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Environmental drivers of trait changes in *Photorhabdus luminescens*

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HIGHLIGHTS

- We studied trait deterioration in *P luminescens* isolated from *H. floridensis*.
- We examined nutritional effects on deterioration using three different media types.
- There was no overall trait deterioration in *Photorhabdus luminescens* over time.
- Any observed trait changes were reversible based on nutrition or environment.
- To prevent changes in biocontrol agents, testing proper growth media is necessary.

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ABSTRACT

Biological control agents have become increasingly important in integrated pest management programs. However, certain traits of these agents that are needed for efficient biocontrol often decrease or are lost during *in vitro* rearing. Entomopathogenic nematodes (EPNs) often exhibit trait deterioration when reared under laboratory conditions. EPN trait deterioration has been attributed (at least in part) to genetic causes; however, the underlying causes of trait deterioration in its bacterial endosymbiont have not been explored. In this study the EPN symbiont *Photorhabdus luminescens* was monitored for the deterioration of three traits; inclusion body production, reproductive potential, and virulence, in three different nutritional environments; lipid liquid medium (LLM), nutrient broth (NB), and tryptic soy broth + yeast extract (TSY). Significant trait deterioration did not occur for any of the traits in any environment. There was an increase in inclusion body production in TSY. Additionally, there was variation in growth within NB and TSY sub-cultured population lines and one TSY sub-population line was less virulent than the other two. However, returning bacteria to LLM restored all traits to wild-type levels. We infer the observed trait deterioration in *Photorhabdus* was minimal and appeared to be driven by environmental conditions as opposed to stable genetic changes. Our data suggest that variation among traits of *in vitro* cultures of *Photorhabdus* is more likely due to environmental variation than inadvertent laboratory selection or other genetic processes.

1. Introduction

Chemical pesticides are commonly used against agricultural insect pests; however, in recent decades pest control efforts have refocused on finding new methods (Chandler et al., 2011). Chemical pesticides are effective but can have negative consequences on the environment and human health, as well as promote secondary pest outbreaks and the evolution of resistance (Coppel and Mertins, 1977; Pimentel et al., 1992). To combat these harmful effects individuals and agencies have implemented integrated pest management (IPM) programs. IPM programs utilize biological agents for insect control including bacteria, fungi, viruses, nematodes, and entomophagous insects (Chandler et al., 2011).

When a biocontrol agent is isolated from the environment and repeatedly cultured for experimental or commercial purposes essential traits or phenotypes, particularly virulence, host-finding abilities, and environmental tolerance (Kaya and Gaugler, 1993; Shapiro-Ilan et al., 2003), can be lost. These trait losses, or deterioration, are due to genetic processes such as drift, inbreeding, or inadvertent selection (Bai et al., 2005; Hopper et al., 1993; Hoy, 1985; Roush, 1990). However, nutrition plays a significant role in the efficiency of a mass-produced biocontrol agent (Cabrefiga et al., 2011; Shapiro and McCoy, 2000; Shapiro-Ilan et al., 2008).

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Therefore, changes in biocontrol traits may also arise from non-genetic factors such as poor nutrition and disease (Hopper et al., 1993).

Entomopathogenic nematodes (EPNs; genera *Heterorhabditis* and *Steinernema*) are important biocontrol agents that kill their invertebrate hosts with the aid of a mutualistic bacterium (Gaugler, 2002). The bacteria (*Xenorhabdus* spp. for steinernematids and *Photorhabdus* spp. for heterorhabditids) are primarily responsible for killing the host (Bilgrami et al., 2006; Gerritsen and Smits, 1993; Han and Ehlers, 2000) and providing the nematodes with nutrition and defense against secondary invaders (Poinar, 1990). For example, *Photorhabdus* spp. produce crystalline protein inclusion bodies that are crucial for supporting nematode growth (Bintrim and Ensign, 1998; Bowen and Ensign, 2001) and antimicrobial molecules that prevent other microbes from occupying the same insect (Eleftherianos et al., 2007; Williams et al., 2005). Efficient reproduction and high virulence are also important *Photorhabdus* spp. traits needed for their use as effective biocontrol agents (Han and Ehlers, 2000).

EPNs are amenable to laboratory rearing and mass production using *in vivo* or *in vitro* methods (Ehlers and Shapiro-Ilan, 2005; Shapiro-Ilan and Gaugler, 2002). Regardless of the culture method both the nematode and the symbiotic bacteria exhibit trait deterioration. While there have been investigations on trait deterioration in EPNs, most research has focused on the underlying causes in the nematode, which have suggested genetic sources for deterioration (Bai et al., 2005; Chaston et al., 2011). Furthermore, only one study has demonstrated trait deterioration in the bacterial symbionts without their nematode partner (Wang et al., 2007). This study examined inclusion body production and size, reproductive potential, phase switching, and virulence in two strains (Hb-NJx and Hb-GA) of *Photorhabdus luminescens* (unknown subspecies) before and after repeated sub-culturing in tryptic soy broth. *P. luminescens* exhibited trait deterioration in all traits except reproductive potential. Both strains demonstrated an increase in reproductive potential and one of the two strains also increased in virulence.

To our knowledge, there are no published results on the underlying causes of trait deterioration in *Photorhabdus* spp.; therefore, the purpose of this study was to understand the role of the environment in trait changes of *Photorhabdus* sp. observed *in vitro*. We hypothesized that environment affects trait deterioration of *Photorhabdus* spp. Therefore, different nutritional sources would result in varying levels of deterioration. Using *P. luminescens* subsp. *luminescens* isolated from *Heterorhabditis floridensis* K22 (Rhabditida: Heterorhabditidae) (Nguyen et al., 2006; Shapiro-Ilan et al., 2014) we monitored changes in important biocontrol traits before and after repeated sub-culturing in three different nutritional regimes. The traits we investigated were crystalline inclusion body production, reproductive potential, and virulence because these are biocontrol traits specific to the bacterial symbiont and were previously shown to significantly change after repeated sub-culturing (Wang et al., 2007). Our results show that trait changes were not as drastic as previously described (Bilgrami et al., 2006; Wang et al., 2007); however, there was some nutritional effect on exhibited trait changes.

2. Materials and methods

2.1. Cultures and growth conditions

In this study, we used *P. luminescens* subsp. *luminescens* previously isolated from fresh cultures of *H. floridensis* K22 (Shapiro-Ilan et al., 2014). We recovered *P. luminescens* subsp. *luminescens* K22 by spreading the hemocoel from infected insects onto lipid agar (nutrient broth, 5 g l^{-1} yeast extract, 2 g l^{-1} $MgCl_2$, 0.004% corn oil, and 0.007% karo syrup). Using *Photorhabdus* spp. from recently isolated EPNs is crucial since any established lab strain has a high likelihood of already being appreciably deteriorated. The bacteria can exist in two phases (primary and secondary), but the primary form produces antibiotics, proteases, crystalline inclusion protein bodies, and is preferable for nematode growth (Akhurst, 1980). Growth on NBTA (nutrient agar, 25 mg l^{-1} of bromothymol blue, 40 mg l^{-1} of triphenyl-2,3,5-tetrazolium chloride) (Akhurst, 1980) and lipid agar plates confirmed primary phase bacteria based on color (green/blue on NBTA and red/orange on lipid

Table 1
Comparison of methods in trait deterioration studies.

	Blackburn et al.	Wang et al. (2007)	Bilgrami et al. (2006)
Organism	*P. luminescens* susp. luminescens (strain K22)	*P. luminescens* (strain Hb-NJx and Hb-GA)	*H. bacteriophora–P. luminescens* complex (strains Hb-NJx and Hb-GA)
Sub-culture method			
Culture medium	*In vitro* 50 mL liquid culture	*In vitro* 50 mL liquid culture	*In vivo* using *G. mellonella*
Broth	LLM, NB, TSY	TSY	N/A
Inoculum	50 CFU	20–30 CFU	N/A
Phase selection	Primary phase selection	Primary phase selection	N/A
Culture time	48 h	48 h	2–3 EPN generations
Bioassays			
Cell and inclusion body size	N/A	1:50 dilution 1000× with 3 fields of view Unknown method of microscopy	N/A
Inclusion body production	1:50 dilution 1000× with 3 fields of view Phase-contrast microscopy	1:50 dilution 1000× with 3 fields of view Unknown method of microscopy	N/A
Cell type (phase switching)	N/A	Repeated sub-culture without primary phase selection	N/A
Reproductive potential	10^6 inoculum in 50 mL	10^9 inoculum in 50 mL	Investigated reproductive potential of nematode
	OD_{600}-values Every 4 h for 48 h	OD_{600}-values Every 4 h for 48 h	
Virulence	*G. mellonella* LT_{50} Number of cells injected based on CFUs	*T. molitor* LD_{50} Number of cells injected based on counting total cells with a Petroff–Hausser chamber	*G. mellonella* % Mortality at 72 h