

**EÖTVÖS LORÁND UNIVERSITY
FACULTY OF SCIENCE
DEPARTMENT OF GENETICS**

**Identification and characterization of antibiotics producing
entomopathogenic bacteria**

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THESES OF PH.D DISSERTATION

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PROGRAM: CLASSIC AND MOLECULAR GENETICS

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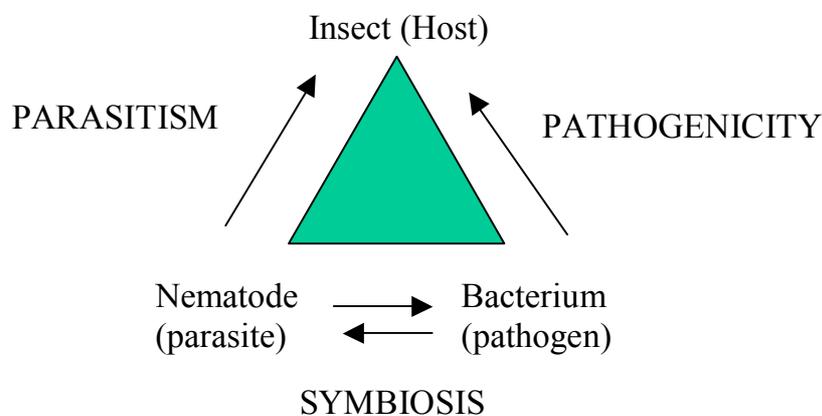
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INTRODUCTION

The conventional chemical pest control has recently been heavily criticised for adverse environmental effects. As a consequence of this, there are new trends in the agriculture, aiming to reach an optimal way of integrated plant protection. An important aspect of the integrated plant protection is to replace the harmful chemicals with the different techniques of biological control. The biological pest control of the insect pests is based on using natural enemies, such as viruses, bacteria, fungi and nematodes. The analysis of the entomopathogenic nematode bacterium symbiotic complexes, as one of the most powerful tool of biological insect pest control has been the main line of research in our laboratory in the past 15 years. The tripartite system of the insect – entomopathogenic nematode – entomopathogenic bacterium includes pathogenicity, parasitism and symbiosis, the bacterium should be considered as the pathogen; the nematode as a parasite and the insect as a host. (Fig 1).

Fig 1. Tripartite mutualism of the insect and the entomopathogenic nematode/bacterium symbiotic complex



Apart from the insect host, the tripartite mutualistic system includes entomopathogenic bacteria belonging to the *Xenorhabdus* and *Photorhabdus* genera, obligate symbionts of entomopathogenic nematodes, belonging to the *Steinernema* and *Heterorhabditis* genera, respectively. The infective dauer juveniles (IJ) of the entomopathogenic nematodes carry bacterial symbionts colonizing a special segment of their gut. The non-feeding, non-ageing IJ is the only developmental variant of the nematode which can be found outside the insect. It is capable of searching for and find the insect host and enter it through natural openings. After

entering the hemocoel, it breaks through the first defension line of the insect immune sytem; melts and release the bacteria. During a couple of hours, meanwhile the nematode switch back to a feeding form (fourth larval stage, J4) the bacteria cause septicemia by its toxins and propagate. Then the role of the biological role of the bacterial symbiont changes. The bacteria serve as a food and also as a protector the dixenic system (insect, nematode and bacterium) by producing antibiotics of a large spectrum. The bacteria made the insect tissues consumable for the insects (by using several exo-enzymes, such as proteases, lipases, phospholipases). After a few generations when the cadaver becomes overpopulated, the actual first stage nematode larvae (J1) adopt an alternative developmental pathway and grow to non-feeding, non-ageing IJs, which leave the cadaver and search for new hosts.

In our Laboratory Ph.D. student Emília Szállás made an internationally recognized contribution to phylogenetic analysis of genus *Photorhabdus* (Szállás et al. 1997) with co-guidance of Prof. Erko Stackebrandt (Braunschweig, Germany). Two other Ph. D. Theses (Horolma Pamjav and Dimitra Triga) were dedicated to elaborate a unique method for identication of a *Heterorhabditis* and *Steinernema* species, based upon the PhastSystem PAGE PCR-RFLP analysis of the ITS region of the *rRNA* operon (Pamjav et al., 2000, Triga et al., 1999) with the professional significant help and co-guidance of Dr. Zsuzsanna Buzás (Agrobiotechnology Research Center, Gödöllő, Hungary). Several students, including Orsolya Oravec, Erzsébet Böszörményi, Ghazala Furgani and myself had been dealt with the problems of symbiosis between different nematode and bacterial taxa (gnotobiology) in our Laboratory.

The different strains of the *Xenorhabdus* and *Photorhabdus* genera produce antibiotics of different chemical structure. These antibiotics are usually effective against both Gram negative and Gram pozitiv bacteria, as well as against several fungi. We have promising data concerning control of the fireblight disease in apple trees, caused by *Erwinia amylovora*, in co-operation between the University of Debrecen (Profs. Attila Szentirmai anf Ferenc Sztaricskai); the Szent István University (Dr. Mária Hevesi) and field experts.

I have been interested in the genetic regulation of the biosynthesis of antibiotics in entomopathogenic bacteria. I have isolated mutants defective in antibiotics production and got a great honour that Prof. Heidi Goodrich-Blair was so kind to identify the mutation sites in three of my transposon induced mutants at her Laboratory (University of Wisconsin – Madison, USA).

I have also been interested in the mechanisms of the so-called 1^o - 2^o transition. There are two variants – called primary (phase 1 or 1^o) and secondary (phase 2, or 2^o) forms - of each

entomopathogenic bacterium strain. Both produce toxins, but only the phase 1 is capable of producing antibiotics and existing as the only inhabitants in the gut of IJs. The 1^o - 2^o transition – with only one known exception is a one-way street, and seems to happen occasionally. In our Laboratory Antónia Völgyi made an internationally accepted contribution toward the genetic analysis of the primary – secondary transition *Xenorhabdus nematophila* (Völgyi et al., 1998, 2000), under the co-guidance of Prof. Steven Forst (University of Madison, Milwaukee, USA).

OBJECTIVES

1. Taxonomic identification of some entomopathogenic bacteria isolated in our laboratory by using molecular tools such as 16S rDNA sequencing and ribotyping and phenotypic characterization.
2. Screening our bacterial strain collection for antibiotics production.
3. A mutant hunt and genetic analysis of the antibiotics production in *Photorhabdus temperata subsp. temperata* K122 strain.
4. A phenotypic analysis of primary secondary phase transition of *Photorhabdus* species.

MATERIALS AND METHODS

I was working on entomopathogenic *Photorhabdus* and *Xenorhabdus* (EPB) strains, majority of which had been isolated by us in our laboratory directly from the gut of the infective dauer juvenile (IJ) individuals of entomopathogenic nematodes (EPN), belonging to the *Heterorhabditis* and *Steinernema* genera, respectively. This technique was first used by Akhurst (1980) and significantly improved by Attila Lucskai (1999).

As for the molecular identification and characterization of the EPB strains I used standard techniques for DNA isolation, PCR, sequencing; automatized ribotyping (Allerberger and Fritschel, 1999), as well as for biochemical characterization I used Api, BIOLOG and conventional biochemical techniques (Smibert and Krieg, 1994). As for the phenotypic characterization of the primary – secondary variants, I used the methods summarized by Boemare et al. (1997).

As for genetic analysis of the antibiotics production I used the protocol of pLOF mutagenesis (Hererro et al.,1990) successfully adapted to EPB strains by Antónia Völgyi (Völgyi et al., 2000) and others (Williams et al., 2005; Bennett and Clarke, 2005; Joyce and Clarke, 2003) for mutant hunt.

RESULTS

1. I have determined the complete sequence of the 16S rRNS gene of 8, and the partial sequence of 3 additional *Xenorhabdus* isolates. The sequences were compared to each other and to the sequences of reference strains of *Xenorhabdus* species. 7 of the strains were identified as member of described *Xenorhabdus* species, while 4 of them were considered to represent four new, independent species.
2. The bacteria isolates from *S. scapterisci*, *S. bicornutum*, *S. rarum* and *S. serratum*, could not be placed in any of the described *Xenorhabdus* species on the basis of their 16S rDNA sequences. Another molecular, biochemical and microbiological tests including automated rypoprint analysis, biochemical tests, cell and colony morphology etc., also confirmed that these strains can not be placed in any known species. Therefore I had suggested to establish 4 new *Xenorhabdus* species: *X. budapestensis*, *X. ehlersii*, *X. innexi* and *X. szentirmaii*, respectively. My suggestions had been accepted and published (Lengyel *et al.*, 2005). My taxonomy work was co-supervised by Professor **Erko Stackebrandt** (DSMZ, Braunschweig, Germany).
3. I confirmed that the symbionts of the EPN species *S. kraussei*, *S. affinis*, *S. bibionis* és *S. intermedium* belong to the *X. bovienii* bacterium species. I also proved, that the symbiont of *S. cubanum* is a strain of *X. poinarii*, another strain of which is a symbiont of the known (NC513) as well as a recently identified (KMD15, Attila Lucskai and Michael G. Klein) strains of *S. glaseri*.
4. As a result of screen of the 102 strains of EPB stock collection for antibiotics production, we found more than 20 good antibiotics producers. We have found the antibiotics production of three of my four new species (*X. budapestensis*, *X. szentirmaii* and *X. innexi*) is reproducibly much higher than those of the others.

5. As for my attempt of analysing the antibiotics production genetically, I have established a mutant library including 9000 Km^R transposon-induced mutants of *P. temperata* ssp. *temperata* K122 strain. I choose this strain since we had some cooperation with British laboratories (Richard French-Constant and David Clarke) at that time, who used to work on this strain. I characterized each of my mutants for antibiotics production. I present the data concerning the antibiotics production and phenotypic features with special attention to the secondary-like features in my thesis. The locations of the insertions were identified in the laboratory of **Prof. Heidi Goodrich-Blair** (University of Wisconsin – Madison, USA) in three cases. One of my mutants was inserted at a gene encodes a protein similar to the LysR family of transcriptional regulators; another one at a gene encodes an ABC transporter-like protein another one in a gene, playing a role in carbapenem (antibiotics) production. I would like to continue and explore this analysis in the future.

6. I characterized the primary and secondary variants of 20 *Photorhabdus* strains phenotypically. This part was a kind of co-operation between students working in our Laboratory at this sub-line of research. My analyses included dye adsorbance, kótés, pigment production, bioluminescence, motility, hemolysis, exoenzyme (protease, lecithinase, lipase) production as well as antibiotics production. At the same time, Erzsébet Böszörményi carried out gnotobiological analysis; Dr. Antónia Völgyi compared the outer membrane protein patterns; while Janette Stenroos-Ek compared the intracellular protein crystal production variants of primary and secondary forms of *Photorhabdus* strains. We were also consulting with **Prof. Steven Forst** (University of Wisconsin – Milwaukee, USA). We found, that only the primary variant supports the growth and / or development the respective symbionts to fertile adult. Also, the capability of retention of the bacterium in the gut of the IJ similarly to producing intracellular protein crystals is a privilege of the primary variant. Contradicting to some concerning references, I found that in the exo-enzyme and antibiotic production, and motility there is no general rule concerning primary – secondary variation. In general there is quantitative, instead of qualitative difference between the two variations.

DISCUSSION

My first objective was the taxonomic identification of some entomopathogenic bacteria isolated in our laboratory by using molecular tools such as 16S rDNA sequencing. I managed to identify four new species successfully; three of which are of excellent antibiotics producers. My researches open the door for explore the potential of the recently known antibiotics producing *Xenorhabdus* species.

My taxonomic studies allow to have sight into the problem of co-evolution. It has been widely accepted that the EPN/EPB symbiosis is a school example of co-speciation. Some of my data supports this hypothesis but others (bacterial symbionts of different *Steinernema* species are proven to belonging the same *Xenorhabdus* species) strongly contradict to this hypothesis.

As for the another objectives, I manage to screen our EPN library for antibiotics production and found some excellent and some weak antibiotics producing strains. I also proved that while the *X. nematophila* strains are rather uniform, the *X. bovienii* strains are heterogeneous concerning their antibiotics production and profile. *X. bovienii* as well as the *S. anomali* symbionts proved weak antibiotics producers in repeated tests. This part of my work has some practical applicability. The 20 EPB strains, which were considered as good antibiotics producers, were tested in the Laboratory of Dr. Mária Hevesi in the Horticultural Faculty of Szent István University of Budapest, Hungary, for testing their antibiotics on *Erwinia amylovora*, causing the fire blight disease in apple trees. One of the antibiotics isolated from one my new species a *X. budapestensis* proved extremely powerful against this bacterial pest both *in vitro* and *in vivo*. It have then been isolated and almost completely identified by Prof. Attila Szentirmai and Prof. Ferenc Sztaricskai at University of Debrecen, Hungary.

I accomplished a successful mutant hunt by using transposon mutagenesis. My genetic analysis of the antibiotics production may contribute to reveal the details of the synthesis of antibiotics of entomopathogenic bacteria. There are two new scientific information concerning this: (i) the role of a transporter gene in the biosynthesis of the antibiotics; (ii) the discovery of a regulator protein playing a role in the antibiotics biosynthesis. I have also proved the evidence of the role of the carbapenem (*cpm*) operon in the biosynthesis of the antibiotics in *Photorhabdus*.

As for one of the objectives, the phenotypic analysis of primary secondary phase transition provided some new information, allowed us to distinguish between the characters of *Steinernema / Xenorhabdus* and *Heterorhabditis/Photorhabdus* symbioses. The most important differences are as follows: the capability of retention and supporting normal nematode development is an exclusive privilege of the primary variant of *Photorhabdus* strains, while in case of *Xenorhabdus* there are exceptions.

One of the reasons why I am interested in the primary - secondary transition is, that only the phase 1 cells are capable of producing antibiotics. It seems reasonable to suppose that the phase 1 – phase 2 transition and the antibiotics production are co-regulated. There has been “more anecdotes than facts” (Richard French-Constant) concerning primary - secondary transition. In the light of our systematic analysis it could be concluded, that in the case of *Photorhabdus*, only the capability of the retention of the bacteria and the intracellular protein production as well as of the supporting nematode development could be considered as exclusive primary state characters. In the cases of characters which can be quantified it seems that there is quantitative difference between the variations.

PUBLICATIONS

Publications in peer review Journals

Lengyel K, Lang E, Fodor A, Szallas E, Schumann P, Stackebrandt E. (2005) Description of four novel species of *Xenorhabdus*, family *Enterobacteriaceae*: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. *Syst Appl Microbiol.* 2005 28(2):115-22. IF: 1,91

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**NOVEL SCIENTIFIC RESULTS IN THE PH. D.
DISSERTATION OF KATALIN LENGYEL**

I. The Candidate established 4 new *Xenorhabdus* species: *X. budapestensis*, *X. ehlersii*, *X. innexi* and *X. szentirmaii*, respectively by using molecular tools, 16S rDNA sequence analysis and automated ribotyping combined with traditional microbiological and biochemical methods. (Lengyel *et al.*, 2005; 2006).

II. The Candidate confirmed that the symbionts of the EPN species *S. kraussei*, *S. affinis*, *S. bibionis* és *S. intermedium* belong to the *X. bovienni* bacterium species. She also proved, that the symbiont of *S. cubanum* is a strain of *X. poinarii*, another strain of which is a symbiont of the known (NC513) as well as a recently identified (KMD15, Attila Lucskai and Michael G. Klein) strains of *S. glaseri*. These taxonomic findings allow to have sight into the problem of EPN-EPB co-evolution. It has so far been widely accepted that the EPN/EPB symbiosis is an school example of co-speciation. Some data (bacterial symbionts of different *Steinernema* strains are proven to belonging the same *Xenorhabdus* strains while different symbiont bacteria from different strains of *H. bacteriophora* belong to different *Photorhabdus* species) of the Candidate strongly contradicts to this hypothesis. Her conclusion is that in the bacteria the capability of building up symbiosis with a given nematode is a phenotypic consequence of the mutations of only a limited number of genes, which themselves are not playing significant role in the evolution, seems reliable. Once a symbiosis established, this meant also an isolation of one group of bacteria from the others of the same taxon. Naturally the mutations occurring independently of the isolated population as a function of time, are leading finally to microevolutionary differences between them and finally to speciation. But this speciation is almost completely independent of the speciation of the respective nematode (*Steinernema* or *Heterorhabditis*) taxon.

III. The genetic analysis of the antibiotics production of the Candidate may contribute to reveal the details of the synthesis of antibiotics of entomopathogenic bacteria. There are two new scientific information concerning this: (i) the role of a transporter gene in the biosynthesis of the antibiotics; (ii) the discovery of a regulator protein playing a role in the antibiotics biosynthesis; (iii) the evidence of the role of the carbapenem (cpm) operon in the biosynthesis of the antibiotics in *Photorhabdus*.

IV. The phenotypic analysis of primary secondary phase transition provided some new information, allowed us to distinguish between the characters of *Steinernema / Xenorhabdus* and *Heterorhabditis/Photorhabdus* symbioses, which had been questioned in the literature. The most important differences are as follows: the capability of retention and supporting normal nematode development is an exclusive privilege of the primary variant of *Photorhabdus* strains, while in case of *Xenorhabdus* there are exceptions.