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ORIGINAL PAPER

Stability of entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus luminescens*, during in vitro culture

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Abstract The entomopathogenic nematode–bacteria complexes *Heterorhabditis bacteriophora/Photorhabdus luminescens* and *Steinernema carpocapsae/Xenorhabdus nematophila* are mass produced for use as biological insecticides. Stability of the bacterial partner in culture is essential for maintaining traits important for both biological control and production. Two geographically distinct strains of each bacterial species were isolated from their nematode partners and serially subcultured on in vitro media to assess trait stability. Subculturing resulted in a shift to secondary cell production in one *P. luminescens* strain and both *X. nematophila* strains within ten in vitro culture cycles. However, when cell phenotypic variation was controlled in *X. nematophila* strains by regular selection for primary variants, no trait change was detected in the primary variant after prolonged subculture. When *P. luminescens* cell phenotypic variation was controlled by selection for primary variants, changes in the primary variant of both strains were noted including reductions in cell and inclusion body size and inclusion body prevalence. Bacterial ability to cause lethal infections following injection into the hemocoel of *Tenebrio molitor* larvae declined by more than half in primary variants of one *P. luminescens* strain. Conversely, yield was enhanced, with the subcultured *P. luminescens* strains showing 53.5 and 75.8% increases in primary cell density. Field adapted traits of primary variant *P. luminescens* strains tend to deteriorate during in vitro culture as tradeoffs for gains in yield. In vitro producers of the *P. luminescens/H. bacteriophora* complex must weigh the need for superior bacterial yield against the need to preserve traits important for biological control.

Keywords Stability · Entomopathogenic nematodes · *Xenorhabdus nematophila* · *Photorhabdus luminescens*

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Introduction

Photorhabdus luminescens and *Xenorhabdus nematophila* are Gram-negative, non-spore forming bacteria mutualistically associated with the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*, respectively. The symbiotic bacteria are carried in the gut of their associated nematode infective juveniles, a specialized free-living stage. The infective juveniles search for insect hosts in the soil environment, and penetrate into the host hemocoel via natural openings or the cuticle. The nematodes then release their symbiotic bacteria, which multiply to kill the insects in 1–2 days. The nematodes feed upon the bacteria and host tissues to complete their development. When the insect cadaver is depleted of nutrients and nematode densities have reached high levels after 2–3 generations, infective juveniles carrying their symbiotic bacteria emerge from the cadaver to search for new insect hosts. These bacteria are unique in the bacterial world in forming a mutualistic association in one host and an aggressively pathogenic association against another phylum [14].

The nematodes benefit from this cyclic association by using their symbiotic partner to kill the insect host quickly and to provide essential nutrients, antibiotics to provide protection against secondary invaders, and anti-immune factors to overcome insect defenses [14]. Conversely, the bacteria benefit by using their nematode partner to provide protection from biotic and abiotic soil factors, and as a transmission vector to find, recognize, and penetrate suitable insect hosts. The elucidation of this complex symbiotic relationship [26, 27] eventually made mass production of the nematode-bacterium complex possible [16], and led in turn to their development and use as biological insecticides against citrus root weevils, billbugs, white grubs, black vine weevils, mushroom flies, mole crickets, and many other soil insect pests [17]. The bacteria also yield a diversity of compounds with antimicrobial, nematicidal, insecticidal, and other bioactivities of industrial interest [35].

Industrial producers of the entomopathogen complex employ either in vivo or in vitro methods [16]. In either system, trait deterioration is of concern. Reports of deterioration have focused on the nematode partner and include declines in storage stability in *S. carpocapsae* [15], virulence in *H. bacteriophora* [19, 34], and infectivity and reproductive capacity in *Steinernema glaseri* [32] following repeated in vivo subculturing. Subculturing of *S. carpocapsae* and *H. bacteriophora* resulted in the decline of multiple important traits relevant to biological control including virulence, heat tolerance, fecundity, host finding, and nictation, with the bacterial partner implicated in trait change [7]. Strain deterioration ranks behind only contamination as a problem in entomopathogenic nematode culture [16].

The symbiotic bacteria of entomopathogenic nematodes play an important role in nematode fitness, so any change in the bacteria during culture may affect nematode fitness. For example, the bacteria are prone to phenotypic variation [8, 14] and show two extremes of variant cell types, primary and secondary. Prolonged culturing induces formation of secondary cell variants with a concurrent reduction in nematode yield [1].

Trait change in other species of laboratory-cultured bacteria has been reported [23, 33]. Change in entomopathogenic nematodes/bacteria has focused on the nematode partner, whereas only alteration in cell variants (i.e., phase shift) has been studied in the associated bacteria. We investigated the stability of the bacterial partner in the entomopathogenic nematode–bacterium complexes *H. bacteriophora/P. luminescens* and *S. carpocapsae/X. nematophila* during in vitro subculturing. Previously we examined how the nematode-bacteria complex changes during serial subculture [7]. Here we focus exclusively on deterioration in bacteria in serial culture without their nematode partners as is the case during routine culture as well as early scale up for industrial production. Trait change associated with phase shift has already been well characterized, thus we investigate change when phase shift is controlled; that is, change in primaries.

Phase shift from primary to secondary in the bacterial symbionts was observed as early as 1980 (1) and is associated with phenotypic changes, often unfavorable from a production standpoint, in yield, antibiotics, luminescence, dye absorption, colony morphology, pigmentation, and other traits (1, 27). Thus, industrial producers control phase by inoculating the primary variant. Phase shift, however, is not our focus here as the shift to secondary variants is not only well documented but is a stress response rather than the origin of trait deterioration. Here we show for the first time that even when phase shift is controlled by repeated selection for primary colonies, changes can nevertheless result *within* the primary variant.

Materials and methods

Cultures

Soil samples were collected from New Jersey and Georgia, USA, to extract two fresh isolates of *H. bacteriophora*, and from New Jersey and Arkansas, USA, to extract two fresh isolates of *S. carpocapsae* using the insect bait method [5]. Symbiotic bacteria were isolated from the hemolymph of infected *Galleria mellonella* larvae and plated onto NBTA plates (nutrient agar supplemented with 25 mg of bromothymol blue and 40 mg of triphenyl-2,3,5-tetrazolium chloride per liter), according to procedures previously described [12]. The resulting single colony *P. luminescens* strains were designated as Hb-GA and Hb-NJx, and the *X. nematophila* strains as Sc-Cxrd and Sc-NJ, denoting their nematode partner and geographical origins.

A base population of each strain was established in modified TSY media (40 g tryptic soy broth and 5 g yeast extract per liter) and a portion of the culture was stored at −80°C in 20% glycerol [28]. All bacterial culture was in vitro per standard practice in entomopathogenic nematode mass production [16]. From the base culture, three culture replicates for each of the four strains were setup in 250 ml shaker flasks (1×10^9 cells of inoculum in 50 ml of TSY). The flasks were incubated in darkness at 25°C and 200 rpm for 48 h per cycle [31]. With the exception of the experiment on cell type detailed below, inoculum for every liquid culture

cycle was initiated by plating the previous cycle onto NBTA plates and selecting 20–30 primary cell type colonies. This complied with practices in the entomopathogenic nematode industry in which in vitro production is initiated with primary cells.

Changes resulting from serial laboratory culture in cell type, cell and inclusion body size, and inclusion body prevalence were assessed every fifth cycle for 25 cycles (10 cycles for the study in cell variants) in TSY medium. Yield and virulence assays were conducted by comparing 25th cycle bacteria with base bacteria thawed for 24 h at room temperature and grown for 24 h in TSY medium. All experiments were conducted at 25°C.

Cell type

A separate culture was used in an experiment designed to test cell type stability. This experiment was as described above except without intermediate plating and selection for primary cell colonies between cycles. Each fifth cycle culture was inoculated onto NBTA plates (three plates per strain), incubated for 48 h, and variant colonies differentiated according to dye absorption, pigmentation, swarming, colony stickiness (*Photorhabdus* only), colony and cellular morphology [1, 36]. The experiment was terminated after ten cycles. Cell phenotype was controlled for primary cells in all subsequent experiments.

Cell and inclusion body size

Three glass microscope slides were prepared from each fifth cycle flask after 1:50 dilution with Ringer's solution. Three photos were taken at 1,000× magnification for each flask and image analysis software (http://www.jimage-mosaic.sourceforge.net/) used to measure cell and inclusion body sizes of 30 stationary-phase cells per replicate.

Inclusion body prevalence

A glass microscope slide was prepared with a sample taken from every fifth cycle flask after 1:50 dilution with Ringer's solution. Three fields-of-vision at 1,000× magnification, each containing from 37 to 74 cells, were randomly selected and the proportion of cells containing inclusion bodies was determined.

Yield

Approximately 1×10^9 cells from the control (base) and 25th cycle populations for each strain were inoculated into 50 ml of TYS medium as described above. Optical densities (OD^{600}) were recorded at 4 h intervals over 48 h and used to determine cell density with a previously established standard growth curve. Three flask replicates were measured for each strain and control.

Virulence

Tenebrio molitor was used for virulence assays rather than *G. mellonella* because the high susceptibility of the latter host rendered it unsuitable for this assay. Larvae of *T. molitor* from Superworm Farm (Kempton, PA, USA) were held with oatmeal as food for 3 days before bacterial injection. Late instar larvae weighing from 0.09 to 0.15 g were used.

Stationary-phase cells from each strain of the 25th culture cycle were washed in M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 1 ml $MgSO_4$ per liter) by centrifugation (1,500 rpm for 15 min) twice followed by a single wash and resuspension in sterile Ringer's solution. Bacterial density was determined with a Petroff–Hausser counting chamber and the cells diluted with Ringer's to concentrations of 0, 10, 20, 50, and 100 cells per 3 µl for injection.

A 25 µl glass capillary tube was heated and drawn out to a fine point to form a glass microsyringe injection needle. We injected 3 µl of cell suspension per larva via microinjector (Drummond Scientific, Broomall, PA, USA) by inserting the glass needle between the sixth and seventh dorsal sclerites. Three replicates of ten larvae each were injected per strain and the experiment was repeated twice. Sterile Ringer's solution was used as the control. Injected larvae were transferred to a 60-mm Petri dish with oatmeal. The dishes were incubated at 25°C and mortality assessed at 8 h intervals for 48 h.

Statistical analysis

Treatment effects were compared against the controls using the Student '*t*' test at $P \leq 0.05$ and LD_{50} values were calculated [29]. Tukey's multiple range test was applied to make comparisons between means. Untransformed data was subjected to analysis using Kyplot software (http://www.kyenslab.com/en).

Results

Cell type

In cultures designed to assess cell type stability and where cell phenotypic variation was therefore not

controlled, more than 90% of colonies shifted from primary to secondary cell formation within five cycles for the Hb-GA strain of *P. luminescens* and nearly 70% ($P < 0.05$; $t_4 = 139.9$) for the Sc-NJ strain of *X. nematophila* ($P < 0.05$; $t_4 = 44.9$) (Fig. 1). When the study was terminated after ten cycles, *P. luminescens* Hb-NJx still produced exclusively primary colonies whereas the other three strains had shifted heavily (88.9–95.4%) to the secondary cell variant.

This was the only experiment in our study in which cultures were permitted to undergo phase shift, as in all subsequent experiments phenotypic variation was controlled for primaries. Efforts to maintain the cultures with the primary variant via selection, and therefore study primary deterioration rather than phase shift, were successful in all cycles including the 25th.

Cell and inclusion body size

When cell phenotypic variation was controlled by selection for primary cells, major reductions of *P. luminescens* cell size resulted from prolonged culture (Fig. 2). Reduced cell size was not observed until cycle 20 in both strains. By the 25th cycle, the volume of *P. luminescens* Hb-NJx cells was diminished by 66.5% from 2.6 ± 0.4 to 0.87 ± 0.2 μm^3 ($P < 0.05$; $t_4 = 7.4$), and in Hb-GA by 86.5% from 2.6 ± 0.4 to 0.35 ± 0.04 μm^3 ($P < 0.05$; $t_4 = 7.7$). Cell size in *X. nematophila* strains did not change during the study (Fig. 2).

Even more severe reductions were detected in the size of *P. luminescens* inclusion bodies, starting from cycle 20 ($P < 0.05$; $t_4 = 6.9$) for Hb-GA (Fig. 3). The reduction in inclusion body volume after 25 cycles of liquid culture for Hb-NJx and Hb-GA were 84.9

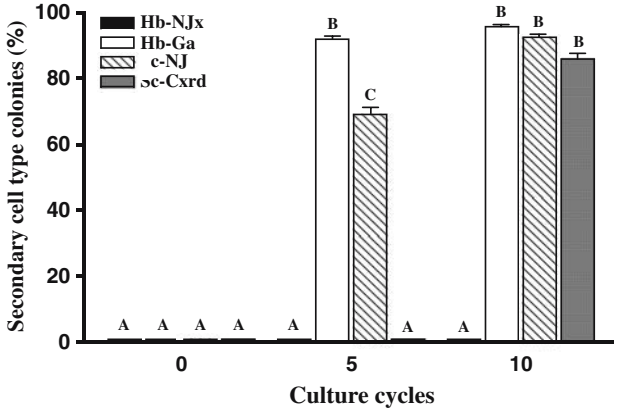

Fig 1 Percentage of secondary cell type colonies of *Photorhabdus luminescens* (Hb-NJx and Hb-GA strains) and *Xenorhabdus nematophila* (Sc-NJ and Sc-Cxrd strains) over ten continuous cycles in liquid culture. Tukey's multiple range test applied for comparison. Bars (±SD) with different letters show significant differences at $P < 0.05$

($P < 0.05$) and 96.4%. No change in inclusion body size was observed in *X. nematophila* (Fig. 3).

Inclusion body prevalence

The proportion of *P. luminescens* strains producing inclusion bodies declined beginning from cycles 10 and 15 for Hb-GA ($P < 0.05$; $t_4 = 5.9$) and Hb-NJx ($P < 0.05$; $t_4 = 5.28$), respectively (Fig. 4). By the 25th cycle, the reduction of cells producing at least one inclusion body reached 20.1 and 29.3% for Hb-GA and Hb-NJx. In addition, the proportion of cells producing two or more inclusion bodies had declined 60.9% for Hb-GA from 33.34 ± 3.11 to 13.04 ± 3.34%, and 56.8% for Hb-NJx from 22.0 ± 4.36 to 9.5 ± 2.72% after 25 cycles (data not shown). The *X. nematophila* strains showed no change in inclusion body prevalence (Fig. 4).

Yield

Yield curves for the two strains of *P. luminescens* showed similar patterns with cycle 25 bacteria providing more rapid population growth and greater final cell densities than the control (base) populations (Fig. 5a, b). Differences were evident within 12 h of flask culture for the Hb-GA strain where 25th cycle bacteria produced 169.4% more cells than the control flasks ($P < 0.05$; $t_4 = 89.1$). The 25th cycle Hb-NJx strain also outperformed the control, albeit less spectacularly, with differences becoming evident 32 h post-inoculation where 25th cycle bacteria produced 53.5% more cells than the controls ($P < 0.05$; $t_4 = 48.2$). After 48 h of culture, Hb-GA cell density averaged 3.685×10^6 cells/μl in the control flasks compared with 6.478×10^6 cells/μl in the experimental 25th cycle flasks, a 75.8% difference. Similar 48 h results were obtained with Hb-NJx where cell density averaged 1.71×10^6 cells/μl in the controls compared to 2.9×10^6 cells/μl in the 25th cycle flasks, a 69.6% difference. The two *X. nematophila* 25th cycle strains showed virtually identical curves relative to their controls (Fig. 5c, d).

Virulence

A decline in bacterial virulence was detected in the Hb-GA strain of *P. luminescens* where a 2.33-fold increase in LD_{50} from 9.73 ± 1.7 to 22.67 ± 1.6 cells was recorded ($P < 0.05$; $t_4 = 14.0$) (Fig. 6). The Hb-NJx strain of *P. luminescens* showed an apparent reduction in LD_{50} after 25 cycles of liquid culture as compared to the control but this difference was not statistically significant. LD_{50} values remained stable for the *X. nematophila* strains (Fig. 6).

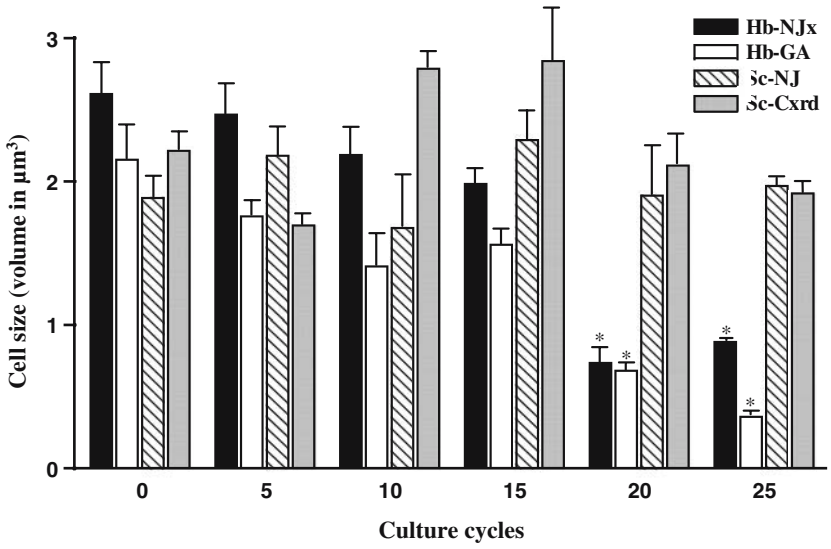

Fig. 2 Cell size of *Photorhabdus luminescens* (Hb-NJx and Hb-GA strains) and *Xenorhabdus nematophila* (Sc-NJ and Sc-Cxrd strains) over 25 culture cycles in liquid culture (cell type was controlled by plating and selection for primary cell colonies between liquid culture cycles). Bars (±SD) with an "*" are significantly different from the control (0) at $P < 0.05$

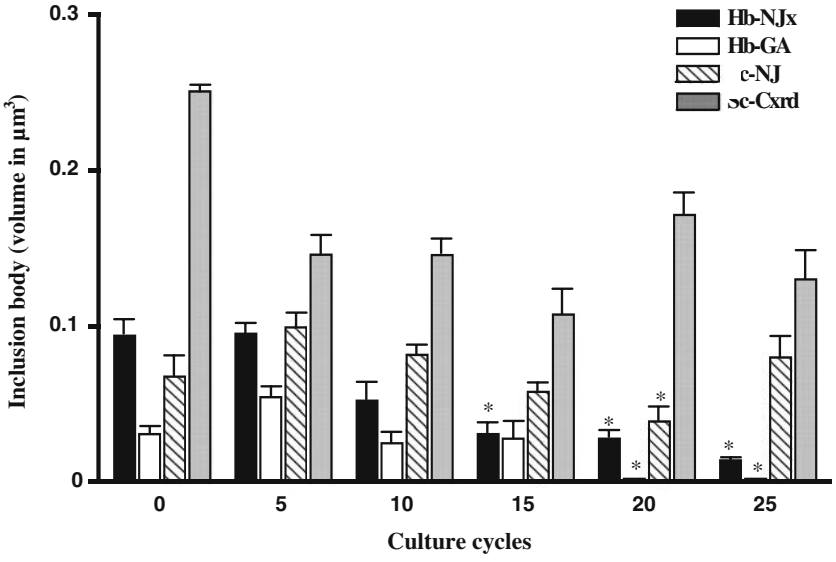

Fig 3 Inclusion body size of *Photorhabdus luminescens* (Hb-NJx and Hb-GA strains) and *Xenorhabdus nematophila* (Sc-NJ and Sc-Cxrd strains) over 25 culture cycles in liquid culture (cell type was controlled by plating and selection for primary cell colonies between liquid culture cycles). Bars (±SD) with an "*" are significantly different from the control (0) at $P < 0.05$

Discussion

Adaptive evolution occurs when a population encounters a new environment, permitting beneficial variants showing fitness (yield) gains to emerge. In our controlled in vitro environment, when cell type variation was controlled, we observed relative fitness gains in the *P. luminescens* strains with the derived lines growing faster with greater yields than their wild type ancestors adapted for in vivo environments. In competing for limited resources, some variants adapted to the new in vitro environment and established optimal growth, replacing the original base population.

Populations often show tradeoffs in relative fitness across different environments, especially in traits where selection pressure has been removed [6]. Thus DeBach's [11] warning that in modifying one character favorably, others may go in the opposite direction. Some beneficial traits, from the symbiotic relationship with their nematode partners and biological control points-of-view, deteriorated in our study including cell and inclusion body size, and prevalence of inclusion bodies in the two *P. luminescens* strains, as well as virulence in one strain (Hb-GA). The Hb-NJx strain of *P. luminescens* was less susceptible than Hb-GA to alteration from subculturing as demonstrated in nearly every trait measured but was especially notable for virulence, cell type, and cell size. The tradeoff, however, was that Hb-NJx did not receive as great a boost in relative fitness (yield) as its counterpart.

Phenotypic variation is controlled during production because the primary cell variant best supports nematode reproduction. Prolonged culture results in secondary cells [1, 2]; thus the least surprising change resulting

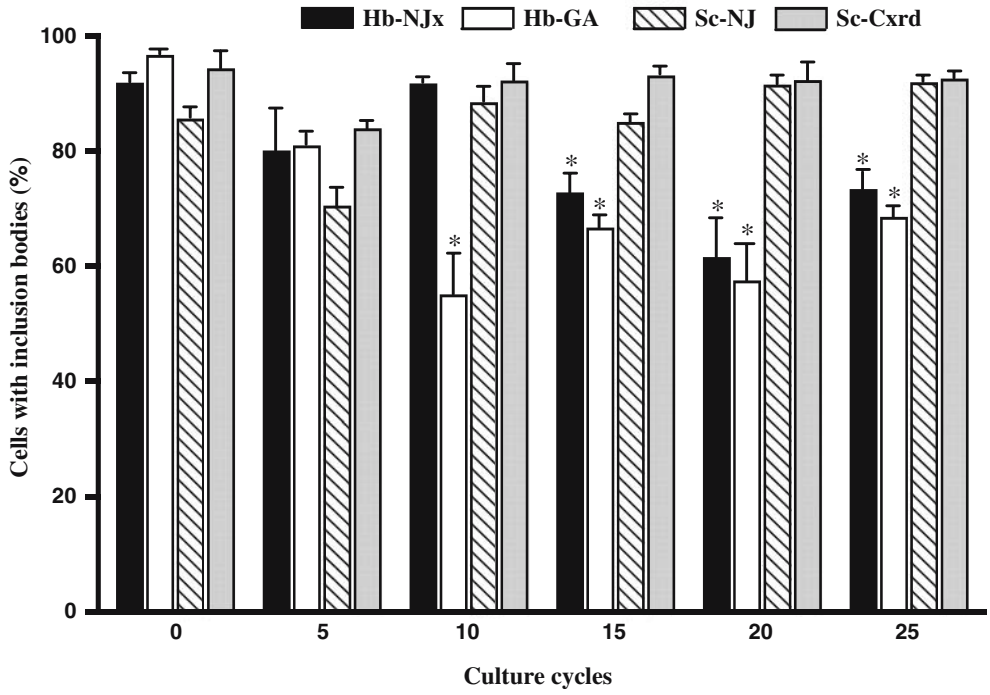

Fig 4 Prevalence (%) of cells of *Photorhabdus luminescens* (Hb-NJx and Hb-GA strains) and *Xenorhabdus nematophila* (Sc-NJ and Sc-Cxrd strains) producing inclusion bodies over 25 culture cycles in liquid culture (cell type was controlled by plating and selection for primary cell colonies between liquid culture cycles). Bars (±SD) with an "*" are significantly different from the control (0) at $P < 0.05$

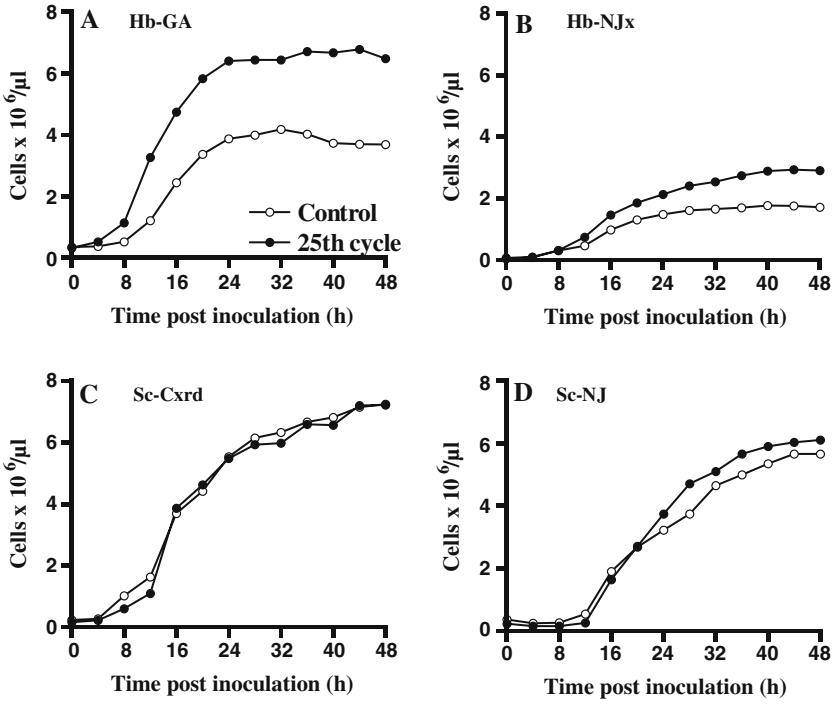

Fig. 5 Growth curves for 25th cycle and control cultures of **a** *Photorhabdus luminescens* Hb-GA, **b** *P. luminescens* Hb-NJx, **c** *Xenorhabdus nematophila* Sc-Cxrd, and **d** *X. nematophila* Sc-NJ (cell type was controlled by plating and selection for primary cell colonies between liquid culture cycles)

from subculturing was phase shift from primary to secondary cell in three of the four strains tested in the single experiment where cell type was not controlled by selection for primary colonies. Interestingly, the Hb-NJx strain of *P. luminescens* maintained cell type integrity for ten cycles, an outcome supporting suggestions to exploit variation by screening for superior strains even within species [16].

Intermediate and small colony variants occur in *Photorhabdus* but not *Xenorhabdus* [18, 21, 22]. We

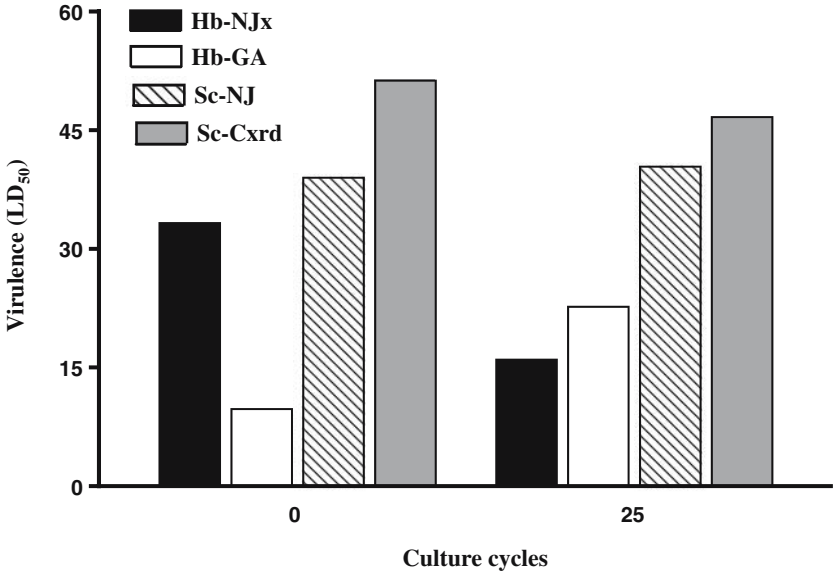

Fig. 6 Virulence expressed as LD_{50} (±SD) for *Photorhabdus luminescens* (Hb-NJx and Hb-GA strains) and *Xenorhabdus nematophila* (Sc-NJ and Sc-Cxrd strains) after 25 cycles in liquid culture via injection into *Tenebrio molitor* larvae (cell type was controlled by plating and selection for primary cell colonies between liquid culture cycles). Bars (±SD) with an "*" are significantly different from the control (0) at $P < 0.05$

did not note intermediate colonies for our *P. luminescens* strains until the 25th cycle. These can occur frequently in intensively subcultured bacterial strains [20, 25], but their significance and genetic base are unclear in entomopathogenic bacteria. In *E. coli*, mutations from transposable elements have been confirmed as responsible for intermediate variants [24] and were assumed to be advantageous in accelerating adaptation to changing environments [3]. This advantage is especially important to entomopathogenic bacteria because they are challenged to maintain the relationship with their nematode partners as well as to survive the environments the partners bring to them.

Deterioration of beneficial traits has been documented for entomopathogenic nematodes subcultured under laboratory conditions. When *H. bacteriophora* was repeatedly subcultured in *G. mellonella* larvae, Wang and Grewal [34] found nematode fecundity, storage stability, UV resistance, heat tolerance, and desiccation tolerance were reduced, although virulence increased. In their in vivo culture system, selection pushed the nematode–bacteria complex toward virulence gains and, in return, other traits were lost as tradeoffs. Similar gains with accompanying beneficial trait tradeoffs were reported in efforts to increase host finding ability or heat tolerance but with parallel losses in traits beneficial for biological control such as storage stability and longevity [15, 30].

Symbiotic bacteria help their nematode partners kill insect hosts but also provide food and a suitable environment for the nematodes to complete their life cycle. The changes we observed in *P. luminescens* presumably impact their nematode partners. In an in vivo study on nematode trait stability, Bilgrami et al. [7] found losses in nematode virulence, host finding ability, reproduction, and heat tolerance after subculturing the *H. bacteriophora/P. luminescens* complexes in *G. mellonella* larvae. Significant recovery of most deteriorated traits could be obtained by feeding the deteriorated nematodes on their non-deteriorated (base population) bacteria and therefore, nematode deterioration was wholly or partially attributed to the associated bacteria. Selection pressures and therefore changes in bacteria differed between the two studies because we cultured the bacteria in novel nutrient environments (alternating solid and liquid media) without the presence of its nematode symbiont and without selection for alleles related to maintaining the symbiosis. Nevertheless, the changes noted in *P. luminescens* cells and inclusion bodies in the present study may result in declines in bacterial quality that would consequently impact nematode quality. The inclusion bodies, for example, makeup 40% of bacterial total protein [9] and may support nematode symbiosis [8, 10, 37]. If the hypothesis that crystals are an important protein source needed by heterorhabditid nematodes for development and growth is correct [9, 22], then the large reduction in *P. luminescens* inclusion body size we observed may negatively impact the nematode partner. Support is found in a related study [7] wherein the *S. carpocapsae/X. nematophila* and *H. bacteriophora/P. luminescens* complexes were subcultured in vivo for 20 cycles. Pairing the 20th cycle bacteria with the control nematodes, resulted in reduced reproduction in both species relative to pairing control bacteria with control nematodes.

Xenorhabdus nematophila was resistance to change for all traits assayed and no evidence of adaptation to

laboratory conditions was detected. Bilgrami et al. [7], however, showed losses in traits relevant to biocontrol during subculture of *S. carpocapsae* that were attributed in part (i.e., heat tolerance) or mostly (i.e., reproduction) to the bacterial partner. Differences in the experimental design of the two studies confound close comparisons, most significantly in vivo culture of the partners concurrently for Bilgrami et al. [7] and in vitro culture of the bacterial partner in isolation for the present study.

We generated each base population from a single colony, an approach that restricts genetic variation and therefore may impact the outcome of adaptation [13]. Moreover, plating between cycles to control cell type may have created a bottleneck effect, resulting in genetic drift where variation would be further reduced. To reduce bottlenecking, we picked up to 30 colonies as inoculum for the next liquid culture cycle but even this may have been insufficient.

Further study is required to determine the basis for the observed changes. Are the changes purely genetic or to a degree nutritional and thus reversible? If the changes are genetic, which genes are altered in nature or protein expression during serial culture? Bai et al. [4] reported alterations of protein electrophoretic profiles after 21 in vitro culture cycles of *P. luminescens* strain HB-GA, with the extent of change increasing with the number of cycles. Most changes were related to biologically active compounds including membrane proteins, alkaline phosphatase and other proteins likely to be important to bacterial virulence or survival.

Field adapted traits of *P. luminescens* declined during in vitro culture as tradeoffs for relative fitness gains. Conversely, neither losses nor gains were observed for the *X. nematophila* strains associated with *Steinernema* nematodes when cell type was stabilized via selection; the genetic basis for this stability is worthy of investigation. In vitro production of the *P. luminescens/H. bacteriophora* complex requires consideration of the need for superior bacterial yield (i.e., reduced scale-up and culture time) against the need to preserve traits important for biocontrol.

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