TAXONOMIC IDENTIFICATION OF BACTERIA, ASSOCIATED WITH BULGARIAN POPULATIONS OF ENTOMOPATHOGENIC NEMATODES FROM GENUS STEINERNEMA (RHABDITIDA, STEINERNEMATIDAE) II

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ABSTRACT
The entomopathogenic nematode from the genera Steinernema and Heterorhabditis are symbiotically associated with bacteria from the genera Xenorhabdus and Photobacterium. The bacteria belong to the family Enterobacteriaceae; pathogenic when injected into the hemocoel of Galleria mellonella (Lepidoptera) larvae. All Xenorhabdus and Photobacterium isolates have been shown to produce two forms when cultured in vitro. These colony forms indicate two phases that differ in their morphological and biochemical characteristics. Each nematode species has a specific association with one species of bacterium. Xenorhabdus or Photobacterium species may be associated with more than one species (1, 2). Entomopathogenic nematodes represent one important part of the spectrum of biocontrol agents. They are used to control insect pests in high-value crops and potentially they could be used in integrated pest management, organic farming and sustainable agriculture systems to control soil-borne insect pests. During our study we found bacteria belonging not only to the family Enterobacteriaceae. The results of an investigation on taxonomic identification of bacteria, isolated from three different entomopathogenic populations are presented in this paper.

Introduction
Entomopathogenic nematodes from the genera Heterorhabditis and Steinernema are symbiotically associated with bacteria Photobacterium and Xenorhabdus. Xenorhabdus bacteria are carried in a special vesicle in an interior part of the intestine (6), whereas Photobacterium were found throughout the whole intestine (13). In the hemolymph, the nematodes found optimal conditions for reproduction.

The infective juveniles of the nematode symbiont release the bacterial cells into the insect’s natural openings (Steinernema spp.) or by boring directly through the insect cuticle (Heterorhabditis spp.). The bacterial cells multiply in the hemocoel, overcome the insect’s defense system and kill the insect host within 24-48 hours. The nature of this bacterial-nematode-insect relationship is not fully understood. Xenorhabdus spp., and Photobacterium spp. are Gram negative, facultative anaerobic rods, belong to the family Enterobacteriaceae (13, 7). In older cultures the cells contain crystalline inclusions. They are motile, facultative anaerobic, do not form spores and the optimal temperature for their growth is between 25-30°C.

The bacteria occurs in two major phases;
the phases I variant is most ideal for nematode development, probably because it furnishes a good source of nourishment and produces an assortment of antibiotics which prohibit the establishment of other microorganisms (3). The forms which differ in their effect on nematode reproduction in vivo and in vitro were distinguished by their different pigmentation, adsorption of bromthymol blue from agar media (14). The form isolated from the infective nematode (the primary form) was unstable, producing the secondary form, which could revert to the primary. Phase I absorbs dyes on agar media, produce several antibiotics, secrete a variety of proteins and produce fimbriae and flagella, while these properties are either apparently absent or reduced in phase II (8).

Many *Xenorhabdus* species produce secondary metabolites with antimicrobial properties, small molecular weigh antibiotics, extracellular enzymes like proteases, lipases, phospholipases and DNA-s involved in breaking down insect tissues to provide nutrients for both the nematode and bacterial symbionts (14). Most species of *Xenorhabdus* and *Photorhabdus* produce more than one group of bioactive secondary metabolites and the metabolites from *Xenorhabdus* species are more diverse from those from *Photorhabdus*. These metabolites not only have diverse chemical structures, but also a wide range of bioactivities of medical and agricultural interest such as antibiotic, antymycotic, insecticidal, nematicidal, antineoplastical and antiviral (20). The reason for this production is not clear yet.

This study focused on the isolation of bacteria from three different species of entomopathogenic nematode, isolated from different places in Bulgaria. The nematodes belong to the genus *Steinernema* (Rhabditida; Steinernematidae) – *S.kraussei*, *S.intermedium* and *S.carpocapsae* respectively (21, 22). The nematode populations are soil inhabitants and they have been taken from different places in Vitosha Mountain near Sofia. The lab of Entomology and Biological control (Department of Zoology and Anthropology) kindly supplied the samples for this research work.

**Isolation of bacteria.** The bacterial strains were obtained indirectly from the nematodes by sampling the *Galleria mellonella* larvae infected by the nematode populations of *S.kraussei*, *S. intermedium* and *S.carpocapsae* respectively. Following the death of the insect larvae that had exhibited signs of nematode infection, the larvae were left for 10 min at 70% ethanol for surface sterilization and then left in lab conditions for drying (25ºC, light-16h). Probes of hemolymph were grown on Standard I agar (peptone-15.0g, yeast agar-3.0g, NaCl-6.0g, D(+)glucose-1.0g, agar-agar-12.0g, demi water-1 liter, pH=7.5; Merck) for 24 hours at 25-27ºC. Each colony was transferred in an agar plate as a single strain.

**Macro morphological tests.** To check the possibility of the strains to grow on different media, Standard I agar, Endo, media of Levin and Mac Conkey were used. The cultivation conditions were 27ºC and pH=7.0. Based on these tests, size, consistence, color, form, margin of the colony and pigmentation were described.

**Micro morphological tests.** 24-hours old cultures were used to describe shape of the cells and motility. The strains were colored to check their response to Gram and possibility to form spores and capsule.

**Results and Discussion**

Dutky (12) and Bovien (9) first pointed out association between nematodes, parasitic for insects and their specific bacteria. Insect larvae possess a system resembling the mammalian complement (5). Foreign bac-
The results received in our investigation are placed in three groups according to the nematode population by which the larvae of *G. mellonella* were infected.

**Group I** - this group includes bacterial strains, isolated from the insects larvae of *Galleria mellonella*, infected by the nematode population of *Steinernema kraussei* in the period of time October-November 2002. The isolated strains were cultured on media Standard I agar (25°C, 3 days). All bacterial strains were checked on many micro-macro and physiological tests for their final identification. The bacterial strains belong not only to the expected family *Enterobacteriaceae*.

From all strains in this group 46% belong to the genus *Bacillus* (family *Bacillaceae*). They are rod shaped cells with length between 0.5-2.5 x 1.2-10.0 µm, Gram positive, motile, with oval spores, aerobes or facultative anaerobes. They can grow in big limits of temperature, pH and NaCl concentration. They have been found in different habitats. Some of them are serious pathogens for animals and people (10).

22% from the strains belong to the genus *Sporosarcina*. They are Gram positive, motile, endospore forming bacteria. The colony color is changed between yellow and orange. The cell size is between 1.0-2.0 -x 2.0-3.0 µm. They easily grow in the soil and in salty marshes.

18% from all strains are from the genus *Micrococcus* (Micrococcaceae). They are Gram positive, motile, non-spore-forming, anaerobes with length between 0, 5-2, 0 µm. The colony color is usually yellow or red, when growing on a simple media, catalase positive and oxidase negative. They have been found on the skin of different animals, in the soil, in the air and in different food products.

Only 14% from the isolated bacteria are members of the genus of the symbiotic bacteria *Xenorhabdus* (family *Enterobacteriaceae*). The presence of bacteria from genus *Bacillus* and *Sporosarcina* is not typical for the population of *S. kraussei*, but they have been found in the population of *Steinernema spp.* (15). Bacteria from genus *Micrococcus* have been isolated from the population of *Steinernema spp.* and *S. feltiae* (15). There is a big percent of non-symbiotic bacteria and large diversity of bacterial genera. Bacteria from the unexpected genus *Bacillus* and *Sporosarcina* were not isolated from other workers in the tropical or other kind of areas. It could be possible that there are specific for the nematode population in the region of Bulgaria (Fig. 1).

**Group II** - this group includes bacterial strains isolated from the insect larvae of *G. mellonella*, infected by the nematode population of *Steinernema intermedium* isolated in the period of time November-December 2002. The isolated bacterial strains were checked on micro-macro and physiological tests.

According to the final identification, the bacteria belong to the same families and genera as the strains isolated from the previous species.

22% from the isolated strains are from the genus *Bacillus*, 48% genus *Sporosarcina*, 4% genus *Planococcus* and 26% - *Xenorhabdus* (Fig. 2).

**Family Micrococcaceae-genus Planococcus**

They are cocci, with length 1.0-1.2 µm, Gram positive, catalase positive, oxidase negative, do not reduce nitrate. They do not grow or grow slow when the concentration of NaCl is below 1% or more than 15%. They are mainly marine habitants (10).

The microbial pathogens of insects are common and it is logical to expect that an insect’s immune system can prevent the
invasion of a variety of microorganisms. Galleria mellonella has a relatively weak immune system (9, 19) consequently, those bacteria have an opportunity for growth and some of them may be resistant to the antibiotics produced by the symbiotic bacteria. It is obvious the similarity of the bacterial micro flora of the larvae of two different nematode species. There is a difference in the per cent by which these bacterial genera are presented. We do not have explanation on this result.

**Group III** - this group includes bacterial strains, isolated from the insect larvae, infected by the nematode population of Steinernema carpocapsae in the period of time December 2002-January 2003.

Less bacterial strains were isolated as inhabitant of the hemolymph of the insect larvae infected by S.carpocapsae. They are bacterial strains belonging almost to the same families as the bacteria isolated from the nematode S.kraussei and S.intermedium. 59% are from genus Sporosarcina, 29% genus Planococcus, and 12% from the symbiotic genus Xenorhabdus. (Fig. 3)

Genus Planococcus are cocci, 1.0-1.2µm, usually are separated, but sometimes they could stay in tetrads, Gram positive, motile with 1-2 flagella, aerobes, do not form spores, do not reduce nitrate, oxidase and catalase negative. The colony color is yellow-orange.

Although not typical, it is possible that either these bacteria or the bacteria from the other isolated non-symbiotic genera might influence the insect and cause death. They could be reason for specific relations between the isolated bacterial genera, between them and the nematode population and between the bacteria, the nematode and the insect hosts (1, 5).

The majority of bacteria isolated from insects can be regarded as facultative pathogens. There is still inadequate experience to provide the evidence to prove that the microbe, isolated from dead insects was the true cause of a disease and the death of the specimen (18).

Members of the family Enterobacteriaceae have frequently been described in the micro flora of insects. There exist a similarity between entomogenous bacteria and those occurring in other habitats. It seems reasonable to assume that strains of one species might differ in their phenotypic characteristics from those of an identical species found in another habitat. There probably exist types of bacteria that are found associated with insects regularly, but less frequently than in other habitats. This
does not imply that they are insect-specific bacteria.

Taxonomic studies confirm that each species of entomopathogenic nematode has a specific natural association with only one *Xenorhabdus* species (though a *Xenorhabdus* spp. may be associated with more than one nematode species) (4).

The results from the present study and those described above suggest that the symbiotic relationship between nematodes and bacteria are not free of competition. The large percent of non-symbiotic bacteria, as well as the diversity of the isolates were unexpected. It may explain why some species *Steinernema* kill the host-insect larvae more slowly (for four days) than the nematode populations with *Xenorhabdus* as symbiont bacteria. The large percentage of some bacterial species in these nematodes could be also responsible for the fast killing of the larvae of *Galleria mellonella*.

The bacteria from the genus *Bacillus* were isolated also from *Steinernema* spp. (15).

Jackson et al. (16) reported association between *Photorhabdus* sp. and the bacterium *Providentia* (= Proteus) rettgeri. From the larvae of *Galleria mellonella* infected with *Steinernema* spp., several Gram negative bacteria other than *Xenorhabdus* were isolated as well as the Gram positive *Enterococcus* sp. Lysenco & Weiser (17) isolated bacteria such as *Alcaligenes*, *Pseudomonas* and *Acinetobacter* spp. from *Steinernema* spp. and S. *carpocapsae*.

Conclusions

According to the results from our investigations we can conclude that in the population of *S. kraussei* and *S.intermedium* bacteria from same families were found. In the population of *S. carpocapsae* bigger diversity of bacterial genera were isolated. The untypical genera *Bacillus* and *Sporosarcina* could be reason for the death of the larvae of *Galleria mellonella*. Bacteria from genus *Sporosarcina* were isolated from the population of *S. kraussei*, *S. intermedium* and *S. carpocapsae*. The biggest percent of bacterial genus *Xenorhabdus* were isolated from the population of *S. intermedium* (26%) and the smallest (14%) in the population of *S. kraussei*.

Biological control offers a tremendous opportunity to supply agriculture with effective tools for the development of production techniques which minimize impact on human health and the environment. EPN represent one important part of the spectrum of biocontrol agents.

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REFERENCES