Evaluation of primary and mutant forms of *Photorhabdus luminescens* on *in vitro* growth and multiplication of their nematode symbiont *Heterorhabditis indica*

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Summary. The P and M form cells, isolated from the infective juveniles of *Heterorhabditis indica* and from the first-stage juveniles emerging from the hermaphroditic females, respectively, proliferated on lipid agar media to provide a rich nutrient base for the efficient multiplication of the nematode symbiont. A significant difference was observed between the two variants in the development of the hermaphrodites followed by the second generation stages between 10 and 30 days. The hermaphrodites developed on the P form lawns were larger in size and produced a greater number of eggs than those recovered from the M form lawns. Both amphimictic and automictic reproduction was observed in the second generation, in both the colonies, between 25 and 35 days. *Endotokia matricida* was common feature to both nematode generations.

Key words: enterobacterium, entomopathogenic nematode, life cycle, M form, P form.

Photorhabdus luminescens is a Gram-negative enterobacterium found in the gut of free-living infective juveniles (IJ) of entomopathogenic nematodes (EPN), *Heterorhabtditis* spp. In the past decade, *P. luminescens* has emerged as a useful model organism to study bacteria-host interactions because it shows symbiosis and pathogenicity with different invertebrate hosts during a normal life cycle (Ffrench-Constant *et al.*, 2003; Goodrich-Blair & Clarke, 2007).

The *H. indica* and *P. luminescens* pair are commercially reared as biological control agents of a wide range of insect pests of agricultural crops (Shapiro-Ilan *et al.*, 2012). The dual attributes of symbiosis with the nematode and pathogenicity towards the insect larvae are essential for the *in vivo* survival of *Photorhabdus* (Dowds & Peters, 2002). *Photorhabdus* exists as two phenotypically distinct variants – the primary (P form) and the secondary variant (P II form). Only the P form found in association with the nematode, can support nematode growth and development, both in the insect cadaver (*in vivo*) and *in vitro* (Akhurst, 1980; Boemare & Akhurst, 1988). Once the P form variants enter the post-exponential phase of growth, the P II variants are expressed which do not support nematode growth and development. A reversion from the P II to the P form variant has not been observed in *Photorhabdus* (Clarke & Dowds, 1995; Hu & Webster, 2000; Daborn *et al.*, 2001). The recently discovered maternal transmittal of *Photorhabdus* to the offspring *via* the rectal gland cells of the maternal nematodes disproves the widely accepted hypothesis that symbionts establish only through oral feeding inside the insect haemocoel (Ciche *et al.*, 2008; Somvanshi *et al.*, 2010).

In contrast to the P form bacteria, the maternally linked 'M form' bacterial cells are not pathogenic nor released into the insect haemolymph. M form cells are avirulent smaller-cell variants produced from P-form cells to initiate mutualism in the intestines of the host nematode (Somvanshi *et al.*, 2012). Although maternally transmitted in the symbiotic relationship, the role of the M form variants is in providing a suitable nutrient rich environment to support nematode growth and development is unclear.

Intra-uterine birth causing maternal death (*endotokia matricida*) is relatively common in

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rhabditid nematodes (Chaudhuri *et al.*, 2011) and typical for entomopathogenic nematodes of the genus *Heterorhabditis*. The main function of this intra-uterine birth is to provide optimal conditions for IJ development when the environmental conditions are becoming detrimental (Serycynska *et al.*, 1974; Baliadi *et al.*, 2001). Low food supply, high population densities, non-functional vulva, and nematode ageing have been found to induce the forming of this phenomenon (Poinar, 1990; Kondo & Ishibashi, 1991; Johnigk & Ehlers, 1999a). Under the present study, we compared the ability of P and M form variants of *P. luminescens* to support the growth and reproduction of their nematode symbiont by supplementing *in vitro* cultures of axenic *H. indica* with these two form variants. As the commercial scale fermenter-based *in vitro* production technology of *Heterorhabditis* relies completely on the monoxenic environment created by the P form variants, a study of the role of the M form variants may provide new insights into existing fermentation methods technology.

MATERIAL AND METHODS

Isolation of P form from IJ. The bacteria were isolated from the haemolymph of *Galleria mellonella* cadavers infected with *H. indica* by using the protocol described elsewhere (Akhurst, 1980). Five 4th instar *G. mellonella* larvae were placed in a Petri dish lined with moist filter paper and inoculated with 100 IJ of *H. indica*. After 48 h the cadavers were surface sterilised by dipping in 95% ethanol, followed by flaming for 3 to 4 s, and finally dipped in sterile distilled water. One cadaver from each nematode isolate was cut open from the ventral side using sterile scissors and forceps without rupture to the midgut. A drop of haemolymph was removed with a sterile loop and streaked onto a nutrient agar (NA) plate (Himedia, cat no. 012). The plates were incubated at 28°C for 48 h. The single-cell colonies were sub-cultured on fresh media 5 to 6 times or until a purified culture was obtained.

Isolation of M form from first-stage juveniles (J1). *Galleria mellonella* infected with *H. indica* were dissected on the third day in Ringer's solution. Hermaphrodite females were collected and rinsed twice in Ringer's solution to remove adhering debris followed by surface sterilised in a solution comprising 2.5 ml 4 M NaOH, 1.5 ml 4% NaOCl and 21.5 ml distilled water for 10 min. Care was taken not to burst the females. The females were rinsed again in Ringer's solution and then transferred to lipid agar (LA) media (Nutrient broth [Himedia, cat no. M 244S]: 16 g + agar 12 g + sunflower oil 5 ml + distilled water 1 l) and incubated at 28°C for the emergence of J1. Emerging J1 were collected in Eppendorf tubes and rinsed twice in sterile distilled water using a tabletop centrifuge. Five J1 were transferred separately in 5 µl drop of sterile distilled water on a clean glass slide. Under a binocular microscope, they were crushed and teased apart to release the body contents. The entire content was then transferred to nutrient agar plates and spread uniformly with a spreader. The plates were sealed and incubated in the dark for 48 h at 27°C to culture the M form *Photorhabdus* cells.

Preparing liquid cultures of P and M forms of *P. luminescens*. Parent cultures from single-cell colonies of both the bacterial isolates were collected on a disposable plastic loop from nutrient agar plates and inoculated in conical flasks containing 50 ml of nutrient broth. The flasks were placed on a shaker at 250 rpm at 28°C in the dark. The cultures were incubated for 24 h and bacterial cell counts made using a haemocytometer. The flask containing the least number of cells (1.3×10^7 ml^{-1}) was selected. The bacterial cell counts of the remaining flasks were adjusted to the same level by diluting the culture with sterile water. The cultures were stored in a refrigerator and used as stock for further studies.

Nematode development on P and M forms of *P. luminescens*. Two ml of sterile LA media was poured in 3 cm diam. Petri dishes and inoculated with 100 µl of liquid stock cultures of either the P or M forms of *P. luminescens*. The plates were sealed and incubated at 28°C overnight in the dark to allow the respective bacterial colony to grow. Fifty sterile eggs obtained as per Lunau *et al.* (1993) were transferred to each dish. The Petri dishes were sealed and incubated at 28°C in dark condition to allow the nematode life cycle to commence. Observations on nematode development and life cycle were taken after 5, 10, 15, 20, 25, 30 and 35 days. Three replicates were maintained for each observation. The lipid agar media with no bacteria served as control and the experiment was repeated once.

Statistical analysis. The growth and reproduction of *H. indica* on lipid agar media supplemented with P and M form variants of *P. luminescens* was analysed by SAS software. The significant difference between different growth stages of *H. indica* on P and M form variants were calculated using Tukey's test.

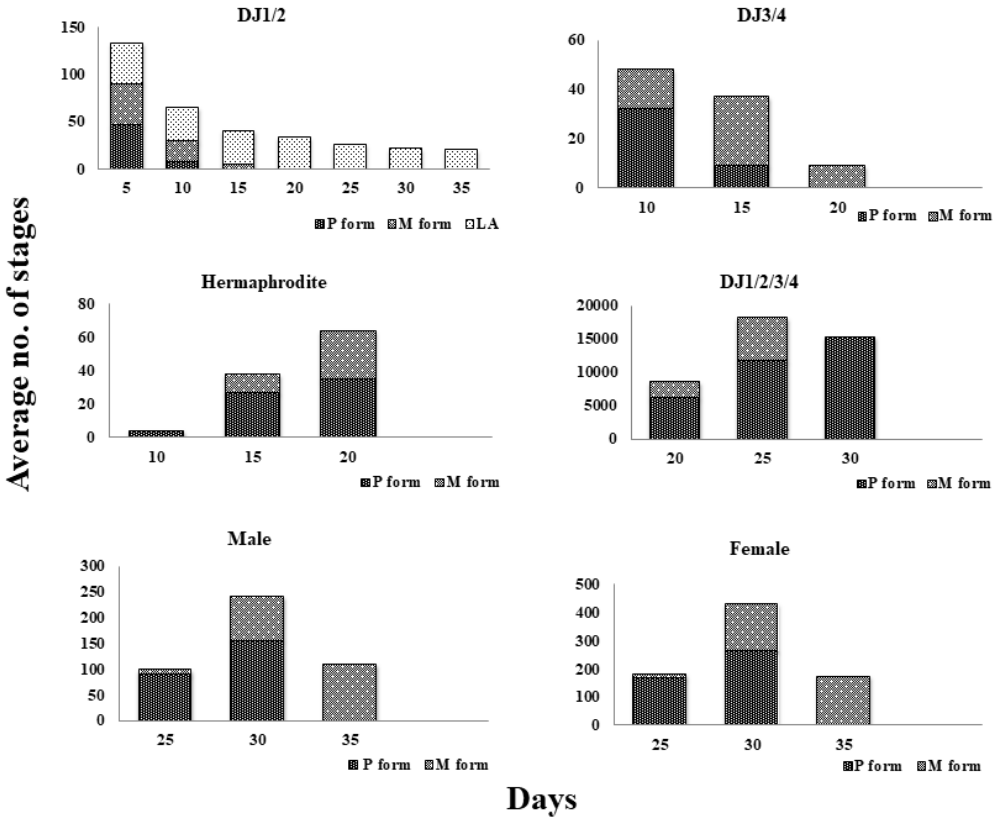

Fig. 1. *In vitro* growth and reproduction of *Heterorhabditis indica* on lipid agar media supplemented with P and M form variants of *Photorhabdus luminescens*.

RESULTS

All stages of *H. indica* propagated successfully on LA media supplemented either with P form *Photorhabdus* (isolated from the *Galleria* haemolymph) or M form *Photorhabdus* (isolated from the J1 hatched from the sterile eggs recovered from the second-generation females of *H. indica*) within 35 days.

First generation J1 and J2. Hatching was initiated within overnight incubation of the plates at 28°C (Fig. 1). The eggs exhibited asynchronous embryogenesis, as different levels of cell developmental stages could be seen. After 5 days nearly 85% of the nematodes had hatched in all the three treatments. After 10 days only 7 nematodes recovered from plates supplemented with P form bacteria were in J1/second-stage juveniles (J2) stage as majority of them had moulted into third- and fourth-stage juveniles (J3/J4), while after 15 days all had transformed into J3/J4. In M form bacterial lawns, 22 nematodes were still in J1/J2 stages on day 10 as compared to 4 after day 15. In control plates (lipid agar alone) where there were no bacteria, the nematodes remained in J1/J2 stages even 35 days after inoculation of eggs, which remained significantly different, throughout, compared with the treated plates.

First generation J3 and J4. Between 10 to 15 days, 41 J1/J2 stages moulted into J3/J4 in plates supplemented with P form cells as compared to 44 in plates with M form cells. After 20 days, however, there was no recovery of J3/J4 in P form as compared to 9 J3/J4 stages in M form, suggesting a relatively slow development of the nematodes in the later. Statistically, there was no significant difference between the two; after day 15 there have been overlapping of J1, J2, J3 and J4 in addition to the hermaphrodites in both P and M form bacterial lawns (Fig. 1).

Hermaphrodites. The first hermaphrodites were recovered after day 10 in P form lawns as compared to none in M form lawns. The numbers of hermaphrodites gradually increased to 27 (day 15) and 35 (day 20) in P form lawns as compared to 11 (day 15) and 29 (day 20) in M form lawns, which were significantly different. The hermaphrodites in P form plates were larger in size (average 2510 μm) and produced more eggs (average 346 eggs per

hermaphrodite) compared with those recovered in M form lawns, which averaged 1390 µm carrying 237 eggs per hermaphrodite. Interestingly in both the forms, the hermaphrodite females were observed to be laying eggs in the media.

Second generation J1/J2/J3/J4. The hermaphrodites recovered between 10 to 20 days started producing the second generation progeny between 20 to 30 days which was statistically higher in P form lawns as compared to the M form. Due to asynchronous development, large numbers and difficulty to differentiate the four developmental stages (J1/J2/J3 and J4) in the lawns, a cumulative count was taken (Fig. 1).

Males and females. The first set of males was recorded on day 20 in both bacterial lawns and were produced through day 35 with no statistical difference between lawns. In the P form lawns the average number of males was 89 (day 25), which increased to 156 (day 30), whereas in M form plates initially only 10 males (day 25) were recorded but increased to 85 (day 30) and 110 (day 35). The second generation female nematodes were recorded between 25 to 35 days and they comprised both amphimictic and hermaphroditic stages. The production of females was similar as in the case of males with no statistical difference between the P and M form lawns. In the P form, 168 females (day 25) increased to 266 (day 30) in comparison to 16 (day 25), 166 (day 30) and 175 (day 35) in M form. Amphimictic mode of reproduction was most probable as the males were observed in coiled position around the female vulva.

***Endotokia matricida*.** *Endotokia matricida* was clearly observed in the hermaphrodites between 10 and 20 days where several females were observed carrying mature infective juveniles inside their body. Between 25 and 35 days, this phenomenon was also exhibited by the second-generation females, which were found to contain not only mature IJ but also female nematodes that were also carrying eggs.

DISCUSSION

In this study we have evaluated the growth and reproduction of *H. indica* on both P and M forms of *P. luminescens*. Following the inoculation of eggs *in vitro*, the growth rate of the first generation J1, J2, J3, J4, males and the second generation females varied among both the M and P form supplemented plates. However, the moulting was initially slow in the M form supplemented plates. This initial slow moulting rate may reflect the fact that the M form bacteria, which naturally persist in the maternal nematode intestines, were grown on artificial LA and thus took longer to exit the G_o phase (resting/senescent phase) and enter the S phase (cell growth phase).

In comparison, the P form symbionts are known to establish faster under *in vivo* or *in vitro* conditions (Forst *et al.*, 1997; Forst & Clarke, 2002; Clarke, 2008; Waterfield *et al.*, 2009). Functionally, the M form variants are much smaller (one-seventh the volume), slower growing, and less bioluminescent than the P form cells. They are also avirulent and produce fewer secondary metabolites essential for nematode growth and multiplication (Serycynska *et al.*, 1974). The P form symbionts produce crystalline inclusion proteins (Cips) containing high levels of essential amino acids that are required for nematode reproduction (Bintrim & Ensign, 1998; Somvanshi *et al.*, 2011) and some feeding studies suggest that Cips have a role in nematode nutrition (You *et al.*, 2006). Thus, the P form variants may have provided a nutrient rich environment resulting in the development after 30 days of a greater number of hermaphrodites, larger in size, and carrying more eggs as compared to the M form variants. The M form variants are reported to lack visible Cip A and Cip B inclusions (Bintrim & Ensign, 1998; Somvanshi *et al.*, 2012). The amphimictic (sexual) and automictic (hermaphroditic) mode of reproduction occurred between 25 and 35 days in the second generation in both the P and M form bacterial lawns. The offspring of the first-generation hermaphrodite have the possibility of developing into amphimictic adults or automictic hermaphrodites, or into both simultaneously (Strauch *et al.*, 1994). The pathway leading to the development of amphimictic adults is induced by favourable nutritional conditions, whereas the development into automictic hermaphrodites is induced by low concentrations of nutrients (Strauch & Ehlers, 1998; Han & Ehlers, 2000, 2001). In the present experiment, as the number of days increased, there was a gradual decrease in the available nutrients that presumably created a stress to trigger the development of hermaphrodites.

Intra-uterine birth causing maternal death (*endotokia matricida*) is relatively common in rhabditid nematodes and typical for EPN of the genus *Heterorhabditis*. When egg laying ceases, the nematodes develop inside the maternal body cavity (Johnigk & Ehlers, 1999b). Interestingly, the nematodes that develop by *endotokia matricida* are predominantly hermaphroditic IJ (Dix *et al.*, 1992). It is reported that *endotokia matricida* is triggered by reduced food supply, high population densities and

nematode ageing in order to provide optimal conditions for the development of IJ (Serycynska *et al.*, 1974; Poinar, 1990; Kondo & Ishibashi, 1991; Johnigk & Ehlers, 1999a; Baliadi *et al.*, 2001). The phenomenon of *endotokia matricida* was observed in the hermaphrodites between 10 and 20 days and between 25 and 35 days. This phenomenon was also exhibited by the second-generation females and many were found to be carrying not only mature IJ but also female stages which were further carrying eggs.

The present study suggested that the maternally transmitted M form variants, which are the mutant forms of P variants, are not required to support nematode multiplication as their intrinsic role in nematode-bacteria symbiosis; they can do so at a lesser degree, if required, under *in vitro* conditions. However, a specialised biological function of M form variants in symbiosis needs further in-depth investigations.

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K. Kiran Kumar and S. Mohan. Оценка воздействия первичнных и мутировавших форм *Photorhabdus luminescens* на рост и размножение *in vitro* их нематоды-симбионта *Heterorhabditis indica*.

Резюме. Первичные (P) и мутировавшие (M) формы бактерий *Photorhabdus luminescens*, изолированные из ивазионных личинок и личинок первой стадии *Heterorhabditis indica*, полученных от гермафродитов первого поколения, соответственно, культивировали на липидном агаре. Эти две формы бактерий на липидном агаре использовали как питательную среду для размножения нематоды *H. indica*. На 10-й и 30-й дни после инокуляции, наблюдали существенные различия в развитии гермафродитов и особей второй амфимиктической генерации. Гермафродиты, развивавшиеся на P-формах, образовывали меньшее число яиц, чем те, что развивались на M-формах. Как амфимиктическое размножение, так и самооплодотворение наблюдали при развитии на P и M-формах на 25-35 день развития. *Endotokia matricida* была отмечена при развитии на каждой из форм.