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Genetic Enhancement of Environmental Stability and Efficacy of Entomopathogenic Nematodes for Biological Control

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Genetic Enhancement of Environmental Stability and Efficacy of Entomopathogenic Nematodes for Biological Control

ABSTRACT

The overall objective of the research project was to enhance the intrinsic biological control potential of entomopathogenic nematodes through genetic manipulation. We have chosen heat and desiccation tolerance as prime traits to be enhanced in order to increase the overall efficacy of these nematodes against insect pests under harsh conditions. Initially, we used mutagenesis and selection approaches to enhance these traits. In the mutagenesis experiments several morphological mutants of *Heterorhabditis bacteriophora* HP88 were isolated and characterized phenotypically and genetically. Infective juveniles of *H. bacteriophora* HP88 were subjected to heat and desiccation selection regimes for several generations. Small increase was recorded, after 4 and 6 rearing cycles for both traits. However, in both selection regimes a significant deterioration in the reproductive capability of the nematodes was observed.

In a screen of new nematode populations, from arid regions in Israel, a heat tolerant (IS₅ strain) and desiccation tolerant (IS₆ strain) were isolated. Both strains were taxonomically identified and their beneficial characteristics (environmental tolerance, insecticidal virulence and reproduction) were determined. We further investigated the stability of the enhanced heat tolerance trait in, and the storage capacity of, the newly discovered IS₅ strain. Genetic studies demonstrated that the heat tolerance of the IS₅ strain is genetically based and is dominant. The trait for heat tolerance was transferred from the IS₅ strain to the HP88 strain of *H. bacteriophora*. The transfer was accomplished by allowing the heat tolerant strain (IS₅) to mate with the commercial strain (HP88). The hybrid nature of the progeny was confirmed using a recessive marker mutant of the HP88 strain (*H-dpy-2*). We have used (RAPD-PCR) to compare genetic variation in the IS₅ and the HP88 strains of *H. bacteriophora*. The results indicated that genetic variation in the HP88 strain was significantly less than in the IS₅ strain which was recently isolated from the field.

The new IS₅ strain may be used as an effective biological control agent in warm environments. In addition, IS₅ can be used as a genetic source for cross-hybridization with other *H. bacteriophora* strains.
BARD PROJECT # IS-2099-92C

Genetic Enhancement of Environmental Stability and Efficacy of Entomopathogenic Nematodes for Biological control

OBJECTIVES

The overall objective of the research project was to enhance the intrinsic biological control potential of entomopathogenic nematodes through genetic manipulation and to understand the genetic architecture underlying the phenotypic variation of beneficial traits. We have chosen heat and desiccation tolerance as prime traits to be enhance in order to increase the overall efficacy of these nematodes against insect pests under harsh conditions.

The Specific Objectives set for this project were:

1) To enhance the desiccation and heat tolerance of Heterorhabditis bacteriophora infective juveniles through selection and mutagenesis.

2) To characterize the improved strains phenotypically biochemically and genetically.

3) To validate efficacy of improved strains in greenhouse and field microplots trials.
RESULTS AND DISCUSSION

ACTIVITY IN ISRAEL

Initially we developed bioassays to select for heat and desiccation tolerance. Nematodes of *Heterorhabditis bacteriophora* HP88 were subjected to the selection regimes for several generations. At each cycle the surviving nematodes were re-cultured and the progeny were exposed to the same selection conditions. Small increase was recorded, after 4 and 6 rearing cycles in the heat tolerance (Fig. 1) and in the desiccation tolerance (Fig. 2).

![Graph showing effect of selection on survival](image1)

**Fig. 1.** Effect of selection for heat tolerance (6 h exposure at 37°C) on survival of the nematode *H. bacteriophora* reared on *G. mellonella* larvae. WT = non selected wild type population.

![Graph showing effect of selection on survival](image2)

**Fig. 2.** Effect of selection for desiccation tolerance (85% RH, 25°C for 3 days) on survival of the nematode *H. bacteriophora* reared on *G. mellonella* larvae. WT = non selected wild type population.
However, in both selection regimes a significant deterioration in the reproductive capability of the nematodes was observed (e.g. see Fig. 3). We concluded that the specific HP88 strain of *Heterorhabditis bacteriophora* which has been reared in the lab for many generations (>150 generations, during a period of 6-7 years) may have adapted to laboratory conditions and is not suitable for selection to harsh conditions.

![Graph showing effect of selection for heat tolerance](image)

Fig. 3. Effect of selection for heat tolerance (6 h exposure at 37°C) on reproductive capacity of the nematode *H. bacteriophora* reared on *G. mellonella* larvae. WT = non selected wild type population.

At this stage we turned to our unique collection of entomopathogenic nematode populations which we have isolated from different natural sites in Israel. Most populations were recently isolated (during the course of the present project) from soil samples at the northwestern part of the Israeli desert, the Negev. They are maintained at the laboratory of Dr. Galzer at the Volcani Center. We tested these isolates in the desiccation and heat tolerance assays (Fig. 4a). The majority of the populations were found to have poor heat tolerance, similar to the HP88 lab strain. However, the isolate designated IS5 showed ten fold higher tolerance to extreme heat conditions. The identification of this new strain, the characterization of its heat tolerance, and the determination of the stability of this trait are described below.

Another isolate (of the nematode *S. feltiae*) designated IS6 showed high tolerance to desiccation (Fig. 4b). Its characterization is described later in this report.
Fig. 4. Survival of different newly isolated Israeli populations of entomopathogenic nematodes following exposure to desiccation (induction of anhydrobiosis at 97% RH for 72 h followed by exposure to 85% RH, for 3 days–25°C), and heat (6 h exposure at 37°C) treatments. a) Heterorhabditid nematodes. HP88 = commercial strain of *H. bacteriophora*. b) Steinernematid nematodes.

**Characterization of the heat tolerance of the IS5 strain**

Morphological measurements of nematodes from the IS5 isolate indicated high similarity to *Heterorhabditis bacteriophora* Poinar 1976. DNA extracted from four different species: *Heterorhabditis* sp. IS5, *H. bacteriophora* HP88, *H. bacteriophora* IS3 (another isolate from the Negev with poor heat tolerance) and *H. megidis* HSH2, were screened with six random PCR primers for molecular characterization. We scored products from different primers for the presence or absence of an amplification product. Average percentage of similarity was calculated among these four different species/strains on the basis of shared DNA fragments. The similarity between IS5 and *H. bacteriophora* HP88 was 20%. Strain IS3 showed higher similarity (85%) to *H. bacteriophora* HP88, but only 15.6% to *H. megidis*. Despite the difference between the IS5 strain and the other *H. bacteriophora* strains in RAPD analysis, the new isolate could not be distinguished from other populations of *H. bacteriophora* on morphological grounds, following the description of this species by Poinar (1976). Therefore, it was considered as a new strain of *H. bacteriophora*. This conclusion was substantiated by our finding (described later in this report) that IS5 and HP88 can cross-breed to produce fertile progeny.
We have conducted a series of experiments to characterized the heat tolerance of IS₅:

Following two hours exposure of infective juveniles (IJ’s) of *H. bacteriophora* HP88 to 37°C survival was reduced by 79%, whereas only 18% mortality was recorded among IJ’s of the IS₅ strain. After 8 h of exposure to 37°C the HP88 strain experienced further reduction in survival to 13%, whereas survival of IS IS₅ was only slightly reduced to 72%.

Exposure of infective juveniles from the HP88 strain for 1 h to 40°C resulted in complete mortality. Among the IJ’s of the IS₅ strain 19.1% survived these high temperature conditions.Giving a heat-shock treatment to the nematodes prior to exposure to the high temperature conditions in the bioassay, enhanced the survival of the IS₅ strain 2.3 fold as compared with non non pre-treated. These findings suggest that a biochemical mechanism involving heat shock proteins may be involved in the heat tolerance of IJ’s from the IS₅ strain. Heat shock proteins are responsible for heat tolerance in many organisms including nematodes.

At 25°C complete insect mortality of *G. mellonella* larvae was recorded 36 h after they were exposed to IJ’s of the IS₅ strain, and after 48 h exposure to the HP88 strain. Infection by the IS₅ strain at 30°C shortened the period required to achieve complete insect mortality to 24 h On the other hand, infection by HP88 at 30°C resulted in complete insect mortality only after 16 hrs). The average number of nematodes found in cadavers infected by the IS₅ strain ranged between 15 to 16.3 as compared to 7.3-10.8 nematodes/cadaver for HP88. Although the penetration rate of the IS₅ strain was 1.5-2 times higher than that of the HP88 strain, this difference can not account for the rapid rate of mortality of *G. mellonella* larvae infected with the IS₅ at 30°C. Perhaps this effect can be attributed to fast establishment and propagation of the symbiotic bacterium associated with the IS₅ strain.

At 25°C both the HP88 and the IS₅ strains produced an average of 90,000 IJ’s per cadaver with no significant difference between them. At incubation temperature of 30°C and at 33°C a 10% increase in the number of progeny was recorded among the cadavers infected with the IS₅ strain. At these temperatures reproduction of the HP88 strain was reduced 10
fold. In all temperature conditions the IJ’s of the IS_5 strain emerged 2-3 days earlier than IJ’s of the HP88 strain.

**Note:** The results of this study have been published (see Appendix 1).

We further investigated the stability of the enhanced heat tolerance trait in, and the storage capacity of, the newly discovered IS_5 strain. After 12 passages through *G. mellonella* larvae, trait stability was determined in terms of survival, virulence, infectivity, and reproduction at elevated temperatures. These assays were conducted on IS_5 populations reared with and without selection pressure (at 30°C and 25°C, respectively). Relative to the HP88 strain, the IS_5 strain exhibited greater heat tolerance after 12 passages regardless of selection pressure. The IS_5 strain reared at 30°C exhibited greater heat tolerance than when reared at 25°C, and greater survival ability at elevated temperatures than the original isolate. These results indicate that the heat tolerance trait is genetically based but is influenced by environmental conditions. Storage capacity of the IS_5 and HP88 strains was measured at 10 and 25°C over a four week period. The IS_5 strain survived significantly longer at 25°C than at 10°C, indicating that these nematodes may be cold sensitive. Because the heat tolerance trait was found to be stable, genetic and biochemical characterizations, as well as field efficacy studies can now be initiated.

**Note:** The results of this study have been published (see Appendix 2).

The trait for heat tolerance was transferred from the IS_5 strain to the HP88 strain of *H. bacteriophora* Poinar. The transfer was accomplished by allowing the heat tolerant strain (IS_5) to mate with the commercial strain (HP88). The hybrid nature of the progeny was confirmed using a recessive marker mutant of the HP88 strain (*H-dpy-2*). Progeny from the cross were examined for heat tolerance by measuring their survival after 2 h exposure to 40°C. After three and six passages through *Galleria mellonella* (L.), heat survival of the hybrid nematodes was significantly greater than survival of the HP88 strain and was similar to the survival of the IS_5 strain. Efficacy, in terms of virulence, reproduction and storage capacity was compared among
the IS₅, hybrid, and HP88 strains. At 32°C, the IS₅ and the hybrid strains caused mortality of *G. mellonella* at a faster rate than the HP88 strain. Similar to the IS₅ strain, the hybrids exhibited sensitivity to cold. After one week of storage at 10°C the survival of the hybrid and IS₅ strains was significantly reduced relative to the HP88 strain. No difference was detected in reproductive potential among the strains. This study demonstrates the potential of using cross-hybridization to genetically improve entomopathogenic nematodes, and illustrates the advantages of using marker mutations in this endeavor.

*Note: The results of this study have been published (see Appendix 3).*

Genetic variation in laboratory reared biocontrol agents may be reduced due to founder effect, inbreeding, and selection. This may hinder their efficacy. Entomopathogenic nematodes in the genus *Heterorhabditis* have been hypothesized to have low genetic variation due to hermaphroditic reproduction. We have used random amplified polymorphic DNA (RAPD-PCR) to compare genetic variation in two strains of *H. bacteriophora*. One strain (IS₅) was recently isolated from the field and expected to be relatively genetically heterogeneous. The other strain (HP88) has been reared under laboratory conditions for over 10 years. For each strain, 15 inbred lines were generated by > eight cycles of selfing of a single hermaphrodite (reaching > 90% homozygosity). Genomic DNA from each of the inbred lines was screened with 14 decamer primers. Genetic variation was calculated based on average percentage similarity of DNA banding patterns and cluster analysis. The results indicated that genetic variation in the HP88 strain was significantly less than in the IS₅ strain. A considerable level of within population variation was detected in both strains. This is the first study to use molecular markers to characterize overall within population for an entomopathogenic nematode. More studies will be needed to determine if genetic variation of biological control agents decrease when they are reared under laboratory conditions over a long period. Using molecular markers, future research will also determine changes in genetic diversity laboratory reared biocontrol agents after they are released into the field.

*Note: The results of this study have been submitted for publication (see Appendix 4).*
The new IS$_5$ strain may be used as an effective biological control agent in warm environments where the activity of other commercial strains is hampered by high temperature conditions. Furthermore, it is likely to better withstand the relatively warm conditions of storage in warehouses than the commercial HP88 strain. Thus, IS$_5$ should have longer shelf-life. In addition, IS$_5$ can be used as a genetic source for cross-hybridization with other $H.\textit{bacteriophora}$ strains.

The research directions currently under investigation regarding heat tolerance are: A) Determination of the physiological basis for heat tolerance with emphasis on of heat shock proteins. B) Examination of the molecular basis for the heat tolerance of IS$_5$ by cloning and examination of the expression of genes encoding heat shock proteins.

**Characterization of the desiccation tolerance of the IS$_6$ strain**

The IS$_6$ isolate showed higher desiccation tolerant then all other population tested (Fig. 4b). This population was identified as a steinernematid (strain of $S.\textit{feltiae}$) while all other isolates were identified as heterorhabditids. Indeed nematodes belonging to Heterorhabditidae are known to have lower desiccation tolerance. We have characterize this particular trait of IS$_6$. Exposure of this strain to 75% relative humidity (RH) for 72 h, after induction of anhydrobiosis, resulted in 37% reduction in survival of the IU's whereas same treatment to the HP88 strain of $H.\textit{bacteriophora}$ caused complete mortality. Survival of the IS$_6$ strain at RH of 85%, 75%, 63%, or 45% for 48 h was 90%, 83%, 70%, and 49% respectively, while survival of the HP88 strain under same conditions was 47%, 32%, 4%, 0% respectively.

Obtaining a desiccation tolerance strain is particularly important since unlike the heat tolerance phenomenon, very little is known about desiccation tolerance even in other organisms. Now we have a tool to investigate he physiological and genetic aspect of this interesting biological process. The research directions currently under investigation are:

a) Optimization of the processes of induction of and recovery from anhydrobiosis.
b) Morphological and behavioral characterization of desiccated nematodes.
c) Determination of the biochemical and physiological processes involved in the desiccation and rehydration processes.
d) Isolation and characterization of genes involved in desiccation tolerance from steinernematid nematodes.

ACTIVITY IN US

The US Team conducted a series of mutagenesis experiments to create a homozygous line with *H. bacteriophora* wild-type strain HP88. Several morphological mutants were isolated after routine mutagenesis with the chemical mutagen ethyl methyl sulfonilic acid (EMS). None of these mutants was able to sustain viability: the majority did not produce viable progeny and the rest died immediately after transfer to fresh medium. Presumably these nematodes had additional metabolic impairment that rendered these mutations lethal.

In subsequent experiments, a homozygous line was created after several cycles of selfing wild-type nematodes (15 cycles). Mutagenesis experiments using nematode cultures of the homozygous line produced mutants that were able to sustain viability and produce progeny. Two mutants, identified by phenotypic characterization were isolated.  
1) Morphological: distinguished by having long thin first generation hermaphrodites (*lon1*). Body length 30-50% longer than wild-type.

2) Behavioral: characterized by slow movement which is possibly due to a deformed cuticle or a deformation in sustaining a hydrostatic pressure.

These mutants have not been previously demonstrated in entomopathogenic nematodes but have some similarity with members of the unc mutants of *C. elegans*. Success in induction of these mutants is probably in part due to using a homozygous line that is easily obtainable using a hermaphroditic nematode species (*H. bacteriophora*).

 Cultures of mutant (*lon1*) and wild-type *H. bacteriophora* HP88 were initiated from infective juveniles on lipid agar plates. Reciprocal crosses between wild-type and mutant nematodes were conducted by first transferring at least five males and one virgin hermaphrodite to lipid agar plate. After conducting 21 crosses we analyzed the data. The phenotypes obtained from the crosses shows the following pattern: Cross- *lon1* female X wt *H. bacteriophora* HP88 male Progeny:

1) Wild-type females 107.318.3
2) Mutant females 25.212.2
3) Wild-type males 6.711.2
4) Mutant males 1.410.33
The analyses of the results in cooperation with Dr. Danny Segal (the Israeli Co-PI) indicate that:

a) The ratio of mutant to wild-type is roughly 1.3 in both males and females. This indicates that the trait is not sex linked (i.e. not on the X chromosome).

b) Males constitute about 6% of the population in both mutant and wild-type adults. This correlates with the conclusion in (a).

c) The 1:3 ratio is a typical of a cross of heterozygous with homozygous recessive.

d) This would mean that both parents of this cross would be having the same genotype that give similar phenotype which contradicts the data of the phenotype mutant characters.

e) The above analysis assumes that all progeny (=F1 generation) are hybrids (i.e., the result of cross fertilization, with NO self fertilization of the mutant females).

Alternative explanation would be as flows:

The long trait is recessive (and not sex linked). All wild-type looking F1 are hybrids (i.e. result from cross fertilization of the mutant female parent, which implies that the sperm from the partner have a 3:1 preference over self sperm fertilizing the female's egg).

We are testing this hypothesis by:

1) Allowing self fertilization of F1 wild-type-looking females (obtained from the previous crosses) then recording percentage of progeny phenotype.

2) Conducting reciprocal crosses between mutant males to wild-type females, then allow for self fertilization of the F1 wild-type-looking females and score percentage of progeny phenotype.

3) Conducting a back cross of the F1 mutant-looking males to wild-type females. and report percentage of progeny phenotype.

These experiments should help determining the genotype of the mutants.
DESCRIPTION OF THE COOPERATION

The success and fruitfulness of this project, as reported herein, are due to intensive and continuous collaboration between the partners. The screen for new nematode isolates, and their initial characterization were done by the Israeli partners. The morphological, physiological and initial molecular (RAPD) characterizations of the heat tolerant IS$_5$ strain were conducted together by the Israeli partner (Dr. Glazer) and the American partners. Dr. Glazer spent some time in the American lab to do that work. The studies demonstrating the stability of the heat tolerant trait in the IS$_5$ strain were done in team-work between the Israeli partners with the help of a US postdoc (Dr. Shapiro). The crosses demonstrating that the heat tolerance trait is genetically based, is dominant and can be transferred to the HP88 strain were also conducted as a team work of the Israeli partners. The evaluation of the genetic heterogeneity between the lab strain (HP88) and the newly isolated IS$_5$ strain were likewise done by the Israeli team.

Identification of the dessication tolerant strain were done primarily by Glazer's group in Israel. Induction of the mutant strain (Ion1) and its characterization were done by the American team and the analysis of their results was conducted together with teh Israeli collaborators. Throughout this period close daily contact was maintained between the Israeli partners, and communication with the American partner was extensive. Dr. Glazer's stay in the Rutgers lab, and Dr. Segal's visit there served greatly to enhanced the collaboration.
EVALUATION OF RESEARCH ACHIEVEMENTS

The objectives of this project were:

1) To enhance tolerance of H. bacteriophora to heat and dessication. Our results described above, and the corresponding publications of them, demonstrate that this goal has been fully accomplished. Through discovering of the natural isolates IS₅ and IS₆ we obtained a heat and desiccation tolerant strains respectively.

2) To characterize the tolerant strains phenotypically, genetically and biochemically. This objective has been accomplished to great extent for the heat tolerant strain where we have described in detail its phenotype, and demonstrated conclusively its genetic basis. We are presently in the process of looking into its molecular basis through analysis of heat shock genes and their proteins in HP88 and in IS₅. For the desiccation tolerant strain we have completed its phenotypic description. The genetic and biochemical studies of this strain will follow shortly.

3) Validate teh efficacy of the improved strains. This objective has been fully accomplished for the heat tolerant strain IS₅ where we showed that it is indeed more pathogenic at elevated temperatures, and further demonstrated that this improved efficacy is heritable and can be transferred to the commercial strain HP88. These efficacy studies were done in the lab and remain to be confirmed in greenhouse and microplot experiments. Comparable studies with the desiccation tolerant strain IS₆ remain to be done for this project.
CONCLUSIONS

The long term goal of our studies is to improve the efficacy of entomopathogenic nematodes as safe and efficient biological control agents. The present project marks a significant step ahead in this endeavor. We demonstrated that genetic improvement of resistance to environmental stress is feasible in these nematodes. We have made pioneering studies in deciphering the genetic basis of the improved beneficial traits. We have demonstrated that these improved traits are stable, whereas in most previous studies improved traits deteriorated over time. Most importantly, we have shown that efficacy of the novel strains has not been compromised. Finally, we made exciting demonstration of the feasibility of transferring the improved beneficial trait from one strain to another, making use of marker mutations which we have previously generated. This opens the way for generating 'super' hybrid strains by combining beneficial traits from different parental strains. The power of molecular biology which we have recently started to apply for the study of the basis of the improved traits will undoubtedly be instrumental in using genetic engineering approaches for generating improved entomopathogenic nematodes.

LIST OF PUBLICATIONS


