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Novel Anti-Microbial Peptides of *Xenorhabdus* Origin Against Multidrug Resistant Plant Pathogens

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1. Introduction

The discovery and introduction of antibiotics revolutionized the human therapy, the veterinary and plant medicines. Despite the spectacular results, several problems have occurred later on. Emergence of antibiotic resistance is an enormous clinical and public health concern. Spread of methicillin-resistant *Staphylococcus aureus* (MRSA) (Ellington et al., 2010), emergence of extended spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* (Pitout, 2008), carbapenem resistant *Klebsiella pneumoniae* (Schechner et al., 2009) and poly-resistant *Pseudomonas* (Strateva and Yordanov, 2009) and *Acinetobacter* (Vila et al., 2007) causes serious difficulties in the treatment of severe infections (Vila et al., 2007; Rossolini et al., 2007). A comprehensive strategy, a multidisciplinary effort is required to combat these infections. The new strategy includes compliance with infection control principles: antimicrobial stewardship and the development of new antimicrobial agents effective against multi-resistant gram-negative and gram-positive pathogens (Slama, 2008). During the last few decades, only a few new antibiotic classes reached the market (Fotinos et al., 2008). These facts highlight the need to develop new therapeutic strategies. The increasing incidence of serious infections caused by antibiotics-resistant and multi-resistant microorganisms such as the methicillin-resistant *Staphylococcus aureus* (MRSA) in human; streptomycin-resistant *Erwinia amylovora* (the bacterial pathogen causing fireblight disease) in Rosaceae make it imperative to develop new antimicrobial agents to face the new challenges Kocsis et al., 2009). It has also become obvious that, at least in Europe, antibiotics are not allowed to use for plant protection. Consequently, new antimicrobial compounds of different mode of action are needed which justify research efforts toward new sources. This chapter should be considered as a modest contribution to these efforts.

Microbes often live in polymorphic environments wherein they have to compete for nutrients, space and overcome toxins in order to survive and flourish. Amongst the chemical toolkits, antibiotics play an important role. The vast majority of recently used antibiotics are of eukaryotic origin, and the vast majority of antibiotics researchers focus on antibiotics producing fungi. However, some bacteria also produce compounds of antimicrobial activity. Consequently, these bacteria may be potential sources of novel antimicrobial compounds acting through novel molecular action mechanism. Our aim is to find bacterial sources for novel antibiotics effective against pathogens, which are resistant to antibiotics used in clinical practice and plant medicine.

The entomopathogenic nematode/bacterium (EPN/EPB) symbiotic associations are considered as model systems to address broad biological questions of mutualism, co-evolution and pathogenesis. As an indispensible part of keeping this system competitive in nature, broad spectral antibiotics produced by symbiotic bacteria (EPB) of entomopathogenic nematodes (EPN) and keep monoxenic conditions within insect cadavers in soil conditions such a way. Recently, the genomic sequences of bacterial symbionts, *Photorhabdus luminescens* (Duchaud et al., 2003) and two *Xenorhabdus* species have been completed, and the latter are being analyzed (Ogier et al., 2010). An area with ramifications in plant pathology, veterinary science, and even human health, is the primary and secondary gene products of antimicrobial activity. Webster et al. (2002) and later Brachmann et al. (2006) and Bode (2009) reviewed the results of antibiotics studies in EPB since the work of Akhurst (1982).

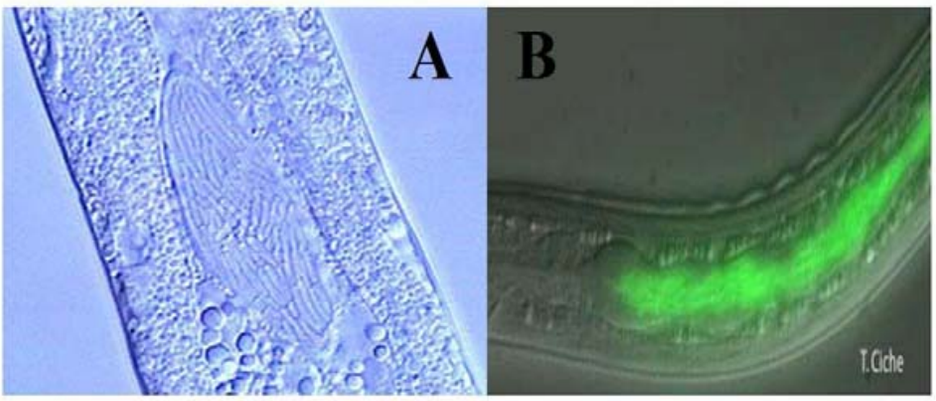

Fig. 1. Localization of *Xenorhabdus* (A) and *Photorhabdus* (B) entomopathogenic nematode symbiotic bacteria (EPB) in the gut of the infective dauer juvenile form of their respective entomopathogenic nematode (EPN) symbiotic partner, *Steinernema* (A) and *Heterorhabditis* (B). After the entering the insect the bacteria kill the host and colonize the cadaver, and serve as food source as well as antimicrobial-producing "safe-guard" for the nematode in soil condition (Courtesy of Dr. Todd Chiche he University of Michigan, USA).

EPB antibiotics have broad target spectra, which provide monoxenic conditions within insect cadavers. Despite descriptions and patenting of antibiotic molecules from *Xenorhabdus* (Webster et al. 1996; Thaler et al. 2001), nothing has been published on their commercial use. Our joint efforts revealed that antimicrobials new profiles are mainly of oligopeptide nature, such as the hexapeptide bicornutin A (Furgani et al., 2008 Böszörményi et al., 2009) which is

effective against prokaryotic (*Erwinia amylovora*) and eukaryotic (*Phytophthora nicotianae*) plant pathogens (Böszörményi et al., 2009). In this chapter, we intend to summarize our results since 2009, which mainly include the search of the target specificity of the cell-free conditioned media (CCFM) of two species, *Xenorhabdus szentirmaii* and *X. budapestensis* (Lengyel et al., 2005). Some of the related (sometimes poly-resistant) pathogens belonging respectively to *Pantoea, Klebsiella, Escherichia, Staphylococcus Salmonella, Candida* and *Alternaria* are also of human clinical and/or veterinary importance.

2. Aim, objective and rationale

2.1 Aim

The problems, which should be solved, are related with the increasing poly-resistance of pathogenic microorganisms of clinical, veterinary and plant medicine significance. Strong efforts of the scientific community have been exerted toward finding new antibiotics with novel action mechanisms. Antimicrobials of peptide nature, which induce apoptosis in target cells, have a great potential in control eukaryotic pathogens. Our contribution to the field is the introduction two novel organisms, *X. szentirmaii* and *X. budapestensis* what we have found excellent sources of compounds of strong antimicrobial activity. Their antimicrobial compounds proved antagonistic towards bacteria resistant to other antibiotics (Furgani et al., 2008; Böszörményi et al., 2009) and also toward some eukaryotic pathogens such as oomycetae, fungi and, according to some preliminary data, against pathogenic protozoon (McGwire et al., 2010, personal communication). Thus, importance and possible application of these compounds in agriculture and veterinary medicine was already thoroughly investigated and proved in several studies. Many of them have been patented but none of them is used in the practice. We considered the possible explanations and introduced two novel EPB species of excellent antimicrobial activities. Efficacy of these components against human clinical isolates has never been investigated before. The aim of our study is to investigate the antimicrobial potential of compounds produced by two entomopathogenic bacteria (EPB) as potential tools of controlling poly-resistant pathogens. Within this joint study, identification and purification of the bioactive molecules of the *Xenorhabdus* strains will be performed in different laboratories in Hungary and Ohio (USA).

2.2 Objective

The final goal of this study is to provide a view about the future perspectives of using antimicrobial peptides produced by *Xenorhabdus szentirmaii* and *X. budapestensis* in plant, - veterinary, - and may be in human medicine.

2.2.1 *Xenorhabdus budapestensis* and *X. szentirmaii* as sources of novel pathogen antagonists different from conventional antibiotics concerning their action mechanism

Our previous studies (Furgani et al., 2008; Böszörményi et al., 2009) confirmed that there might be new perspectives concerning the potential of some *Xenorhabdus* antibiotics, and for their use as alternative tools of pathogens of veterinary and plant medicine importance. We

found that, *X. budapestensis* and *X. szentirmaii* are the best of theses organisms. We also proved that antibacterial activities could mostly be adsorbed by Amberlite, and could be eluted without significant loss of activity. All of them gave a ninhydrin positive reaction, indicating that the most important compounds are of peptide nature. One of our main objectives is to determine their target specificity and then further purification after that. Our first steps toward this direction resulted in the discovery a hexapeptide (called Bicornutin A) of strong antibacterial activity.

Before going further toward biochemical purification, we wanted to test the target spectrum of the intact CFCM of the two species. This is what we are reporting about in this chapter. We provide new data about their control potential on plant pathogens as well as against multiple resistant human and animal pathogens.

We are also going to give an account on the very strong anti-oomycetal activity, providing a potential tool for plant medicine in nursery and forestry. Finally, they have a selective antifungal effect, which might be exhausted in behalf of plant medicine and human clinical practice.

We have also made the first steps toward a genetic approach of better understanding and improving the antimicrobial potential of by *Xenorhabdus szentirmaii* and *X. budapestensis*.

2.2.2 Potential pathogen targets of EPB antimicrobials in nature

At this point, we included into our Introduction vast majority of the potential target organisms, not only those that have already been analyzed. The reason is to draw the attention of the Reader of the perspectives and potential of using EPB antimicrobial compounds of apoptotic activity in the veterinary and plant medical, and potentially in the clinical practice, in the future.

2.2.2.1 Plant pathogenic bacteria

According to the data presented in the Widely Prevalent Bacteria Lists of Plant Pathology at Bugwood Websites in 2009, Ohio, as far the most infected state of the United States of America, is represented by 62 plant pathogenic bacteria. The climate as well as the plant pathogenic bacterium flora of Hungary and Ohio is rather similar. The most prevalent plant pathogenic bacteria belong to eight different groups. The general aim is to discover efficient antibacterial compounds, which antagonize agriculturally important pathogens in an environmentally friendly way. Our way is to test antibacterial activity of some entomopathogenic (nematode-symbiotic) bacteria on plant pathogenic bacteria. Our target bacteria in this study were as follows: *Agrobacterium, Burkholderia, Clavibacter, Curtobacterium, Dyckeya, Erwinia, Pectobacterium, Ralstonia, Pseudomonas,* and *Xanthomonas* species.

2.2.2.1.1 Agrobacterium species, biovars, strains

Agrobacterium species are Gram-negative, non-spore forming, rod-shaped bacteria attacking 643 dicotyledonous (broad-leaved) plant species. Neither monocotyledons, nor Liliales, nor Arales are *Agrobacterium* targets. The target organs are the roots and the stalks. The most characteristic symptoms are hypertrophies in most of their host plants. Disease symptoms:

formation of tumor-like swellings called galls that can generally be found on the crown of the plant just above the soil. The most important *Agrobacterium* pathogens are listed in **Table 1.** The most widely known species cause crown gall disease. *Agrobacterium* cells have one circular and one rode-shape chromosome. As extra-genomic DNA, they contain plasmids of different biological role (Fig 2, Left).. The most important is the ***Ti*** (tumor inducing) plasmid including a sequence (called TDNA) of eukaryotic nature. The TDNA is excised after infection and is capable of inserting randomly to the chromosomes of the plant. The expression of genes located on the TDNA result in tumor. The plant tumor cells produce compounds that are normally not produced by the plant. These compounds are used as a form of energy by the infecting bacteria. *A. tumefaciens* strains use different carbohydrates. They are classified into three main biotypes called biovars. There are allelic differences of genes located on the circular chromosome resulting in phenotypic differences of biovars concerning host preference and antibiotics sensitivity. Crown gall disease in grapevines (Fig 2, Right), is caused by bacteria belonging to Biovar 3, and (recently renamed as *A. vitis*). As for ecology, *A. tumefaciens* can generally be found on and around root surfaces known as the rhizosphere, where they might be attacked either by chemical or biological antagonists; as well as in the cambria (within the plant) as innocent saprophytes. They seem to use nutrients that leak from the root tissue. They invade the plant if it becomes conditioned (susceptible, wounded).

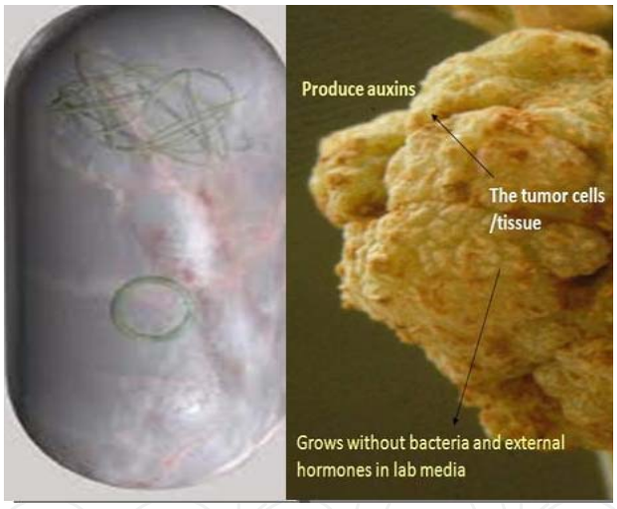

Fig. 2. *A. tumefaciens* cell with visible Ti plasmid ("North") and the genome ("South"); Crown gall disease (on the LEFT) Crown gall disease (on the RIGHT) Courtesy of Prof. George P. Rédei, (University of Missouri-Columbia, MO, USA)

Scientific name	Disease
Agrobacterium tumefaciens	crown gall of dicotyledonous plants
Agrobacterium vitis	crown gall of grape
Agrobacterium rhizogenes	hairy root disease
Agrobacterium rubi	cane gall of *Rubus*

Table 1. ***Agrobacterium*** species and the diseases they cause. (Higher order taxa**:** full lineage: root; cellular organisms; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Rhizobium/Agrobacterium group). SOURCE: Plant Pathology Bugwood Websites - Widely Prevalent Bacteria Lists

2.2.2.1.2 Clavibacter species, subspecies, and strains

Bacterial canker of is one of the most important and most difficult-to-control tomato diseases. Bacterial canker and wilt disease of tomato caused by ***Clavibacter michiganensis*** subsp. *michiganensis* are spread into different countries all over the world resulting in considerable yield losses (up to 70%), mainly in field-grown tomato production. The bacterium has been present in North America, Canada, Asia, and Africa. In European countries, it is on the list of quarantine pest. Symptom of the disease is wilting and desiccation of the plant both in field. Infected fruits show characteristic "bird's eye" spots; fail to develop and fall or ripen unevenly. Whenever it has been established in vascular tissues of the crop, it and becomes seed-born. The disease has also been recorded in greenhouses (Shoemaker and Echandi, 1976; Agrios, 1997). No sufficient chemical control exists. Biological control agents directly used against bacterial canker such as *Pseudomonas fluorescens, Bacillus* and S*treptomyces* species has been tried (Nishioka et al., 1997). The harmful *Clavibacter* bacteria are listed in **Table 2.**

Scientific name	Disease
Clavibacter michiganensis ssp. michiganensis	bacterial canker and wilt of tomato
Clavibacter michiganensis ssp. nebraskensis	Goss' bacterial wilt on cora
Clavibacter michiganensis ssp. sepedonicus	ring rot of potato
Clavibacter michiganensis ssp. insidiosus	bacterial wilt of alfalfa

Table 2. ***Clavibacter*** species and the plant diseases they cause. Taxonomic position: full lineage: root; cellular organisms Bacteria; Actinobacteria; Actinobacteria (class); Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Clavibacter; *Clavibacter, C. michiganensis*. (SOURCE: Plant Pathology Bugwood Websites Widely Prevalent Bacteria Lists)

2.2.2.1.3 Erwinia species and strains

Fire blight disease caused by *Erwinia amylovora* is one of the most destructive plant diseases that cause severe crop losses in many countries for a long time (Rosen, 1929). At present, the disease threatens commercial fruit industries worldwide. It occurs in many fruit species, especially those belonging to the *Pomaceae* and *Rosaceae* families, such as *Malus domestica*, *Pyrus communis*, *Cydonia oblonga* and *Cotoneaster spp*. It appears in the central, southern, and eastern regions of the European continent (Németh, 1998) and present in many states of the USA (Steiner and Zeller, 1996; Paulin, 1997). It appeared in Hungary first in 1995 (Hevesi, 1996). Right after these finding epidemics spread over in many fruit tree orchards (Németh, 1999; Jones et al., 1996). Chemical control and cultural practices did not prove sufficiency to arrest epidemics. Several research programs have been developed continuously to reduce the incidence of this serious disease or overcome the potential of the pathogen (van der Zwet and Beer, 1995). We have provided the first evidence that conditioned cell-free medium of *Xenorhabdus budapestensis* was capable of reducing the spreading of fire blight inflammation on apple trees after artificial infestation in green house conditions. The effects proved unambiguously cytotoxic in laboratory experiments (Böszörményi et al., 2009). **Table 3** provides a list of some plant pathogenic bacterium species belonging to *Erwinia* and *Pantoea* genera.

Scientific name	Disease
Erwinia amylovora	fire blight of Rosaceae
Erwinia tracheiphila	bacterial wilt on corn
Pantoea ananatis	center rot of pineapple
Pantoea stewartii pv. stewartii	Stewart's wilt of maize

Table 3. ***Erwinia*** species and the diseases they cause. SOURCE: Plant Pathology Bugwood Websites Widely Prevalent Bacteria Lists) Taxonomy: Full lineage: cellular organisms; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; *Erwinia*

2.2.2.1.4 Xanthomonas species, subspecies and strains

Bacteria belonging to genus *Xanthomonas* cause numerous plant diseases with diverse symptoms, including vascular wilts, cankers, soft rots, blights, leaf spots, tumors or galls. ***X. euvesicatoria*** strains are pathogens causing the bacterial spot diseases of *Capsicum annuum* and *Lycopersicon esculentum* a consequence of which is destructive loss in these two economically important crops. Control measures are applied yearly but no complete eradication of the disease has been achieved so far. Biological control provided some promising evidence concerning protection against the disease in small-scale plots. For example, *Pseudomonas fluorescens* gave promising inhibitory effects (Colin et al., 1984; Tzeng et al., 1994). *Xanthomonas* pathogens are listed in **Table 4.**

Scientific name	Disease
Xanthomonas vesicatoria	bacterial spot of tomato and pepper
Xanthomonas campestris pv. papavericola	bacterial blight of poppy
Xanthomonas campestris pv. pelargonii	bacterial blight of geranium
Xanthomonas campestris pv. raphani	bacterial leaf spot of radish and turnip
Xanthomonas campestris pv. vesicatoria	bacterial spot of tomato and pepper
Xanthomonas campestris pv. zinniae	bacterial leaf spot of zinniae
Xanthomonas fragariae	angular leaf spot of strawberry
Xanthomonas gardneri	bacterial spot of tomato & pepper
Xanthomonas hortorum pv. hederae	bacterial leaf spot of ivy
Xanthomonas perforans	bacterial spot of tomato & pepper
Xanthomonas translucens pv. undulosa	wheat leaf streak/black chaff
Xylella fastidiosa ssp. fastidiosa	Pierce's disease of grapevine
Xanthomonas arboricola pv. juglandis	walnut blight
Xanthomonas arboricola pv. pruni	bacterial spot of stone fruits
Xanthomonas axonopodis pv. vitians	bacterial leaf spot of lettuce
Xanthomonas campestris pv. armoraciae	bacterial leaf spot of crucifers
Xanthomonas campestris pv. campestris	black rot of crucifers

Table 4. ***Xanthomona****s* species and the diseases they cause. SOURCE: Plant Pathology Bugwood Websites Widely Prevalent Bacteria Lists Taxonomy, full lineage: root; cellular organisms; Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae, *Xanthomonas*.

2.2.2.1.5 Pectobacterium species, subspecies and strains

There is a group of plant pathogens closely related to *Erwinia* called ***Pectobacteria*** In Hungary, ***Pectobacterium chrysanthemi*** and ***P. carotavora*** cause bacterial spot of peach, tobacco wildfire, citrus canker, and some ornamental diseases as well Antibiotics were used to control black rot of cabbage. The list of these bacteria is given in **Table 5.**

Scientific name	Disease
Pectobacterium atrosepticum	black leg of potato
⁺Pectobacterium carotovorum ssp. carotovorum	bacterial soft rot
Pectobacterium chrysanthemi	bacterial wilt and soft rot
Pectobacterium chrysanthemi pv. dieffenbachiae	stem rot
Pectobacterium chrysanthemi pv. zeae	stem rot of maize

Table 5. ***Pectobacteria*** species and the diseases they cause. SOURCE: Plant Pathology Bugwood Websites Widely Prevalent Bacteria Lists. Taxonomy: cellular organisms; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Pectobacterium; Pectobacterium carotovorum. ⁺Synonyms: *Erwinia carotovora (ssp.) carotovora.*

2.2.2.1.6 Ralstonia and Burkholderia species, subspecies (plant variants), biovars and races

Ralstonia solanacearum attacks all the solanaceous crop plants. Over 200 hosts are known. Some are listed in **Table 6**. Strains show varying degrees of host specificity. The high economic and social impact of this organism results from its wide geographical distribution in all warm and tropical countries of the globe. Recently, distribution of the pathogen has been extended to more countries that are temperate from Europe and North America as the result of the dissemination of strains adapted to cooler climates. Nevertheless, the diseases are common in Europe but the pathogen is on the list of quarantine pest. Only complex and integrated control strategy can succeed in reducing the disease incidence.

Scientific name	Disease
Ralstonia solanacearum (excl. Race 3 Biovar 2)	southern bacterial wilt
Burkholderia andropogonis	gummosis of grasses
Burkholderia caryophylli	vascular wilt
Burkholderia gladioli pv. gladioli	scab of flower bulb

Table 6. ***Ralstonia*** and ***Burkholderia*** species and the diseases they cause. (SOURCE: Plant Pathology Bugwood Websites Widely Prevalent Bacteria Lists) Taxonomy: **Lineage** (full): root; cellular organisms; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; *Ralstonia / Burkholderia*.

2.2.2.1.7 Pseudomonas species, subspecies and strains: plant, animal and human pathogens

Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. Almost all strains are motile by means of a single polar flagellum. Its optimum growth temperature is 37 °C, but is also able to grow at 42 °C. *Pseudomonas* may form biofilm or exists in a planktonic form. Genus *Pseudomonas* is cleaved into eight groups. *P. aeruginosa* is the type species and there are another 12 members. Each is well known to plant microbiologists. They cause numerous plant diseases with diverse symptoms, including vascular wilts, cankers, soft rots, blights, leaf spots, tumors or galls. *P. aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its occurrence as a nosocomial pathogen and indicate that antibiotic resistance is increasing in clinical isolates. *P. aeruginosa* is an opportunistic pathogen; it exploits some break in the host defenses to initiate an infection. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients who are immunosuppressed. The case fatality rate in these patients is nearly 50 percent. *P. aeruginosa* isolates may produce three colony types. Natural isolates from soil or water typically produce a small, rough colony. Clinical samples yield one or another of two smooth colony types. One type has a fried-egg appearance, which is large, smooth, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a mucous appearance, which is attributed to the production of alginate slime. The smooth and mucous colonies are presumed to play a role in colonization and virulence.

Being Gram-negative bacteria, most *Pseudomonas spp.* are naturally resistant to penicillin and to the majority of related beta-lactam antibiotics, but a number of those is sensitive to piperacillin, imipenem, ticarcillin, tobramycin, or ciprofloxacin. This ability to thrive in harsh conditions is a result of their hardy cell wall that contains porins. Their resistance to most antibiotics is attributed to efflux pumps, which pump out some antibiotics before the antibiotics are able to act (Poole, 2004). *Pseudomonas aeruginosa* is a highly relevant opportunistic human pathogen. One of the most worrying characteristics of *P. aeruginosa* is its low antibiotic susceptibility. This low susceptibility is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (e.g. *mexAB-oprM*, *mexXY*, etc.) and the low permeability of the bacterial cellular envelopes. Besides intrinsic resistance, *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally encoded genes, or by the horizontal gene transfer of antibiotic resistance determinants. Development of multidrug resistance by *P. aeruginosa* isolates requires several different genetic events that include acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes. Hypermutation favors the selection of mutation-driven antibiotic resistance in *P. aeruginosa* strains producing chronic infections, whereas the clustering of several different antibiotic resistance genes in integrons favors the concerted acquisition of antibiotic resistance determinants. Some recent studies have shown phenotypic resistance associated to biofilm formation or to the emergence of small-colony-variants may be important in the response of *P. aeruginosa* populations to antibiotic treatment. This justifies testing new natural compounds of antibacterial activity against them. The most important plant pathogenic species are listed in **Table 7**.

Scientific name	Disease
Pseudomonas syringae pv. berberidis	leaf spot/fall of Berberis
Pseudomonas syringae pv. coronafaciens	halo blight of oats
Pseudomonas syringae pv. delphinii	Bacterial leaf spot of delphinium crop
Pseudomonas syringae pv. glycinea	bacterial blight of soybean
Pseudomonas syringae pv. lachrymans	angular leaf spot of cucumber
Pseudomonas syringae pv. maculicola	bacterial leaf spot of cauliflower
Pseudomonas syringae pv. apii	leaf spot of parsley
Pseudomonas syringae pv. atrofaciens	basal glume rot of wheat
Pseudomonas syringae pv. atropurpurea	leaf spot of Italian ryegrass
Pseudomonas syringae pv. mori	bacterial blight of mulberry
Pseudomonas syringae pv. morsprunorum	bacterial canker of stone fruit tees
Pseudomonas syringae pv. papulans	blister spot of apple
Pseudomonas syringae pv. phaseolicola	halo blight of bean
Pseudomonas syringae pv. pisi	bacterial blight of pea
Pseudomonas syringae pv. syringae	bacterial brown spot of bean/canker of stone fruit
Pseudomonas syringae pv. tabaci	wildfire of tobacco
Pseudomonas syringae pv. tomato	bacterial speck
Pseudomonas tolaasii	bacterial blotch on mushrooms
Pseudomonas viridiflava	spots/soft rots on leaves/fruits on many plant species

Table 7. Plant pathogenic ***Pseudomonas*** species and the diseases they cause. (SOURCE: Plant Pathology Bugwood Websites - Widely Prevalent Bacteria Lists) Taxonomy: **Lineage** (full): root; cellular organisms; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae *Pseudomonas*

2.2.2.2 Plant pathogenic Oomycetae

The most harmful plant pathogenic oomycetae are the downy mildew, *Phytophthora* and *Pythium* species, causing planting-off and root-rot (decay) diseases. *Phytophthora* species are well known in agriculture, limiting production of many crops from potatoes to citrus (Erwin and Ribeiro, 1996). Classically, this is a genus of agricultural plant pathogens especially destructive in poorly drained soils or cool wet climates. The name derives from Greek for „plant killer". These are oomycetae, water molds, related to algae and are not true fungi (Mycota). Consequently, they are quite resistant to fungicide, but sensitive to antibiotics (Érsek, 1975). Despite their unique phylogeny, they grow as filamentous hyphae and reproduce by spores, like fungi. They disperse and infect by motile zoospores, and survive unfavorable conditions especially drying, as thick-walled chlamydospores or oospores. Most cause root diseases, but especially on trees, some cause some lethal stem cankers, or infect foliage. *Phytophthora* species are one of the most harmful agricultural

pathogens all around the world, often causing serious damages. *Phytophthora infestans* used to cause the Irish potato famine 1845 to about 1860. Although the importance of potato as a staple food to countries lies only with the European countries since then but it extends all the way to the developing countries and especially in Africa. Farmers have tried to stop the disease using synthetic chemicals but it seems to exacerbate from time to time. Even where fungicides are used, continued wet spells often lead to major epidemics, as occurred for example in 2004 in Egypt and 2006 in Northern Peru and Kashmir. Even when epidemic conditions are not widespread, farmers in developing countries may get behind on spraying and lose control of the disease. This increases the chances of developing more resistance to these synthetic chemicals. Hence, the need for us to search for potential biological control for the *Phytophthora* strains which are becoming resistant to fungicides but are sensitive to antibiotics. *P. infestans* is a re-emerging pest ever since its discovery. It still causes major epidemics in potato and tomato crops worldwide (Forbes et al., 1994). For example, in 2000, 15% of the total potato crop of Russia was destroyed due to late blight. Such severe epidemics could trigger a new, potentially catastrophic potato famine (Schiermeier, 2001). Worldwide losses in potato production caused by late blight and measures to control the disease have been estimated at a billion dollar level annually (Duncan, 1999).

There are about 60 species in the genus *Phytophthora* that cause various disease symptoms, including root rot, fruit rot, foliar blight and stem blight on many economically important plants (Erwin and Ribeiro, 1996). A few examples of root rot pathogens are as follows: *Phytophthora sojae* (living on soybean); *P. fragariae* (on strawberries); *P. cryptogea* (on many plant species including tomato and cucumber); and *P. cinnamoni* (on various woody plant species). Other species cause leaf blight symptoms. *Phytophthora* is a good example of such a pathogen along with *P. porri* on leek. There are also many fruit rot pathogens such as *P. capsici* on various plants, and *P. palmivora* and *P. megakarya* on cocoa pods. The classification of *Phytophthora* species based on which part of the plant it predominantly infects is quite arbitrary. Disease symptoms are represented on all plant tissues, above and below ground. The environmental damage caused by *Phytophthora* diseases in natural ecosystems can be tremendous, due to difficulties in controlling the spread of the disease. An example of a severe ecological tragedy is sudden oak death, a disease caused by *P. ramorum*, which has emerged recently along the pacific coast of the United States. *P. ramorum* is destroying oak trees and is probably also spreading to other trees, such as redwoods and to other regions in North America (Knight, 2002). Likewise, *P. cinnamoni*, which has a very wide host range, infecting over 900 species of plants (Zentmyer, 1980), has caused severe epidemics in the jar rah tree forests Western Australia (Podger et al., 1965; Podger, 1972) as well as more recent outbreaks across the world. Hence, one can say the Irish potato famine is therefore not limited to a historic reference. In reality, many *Phytophthora* epidemics are just being kept under control by the use of prophylactic oomycetae (Talbot, 2004). When forest trees are grown in agricultural settings, such as in nurseries, they are vulnerable to agricultural diseases, including *Phytophthora* root rots. The *Phytophthora* species commonly involved in forest nurseries are often the same species affecting agricultural commodities in the area. Douglas-fir seedlings, for example, are affected by *P. megasperma* and other six *Phytophthora* species when raised in poorly drained nursery soils (Hansen et al., 1979). Tree seedlings that had been infected but survived in the nursery are likely to die in the first year after planting into forest sites. These nursery *Phytophthora* species, however, do not survive long in the

forest soils and do not spread to surrounding trees (Roth and Kuhlman 1996; Hansen et al., 1980). They are adapted to agricultural soils, and cannot compete in the more complex forest soil microbial community and generally dryer, better-drained forest soils. Other *Phytophthora* species do very well in forests, however. In recent years, it has become clear that, there is a very different community of *Phytophthora* species resident and probably indigenous in more or less undisturbed temperate forests. Many of these are new to science. For example, eight *Phytophthora* species were isolated from oak forest soils in the Forét de Amance in NE France (Hansen and Delatour, 1999). There were no obvious symptoms of *Phytophthora* root rot in this healthy mature stand, yet 12 of 14 soil samples from one site yielded one or more species. Five of the eight *Phytophthora* species were not described or only very recently described. Similar results have been obtained in Germany (Jung et al., 1996, 2002), eastern deciduous forests in the United States (Balci et al., 2007), and the western US (authors' unpublished data). The indigenous forest *Phytophthora* community is numerous and diverse. In most cases, the *Phytophthoras* are confined to the fine roots of the trees, and while they kill fine roots, in normal soil conditions, the trees replace the roots without dramatic growth loss. In Europe *Phytophthora,* species may contribute to the recurrent, chronic disease called oak decline. Oak decline, however, is primarily associated with periods of unusual drought, often coupled with outbreaks of defoliating insects. Under these stressful conditions, loss of additional rootlets to *Phytophthora* contributes to the decline (Hansen and Delatour 1999; Jung et al., 2000). In contrast to the nursery soil *Phytophthora* species that are generally poorly adapted to forest soils, and to the indigenous *Phytophthora* community that persists in a dynamic equilibrium with its host trees, a few species qualify as truly destructive in the forests. These exotic, invasive species can threaten the economic viability and ecological sustainability of the forests they attack. Distinguishing exotic from indigenous organisms is sometimes difficult. The complex processes of coevolution assure, that the host and the pathogen coexist without either consistently affecting the reproductive fitness of the other. It is often presumed that the resulting disease symptoms will be subtle and perhaps difficult to detect, and the ecological impact will be slight. By this thinking, a *Phytophthora* species that kills trees rapidly and over an expanding area must be exotic. This line of reasoning, while compelling in some situations, must be used with caution. *Phellinus weirii* is a pathogenic fungus that causes laminated root rot, a lethal disease of Douglas fir in North American forests (Childs, 1963). The disease is dramatic, altering forest structure and composition and pathways of succession (Hansen and Goheen, 2000), yet the pathogen is indigenous to the forests where it is found. Another presumed characteristic of an alien population is genetic uniformity. An invading population likely started as one or a few individuals making the first beachhead. This would be an evolutionary „bottleneck", and should result in reduced genetic diversity in the new population. *P. cinnamomi* (Fig 3) occurs worldwide and causes severe root rot and dieback on Fraser firs, shortleaf and loblolly pines, azaleas, camellia, boxwood, and many other trees and woody ornamentals (Ferguson & Jeffers, 1999). The disease impacts a range of economic groups including nursery crops managed forests, and Christmas tree farms. Root infected rhododendrons and azaleas and tree saplings develop above ground leaf chlorosis, necrosis, wilt, leaf curl, and death. Stem necrosis may not occur for many weeks after the development of wilting symptoms. Belowground symptoms are most severe in poorly drained soils and include necrosis of young feeder roots and the lower vascular tissues around the crown and just below the soil line. Cankers may become visible at the base of 1-2 year old plant.

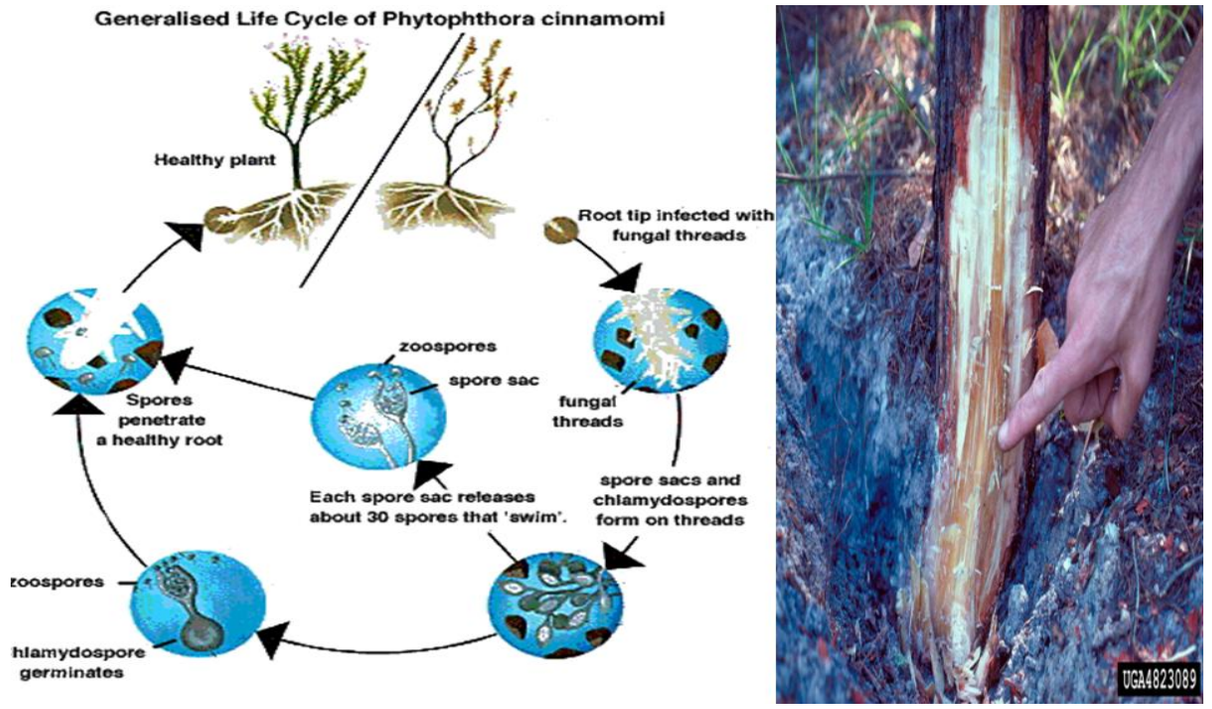

Fig. 3. Life cycle of (Left) and a symptom called resin soaked tissue, caused by *P. cinnamomi* Rands. The host is sand pine (*Pinus clausa* (Chapman ex Engelm.) Vasey ex Sarg) Image location: USA. The website of the picture: *Phytophthora* root rot. *Phytophthora cinnamomi* Rands forestryimages.org. Google pictures. Image No: 4823089. From Edward L. Barnard, Florida Department of Agriculture Consumer Services, Bugwood.org.

Phytophthora species reproduce both clonally and sexually. Many sexual species have in-breeding rather than outbreeding mating systems. Genetic diversity can be expected relatively low regardless of origin. As there have been no studies of diversity in indigenous clonal or in-breeding species, there is no diversity baseline against which to compare a suspected invading population (Érsek et al., 1995). *Phytophthora* species and the diseases caused by them are listed in **Table 8.**

2.2.2.3 Plant pathogenic fungi

Plant pathogens provide new challenge because of resistance problems. Necrotrophic plant pathogens have received increasing attention over the past decade. Initially considered to invade their hosts in a rather unsophisticated manner, necrotrophs are now known to use subtle mechanisms to subdue host plants. The gray mould pathogen *Botrytis cinerea* is one of the most comprehensively studied necrotrophic fungal plant pathogens. The genome sequences of two strains have been determined. Targeted mutagenesis studies are unraveling the roles played in the infection process by a variety of *B. cinerea* genes that are required for penetration, host cell killing, and plant tissue decomposition or signaling. Our increasing understanding of the tools used by a necrotrophic fungal

pathogen to invade plants will be instrumental to designing rational strategies for disease control (van Kan, 2006). *Alternaria alternata* (Keissl, 1912) has been recorded causing leaf spot and other diseases on over 380 host species. It is opportunistic pathogen on numerous hosts causing leaf spots, rots and blights on many plant parts. It can also cause upper respiratory tract infections and asthma in people with sensitivity (Wiest et al., 1987).

Phytophthora sp.	Disease	Host	Disease Management
P. infestans	Late blight	Potato	Sanitation and fungicides
P. sojae	Root and stem rot	Soybean	Resistant varieties
P. palmivora	Black pod	Cacao	Sanitation
P. alni	Collar rot	Alder	Clean nursery stock
P. cinnamoni	Jar rah dieback	Jar rah eucalyptus	Sanitation
	Little leaf disease	Shortleaf pine	Change species
	Ink disease	Chestnut	Uncontrolled
	Avocado root rot	Avocado	Fungicides, Soil management
P. lateralis	Cedar root disease	Port-Oxford-cedar	Sanitation, avoidance and resistance
P. ramorum	Sudden oak death, Ramorum blight	Fagaceae and Ericaceae	Quarantine and eradication
P. cactorum	Crown rot	Strawberry	Prevention and sanitation
P. parasitica	Phytophthora stem-rot	Snapdragon	Prevention and sanitation
P. capsici	Phytophthora blight	Cucumber, Squash, Melons, Pumpkin,	Practice crop rotation
P. fragariae	Red stele	Strawberry	Proper site selection and preparation
P. megakarya	Black pod disease	Cocoa trees	Chemical control-fungicides
P. syringae	Fruit-rot	Apples	Chemical control
P. primulae	Root and stem rot	Parsley	Quarantine and eradication

Table 8. Some destructive ***Phytophthora*** species and the diseases they cause and available disease management.

2.3 Rationale

The target organisms discussed above are all harmful plant pathogen of economic significance. The options of using chemicals (traditional antibiotics or fungicides) against them are rather limited, because of the realistic danger of environmental pollution. Furthermore, the resistance problems (discussed in the Introduction) also should be overcome by introducing novel antimicrobials of different action mechanisms. EPB could also be used in vivo as an antagonistic biocontrol agent, in some niche like rhizosphere in forest threes.

3. Materials and methods

3.1 Materials

3.1.1 Entomopathogenic Bacteria (EPB)

The colonies of *Xenorhabdus* and *Photorhabdus* on indicator LBTA plates are demonstrated in Fig 4.

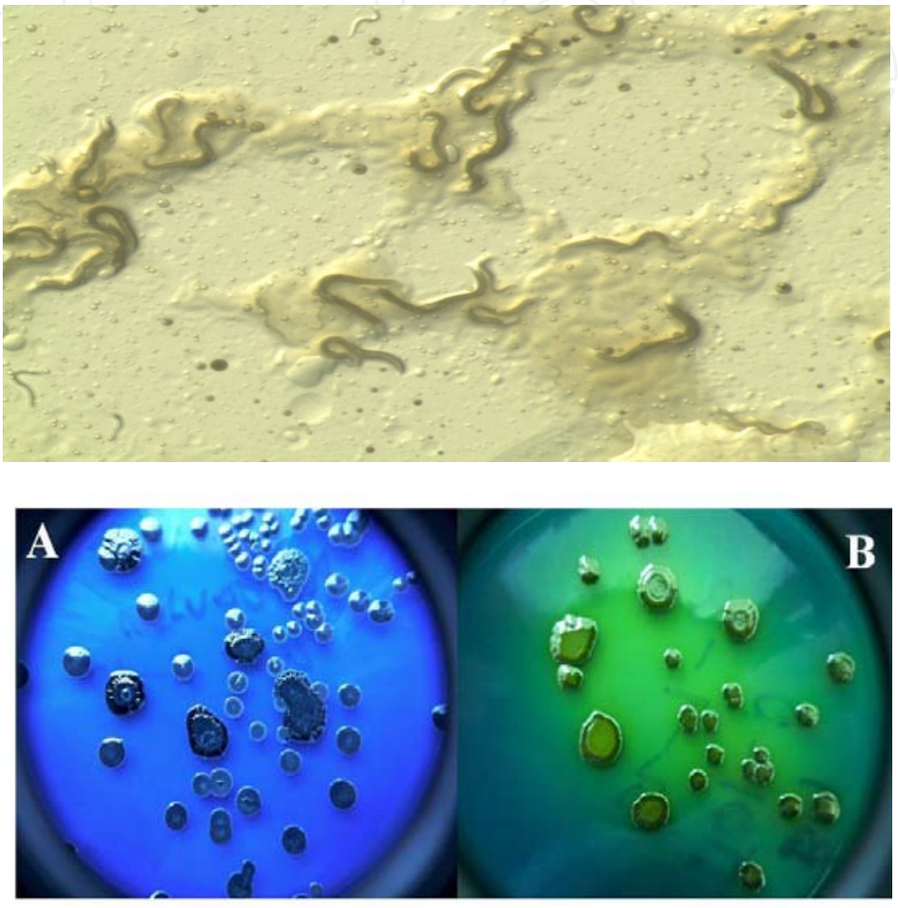

Fig. 4. Colonies of antibiotics-producing primary (1º) cells of a *Xenorhabdus* (A), and a *Photorhabdus* on LBTA indicator plates The bacteria were freshly isolated by Abate Birhan Addisie from the respective nematodes (above). Photo: Andrea Máthé-Fodor (nematodes) and Dr. Csaba Pintér (bacteria)

The list of EPB strains used in our experiments is given in **Table 9**. Some strains were provided by the COST 619 Research Community. Each of them had been deposited at the Georgikon Stock Center of Entomopathogenic Nematodes and Bacteria in Keszthely, Hungary. As for geographic origin, the AZ strains are from St. Miguel Island, Portugal-Azores, HP 88 from the USA, NC19 from North Caroline, USA, Brecon and Q614 from Australia. As for geographic origin, the AZ strains are from St. Miguel Island, Portugal-Azores, HP 88 from the USA, NC19 from North Caroline, USA, Brecon and Q614 from Australia. For further details, see references: Szállás et al., 1997, 2001 Peat et al., 2010; Völgyi et al., 1998, 2000; Lengyel et al., 2005; Brachmann et al., 2006; Furgani et al., 2008; Böszörményi et al., 2009).

Genus, Species	Subspecies	Strain	Reference
Xenorhabdus budapestensis		DSM 16342^{T*}	Lengyel *et al..*, (2005); Furgani *et al..,* (2008);; Böszörményi *et al..,* (2009)
Xenorhabdus .szentirmaii *		DSM 16338^{T}	Lengyel *et al..*, (2005); Brachmann et al.; 2006; Fodor *et al..,* (2007); Furgani *et al..,* (2008); Böszörményi *et al..,* (2009); Bode, (2009)
Xenorhabdus nematophila		ATTC 19061^{T*}	Völgyi et *al..,* .(1998), Völgyi *et al..,* 2000)
Xenorhabdus nematophila		N2-4	Szállás et al.., (1997); Völgyi *et al..,* (1998),
Xenorhabdus innexi		DSM1 6337^{T*}	Lengyel *et al..*, (2005); Furgani *et al..,* (2008);;
Xenorhabdus ehlersii		DSM 16336^{T*}	Lengyel *et al..*, (2005); Furgani *et al..,* (2008);;
Xenorhabdus cabanillassi		RIO-HU	Lengyel *et al..*, (2005); Furgani *et al..,* (2008);;
Photorhabdus luminescens	ssp. l*aumondii*	HP88	
Photorhabdus luminescens	ssp. l*aumondii*	Brecon	Szállás *et al..*, (1997),; Peat *et al..*, (2010); Marokházi et al; (2003);
Photorhabdus luminescens	ssp. l*aumondii*	Az35	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	Az36	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	Az37	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	Az39	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	Q614	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	Az29	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	NC19	Szállás *et al..*, (1997),; Peat *et al..*, (2010);
Photorhabdus luminescens	ssp. l*aumondii*	HP88	Szállás *et al..*, (1997),; Peat *et al..*, (2010);

Table 9. The list of the EPB strains used in this study

3.1.2 Plant Pathogenic Bacteria (PPB)

Plant pathogenic (PP) bacterium strains used in this study as test organisms are listed in Table 10 and 11.

SCIENTIFIC NAME	**NCAIM * CODE**	**BIOASSAY**		Origin
Erwinia amylovora	NCAIM B 01728	+	+	Hungary, apple; Hungary, M. Hevesi
	Ea 88 *str*R	+	+	USA,
	Ea110 *rif* R	+	+	McGhee & Jones, 2000
	Ea Ca 11 *str*R	+	+	McGhee & Jones, 2000
Klebsiella pneumoniae	HIP32 *chl*R	-	+	Human isolate
Kle. pneumoniae # 696	*carb*R	+	+	Mastitis isolate
Pantoea agglomerans	NPHMOS			Human pathogenic,

Table 10. List of antibiotics resistant bacteria used in overlay and /or agar diffusion bioassays of EPB antimicrobials. Abbreviation: * National Collection of Agricultural and Industrial Microorganisms, Hungary (NCAIM); http://ncaim.uni-corvinus.hu; *Kle* = *Klebsiella*

Altogether twelve *Erwinia amylovora* isolates (isolated by Dr. M. Hevesi, Hungary) were studied, but only *Ea* 1 (NCAIM B 01728) is listed here, since all the others reacted very similarly to all EPB CFCM tested. There are three antibiotic resistant strains: CA11 (rif S, *str* R); Ea88 (*rif* S, *str* R); and Ea110 Ea 110 (*rif* R, *str*S), (McGhee and Jones, 2000) also involved.

Dr. A.L. Jones (Michigan State University) kindly provided them in the list. Plant pathogenic bacteria isolated from different host plants are deposited in National Collection of Agricultural and Industrial Microorganisms, (NCAIM); Hungary. Home page: http://ncaim.uni-corvinus.hu. Other additional bacteria used in the second set of experiments were as follows: *P. agglomerans* 83873, obtained from the National Public Health and Medical Officer Service, Hungary.

SCIENTIFIC NAME	NCAIM * CODE	BIOASSAY		Origin
		Overlay	Agar diff.	Agar diff.
Erwinia amylovora	NCAIM B 01728	+	+	Apple; Hungary, M. Hevesi
Clavibacter michiganense ssp. michiganense	NCAIM B 01531.	+	+	Tomato; Hungary causing wilting and canker
Curtobacterium flaccumfaciens pv. betae	NCAIM B 01612.	+	+	Beans; UK causing leaf spot
Agrobacterium tumefaciens	NCAIM B 01681	+	-	Grapes; Hungary, crown gall disease
Bacillus subtilis	NCAIM B 01623.	+		Soil; Hungary, seed decay
Xanthomonas campestris pv. vesicatoria	NCAIM B 01857.	+	-	Pepper, Hungary, bacterial spot
X. campestris pv. carotae	NCAIM B 01699	+	-	Carrot, Hungary, carrot; bacteriosis
X. arboricola pv. juglandis	BK4	-	+	Hungary, walnut,
X. arboricola pv. juglandis	Szen 10	-	+	Romania, walnut,
X. axonopodis pv. phaseoli	B 01523	-	+	Hungary, beans
Pseudomonas fluorescens	NCAIM B 01670.	+	-	Hungary, soil, saprophytic
Pseudomonas fluorescens	NCAIM B 01667.	+	-	Hungary, soil, saprophytic
Pseudomonas syringae pv. syringae	NCAIM B 01556.	+	+	Hungary, rice, brown spot
Pseudomonas syringae pv. phaseolicola	NCAIM B 01689.	+	-	Hungary, beans, halo blight
Pseudomonas syringae pv. phaseolicola	NCAIM B 01715.	+	-	Hungary, beans, halo blight
Pseudomonas syringae pv. phaseolicola	NCAIM B 01776.	+	-	Hungary, beans, halo blight
Pseudomonas syringae pv. savastanoi	NCAIM B 01823.	+	+	Hungary, oleander, olive knot
Pseudomonas corrugata	NCAIM B 01637.	+	+	Hungary, tomato, pith necrosis
Pseudomonas syringae pv. lachrymans	NCAIM B 01537	-	+	Hungary, Cucumber,
Pseudomonas syringae pv. morsprunorum	NCAIM B 01684	-	+	Hungary, apricot,
Dyckeya chrysanthemi	NCAIM B 01839	-	+	Hungary, tomato
Burkholderia cepacia (syn. Pseudomonas cepacia	NCAIM B 01621	-	+	Italy, onion
Ralstonia solanacearum 879	PD2762	-	+	The Netherlands, potato
Ralstonia solanacearum	1070	-	+	Hungary, potato
Ralstonia solanacearum	1226	-	+	Hungary, potato,

Table 11. List of plant pathogenic bacteria used in overlay and / or agar diffusion bioassay tests. Abbreviation: * National Collection of Agricultural and Industrial Microorganisms, Hungary (NCAIM); http://ncaim.uni-corvinus.hu; *X.* = *Xanthomonas*

3.1.3 Eukaryotic plant pathogens: Phytophthora, Pythium, Botrytis, Alternaria and Fusarium species

Eukaryotic plant pathogens used in this study were oomycetae, such as**;** Phytophthora nicotianae; P. infestans, P. ramorum, P. cinnamoni, P. citricola; P. citrophthora and P. pelgrandis; and fungi, namely: Botrytis cinerea; Alternaria alternata; Fusarium gramineae. The oomycetal strains used in this study are listed in **Table 12.** All but K-39 had been isolated by András Józsa from Hungary and identified by Józsa & Bakonyi (in preparation) with classical and molecular tools. The fungus isolates Botrytis cinerea; Alternaria alternata and Fusarium gramineae (they are not separately listed) were also isolated in Hungary and kindly provided by Dr. Sándor Kadlicskó (University of Pannonia, Keszthely, Hungary).

SCIENTIFIC NAME	#Strain	Reference
Pythium sp.	JA 317	András Józsa – József Bakonyi
Pythium sp.	JA 319	András Józsa – József Bakonyi
Pythium sp.	JA 301	András Józsa – József Bakonyi
P. citricola	JA 74	András Józsa – József Bakonyi
P. citrophthora	JA 479	András Józsa – József Bakonyi
P. nicotianae	JA 168	András Józsa – József Bakonyi
P. nicotianae	JA H 1/100	András Józsa – József Bakonyi
P. cactorum	JA 163	András Józsa – József Bakonyi
P. pelgrandis	JA 337	András Józsa – József Bakonyi
P. cinnamoni	JA 153	András Józsa – József Bakonyi
P. megaspermium	JA 209	András Józsa – József Bakonyi
P .infestans	K-39	József Bakonyi

Table 12. List of plant pathogenic oomycetae used as test organisms in studying anti-oomycetal activity of Xenorhabdus CFCM agar diffusion tests. #Strains are deposited at the Institute of Plant Protection, Hungarian Academy of Sciences by József Bakonyi. As for Botrytis cinerea, see Rosslenbroich & Stuebler (2000).

3.2 Media and cultures

3.2.1 Bacterium media and cultures

Ingredients of bacterial media used in this study are summarized in Table 13.

Basic components were autoclaved (20', at 121 ^{o}C). Some ingredients should be added after autoclave, such as are BTB, TTC, antibiotics and (Leclerc and Boemare, 1991) CFCM. Both EPB and PPB strains grew on LB, and LBA. In overlay bioassay a 5 µl O/EPB culture s dropped to the center of a plate. After 5 days, it was overlaid with text bacteria suspended in a soft (0.6 w/v) agar. The size of the inhibition zone was measured around the colony. When LBA was used as "poisoned agar" 500 ml water was added before, and + 500 ml of diluted CFCM after autoclave.

Name of the media	Final ml	INGREDIENTS						USED FOR
		Before autoclaving				After autoclaving		
	Water	Agar	NaCl	Trypton	Yeast extract	BTB+	TTC++	
LB	1000	-	5 g	10 g	5g	-	-	Culturing EPB and PPB
LBA	1000	15 g	5 g	10 g	5g	-	-	Culturing EPB and PPB; For overlay bioassay
LBTA	1000	15 g	5 g	10 g	5g	1 ml Stock	1 ml Stock	Indicator plate
Mc Conkey	1000	35 g of Commercially available powder						

Table 13. Bacterium media used in this study. Abbreviations: LB = Luria Broth; LBA = Luria Broth Agar; LBTA= Luria Broth Agar supplemented with indicators stains, bromothymol blue (BTC) and triphenyltetrazolium chloride; + and ++: Stock solutions of BTB and TTC, 25 mg/ml and 40 mg/ml chloride (TTC) dissolved in ethanol (Leclerc & Boemare, 1991; Ausubel et al., 1999); EPB and PPB = entomopathogenic (antagonist producing) and plant pathogenic (test) bacteria. On indicator plates antibiotic producing 1^{o} can be distinguish from 2^{o} variants and from contaminants.

3.2.2 Preparation of Cell-Free Conditioned Xenorhabdus Media (CFCM)

Xenorhabdus szentirmaii and *X. budapestensis* had been isolated from their nematode symbiont, *Steinernema rarum* and *S. bicornutum* and identified by us (Lengyel et al., 2005**).** *Xenorhabdus szentirmaii* and *X. budapestensis* were maintained Luria Broth (LBA) medium and sub-cultured freshly. Because of the instability of the phase I under normal culture condition, glycerinated stocks of the bacteria frozen at $-80^{o}C$ were used as starting material for culture. To ensure the presence of phase I, the glycerinated stocks were incubated in the dark at 28 on LBTA (see above). Phase I is distinguished from phase II by its adsorption of LBTA to produce a blue colony with a clear zone around bacteria culture for antibiotic production was prepared by inoculating a loopful of phase I of *Xenorhabdus szentirmaii* or *X. budapestensis* growing on an LBTA plate into a test tube 5 ml fresh LB medium. At the and of the log phase this volume of cell suspension was added into baker containing 100-300 ml of sterile LB and was cultivated at $28^{0}C$ on a laboratory shaker at 200 rev min^{-1} for 24-48 h, during which time the optical density respectively. A starter culture was produced first in 5.0 ml of LB media. 5-ml test tubes cultured were used as inoculum for scaling up larger cultures. The liquid cultures were scaled up in to 250, 500 and 1000 ml volume by using the 5 ml overnight cultures as inocula and grown in Erlenmeyer flasks, shaken for a couple of days. Finally, in the mid stationary phase, cultures were centrifuged at 13,000 g and filtered through a 0.22 μm pore size filter. After cooling down, the liquid medium was measured into 5 ml test tubes. A clean growing colony from a Petri-dish that had been grown with the bacterium, a loop-size colony was suspended into 5-ml volume of liquid medium in a test-tube. For each bacterium grown in liquid a non-inoculated control (a test-tube with liquid medium) was left for comparison after the growing period. Aliquot samples were taken regularly to check the antibiotics activity. Not only the antibiotics producing but also the test bacteria were growing on LB or LBA as well.

3.2.3 Cultures for eukaryotic plant pathogens

Ingredients of media used for studying Oomycetae and Fungi are summarized in Table 14.

Name of the media	Final ml	INGREDIENTS				USED FOR
		Before autoclaving				
	Water	Agar	Chopped carrot^{+}	Chopped Potato^{++} or Potato infusion	Ford hook 242 Dried lima bean seed	
CA (Carrot Agar)	1000	15g	200 g			Culturing *Phytophthora, Pythium*
PDA (Potato Dextrose Agar)	1000	20 g		200 g		Culturing eukaryotic plant pathogens
LiBA (Lima Bean Agar)	1000	20g			5 g	

Table 14. Ingredients of media used for culturing of and assaying on plant pathogenic oomycetae and fungi in this study. For bioassay, the "poisoned agar" version is used: instead of 1000 ml DW only 500 ml DW is to be added to the media before autoclaving and 500 ml cell free conditioned media (CFCM) of different dilution after autoclaving, before pouring the media into plates.

As for CA, carrots were washed and using a knife peeled and cut into small pieces. 200g of the chopped carrots were put into a grinder. Some water was added in to the chopped carrots during the procedure. The carrots were ground into a fine mixture of water suspension and filtered through a cloth in funnel into a new beaker. 30g agar was added. The mixture was filled up with distilled water (DW) up to a 1000 ml before autoclaved (20'; at 121^{0}C) and dispensed into Petri plates. A. Józsa established this technique in our lab. Lima Bean (LiBA) Agar Media was described) and used for growing stock cultures of *Phytophthora megasperma* var. *sojae* by Hilty & Schmitthenner (1962).

As for PDA, 200 g sliced unpeeled potatoes are boiled in 1 liter DW for 30' and filtered through cheesecloth, or commercially available potato infusion dehydrated form was used. Filtered (sterile) ingredients were added after autoclave (15', 121^{0}C) before dispensed 20-25 ml portions into sterile Petri dishes. Final pH= 5.6 ± 0.2. Medium should not be re-melted more than once. Commercially available media require extra agar supplement with to a final concentration of 20 g/liter. These media could also be used as "poisoned agar" if only 500 ml water is added before autoclave and it is it is supplemented with 500 ml of different dilutions of the CFCM after autoclave.

3.3 Bioassays

3.3.1 Bioassays of antibacterial activity

3.3.1.1 Overlay bioassay

When using overly tests in plates, we incubated plates with a 5 µl of overnight (O/N) liquid culture of the antibiotics producing bacteria for 5 days in a 25-oC incubator. The

antibacterial activities of the *Xenorhabdus* and *Photorhabdus* species have routinely been tested against laboratory standard *E. coli* strains. In these tests one colony were suspended in 5 ml of LB and incubated for 24 h (up to nearly stationary phase). The plates were then overlaid with 55 ^{0}C soft (0.8 % w/v) agar containing the suspension of the liquid culture of test bacteria) in 3%. The diameters (given in mm) of the inhibition circles were measured after five days. In later sets of experiment plant, pathogenic bacteria were used as test organisms (various *Pseudomonas spp., Erwinia spp., Clavibacter spp., Agrobacterium tumefaciens, Xanthomonas spp., Curtobacterium flaccumfaciens spp.*). The plates were then incubated at 37 ^{0}C for 24h. The diameter of the inactivation zone around the antibiotics producer *Xenorhabdus* colony was measured.

3.3.1.2 Agar diffusion test

A. Antimicrobial disc assay: In plastic Petri plates of 8.5 cm diameters 15 ml LBA media were poured producing a 7 mm thick solid media. 3 ml of *Erwinia* or other test bacterium cells suspended in soft (0.6 w/v) agar in 50 ^{o}C was poured onto the surface of the media. The density of the bacterial cells was 1:100 of an O/N (stationary phase) *Erwinia* cultures. BBL* blank paper discs had been submerged in the solutions to be tested and then placed on the surface of the agar. The plates were scored on the next day and the diameters of the inactivation zones were measured around each disc. There were three replicates made for each test.

B. Antimicrobial hole test assay: *Erwinia* or other test bacterium cells (1:100 of an O/N (stationary phase cultures) were given to LBA media of 50 ^{o}C temperature (10 V/V %), mixed carefully and poured into plates (18 ml/plate). Right after the agar, solidified holes of 1 cm diameter were made by using a cork borer. 100 µl volume of each solution to be tested was pipette into one hole. The plates were scored on the next day. The diameters of the inactivation zone were measured around each disc. There were at least two replicates made for each test. This technique was standardized in Dr. Hogan's lab this summer (in preparation). The pictures of overlay and of an agar diffusion assays are shown in **Fig 5** and **Fig 6**, respectively.

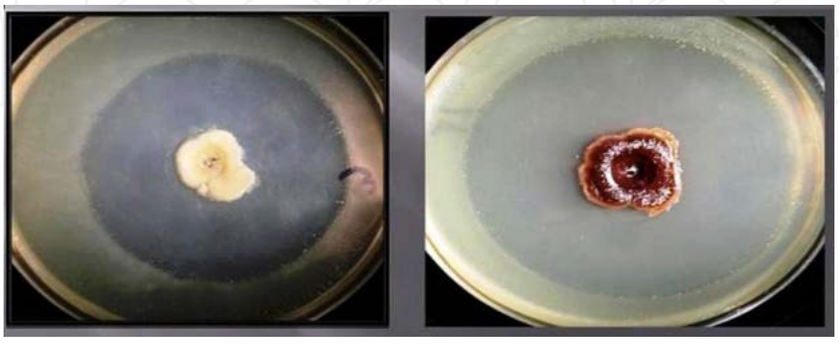

Fig. 5. Antibacterial potential of 5-d old colonies of *Xenorhabdus budapestensis* (Left) and *X. szentirmaii* (Right) on Gram-negative target, *Escherichia coli* OP50. Overlay bioassay on LBA plates. Photo: Dr. Cs. Pintér

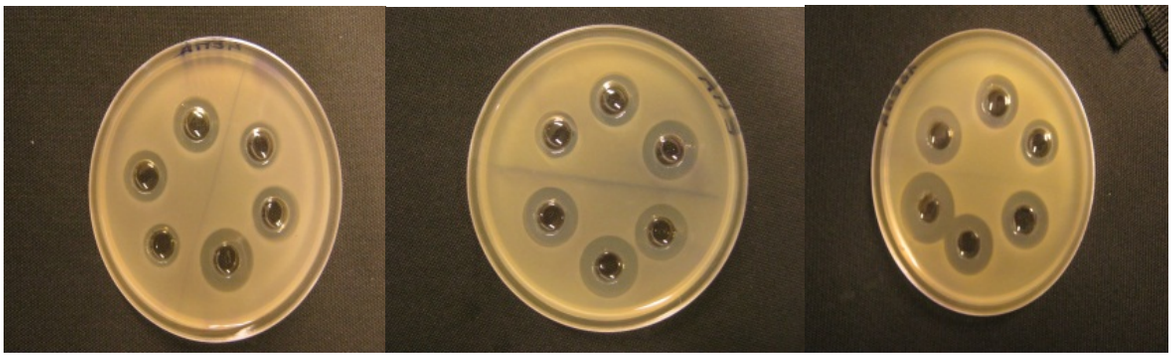

Fig. 6. Agar-diffusion plate diffusion plates Test bacteria: LEFT: Pseudomonas syringae pv. syringae CFCM of Xenorhabdus budapestensis ("West") and X. szentirmaii ("East); MEDIUM: Curtobacterium flaccumfaciens CFCM of X. budapestensis ("North") and X. szentirmaii ("South); RIGHT Xanthomonas axonopodis CFCM of X. budapestensis ("North") and X. szentirmaii ("South). The dilutions of CFCM administered into the holes were 10, 20 and 40 V/V respectively. The sizes of the inhibition zones proved reproducibly dose dependent. Photo: M. Hevesi.

3.3.1.3 Determination of MIC 95 (MID95) values

When exact concentration of bacterial cells needed, it was determined using a spectrophotometer at 600 nm wavelength. Alternatively, for antibiotic production we found that the optimal length of the incubation in liquid is 3-5 days. Considering that the cell-free conditioned *Xenorhabdus* media (CFCM) is a mixture of biologically active compounds of unknown nature, we did not use the term of EC50 and EC95, (concentration of the antibiotics resulting it 0 or 50% growth of the test bacteria). We used the term of "Maximum Inhibiting Dilution" 95 given in V/V % (see Furgani et al., 2008) instead. (100 V/V% is the undiluted CFCM, and 0 V/V% is the LB media). To determine MID 95 we used LB media in 200 µl volume containing 2 µl of test bacterium suspension. It contained of 2 µl test bacterial O/N culture of 1:10 dilution. We worked with 96-hole cell culture or microtiter plates. EMC and EMA cultures were tested in 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 V/V % dilutions. There were 4 replicates for each dilution. We considered the putative MID 95 (Maximum Inhibitory Dilution, if no bacterial growth was observed by using Sensititre Manual Viewer in 24 h (or by bare eye) at a given concentration. To make determine whether the bacteriostatic affect were complete 100 µl volume of each (visually clean) mixture were plated out. We plated a serial dilution of the culture from – 10^5 dilutions and counted the colonies grown on the plates. The largest dilution (the smallest concentration) of the media was considered as MID95 (practically MID100), if no colony grew in these plates. The untreated controls were diluted and plated similarly. The colonies could be counted only at 10^{-7} dilutions in the control. The LC95 of the commercial antibiotic was determined very similarly using 96-hole cell culture or microtiter plates.

3.3.1.4 In planta bioassays of antagonistic activity on fire blight inflammation

This method was described before (Böszörményi et al., 2009).

3.3.2 Bioassay techniques for quantifying anti-oomycetal and anti-fungal activities

3.3.2.1 Bioassay of Anti-Oomycetal Activities

The antagonistic activities of the CFCM of two *Xenorhabdus* species (*X. budapestensis, X. szentirmaii*) have been tested on four *Phytophthora* and three *Pythium* isolates and evaluated qualitatively and quantitatively. In both experiments, we used "poisoned" carrot agar (PCA, see below) plates of 10 ml volume in 5.5-cm diameter sterile Petri plates or of 20 ml volume in 8.5-cm diameter sterile Petri plates. These media contained double carrot agar and different concentrations (dilutions) of cell-free conditioned media (CFCM) of *Xenorhabdus szentirmaii* (or *X. budapestensis*) in 1:1 ratio. We ran several sets of experiments. In the first run, we compared the dilutions of 0, 25, 50 and 75% v/v dilutions. In the second run, we compared 0, 10, 30 and 40% v/v dilutions of both antibiotics producing bacteria. For confirmation, we ran a third set by using 0, 25, 37.5 and 40% v/v volumes. Each assay carried out on a 1-cm diameter circular-shape carrot-agar disc with *Phytophthora* (or *Pythium*) obtained from culture plate by sterile cork borer, put into the center of each plate. Evaluation: The diameter of the growing mycelia of the *Phytophthora* on the carrot-agar was measured after different days following inoculation

3.3.2.2 Bioassay of Anti-Fungal Activities

The antagonistic activities of the CFCM of two *Xenorhabdus* species (*X. budapestensis, X. szentirmaii*) on one *Botrytis cinerea*, *Alternaria alternata* and *Fusarium graminearum* isolates were studied in a very similar manner as t Agar (LiBA) instead of CA. The fungi were cultured on PDA plates. The anti-oomycetal studies, with the only difference that media was Potato Dextrose Agar (PDA) or Lima Bean Agar (LiBA).

3.4 Methods of genetic analysis

3.4.1 Transposon tagging of genes of interest

The principle of STM is that a mobile genetic element (Tn10) should be randomly inserted into the genome with the help of the transposon via conjugation. The gene in question is inactivated by insertional mutation. a transposon is used which inserts itself into the gene sequence. When that gene is transcribed and translated into a protein, the insertion of the transposon affects the protein structure and (in theory) prevents it from functioning. In STM, mutants are created by random transposon insertion and each transposon contains a different 'tag' sequence that uniquely identifies it. If an insertional mutant bacterium exhibits a phenotype of interest, such as loosing the ability to produce antibiotics then we will sequence its genome and run a search (on a computer) for the tag used in the experiment. When a tag is located, the gene that it disrupts is also thus located (It will reside somewhere between a start and stop codon which mark the boundaries of the gene).

In our experiments the donor stain was *E. coli* S17 *E. coli* S17-pir pLOF (came from Dr. János Kiss (Agricultural Biotechnology Center, Gödöllő, Hungary), and the recipient was *Xenorhabdus*. The antibacterial activity of population was tested on *E. coli* OP 50 in LBA plates or LB liquid media.

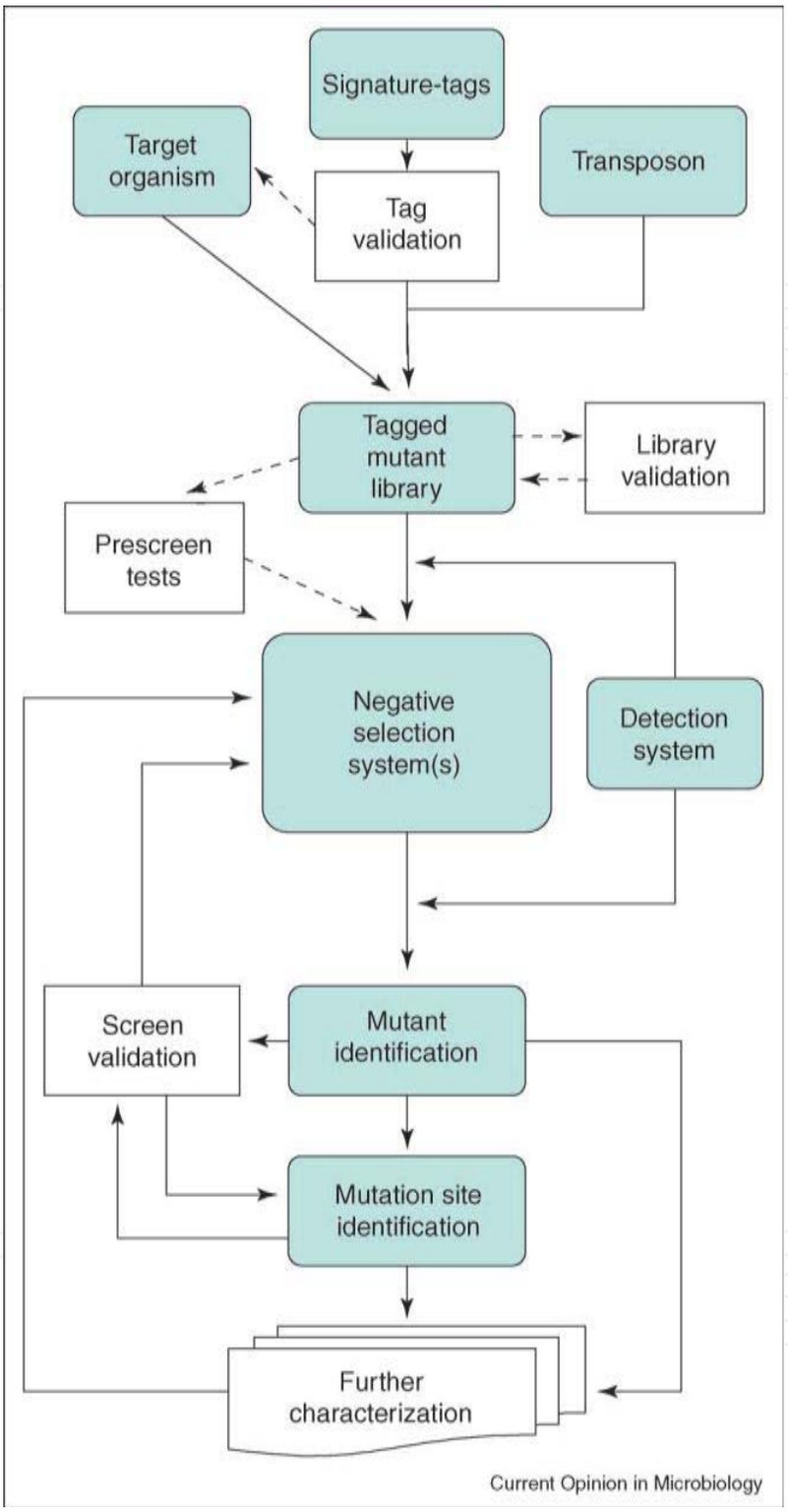

Fig. 7. Flow chart overview depicts Signature Tagged Mutagenesis (STM) of the different modules and their interconnectivity (kindly provided by Prof. Heidi Goodrich-Blair, Univ. Wisconsin. Madison). By using this technique, the genetic background of several important functions related to colonization, pathomechanisms and symbiotic behavior of Xenorhabdus nematophila have been revealed (Heungens et al., 2002; Martens et al., 2003; Richards et al., 2009) in her laboratory.

3.4.1.1 Isolation of spontaneous rifampicin resistant Xenorhabdus mutants

Spontaneous rifampicin resistant (rif^R) mutants of the target organisms are recipients. They were obtained from both *X. budapestensis and X. szentirmaii* by selection on LBA plates containing 50 μg / ml (rif_{50}). The individual colonies were picked up by using sterile loop and transferred to sterile test tube into 5 ml LB containing 50 μg / ml rifampicin. The cultures were grown over night (O/N) on rotary shaker at 200 rpm (at ~ 25 ^oC for further experiment. Using the same protocol, *E. coli* S17- λ^{pir} pLOF (which carries the transposon gene) was grown in LB containing kanamycin 30μg/ml (kan_{30}).

3.4.1.2 Conjugation

The insert of plasmid was subjected to transposon mutagenesis by the use of a genetically engineered derivative of Tn*10*, which will be referred to as mini-Tn*10*pLOF/Km (Simon et al., 1983; Herrero et al., 1990). This construction behaves as a mobilizable plasmid could be replicated only in the in S17 λ^{pir} background. Consequently, if it could be transferred from a conjugative *E. coli* strain to *Xenorhabdus*, where it is be capable of being inserted into the chromosome but unable for further "jump". The easiest and way is to transfer Tn10 via conjugation from *E. coli* to *Xenorhabdus*. There are several technical alternatives for promoting conjugation, either in a LBA plates or in a filter. One then could select for the respective mutant by double antibiotics selection. The technique what was establishes in our laboratory are given below (**Table 15).** This is a modification of the original protocol of Herrero et al. (1990) by A. Máthé-Fodor.

3.4.1.3 Mutant hunt: Selecting for transconjugants

Each survivor on the selective media was tested for the differential dye uptake on indicator plates. The spontaneous secondary (2^o) variants (Völgyi et al., 2000) were discarded. We considered "transconjugants" those cells from the conjugation mixture, which survived and develop colony in the presence of 50μg /ml rifampicin and 30 μg /ml kanamycin. They were tested for antibiotic activity in overlay bioassay (see above) and *E. coli* OP50 was used as test organism. Putative pigment mutants of *X. budapestensis* were identified visually. The putative mutants have been stored in 20% glycerol / LB solution at -80^OC as described by Miller (1972). Crystal mutants were screened based on colony color morphology.

BEFORE STARTING THE TRANSPOSON MUTAGENESIS EXPERIMENT	***NOTES***
Choose a donor: **Donor**: S17-1 pLOF (*Km*®) or ChlR®)	
Choose a recipient: *EPB* strain **(Xenorhabdus szentirmaii, X. budapestensis**)	
PREPARATION 1	
ISOLATION OF SPONTANEOUS RIFAMPICIN RESISTANT (rif®) MUTANTS	
Make an overnight (O/N) LB liquid culture of he chosen EPB bacterium	
Seed at least 30 LB plates containing Rif100 with 0.1 ml EPB bacterium suspension	***Keep the plates in dark, Use aluminum folia***
Isolate single colonies, which had been grown for 48-72 hrs. (Not later, because the rifampicin can be decayed)	
Prepare LB rif100 and LB Km30 LB plates	
PREPARATION 2	
Prepare 50 ml of LB rif100 LB liquid media	
Prepare 50 ml Km 30 LB liquid media	
Prepare 30 LB rif100 plates.	***Keep plates at 4 ^oC***
Prepare 30 Km 30 LB plates	

MUTAGENEZIS PROTOCOL

1st DAY

Pick 2-3 day-old SINGLE colonies from your donor on LB-Km30.
Pick 2-3 days old SINGLE colonies from your recipient on LB-Rif100 plates.
Inoculate 5-ml liquid LB Km30 with 1 colony of the donor
Inoculate 5-ml liquid LB rif100 with 1 colony of the recipient
Make overnight cultures (O/N) from both.

Use fresh plates! In case, keep the plates at 4 °C.

2nd DAY

Inoculate 50 ml LB Km30 liquid culture with 1ml O/N Donor culture.
Inoculate 50 ml LB rif100 liquid culture with 1 ml O/N Recipient culture
Incubate the donor at 37 °C or 30 °C
Incubate the recipient at 30 °C

Do not shake or roll!

Stop growing the recipient at OD (600nm) 0.2-0.3.

Calculation:
[(OD of the donor) x 2]/ (OD of the recipient) = (ml of recipient) to be added to each ml of donor

Mixed the parents as follows:
DONOR / RECIPIENT cells is 1:2
Take a sterile glass Petri plate
Pipette 1 ml of the donor onto the plate.
Pipette 4 ml of LB liquid media to dilute it.
Take a sterile syringe, to which bacterium filter could be adjusted
Take out the 5 ml diluted donor cell suspension with the sterile syringe
Adjust sterile 0.22 µm filter to it.
Push through the filter and discard supernatant
Disconnect the filter and the syringe
Wash donor cells. Take 5 ml of sterile LB media into the syringe (use a new sterile one).
Adjust the sterile 0.22 µm filter containing the donor cells to it.

Repeat this step twice or 3X

Do the same with the recipients:
Put the calculated volume of recipient suspension onto another sterile plate.
Dilute it up to 5 ml by adding sterile LB.
Take up the 5 ml diluted recipient suspension with ANOTHER sterile syringe
Adjust the SAME FILTER you had used with the donors to the syringe with the recipient cell suspension
Push the suspension through the filter.
Disconnect the filter and the syringe.
Take 5 ml of sterile LB media into the syringe (you should use a new sterile one)
Take out the 0.22 µm filter paper of which the donor and recipient cells are with a forceps onto the surface of a sterile LB agar plate for a couple of hours
After a couple of hours transfer the filter paper onto the surface of an LB plate containing IPTG35
Incubate overnight, let the cells make love (conjugate)

3rd DAY

Wash the cells off filter with 4 ml of LB liquid media
Each of 100 µL aliquots should be spread on separate Km30 + Rif100 LB agar plates
Incubate them O/N at 30 °C

Only trans-conjugants will grow

4th DAY

Prepare about 60 fresh LB Km30 plates what you need to use on the fifth day

5th DAY

Transfer individual colonies grown up on the surface of the Km30Rif100 LB plates onto Km30 plates in a replicable order by using sterile toothpicks. We usually put 25 colonies onto one plate

Table 15. Transposon tagging (mutagenesis) protocol used in our laboratory by A. Máthé-Fodor.

4. Results and discussion

4.1 Results on plant pathogen bacteria

4.1.1.1 Effects on Xanthomonas strains

The CFCM of both *X. budapestensis* and *X. szentirmaii* exerted strong growth-inhibiting effects against the *Xanthomonas* strains studied. Part of the results came from overlay bioassays others from Agar Diffusion tests.

Data are presented in **Table 16**

SCIENTIFIC NAME	NCAIM * CODE	Overlay bioassay, Diam. inactivation zone (mm)		Agar diffusion assay – diam. the inactivation zones in mm	
		EMA	EMC	EMA	EMC
Xanthomonas campestris pv. carotae	NCAIM B 01669.	51.00±1.22	48.50±11.67	NT	NT
Xanthomonas campestris pv. vesicatoria	NCAIM B 01857.	58.50±0.50	19.00±2.60	NT	NT
Xanthomonas arboricola pv. Juglandis (Szen 10)	-	NT	NT	17.94±1.83	24.77±1.40
Xanthomonas axonopodis pv. phaseoli	-	NT	NT	20.52±3.18	22.04±2.76
Xanthomonas axonopodis pv. phaseoli	-	NT	NT	20.52±3.18	22.04±2.76

Table 16. Growth inhibiting effects of *Xenorhabdus* antimicrobials on *Xanthomonas* strains in overlay bioassay and agar-diffusion tests. In different degree, but each strain proved sensitive. Abbreviations: EMA = *X. budapestensis*; EMC = *X. szentirmaii*.; NCAIM: National Collection of Agricultural and Industrial Microorganisms, Hungary.

4.1.1.2 Effects on Erwinia, Pectobacteria and Pantoea strains

Each *E. amylovora* strain was equally sensitive to antimicrobials of different EPB strains, especially to those of *X. budapestensis* and *X. szentirmaii* regardless their resistance to any other conventional antibiotics (**Table 17).**

SCIENTIFIC NAME	NCAIM * CODE	Streptomycin, 200 ug/ml	*X. nematophila*	*Photorhabdus luminescens* ssp.		
			N2-4	*laumondii* ARG	*akhurstii* IS5	*akhurstii* EG2
Erwinia amylovora	NCAIM B 01728	40±0.3	36±0.5	23±0.5	21±0-5	13±05
	Ea 88 *str*R	40±0.5	35±0.5	24±0.5	22±0.5	13±0.5
	Ea110 *rif* R	13±0.2	35±0.5	24±0.5	23±0.5	14±0.5
	Ea Ca 11 *str*R	26±02	36±0.5	30±0.5	22±0.5	13±5

Table 17. Growth inhibiting effects of different EPB antimicrobials on *E. amylovora* strains resistant to different conventional antibiotics - results of overlay bioassays

EPB antimicrobials were active against chloramphenicol resistant human pathogenic *Pantoea, Klebsiella pneumoniae* and mastitis isolation *Kle. pneumoniae* #696 (see Table 18). This facts support the idea of considering *Xenorhabdus*, antimicrobials as potential tools against poly-resistant harmful plant pathogens, belonging to *Agrobacterium tumefaciens, Clavibacter michiganense ssp michiganense, Curtobacterium flaccumfaciens pv. betae* and *Dyckeya chrysanthemi* species (see Fig. 8, Table 18).

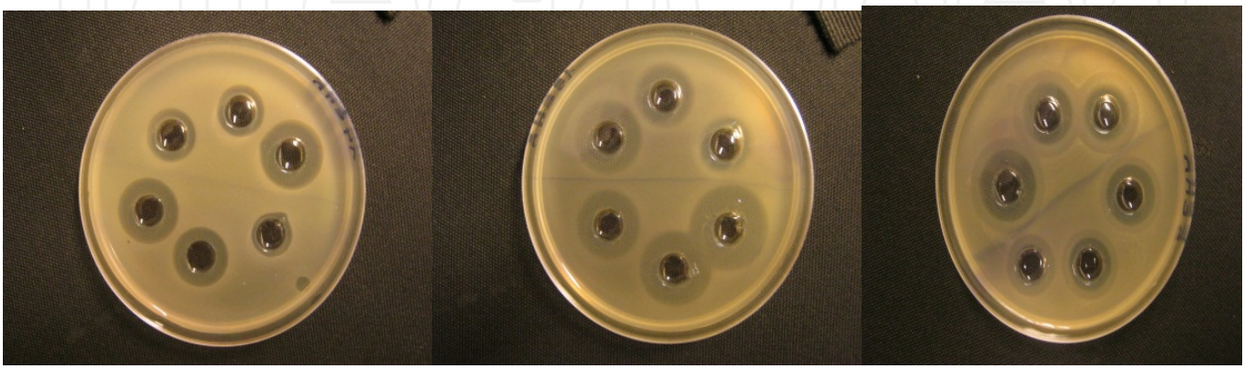

Fig. 8. Sensitivity of antibiotics resistant Gram-negative bacteria to the antibacterial activity of *Xenorhabdus budapestensis* ("North", "North" and "South-East", respectively, and *X. szentirmaii*. Test organism from left to right: Human pathogen *Klebsiella pneumoniae (HIP32)* (chloramphenicol resistant); *Erwinia amylovora* Ca 11(rif S, str R); *Pantoea agglomerans (*human pathogen, closely related to *Erwinia* species). See data in Tables 17 and 18.

SCIENTIFIC NAME	**NCAIM * CODE**	Overlay bioassay, Diam. inactivation zone (mm)		Agar diffusion assay – diam. the inactivation zones in mm	
		EMA	EMC	EMA	EMC
Erwinia amylovora Ea1	Ea1	44.00±0.50	43.33±1.44	20.79±0.69	24.82±1.13
Erwinia carotavora ssp. atroseptica	NCAIM B 01611	51.33±1.22	49.00±3.28	NT	NT
Pantoea agglomerans (related human pathogen)		NT	NT	17.43±4.81	17.84±0.83
Klebsiella pneumoniae (HIP32) chloramphenicol resistant		NT	NT	18.59±2.96	20.05±0.43
Dyckeya chrysanthemi		NT	NT	19.24±0.84	24.64±0.83
Agrobacterium tumefaciens	NCAIM B 01681	36.67 ±1.15	24.33 ±4.04	NT	NT
Clavibacter michiganense ssp michiganense	NCAIM B 01531.	47.33±2.33	NT	NT	NT
Curtobacterium flaccumfaciens pv. betae	NCAIM B 01612	34.16±0.76	47.67±2.08	19.18 ±0.88	23.10 ±0.09

Table 18. Growth inhibiting effects of *Xenorhabdus* CFCM on sensitive plant pathogenic and related bacteria. Abbreviations: EMA = *X. budapestensis*; EMC = *X. szentirmaii.*; NCAIM: National Collection of Agricultural and Industrial Microorganisms, Hungary. Home page: http://ncaim.uni-corvinus.hu Number of replicates: 3

4.1.1.3 Effects on Pseudomonas strains

The *Pseudomonas* strains reacted rather differently. Two independent strains *P. fluorescens* proved sensitive to *X. budapestensis* but hardly reacted to *X. szentirmaii* in overlay bioassay. Off the *Pseudomonas syringae* variants *pv. glycineae* proved far the most reactive and the reaction was similar toward the two CFCM. There were quantitative differences between the reaction of three *P. syringae pv. phaseolicola* isolates to the two CFCM. *P. syringae pv. syringae* double more sensitive to *X. budapestensis* than to *X. szentirmaii* in overlay bioassay but the results of agar diffusion assays did not differed significantly. Data are presented in **Table 19.** *P. syringae pv. savastanoi* proved completely inactive in repeated agar diffusion tests, but showed significant activity in overlay bioassays. *Pseudomonas corrugata, Pc 12, Burkholderia cepacia (syn. Pseudomonas cepacia),* as well as *Ralstonia solanacearum 1070, Ralstonia solanacearum 1240P. corrugata Pc 12* proved completely resistant to the antibacterial potential of both *X. szentirmaii* and *X. budapestensis* in agar diffusion tests.

4.2 Results on eukaryotic plant pathogens

4.2.1 Results on Oomycetales: Phytophthora and Pythium strains

In order to see the potential EPB antibiotics on eukaryotic plant pathogens, the anti-microbial activities of *X. szentirmaii* DSM 16638 (EMC) were assayed on some different *Phytophthora* and *Pythium* isolates qualitatively.

SCIENTIFIC NAME	**NCAIM * CODE**	Overlay bioassay, Diam. inactivation zone (mm)		Agar diffusion assay – diam. the inactivation zones in mm	
		EMA	EMC	EMA	EMC
Pseudomonas fluorescens	NCAIM B 01670	38.33 ±4.00	26.00 ±5.29	NT	NT
Pseudomonas fluorescens	NCAIM B 01667	41.00±2.00	19.33±3.05	NT	NT
Pseudomonas syringae pv. syringae	NCAIM B 01556	45.50±0.50	23.83±2.02	16.88±0.88	20.13±4.3
Pseudomonas syringae pv. phaseolicola	NCAIM B 01689	49.00±4.00	42.33±3.21	NT	NT
Pseudomonas syringae pv. phaseolicola	NCAIM B 01776	27.50±4.00	24.00±5.20	NT	NT
Pseudomonas syringae pv. phaseolicola	NCAIM B 01715	NT	45.17±4.25	NT	NT
Pseudomonas syringae pv. glycineae	NCAIM B 01574	48.75±10.04	45.33±3.05	NT	NT
Ps. syringae pv. lachrymans		NT	NT	22.54±1.58	23.97±1.75
Ps. syr. pv. morsprunorum		NT	NT	14.60±0.78	14.88±1.27

Table 19. Effects of *Xenorhabdus* CFCM on sensitive plant pathogenic *Pseudomonas*. Abbreviations: see Table 16.

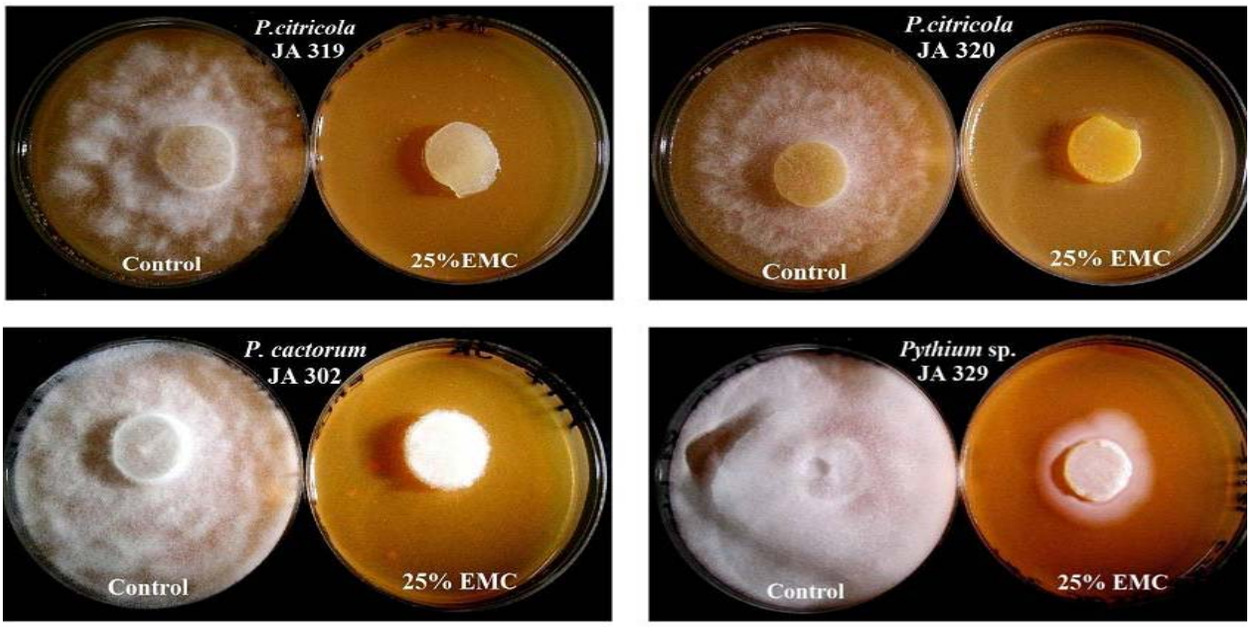

Fig. 9. Three different "Petri-plate phenotypes" showed of the extremely sensitive plant pathogen oomycetes treated with a low dose of *X. szentirmaii* CFCM on "poisoned carrot agar (PCA) plates.

As demonstrated on **Fig 9** each strain proved extremely sensitive to the presence of EPB antimicrobials in the "poisoned agar", even at low doses as 25 V/V%. There were three "phenotypes" of the plates. A). There were no mycelial growth (shown by in two independent isolate of *P. citricola*; B) There were some vertical growth from the disc, but no horizontal growth, (*P. cactorum*) indicating, that the antimicrobial compound was so cytotoxic, that it killed each cell which got contact to the agar, but the active compounds could not penetrate through the disc; C). Some *Pythium* was able for a horizontal growth. These data may provide information about reversibility or irreversibility of the treatment concerning a given pathogen species. In these studies we used 25 V/V % of CFCM in Petri plates of 5.5cm and 8.5 mm diameter, respectively. A CA-disc of 1-cm diameter with *Phytophthora* (*Pythium*) mycelia was put in the center of the plates and the growth of the colonies was evaluated 5 days thereafter. The radial mycelial growth was completely inhibited. Once a mycelium got in contact with the agar containing the antibiotics it died. The vertical growth of the mycelia was not inhibited, indicating, that agar disc on which the colonies were grown acted as a barrier (**Table 20**). The different doses were also compared.

We found that 75 V/V %- of the conditioned media resulted in complete inhibition.

50 V/V %- of the conditioned media did not result in complete inhibition of isolates JA 320 (*P. citricola*) and JA 301-es (*Pythium* sp.). The growth retardation were ~ 35% in comparison to the control (100 %). 25 V/V %- of the conditioned media did result in complete inhibition of three isolates: JA 320 (*P. citricola*) and JA 301-es (*Pythium* sp.) growth retardation were ~ 54%, while that of JA 317 Pythium 34 % in comparison to the control (100 %).

Phytophthora Pythium strains	Treatment					
	Control			25% EMC Carrot-Agar		
P. citricola JA 320	+++	+++	+++	++	+	-
P. citricola JA 319	+++	+++	+++	-	-	-
P. plurivorum JA 309	+++	+++	+++	-*	-*	-*
P. cactorum JA 302	++++	++++	+++	-**	-**	-**
Pythium sp. JA 317	++++	++++	++?	-*	+*	-*
Pythium sp. JA 329	++++	+++	+++	-*	+*	-*
Pythium sp. JA 301	++++	++++	+++	++	+	-

Table 20. Qualitative analysis of the antimicrobial activity of EMC on the isolates on the 5th day

The quantitative data (diameters of the growing colony) determined on the 5th day of the experiment concerning *X. budapestensis* are summarized in **Table 21.** The original diameter of the mycelia disc that had been initially placed at the centre of the Petri-dish was 10.0 mm on the start (0) day. We found that 75 V/V %- of the conditioned media resulted in complete inhibition. 50 V/V %- of the conditioned media did not result in complete inhibition of two isolates: In case of JA 320 (*P. citricola*) the retardation rate was ~39 %, while that of and JA 301 (*Pythium* sp.) growth retardation were ~ 28% in comparison to the control (100 %).

Isolate	Scientific name	EMA experiment: Diameter of the CA discs with mycelia			
		0 (Control)	25 V/V%	50 V/V %	75 V/V%
JA319	*Phytophthora citricola*	37.0	10.0	10.0	10.0
JA320	*Phytophthora citricola*	44.5	21.0	18.0	10.0
JA302	*Phytophthora cactorum*	52.0	10.0	10.0	10.0
JA309	*Phytophthora plurivora*	49.5	16.0	10.0	10.0
JA317	*Pythium sp*	52.5	10.0	10.0	10.0
JA301	*Pythium sp.*	42.0	10.0	10.0	10.0
JA301	*Pythium sp.*	51.0	24.0	14.0	10.0

Table 21. Effects of CFCM of *X. budapestensis* on the growth of *Phytophthora* and *Pythium* isolates (on the size of colonies of the test organisms) on the 5th day.

4.2.1.1 The kinetics of mycelial growth of Phytophthora and Pythium strains

Altogether 8 *Phytophthora* species (see **Tables 21** and **22**) were involved in the experiments aimed at determining the time and dose dependence of the anti-oomycetal activities of the intact CFCM of *X. szentirmaii* and *X. budapestensis*.

As for *Xenorhabdus szentirmaii* (EMC), on the third day, its CFCM exhibited complete inhibition in all dilutions except for *P. nicotianae* JA 168, *P. pelgrandis* JA 337 and *P. megasperma* JA 209 which showed some growth at 10% v/v. *P. pelgrandis* JA 337 appeared to depict much growth when compared to the control. On the 6th day, there were some little growths with *P. nicotianae* JA 168 and *P. megasperma* JA 209 at 20% v/v but *P. citrophthora* JA 479, *P. nicotianae* H-1/00, *P. cactorum* P163 and *P. cinnamoni* JA 153 could not show any growth even at 10 V/V %. On the 9th day, some growth could be seen with the *P. nicotianae* JA 168, *P. nicotianae* H-1/00, *P. cinnamoni,* and JA 153 and *P. megasperma* JA 209 at 20% v/v. *P. citrophthora* JA 479 and *P. pelgrandis* JA 337 could not show any growth even at 10% v/v. On the 14th day, only *P. cinnamoni* JA 153 and *P. megasperma* JA 209 could show some growth at 20% v/v and above but no strain exhibited any growth at 40% v/v.

As for *Xenorhabdus budapestensis* (EMA), on the third day its CFCM allowed some growth for *P. citrophthora* JA 479, *P. pelgrandis* JA 337, *P. cinnamoni* JA 153 and *P. megasperma* JA 209 could at 10 and 20 % v/v but no strain exhibited any growth at 30% v/v and above. On the 6th day only *P. citrophthora* JA 479, *P. cinnamoni* JA 153 and *P. megasperma* JA 209 had showed some growth at 20% v/v with *P. nicotianae* JA 168 and *P. cactorum* P163 not depicting any growth at all even at 10% v/v. On the 9th day, only *P. nicotianae* JA 168 and *P. nicotianae* H-1/00 did not show any growth at 20% v/v but there was no growth at all at 30 and 40% v/v. On the 14th day, only *P. citrophthora* JA 479, *P. nicotianae* JA 168 and *P. megasperma* JA 209 showed growths at 30 and 40% v/v.

4.2.1.2 Anti-oomycetal activity quantitatively be adsorbed by Amberlite XAD 1148R

We found that autoclaved sample of Amberlite XAD 1148R could quantitatively adsorb the anti-oomycetal activities from the CFCM of *X. szentirmaii* when used according to the manufacturer's suggestion (**Fig 10**).

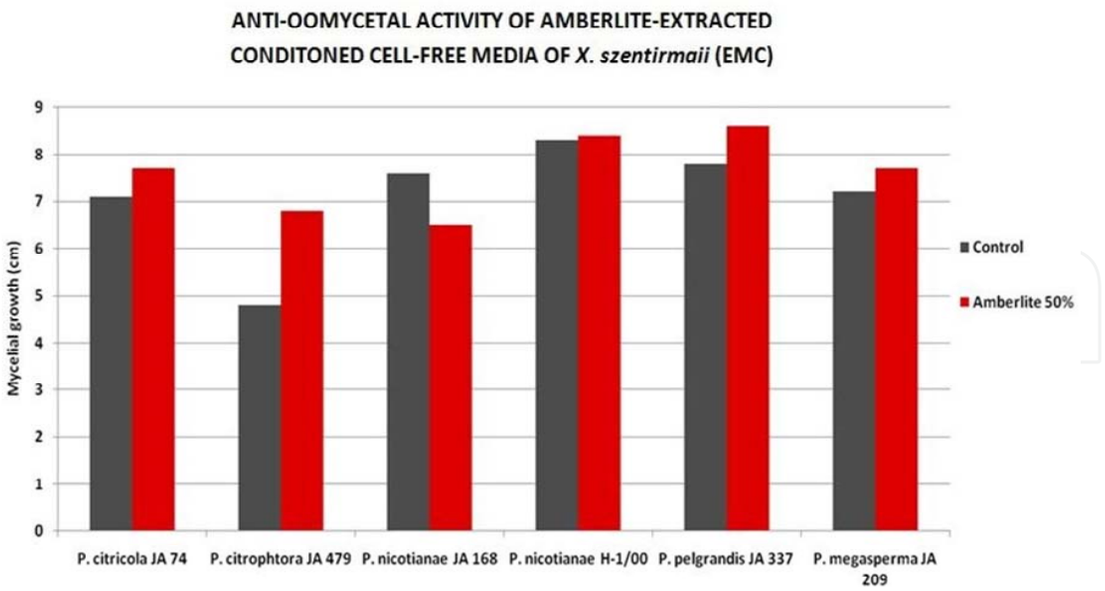

Fig. 10. Growth of *Phytophthora* citricola (JA74, JA 479), *P. nicotianae* (JA168 and H-1/100), *P. pelgrandis* (JA337) and *P. megasperma* (JA 209) on untreated CA plates (control, black) and CA media containing 50 V/V% CFCM from *Xenorhabdus szentirmaii* which had been incubated with sterile autoclaved Amberlite-XAD 1148R O/N.

	Treatments: ***X. budapestensis*** CFCM in 2X LB liquid media (V / V %)				
3rd day					
	0	10	20	30	40
P. citricola JA 74	48.0	10.0	10.0	10.0	10.0
P. citrophthora JA 479	54.0	14.0*	15.0*	10.0	10.0
P. nicotianae JA 168	37.0	10.0	10.0	10.0	10.0
P. nicotianae H-1/00	52.0	10.0	10.0	10.0	10.0
P. cactorum P163	48.0	10.0	10.0	10.0	10.0
P. pelgrandis JA 337	46.0	14.0*	10.0	10.0	10.0
P. cinnamoni JA 153	55.0	18.0*	14.0*	10.0	10.0
P. megasperma JA 209	42.0	18.0*	10.0	10.0	10.0
6th day					
P. citrophthora JA 479	48.0	10.0	10.0	10.0	10.0
P. nicotianae JA 168	54.0	14.0*	15.0*	10.0	10.0
P. nicotianae H-1/00	37.0	10.0	10.0	10.0	10.0
P. cactorum P163	52.0	10.0	10.0	10.0	10.0
P. pelgrandis JA 337	48.0	10.0	10.0	10.0	10.0
P. cinnamoni JA 153	46.0	14.0*	10.0	10.0	10.0
P. megasperma JA 209	55.0	18.0*	14.0*	10.0	10.0
9th th day					
P. citricola JA 74	80.0	29.0*	26.0*	10.0	10.0
P. citrophthora JA 479	82.0	55.0*	35.0*	10.0	10.0
P. nicotianae JA 168	88.0	18.0*	10.0	10.0	10.0
P. nicotianae H-1/00	88.0	37.0*	10.0	10.0	10.0
P. cactorum P163	78.0	23.0*	15.0*	10.0	10.0
P. pelgrandis JA 337	82.0	43.0*	19.0*	10.0	10.0
P. cinnamoni JA 153	88.0	48.0*	42.0*	10.0	10.0
P. megasperma JA 209	88.0	32.0*	10.0	10.0	10.0
14th day					
P. citricola JA 74	84.0	38.0*	30.0*	10.0	10.0
P. citrophthora JA 479	88.0	64.0*	44.0*	38.0*	29.0*
P. nicotianae JA 168	88.0	25.0*	35.0*	15.0*	15.0*
P. nicotianae H-1/00	88.0	49.0*	47.0*	10.0	10.0
P. cactorum P163	80.0	35.0*	19.0*	10.0	10.0
P. pelgrandis JA 337	88.0	55.0*	28.0*	10.0	10.0
P. cinnamoni JA 153	88.0	62.0*	59.0*	10.0	10.0
P. megasperma JA 209	88.0	54.0*	52.0*	49.0*	50.0*

Table 22. Diameter of the *Phytophthora* colonies (mm) at different *X. budapestensis* CFCM doses at different days of the experiment

	Treatments: ***X. szentirmaii*** CFCM in 2X LB liquid media (V / V %)				
3rd da					
	0	10	20	30	40
P. citricola JA 74	48.0	10.0	10.0	10.0	10.0
P. citrophthora JA 479	60.0	10.0	10.0	10.0	10.0
P. nicotianae JA 168	36.0	19.0*	10.0	10.0	10.0
P. nicotianae H-1/00	60.0	10.0	10.0	10.0	10.0
P. cactorum P163	50.0	10.0	10.0	10.0	10.0
P. pelgrandis JA 337	45.0	22.0*	10.0	10.0	10.0
P. cinnamoni JA 153	50.0	10.0	10.0	10.0	10.0
P. megasperma JA 209	40.0	15.0*	10.0	10.0	10.0
6th day					
P. citrophthora JA 479	84.0	10.0	10.0	10.0	10.0
P. nicotianae JA 168	58.0	28.0*	13.0*	10.0	10.0
P. nicotianae H-1/00	88.0	10.0	10.0	10.0	10.0
P. cactorum P163	82.0	10.0	10.0	10.0	10.0
P. pelgrandis JA 337	70.0	22.0*	10.0	10.0	10.0
P. cinnamoni JA 153	80.0	10.0	10.0	10.0	10.0
P. megasperma JA 209	86.0	22.0*	16.0*	10.0	10.0
9thth day					
P. citricola JA 74	62.0	30.0*	10.0	10.0	10.0
P. citrophthora JA 479	88.0	10.0	10.0	10.0	10.0
P. nicotianae JA 168	62.0	36.0*	26.0*	10.0	10.0
P. nicotianae H-1/00	88.0	24.0*	11.0*	10.0	10.0
P. cactorum P163	88.0	10.0	10.0	10.0	10.0
P. pelgrandis JA 337	76.0	35.0*	10.0	10.0	10.0
P. cinnamoni JA 153	88.0	19.0*	14.0*	10.0	10.0
P. megasperma JA 209	88.0	28.0*	21.0*	10.0	10.0
14th day					
P. citricola JA 74	72.0	45.0*	10.0	10.0	10.0
P. citrophthora JA 479	88.0	21.0*	10.0	10.0	10.0
P. nicotianae JA 168	66.0	43.0*	10.0	10.0	10.0
P. nicotianae H-1/00	88.0	34.0*	10.0	10.0	10.0
P. cactorum P163	82.0	13.0*	10.0	10.0	10.0
P. pelgrandis JA 337	82.0	44.0*	10.0	10.0	10.0
P. cinnamoni JA 153	88.0	33.0*	29.0*	20.0*	10.0
P. megasperma JA 209	88.0	41.0*	36.0*	30.0*	10.0

Table 23. Diameter of the *Phytophthora* colonies (mm) at different *X. szentirmaii* CFCM doses at different days of the experiment

4.2.2 Results on fungi: Botrytis, Alternaria and Fusarium strains

The *in vitro* antifungal activities of the cell-free filtrates of *X. szentirmaii* and *X. budapestensis* were assayed against different species of phytopathogenic fungi that are common in plant fungal infections and are known as major causes of disease in agriculture crops.

These include *Botrytis cinerea, Alternaria alternata,* and *Fusarium graminearum*. The three fungi reacted rather differently.

Depending on the concentration, the mycelial growth ceased. *Botrytis cinerea* ceased growing at 25 V/V % of the media, while the controls grew over the plates (of 9.5 mm diameter).

The reaction of *A. altera* to the antifungal compounds of *X. budapestensis* was different (**Fig 11**). While the untreated controls, similarly to those of *Fusarium* overgrew the plates, the *A. altera* colonies on the "poisoned" agar plates were less then a third in size.

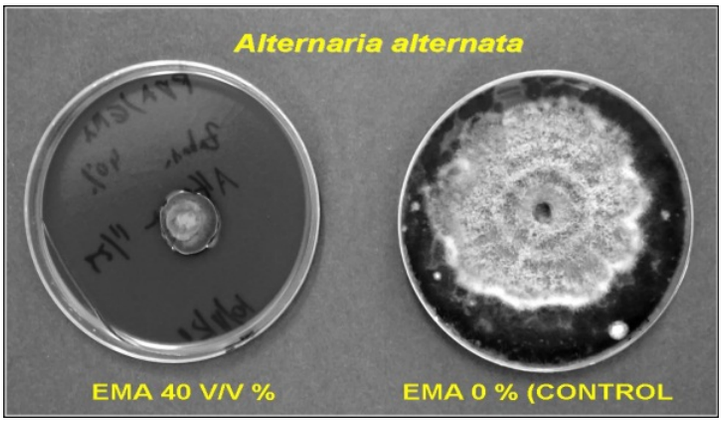

Fig. 11. 3-day-old poisoned (left) and control PDA plates containing 40 V/V % of *X. budaestensis* (EMA) CFCM and an *Alternaria aternata* inoculum (Photo: Dr. Csaba Pintér).

The mycelial growth was very poor, but the number of conidia was significantly higher in the treated plates than in the control plates in one week. Both the growth retardation and the conidial production was dose dependent.

F. gramineae controls overgrew the Petri plates within 3 days. During this time there is only a retarded growth could be observed at higher CFCM concentrations (**Fig 12)**

Fig. 12. 3-day-old poisoned (left) and control PDA plates containing 40 V/V % of *X. budaestensis* (EMA) CFCM and an *Fusarium graminarum* inoculum (Photo: Dr. Csaba Pintér).

Finally, the growth was complete at each concentration, but speed of the growth depended on the concentration, as demonstrated in **Fig 13**.

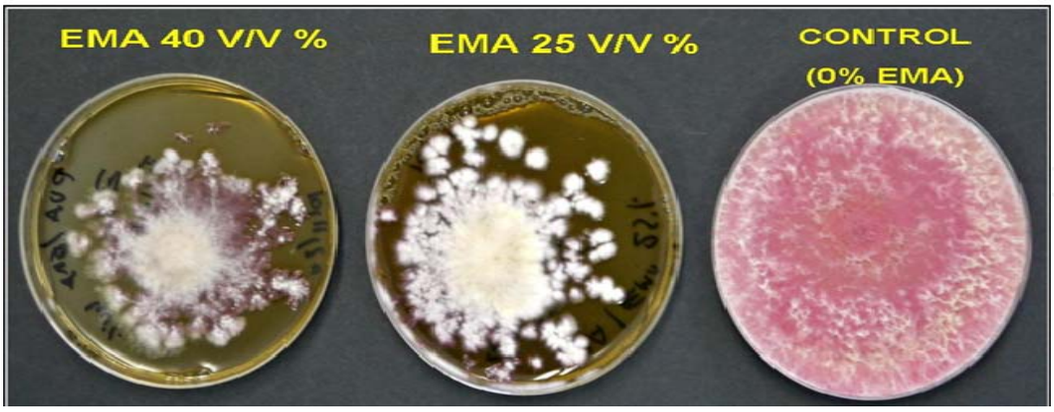

Fig. 13. Demonstration the reversible antifungal potential of the CFCM of *Xenorhabdus budaestensis* (EMA) on *Fusarium gramineae* on "poisoned" Potato Dextrose Agar Plate. (Photo: Dr. Csaba Pintér).

4.2.2.1 Reversible and irreversible antifungal activity

Our data suggest that either the active compounds in the CFCM, which exert antibacterial, anti-oomycetal or anti-fungal activities are probably different. An argument for this hypothesis is that the antibacterial activity of *X. budapestensis* was stronger but the anti-oomycetal effects were somewhat weaker then that of *X. szentirmaii*. Both compounds exerted an irreversible and strong fungicide effects on *Botrytis* while the effect on the growth of *Fusarium* proved temporary and reversible. In general, the antifungal effects of *X. szentirmaii* were more convincing. The effects concerning *X. szentirmaii* are shown on **Figs 14, 15, and 16.** An the basis of data below, we concluded that *Xenorhabdus szentirmaii* produces compound(s) which might be useful in controlling both *Botrytis* and *Alternaria* in a proper concentration, in proper way. *Alternaria*, however, may have a potential of adapting a survival strategy through sporulation.

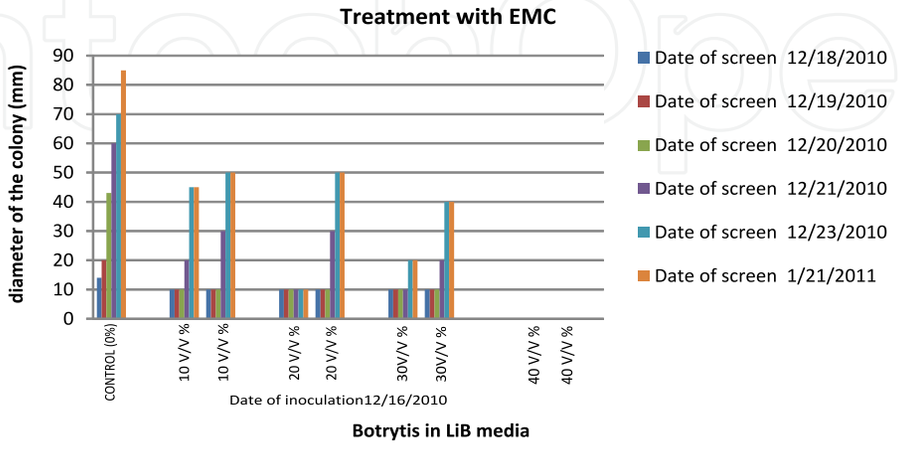

Fig. 14. Intact CFCM of *X. szentirmaii* inhibited completely the growth of *B. cinerea* at least for two weeks up to 30 V/V%. The largest dose (40 V/V %) quantitatively destroyed the pathogen.

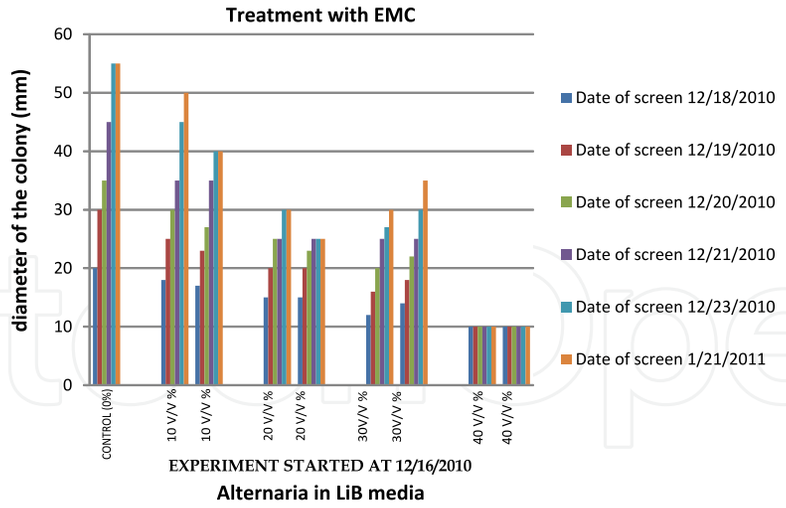

Fig. 15. Intact CFCM of *X. szentirmaii* significantly slowed down the growth of *Alternaria alternata* in Lima Bean agar media.

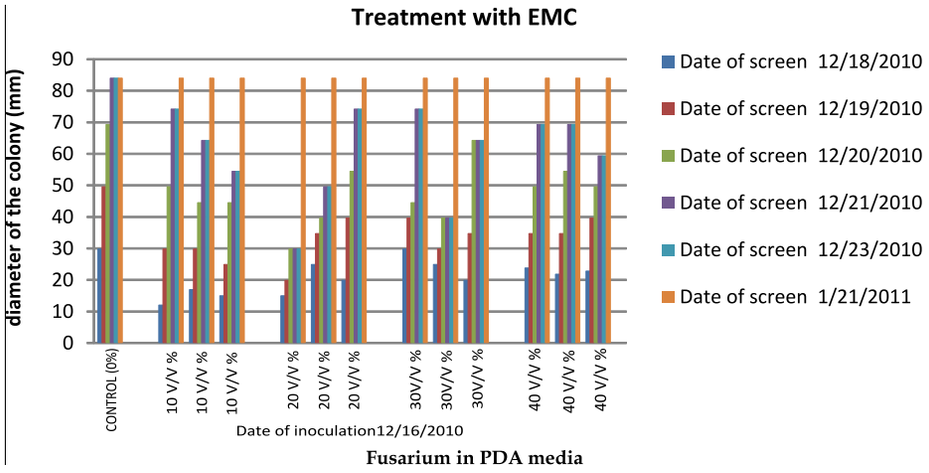

Fig. 16. Intact CFCM of *X. szentirmaii* exerted only temporary slowing effects on the growth of *F. gramineae* in As for *Alternaria alternata*, the speed of the growth as well as the final size of the colony was more or less dose-dependent up to 30 V/V %. The largest dose (40 V/V %) quantitatively destroyed the pathogen. The results are slightly influenced by the media. The antifungal effects are unambiguously (significantly) stronger in PDA than in LiBA media (data not shown).

As for *Fusarium graminearum*, only the speed but not the final size of the colonies proved dose-dependent. Even the largest dose (40 V/V %) could not destroy the pathogen. The results are slightly influenced by the media. The antifungal effects are unambiguously (significantly) stronger in PDA than in LiBA media (data not shown).

4.4 Preliminary results of genetic analysis

4.4.1 A search for antibiotics non-producer mutants in *X. budapestensis*

As for our mutant hunting in *X. budapestensis* we altogether tested 38 of the 177 *rif*R *k*anR transconjugants for antibiotics their antibiotics hypo, - or hyper-production **(Fig 17).**There were 26, which could not show any antibiotics production in our experimental conditions.

We considered them as antibiotics non-producers with inactivate structural gene(s). There were also 13 (# 15, 69, 70, 73, 74, 75, 76, 88, 90, 91, 92, 93 and 135) overproducers. They should be carefully retested. Amongst them, we are searching for regulatory mutants. Those which antibiotics production was uncertain discarded.

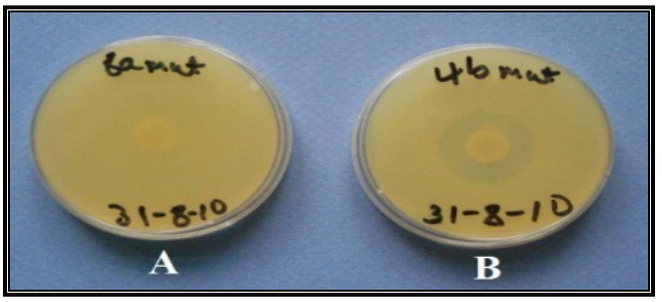

Fig. 17. Overlay bioassay mutant antbiotics non-producing mutant (left, A), and a low producing mutant (B)

4.4.2 A search for exocrystal mutants in *X. szentirmaii*

This unique natural product of *X. szentirmaii* (**Fig 18 and 19)** was made accessible from the recently described *X. szentirmaii* (Fodor et al., 2007). The surface of the colonies of *X. szentirmaii* has a striking phenotype by their purple metallic color.

Fig. 18. *Xenorhabdus szentirmaii* colonies on LBA (Left)) and LBTA (Medium) plates; Crystals on the surface of a colony, 40X magnification in through Leica stereomicroscope (Right) Photo: A. Máthé-Fodor

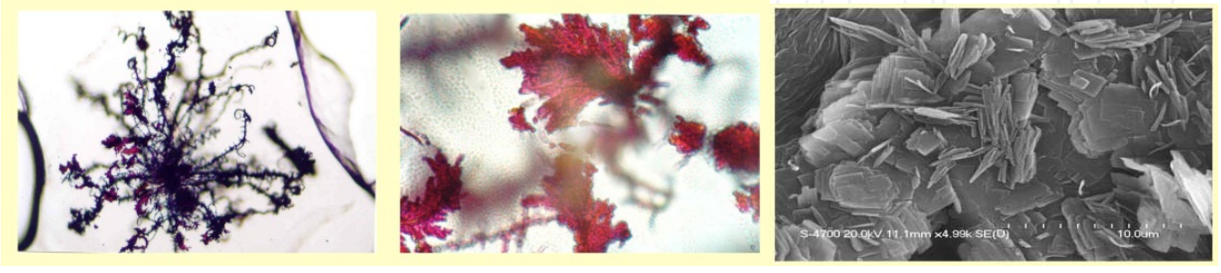

Fig. 19. Isolated antibiotic poly-iodinin crystal from the agar light microscopy, 125 X (D) and 1,000X (E) and with SEM (S-4700 20.0 kV 11.1 mmX4.99 SE. Photo: A. Máthé-Fodor. The crystal was isolated by using a double layer of sterile cellophane covering an LB plate and over-layered with bacterium suspension. This pigment crystal was identified by Haynes and Zeller as iodinin, (Fodor et al., 2008.

We altogether isolated 22 crystal antimicrobial crystal mutants from *X. szentirmaii* **(Fig 20).**

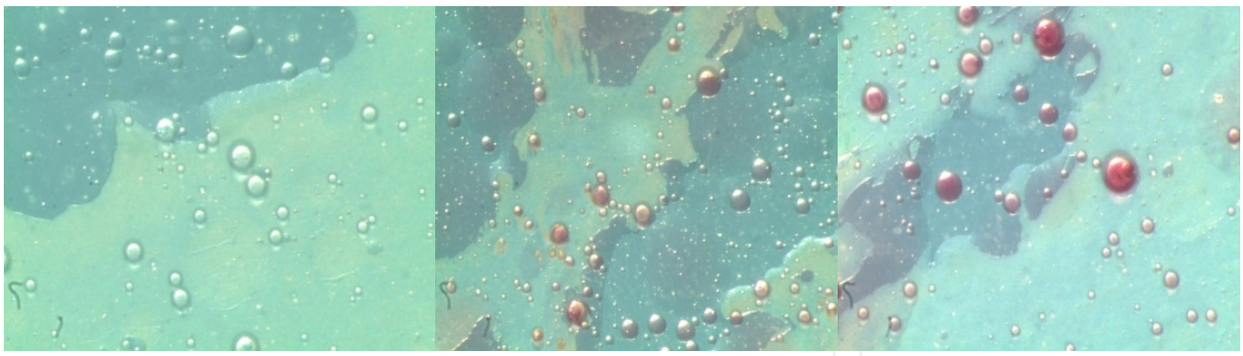

Fig. 20. Light mutant produce colorless (Left); WT produces colored (Medium) and dark mutant (Right) produce dark oligomer soluble in the oil of ENGM plate (Fodor et al., 2010b). The antibiotic pigment crystal was discovered and isolated by A. Máthé-Fodor in 2003). The structure was identified by Haynes and Zeller as iodinin, (see: Fodor et al., 2007).

5. Conclusions and perspectives

We have to think about new perspectives of antimicrobials produced by entomopathogenic bacteria. It should be a rational explanation why the intact cell-free media of some *Xenorhabdus* strains is so powerful against different microorganisms, while the isolated (and patented, Webster et al., 1996) compounds are useless from practical aspect (Fodor et al., 2008). The cell-free conditioned media (CFCM) of both *Xenorhabdus szentirmaii* and *X. budapestensis* is a mixture of natural compounds, which are successfully used in soil condition against the most different pathogens. Interestingly, none of the single isolated, identified, patented and re-synthesized compound reached the market. The list of them is given by Dr. Bode (2009, **Fig. 21**). The authors admitted (Brachmann et al., 2006; Susa et al., 2008) that the isolated "antibiotics" proved rather weak in different bioassays.

On the other hand, the intact media, at least those of *X. szentirmaii* and *X. budapestensis* are quite effective against different bacteria, including multi-resistant plant, human pathogens (including MRSA, data are not given), mastitis pathogens (Furgani et al., 2008), plant parasitic oomycetae and fungi. They are effective against the plant pathogenic protozoon *Leichmania donovani* (McGwire et al., unpublished data). We also found that the different Xenorhabdus species uses different chemical "weapons" when competing with each other (Fodor et al., 2010a) indicating that the "toolkit" needed for survival in the nature is more complex and probably need synergistic interactions. The data presented here seen, that majority of the PPB was rather sensitive, they proved some resistant. All the tested *Xanthomonas, Erwinia* and *Pectovora* strains were extreme sensitive, while *Ralstonia* proved resistant and so some *Pseudomonas* strains. The availability of both sensitive and resistant *Pseudomonas* strains pride an option of genetic analysis of action mechanism of EPB antimicrobial in plant pathogen bacteria.

Badosa et al (2007) developed and screened a 125-member library of synthetic linear undecapeptides based on a previously described peptide H-K(1)KLFKKILKF(10)L-NH(2) (BP76) that inhibited *in vitro* growth of the plant pathogenic bacteria *Erwinia amylovora, Xanthomonas axonopodis pv. vesicatoria*, and *Pseudomonas syringae* pv. *syringae* at low

micromolar concentrations. Peptides were designed using a combinatorial chemistry approach by incorporating amino acids possessing various degrees of hydrophobicity and hydrophilicity at positions 1 and 10 and by varying the N-terminus. Library screening for *in vitro* growth inhibition identified 27, 40 and 113 sequences with MIC values below 7.5 microM against *E. amylovora, P. syringae* and *X. axonopodis*, respectively. Cytotoxicity, bactericidal activity and stability towards protease degradation of the most active peptides were also determined. Seven peptides with a good balance between antibacterial and hemolytic activities were identified. Several analogues displayed a bactericidal effect and low susceptibility to protease degradation. The most promising peptides were tested in vivo by evaluating their preventive effect of inhibition of E. amylovora infection in detached apple and pear flowers. The peptide H-KKLFKKILKYL-NH(2) (BP100) showed efficacies in flowers of 63-76% at 100 microM, being more potent than BP76 and only less effective than streptomycin, currently used for fire blight control. We suppose that our natural compounds are chemical relations with this peptide family. The only sequenced oligopeptide (bicornutin A, Böszörményi et al., 2009) what we found in *X. budapestensis* is a hexapeptide but quite different from those described by Badosa et al (2007). Linear and cyclic peptides have recently been discovered in *Xenorhabdus* species by Lang et al (2008).

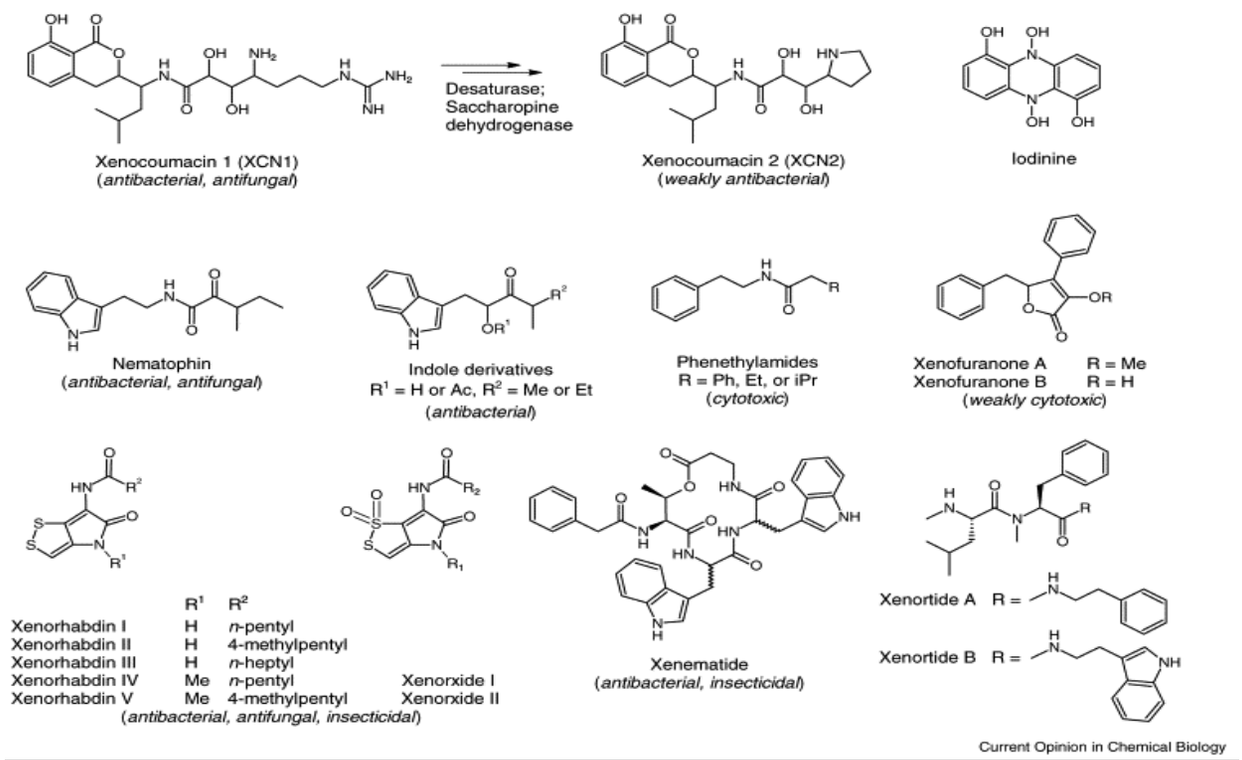

Fig. 21. EPB antibiotics, which had so far been identified (Bode, 2009)

Oomycetes from the genus *Phytophthora* are fungus-like plant pathogens that are devastating for agriculture and natural ecosystems. Due to their particular physiological characteristics, no efficient treatments against diseases caused by these microorganisms are presently available. To develop such treatments, it appears essential to dissect the molecular mechanisms that determine the interaction between *Phytophthora* species and host plants.

Available data are scarce, and genomic approaches were mainly developed for the two species, *Phytophthora infestans* and *Phytophthora sojae*.

However, these two species are exceptions from, rather than representative species for, the genus. *P. infestans* is a foliar pathogen, and *P. sojae* infects a narrow range of host plants, while the majority of *Phytophthora* species are quite unselective, root-infecting pathogens Attard et al., 2008). *Phytophthora ramorum*, causal agent of sudden oak death, is an emerging plant pathogen first observed in North America (Knight, 2002); associated with mortality of tanoak (*Lithocarpus densiflorus*) and coast live oak (*Quercus agrifolia*) in coastal forests of California during the mid-1990s. (Rizzo et al., 2005). The pathogen is now known to occur in North America and Europe and have a host range of over 40 plant genera. (The anti-oomycetal of the CFCM of *X. szentirmaii* and *X. budapestensis* make these two bacteria as potential tools in fighting Phytophthora, especially in forests, in the rhizosphere of the trees.

In the last half century, antibiotics revolutionized the human medicine, veterinary science, animal breeding and agricultural plant medicine. The use of antibiotics without any control, however, resulted in serious problems, mainly due to the massive selection for antibiotics resistant and multi-resistant pathogens. Gram-positive organisms are the most common bacterial pathogens that cause diseases in humans, with streptococci and staphylococci occurring most frequently. Immunization has been extremely successful in eradicating some Gram-positive infections, such as diphtheria and tetanus, and relatively successful for pneumococci. *Staphylococcus aureus* vaccines are under investigation. In terms of antimicrobial susceptibility, some Gram-positive organisms have remained sensitive to most antimicrobials, whereas others, including staphylococci, pneumococci and enterococci, have developed clinically relevant resistance. Extensive exposure to antimicrobials in the hospital setting has caused the spread of clones mainly in the hospital environment, yet multiresistance is now also found in community-acquired diseases. Community-acquired methicillin-resistant S. aureus (CA-MRSA) and resistant pneumococci are the most important examples, but even viridans streptococci are becoming resistant to some antibiotics. Moreover, MRSA and vancomycin-resistant enterococci (VRE) are found in pets and farm animals. Because of these concerns, new antimicrobials have been developed during the past decade, including quinupristin/dalfopristin, linezolid, trigecycline, daptomycin and dalbavancin. Also under investigation are beta-lactams, streptogramins and quinolones with activity against MRSA, penicillin-resistant pneumococci and VRE. Finally, infection-control measures, including the identification of carriers of multiresistant organisms and appropriate isolation, must continue to be implemented (Metzger et al., 2009). Increasing incidence of infections caused by poly-resistant organisms is associated with prolonged hospital stays, increased health care costs, and increased morbidity and mortality (Dötsch et al., 2009). When using a molecule antibiotics, it is only the matter of time when the first resistant pathogen arises and then the use of the antibiotics will be as a selective factor. Biofilm formation of some pathogens also act against the efficacy of traditional antibiotics (Kustos et al., 2005).Furthermore, in the past antibiotics were also used in an uncontrolled manner in animal husbandry. For instance, antibiotics were used in poultry for many years for their growth promoting effects.

Antibiotics growth promoters mostly have their effects by modifying the intestinal microbiota, targeting mainly Gram-positive bacteria, which are associated with poor health and performance of the animals (Bredford, 2000). Taking into account this typical spectrum

of activity of the antibiotics used as growth promoters, and taking into account that the concentrations used in feed are below the minimum inhibitory concentrations as tested *in vitro*, one may expect any beneficial effect of growth promoter against intestinal carriage of zoonotic agents. Most of zoonotic agents found, for instance, in the chicken gastro-intestinal track are Gram-negatives.

Antibiotics have been used since the 1950s to control certain bacterial diseases of high-value fruit, vegetable, and ornamental plants. Today, the antibiotics most commonly used on plants are oxytetracycline and streptomycin. In the USA, antibiotics applied to plants account for less than 0.5% of total antibiotic use. Resistance of plant pathogens to oxytetracycline is rare, but the emergence of streptomycin-resistant strains of Erwinia amylovora, *Pseudomonas* spp., and *Xanthomonas campestris* has impeded the control of several important diseases. A fraction of streptomycin-resistance genes in plant-associated bacteria are similar to those found in bacteria isolated from humans, animals, and soil, and are associated with transfer-proficient elements. However, the most common vehicles of streptomycin-resistance genes in human and plant pathogens are genetically distinct. Nonetheless, the role of antibiotic use on plants in the antibiotic-resistance crisis in human medicine is the subject of debate (MacManus et al., 2002)

The multi-resistance in Gram-negative bacteria is more frequently efflux-mediated (Poole, 2004). The integral inner membrane resistance-nodulation-division (RND) components of three-component RND-membrane fusion protein-outer membrane factor multidrug efflux systems define the substrate selectivity of these efflux systems. To gain a better understanding of what regions of these proteins are important for substrate recognition, a plasmid-borne mexB gene encoding the RND component of the MexAB-OprM multidrug efflux system of Pseudomonas aeruginosa was mutagenized *in vitro* by using hydroxylamine and mutations compromising the MexB contribution to antibiotic resistance identified in a ΔmexB strain (Sobel et al., 2003). Of 100 mutants that expressed wild-type levels of MexB and showed increased susceptibility to one or more of carbenicillin, chloramphenicol, nalidixic acid, and novobiocin, the mexB genes of a representative 46 were sequenced, and 19 unique single mutations were identified. While the majority of mutations occurred within the large periplasmic loops between transmembrane segment 1 (TMS-1) and TMS-2 and between TMS-7 and TMS-8 of MexB, mutations were seen in the TMSs and in other periplasmic as well as cytoplasmic loops. By threading the MexB amino acid sequence through the crystal structure of the homologous RND transporter from Escherichia coli, AcrB, a three-dimensional model of a MexB trimer was obtained and the mutations were mapped to it.

Unexpectedly, most mutations mapped to regions of MexB predicted to be involved in trimerization or interaction with MexA rather than to regions expected to contribute to substrate recognition. Intragenic second-site suppressor mutations that restored the activity of the G220S mutant version of MexB, which was compromised for resistance to all tested MexAB-OprM antimicrobial substrates, were recovered and mapped to the apparently distal portion of MexB that is implicated in OprM interaction. As the G220S mutation likely impacted trimerization, it appears that either proper assembly of the MexB trimer is necessary for OprM interaction or OprM association with an unstable MexB trimer might stabilize it, thereby restoring activity (Middlemiss & Poole (2004).

Due to the fatal consequences the prophylactic or curative use of antibiotics more and more prohibited by EU regulations, such as Nº 2160/2003, amended by EU Nº 1091/2005 regulation in the agriculture since January, 2006. The use of "ionophore" class of antibiotics (stereo isomers of sacred water) is permitted, but their efficacy is rather questionable (Van Immerseel et al., 2002). The non-digestible feed ingredients, mainly saccharids, called prebiotics are used to make the host less vulnerable from some pathogens. Probably cannot replace antibiotics either. The tendency is to use complex materials with more than one mode of action. The permission of using probiotics, defined as living microbial feed supplements, which beneficially affect the host by improving its microbial balance (Van Immerseel et al., 2010) however, may provide an option to replace antibiotics by some natural factors with similar efficacy and reducing the risk of selecting for poly-resistant pathogens. Herein, we provided an example of a natural product of potential use against different plant, veterinary and even human pathogens. The perspective is to develop a "probiotic" microorganism, which carries (but cannot transmit) those genetic units of *Xenorhabdus budapestensis* and /or *X. szentirmaii*, which were a natural component, such as *L. salivations* (Pascual et al., 1999) of the niche where should be applied, and the genetic construction would be cloned downstream of an inducible bacteria promoter. As a first step toward this direction, we made the following: We have altogether isolated 22 antimicrobial crystal mutants from *X. szentirmaii* and a few antibiotics non-producers from *X. budapestensis*. The antibiotics mutants should be retested. As for the crystal mutants, we followed the protocol of Prof. Heidi Goodrich-Blair (University of Wisconsin-Madison). We have started the following experiment:

We have isolated genomic DNA from each and digested with restriction enzymes *Xba*I, *Knp*I, *Spe*I, *and SalI* respectively. We also digested pBluescript SK vector with the same enzyme. We ligated the fragments into pBluescript SK vector, which is AmpR. The fragments digested with different enzyme from a given mutants were united. The salts were removed by dialysis. The DNA was electroporated into *E. coli* DH5 alpha. We were selecting on ampicillin (150) kanamycin (50) LB plates. The-double resistant clones were grown in 3 ml LB + Km and plasmids were isolated, digested with *Eco*RI. The lengths of the fragments were determined and then sequenced.

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