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Selection of a bacterium for the mass production of *Phasmarhabditis hermaphrodita* (Nematoda : Rhabditidae) as a biocontrol agent for slugs

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Summary – Dauer larvae of the slug-parasitic nematode, *Phasmarhabditis hermaphrodita,* were found to carry a mean of *ca* 70 bacterial colony forming units with up to five different bacterial types present in individual nematodes. Nine bacterial isolates found associated with *P. hermaphrodita,* were injected into the body cavity of the slug, *Deroceras reticulatum.* Only two isolates (*Aeromonas hydrophila* and *Pseudomonas fluorescens,* isolate 140), were found to be pathogenic. *Phasmarhabditis hermaphrodita* grown in monoxenic culture with five bacterial isolates (*Moraxella osloensis, Providencia rettgeri, Serratia proteamaculans, P. fluorescens,* isolate 140 and *P. fluorescens,* isolate 141) which supported strong growth were bioassayed against *D. reticulatum.* Nematodes grown with *M. osloensis* or *P. fluorescens* (isolate 141) were pathogenic, those grown with *P. rettgeri* gave inconsistent results and those grown with the other two bacteria were non-pathogenic. These results demonstrate that differences in pathogenicity between nematode/bacterium combinations did not result from differences in bacterial pathogenicity alone. *M. osloensis* was selected as the bacterium for rearing *P. hermaphrodita* as a biocontrol agent for slugs.

Résumé – ***Sélection d'une bactérie pour la production en masse de* Phasmarhabditis hermaphrodita *(Nematoda : Rhabditidae), agent de contrôle biologique des limaces*** – Il a été observé que le nématode parasite des limaces *Phasmarhabditis hermaphrodita* était porteur d'environ 70 « unités formant une colonie » (CFU) appartenant jusqu'à cinq types différents de bactéries par individu. Neuf isolats bactériens associés à *P. hermaphrodita* sont injectés dans la cavité du corps de la limace *Deroceras reticulatum.* Seuls deux isolats – *Aeromonas hydrophila* et *Pseudomonas fluorescens* isolat 140 – se sont révélés pathogènes. *Phasmarhabditis hermaphrodita* en élevage monoxénique avec cinq isolats bactériens (*Moraxella osloensis, Providencia rettgeri, Serratia proteomaculans, P. fluoresens* isolat 140 et *P. fluorescens* isolat 141) permettant un taux élevé de croissance a été testé envers *D. reticulatum.* Les nématodes élevés avec *M. osloensis* et *P. fluorescens* isolat 141 se sont montrés pathogènes, ceux élevés avec *P. rettgeri* donnent des résultats inconstants et ceux élevés avec les autres bactéries ne sont pas pathogènes. Ces résultats prouvent que les différences dans la nocuité des combinaisons nématode/bactérie ne proviennent pas seulement de la nocuité de la bactérie. *M. osloensis* a été sélectionné en vue de l'élevage de *P. hermaphrodita* comme agent de contrôle biologique envers les limaces.

Key-words : *Phasmarhabditis hermaphrodita,* rhabditid nematodes, bacteria, parasite, slug, *Deroceras reticulatum,* monoxeny, pathogenicity, biological control.

The rhabditid nematode, *Phasmarhabditis hermaphrodita* (Schneider), is a parasite capable of killing several species of pest slugs and has the potential to be used as a biocontrol agent (Wilson *et al.,* 1993 *a*). It is a bacterial feeder and has been mass-produced in xenic culture on nutrient media supporting a mixed bacterial flora (Wilson *et al.,* 1993 *b*) using similar techniques to those developed to mass-produce entomopathogenic nematodes of the families Heterorhabditidae and Steinernematidae, which have been used successfully to control several insect pest species.

Heterorhabditid and Steinernematid nematodes are symbiotically associated with bacteria of the genera *Photorhabdus* (Boemare, 1993) and *Xenorhabdus* (Thomas & Poinar, 1979), respectively, which are carried monoxenically in the anterior portion of the nematode gut. These mutualistic bacteria are thought to play an important role in the pathogenicity of the nematode/bacterium complex and when these nematodes are mass-produced for commercial use, they are grown in monoxenic culture with their preferred species of *Xenorhabdus* or *Photorhabdus.* This generally results in consistent and predictable yields of reliably virulent nematodes. These bacteria, however, can spontaneously alternate between two phases, and phase two provides a less suitable environment for nematode growth than phase one (Akhurst & Boemare, 1990).

There are many ways in which nematodes can interact with bacteria (Poinar & Hansen, 1986), but little is known about such interactions for *P. hermaphrodita*. Wilson *et al.* (1995) found that many species of bacteria supported growth of *P. hermaphrodita* in monoxenic cultures, but that different bacterial isolates caused differences in the total numbers of nematodes produced in culture and influenced the proportion of the population which formed dauer larvae after three weeks. As dauer larvae are the stage of *P. hermaphrodita* which infect slugs, this has important implications for production of *P. hermaphrodita* as a biocontrol agent. Wilson *et al.* (1995) did not investigate differences in the quality of *P. hermaphrodita* grown in monoxenic culture with different bacteria, in particular, any effect that the bacterial food-source might have on the ability of dauer larvae to infect and kill slugs.

The investigations described herein into the relationships between *P. hermaphrodita* and bacteria had two related aims. First to determine the direct effects of bacteria associated with *P. hermaphrodita* on the field slug, *Deroceras reticulatum* (Müller). And, second, to determine whether the pathogenicity of *P. hermaphrodita* to *D. reticulatum* is influenced by growth with different bacteria. Bacteria were isolated from dauer larvae of *P. hermaphrodita,* from xenic cultures of *P. hermaphrodita* which were producing highly pathogenic nematodes and from living or dead individuals of *D. reticulatum* infested by *P. hermaphrodita*. The effects of nine isolates on slugs were tested by injecting cultures of bacteria directly into the haemocoel of *D. reticulatum*. Five of these bacteria which had been found to support good monoxenic growth of *P. hermaphrodita* (Wilson *et al.,* 1995) were used to grow monoxenic cultures of *P. hermaphrodita* which were then bioassayed for their effects on *D. reticulatum*.

Materials and methods

Collection and maintenance of slugs

Slugs (*Deroceras reticulatum*) were collected from traps consisting of inverted plastic flowerpot saucers baited with bran in rough grassland at Long Ashton Research Station (LARS). They were kept at 10 °C with a 12 h night and day cycle in non-airtight plastic boxes lined with moistened absorbent cotton wool.

Collection and maintenance of nematodes

In the initial phase of the investigation, dauer larvae of *P. hermaphrodita* were either produced in xenic cultures using foam culture and harvested as described by Wilson *et al.* (1993 *b*), or they were isolated from individuals of *D. reticulatum* that were found to be infected with *P. hermaphrodita* when collected from the field or from *D. reticulatum* infected in the laboratory with xenically cultured *P. hermaphrodita*.

To obtain monoxenic cultures of *P. hermaphrodita,* xenically-cultured individuals were first freed of bacteria then grown with the selected bacterium on a kidney-based medium on 3 cm diameter Petri dishes, as described by Wilson *et al.* (1995 *a*). Gravid adult *P. hermaphrodita* were transferred, through two changes of sterile water, to a sterile watch glass containing water with 0.02 % sodium ethyl mercurithiosalicylate (" Thimerosal ", Sigma) where they were left for 16 h at 10 °C. After this period, the larvae (L1/L2) produced by the adults were transferred to centrifuge tubes containing 10 ml quarter strength Ringer's solution (Poinar & Thomas, 1984) with 500 units ml^{-1} of both benzyl penicillin and streptomycin sulphate (Sigma). The larvae were kept in this solution at 10 °C for 24 h then concentrated by centrifugation at 90 *g* for 10 min, re-suspended in sterile quarter strength Ringer's solution and centrifuged again. Re-suspension and centrifugation were repeated once more to remove any traces of antibiotics, then sterile (axenic) larvae were transferred to a sterile watch glass, where they could be handled individually using sterile micro-pipettes. For larger scale production of monoxenic nematodes for use in bioassays, monoxenic *P. hermaphrodita* from Petri dish-cultures were introduced into 250 ml flasks by pippeting 1 ml of sterile broth over the surface of the agar, then re-pippeting the liquid containing a suspension of bacteria and 50 000-100 000 nematodes into each flask, containing either a solid or liquid medium. For solid phase cultures, flasks were filled with foam chips impregnated with kidney medium (Wilson *et al.,* 1993 *b*) and incubated at 15 °C for 3 weeks; dauer larvae were then harvested by placing the foam on sieves in water (Wilson *et al.,* 1993 *b*). For liquid cultures, nematodes were introduced into 250 ml flasks containing 50 ml of liquid kidney medium (KYO) (Wilson *et al.,* 1995) which were incubated at 15 °C in a gyrotatory flask shaker/incubator at 200 rpm for 3 weeks. Dauer larvae were harvested by allowing the nematodes to settle out in tap water.

Isolation and maintenance of bacterial cultures

Bacteria were isolated from within dauer larvae of *P. hermaphrodita,* from living and dead *D. reticulatum* infected with *P. hermaphrodita,* and from xenic foam chip cultures of *P. hermaphrodita,* as described below.

Dauer larvae were first surface-sterilised by immersion in 0.1 % (w/v) sodium ethyl mercurithiosalicylate (" Thimerosal ", Sigma Ltd) for 1 h, transferred to fresh Thimerosal for a further 3 h, then transferred into sterile quarter strength sterile Ringer's solution (Poinar & Thomas, 1984). Bacteria were liberated from these surface-sterilised nematodes in one of two ways : *i)* 48 individual dauer larvae were transferred into a drop of sterile saline on a flame-sterilised microscope slide, and then cut at several sites along their bodies. The drop of saline complete with the dead nematode was transferred to a

nutrient agar plate where it was spread over the agar surface with a glass spreader; *ii*) an estimated 20 000 dauer larvae were suspended in 1 ml of sterile Ringer's solution in a 5 ml Teflon tissue homogeniser, ground for *c.* 1 min then added to 9 ml of sterile nutrient broth. This suspension was serially (ten-fold) diluted and 0.1 ml aliquots of the resulting bacterial suspensions were plated out onto nutrient agar, using standard micro-biological techniques.

Bacteria were isolated from xenic foam-chip cultures by transferring foam chips, using sterile forceps, to nutrient broth tubes which were shaken, serially diluted and plated out onto nutrient agar.

Bacteria were isolated from within the mantle of living nematode-infected *D. reticulatum*, and from cadavers of *D. reticulatum* which had died following nematode infection and were supporting growth of large numbers of nematodes. The surface of the mantle of living *D. reticulatum* was first swabbed with 70 % (v/v) ethanol to remove surface bacteria. The mantle was then pierced with a sterile needle and drops of the exuding fluid spread over the surface of nutrient agar plates. Loopfulls of bacterial mix were taken from the slug cadaver and transferred to nutrient broth which was serially diluted and plated out on nutrient agar plates.

In all cases, nutrient agar plates were incubated at 25 °C for 2 days. Numbers of colony-forming units were then estimated and different bacterial isolates, as distinguished by colonial morphology, were subcultured and maintained on nutrient agar slopes.

Injection of slugs with bacteria

Nine bacterial isolates were grown in static cultures of nutrient broth for 24 h at 25 °C before their effects on *D. reticulatum* were tested. *D. reticulatum* were anaesthetized by exposure to carbon dioxide for 15 min (Henderson, 1969) prior to injection with 10 µl of sterile nutrient broth or bacterial culture, using a micro-applicator fitted with an all-glass syringe. After injection, twenty slugs per treatment were kept in plastic boxes (ten slugs per box) lined with moist absorbent cotton wool and mortality was recorded after 8 days. Three different experiments were done in which at least three different bacteria were screened. In the first two experiments, slugs injected with sterile nutrient broth and untreated slugs were used as controls, whereas in the third experiment only slugs injected with sterile nutrient broth were used as controls.

Bioassay of effects of *P. hermaphrodita* on *D. reticulatum*

Bioassays were done at 10 °C and 12 h daylength using a soil-based bioassay system as described by Wilson *et al.* (1993 *b*). Ten individual *D. reticulatum* were placed in plastic boxes of moistened soil aggregates containing either 0, 15 000, 23 000, 35 000, 55 000, or 75 000 dauer larvae of *P. hermaphrodita*. The slugs were removed from these boxes after five days and kept individually in Petri dishes each containing a 3 cm diameter leaf disc of *Brassica chinensis* (L) which was renewed every 3 days. Slug mortality was recorded after 9 days in the Petri dishes (fourteen days after initial exposure to nematodes).

Three experiments were done. In the first, nematodes grown in monoxenic foam-chip cultures with five bacteria (*Moraxella osloensis, Providencia rettgeri, Serratia proteamaculans* and two isolates of *Pseudomonas fluorescens* (numbers 140 and 141) were bioassayed using 50, 70, 50, 30 and 70 slugs per nematode dose, respectively, for each nematode/bacterium combination, as permitted by yields of dauer larvae from flasks of different monoxenic cultures. The bacterial flora in all flasks was examined immediately before harvest to ensure that cultures were still monoxenic. Foam chips were removed from each flask and suspended in nutrient broth, which was diluted serially and plated out onto nutrient agar so that individual colonies could be observed. Any cultures with obvious contamination were discarded.

Two further experiments used nematodes grown in monoxenic liquid culture with either *M. osloensis* or *P. rettgeri*. In both experiments, 30 slugs were used for all six nematode doses of each nematode/bacterial combination. As before, bacteria present in the liquid cultures were tested prior to harvesting to ensure monoxenicity.

For all bioassays, slug mortality data were analyzed by probit analysis using the GENSTAT 5 (Payne *et al.*, 1987) Probitan procedure (Payne, 1991).

Results

Isolation of bacteria

Over 150 bacterial isolates were obtained, 54 from the intestine of dauer larvae, 7 from infected slugs and *ca* 90 from xenic nematode cultures. Results of tests used in identifying bacteria have been deposited with the editor of *Fundamental and Applied Nematology*. *Moraxella osloensis* was described as *Moraxella phenylpyruvica* in an earlier paper (Wilson *et al.*, 1995) but further tests have identified it as the former species (A. von Graevenitz, pers. comm.).

Nine individual bacterial isolates were selected and identified for use in this study (Table 1). The mean number of bacterial colony-forming units, estimated by cutting up individual dauer larvae, was 26 (s.e. = 22.2) and the number estimated by the tissue homogeniser method was 71 (s.e. = 8.5). The number of different bacterial types from individual nematodes, as distinguished by colonial morphology, ranged from one to five.

Injection of *D. reticulatum* with bacteria

Two of the nine species of bacteria tested for pathogenicity to *D. reticulatum, Aeromonas hydrophila* and *Pseudomonas fluorescens* (isolate number 140), were

Table 1. *Species and sources of bacteria tested for their effects on* Deroceras reticulatum. *All bacteria were isolated at Long Ashton Research Station.*

Bacterium	Isolation source
Aeromonas hydrophila	Intestine of *P. hermaphrodita* dauer larvae
Aeromonas sp.*	Cadaver of field collected *D. reticulatum* which had died following infection with *P. hermaphrodita*
Flavobacterium breve	Xenic foam-chip culture of *P. hermaphrodita*
Flavobacterium odoratum	Xenic foam-chip culture of *P. hermaphrodita*
*Moraxella osloensis***	Xenic foam-chip culture of *P. hermaphrodita*
Providencia rettgeri	Xenic foam-chip culture of *P. hermaphrodita*
Pseudomonas fluorescens (isolate no. 140)	Cadaver of field-collected slug (*D. reticulatum*) which had died following infection with *P. hermaphrodita*
Pseudomonas fluorescens (isolate no. 141)	Mantle cavity of living field-collected slug (*D. reticulatum*) infected with *P. hermaphrodita*
Serratia proteamaculans	Xenic foam-chip culture of *P. hermaphrodita*

* Similar to *Aeromonas salmonicida* National Collection of Industrial and Marine Bacteria NCIMB, pers. comm.

** This was identified by NCIMB as an atypical isolate of *Moraxella phenylpyruvica,* but has since been identified by A. von Graevenitz (pers. comm.) as *M. osloensis.*

found to be highly pathogenic when injected into *D. reticulatum* (Fig. 1), causing significantly ($P < 0.01$) greater mortality than that for untreated slugs or slugs injected with sterile nutrient broth. Injection with other bacteria, including another isolate of *P. fluorescens* (No. 141), or sterile nutrient broth, did not cause mortality significantly greater than that in untreated slugs.

BIOASSAYS OF THE EFFECTS OF NEMATODES, GROWN IN MONOXENIC CULTURE WITH DIFFERENT BACTERIA, ON *D. RETICULATUM*

Aeromonas hydrophila, which was found to be pathogenic when injected into *D. reticulatum,* did not support good growth of *P. hermaphrodita* in monoxenic cultures (Wilson *et al.,* 1995) and it was not therefore possible to test nematodes grown on this bacterium for their effects on *D. reticulatum.* However, *P. fluorescens,* isolate No. 140, which was pathogenic when injected into *D. reticulatum,* supported good growth of *P. hermaphrodita* in monoxenic cultures and nematodes grown in cultures with this bacterium were tested, together with nematodes grown monoxenically on four other bacteria which supported good nematode growth (Wilson *et al.,* 1995) but which were not themselves pathogenic when injected into *D. reticulatum.*

Nematodes grown in foam chip cultures

There was no significant increase in slug mortality with increasing nematode dose for nematodes grown with *P. rettgeri, P. fluorescens* (isolate 140) or *S. proteamaculans* (Fig. 2). However, probit analysis showed a significant ($P < 0.05$) positive dose-response relationship for nematodes grown in monoxenic culture with *M. osloensis* and *P. fluorescens* (isolate 141) (Fig. 2). The ED_{50} values (effective dose required to kill 50 % of slugs, 14 days after treatment) for these two nematode/bacterium combinations were 28 900 ($\log_{10}$ s.e. = 0.0605) and 49 700 ($\log_{10}$ s.e. = 0.0947), respectively. There was no evidence of lack of fit for either probit model.

Nematodes grown in liquid cultures

In the first experiment (Fig. 3A), nematodes grown with *M. osloensis* were found to be pathogenic with an estimated ED_{50} value of 35 900 ($\log_{10}$ s.e. = 0.0583), similar to that for nematodes grown with this bacterium on foam chips. However, nematodes grown with *P. rettgeri* were not pathogenic, because slug mortality did not increase with nematode dose for this nematode/bacterium combination. Again, this is similar to the results obtained using foam chip cultures.

In the second experiment (Fig. 3B), nematodes grown with *M. osloensis* were again highly pathogenic with an ED_{50} value of 20 000 ($\log_{10}$ s.e. = 0.0355) but, in contrast to earlier experiments, nematodes grown with *P. rettgeri* were also pathogenic with an estimated ED_{50} of 32 300 ($\log_{10}$ s.e. = 0.0511).

Discussion

Although the studies on the gut flora of *P. hermaphrodita* presented in this paper are limited in their scope, they have established that *P. hermaphrodita* can retain many different types of bacteria within its gut and that no one isolate seemed to predominate. Since *P. hermaphrodita* can grow and reproduce on a wide range of bacteria (Wilson *et al.,* 1995), and it is known that ingested bacteria may sometimes survive within the gut of bacteriophagous nematodes (Poinar & Hansen, 1986), this finding is not surprising. However, it contrasts with entomopathogenic nematodes, which are generally considered to retain only their symbiotic *Xenorhabdus* or *Photorhabdus* bacteria. Nevertheless, it should be noted that Boemare *et al.* (1983) found eight species of non-symbiotic bacteria within *Steinernema carpocapsae* strain DD-136 and that Lunau *et al.* (1993) found evidence of non-symbiotic bacteria associated with various *Steinernema* spp. and *Heterorhabditis* spp.

It is difficult to quantify accurately retention of bacteria within the gut of dauer larvae of *P. hermaphrodita,*

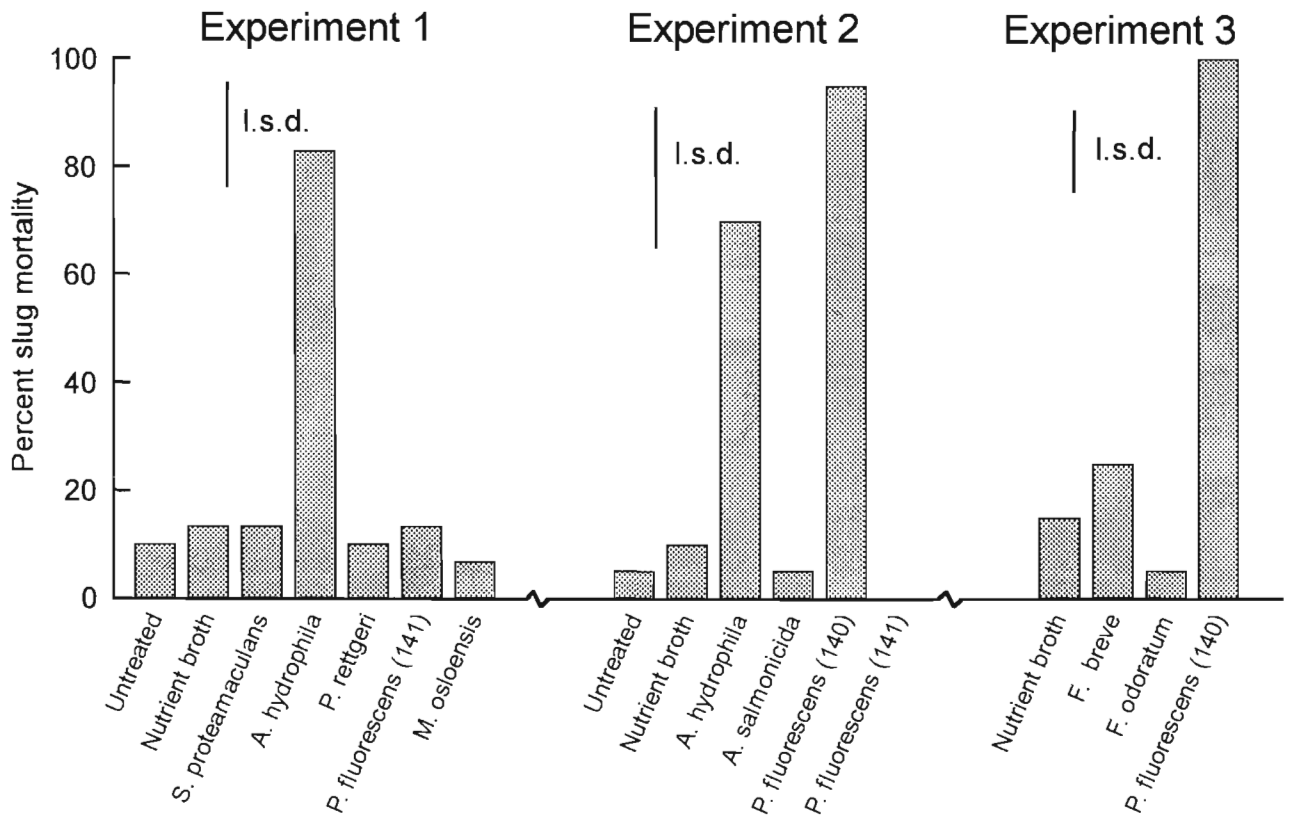


Fig. 1. *Percentage slug mortality after 8 days at 10 °C in three experiments in which* D. reticulatum *was injected with (10 µl of sterile nutrient broth or overnight cultures of different bacteria.* l.s.d. = least significant difference, $P < 0.05$).

because the double cuticle is difficult to disrupt completely. However, numbers of bacteria per *P. hermaphrodita* larva are similar to estimates made for entomopathogenic nematodes, e.g., Poinar (1975), states that 20-250 bacterial cells are associated with each dauer larva of *Steinernema carpocapsae.*

Of nine selected bacterial strains associated with *P. hermaphrodita,* only *A. hydrophila* and one strain of *P. fluorescens* (isolate number 140) were found to be pathogenic to *D. reticulatum* when injected into the haemocoel. It is, perhaps, surprising that other bacterial strains were not pathogenic considering the large numbers of bacteria which were injected. Little is known about the immune systems of gastropod molluscs, but phagocytic cells and agglutinins are thought to be important (South, 1992) and the present study indicates that antibacterial mechanisms are very effective. It should be noted that while *A. hydrophila* and *P. fluorescens* (isolate 140) were pathogenic when injected, they are only opportunistic pathogens. Neither bacterium can cause disease in *D. reticulatum* as a result of skin contact or ingestion (Wilson, 1992). However, interestingly, *A. hydrophila* has been reported to be associated with disease of the giant African snail, *Achatina fulica* (Mead, 1961), and in snails reared in snail farms in the U.K. (Runham, 1989).

Our study clearly shows that growing *P. hermaphrodita* in monoxenic culture with different bacteria can dramatically affect its pathogenicity to *D. reticulatum* and that healthy dauer larvae of *P. hermaphrodita* grown monoxenically with certain bacteria are unable to kill slugs. The bacterium which produced the most pathogenic nematode/bacterium combination (*M. osloenis*) was not itself pathogenic when injected into *D. reticulatum,* and, furthermore, nematodes grown in monoxenic culture with *P. fluorescens* (isolate 140) were not pathogenic to slugs, even though this bacterium was itself pathogenic when injected into slugs. It is possible that the way in which the bacteria interact with both *P. hermaphrodita* and the immune system of the host slug is important in determining the pathogenicity of different combinations of nematodes and bacteria. Dunphy *et al.* (1985), showed that *Steinernema glaseri* was more virulent when grown on certain subspecies of *Xenorhabdus nematophilus* than others and found that virulence correlated well with the proportion of infective juveniles which retained bacteria. Also, Akhurst (1986) observed

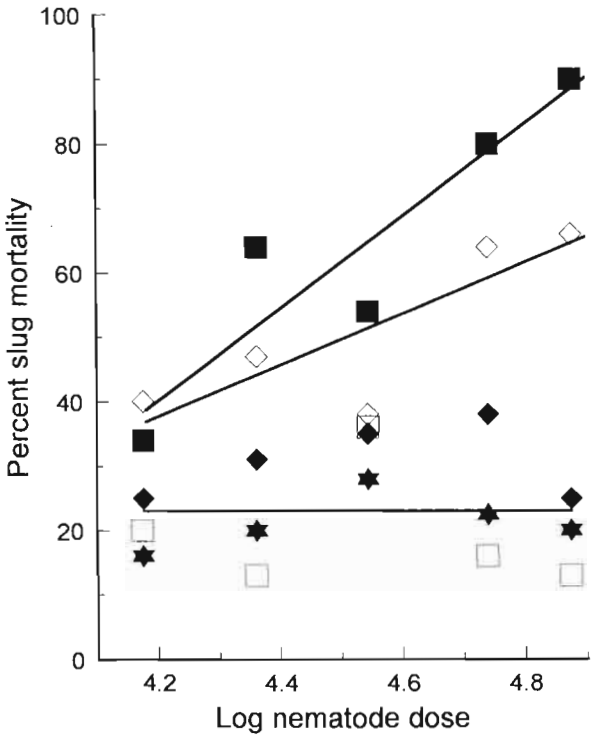
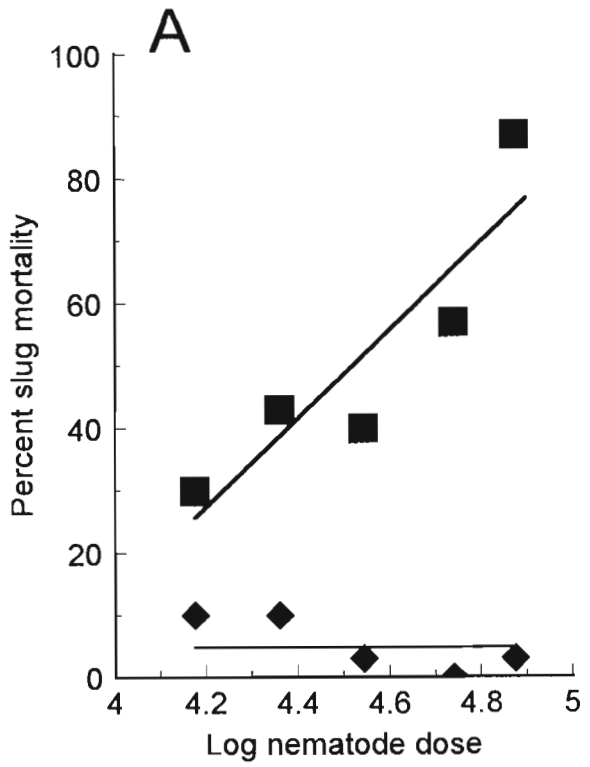


Fig. 2. *Percentage mortality of* D. reticulatum *measured 14 days after initial exposure to five different doses of* P. hermaphrodita *dauer larvae grown in monoxenic culture on foam chips with* M. osloensis (■), P. rettgeri (♦), S. proteamaculans (★), P. fluorescens *(isolate 140)* (□), *or* P. fluorescens *(isolate 141)* (◇).

that neither axenic *S. glaseri* nor its symbiont, *X. poinarii* were pathogenic to *Galleria mellonella* on their own, but were in combination.

Growth on different bacteria may influence the ability of the nematode to find and penetrate slugs. Curran (1993), referring to entomopathogenic nematodes, states that under controlled conditions, apparently favourable for infection, only a proportion of individual nematodes are capable of establishing in a host insect. This proportion ranges from 13 to 71 % for different species of *Steinernema* and *Heterorhabditis*. Curran (1993) also states that the proportion of the population which is capable of infecting and establishing within host insects can be significantly altered by the nematode culture conditions. It is possible that different conditions in monoxenic cultures with different bacteria influenced the proportion of *P. hermaphrodita* capable of infecting



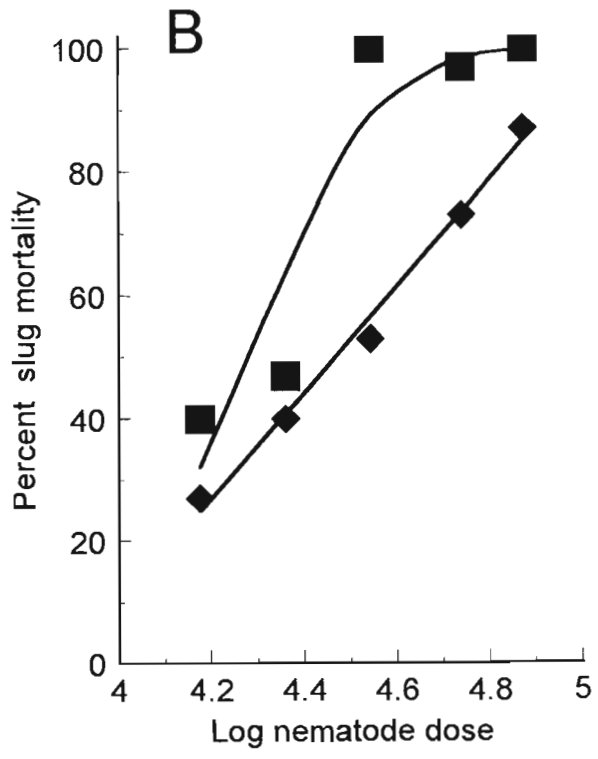


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Fig. 3. *Percentage mortality of* D. reticulatum *measured 14 days after initial exposure to five different doses of* P. hermaphrodita *dauer larvae grown in liquid monoxenic culture with* M. osloensis (■) *or* P. rettgeri (♦) *in (A) the first and (B) the second bioassay experiment using nematodes produced in liquid cultures.*

D. reticulatum in our experiments. *Providencia rettgeri,* the bacterium which produced the highest yields of dauer larvae of *P. hermaphrodita* in an earlier study (Wilson *et al.*, 1995) was found to produce nematodes which were inconsistent in their effects on *D. reticulatum,* showing no detectable effects in two experiments, but a strong dose response in a third experiment. The two experiment were done using identical methods, each with five doses of nematodes and 30 slugs per dose. The results of both experiments were statistically valid, but contradictory, for reasons which are unknown. *Moraxella osloensis* produced nematodes which were consistently pathogenic to *D. reticulatum* in all three experiments reported here and since. It produced yields of dauer larvae only slightly less than those achieved with *P. rettgeri* (Wilson *et al.*, 1995). Therefore, it was selected as the preferred bacterial species to produce *P. hermaphrodita* for further studies and for commercial production as a biocontrol agent for slugs.

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