	A. Fodor	Uruguay	

5.2 Methods

5.2.1 Gelatin hydrolysis plate assay (Frazier's method) (57)

Gelatin nutrient agar plates (12 g/l gelatin in nutrient agar) were spot inoculated with 10 µl cell suspension for 24-huor cultures (four spots from each strain). After 2-day incubation at 28°C, the plats were overlaid with 5.0 mL mercuric chloride reagent (12 g $HgCl_2$ dissolved in 96 mL 2.0 M HCl solution) to reveal gelatin hydrolysis as clear, no precipitation zones around the colony area) was determined densitometrically with Molecular Analyst software (BioRad).

5.2.2 *G. mellonella* infection experiments

An overnight LB culture of *X. kozodoii* Morocco strain, which was started with several colonies from a fresh LB plate, was 20-fold diluted into LB medium and grown to OD_{600} = 0.3 (2-3 hours incubation at 30°C). Then the cells in 7.0 mL of such a culture were sedimented, washed twice in 1.0 mL phosphate-buffered saline (PBS, 137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 6.5), and finally resuspended in 1.0 mL PBS. To determine cell counts, serial dilutions (10^1-10^6) were made in PBS, and 20 µL of the dilutions were plated onto LB plates. Three groups of fifth-instar *G. mellonella* larvae (5 larvae in each) were injected with 5 µL of appropriately diluted *Xenorhabdus* cell suspension (~ 50 cells). In the injection control group, insects were injected with 5 µL PBS. Body homogenates or hemolymph samples (from 5-8 µL hemolymph) were prepared as described under 5.2.3. For zymographic analysis and enzyme assays 4.0 and 7.0 µL of the samples were used, respectively.

5.2.3 Preparation of hemolymph and body homogenate samples

In the case of *G. mellonella* larvae, hemolymph samples were taken through prolegs and diluted immediately 10-fold with ice cold PBS that contained 0.25 µg/mL phenylthiourea (PBS-Ptu). Then the cellular fraction was sedimented in an Eppendorf centrifuge at 2,800 ×g for 20 minutes, and the supernatants were used for experiments. Body homogenates were prepared by homogenizing ice chilled larvae (~ 0.1 g each) in 1.0 mL PBS-Ptu. The homogenates were centrifuged in an Eppendorf centrifuge at 2,800 ×g for 20 minutes, and the supernatants were used for analysis of enzyme activity.

The hemolymph from non-immunized fifth instar *Manduca sexta* larvae were provided by G. Felföldi from our laboratory. The larvae were bled through their horn cut and their hemolymph was treated as above. The samples were stored at -80 °C.

5.2.4 Preparation of bacterium culture supernatants and bacterium cell lysates

Culture supernatants and cell lysates were prepared from 1.0 mL bacterium cell suspension. The cells were sedimented at 16,000 ×g for 5 minutes. To prepare cell lysate, the cellular pellet was washed twice in 1.5 mL LB and resuspended in 200 µL 0.1 M TRIS buffer (pH 8.0) containing 20 % sucrose. After incubating on ice for 10 minutes, the suspension was centrifuged as above, the supernatant was discarded and the cells were resuspended in 1 mL distilled water.

5.2.5 Polyacrylamide gel electrophoresis and zymography

For native gel electrophoresis 10 % acrylamide and 0.26 % bis-acrylamide gels were made in 0.38 M TRIS-HCL buffer (pH 8.8). For zymography, gelatin (Bloom 300, Sigma) or casein (Sigma) was copolymerized in the gels at 0.025 % final concentration. Running of native gels was continued after the dye front reached the bottom of the gel for further 90 minutes. SDS gel electrophoresis was performed using a 10 % acrylamide, 0.26 % bis-acrylamide separation gel, which contained 0.025% copolymerized casein (Sigma) in the case of zymography. To test the effect on zymographic activity of PMSF and the complex forming compounds, EDTA and 1,10-phenatroline, they were applied in 5.0 mM concentration during both electrophoresis sample preparation and incubation of gels after running. In these cases, the samples were loaded on the gel in a distance of three wells from each other to prevent their cross contamination during electrophoresis due to the diffusion of inhibitors. The composition of the sample buffers was the following: 2×sample buffer for native-PAGE. (Sample buffer SDS free: 40% Glycerin, 0.2M Tris-HCl of PH: 8.5, 0.1% bromophenol blue).

For zymographic detections, the protein samples (even for SDS-PAGE) were not boiled and did not contain DTT. After running, the gels were soaked in 2 - 4 changes of 100 mL buffer solution containing 50 mM TRIS-HCL (pH 8.0), 10 mM $CaCl_2$ in order to develop the proteolytic bands. Native gels were incubated in two changes, the first for 20 minutes and the second for 60 minutes. SDS gels were incubated in four changes, the first three for 20 minutes each, and the last one for 120 minutes. The proteolytic bands were visualized with coomassie R250 staining.

5.2.6 Purification of *Xenorhabdus* protease B

Four liters of LB medium was inoculated from an overnight LB culture of *X. kozodoii* Morocco strain such that the OD_{600} of the resulting cell suspension was ~0.05. It was grown at 30 °C until the OD_{600} was ~0.3 (20-22 hours, late logarithmic phase). The pH of the culture supernatant was set to 8.0 with NaOH then 150 ml slurry of QAE Sephadex A-50 resin (Pharmacia) was added to it, which had previously been equilibrated with buffer A (50 mM TRIS-HCl, pH 8.0, 10 mM $CaCl_2$). After shaking for 2-3-hours in the cold room, the resin was filtered off and washed three times with 600 ml of buffer A. Before elution of proteins, the resin was filled into a column and was washed with a column volume of buffer A. The elution was performed with 200 mL of a linear NaCl gradient (0-1.0 M) in buffer A at 1.6 mL/min flow rate. The zymographically active fractions were pooled and dialyzed against buffer S (50 mM TRIS-HCl, pH 8.0, 1.0 mM $CaCl_2$). The dialyzate was centrifuged (71×g for 15 minutes), then applied to a PAE 300 (Millipore) anion exchanger column (1.6×2.0 cm) equilibrated with buffer S. After loading, the column was washed with 20 mL buffer S, then the proteins were eluted with 40 mL of a linear NaCl gradient (0-0.1 M) in buffers S at 0.2 mL/min flow rate. The protein composition and purity of chromatography fractions and enzyme preparations were checked with SDS-PAGE. The occurrence of degraded or variant molecular forms was investigated also with zymography following native or SDS –PAGE as described [77, 87]. The enzyme preparations were stabilized with the addition of 50 g/ml bovine serum albumin, and they were stored at -20 °C.

5.2.7 Measurement and calculation of protease activities

Enzyme activities were measured in 1.0 mL final volume, at 30 °C in an enzyme assay buffer (50 mM TRIS-HCl (pH 8.0), 10 mM $CaCl_2$, 0.1 M NaCl and 50 μg/mL bovine serum albumin). The activities on fluorometric substrates were measured at 380 nm excitation and 460 nm emission (substrates with AMC and 4-methoxy-naphtylamide fluorophore), or at 340 nm excitation and 495 nm emission (substrates with Dabcyl quencher and Edans fluorophore groups). The activities on photometric substrates with p-nitroanilide leaving group were measured at 410 nm, whereas the 2-furylacryloyl (Fua) group blocked substrates and the substrate with thiobenzyl leaving group were measured at 324 nm. In the case of the latter substrate, the enzyme assay buffer was supplemented with a SH reagent, 4,4-dithiodipyridine (25 mM final concentration). The reactions were started with the addition of the appropriate substrate.

Enzyme activities in culture supernatant and hemolymph samples from *G. mellonella* were measured at 50 μM final substrate concentration using 50 μL culture supernatant and cell lysate or 7.0 μL *G. mellonella* 10×diluted hemolymph samples. The hydrolysis rate of Succ.-Ala-Ala-Pro-Phe-SBzl and the furylacroyl substrates were calculated from the first, linear part of the time dependence curves, using the values ε = 19,000 $M^{-1}cm^{-1}$ and ε = 2,510 $M^{-1}cm^{-1}$, respectively. The precise concentration of furylacryloyl substrates was determined spectrophotometricaly using ε = 24,700 $M^{-1}cm^{-1}$ [78].

For the determination of the kinetic parameters of *Xenorhabdus* protease B (and *Photorhabdus* protease A) the purified enzymes were used at 5-30 nM final concentration. For the calculation of the activities on substrates with Dabcy quencher and Edans fluorophore the same procedure was applied as described in ref. [79] The determination of the molar fluorescence gave 4.36×10^{11} ΔCPS/M for the hepatitis A protease C substrate, which is essentially the same value as that of the substrate of PrtA [79]. The activities on substrates containing AMC chromophore were calculated with 1.2×10^{13} ΔCPS/M molar fluorescence, which was determined with AMC calibration. In the case of the substrate with pNA chromophore the ε = 8,100 $M^{-1}cm^{-1}$ absorption coefficient was used in the calculations.

5.2.8 Measuring the effect of inhibitors, metal ions and pH on the activity of *Xenorhabdus* protease B

20 nM enzyme was incubated with inhibitors at room temperature for 20 minutes in the assay buffer (in the case of EDTA, Ca^{2+} was omitted from the assay buffer) before measuring the remaining activity on Boc-Val-Leu-Lys-AMC substrate at 40 μM final concentration as above. As a control, protease B was incubated under the same conditions, but without the presence of inhibitor. To test the effect of metal ions, they were added to the 1,10-phenantroline or EDTA inhibited enzyme and incubated for 5.0 minutes prior to activity measurement. The activities were calculated from the initial substrate hydrolysis rates (the first, linear part of the curves) where it is proportional to k_{cat}/K_M (at the substrate concentration well below K_M).

To determine the pH profile of *Xenorhabdus* protease B, the activity was measured at 40 μM substrate - (Boc-Val-Leu-Lys-AMC) and 50 nM enzyme concentration (as above), in the following buffer solutions: sodium acetate (pH 4.5, 5.0, 5.5), MES-HCl (pH 6.0, 6.5), MOPS-HCl (pH 7.0, 7.5), HEPES-HCl (pH 8.0), TRIS-HCl (pH 8.5, 9.0) and CAPS-HCl (pH 10.0). The concentration of the buffer systems was 50 mM containing 50 μg/ml bovine serum

albumin 10 mM $CaCl_2$ and 0.1 M NaCl. The enzyme activities were calculated as above. Data points were fitted with Origin 5.0 software using the following equation:

$$k_{cat}/K_M = L_1\left(\frac{1}{1+\frac{10^{-pH}}{10^{-pK_1}}}\right)\left(\frac{1}{1+\frac{10^{-pK_2}}{10^{-pH}}}\right)+L_2\left(\frac{1}{1+\frac{10^{-pH}}{10^{-pK_3}}}\right)\left(\frac{1}{1+\frac{10^{-pK_4}}{10^{-pH}}}\right) \qquad (1)$$

Where L_1 and L_2 are amplitude factors, $(k_{cat}/K_M)_{lim1}$ and $(k_{cat}/K_M)_{lim2}$, respectively.

5.2.9 Protein digestions with purified *Xenorhabdus* protease B *in vitro*

To test whether *Xenorhabdus* protease B cleaves bovine serum albumin, fibrinogen, and two collagen types (III and IV), ~10 μg of these proteins was digested with 1.0 pmole (~0.06 μg) purified protease B for 90 minutes, at 30°C, in 50 μL final volume of the assay buffer (above), which did not contained bovine serum albumin. The reactions were stopped by the addition of 25 μl of 2×sample buffer (containing DTT, above) and boiling for five minutes. The samples were analyzed in 10% SDS polyacrylamide gel.

When *M. sexta* hemolymph proteins were digested they were previously fractioned with DEAE anion exchange chromatography, according to Felföldi et al. [50]. Six separated fractions of distinctive protein compositions were obtained (fractions A-F). For digestion 1-16 μg hemolymph protein (depending on the protein content of the fraction) was incubated at 30°C, in 50 μL final volume of the assay buffer (above), which did not contained bovine serum albumin, for 45 or 90 minutes in the presence of 1.0 pmole (~ 0.06 μg) of the following proteases: bovine pancreatic trypsin, chymotrypsin and elastase, *Clostridium histolyticum* collagenase and purified protease B. The reactions were stopped by the addition of 25 μl of 2×sample buffer (containing DTT, above) and boiling for five minutes. The samples were analyzed in 10% SDS polyacrylamide gel.

5.2.10 N-terminal sequence determination of *Xenorhabdus* protease B

For N-terminal sequencing samples of purified *Xenorhabdus* protease B were run in 10 % acrylamide SDS gel under reducing conditions. After running, the gels were soaked for 10 minutes in transfer buffer (10 mM CAPS (Sigma) pH 11.0, 10 % methanol), then blotted onto Immobilon-P PVDF Transfer Membrane (Millipore) at 200 mA for 2 hours. Proteins on the membrane were visualized by coomassie staining. The bands corresponding to the proteases were cut out and subjected to Edman-sequencing in a Microtec-protein sequencer (Applied Biosystems) by Dr. András Patthy at the ELTE-MTA.

6. Results

6.1 Searching protease activities in *Xenorhabdus* cultures

As an initial step in the investigation of secreted proteolytic activities, I screened the cultures of 17 *Xenorhabdus* strains using seven detection methods that had employed been earlier on *Photorhabdus* strains [77]. The results of screening are summarized in **Table 3**.

6.1.1 Gelatin hydrolysis plate assay

In this non-specific, semi-quantitative plate assay *Xenorhabdus* strains performed similarly to *Photorhabdus* strains: the majority of activities differed by only several fold. However, unlike *Photorhabdus*, each of the studied *Xenorhabdus* strain proved positive regardless if they were primary or secondary phase variants (or phenotype variant). The strongest activity (by *X. nematophila* DSM 4768 was twenty-fold higher than the weakest one (*X. nematophila* AN6/1).

6.1.2 Detection of activities with zymographies

Another, non-specific and semi-quantitative method is zymography after gel electrophoresis which, however, is able to resolve activities according to their molecular properties. I applied this method for the 17 strains after both SDS and native PAGE, and using gelatin and casein as substrate. Gelatin allowed a more sensitive detection of *Xenorhabdus* proteases especially after SDS-PAGE (**Fig. 2**). I could separate three different activity bands with SDS-PAGE, that I distinguished with letters (A-C, see **Fig. 2A, B**), and two activities with native PAGE that I labeled with numbers (1, 2, see **Fig.2C, D**). When the lysed cell fractions were analyzed the activities remained below the level of detection indicating that they are not from intracellular proteases. Sometimes activity bands 2 and B were split which I attributed them either to molecular variants or degradation products of the enzymes. At the same time activity 1 usually was a wide band which - on casein substrate - could often be resolved in 4-6 component bands. Molar mass estimation gave 90, 55 and 35 kDa for activities A, B and C, respectively. I could even observe different substrate preferences such that activities A and C were mainly detectable with gelatin and casein substrate, respectively, while activity 1 had an apparently stronger affinity to substrates than activity 2, as indicated by substrate cleavage during native gel electrophoresis (generating a long stripe of activity) and the concomitant dependence of migration on protein amount.

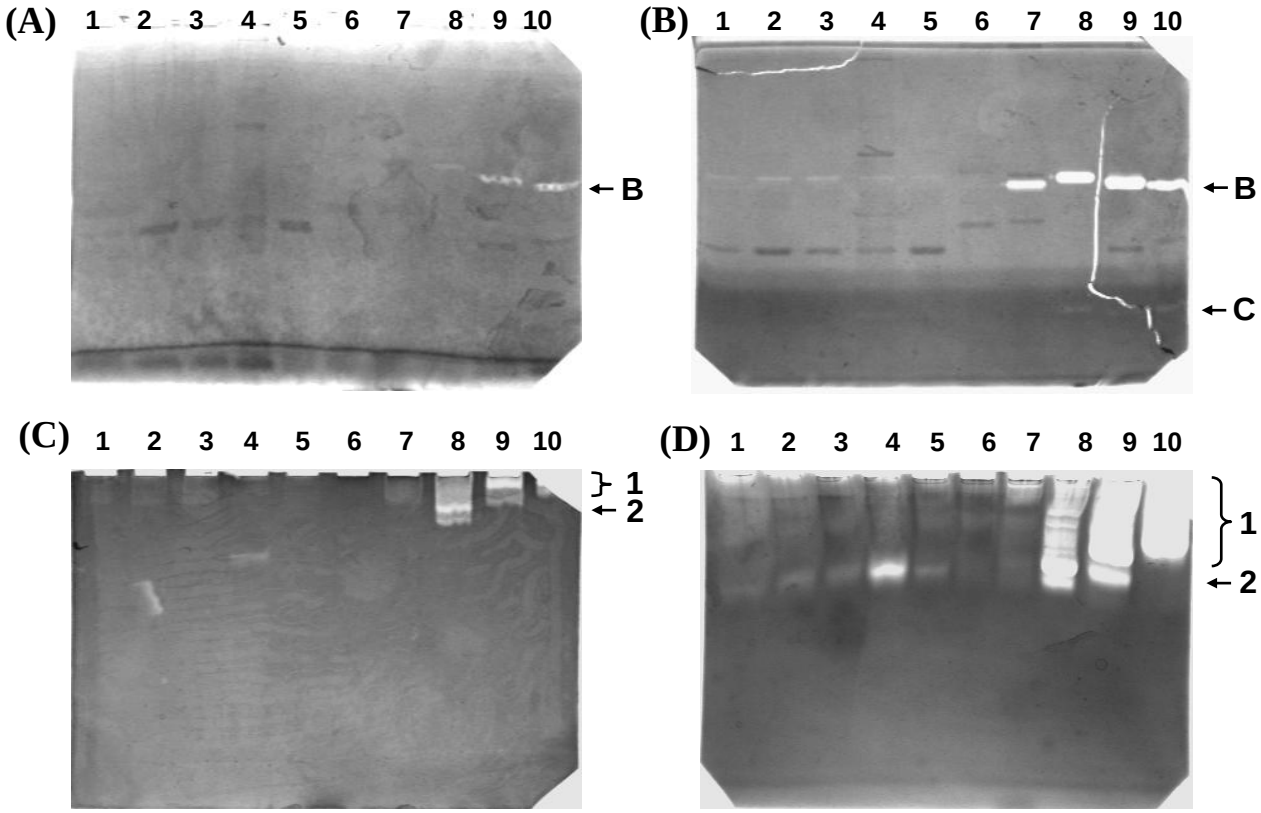


Figure 2 Protease secretions of ten *Xenorhabdus* ssp. strains
Protease activities were analyzed with zymographies after SDS PAGE (panels (**A**) and (**B**)) and native PAGE (panels (**C**) and (**D**)) using gelatin (panels (**A**) and (**C**)) and casein (panels B and D) as substrates. Strains shown are: 1, DSM 3370, 2, AN6/1, 3, AN6/2, 4, Riobave/1, 5, Riobave/2, 6, DSM 4766, 7, Intermedium, 8, Kraussei, 9, Anomali, 10, Morocco. The samples were prepared from 11 h cultures were activities B, C, 1 and 2 are visible but not A.

To investigate which of the activities detected after native- and SDS-PAGE were from the same enzyme I employed inhibitors and run gels with the purified protease generating the activity B (**Fig 3).** (I used the *X. kozodoii* Morocco and intermedium strains in these experiments because they expressed the most stably activities A and C). I found that activity B as well as activity band 1 on gelatin substrate and activity band 2 on casein substrate were from the same enzyme. I called this enzyme (*Xenorhabdus*) protease B. (The *X. kozodoii* Morocco and intermedium strains were used in these experiments because they expressed the most stably activities A and C). The inhibitor sensitivity of all the zymographic activities was similar: they were inhibited by complex forming compounds (EDTA and 1,10-phenantroline), but not with serine protease inhibitor (PMSF, data not shown). Therefore the question whether the protease generating band 1 (on casein substrate) and any of those generating band A or C are the same cannot be answered with certainty. However, because no correlation can be

observed between them in their occurrence (see **Table 3**) I suppose they are different enzymes, and there are only technical reasons why activities A and C cannot be detected after native-PAGE and activity 1 (on casein) after SDS-PAGE. Important to note that, interestingly, EDTA did not inhibited protease B activity completely. Therefore I repeated the zymography also with purified protease B using a reduced amount of the enzyme (~5.0 pmole), but I got the same result (**Fig. 3, lanes a-d)**.

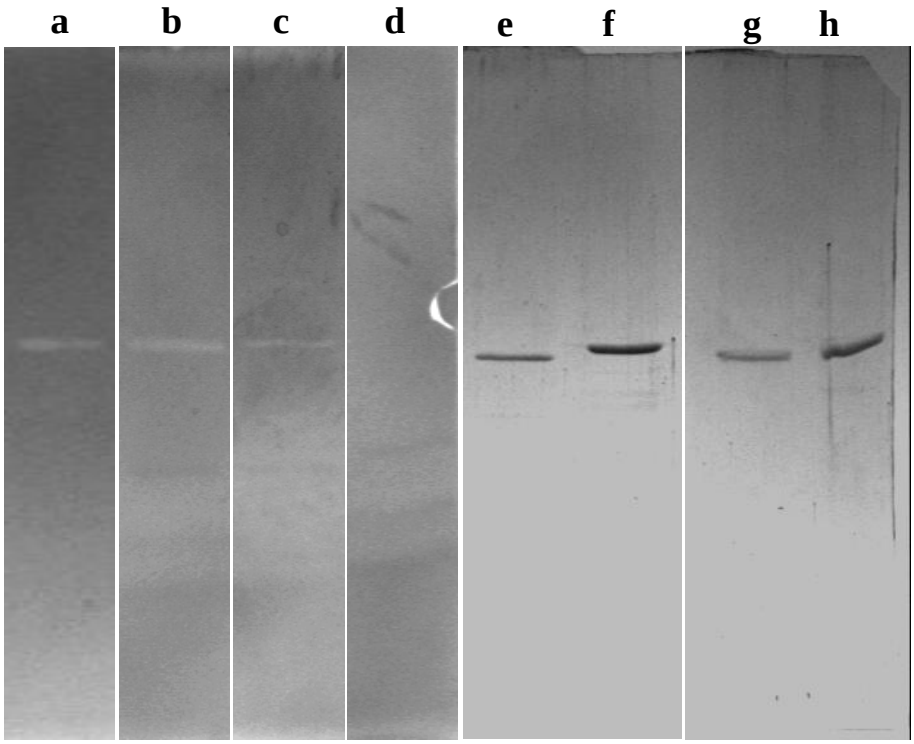


Figure 3 The effect of three inhibitors on purified *Xenorhabdus* protease B.
The protease activity and autoproteolytic stability was investigated with zymography following SDS-PAGE (using casein substrate, lanes a-d), and SDS-PAGE (lanes e-h), respectively. Inhibitor treatments: none (a), PMSF (b), EDTA (c, e, f), 1,10-phenantroline (d, g, h). For a comparison (see text) the treatment of *Photorhabdus* PrtA is also shown (lanes e and g).

The tested strains exhibited large differences in both the number and intensity of protease production (**Table 3**). AN6/1, RIO –HU /1 and DSM 16337 were the weakest, while Morocco, Anomali AZ, Kraussei and Intermedium were the strongest in this regard, indicating that this property was strain and not species specific. The cross-strain comparisons also showed that the production of protease B (activity B) was the most intensive and the most common (being produced by all the strains), and that the molecular properties of the enzymes, which produced activities A, B, C as well as 1 and 2, were different (reflected by their relative mobility). The appearance of zymographic activities allowed an estimation of the dynamics in the secretion of the corresponding enzymes (**Table 3** and **Figure 4**), except

for the heterogeneous activity-band 1 (on casein - see above). According to this protease B was produced the earliest (detected even in the early logarithmic phase i.e. in 4 h cultures), while all the others were produced from the late logarithmic or stationary phases. Protease B was different from the other enzymes also in that its secretion declined in the stationary phase.

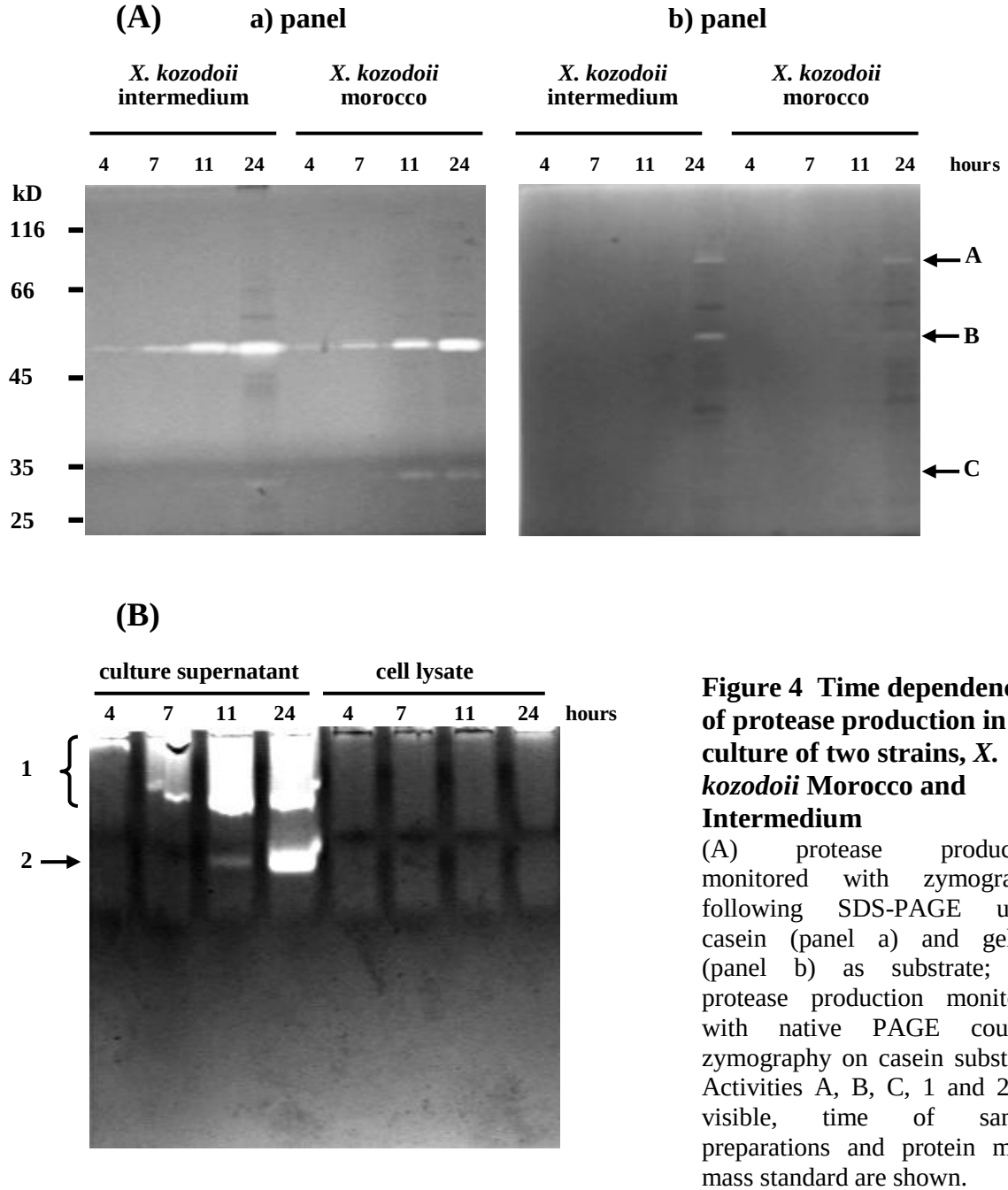


Figure 4 Time dependence of protease production in the culture of two strains, *X. kozodoii* Morocco and Intermedium
(A) protease production monitored with zymography following SDS-PAGE using casein (panel a) and gelatin (panel b) as substrate; (B) protease production monitored with native PAGE coupled zymography on casein substrate**.** Activities A, B, C, 1 and 2 are visible, time of sample preparations and protein molar mass standard are shown.

Thus altogether I could distinguish at least four proteolytic activities with zymographies in the culture supernatant of *Xenorhabdus* strains.

6.1.3 Exploring protease production with chromogenic substrates

For a quantitative detection of proteolytic activities I used several oligopeptide substrates, the cleavage of which can also be informative to the type of the enzyme. For example, Succ-AAPX-AMC (where X was Phe and Lys) and Succ-AAPF-SBzl are used to measure the activity of serine proteinases, while Fua-LGPA is readily hydrolyzed by collagen peptidases. Every strain showed activity on Succ-AAPF-SBzl (**Table 3**) but I could not find activity with the Succ-AAPX-AMC substrates (where X was Phe, Lys, Arg, Ala, Leu and Asp – data not shown). This indicates that the Succ.-AAPF-chromophore is a suboptimal substrate therefore when the easily hydrolysable (thiol) ester bond is replaced with the more stable amide and/or the P1' position is occupied by aminomethyl-coumarin and not thio-benzyl group in the substrate the enzyme is unable to cleave it. I also employed two other substrates, Fua-LGPA and Fua-ALVY. We had previously found the former to be hydrolyzed by oligopeptidase A of *Photorhabdus* [78], while the latter was cleaved an as yet uncharacterized enzyme in *P. luminescens* Brecon strain [77]. Essentially every *Xenorhabdus* strains showed activity on these substrates (only Fua-LGPA was not cleaved by AN6/1 (**Table 3**). The Fua-LGPA-ase activity was supposedly from the *Xenorhabdus* orthologue of oligopeptidase A. The dynamics of production was different for the three activities observed with the chromogenic substrates. While the Fua-LGPA-ase activity exhibited a steady increase from its first appearance in the logarithmic growth phase (similarly to that in *Photorhabdus* strains), the other two showed a peak, the Fua-ALVY-ase activity at the beginning of the logarithmic phase (**Figure 5**) and the Succ.-AAPF-SBzl-ase activity in the late logarithmic or early stationary phase (data not shown).

When I compared cell lysates to the culture supernatants the Fua-LGPA-ase and the Succ-AAPF-SBzl-ase activities were an order of magnitude higher in the lysates than in the culture supernatant (**Table 4**). This indicated intracellular enzymes that leaked out to the medium on cell death. This conclusion was supported for the Fua-LGPA-ase activity by the dynamics of its appearance in the medium, which was also the same for the Fua-LGPA hydrolyzing intracellular enzyme, oligo-peptidase A, of *Photorhabdus*. However, leaking cannot explain either the early appearance of or the peak in the activity of the Succ-AAPF-SBzl hydrolyzing enzyme. Instead, the higher activity in the cellular faction might be explained by a periplasmic localization of the Succ-AAPF-SBzl-ase enzyme, or by enzyme(s)

Table 3 Protease secretions of 17 *Xenorhabdus* ssp. strains and phase variants: summary of results with seven detection methods

strain[1]	gelatin liquefaction assay[2]	SDS-PAGE[3] A	SDS-PAGE[3] B	SDS-PAGE[3] C	native-PAGE[3] 1	native-PAGE[3] 2	Succ.-AAPF-Sbzl[4] $\times 10^{-7}$M min^{-1}	Fua-LGPA[5] $\times 10^{-7}$M min^{-1}	Fua-ALVY[5] $\times 10^{-7}$M min^{-1}
X. nematophila DSM 3370	+	(+) (s) -	- (x) (l)*	- -	(+) (ll) -	- (x) (ll)	2.5 (el)	10.5 (l)	6.0 (el)
X. nematophila AN6/1	+	- -	- (x) (ll)*	- -	- -	- -	2.3 (el)	0.0 (l)	7.1 (el)
X. nematophila AN6/2	+	(+) (s) -	(+) (l) xx (l)*	- -	- -	- (x) (ll)	3.5 (el)	6.5 (l)	8.2 (el)
X. cabanillassii Riobrave/1	+	(+) (ll) -	- (x) (l)*	- -	- -	- x (ll)	23.0 (el)	23.5 (l)	11.0 (el)
X. cabanillassii Riobrave/2	+	(+) (ll) -	- (x) (l)*	- -	- -	- -	2.1 (el)	10.6 (l)	13.4 (el)
X. bovienii DSM 4766	+	(+) (ll) -	+ (s) x (ll)	- (x) (s)	(+) (s) -	- (x) (s)	22.0 (el)	10.1 (l)	10.4 (el)
X. bovienii Kraussei	+	- -	(+) (ll) xx (ll)	- (x) (ll)	+ (ll) x (ll)	- xx (ll)	67.0 (el)	15.6 (l)	31.9 (el)
X. kozodoii Intermedium	+	(+) (s) -	(+) (ll) xx (el)	- (x) (ll)	(+) (ll) -	- x (ll)	6.1 (el)	8.5 (l)	9.3 (el)
X. kozodoii Anomali AZ	+	- -	(+) (l)* (x) (el)*	- (x) (l)	+ (ll) xx (ll)	- xx (ll)	3.1 (el)	26.7 (l)	39.0 (el)
X. kozodoii Morocco	+	(+) (ll) -	+ (l) xx (el)*	- (x) (l)	+ (l) xx (el)	- xx (ll)	18.0 (el)	9.6 (l)	8.7 (el)
X. poinarii DSM 4768	+	(+) (s) -	++ (l)* xx (l)*	- -	(+) (ll) -	- (x) (ll)	1.8 (el)	11.3 (l)	15.4 (el)
X. poinarii Cubanum	+	(+) (s) -	+ (l) xx (el)	- (x) (ll)	++ (ll) -	- xx (ll)	7.9 (el)	10.0 (l)	11.9 (el)
X. beddingii DSM 4764	+	- -	++ (el)* xx (el)*	- -	(+) (ll) -	- (x) (ll)	2.0 (el)	8.3 (l)	12.9 (el)
X. budapestensis DSM 16342	+ -	- xx	+ (s) (ll)	- -	++ (ll) xx	- (ll)	2.8 (el)	9.2 (l)	13.7 (el)
X. szentirmaii DSM 16338	+	- -	(+) (ll) xx (el)*	-	+ (ll) -	- x (ll)	4.7 (el)	31.2 (l)	11.9 (el)
X. ehlersii DSM 16337	NT	- -	+ (el) x (el)*	- -	++ (l) -	- (x) (l)	2.8 (el)	14.4 (l)	27.0 (el)
X. innexi DSM 16336	NT	- -	+ (ll) xx (el)*	- -	++ (ll)	- xx (ll)	4.5 (el)	4.0 (l)	10.6 (el)

Table 3 Protease secretions of 17 *Xenorhabdus* ssp. strains and phase variants

[1] the identity of strains is given in Table 1.

[2] +, clearing zone is visible, -, clearing zone is not visible.

[3] The semi-quantitative rating of the strongest activities in zymographies were the following: on gelatin substrate (+) weak, + medium (well detectable) and ++ strong, and on casein substrate (x) weak, x medium and xx strong. The time of the first zymographic detection of an activity is shown in parenthesis behind the ratings as follows: el, early logarithmic phase (0-4 h culture), l, logarithmic phase (4-8 h culture), ll, late logarithmic or early stationary phase (8-20 h culture) and s, stationary phase (longer than 20 h growth). (For the optical densities in these phases see the growth curves of two strains in Fig. 5.)

[4] The activities were measured using 50 μL supernatant of 4 hour cultures and 50 μM substrate concentration.

[5] The activities were measured using 50 μL supernatant of 24 hour cultures and 50 μM substrate concentration. For the dynamics of production in the case of two strains see Fig. 5. The calculation of enzyme activities is given in Methods.

* When the cultures were incubated longer than 20-24 hours, the production if these activities declined.

NT, not tested.

in the cytosol that can hydrolyze this substrate. (Succ-AAPF-Bzl is an easy to hydrolyze, ester type substrate).

Since the distribution among strains of the activities on chromogenic substrates was different from both each other and the zymographic activities I suppose that they were produced by different enzymes. To investigate this question further I tested the inhibitor

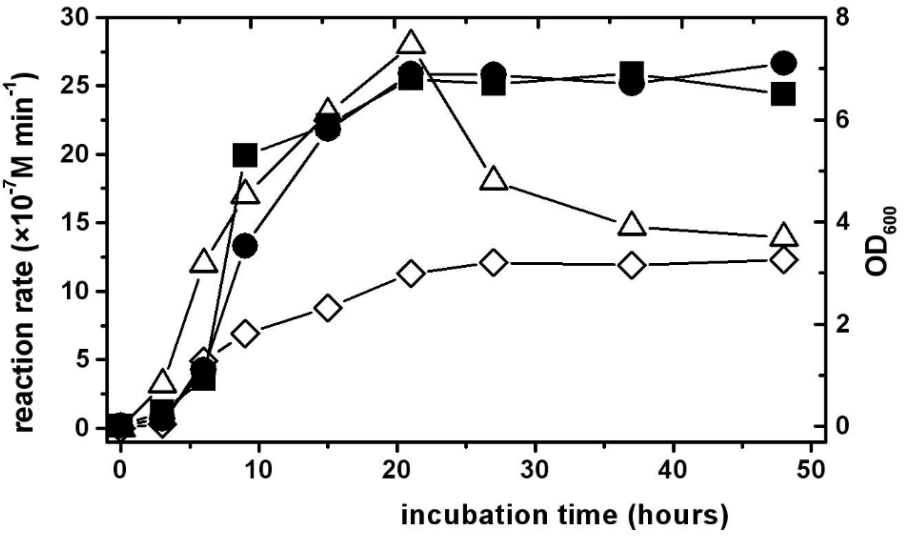


Figure 5 The cell growth of and two enzyme activities as the function of time.
Shown are cell densities (OD_{600}, filled symbols) in the culture of *X. kozodoii* Morocco and Intermedium strains (circles and squares, respectively) and Fua-LGPA-ase (open diamonds) and Fua-ALVY-ase (open triangles) activities. The activity values for both substrates are representatives of data from measurements in the two cultures of Morocco strain (measured in 50 μL culture sample at 50 μM substrate concentration).

strain	sample type	reaction rate (M min^{-1}) on		
		Fua-ALVY	Fua-LGPA	Scc.-AAPF-SBzl
Morocco	SN	3.5×10^{-6}	7.5×10^{-7}	4.5×10^{-8}
	CF	1.8×10^{-7}	3.3×10^{-6}	1.2×10^{-7}
Kraussei	SN	1.1×10^{-6}	3.2×10^{-7}	5.8×10^{-8}
	CF	1.0×10^{-8}	1.2×10^{-6}	3.4×10^{-7}
Serratum	SN	6.5×10^{-7}	2.1×10^{-7}	5.0×10^{-8}
	CF	1.1×10^{-7}	6.9×10^{-7}	2.1×10^{-7}

Table 4 Comparison of activities on three substrates between cell fraction and culture supernatant.

The activities were measured from 11 hour cultures under the same conditions as in given in the legend to Figure 5. CF, cell fraction, SN, culture supernatant.

of these activities from *X. kozodoii* Morocco and Intermedium BIO strains (**Table 5**). The activity on Succ.-AAPF-SBzl was dependent on active serine since it was inhibited by PMSF, while – being inhibited by EDTA and 1,10-phenantroline - the Fua-LGPA-ase and Fua-ALVY-ase activities proved metal ion dependent. These, at the same time, differed from each other in their sensitivity to thiol reagents (Cys, Thiomerosal and DTT). These experiments could not exclude completely the possibility, that either the Fua-LGPA-ase or the Fua-ALVY–ase activity is from protease B, however, the purified protease B did not cleave either of these substrates (see **Table 7**).

Inhibitor	remaining activity[1] on		
	Succ.-AAPF-Sbzl	Fua-LGPA	Fua-ALVY
EDTA	99.1 ±11.2	1.3 ±2.2	0.1 ±0.1
1,10-phenantroline	105.3 ±15.1	0.1 ±1.5	0.9 ±0.5
PMSF	14.8 ±7.5	86.3 ±5.4	16.6 ±6.2
Cys	NT	29.7±11.0	0.2 ±0.3
Thiomerosal	NT	97.0 ±5.2	56.3 ±8.8
DTT	NT	68.5 ±7.8	0.5 ±0.2

Table 5 The effect of several inhibitor types on the activities found on three chromogenic substrates.

[1] Activities expressed in % of control. The results are from measurements on two different culture supernatant, and the data shown are the average of at least three measurements. The control (100%) values for the Succ.AAPF-SBzl-ase, the Fua-LGPA-ase and Fua-ALVY-ase activities are $2.90 \pm 0.21\times10^{-7}$ M/min, $7.6 \pm 0.5\times10^{-7}$, and $3.4 \pm 0.42\times10^{-6}$, respectively.

NT, not tested.

Taken together the results of the investigation of proteolytic activities detectable in *Xenorhabdus* strains, I observed that at least six protease activities: protease B, as well as activities A, C, 1 (on casein substrate), and those on Fua-ALVY and (possibly) Succ.-AAPF-SBzl substrates were secreted. This number is three times more than what have been found for *Photorhabdus* strains using the same detection methods [77].

6.2 Purification and identification of *Xenorhabdus* protease B

In an earlier study, Caldas et al. had detected two proteases in *X. nemtophila* culture that they distinguished as protease I and II [31]. I supposed that protease I and protease A (activity A) are the same because of their similar molar masses (both are ~90 kDa). For the same reason protease II and protease B (molar masses 60 and 55 kDa, respectively) might correspond to each other. The identity of the latter two is supported also by the fact that the production of protease II, like that of protease B, declines on longer incubation. However, the relationship of protease B from *X. kozodii* var. Marocco with protease II from *X. nemtophila* and with PrtA from *Photorhabdus* requires further investigations because protease II but not protease B (or PrtA) was inhibited with serine proteinase inhibitors [31] and because protease B (similarly to protease II) could not be inhibited with EDTA (an inhibitor of metallo enzymes including PrtA). I investigated these questions with a detailed enzymological characterization of protease B and with a comparison of the available amino acid sequences and tertiary structures.

6.2.1 Development of a purification procedure for protease B

I purified protease B from 20-22 hour culture of *X. kosodoii* Marocco strain for which developed a short, two-step procedure. The summary of purification is shown in **Table 6**,

Step	vol. (ml)	total protein (mg)	spec. activity ($M\ s^{-1}mg^{-1}$)	total activity ($M\ s^{-1}$)	yield (%)
culture supernatant	3000	~100	9.2×10^{-9}	9.2×10^{-7}	100
QAE Seph. ion exchange	35	7.8	3.3×10^{-6}	2.6×10^{-5}	283
PEI Silica ion exchange	8.4	1.9	8.3×10^{-6}	1.6×10^{-5}	174

Table 6 Summary of purification of protease B
The activities were measured on hepatitis A virus protease C substrate (Dabcyl-Gly-Leu-Arg-Thr-Gln-Ser-Phe-Ser-Edans). For details see section 5.2.6 in Methods.

while the details of the procedure are described in the section 5.2.6 of Methods. The first step was a preliminary purification on a QAE Sephadex column. I noted that – despite the huge loss in total protein - there was a substantial increase in the total enzyme activity after this step, similarly to what had been observed during the purification of PrtA. I suppose the explanation is the same: during QAE ion-exchange chromatography the preparation got rid of an inhibitor, which is a well known phenomenon in the purification of PrtA. The second purification step on PAE Silica anion exchanger eliminated all the contaminating proteins present in the fractions from the QAE Sepharose column. On testing the purity of the preparation with SDS-PAGE after the second step, one single protein band was visible with coomassie staining

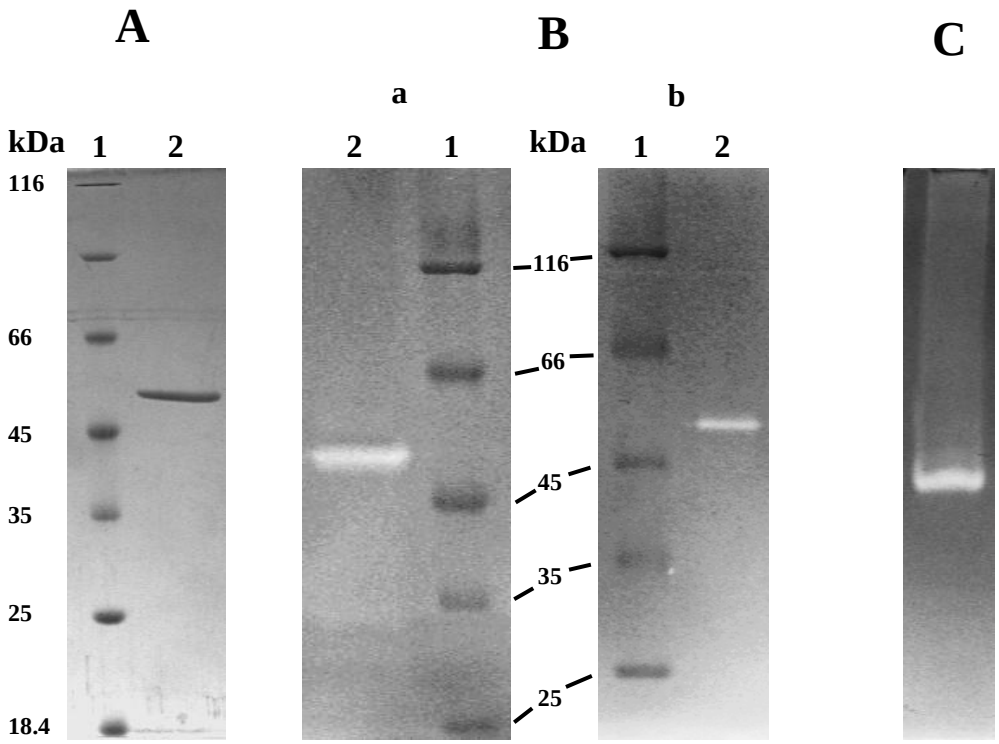


Figure 6. Checking the purity of protease B preparations.
A, SDS-PAGE with coomassie staining; B, zymography following SDS-PAGE using casein (a panel) and gelatin (b panel) as substrate; C, zymography following native page using gelatin as substrate.

that of protease B (**Figure 6A**). When I checked the preparations with the sensitive and non-specific zymographic method following SDS-PAGE or native PAGE I found one activity band, that of protease B (**Figure 6B and C**). Therefore I concluded that our preparations pure enough for enzymatic characterization since they did not contain detectable amount of contaminating protein(s) or protease(s). The single, sharp band of protease B also excluded

both the degradation by (self) cleavage and the presence of alternative form(s). (PrtA peptidase of *Photorahbdus* is produced in several variant forms [77, 79].) The protease B preparations remained stable up to one year on storage at -20°C without signs of degradation or loss of activity. Therefore I supposed that no inactivation occurred during preparation either and so I considered our preparations pure and fully active during enzyme activity calculations.

6.2.2 N-terminal sequence analysis of protease B

The N-terminal sequence determination was performed by Dr. András Patthy in the ELTE-MTA Molecular Biology Group.

The interrogation of MEROPS database of proteases with the N-terminal sequence resulted in two hits with *Xenorhabdus* metalloproteases (**Figure 7**). The match of these is not perfect, however, it was far the best if the comparison included all the 15 amino acids of protease B. Thus the sequence similarity is in accordance also with the metalloprotease character of protease B and with the similarities between protease B and *Photorhabdus* PrtA peptidase in molar mass, enzymatic properties and target proteins – see section 6.3.2, 6.3.3 and 6.4.3). The similarity between the *Xenorhabdus* proteases and *Photorhabdus* PrtA is not seen in the N-terminal sequences, however, it is obvious in other segments (see **Figure 11** below). Both PrtA peptidase and the *X. nematophila* enzymes are metzincins, and belong to the M10 subfamily of Zn-metalloproteases, in the M10B group. (These are the serralysins, the bacterial group of intersticial collagenases [83, 101]).

Protease	**N-terminal sequence**
Xenorhabdus kozodoii Protease B	**LSDSsyVQDVNsLLk**
Xenorhabdus bovienii prtA protein, (CBJ79640)	**LSDSksVQDVNaLLt**
Xenorhabdus nematophila prtA protein, (Q5D1B7)	**LSDShsaQDVNaLLt**
Photorhabdus sp. AZ29 prtA protein (P82115)	**LigSakanelqtqLq**
Photorhabus luminescens prtA protein (Q7N8R3)	**vSgSekanellkwLq**

Figure 7. Comparison of the N-terminal sequence of protease B to several N-terminal sequences in the Serralysin (M10B) subfamily of metalloproteases

Capital letters show amino acids identical to the amino acid in protease B. UniProt identifiers are given in parenthesis.

6.3 Enzymatic characterization of protease B

6.3.1 Searching for suitable substrate and characterization of cleavage site preference

With the aim of finding good substrate(s) for the enzymatic characterization I initially screened twenty one relatively simple oligopeptide substrates which contained chromophores as leaving group on the N-terminal side of the scissile bond. Seventeen of these substrates, which were fluorescent by aminomethyl-coumarine (AMC) or β-methoxy-naphtylamine and SBzl chromophore, permitted sensitive measurement. I also tested a less sensitive, photometric substrate, DL-Val-Leu-Arg-pNA, because it had been reported to be cleaved by the *X. nematophila* protease, protease II [31], which I supposed to be related to (or the same as) my enzyme (see the introduction to section 6.2). Since this substrate contains a basic residue at the P1 position (on the C-terminal side of the scissile bond [104]), and serralysins were found to prefer such residues at this position, I selected a variety of substrates that contained Arg, or Lys at the P1 site.

Being different only in the P1 residue (the Xaa site), the Succ.-Ala-Ala-Pro-Xaa-AMC substrates (#2-8, #10, #12-14 and #16-19 in **Table. 7**) made possible an investigation of the P1 residue discrimination of *Xenorhabdus* protease B. As it is evident from the activity data the positively charged basic residues were the preferred ones because only those substrates were cleaved that contained Lys and Arg at this site and not those that contained aliphatic (Ala, Leu), aromatic (Phe) or negatively charged (Asp) amino acids. The absence of basic amino acids might be a reason also why protease B did not cleave further two oligopeptide substrates, Fua-Leu-Gly-Pro-Ala and Fua-Ala-Leu-Val-Tyr. (These substrates were hydrolyzed by unidentified enzyme(s) in the culture supernatant of *Xenorhabdus* strains – see Table 3.) Given the fact that *X. nematophila* protease II cleaved substrate #15 [31], this P1 residue preference of protease B was the same as that of protease II, but differed from that of *Photorhabdus* PrtA, which preferably cleaves in hydrophobic peptide segments, C-terminal to mostly polar but not charged residues [79]. At the same time, the (P1) Lys and Arg specificity of protease B matched that of other serralysins (serralysin of *Serratia marcescens* [76], alkaline proteinase of *Pseudomonas aeruginosa* [76, 86] and ZapA of *Proteus mirabilis* [14, 17, 51, 74 and 75] which - when were tested on a number of biological and synthetic substrates - were found to cleave mainly next to (P1) Arg or Lys.

The performance of synthetic substrates is influenced by both their sequence and length. The number of substrates in our set was small for a detailed analysis of these effects

but permitted several conclusions. The shortest substrates N-benzoyl-Arg-AMC, N-CBZ-Lys-SBzl, L-Ser-AMC (#8-10), are able to interact with the enzyme mainly through the (P1) Arg or Lys residues and much less by the flanking groups. Since the thioester bond in substrate #9 is much easier to hydrolyze than the amide bonds in the other substrates, the inactivity of protease B on this substrate documents the best that the restriction of enzyme-substrate interactions mainly to those between merely the P1 substrate and the S1 enzyme sites is not sufficient for this enzyme. The number of enzyme substrate interactions is extended in the other, easy to hydrolyze substrate, Succ.-Ala-Ala-Pro-Phe-SBzl (#1 in Table 7), but protease B was inactive on this also, illustrating how much the bulky aromatic is unfavorable in the P1 position. In substrates #12 and 13 the P1 residue is basic and they are longer than substrates #8-11 thus permitting interactions which include positions (P4) P3-P1 also. The inactivity of protease B on these substrate can probably be explained with unfavorable stereochemistry of D-Ala and interactions of the bulky aromatic groups at sites P3 (benzyl) and P4 (benzoyl). This explanation is supported by the fact that the other simple substrates of the same length (substrates #14-18), which do not contain aromatic ring in the P1-P3 positions, were cleaved by protease B.

The specific activities (k_{cat}/K_M values) of protease B on all the hydrolyzed simple substrates (#6 and 7 and #14-19) were similar: the differences remained within one order of magnitude. The small differences between the values do not allow any conclusion regarding the residue discrimination of protease B at the P2 and P3 substrate sites. However, the differences in K_M and k_{cat} values were larger, up to two and four orders of magnitude, respectively. The 10^{-4}-10^{-3} $sec.^{-1}$ values for k_{cat} generally show a positioning of the scissile bond which is not good enough for efficient catalysis. But substrate #16, exhibiting a relatively high k_{cat} value, was substantially better in this regard which can probably be ascribed to the contribution of (P2) Leu and (P3) Val residues (and not P1 Lys). This might be seriously compromised in substrate #15 by the bulky tert-butoxycarbonyl group at the P4 position. The acceptance by protease B of non-polar aliphatic side chains at the P2 and P3 positions is a feature which was observed in most of the substrates of serralysins [13, 14, 51, 74, 75, 76, 79, 86 and 118].

Generally a significant enhancement of proteolytic activity can be reached if the enzyme can bind the substrate also C-terminal to the scissile bond (sites P1'-P4'). Earlier this effect of the P1'-P4' interactions has been observed on another serralysin, *Photorhabdus* PrtA activity, too [79]. Here I tested this on *Xenorhabdus* protease B using two longer fluorescence-quenching-type substrates (#22 and 23 - **Table 7**) that allow such extended

enzyme-substrate interactions. Indeed, the specific activity on substrate #22 increased almost a hundred-fold compared to the best of the simple substrates (substrate #16) through a hundred-fold improvement in K_M, indicative of a stronger substrate binding. The catalytic efficiency was also more than two orders of magnitude higher relative to those on the simple substrates except for substrate #16, compared to which it remained slightly lower. This might be because substrate #22 – unlike the others - did not contain Arg or Lys residue, found to be the needed ones in P1 site. Substrate #23 eliminated this problem, too, but there was only a modest, several-fold increase in the catalytic efficiency and in the specific activity (relative to those on substrate #22). They still were two orders of magnitude less than those of proteases on their best substrates (typically in the range of 10^2 sec^{-1} and 10^6 $sec.^{-1}M^{-1}$, respectively, at 10^{-5} M K_M value), including *Photorhabdus* PrtA on substrate #22. To confirm the significance of the presence of basic residue in substrate #23 I investigated the site of cleavage in this substrate with a mass spectrometric analysis of the reaction products. Unexpectedly, however, the result proved that the single bond hydrolyzed was the Ser-Phe and not the Arg-Thr bond. Selection for Ser in P1 site has been observed so far in two rare cases among many reported cleavage sites of serralysisns: in α1-antichymotrypsin by serralysin [100], and it is in an interesting agreement with the occurrence of Ser residue in this position of almost all of the known serralysin inhibitors of prokaryotic origin. Unfortunately I was unable to do this analysis on the hydrolysis products of substrate #22 because too many fragments formed from cleavages at probably three or more sites. This indicates that protease B did not prefer any of the sites over the others in this substrate.

Though substrate #23 proved suboptimal to protease B I tested its enzyme selectivity using two general digestive enzymes, trypsin and chymotrypsin, as well as the supposedly closest relative, PrtA. The high k_{cat}/K_M values, 1.6×10^7 and 2.8×10^6 $sec^{-1}M^{-1}$, that I obtained for pancreatic trypsin and chymotrypsin, respectively, showed that substrate #23 is not selective. Indeed, unlike substrate #22, which proved very selective for PrtA activity in biological samples [79], substrate #23 was not good enough for such measurements of protease B activity (in insect hemolymph) due to high background activity (see section 6.4.1). Since substrate #22 was found in a substrate sequence optimization and proved very sensitive and specific for *Photorhabdus* PrtA [79], a comparison of the kinetic parameters of PrtA and protease B on each other's best substrate (substrates #22 and #23, respectively) can be informative also about how these two, supposedly very closely related serralysin-type enzymes discriminate between cleavage sites. When I compared them on substrate #22 the difference in specific activity was two orders of magnitude. This aroused almost entirely from

Table 7. The kinetic parameters of *Xenorhabdus* protease B on 23 synthetic substrates

	Substrate P4 P3 P2 P1 P1′	k_{cat} (sec^{-1})	K_M (M)	k_{cat}/K_M (sec^{-1}/M)
1	Succ.Ala-Ala-Pro-Phe-SBzl		no activity	
2	Succ.Ala-Ala-Pro-Phe-AMC		no activity	
3	Succ.Ala-Ala-Pro-Ala-AMC		no activity	
4	Succ.Ala-Ala-Pro-Leu-AMC		no activity	
5	Succ.Ala-Ala-Pro-Asp-AMC		no activity	
6	Succ.Ala-Ala-Pro-Lys-AMC	$7.7\pm1.70\times10^{-3}$	$1.1\pm0.4\times10^{-4}$	7.7×10^{1}
7	Succ.Ala-Ala-Pro-Arg-AMC	$1.4\pm0.46\times10^{-3}$	$1.7\pm0.6\times10^{-5}$	8.2×10^{1}
8	N-benzoyl-Arg-AMC		no activity	
9	N-CBZ-Lys-SBzl		no activity	
10	L-Ser-AMC		no activity	
11	His-Ser-naphtylamide		no activity	
12	D-Ala-Leu-Lys-AMC		no activity	
13	N-benzoyl-Phe-Val-Arg-AMC		no activity	
14	N-t-BOC-Gln-Ala-Arg-AMC	$2.4\pm0.91\times10^{-4}$	$4.0\pm1.5\times10^{-4}$	5.9×10^{1}
15	N-t-BOC-Val-Leu-Lys-AMC	$6.7\pm0.22\times10^{-3}$	$1.5\pm0.3\times10^{-4}$	4.5×10^{1}
16	DL-Val-Leu-Arg-pNA	$1.1\pm0.19\times10^{0}$	$3.8\pm0.4\times10^{-3}$	2.9×10^{2}
17	Z-Gly-Gly-Arg-AMC	$1.8\pm0.53\times10^{-3}$	$8.9\pm1.6\times10^{-5}$	2.0×10^{1}
18	N-t-BOC-Val-Pro-Arg-AMC	$7.1\pm0.98\times10^{-3}$	$6.5\pm1.6\times10^{-5}$	1.1×10^{2}
19	N-t-BOC-Leu-Ser-Thr-Arg-AMC	$5.7\pm0.37\times10^{-3}$	$6.2\pm0.7\times10^{-5}$	9.2×10^{1}
20	Fua-Leu-Gly-Pro-Ala		no activity	
21	Fua-Ala-Leu-Val-Tyr		no activity	
22	DABCYL-Glu-Val-Tyr-Ala-Val-Glu-Ser-EDANS	$3.3\pm0.2\times10^{-1}$	$3.3\pm1.0\times10^{-5}$	1.1×10^{4}
	↓	$*2.1\pm0.3\times10^{2}$	$*9.0\pm0.2\times10^{-5}$	$*2.3\times10^{6}$
23	DABCYL-Gly-Leu-Arg-Thr-Gln-Ser-Phe-Ser-EDANS	$1.1\pm0.2\times10^{0}$	$2.1\pm0.4\times10^{-5}$	5.1×10^{4}

Table 7. The kinetic parameters of *Xenorhabdus* protease B on 23 synthetic substrates
The (±standard deviation) values are averages of three measurements on two different enzyme preparations. The arrow indicates the site of cleavage. * *Photorhabdus* PrtA activity from ref. [79]

the difference in their catalytic efficiency (k_{cat}), that is, in the scissile bond positioning as the K_M, values were the same. At the same time, the difference between the specific activities was extremely high if I compared them on substrate #23, because *Photorhabdus* PrtA did not cleave this substrate at all. Since the sequence of substrates #22 and 23 are very different, they provide substantially different enzyme-substrate interaction possibilities. PrtA proved rather selective for bonds in P4-P4' peptide segments that contain hydrophobic amino acids, and was unable to cleave any of the Succ.-Ala-Ala-Pro-Xaa-AMC substrates (J. Marokházi personal communication). Relative to these features, the sequence of substrates in **Table 7** that were hydrolyzed by protease B suggest that this enzyme can cleave in more polar environment (also). I suppose this is why protease B could cleave substrate #22, and PrtA was not able to cleave the more polar substrate #23.

6.3.2 The pH profile of the activity of *Xenorhabdus* protease B:

For a further characterization of *Xenorhabdus* protease B I investigated the pH profile of its activity. As seen in **Figure 8** the pH curve showed high activity between pH 7.0 and 8.0

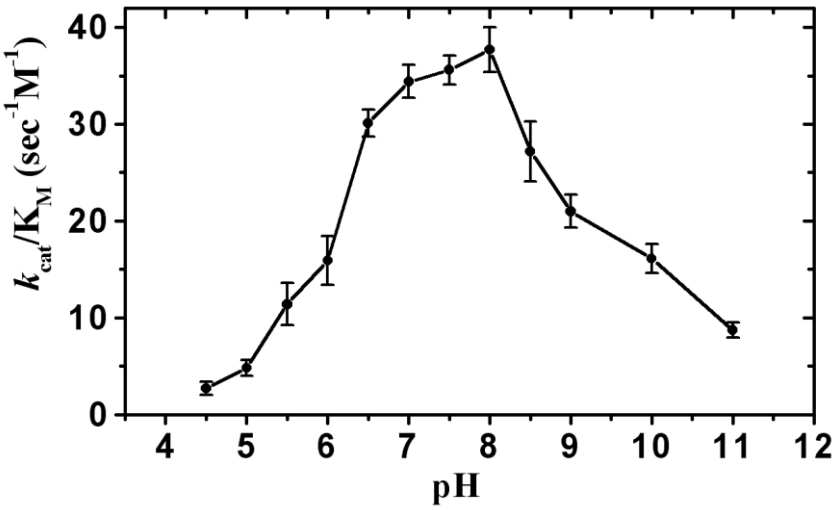


Figure 8 The pH profile of the activity of *Xenorhabdus* protease B.
The activity was measured on Boc-Val-Leu-Lys-AMC substrate at 40 μM substrate and 20 nM enzyme concentration. For the buffers used and other the conditions of measurement are given in Methods. Data points were fitted with equation (1) (see Methods).

which range is narrower than that of *Photorhabdus* PrtA [79] The data points could be fitted the best with equation (1) (see Methods), which indicated four ionization steps. The acidic

values, pK_{a1} and pK_{a3}, were ~5.5 and ~6.5, respectively. The former supposedly belongs to a glutamic acid and an associated water molecule in the catalytic site, as it has been shown in the case of serralysins and some other Zn-metallopeptidases [71, 85, 90, 106] while the latter might show the influence of a histidine residue, three of which are around catalytic zinc. The basic pK_a values, pK_{a2}~8.5 and pK_a4~11.1, are more difficult to interpret. We suppose that the latter belongs to the Lys residue in the substrate, while the former might be related to the ionization state of Tyr216, as it has been proposed for serralysin [90].

6.3.3 The effect of treatment of protease B with inhibitors and metal ions

I measured the effect of inhibitors and metal ions on the enzyme activity, incubating 20 nM enzyme with inhibitor or metal ion at room temperature for 20 minutes in the assay buffer (in the case of EDTA, Ca^{2+} ion was omitted from the assay buffer) before measuring the remaining activity. As a control, protease B was incubated under the same conditions without inhibitor. As **Table 8** shows the activity was slightly increased by Cu^{2+} and Co^{2+} ions. At the same time it was not sensitive for serine protease inhibitors PMSF, SBTI), but was partially inhibited by Zn^{2+} (which has not been reported in the case of serralysins and was not observed on *Photorhabdus* PrtA either). The activity was lost on treatment with thiol reagent DTT, and it was completely lost on 1,10-phenantroline addition, features shared by many metalloproteases. It was therefore unexpected that EDTA had only a slight effect on the activity even upon long incubation with excess amount of this chelator.

addition	**final concentration** (mM)	**remaining activity** (%)
EDTA	1.0	83 ± 10
1,10-phenanthroline	1.0	13 ± 11
PMSF	1.0	90 ± 17
SBTI	10.0	92 ± 12
Thimerosal	1.0	91 ± 9
DTT	1.0	48 ± 5
Cysteine	1.0	87 ± 10
Zn^{2+}	2.0	16 ± 4
Cu^{2+}	2.0	144 ± 17
Co^{2+}	2.0	224 ± 36

Table 8 The effect of inhibitors and metal ions on the purified protease B.
The activities were measured on Boc-Val-Leu-Lys-AMC substrate at 40 μM substrate and 20 nM enzyme concentration. Activity is expressed as % of activity the untreated enzyme.

6.3.4 Rescue of enzyme activity with the addition of metal ions to 1,10-phenantroline and EDTA inhibited protease B

In order to see which metal ions can have catalytic function in protease B I added several of them to the 1,10-phenantroline inhibited protease B. I chose mostly those ions that are known to have or augment catalytic activity in proteolytic enzymes. As seen in **Table 9**, the metal ions, which are usually able to restore the activity of metalloproteases after the removal of their catalytic ions, could also rescue the activity of protease B except for Zn^{2+} (which, however, was also efficient in the case of *Photorhabdus* PrtA). Moreover, the addition of Zn^{2+} increased the partial inhibition by EDTA. I suppose this inhibition was the manifestation of the inhibitory effect of Zn again, as above (**Table 8**). The addition of Cu^{2+} and Co^{2+} caused "supernormal" activities (not observed in *Photorhabdus* PrtA) which roughly

metal ion added (2.0 mM)		**activity** (% of control)[1]	
		1,10-phenathroline[2]	EDTA[2]
-		9 ± 8	62 ± 12
Zn^{2+}	0.1	-	45 ± 11
Zn^{2+}	2.0	10 ± 10	18 ± 10
Cu^{2+}	2.0	156 ± 21	76 ± 6
Co^{2+}	2.0	187 ± 25	206 ± 23
Mn^{2+}	2.0	84 ± 19	94 ± 18
Ca^{2+}	2.0	17 ± 6	-
Fe^{2+}	2.0	15 ± 4	-

Table 9 Rescue of protease B activity with metal ions after inhibition with chelators.

[1] Control was incubated for the same time as inhibited enzyme (20 nM enzyme in both) but in the absence of 1,10-phenantroline, EDTA or metal ion, giving specific constant $4.25 \pm 0.38 \times 10^1$ $s^{-1}M^{-1}$ (at 40 μM Boc-Val-Leu-Lys-AMC substrate). The values (± standard deviation) are the average of at least three measurements on at least three enzyme preparations.

[2] The time of enzyme treatment was 20 and 120 minutes with 1.0 mM 1,10-phenantroline and EDTA, respectively. (See methods for further details.)

corresponded to those that we got when we added Cu^{2+} and Co^{2+} ions without prior removal of the catalytic ion (**Table 8**). Cu^{2+} and Co^{2+} are known not only to restore the activity of a number of metalloproteases (partially or completely [14]) but also to enhance it. For example, Co^{2+} increases the activity of alkaline proteinases of *P. aeruginosa* [76, 86], a serralysin. Similarly, using a tripeptide mimic synthetic substrate, a 20-30-fold increase in specificity constant was found in serralysin upon replacement of Zn^{2+} with either Cu^{2+} or Co^{2+} [90].

6.4 Investigation of the potential physiological role of protease B

On the basis of previous observations in our laboratory on *Photorhabdus* PrtA peptidase [50, 77 and 79], I hypothesized that some of the secreted proteases of *Xenorhabdus* can also

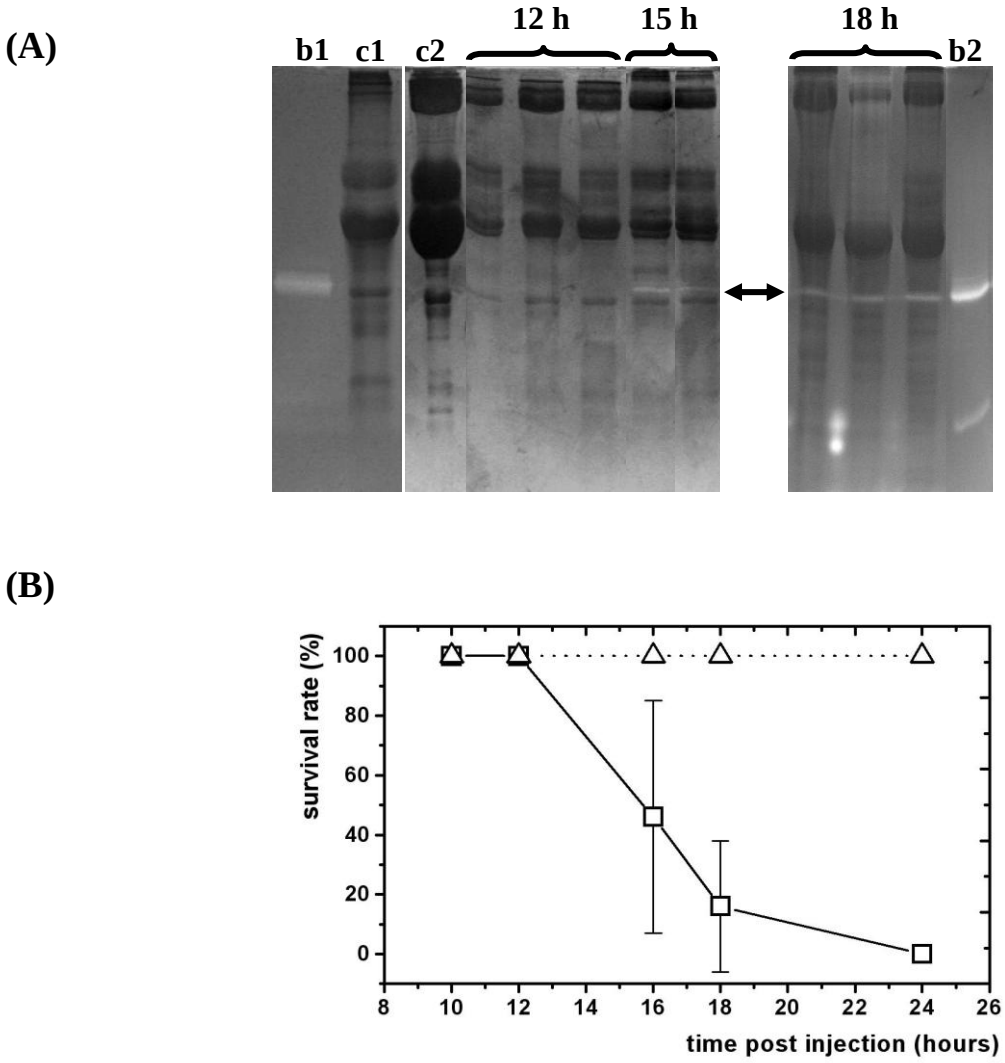


Figure 9 Detection of protease B in insects and insect mortality on *Xenorhabdus* infection

(**A**) SDS-PAGE coupled zymographic detection with casein substrate of protease B production during *G. mellonella* infection (arrow indicates the detected activity). The numbers above the lanes indicate the time of sample takings in hours post infection. Purified protease B was also run in the gels as standard (samples b1 and b2, ~10.0 and ~20.0 pmoles of enzyme loaded, respectively). c1, control hemolymph from PBS injected insects, c2, control hemolymph, from naïve insect. (**B**) Survival rate of *G. mellonella* larvae after injection of ~100 cells of *X. kozodoii* Morocco strain. Data from four experiments are averaged. For the conditions of infection and sample preparation see materials and methods.

take part in the establishment of infection. The best candidate enzymes for such a role are those that are secreted early and by every strain. Of the proteases I have found in *Xenorhabdus*, protease B, the Fua-ALVY-ase enzyme and the Succ-AAPF-SBzl cleaving enzyme met these criteria. Therefore I tried to investigate the dynamics of appearance of these activities in the hemolymph of *Xenorhabdus* infected *G. mellonella* larvae. My attempt was successful only for protease B. I did not observe increase in the activity on the Succ.-AAPF-SBzl and Fua-ALVY substrates, which were rather high in the hemolymph of PBS injected (i.e. control) insects also (data not shown), probably due to the presence of enzymes in the body fluid that can cleave these substrates. So then I focused on only protease B.

6.4.1 Investigation of the production of protease B during infection

I could detect protease B in the hemolymph from the 15th hour of infection when larvae were still alive, but moved only on prodding (**Figure 9A**). According to mortality assay (**Figure 9B**) this is relatively early in the molecular interactions between the pathogen and the host, but relatively late in the pathogenicity phase of infection. (I note that samples from dead insects were not suitable for analysis with gel electrophoresis because of the high content of insoluble material in them that could not be sedimented even with longer and higher speed centrifugation than what was used for pelleting cellular fraction.)

I also tried to measure the activity of protease B in hemolymph samples taken from *Xenorhabdus* infected *G. mellonella* larvae with the most sensitive substrate of protease B (substrate #23). The results in **Table 10** show that this substrate is, unfortunately, not suitable for this purpose because of a high background activity (in the control insects) and a dynamics of activity which is unrelated to that of protease B seen in zymography (**Figure 9A**). This indicates that substrate #23 is probably more sensitive to other enzyme(s), not surprisingly since it was hydrolyzed better by trypsin and chymotrypsin than by protease B (see 6.3.1 above).

Sample	**Activity** (CPS/s)
control (not injected)	1.7×10^3
12 h	4.4×10^2
18 h	4.8×10^2
24 h	1.3×10^3

Table 10 Measuring protease activity with substrate #23 in the hemolymph of *G. mellonella* during infection.
The activities of 7.0 µl 10× diluted samples were measured at 50 µM final substrate concentration. (For further details see Methods.)

6.4.2 Testing the activity of protease B on pure proteins, albumin, fibrinogen, and several collagens

When I exposed several other native proteins (albumin, fibrinogen, collagen types I and IV) to protease B it did not show activity. Earlier, the same observation was made with *Photorhabdus* PrtA [79] along with the selective cleavage of a number of hemolymph proteins [50]. Therefore I continued to find potential substrate proteins to protease B among proteins in the hemolymph of insect.

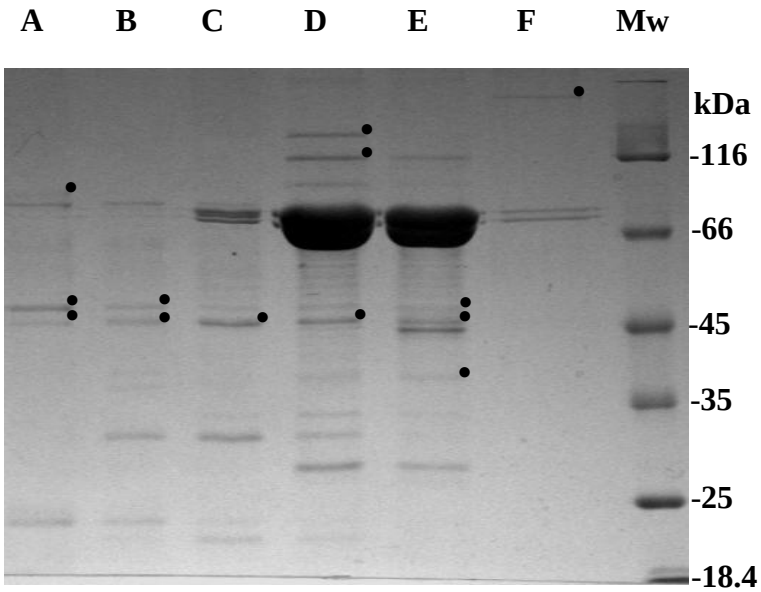
6.4.3 Identification of potential target proteins in insect hemolymph

Prior to digestion, I fractioned the hemolymph proteins with DEAE anion exchange chromatography, according to Felföldi et al.[50] to separate proteins into fractions of distinct protein composition. (Without this the sensitivity of detection is much less because of the very high protein content of the hemolymph.) Six fractions of distinct protein composition can be obtained (fractions A-F, **Figure 10A**). For finding substrate proteins, that might be specific targets of protease B, I exposed these fractions (1-16 μg hemolymph protein depending on their protein content) to digestion by ~1.0 pmole of the following proteases: bovine pancreatic trypsin, chymotrypsin and elastase, *Clostridium histolyticum* collagenase, and purified Protease B. I found nine proteins which were cleaved by protease B **(Figure 10B).** Because these proteins were not digested by the non-specific pancreatic serine proteinases, trypsin and chymotrypsin, or collagenases (data not shown), I concluded that the cleavage of these proteins is selective for protease B, i.e. that the eight proteins are specific protease B targets in the hemolymph. At the same time, all of the eight proteins were cleaved when the hemolymph fractions were exposed to digestion by PrtA peptidase of *Photorhabdus* (data not shown). This made possible an identification of those protease B targets that - in an earlier study - had already been identified as substrate proteins of PrtA peptidase with N-terminal sequencing and database search [50]. According to this the identity of some of the protease B target proteins (the bands in the gels in **Figure 10B**,(**panels a** and **b**) is the following: band #8 serine protease homolog 3 (SPH-3), band #7, Manduca serpin 1, band #5 (or #6) hemocyte aggregation inhibitor protein (HAIP), which all are immune related proteins of *M. sexta*. Such a target protein profile hints more for an enzyme that functions for the enhancement of virulence rather than an enzyme which is involved in the bioconversion of the insect cadaver. The fact that – in culture - the production of protease B declines from about 24 h (a relative late time in infection) also supports this notion.

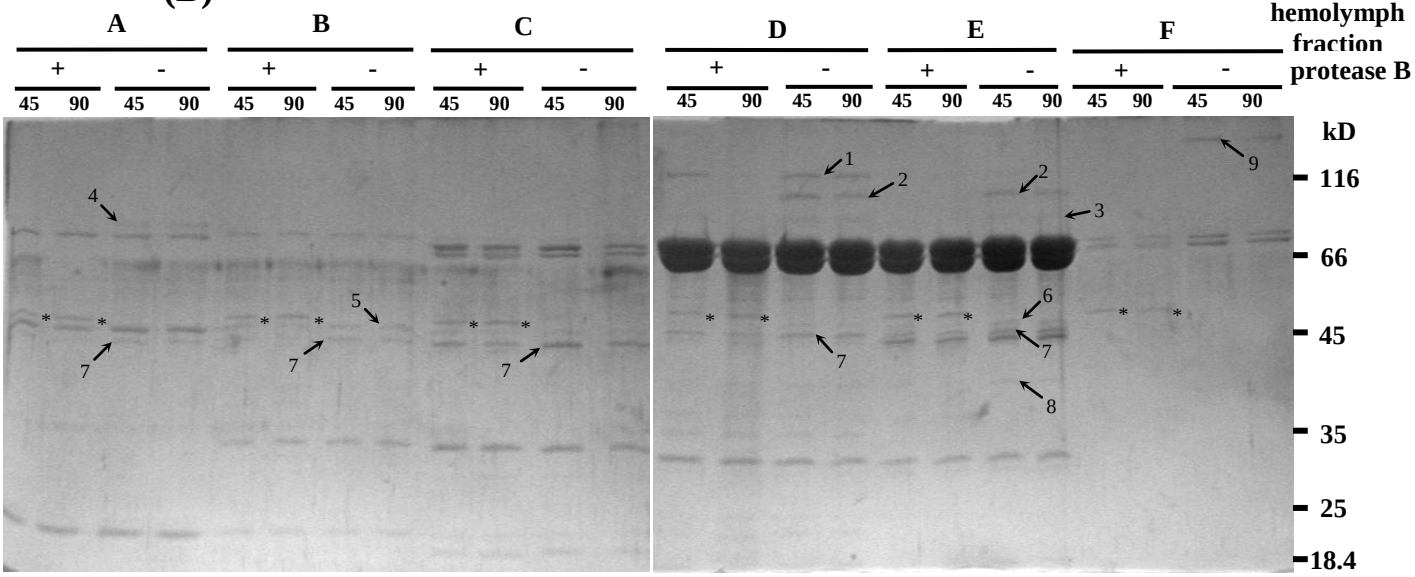
Figure 10 Selective digestion of *M. sexta* hemolymph proteins with purified protease B.

(A)

(**A**) Hemolymph protein fractions A-F of *M. sexta* (lanes A-F). Dots mark protein bands that disappear slectively upon protease B treatment of the hemolymph fraction, as seen in (B). (For the preparation of hemolymph protein fractions see Methods.) (**B**) *In vitro* digestion of hemolymph fractions with purified protease B (panels a and b). The hemolymph fractions A-F and the presence (+) or absence (-) of protease B during incubation are shown above the gels. The incubation times are indicated above the lanes. Arrows show the leaved bands, which are numbered; asterisks indicate bands from protease B. Molar mass scale is indicated by standard (Mw).



(B)



7 DISCUSSION

In my thesis work first I studied the secretion of proteases by an entomopathogenic bacterium *Xenorhabdus*. Then I characterized the enzymatic properties of one of the secreted enzymes, protease B. I made these in order to generate data to a comparison of the role of proteases in the pathomechanism of two closely related bacterium genera, *Xenorhabdus* and *Photorhabdus*. Although these bacteria live in entomopathogenic symbiosis with only distantly related nematodes (*Seinernema* and *Heterorahbditis*, respectively – see chapter 3.1), their very close relationship and identical life strategy supposed close similarity also in the number and type and, therefore, in the role and even many enzymatic properties of proteases they produce during infection. My results show that – surprisingly - the similarity is not so close, it is only partial at both the level of bacterium cells and an enzyme.

7.1 Comparison of the proteolytic virulence factors of the close relatives *Xenorhabdus* and *Photorhabdus*

With seven detection methods I have found six secreted and one intracellular proteolytic activity in the screening of seventeen *Xenorhabdus* strains that included two primary-secondary form variant pairs. Although exhibiting differences in the number and intensity, three of the secreted activities were present in every strain. Similarly to earlier observations on *Photorhabdus* strains [21,77], I did not find characteristic difference between the primary and secondary phenotypic variant pairs of *Xenorhabdus* strains either in their protease production such that the latter ones would be less active. The low number of primary-secondary form variant pairs does not allow drawing further conclusions regarding a form variant specific difference in either the intensity or the number of activity types. A comparison with data in other publications (see refs. [21, 115] is further limited by e.g. the difference in the substrates used, which determines the sensitivity of detection. (For example, casein is less sensitive for digestion than gelatin (a denatured protein), while the amino acid sequence in gelatin provides a smaller variety of cleavage sites.) A further complication might arise from a damage of enzymes caused (in other laboratories) during sample preparation and gel electrophoresis analysis.

The identical methods of detection permit a comparison, which has not been possible before, of the secretion and usage of proteolytic enzymes between *Xenorhabdus* and *Photorhabdus*. As for similarities, the Fua-LGPA–ase activity of *Xenorhabdus* appears to be the same as Oligopeptidase A of *Photorhabdus*. Also, protease B of *Xenorhabdus* appears to

be similar to PrtA peptidase of *Photorhabdus*, despite the dynamics of their production differs. Furthermore, there is a lack in both pathogens of those secreted activities that could be detected with substrates which are sensitive for trypsin- and chymotrypsin-like serine proteinases (enzymes that can cleave the Succ.-AAPX-AMC substrates). On the other hand, there are numerous differences also between the two genera, which are surprising compared to their very close relationship. For instance, unlike *Photorhabdus*, *Xenorhabdus* strains did not to secrete thermolysin-like enzyme, while *Photorhabdus* does not secrete such activities as A and C of several *Xenorhabdus* strains. Similarly, the activities on Fua-ALVY and on Succ.-AAPF-SBzl substrates are common in *Xenorhabdus*, but are missing in *Photorhabdus* making the protease secretion of the former bacterium more abundant in the number of secreted protease types (according to the detection methods we applied). Even if we consider only those four activities that are secreted by almost all of the tested *Xenorhabdus* strains, a larger set of potential proteolytic virulence factors are available for *Xenorhabdus* than for *Photorhabdus*. It is an open question whether this difference influences the mechanism of infection or the toxicity (e.g. the speed of insect killing between the two bacterium genera) needs further investigation. A further investigation of the proteolytic virulence factors of *Xenorhabdus* and *Photorhabdus* through genome analysis has recently become an opportunity with the publication of the genome of *Xenorhabdus nematophila* [61].

If we compare the dynamics of production of the detected activities it is rather different either if we compared them in culture (occurring from the early logarithmic to stationary phase) or if we compared the occurrence in culture and during infection. Unfortunately, for technical reasons I could make the latter comparison only for protease B because of the three earliest secreted activities, Fua-ALVY-ase, Succ.-AAPF-SBzl-ase and protease B (which might be the most interesting for potential role in the early, pathogenicity phase of infection), I could investigate the production of only this enzyme during infection. My observations with zymography of hemolymph samples suggest that this activity is produced later than 12 but earlier than 15 hours post infection, which is significantly later than the occurrence in culture (4-7 hours, early-mid logarithmic phase). According to mortality curves it appears that when I could detect protease B activity in the hemolymph with certainty it might be about three hours before insect death. If we compare this occurrence of protease B to that of *Photorhabdus* PrtA peptidase, the former is relatively later taking into account also the dynamics of infection: on injection of the same amount of bacterium cells *G. mellonella* larvae died 28-34 hours after *Photorhabdus* infection with the first detection of PrtA peptidase at the 18^{th}-21^{st} [77]. At the same time it cannot be ruled out that, similarly to

PrtA peptidase [79], some protease B activity, which might be detectable only with a sensitive and specific substrate, is present in the tissues or in the hemolymph even at 7-9 hours after infection.

7.2 *Xenorhabdus* protease B is an orthologue of *Photorhabdus* PrtA peptidases (*Xenorhabdus* PrtA peptidase) with differences in the enzymatic properties

From the data I could collect about *Xenorhabdus* protease B I conclude that this enzyme and *Photorhabdus* PrtA peptidase are not only of the same type (that belongs to the M10B family, the serralysins) but they are the orthologue of each other. The evidences supporting my conclusion are the following:

1) *Molecular properties*: The size of both enzyme molecules is the same, 55 kDa (the typical size of serralysins). Although the sequence comparison we made is indirect, it reliably shows that the two enzymes have an essentially identical amino acid sequence in the catalytic domain (**Figure 11**) and elsewhere. I have sequence data only for the N-terminal 15 amino acids which are very similar to the N-terminal sequence of two *Xenorhabdus* PrtA peptidases (**Figure 7**) despite the fact that the N-terminal sequence of serralysins are usually quite dissimilar. (This is illustrated by the comparison of *Xenorhabdus* PrtA peptidases to *Photorhabdus* PrtA peptidases – **Figure 7**). However, if we compare the sequence of PrtA peptidases elsewhere, and especially in the catalytic domain, the similarity is higher than 90% (**Figure 11**).

2) *Enzymatic properties*: Both *Xenorhabdus* protease B and *Photorhabdus* PrtA peptidase (like other serralysins) are metalloenzymes using Zn as catalytic ion. The evidence supporting the catalytic role of Zn in *Xenorhabdus* protease B is (again) indirect, but strong: it is the inhibitory effect of Zn^{2+} on the catalytic activity. This, although has not so far been seen in the case of serralysins, was observed only for those enzymes that use Zn for catalysis (e.g. thermolysin, carboxypeptidase and collagenases). The inhibition is caused by the binding of Zn-hydroxide to the catalytic Zn [71]. Thus despite Zn is unable to rescue the activity of *Xenorhabdus* protease B (unlike *Photorhabdus* PrtA [79]), the Zn inhibition of activity identifies the catalytic ion as Zn. The pH profile of activity and - apart from the effect of EDTA (and Zn) - the inhibitor sensitivity of the two enzymes is also very similar (the thiol reagents (DTT, Cys and thimerosal) have milder effect on the *Xenorhabdus* protease).

3) *Target protein specificity/function*: As far as the analysis with SDS-PAGE I made allows, the target proteins of *Xenorhabdus* protease B and *Photorhabdus* PrtA peptidase are largely the same: all of the eight hemolymph proteins cleaved by the *Xenorhabdus* enzyme are among those sixteen that have been found to be cleaved by the *Photorhabdus* enzyme [50] many of them are known to have immune (related) function. Further fractionation (and concentration) of hemolymph proteins would be needed to answer the question whether the other eight substrate proteins of *Photorhabdus* PrtA are also hydrolyzed by *Xenorhabdus* protease B. Thus we cannot say yet that the target protein profile of the two enzymes is (completely) identical, but we can already say that a role of both enzymes (as virulence factors) is immune suppression.

Taken together these similarities it is reasonable to conclude that *Xenorhabdus* protease B and *Photorhabdus* PrtA peptidase are orthologue. Moreover, considering the very close relationship between the two bacterium genera, *Xenorhabdus* and *Photorhabdus*, these PrtA peptidases are very close orthologue. Therefore it is surprising that their enzymological properties are substantially different in several respects: cleavage site preference, effect of Zn^{2+}, Cu^{2+} and Co^{2+} ions and EDTA.

7.3 How can be *Xenorhabdus* and *Photorhabdus* PrtA peptidases different?

1) *Cleavage site preference*: My data indicate a difference in residue selectivity around the scissile bond between *Xenorhabdus* and *Photorhabdus* PrtA peptidases such that the former enzyme prefers a more polar environment than the latter one. In this respect the *Xenorhabdus* enzyme is closer to other serralysins than the *Photorhabdus* enzyme. However, the data I obtained are unfortunately not sufficient to draw further conclusion in this issue. Presently not even the question of why the bond C-terminal to Ser and not to Arg is cleaved in substrate #23 when in the case of other substrates, that do not contain amino acids in the P1'-P3' positions, clearly basic residues are preferred in the P1' position. In the background of this problem the fact might be that no specific P-site – S-site interaction(s) dominate(s) in the alignment of the substrate to the active site of serralysisns. Therefore even subtle sequence differences can result in a large shift in the alignment and, concomitantly, in the site of cleavage. I suppose, that this type of enzyme-substrate interaction might be the explanation to the difference in S1 site preference of protease B between the short and long substrates (substrates #1-7 and #18, 19, respectively) as it is the assumed explanation of the known relaxed cleavage site specificity of serralysins also. Thus although a cleavage of substrate #19

by of *Xenorhabdus* PrtA after Arg would mean the same alignment of P1 and P2 residues as in the case of substrate #12 it might be (energetically) unfavorable if the bulky DABCYL chromophore following Gly) would disrupt the binding at P3 and P4 sites. At the same time, the alignment with the observed cleavage site (P1 Ser) allows the binding of an aromatic P1' residue (Phe), which might energetically better than the binding of the smaller and more polar Thr.

From these considerations it is clear that further systematic analysis of cleavage site(s) using a range of (appropriately designed) synthetic substrates and known peptides of biological origin (e.g. insulin chains or neuropeptides) is needed to understand better my observations on the cleavage site preference of *Xenorhabdus* PrtA.. Even more interesting and informative could be such an analysis with both enzymes on natural substrate proteins for two reasons: i) these substrates are in native conformation, which makes them much harder to cleave; ii) we would compare two enzymes that apparently have identical targets yet different cleavage site preference

2) *The effect of Co^{2+} and Cu^{2+} ions*: The difference between *Xenorhabdus* and *Photorhabdus* PrtA peptidases is that while these ions do not influence the activity of the latter enzyme, and can only (partially) rescue its chelator inhibited activity, they activate the former enzyme, resulting in supernormal activity even after inhibition with chelator. The mechanism of how these ions enhance the activity of some Zn metalloproteases in the serralysin subfamily has not been studied in detail, thus it is a question how and why these ions can increase the specificity constant (mainly through increasing k_{cat}). It is known that Cu^{2+} and Co^{2+} can activate water molecule, similarly to Zn^{2+}, but – to explain the differential effect of these ions - we can only suppose that a specific structure of the active site is needed also, which is provided only by some of the metallo-proteases (present in *Xenorhabdus* but not in *Photorhabdus* PrtA peptidase). Further discussion of this question requires a structure comparison of the active site in two enzymes, which is not possible at present because the 3D structures are no available.

3) *The effect of Zn^{2+} and EDTA*: To explain why Zn^{2+} is inhibitory to *Xenorhabdus* PrtA peptidase but not to *Photorhabdus* PrtA peptidase, and why the former enzyme is less sensitive to EDTA treatment than the latter one, Istvan Venekei made a sequence and structure comparison in the active site of three serralysins and human carboxypeptidase A. The result of this is briefly the following:

The mechanism of Zn^{2+} inhibition was studied in detail in carboxypeptidase A [71] and it was found that it is due to the binding of Zn-hydroxide to the catalytic Zn. This binding needs a stabilization to be inhibitory on the activity. The stabilization is provided by the carboxylate group of a Glu residue such that the bound Zn-hydroxide bridges this group and the catalytic Zn (Zn-hydroxide bridge [72]). In most of the serralysisns (including *Photorhabdus* PrtA) the acylamide group of Asn191 is in analogous position, 5.2 Å from the catalytic zinc, which is not suitable for stabilization. However, out of 145 serralysin sequences in the MEROPS database (which are reliably aligned in the active site region) twelve enzymes contain Asp in this position, and the *Xenorhabdus* PrtA peptidase is among them. Since this is the only difference between *Xenorhabdus* and *Photorhabdus* PrtA peptidases on the first and second shell of interactions around the catalytic Zn (**Figure 11**) it is reasonable to suppose that this is why the former but not the latter enzyme is inhibited by Zn^{2+}.

A possible structural interpretation of the differential sensitivity of *Xenorhabdus* and *Photorhabdus* PrtA peptidases to EDTA treatment is a bit more circumstantial. It cannot be based on other structural difference than the inhibitory effect of Zn^{2+}, i.e. on the Asn191Asp substitution as it is the only difference on the first two shells of interaction around the catalytic zinc Zn^{2+} between *Xenorhabdus* and *Photorhabdus* PrtA peptidases. We suppose that the negative charge in the side chain of Asp191 of *Xenorhabdus* PrtA peptidase is unfavorable for the also negatively charged EDTA in its binding to the catalytic Zn^{2+}. In support of this assumption is the reduced EDTA sensitivity of another, non PrtA peptidase serralysin, SMP6.1, and insecticidal protease from a *Serratia* species, which contains also Asp in position 191 [121].

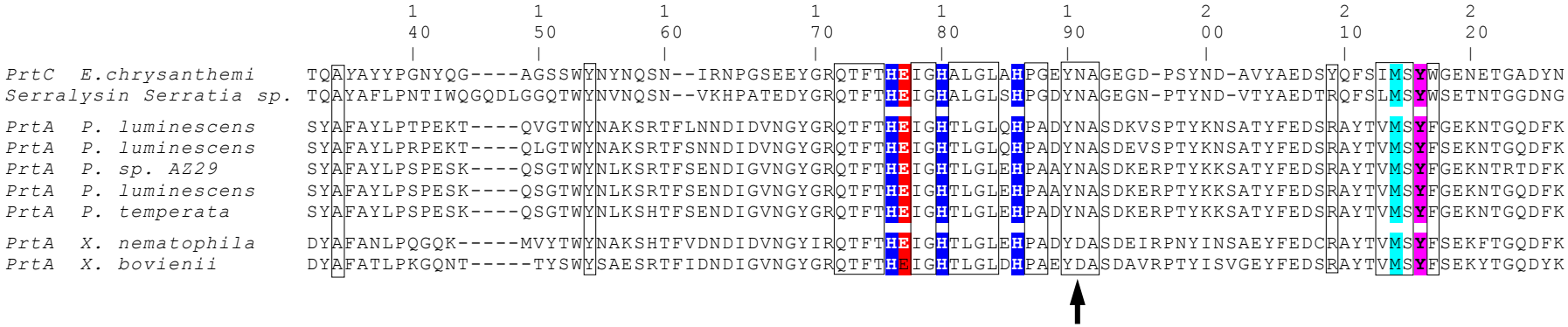


Figure 11 Aligned sequences in the active site region of PrtA peptidases and two other serralysisns

Amino acids on the first interaction shell around Zn-ion are highlighted with blue, red and purple color (His176, His180, His186 and Glu187 are coordinating catalytic Zn, while Tyr216 participates in catalysis), boxed amino acids are on the second shell of interaction in the structure of at least two different serralysin enzymes (The amino acids on the second shell are in interaction with the amino acids on the first shell. Conserved Met214, which is characteristic to metzincins, is highlighted with green color). The amino acid numbering in Serralysin is used. Arrow points to the site on the second shell where the only difference between Xenorhabdus and Photorhabdus PrtA peptidases is found. To identify the interaction shells we compared the 3D structure of serralysin from *Serratia marcescens*, alkaline proteinase from *Pseudomonas aeruginosa* and PrtC from *Erwinia chrysanthemi*, PDB codes are 1SMP, 1KAP and 1K7G, respectively [15, 16,, 67]).

8 Summary

In my thesis work I collected data to a comparison of the proteolytic virulence mechanisms of two closely related bacterium genera, *Xenorhabdus* and *Photorhabdus*, that follow identical life strategy. Using seven detection methods I have distinguished six secreted proteolytic activities (A, B, C, 1, Fua-ALVY-ase as well as Succ.-Ala-Ala-Pro-Phe-SBzl-ase activities) in the culture supernatant of *Xenorhabdus* strains. Although activities A, C and 1 were missing from some of the strains, this number of proteolytic activities is higher compared to *Photorhabdus* (which secretes three activities). Of the six *Xenorhabdus* proteases I identified one of the most common ones as *Xenorhabdus* protease B, which proved the orthologue of *Photorhabdus* PrtA peptidase according to its molecular end enzymological properties. One other activity, the Fua-ALVY-ase one, also has a counterpart in *Phtorhabdus*. At the same time activity corresponding to *Photorhabdus* thermolysin-like enzyme is missing from *Xenorhabdus* strains. These observations indicate that the infection mechanism of *Photorhabdus* and *Xenorhabdus* might be more different at molecular level than what one would expect on the basis of their close relationship and identical life strategy. The conclusion is similar that can be drawn from the comparison of the PrtA peptidases of *Xenorhabdus* and *Photorhabdus*: besides the close similarity in basic molecular and enzymological properties (molecular size, almost identical amino acid sequence, Zn^{2+}-based catalytic mechanism, (in)sensitivity to a range of inhibitors and metal ions) there are differences also, which are surprising with regard to the close relationship of the two enzymes. For example, unlike *Photorhabdus* PrtA peptidase, the *Xenorhabdus* orthologue is activated by Co^{2+} and Cu^{2+}, inhibited by Zn^{2+} and is less sensitive for EDTA inhibition. We suppose that the latter two "specialty" of *Xenorhabdus* enzyme can be ascribed to one single sequence difference between the two enzyme on the first and second structural shell around the catalytic ion, where the side chain of Asp191 (Asn in the *Photorhabdus* enzyme) can provide the stabilization of the inhibitory Zn-hydroxide, and also might interfere with the inhibitory effect of the negatively charged EDTA. An additional difference between *Xenorhabdus* and *Photorhabdus* PrtA peptidase is in their cleavage site preference. Although screening the activity on further substrates is needed for the appropriate comparison, my data suggest that the *Xenorhabdus* enzyme hydrolyze peptide bonds in a more polar environment than where the *Photorhabdus* enzyme does. Considering the fact that the cleavage of native proteins is difficult, a difference in cleavage site preference is interesting because the *Xenorhabdus* enzyme digests the same target protein as the *Photorhabdus* enzyme. The identity of target proteins, at the same time, indicates that a common role of *Xenorhabdus* and *Photorhabdus* PrtA peptidases might be immune suppression.

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