

**Genomics of *in vitro* dauer juvenile recovery of the entomopathogenic
nematode *Heterorhabditis bacteriophora* in monoxenic liquid culture
with its symbiotic bacterium *Photorhabdus laumondii***

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Dissertation
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of the Faculty of Agricultural and Nutritional Sciences
at Kiel University
submitted by

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1 Introduction

The entomopathogenic nematodes (EPN) of the genus *Heterorhabditis* (Nematoda: Rhabditida) are lethal to a broad range of insects and have been widely used as biocontrol agents of pest invertebrates (Grewal et al. 2005). Among several examples, there is a wide use of this EPN against the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), the corn rootworm *Diabrotica v. virgifera* (Coleoptera: Chrysomelidae) and several scarab grubs (Coleoptera: Scarabaeidae) (Ehlers 2003; Grewal et al. 2005; Lacey et al. 2015). The dauer juvenile (DJ) is the only free-living stage of *Heterorhabditis*. It is a non-feeding, developmentally arrested stage, similar to the dauer stage of the model nematode *Caenorhabditis elegans* (Golden and Riddle 1982). The DJ in this stage is relatively resistant to environmental stresses (e.g., high/low temperatures, desiccation). Nematodes of the genus *Heterorhabditis* hold a tight symbiotic relationship with gram-negative bacteria of the genus *Photorhabdus* (Gammaproteobacteria: Morganellaceae) (Adeolu et al. 2016), harbouring cells of its bacterial symbiont in its intestine. The DJ search for insect hosts in the soil and enter the insect via natural openings (e.g., mouth, anus, and spiracles) or penetrate through the insect cuticle by using their anterior tooth, while shedding their secondary cuticle during the invasion (Bedding and Molyneux 1982). Within 15 min after the invasion of insect's hemocoel, the insect's immune reactions begin (Ebrahimi et al. 2011). The DJ respond to the so-called "food signal" in the haemolymph, which induces the exit from the DJ stage and the regurgitation of the symbiotic bacteria from the nematode gut into the insect hemocoel within 30 min after invasion (Ciche and Ensign 2003). Thereafter, the *Photorhabdus* cells override the insect immune system, grow exponentially, kill the host by septicaemia, and act as nutritional source for the nematodes (Ehlers 2001; Han and Ehlers 2000). The process during which the nematodes exit the arrested stage, grow and develop into reproductive hermaphrodites is called DJ recovery (Golden and Riddle 1982). The nature of the insect's chemical signal triggering DJ recovery has not been identified yet (Ehlers 2001; Johnik et al. 2004; Steven et al. 1997; Strauch and Ehlers 1998). Within the insect, the recovered DJ develop to self-fertilizing

hermaphrodites, which lay eggs. The hatching J1 juveniles develop further through four juvenile stages to cross-fertilizing female and male individuals. Sexual reproduction can take place for some generations until the nutrient sources are depleted. When nutrient sources diminish, a new generation of DJ is produced, which leave the insect on the search of new hosts (Grewal et al. 2005).

1.1 Challenges for *in vitro* mass production of *Heterorhabditis bacteriophora*

The mass production of *Heterorhabditis* spp. in artificial liquid medium was established several decades ago (Ehlers et al. 1998; Wouts 1981). Since then, the production of EPN in large bioreactors has been optimized and today they are widely used commercially for the biological control of many insect pests (Ehlers 2001). At the start of each production cycle, *H. bacteriophora* DJ are inoculated into 24 h pre-cultures of the symbiotic *Photorhabdus* bacterium (Ehlers 2001). Artificial culture media used in mass production lack any food signal and thus do not induce DJ recovery. Food signals triggering DJ recovery are in this case excreted by the symbiotic bacteria (bacterial food signal) (Aumann and Ehlers 2001). Inside bioreactors, the inoculated and recovered nematodes develop to hermaphrodites, inside which, the offspring develops to new DJ by the phenomenon known as *Endotokia matricida*. First stage juveniles (J1) hatch from eggs and grow inside the hermaphrodite by feeding on its body content, finally leading to the death of the mother leaving only its cuticle behind (Johnigk and Ehlers 1999). Towards the end of the mass production cycle, readily developed DJ leave the remaining carcass, are harvested, and formulated as a product. Different to the *in vivo* scenario with > 95% recovery within 24 h, the signals of the bacterial symbiont are less effective and trigger DJ recovery between 0 and 81 % scattered over 2 to 5 days, resulting in a non-synchronous population development causing lower yields and occurrence of a second generation of males and females, which are unable to mate in liquid culture, thus not producing offspring (Dolan et al. 2002; Han et al. 2000; Strauch and Ehlers 1998).

For commercial use, *H. bacteriophora* DJ are reproduced in bioreactors of up to 100 m³ capacity within 11 days. After completion of the cycle, a high and homogeneous DJ yield is expected (>

200,000 DJ mL⁻¹, less than 1 % other stages). The DJ yield in mass production depends on a rapid, synchronized and high recovery of the inoculated DJ. A low percentage of DJ recovery leads to low DJ yields and an inefficient final DJ harvest due to the presence of other juvenile and adult stages (Johnigk et al. 2004). Previous works on *in vitro* culturing showed that the optimal timing for DJ inoculation is dictated by a characteristic pH dynamic in the *Photorhabdus* culture, and this timing influences the synchronicity of the DJ recovery (Johnigk et al. 2004). Joyce et al. (2008) determined that *P. laumondii* biosynthesises stilbenes that have significant effects on induction of DJ recovery. Wang et al. (2022) found isopropylstilbene supporting DJ recovery. Different *Photorhabdus* species/strains can vary significantly in their support of DJ recovery and the reproductive potential of *H. bacteriophora*. However, no complete picture about how DJ perceive these signals is available. Thus, understanding the mechanisms behind *in vitro* DJ recovery is essential for a further optimization of the EPN production. The life cycle of *H. bacteriophora* in insect and liquid culture is presented in Fig. 1.

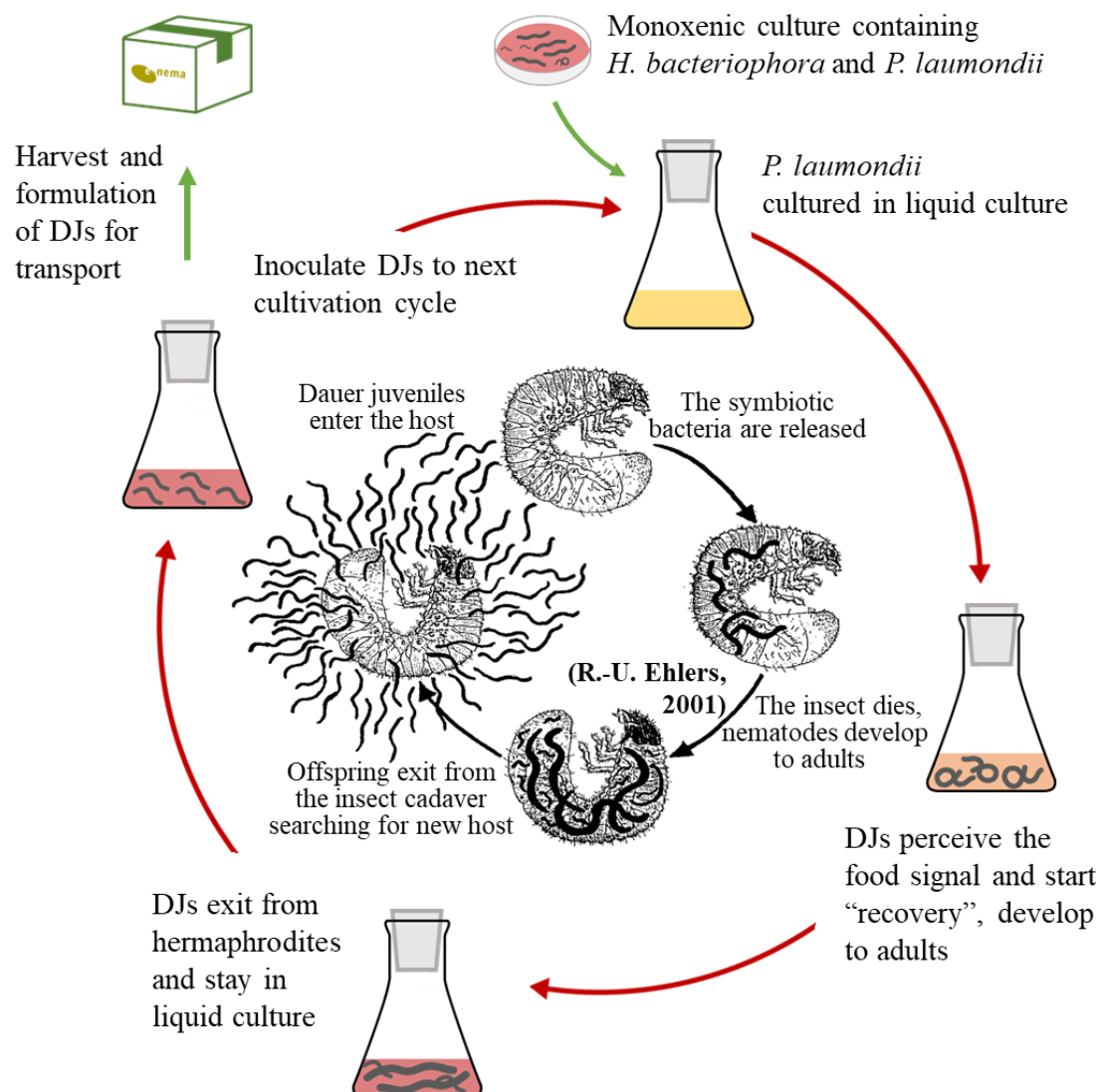


Figure 1. Life cycle of *Heterorhabditis bacteriophora* in the insect host and in *in vitro* liquid culture. The life cycle in insects is taken from Ehlers (2001). Inside the insect, DJ recover upon signals present in the haemolymph. On the other hand, under *in vitro* culturing, DJ recover upon signals released by the pre-cultured *Photorhabdus* bacteria. In the past, it has been observed that generally the so called “insect food signal” is stronger inducing DJ recovery than the “bacteria food signal”.

1.2 *Heterorhabditis-Photorhabdus* symbiotic relationships

The mutualistic interaction of *Heterorhabditis* and *Photorhabdus* is highly coevolved, where the bacteria rely on the nematode for the transmission between insect hosts. The bacteria are lethal pathogens while the nematode growth requires the bacterial cells as nutrition source (Goodrich-Blair and Clarke 2007; Griffin et al. 2005). Bacteria-free *Heterorhabditis* can infect hosts but are non-pathogenic to insects (Han and Ehlers 2000). Until now no free-living *Photorhabdus* have been discovered in the soil (Hapeshi and Waterfield 2016).

Concerning specificity of *H. bacteriophora* to different *Photorhabdus* sp., the nematode appears to be relatively flexible. Until today, more than twenty species of *Photorhabdus* have been discovered, ten of these species and subspecies have been isolated from *H. bacteriophora*, which are: *P. caribbeanensis* (Tailliez et al. 2010), *P. cinerea* (Kazimierczak et al. 2017), *P. kayaii* (Kazimierczak et al. 2017), *P. khanii* subsp. *khaini* (Tailliez et al. 2010), *P. kleinii* (Machado et al. 2018), *P. laumondii* subsp. *clarkei* (Machado et al. 2018), *P. laumondii* subsp. *laumondii* (Saux et al. 1999), *P. luminescens* subsp. *Luminescens* (Saux et al. 1999), *P. stackebrandtii* (Tailliez et al. 2010), and *P. thracensis* (Hazir et al. 2004). The subspecies elevation of *Photorhabdus* spp. was referred to Machado et al. (2018).

Besides, EPN can swap their symbiotic bacteria at intra- and interspecies level. Kazimierczak et al. (2017) found that *Heterorhabditis* (*H. bacteriophora* and *H. megidis*) strains can develop on different *Photorhabdus* (*P. kayaii*, *P. cinerea* and *P. temperata*) isolates. Whether this flexibility concerning the bacterial association can bring advantages to the nematode-bacteria complex has already been demonstrated for nematode virulence. For instance, Machado et al. (2020) improved virulence of *H. bacteriophora* to western corn rootworms (WCR) by increasing benzoxazinoid resistance of *Photorhabdus* through bacterial symbiont engineering.

Concerning *Photorhabdus* natural products, secondary metabolites have been linked to traits like insect pathogenicity, protection of the insect cadaver and support of nematode development (Shi and Bode 2018). Several conservative biosynthetic gene clusters for natural products have been identified from *Photorhabdus* sp. (Shi et al. 2022). Concerning DJ recovery of *H.*

bacteriophora, two compounds of less than 20 kDa (non-identified) were reported to influence recovery (Aumann and Ehlers 2001). Joyce et al. (2008) and Wang et al. (2022) reported that stilbenes produced by *Photorhabdus* trigger *H. bacteriophora* DJ recovery, however, the compounds were not sufficient in absence of bacteria cells or supernatant.

Thus, the bacterial food signal appears to be of complex composition, and individual elements may not fully trigger DJ recovery. To date, the answer to the question whether bacterial or nematode genetic factor play the main role in recovery regulation of DJ populations is up to now not elucidated.

1.3 Breeding activities and genetic variation of *H. bacteriophora*

To improve the efficiency of EPN as biological control agents, intensive breeding activities have focused on enhancing beneficial traits of *H. bacteriophora* strains. To date, several improvements were based mainly on phenotypic selection, hybridization, mutagenesis, and recombinant DNA techniques (Glazer 2015). Phenotypes with traits for enhanced virulence, longevity, desiccation tolerance and stress resistance have been identified e.g., Anbesse et al. (2013); Godina et al. (2022b); Mukuka et al. (2010); Sumaya et al. (2018); Toepfer et al. (2005). Concerning DJ recovery, Johnigk et al. (2002) propagated 30 inbred lines of *H. bacteriophora* in liquid culture and detected no significant genetic variability for their recovery. While plenty of breeding efforts have produced superior strains with enhanced characteristics, the DJ recovery remains to be further investigated for improvement.

Concerning genotypic tools for the improvement of traits in *H. bacteriophora* by marker assisted breeding, single nucleotide polymorphisms (SNPs) are the simplest form of DNA variation and have been widely applied on *C. elegans*, animal, and plant breeding (Davis et al. 2005; Dekkers 2012; Mammadov et al. 2012). Godina et al. (2022a; 2023) and Levy et al. (2020) reported SNPs in dozens of *H. bacteriophora* hybrids and wild-type strains/lines with relation between genotypic and phenotypic differences (longevity, heat & desiccation tolerance, virulence against *Tenebrio molitor*) in distinct *H. bacteriophora* wild type and inbred line populations. To date, the question whether the natural genetic variability in *H. bacteriophora*

can be considered a source for the phenotypic improvement of the DJ recovery in this EPN has not been assessed. Similarly, the use of mutants as potential source for the phenotypic improvement has not been reported.

1.4 Regulatory elements of DJ recovery in *H. bacteriophora* and *C. elegans*

Comparing the model nematode *C. elegans* and *H. bacteriophora*, the feeding habits (free-living vs entomopathogenic) of both species are different. On the other hand, the two species are both rhabditid nematodes in the eurhabditid clade and both have developmentally arrested dauer stage during the life cycle (Ciche 2007; Kim et al. 2017). For this reason, *C. elegans* can be used as source of knowledge to understand *H. bacteriophora* regulatory processes.

In the model nematode *C. elegans*, three main environmental cues influence dauer formation and the development from J1 to dauers: i) dauer-inducing pheromones (DIP), a complex mixture of ascarosides, ii) lack of food, iii) and temperature (Choe et al. 2012; Golden and Riddle 1984). A wide range of mutants with high variability in the dauer-related processes were classified into two groups: i) dauer constitutive mutants (*daf-c*), which do not develop to adults from the J1 stage, but always enter dauer diapause, and ii) dauer defective mutants (*daf-d*) that bypass the diapause and always develop to adults, regardless of the environment conditions (Fielenbach and Antebi 2008). The main signalling pathways that are involved in dauer recovery in *C. elegans* have been identified, including the guanylyl cyclase pathway, transforming growth factor beta (TGF- β) signalling pathway, insulin/insulin-like growth factor-1 (insulin/IGF-1) signalling (IIS) pathway, and the DAF-12 nuclear hormone receptor pathway (Choe et al. 2012; Fielenbach and Antebi 2008; Hu 2018). Several studies have focused on characterizing the recovery-related function of diverse gene/protein types (Chou et al. 2015; Fernández et al. 2021; Gelmedin et al. 2011; Martinez and Gill 2023; Ouellet et al. 2008).

Of the predicted protein-coding genes of *H. bacteriophora*, nearly half of the genes are *C. elegans* homologs (Bai et al. 2013; McLean et al. 2018). In this concern, the McLean and co-authors predicted more than 14,000 protein coding genes including a high proportion EPN exclusive proteins. *H. bacteriophora* has most (19 of 23) of the IIS pathway genes of *C. elegans*,

which are crucial for dauer recovery and formation, longevity regulation, stress resistant and innate immunity (Bai et al. 2013). Moshayov et al. (2013) determined that two genes regulating DJ recovery of *H. bacteriophora* are associated with the IIS pathway. Another benefit from the *C. elegans* research is the possible use of well-developed tools (e.g., genetic modifications and RNAi) that can be transferred to *H. bacteriophora* for genetic studies (Ciche and Sternber 2007; Ratnappan et al. 2016).

Concerning the molecular characterisation of the DJ recovery and DJ infection process, Bai and Grewal (2007) for the first time analysed the gene expression of *H. bacteriophora* DJ upon insect haemolymph contact (1 h) and identified two down-regulated gene clones HbGPS11.C1G02 and HbGPS11.C4C08, encoding a phosphofructokinase (PFK) and a trans-sialidase homologous to a *Trypanosoma cruzi* gene model, respectively. Later, Moshayov et al. (2013) treated *H. bacteriophora* DJ with *Galleria mellonella* (Lepidoptera: Pyralidae) haemolymph, compared the expression after two time points (3 and 6 h), and identified eight candidate genes involved in the DJ recovery process. Two of the genes (*akt-1* and *glb-1*) were associated with the insulin/IGF-1 signalling pathway in *C. elegans*. Vadnal et al. (2017) sequenced RNA from *H. bacteriophora* DJ exposed to *Manduca sexta* (Lepidoptera: Sphinginae) haemolymph for 9 h and found that a large proportion of the differentially expressed genes are unique to parasitic nematodes.

Despite the previous pioneering molecular characterisation works, enhancing the availability of expression information is still crucial to elucidate the mechanisms underlying the fine tuning of the DJ recovery induction in *H. bacteriophora*. Also, the question of whether molecular mechanisms are differentially activated upon exposure to insect- and bacteria-food-signals during the early DJ recovery stages, remains unanswered.

1.5 Objectives

In this study, we aimed to elucidate potential regulators of the *H. bacteriophora* DJ recovery for the improvement of the *in vitro* production process. For this purpose, a collection of 168 *H. bacteriophora* EMS-mutant lines has been generated. All mutants, and a selection of 25 WT lines were evaluated by systematically testing the DJ *in vitro* recovery induced by cell-free bacterial supernatants. Parallel to phenotyping, 96 of the mutant lines were subjected to genotyping by sequencing (GBS) to determine SNPs and run an association analysis between pheno- and genotype data for the identification of SNPs related to DJ *in vitro* recovery.

Concerning possible phenotypic variability related to support of DJ recovery by different *Photorhabdus* strains, fourteen *Photorhabdus* strains from phenotyped WT nematodes were isolated, identified to species level, and their sterile supernatants were produced. The supernatants were tested with five previously characterized *H. bacteriophora* lines/strains and their DJ recovery was evaluated. In parallel, the influence of DJ age (storage at 4 °C) on recovery was assessed.

To deeper understand gene expression changes during *in vitro* DJ recovery, RNA-seq analysis of two mutant lines, M31 & M88, with opposite DJ recovery phenotypes was performed. A time series RNA-seq experiment (0.5, 1, 3 and 6 h after exposure to the *Photorhabdus* supernatant) of both mutants was conducted and the expression profile was clustered into similar patterns, identifying over & under-represented gene ontology (GO) categories and associated genes for each cluster. The gene models linked to recovery-related functional information in *C. elegans*. Also, gene expression information was combined with genotypic data (SNPs) to elucidate possible interactions with potential influence on the DJ recovery.

The present study had the following specific objectives:

- I. To produce a collection of highly homozygous *H. bacteriophora* mutant lines.
- II. To determine the DJ recovery phenotype of *H. bacteriophora* mutant and WT lines upon *P. laumondii* supernatant exposure.
- III. To assess the trade-off effects between beneficial traits of DJ recovery, longevity (survival under oxidative stress), and virulence against an insect host on a subset of mutant lines.
- IV. To identify SNP markers associated with the DJ *in vitro* recovery trait in mutant lines.
- V. To assess the DJ recovery phenotypic variability influenced by different *Photorhabdus* species/strains.
- VI. To determine the influence of DJ age (storage in 4 °C) on recovery.
- VII. To characterize the transcriptome of *H. bacteriophora* mutant lines upon bacterial food signal exposure to postulate potential regulators operating the early stage of the DJ recovery process.

2 Results and Discussion

2.1 Low variability of DJ recovery in *H. bacteriophora* wild type lines

Entomopathogenic nematodes have been widely used against many insect pests in soil and cryptic environments (Grewal et al. 2005). A better understanding of factors influencing DJ *in vitro* recovery during commercial mass production can help stabilize the biotechnical process (Ehlers 2001). In *H. bacteriophora* liquid production, a rapid, high, and synchronized recovery of the inoculated DJ is one of the key points that determine the quality and quantity of the final yield (Ehlers 2007; Ehlers et al. 1998). To investigate the DJ recovery process, we phenotyped 25 WT strains (Godina et al. 2022a) under monoxenic liquid culture conditions and in parallel upon bacterial supernatant exposure. Liquid culture and production of the supernatants were performed using *P. laumondii* (strain DE2) cultured in LM medium. Recovery was assessed three days after DJ inoculation by observing the morphological changes of the DJ head region and esophagus (Publication 1, Fig. 1) following Strauch and Ehlers (1998). Apart from reference lines IL3 and HB4, the DJ recovery in liquid culture ranged from 16.5 ± 8.7 % (line MM141) to 34 ± 17.6 % (IR31). Upon supernatant exposure, DJ recovery ranged from 1.6 ± 0.7 (AU11) to 23.6 ± 4.3 % (HU12) (Publication 1, Fig. 2). Compared to the *in vitro* DJ recovery of IL3 (35.7 ± 12.3 %) and HB4 (25.7 ± 8.5 %), both inbred lines taken from commercially produced strains selected over the years for high recovery, the variability of WT lines was lower under both conditions. This indicates that the natural genetic variability in *H. bacteriophora* may be insufficient as source for DJ recovery improvement.

2.2 Enhanced variability of DJ recovery in *H. bacteriophora* mutant lines

Sumaya et al. (2018) generated a population of mutagenized *H. bacteriophora* nematodes from the commercial donor line IL3, using ethyl methanesulfonate (EMS). Starting on this population, a collection of 168 highly homozygous EMS-mutant lines was generated in the present thesis.

Initial experiments were carried out with a subset of 18 mutant lines to compare DJ recovery

under exposure to *P. laumondii* (strain DE2) supernatant grown in LB (Luria-Bertani broth) and LM (liquid medium) medium, hereinafter referred to as LB supernatant and LM supernatant, respectively. In parallel, seven of these mutant lines were grown in LM liquid culture with pre-grown *P. laumondii* (strain DE2). DJ recovery in LM liquid culture was the highest, followed by LM and LB bacterial supernatant and the differences were significant. The heritability ($h^2 \geq 0.84$) of DJ recovery of mutants within each of the three bacterial growth methods was high, indicating that the genetic differences between mutant lines are the main source of variation. A significantly positive correlation ($R > 0.7$) between the DJ recovery in liquid culture and the two supernatants was evidenced (Publication 1, Fig. 3E).

Based on these results, the full collection of 168 mutant *H. bacteriophora* lines and two reference lines (HB4 and IL3) were inoculated into LM-produced *P. laumondii* supernatant to phenotype the DJ recovery. The phenotypic differences between mutant lines were significant and their DJ recovery ranged from 4.6 ± 0.8 % (M88) up to 67.2 ± 7 % (M31) (Publication 1, Fig. 4). A high heritability was as well identified in the full collection of mutants ($h^2 = 0.68$), which is consistent with the results of the preliminary tests. The high heritability of DJ recovery indicates that the differences between lines must be associated with genotypic diversity. Besides, compared to WT DJ recovery ranging from 1.6 – 23.6 % in LM supernatant, the mutant lines significantly broadened the spectrum of phenotypic variability (4.6 – 67.2 %). What can be the reason for those DJ recovery variations?

In the model nematode *C. elegans*, the dauer entry/exit process starts with the signals binding to amphid chemical receptors (Bargmann 1998; Fielenbach and Antebi 2008; Hilliard et al. 2002). Aumann and Ehlers (2001) also detected substances that are biologically antagonistic to DJ recovery in the bacterial supernatant. EMS mutagenesis normally causes G:C to A:T transitions induced by guanine alkylation (Sega 1984). Considering that certain functions may be destroyed by these point mutations, it can be speculated that pathways responsible for signal transduction for the antagonistic molecule in the supernatant might have been affected and this has resulted in a broader phenotypic spectrum for DJ recovery within the mutant population than reported for the WT population.

2.3 Trade-off effects between DJ recovery and DJ longevity and virulence

To investigate possible interactions between *H. bacteriophora* beneficial traits, the DJ longevity (survival under oxidative stress induced by H_2O_2) and the virulence against the mealworm *Tenebrio molitor*, the subset of 18 mutant lines was phenotyped together with the reference lines IL3 and HB4. The infectivity against mealworms ranged from 22 ± 8 % (M31) to 78.1 ± 1.8 % (M43). The most contrasting mutant lines concerning recovery (M31 and M88) were both low in virulence (22 ± 8 and 35.2 ± 16.3 %, respectively) (Publication 1, Fig. 5A). The mean time survived by 50 % (MTS₅₀) of DJ populations in 70 mM H_2O_2 ranged between 9.3 ± 3 h (M60) and 150.8 ± 68.7 h (M19) (Publication 1, Fig. 5B). In both, DJ virulence and DJ longevity experiments, the phenotypic differences between mutant lines were significant. A high heritability ($h^2 \geq 0.9$) for each phenotypic trait indicates the strong genetic component influencing the traits of the mutant.

No mutant line was observed to show a combined high performance for all beneficial traits. Throughout the analysed mutant subset, DJ survival (MTS₅₀) resulted in a significantly positive correlation with DJ virulence, whereas DJ recovery resulted negatively correlated with virulence and survival, respectively (Publication 1, Fig. 6). Undoubtedly, this will cause serious difficulties for EPN breeding if the antagonism is ubiquitously distributed among EPN populations.

Mutagenesis is a feasible approach to improve traits in *H. bacteriophora*, as has been reported recently by Sumaya et al. (2018) for DJ longevity (survival). Mutant materials may be further considered for a breeding program to enhance *in vitro* DJ recovery, but not before they have been further characterized for the stability of other beneficial traits.

2.4 SNP markers for the DJ recovery phenotype

Considering that the high heritability of the DJ recovery phenotype in *H. bacteriophora* mutants indicates a strong genetic component, we carried out genotyping in a subset of the mutant lines. The determined SNPs in our mutant lines (M1 – 96) were identified by the genotyping by

sequencing (GBS) approach following Godina et al. (2022a). Concerning the number of discovered SNPs, our results are modest in comparison to the previous reports (~ 190 vs ~ 4,800 SNPs). These differences may be explained by the nature of the materials used (closely related mutant lines vs WT lines).

Association analysis between SNP genotype and DJ recovery phenotype was carried out in separate experiments using: i) 18 mutant lines exposed to LB supernatant, ii) 18 mutant lines exposed to LM supernatant, and iii) 96 mutants exposed to LM supernatant. From 20 SNPs with potential association with the DJ recovery trait, the SNPs N1696-4863, N1696-4871, N3144-589 and N3144-606 were recorded throughout all experimental conditions tested (Publication 1, Table 1b). The alleles of these four SNPs can separate mutant lines into two groups with contrasting DJ recovery (Publication 1, Fig. 7).

For the four SNPs with strong association with DJ recovery, we screened for *H. bacteriophora* gene models neighboring (5,000 bp up- and downstream) the SNPs using the publish TT01 genome sequence and the annotations available from McLean et al. (2018). Thereafter, we BLASTed the corresponding gene models against the Swissprot database (BLASTx). Two closely located gene models (g1799 and g1800) were found upstream the SNPs N1696-4863 and N1696-4871 (Publication 1, Fig. 8), which were homologs to the *C. elegans* genes *rig-6* and *acc-1*. These genes are reported to be related to signal transmission and neuron physiology in the model nematode (Publication 1, Table 1) (Katidou et al. 2013; Putrenko et al. 2005). The main factors determining the *C. elegans* dauer formation involve sensory and dietary cues and there are already well-defined regulatory pathways explaining the regulation of dauer entry and exit (Allen et al. 2015; Fielenbach and Antebi 2008). Genes situated close to our associated SNPs may or may not be directly involved in these pathways.

2.5 Nematode factors play a superior role influencing the DJ recovery phenotype in comparison to *Photorhabdus* spp. factors

To date, there is no report concerning phenotypic variability related to different *Photorhabdus* strains causing DJ recovery. We isolated and identified the symbiotic bacteria from fourteen *H.*

bacteriophora WT strains analysing the sequences of five genes: 16S rRNA, *gltX*, *recA*, *dnaN* and *gyrB*. Two isolates (DE6 and HU2) were identified as *P. kayaii*, eleven isolates (AU1, DE2, DE8, HB1, HY1, IT4, MM14, MM8, PT2, SCR1, XX2) as *P. laumondii*, and one isolate (PT1) as *P. thracensis*. The resulting phylogenetic tree topology (Publication 2, Fig. 2 and 3) agreed with previous reports (Kazimierczak et al. 2017; Machado et al. 2018; Orozco et al. 2013; Sajnaga and Kazimierczak 2020).

Subsequently, five *H. bacteriophora* strains/lines were exposed to supernatants of the fourteen identified *Photorhabdus* sp. isolates and their potential supporting DJ recovery was determined. Two nematode commercial strains (EN01 and HB4), the inbred line IL3 selected from EN01 (Sumaya et al. 2018), and two mutant inbred lines M31 and M88 were tested. Significant differences were observed between the recovery of the nematode materials. The highest mean DJ recovery was recorded for the strain EN01 with 86.6 ± 7.7 % (ranging between 69 - 97.7 %), followed by the mutant M31 82.1 ± 6.7 % (69.8 - 92.4 %), HB4 47.7 ± 13.6 % (30.2 - 75.3 %), IL3 33.1 ± 12.5 % (17.1 - 60.8 %), whereas mutant line M88 had the lowest mean recovery 5.1 ± 2.7 % (2.6 - 12.2 %) (Publication 2, Fig. 4).

Within each *H. bacteriophora* material, significant differences were also determined for the recovery in different bacterial strain supernatants. The *P. laumondii* strain HY1 induced the highest DJ recovery on average throughout all nematode lines: EN01 (97.7 ± 2.5 %), HB4 (75.3 ± 16.9 %), IL3 (60.8 ± 17 %), M31 (92.4 ± 5.4 %) and M88 (12.2 ± 8.1 %). On the other side, *P. laumondii* strain S-CR1 supernatant was often the lowest in support of DJ recovery in EN01 with 69 ± 19.1 %, HB4 with 30.2 ± 8.9 % and M88 with 2.6 ± 1.7 %. In *P. kayaii* strain HU2 supernatant, the lowest recovery at 17.1 ± 17.5 and 69.8 ± 16 %, was observed for lines IL3 and M31, respectively.

Kazimierczak et al. (2017) reported that *Heterorhabditis* nematodes can swap their symbionts at intra- and interspecies levels within the genus *Photorhabdus*. Variations within these nematode-bacterial complexes show that nematode performance can be affected (Chapuis et al. 2009; Kazimierczak et al. 2017). However, which if such new combinations are optimal for the function of the nematode-bacterium symbiosis needs further investigation. As the relation is

quite close with several interactions of existential properties it can be assumed that a selection pressure will enhance adaptation to the specific functions of the partnership. Our *P. laumondii* strains MM8 and MM14 were isolated from *H. bacteriophora* undergoing several passages through *Melolontha melolontha* (cockchafer white grub) for 8 and 14 generations, respectively (Berner et al. 2001). The DJ recovery upon exposure to the bacterial supernatant of MM14 was always higher than in MM8 (Publication 2, Fig. 4), regardless of the nematode line. This might already indicate a certain adaptive process, whether of the nematode strains adapting to the natural product environment provided by the symbiont or by the bacterium providing those products favouring the symbiotic interaction by enhancing DJ recovery and the nematode's reproductive program.

Our knowledge of the nature of the bacterial food signal still is rudimentary. More information on its components and biosynthesis might help to improve process conditions in biotechnological mass production. Several proteins and natural compounds have been reported to play an active role in regulating bacteria-nematode-insect interactions (Duchaud et al. 2003; Joyce et al. 2008; Shi and Bode 2018; Shi et al. 2022). Besides conserved core genes that are essential for basic biological functions, unique biosynthetic gene clusters within species also may play an important role in the association with the nematode. Six of the *Photorhabdus* strains analysed in the present study (DE6, HU2, DE2, IT4, HB1 and PT1) were also included in the large analysis reported by Shi et al. (2022). According to the authors, the strains differently synthesized natural products like saccharides and terpenes. Thus, the large contrasts in DJ recovery observed between *P. laumondii* isolates DE2, IT4, and HB1 may be related to strain-specific features in secondary metabolite clusters. However, whether any of these natural products contribute to DJ recovery needs further investigation.

For the same *H. bacteriophora* strain/line, the support of DJ recovery by different *Photorhabdus* strains is variable. However, these differences are less pronounced than the differences between the three nematode lines EN01, HB4 and IL3 (Publication 2, Table 3). The heritability of the trait DJ recovery in bacterial supernatant was $h^2 > 0.6$, despite the differences induced by the different bacterial strains (Publication 2, Fig. 4). This indicates that the nematode genetic

background has a larger influence on DJ recovery than factors from the symbiotic bacterium. The DJ recovery instability is on the side of the nematode.

We can conclude that the future challenge and focus on *H. bacteriophora* DJ recovery improvement will be on the breeding of nematode materials. Besides, the switch of *Heterorhabditis-Photorhabdus* partners can nevertheless be a good complement to optimize the DJ recovery.

2.6 Influence of *H. bacteriophora* DJ aging on its recovery

To find out whether the age of the DJ inoculum has effects on their recovery, DJ of five *H. bacteriophora* materials (EN01, HB4, IL3, M31 and M88) were exposed to the supernatant of *P. laumondii* (strain DE2). The dynamics of recovery over the storage time followed different patterns. The lines EN01, HB4 and IL3 showed the highest DJ recovery at the early observation points (week 1 and 2) followed by a significant decrease over storage time. These lines hardly recovered in Ringer's solution (control). On the other side, both mutants had abnormal reaction to the bacterial supernatant. M31 seems to be recovery-constitutive (79.6 – 85.9 %), exiting the dauer stage even in Ringer's solution, with a DJ recovery significantly increased over the DJ's storage time. Mutant line M88 seems to be recovery-defective (< 5 %) even in bacterial supernatant (Publication 2, Table 4).

In *C. elegans*, worm aging is reported to implicate muscle deterioration, metabolic disorder and accumulation of molecular and cellular damage (Golden and Melov 2007). In EPN, the lipid reserves of *Heterorhabditis* DJ are depleting during storage, and their mobility also decreases (Fitters and Griffin 2004; Menti et al. 2000). This limitation can be overcome by preventing too long storage periods of the nematode inoculum. Given the increasing demand for heterorhabditid nematodes in biological control also during winter periods (Lacey and Georgis 2012), the problem of inoculum storage might be less important in the future, or early precultures should produce inoculum with more predictable DJ recovery before the season starts. The two mutant lines can be used for future studies investigating the genetic background for DJ recovery in *H. bacteriophora*.

2.7 Transcriptome changes during early DJ recovery in *H. bacteriophora*

To further explore potential DJ recovery regulators in *H. bacteriophora*, we selected two EMS-mutant lines with contrasting DJ recovery phenotypes (M31 and M88) and analysed differences in gene expression during the first six hours after exposure to *P. laumondii* (DE2) supernatant. The main findings of this approach are described in the following subsections.

2.7.1 *H. bacteriophora* DJ exposed to bacterial supernatant shed off their secondary cuticle as a primary response

To track the early DJ recovery, we observed the morphological change of DJ incubated in *P. laumondii* (strain DE2) supernatant over 72 h (Publication 3, Fig. 1A). The mouth of unrecovered DJs is closed and presence the DJs are ensheathed in the pre-dauer J2d cuticle. Upon bacterial supernatant exposure, the first morphological reaction is the exsheathment from the secondary cuticle within 1 – 3 h, in both M31 and M88 mutants. Thereafter, further developments of the mouth and intestine were observed. The major morphologic changes in the anterior part were determined after 1-day exposure to supernatant. After ~ 72 h supernatant exposure, the vulva of young hermaphrodites is differentiated.

During the insect host infection process, *H. bacteriophora* DJ are also reported to lose their secondary cuticle while entering the insect. Within 15 min after invasion, DJ react to factors in the haemolymph inducing the release of the symbiotic bacteria within the first 30 min after invasion (Ciche and Ensign 2003; Ebrahimi et al. 2011). Antagonistic proteins involved in the immune response of insects infected with *H. bacteriophora* have been also detected at very early stages after infection (Arefin et al. 2014; Castillo et al. 2015; Kenney et al. 2019; Kenney et al. 2020). To respond to the insect's immune defence, *H. bacteriophora* DJ need a react fast on contact with the insect food signals leading to DJ recovery and bacterial release, which supports the repression of the immune response. In our experiments, the DJ were exposed to bacterial supernatant instead of insect haemolymph. Despite the differences in conditions (bacterial supernatant *vs in vivo* infection and haemolymph contact), the shedding off of the J2d

cuticle appears to be tightly associated with triggering the DJ recovery. Line M31 showed a significantly higher recovery after 72 h than M88 ($P < 0.01$, Publication 3, Fig. 1B). However, we can postulate that the cuticle shedding off and further development appear to be non-coupled events causing the M88 mutants to be recovery defective.

2.7.2 Mutant lines M31 and M88 have different expression dynamics upon bacterial food signal contact

For both mutant lines, 10 RNA-seq libraries including two controls (M31_C, M88_C) and four supernatant exposure treatments per line were produced (Publication 3, Table 1). The expression data obtained after the supernatant treatments were compared against the respective control in Ringer's solution, and the expression (\log_2 transform of fold change, \log_2FC) for gene models at each time point in both mutants was determined (Publication 3, Table S1). The genetic differences between both mutants showed at higher effect on the observed expression patterns than the bacterial supernatant exposure time (Publication 3, supplementary Fig. S1).

Concerning general regulation tendencies, the proportion of *H. bacteriophora* down-regulated was higher than up-regulated gene models (transcripts). The highest up-regulation was detected after 0.5 h of exposure in both mutant lines. Regarding the correlation between M31 and M88 expression levels along the time points, the similarity between both lines increases with the time of supernatant exposure. Early time points (0.5 and 1 h) showed the highest differences (Publication 3, Fig. 3).

Concerning fine regulation dynamics, we determined six distinct clusters of temporal regulation patterns in each line (Publication 3, Fig. 4). The clusters with later peaks of expression show more common gene models between both mutants. We also searched for differential representation of gene ontology (GO) biological processes (BPs) among the determined clusters for both mutants (M31 vs M88) (Publication 3, Fig. 5). In agreement with the morphological results, the cuticle development and molting cycle related functions were enriched in both mutants at early up-regulation clusters (cluster 1 and 2). We therefore can postulate that the *H. bacteriophora* DJ detect the “food signal” stimulus from *Photorhabdus* supernatant within 0 –

1 h, and immediately switch on the molting process. However, there is a timeframe shift (delayed response) of the M88 mutant.

2.7.3 Candidate genes for further research

Based on general GO representation analysis, we determined the gene-concept networks of the most represented GO categories (Publication 3, Fig. 7 and 8). We evidenced the relation between the GO categories and the *C. elegans* homologous gene models within each cluster that were differently regulated (Publication 3, Table 3). Thereafter, we identified the most interactive genes within each network.

Nine gene models (Publication 3, Fig. 9) with distinct regulation dynamics (regulation cluster and gene-concept network) in the two mutant nematodes are postulated for further characterisation and eventually to be related to DJ recovery regulation in the analysed mutants. The homologous genes in *C. elegans* include collagen and cuticle function related genes *col-14* (g3182), *col-40* (g13157) and *sqt-3* (g200) (Johnstone and Barry 1996; Lang and Lundquist 2021; Lažetić and Fay 2017), dauer formation insulin/IGF-1 signalling (IIS) pathway control factor *pqm-1* (g1034) (Tepper. et al. 2013), LIM-homeodomain (LIM-HD)-encoding gene *lim-7* (g8904) (Voutev et al. 2008), *bed-3* (g10031), which is involved in organogenesis (Goh and Inoue 2018; Inoue and Sternberg 2010), sensory neuron control factor *gcy-35* (g7675) (Gray et al. 2004), cellular and embryonic polarity regulator *cdc-42* (g2512) (Wang et al. 2017), and *inx-16* (g8651), related to intestinal muscle contractions (Peters et al. 2007).

Concerning characterized pathways in *C. elegans*, the IIS pathway has been identified as one of the main regulators of dauer formation (Hu 2018). Bai et al. (2013) analysed the *H. bacteriophora* genome and found 19 of 23 *C. elegans* IIS pathway genes. Moshayov et al. (2013) verified two IIS-associated genes (*akt-1* and *glb-1*) that were activated in *H. bacteriophora* during DJ recovery. In our analysis, we found gene models with homology to the *C. elegans* IIS pathway related genes *pqm-1*, *hil-1* and *daf-21*. The observed expression patterns suggest that the physiological importance of several of the genes mentioned above trespasses the species and habit borders in nematodes.

On the other side, compared with previous *H. bacteriophora* reports on DJ recovery upon insect hemocoel contact (Bai and Grewal 2007; Moshayov et al. 2013; Vadnal et al. 2017), no gene related to the host immunosuppressive factors has been found to react upon supernatant exposure in our research. This finding allows to postulate that at an early stage, the food signal from insect and bacteria already lead to distinct regulatory responses in *H. bacteriophora*.

2.7.4 Genotypic differences between M31 and M88

The mutants M31 and M88 are derived from the same donor line IL3 (Sumaya et al. 2018) and the two lines have contrasting DJ recovery phenotypes. Concerning polymorphisms inside or in the vicinity of differentially regulated genes, we compared the common and exclusive SNPs between both mutants and their donor line and found annotated genes nearby each SNP. We determined 22 and 24 exclusive SNPs in the two mutants (Publication 3, Fig. 10A). Within the evidenced gene models, 23 transcripts with differential regulation patterns ($|\log_2FC| \geq 1$, $P \leq 0.05$) in M31 or M88 were determined (Publication 3, supplementary Table S14). The gene model g1974 (Publication 3, Fig. 10B), which is homologous to WR25_03063 from *Diploscapter pachys* can be considered in this context for deeper characterization. The nature of the specific SNP polymorphisms should be further elucidated to find whether new stop codons or reading frame shifts may be related to changes in expression and to a downstream physiologic change.

2.7.5 General remarks

By the morphologic and transcriptomic tracking of *H. bacteriophora* exposure in bacterial supernatant, we confirmed that DJ can rapidly detect and react to a “food signal” to initiate recovery. Moreover, we provide evidence that there is already at this early stage a potential discrimination between the insect- and the bacterial- “food signal”. Thus, DJ recovery in large bioreactors may be improved by breeding and selection of specific nematode genotypes and expression phenotypes favouring the early signal detection and early morphologic responses. Future approaches using the present results should aim to characterize the function of relevant genes and find the compounds activating the early expression induction in crucial regulators.

Summary

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora*, symbiotically associated with *Photorhabdus* spp. bacteria, is a biological control agent against insect pests worldwide. The dauer juvenile (DJ) is a free-living and developmentally arrested stage that carries cells of the symbiont, seeks and invades the insect host. Once inside the insect, the DJ perceive the haemolymph food signal, regurgitate symbiotic bacteria, and re-gain development until sexual maturity. The sequence of events during the exit of the DJ stage in EPN is called DJ recovery. A rapid, synchronized, and high DJ recovery is a major key factor for commercial production of EPN. For culturing in large bioreactors (> 3,000 up to 100,000 L), DJ are used as initial inoculum at the beginning of the process. This takes place after pre-culturing the symbiotic bacterial partner (monoxenic culture) in the same bioreactor for 24 h. Once the DJs are in contact with the *Photorhabdus* bacteria, a “bacterial food signal” is perceived, which induces the DJ recovery. Thereafter, DJs develop to mature hermaphrodites producing hundreds of new DJs inside their body (*Endotokia matricida*). At the end of the process (~ 11 days), the DJ are released and harvested. A high and homogeneous DJ yield is expected (> 200,000 DJ·mL⁻¹, less than 1 % other stages) when recovery is optimal. In cases of suboptimal recovery, low DJ yields are obtained leading to very large energy and material losses (high production costs). Understanding the DJ recovery in *H. bacteriophora* is thus crucial to improve the mass production of EPN. Previous research has already elucidated major conditions influencing the DJ recovery in EPN. However, the following questions remain unsolved: i) which role plays the nematode genetic variability on the DJ recovery phenotype, ii) how heritable is the DJ recovery phenotype, iii) in the nematode-bacteria interplay, which components are crucial or which are subordinate for the recovery success in DJ populations, and iv) can genomic approaches postulate gene models involved in the DJ recovery regulation.

Concerning the first and second questions, I developed a predictor bioassay based on *Photorhabdus* supernatant to evaluate the DJ recovery phenotype of several *H. bacteriophora* WT and more than 160 EMS mutant lines. I characterized more than 150 single nucleotide

polymorphisms (SNPs) within the mutants via high throughput genotyping, conducted association analysis and determined a set of four SNPs with robust association to the DJ recovery phenotype. Sets of mutants with contrasting reproducible DJ recovery phenotype were also revealed. Relevant to the third question, I carried out a detailed geno- and phenotypic characterization of 14 *Photorhabdus* strains and evaluated their influence on the DJ recovery phenotype in a set of *H. bacteriophora* materials. It was evidenced that the bacterial component plays a subordinate role, whereas the nematode genetic pre-disposition is a main factor in the regulation of the DJ recovery in this species. As final approach, I conducted a genome-wide RNA-seq analysis of the early DJ recovery stages (0.5 – 6 h) in two nematode mutant lines with contrasting phenotype (M31 & M88). The expression of more than 14,000 gene models was analysed and the information was combined with functional databases (Gene Ontology) considering the homologies with the model nematode *Caenorhabditis elegans*. As outcome, nine gene models are postulated as potential targets for future approaches. In the following thesis sections the main findings for each approach are described and discussed in detail.

Zusammenfassung

Der entomopathogene Nematode (EPN) *Heterorhabditis bacteriophora*, der eine enge Symbiose mit Bakterien der Gattung *Photorhabdus* spp. unterhält, ist ein biologisches Bekämpfungsmittel gegen Insektenplagen weltweit. Die Dauer Larve (DL) ist dessen freilebendes und in der Entwicklung pausierendes Stadium, das Symbiontenzellen im Darm trägt, und den Insektenwirt infiziert. Einmal im Inneren des Insekts (Hämolymphe) empfangen DL ein Futtersignal, setzen die Bakterien frei und nehmen ihre Entwicklung zur Geschlechtsreife wieder auf. Das Ereignis der Unterbrechung des diapausierenden DL-Stadiums wird Dauer „Recovery“ genannt. Eine schnelle, synchronisierte und hohe DL-Recovery ist ein entscheidender Faktor für die kommerzielle Produktion von EPN. Zur Kultivierung in großen Bioreaktoren (> 3.000 bis zu 100.000 L) werden DL als Inokulum zu Beginn des Prozesses verwendet. Dies geschieht nach einer Vorkultur des Symbionten (monoxenische Kultur) im selben Bioreaktor für 24 Stunden. Sobald sich die DL in Kontakt mit den *Photorhabdus*-Bakterien befinden, wird das bakterielle Futtersignal wahrgenommen, das die DL-Recovery induziert. Anschließend entwickeln sich die DL zu geschlechtsreifen Hermaphroditen, die Hunderte neuer DL in ihrem Körper produzieren (*Endotokia matricida*). Am Ende des Prozesses (~ 11 Tage) werden die DL freigesetzt und geerntet. Ein hoher und homogener DL-Ertrag wird erwartet (> 200.000 DJ·mL⁻¹, weniger als 1 % andere Stadien), sofern die DL-Recovery optimal verläuft. Bei suboptimaler Recovery wird ein niedriger DL-Ertrag erzielt, was zu sehr hohen Energie- und Materialverlusten führt (hohe Produktionskosten). Das Verständnis der DL-Recovery in *H. bacteriophora* ist daher entscheidend, um die Massenproduktion von EPN zu verbessern. In vorherigen Untersuchungen wurden bereits wesentliche Bedingungen identifiziert, die die DL-Recovery von EPN beeinflussen. Allerdings blieben die folgenden Fragen unbeantwortet: i) welche Rolle spielt die genetische Variabilität der Nematoden für den DL-Recovery-Phänotyp, ii) wie stark vererbbar ist der DL-Recovery-Phänotyp, iii) bei der Interaktion zwischen Nematode und Bakterien, welche Komponenten sind entscheidend oder welche untergeordnet für den Erfolg der DL-Recovery in DL-Populationen, und iv) können genomische Ansätze Genmodelle als

Kandidaten zur Regulierung der DL-Recovery postuliert werden.

In Bezug auf die ersten beiden Fragen habe ich ein Bioassay basierend auf *Photorhabdus*-Überstand entwickelt, um den DL-Recovery-Phänotyp von mehreren *H. bacteriophora* WT und über 160 EMS-Mutantenlinien zu ermitteln. Anschließend habe ich über 190 einzelne Nukleotidpolymorphismen (SNPs) in den Mutantenlinien durch Hochdurchsatz-Genotypisierung charakterisiert, Assoziationsanalysen durchgeführt und eine Auswahl von vier SNPs identifiziert, die mit dem DL-Recovery-Phänotyp stark assoziiert sind. Es wurden ebenfalls Gruppen von Mutanten mit kontrastierendem und reproduzierbarem DL-Recovery-Phänotyp ermittelt. Bezüglich der dritten Frage habe ich eine ausführliche geno- und phänotypische Charakterisierung von 14 *Photorhabdus*-Stämmen durchgeführt und deren Einfluss auf den DL-Recovery-Phänotyp in unterschiedlichen *H. bacteriophora* Linien evaluiert. Es wurde festgestellt, dass die Bakterienvariabilität eine untergeordnete Rolle, während die genetische Veranlagung des Nematoden eine Hauptrolle bei der Regulierung der DL-Recovery spielt. Als abschließender Ansatz habe ich eine genomweite RNA-seq-Analyse der frühen DL-Recovery-Ereignisse (0.5 – 6 h) in zwei Nematoden EMS Mutantenlinien mit kontrastierendem Phänotyp (M31 and M88) durchgeführt. Die Expression von mehr als 14.000 Genmodellen wurde analysiert und die Informationen wurden mit funktionalen Datenbanken (Gene Ontology) unter Berücksichtigung von Homologien mit dem Modellnematoden *Caenorhabditis elegans* verglichen. Als Hauptergebnis werden neun Genmodelle als potenzielle Ziele für zukünftige Studien postuliert. Die wichtigsten Erkenntnisse für jeden Ansatz werden in den folgenden Abschnitten beschrieben und ausführlich diskutiert.

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Annex 1: Publication 1

Pheno- and genotyping *in vitro* dauer juvenile recovery in the nematode *Heterorhabditis bacteriophora*

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Pheno- and genotyping in vitro dauer juvenile recovery in the nematode *Heterorhabditis bacteriophora*

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Abstract

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* is an effective biological-control agent of insect pests. The dauer juveniles (DJs) seek for, infect insects, and release cells of the carried symbiotic bacterium of the genus *Photorhabdus*. Inside the host, the DJs perceive signals from the insect's haemolymph that trigger the exit from the arrested stage and the further development to mature adults. This developmental step is called DJ recovery. In commercial production, a high and synchronous DJ recovery determines the success of liquid-culture mass production. To enhance the understanding about genetic components regulating DJ recovery, more than 160 mutant- and 25 wild type inbred lines (WT ILs) were characterized for DJ recovery induced by cell-free bacterial supernatant. The mutant lines exhibited a broader DJ recovery range than WT ILs (4.6–67.2% vs 1.6–35.7%). A subset of mutant lines presented high variability of virulence against mealworm (*Tenebrio molitor*) (from 22 to 78% mortality) and mean time survival under oxidative stress (70 mM H₂O₂; from 10 to 151 h). Genotyping by sequencing of 96 mutant lines resulted in more than 150 single nucleotide polymorphisms (SNPs), of which four results are strongly associated with the DJ recovery trait. The present results are the basis for future approaches in improving DJ recovery by breeding under in vitro liquid-culture mass production in *H. bacteriophora*. This generated platform of EMS-mutants is as well a versatile tool for the investigation of many further traits of interest in EPNs.

Keypoints

- Exposure to bacterial supernatants of *Photorhabdus laumondii* induces the recovery of *Heterorhabditis bacteriophora* dauer juveniles (DJs). Both, the bacteria and the nematode partner, influence this response. However, the complete identity of its regulators is not known.
- We dissected the genetic component of DJ recovery regulation in *H. bacteriophora* nematodes by generating a large array of EMS mutant lines and characterizing their recovery pheno- and genotypes.
- We determined sets of mutants with contrasting DJ recovery and genotyped a subset of the EMS-mutant lines via genotyping by sequencing (GBS) and identified SNPs with significant correlation to the recovery trait.

Keywords *Heterorhabditis bacteriophora* · Liquid culture production · EMS-mutagenesis · Dauer juvenile recovery · *Photorhabdus laumondii* bacterial supernatant · Longevity · Virulence · SNPs

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Introduction

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (Nematoda: Rhabditida) is an effective biological-control agent against insect pests, like for example the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), the corn rootworm *Diabrotica virgifera* (Coleoptera: Chrysomelidae), and several scarab grubs (Coleoptera: Scarabaeidae) (Grewal et al. 2005; Lacey et al. 2015). Nematodes of this species hold a tight symbiotic relationship with gram-negative bacteria of the genus *Photorhabdus*

(Gammaproteobacteria: Morganellaceae). The dauer juvenile (DJ) of *H. bacteriophora* is a developmentally arrested third juvenile stage that harbors the bacteria in its intestine. The DJs seek for insects in the soil and transfer cells of the bacterial symbiont into the body of the host. DJs enter the insect via natural openings (e.g., mouth, anus, and spiracles) or penetrate through the insect cuticle by using their anterior tooth (Bedding and Molyneux 1982). Once inside the insect's haemocoel, the nematodes release the symbiotic bacteria, and both interact to overcome the insect's defense mechanisms. The bacteria grow exponentially killing the insect, while acting as nutritional source for the nematodes (Han and Ehlers 2000). Once in contact with the hemolymph, DJs exit the arrested stage and resume development (process called DJ recovery) and reach reproductive maturity. The nature of the insect's chemical signal (food signal) triggering DJ recovery has not been identified yet (Steven et al. 1997).

The mass production of *Heterorhabditis* spp. in artificial liquid medium has been established several decades ago (Ehlers et al. 1998; Wouts 1981). Since then, the production of EPN in large bioreactors has been optimized and today they are used commercially in biological control of many insect pests (Ehlers 2001).

One of the key factors that determines the success of the liquid culture production processes for *H. bacteriophora* is a rapid, synchronized, and high DJ recovery (Johnigk et al. 2004). Artificial culture media used in mass production lack any food signal and, thus, do not induce DJ recovery. However, liquid media are incubated for 1 day with their specific symbiont before DJs from monoxenic cultures are inoculated. In this case, the DJ recovery is triggered by signals coming from the symbiotic bacteria (bacterial food signal) (Aumann and Ehlers 2001). Different to the in vivo scenario with > 95% recovery within 24 h, the signals of the bacterial symbiont are less effective and trigger DJ recovery between 0 and 81% scattered over 3 to 5 days (Dolan et al. 2002; Han et al. 2000; Strauch and Ehlers 1998). In artificial *Photorhabdus* spp. cultures, factors like the bacteria growth phase, carbon dioxide concentration, nematode pheromone concentration (ascarosides and isopropylstilbene), have been determined as important co-factors in increasing DJ recovery (Jessen et al. 2000; Strauch and Ehlers 1998; Wang et al. 2022). However, no complete picture about how DJs perceive these signals is available.

The DJ recovery process was first molecularly characterized by Bai and Grewal (2007) via identifying the gene expression of *H. bacteriophora* DJs upon contact with the insect hemolymph. In this work, the authors identified two actin-annotated clones (HbGPS11.C1G02 and HbGPS11.C4C08), which were significantly downregulated. Later on, Moshayov et al. (2013) analyzed the development from the dauer to the recovered stage of *H. bacteriophora* through RNA-seq. The authors identified eight genes strongly associated with DJ

recovery and the genes were verified by qPCR and transcription silencing. Two of the genes (*hsp-90*, corresponding to *daf-21* and *hsp-1*) were associated with the dauer formation insulin-like growth factor pathway in *Caenorhabditis elegans*. Several genes and signaling pathways have been identified that are involved in dauer recovery in *C. elegans*, including the insulin/IGF signaling pathway (IIS), transforming growth factor beta (TGF- β) signaling pathway, and the DAF-12 nuclear hormone receptor pathway (Fielenbach and Antebi 2008; Hu 2018). However, the specific gene functions concerning the DJ recovery process in *H. bacteriophora* are still not well understood. In the model nematode *C. elegans*, three main environmental cues influence dauer formation and the development from J1 to dauers: (i) dauer-inducing pheromones (DIP), a complex mixture of ascarosides; (ii) lack of food and, (iii) temperature (Golden and Riddle 1984). A wide range of mutants has been reported with a high variability in the dauer-related processes. These can be classified into two groups: (i) dauer-constitutive mutants (*daf-c*), which always enter dauer diapause, and (ii) dauer defective mutants (*daf-d*) that bypass the diapause, regardless of the environment conditions (Fielenbach and Antebi 2008).

Determining mayor DJ recovery genetic regulators in EPN may allow the selection of wild type (WT) strains with improved in vitro recovery performance and would enable genotypic selection in breeding approaches. Up to date, several beneficial nematode traits have been improved based mainly on phenotypic selection, hybridization, mutagenesis, and recombinant DNA techniques (Glazer 2015). As examples, improvement on DJ virulence, desiccation tolerance (Anbesse et al. 2012; Mukuka et al. 2010), oxidative stress tolerance, and longevity (Sumaya et al. 2018) of *H. bacteriophora* have been achieved.

No information on phenotypic and genotypic variability of WT or mutant strains of *H. bacteriophora* concerning DJ recovery or reproductive potential is available. However, a better understanding of the upstream regulatory processes of in vitro DJ recovery is essential to improve the liquid-culture mass production. Concerning genetic variability, recent works reported on the discovery of thousands of single nucleotide polymorphisms (SNPs) and their potential use for genetic selection and association analyses with beneficial traits (Godina et al. 2022; Levy et al. 2020). This pool of information could also serve to investigate the genetic background regulating DJ recovery in *H. bacteriophora*. In the present work we have produced a collection of more than 160 highly homozygous *H. bacteriophora* EMS-mutant lines starting from an existing EMS population produced by Sumaya et al. (2018). We used 25 existing WT inbred lines (WT ILs) as well developed in previous studies (Godina et al. 2022). All lines were evaluated by systematically testing the DJ recovery induced by bacterial cultures as well as cell-free bacterial supernatants. Additionally, a subset of

mutant lines was evaluated for DJ longevity (survival under oxidative stress tests induced by 70 mM H₂O₂) and virulence against mealworm (*Tenebrio molitor*) to check for possible trade-off effects. Parallel to phenotyping, we carried out genotyping by sequencing (GBS) of 96 EMS-mutant lines to determine SNPs and run an association analysis between pheno- and genotype data for the identification of SNPs related to DJ recovery.

Material and methods

Wild type and mutant inbred lines

A subset of 25 highly homozygous WT ILs was chosen from the lines produced by Godina et al. (2022). Ethyl methanesulphonate (EMS) is commonly used as a mutagenic chemical to induce random-point mutations also in nematodes (Brenner 1974). Sumaya et al. (2018) produced an EMS-mutant population out of the commercial *H. bacteriophora* inbred line IL3. This mutant pool was used to generate inbred lines by initial liquid culturing and subsequent sub-culturing of single hermaphrodites according to Sumaya et al. (2018). In liquid culture, *H. bacteriophora* second generation males and females cannot copulate (Johnigk and Ehlers 1999a); therefore, nematodes can only reproduce through self-fertilization of first-generation hermaphrodites (Strauch et al. 1994). Liquid cultures with the mutant pool were repeated four times. Thereafter, single hermaphrodites culturing on NGG (nematode growth gelrite) solid culture was repeated for three further generations by picking individual mature self-fertilizing hermaphrodite for each subsequent growth cycle into a new NGG plate. A minimum of seven selfing cycles leads to > 95 % homozygosity (Hartl and Clark 1997). First generation nematodes on NGG are only reproducing by self-fertilization. We obtained two groups of inbred lines: (a) 103 mutant inbred lines that had been produced in liquid and solid culture and (b) 65 mutant inbred lines that were only produced on NGG solid medium from the beginning. In total, 168 mutant *H. bacteriophora* inbred lines were generated. The commercial donor line IL3 and commercial hybrid HB4 were used as references for the following experiments. Abnormal visual phenotypes (e.g., dumpy worms) were observed in some of the lines; however, these were not assessed quantitatively.

Nematode monoxenic liquid cultures

For monoxenic liquid cultures of WT ILs and mutant lines, nematode eggs were isolated from early hermaphrodites, surface sterilized and cultured following the procedure described by Lunau et al. (1993). First stage juveniles (J1) hatching from the surface-sterilized eggs were transferred

to Petri dishes containing Wouts agar (16 g Bacto nutrient broth, 12 g Bacto agar, 5 g corn-oil or sunflower oil, and 1 L distilled water), which had been inoculated with *P. laumondii* bacteria and incubated in the dark at 25 °C for 1 day before the transfer of the J1. Culturing *H. bacteriophora* in all experiments used the bacterium *P. laumondii* (strain DE2; e-nema GmbH, Schwentimental, Germany). The subspecies *P. luminescens* subsp. *laumondii* was elevated to species level *P. laumondii* (Machado et al. 2018). Subsequently, resulting hermaphrodites in late *endotokia matricida* (Johnigk and Ehlers 1999a) were transferred directly to *P. laumondii* pre-inoculated (24 h) liquid medium (LM) (15 g yeast extract, 20 g soy powder, 4 g NaCl, 0.35 g KCl, 0.15 g CaCl₂, 0.1 g MgSO₄, 6 g lecithin diluted in same volume of rapeseed oil, 40 g rapeseed oil, and 1 L distilled water, pH = 7 ± 0.1) in Erlenmeyer flasks (Ehlers et al. 1998; Hirao and Ehlers 2010) and incubated at 25 °C on a shaker (180 rpm, rotation diam. 4 cm) for 15 days. Subcultures of monoxenic nematode liquid cultures were carried out in 250-ml flasks (50 ml volume LM) by the same procedure, inoculating DJs at a final density of 4,000 DJs mL⁻¹ using cultures of previous generations. Controls for contaminant growth were carried out by streaking samples on NBTA agar (10 g Bacto tryptic soy broth, 0.025 g bromothymol blue sodium salt, 14 g Bacto agar, 1 L distilled water, and 4 ml of 1 % sterile filtered 2,3,5-triphenyltetrazolium chloride) (Akhurst 1980).

Nematode solid culture

For the production of small amounts of DJs and to maintaining single mutant lines, nematodes were grown on solid NGG medium (3 g·L⁻¹ Gelrite, 2.5 g·L⁻¹ peptone from casein, 51 mM NaCl, 1 mM CaCl₂·2 H₂O, 1 mM MgSO₄·7 H₂O, 1 mM KH₂PO₄, 12 µM filter-sterilized cholesterol in 99 % ethanol) in Petri dishes according to Sumaya et al. (2018). A sterilized semi-solid NGG (similar to solid but containing only 1.5 g·L⁻¹ Gelrite and 2 g·L⁻¹ peptone) was mixed with a *P. laumondii* suspension cultured in LM for 24 h at 25 °C and adjusted to a final cell density of 2 × 10¹⁰ cells·mL⁻¹. The bacterial loan (bacteria in semi solid NGG) was distributed over the solid NGG covering the complete Petri dishes. After 24 h incubation in 25 °C, DJs were inoculated into NGG plates and incubated for 6–7 days at 25 °C until hermaphrodites in late *endotokia matricida* stage (Johnigk and Ehlers 1999b) were obtained. Mature hermaphrodites were washed off with Ringer's solution (9 g NaCl, 0.42 g KCl, 0.37 g CaCl₂·2 H₂O, 0.2 g NaHCO₃, and 1 L distilled water) and stored until DJs hatched from the maternal carcass. Harvested DJs were further stored in Ringer's solution in 25-ml culture bottles (filled up to ~5 ml volume) at 4 to 6 °C for further experiments. For every experiment, DJs were stored for a maximum 30 days.

Production of bacterial supernatant

The ability of *H. bacteriophora* lines to recover in cell-free *P. laumondii* bacterial supernatant was investigated. *P. laumondii* cultures in Luria-Bertani broth (LB) (5 g·L⁻¹ NaCl, 10 g·L⁻¹ tryptone, and 5 g·L⁻¹ yeast extract) and in LM medium were grown at 25 °C on a shaker (180 rpm, rotation diam. 4 cm) for 24 h and then centrifuged at 10,000 rpm (4 °C) for 10 min. Resulting supernatants were passed through 5- and 0.2-μm pore-size filters (in sequential way) and stored at -80 °C until used. The supernatants from two media are hereafter referred to as LM & LB supernatant.

Assessment of DJ recovery in liquid culture and bacterial supernatant

DJ recovery was assessed in liquid culture and in bacterial supernatants. In liquid culture DJ recovery was determined 3 days after inoculation and incubation at 25 °C by counting the developing hermaphrodites under an inverted microscope (Zeiss, Germany). Recovery in supernatants was assessed observing morphological changes of the DJ head region and esophagus (Fig. 1) following (Strauch and Ehlers 1998) 3 days after inoculation in 24 h pre-cultured *P. laumondii* supernatant. For this purpose, DJ populations were incubated in 24-well plates using randomized arrangement. Each cell was filled with 200 μl supernatant and 100–200 DJs, in the presence of 0.2 % streptomycin sulfate. Control assays were carried out in Ringer's solution. The DJ recovery was evaluated within 3 days after incubation at 25 °C by counting recovered and unrecovered DJs in each cell under an inverted microscope.

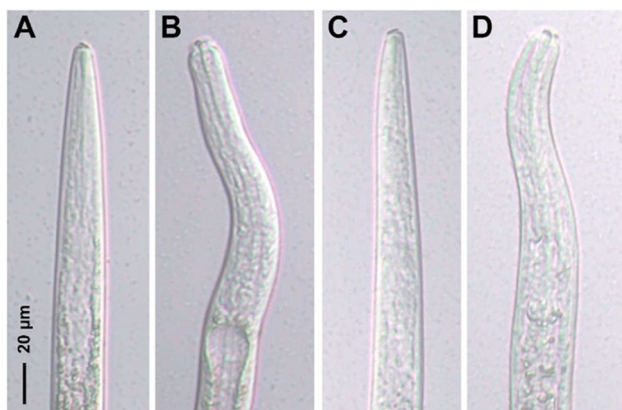


Fig. 1 Morphological changes observed during *Heterorhabditis bacteriophora* (strain HB4) DJ recovery. Unrecovered DJs in Ringer's solution (A and C) are compared to recovered individuals after exposure to supernatant (B and D). Bacterial supernatant is derived from *Photorhabdus laumondii* (strain DE2) culture. DJs were incubated at 25 °C for 3 (A and B) and 5 (C and D) days. The scale bar for all photos is depicted in A

DJ virulence assay

Virulence assays were carried out with a subset of 18 mutant lines and two reference materials (HB4 and IL3). From each line, 600 DJs were incubated in Petri dishes (diam. 15 cm), each filled with 150 g sand (0–2 mm particle size, 5.5–6.0 % moisture) and 40 larvae of the coleopteran mealworm *Tenebrio molitor*. Each treatment and line consisted of four replicates (plates) and three independent trials with different DJ batches. After 7 days incubation in the dark at 25 °C, the mealworm mortality caused by DJ infection was confirmed by measurement of bioluminescence produced by *P. laumondii* in the insect cadaver with a portable tube luminometer (Junior LB 9509, Berthold Technologies, Bad Wildbad, Germany).

DJ survival assay

Sumaya et al. (2017) reported that *H. bacteriophora* DJ oxidative stress tolerance and DJ longevity in Ringer's solution are positively correlated. A subset of mutant 18 mutant lines and two reference materials (IL3 and HB4) was phenotyped in 24-well plates following the same procedure described by Sumaya et al. (2017). DJ populations (100–200 DJs) were disposed in cell wells filled with 70 mM H₂O₂ in 400 μl final volume in four randomly-arranged replications (cells) and kept at a constant of 25 °C. The oxidative stress trials were repeated four independent times. The DJ survival was evaluated by counting all the living and dead DJs under an inverted microscope in each cell every day for the first 3 days and at intervals of 3–5 days until more than 90 % individuals were dead. The mean time survived by 50 % (MTS₅₀) of the DJ-population was calculated by adjusting the experimental data to a logistic model (see statistical analysis).

Nematodes genotyping and association analysis

To screen for single nucleotide polymorphisms (SNPs) among mutant lines, genotyping by sequencing (GBS) was carried out with group (a) mutants (the first 96 lines). All sequencing was done at LGC Genomics (Berlin, Germany). All DNA extractions were started with a minimum of 50 hermaphrodite individuals, and ~100 ng DNA per sample were sequenced in duplicate. At the sequencing source, the genomic DNA was digested with *MspI* restriction enzyme. All DNA samples were sequenced with the Illumina NextSeq 500 (1 × 75 bp) platform with a minimum of 1.2 million reads per sample. The treatment and annotation of the sequencing output followed the recent report of Godina et al. (2022). For sequence comparison against the non-mutagenized donor line, positions of SNPs were also determined in a genome draft of the *H. bacteriophora* commercial inbred line IL3 (unpublished).

Subsequently, the association analysis of SNPs markers and phenotypes was done using the software TASSEL 5.0 (Ithaca, NY, USA). Positions of highly relevant SNPs were also determined in the *H. bacteriophora* strain TT01 genome draft PRJNA13977.WBPS16 deposited at the Wormbase (<https://wormbase.org>). For sequence analysis, the *H. bacteriophora* gene models annotation of McLean et al. (2018) was used to identify genes close to significant SNPs. For this, the region 5000 bp up- and downstream of given SNPs were extracted. Subsequently, we used BLASTx routine ($E\text{-value} < 1.0 \times 10^{-5}$) to determine homologous proteins on the Swissprot/Uniprot database (<https://www.uniprot.org>). All sequence handling and analysis was done with the software CLC Main Workbench 20 (Qiagen, Aarhus, Denmark). After the GBS genotyping and SNP extraction, a HapMap file with the allele calls for each SNP and mutant lines was generated at the sequencing source. Subsequently, the genotypic information from SNPs and the DJ recovery phenotype were combined through association analysis using the general linear models ($P < 0.05$, 100 permutations) of the TASSEL 5.0. Thereafter, for associated SNPs ($P < 0.05$), the strong and weak alleles were determined along the screened mutant lines. Significant differences in DJ recovery between the allelic groups were determined through independent ANOVA tests. All association analyses were carried out using the data from each experimental repetition separately.

Statistical analysis

The DJ recovery was calculated as percentage of recovered DJs in each cell well. DJ virulence was determined by the mortality (%) on the mealworm populations. The MTS_{50} in DJ populations under oxidative stress was calculated by dynamic curve fitting using the 5 parameter logistic standard curve from the Sigmaplot 14.0 software (San Jose, CA, USA). For statistical analyses, the data normality and variance equality were tested by Shapiro-Wilk test and the F -test method. The normal-distributed and equal-varianced data were analyzed by parametric tests (two- and one-way ANOVA followed by Tukey's HSD test, or t test). Non-normally distributed or unequal-variance data were log-transformed or analyzed by non-parametric tests (Kruskal-Wallis or Mann-Whitney U). The correlation between phenotypes was assessed by Pearson correlation coefficient (normal-distributed data) or Spearman's rank correlation coefficient (non-normal distributed data). The general linear model between phenotypes was built with linear regression.

The variation in the DJ phenotypic traits was evaluated by the broad-sense heritability (h^2) method

(Covarrubias-Pazarán 2019; Johnigk et al. 2002), using following equation:

$$h^2 = \frac{V_g}{V_g + \frac{V_{ge}}{L} + \frac{V_e}{RL}}$$

where V_g , V_{ge} , and V_e are representing variation in genotype (lines), intercept (genotype and environment), and residual (environment), respectively; L is the number of independent experiment repeat, and R is the number of technical replications in each repeat.

A principal component analysis (PCA) was performed on the normalized phenotype values of mutant lines. Phenotypic data of these lines were clustered with K -means method, the optimal K was found via the Akaike information criterion. All data were collated and pre-treated in Microsoft Excel. Data normality and variances test, significance analysis, and linear model were performed using GraphPad Prism 12.0 software (San Diego, CA, USA). Correlations, PCA, and cluster were assessed with R v4.2.1 in Rstudio v2022.07.1+554 (Boston, MA, USA) development environment. The R packages factoextra v1.0.7, FactoMineR v2.4, ggplot2 v3.3.6, cluster v2.1.3, lme4 v1.1.30, and Hmisc v4.7.1 were used to implement the analysis.

Results

DJ recovery of wild type lines

The DJ recovery of 25 *H. bacteriophora* WT ILs was evaluated under monoxenic liquid culture and in parallel upon bacterial supernatant exposure. Both cultures were produced in LM medium. In liquid culture, DJ recovery was assessed three days after DJ inoculation to pre-cultured bacteria. The highest recovery was determined for the commercial line IL3 ($58.7 \pm 7.6\%$) whereas the remaining WT ILs ranged from $16.5 \pm 8.7\%$ (line MM141) up to $34 \pm 17.6\%$ (line IR31). Differences between IL3 and the remaining lines were significant (one-way ANOVA; $F = 1.729$; $df = 24, 72$; $P < 0.05$). Concerning DJ recovery upon supernatant exposure, the two reference lines HB4 and IL3 had the highest recovery (25.7 ± 8.5 and $35.7 \pm 12.3\%$, respectively). Except HU12 ($23.6 \pm 4.3\%$), all WT ILs had a significantly lower recovery, ranging from 1.6 ± 0.7 (AU11) to $8.2 \pm 4.5\%$ (TR21) (one-way ANOVA; $F = 17.43$; $df = 24, 75$; $P < 0.01$). The variability of WT ILs is low under both conditions. The heritability (h^2) of DJ recovery in supernatant is higher than in liquid culture (0.96 vs 0.44), indicating that DJ recovery in liquid culture is influenced by environmental rather than genetic factors. The DJ recovery of WT lines in liquid culture and in bacterial supernatant was significantly positive correlated (Spearman's rank correlation, $R = 0.717$; $P < 0.01$). An

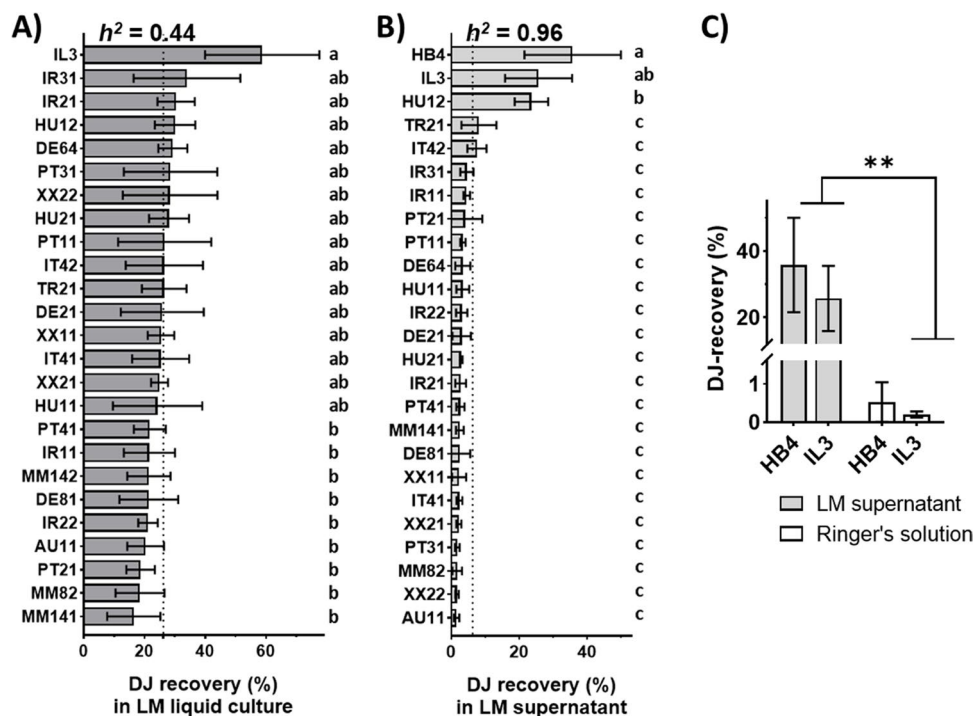


Fig. 2 Average DJ recovery (%) of *Heterorhabditis bacteriophora* WT ILs incubated in *Photorhabdus laumondii* (strain DE2) liquid culture (A) and supernatant produced in LM medium (B). DJ recovery in the commercial reference strains HB4 and IL3 incubated in supernatant and Ringer's solution (C). The DJ recovery was assessed 72 h after DJ incubation. Both experiments were repeated four independent times, each time with four replicates. Error bars depict the

SD. Different letters behind the bars in A and B denote significant differences among WT ILs ($P < 0.05$). Asterisks (*) over the bars in C denote significant differences between treatments and control (** $P < 0.01$). Dash lines in A and B indicate the average of all lines. The heritability (h^2) is provided for each phenotype on top of panels A and B

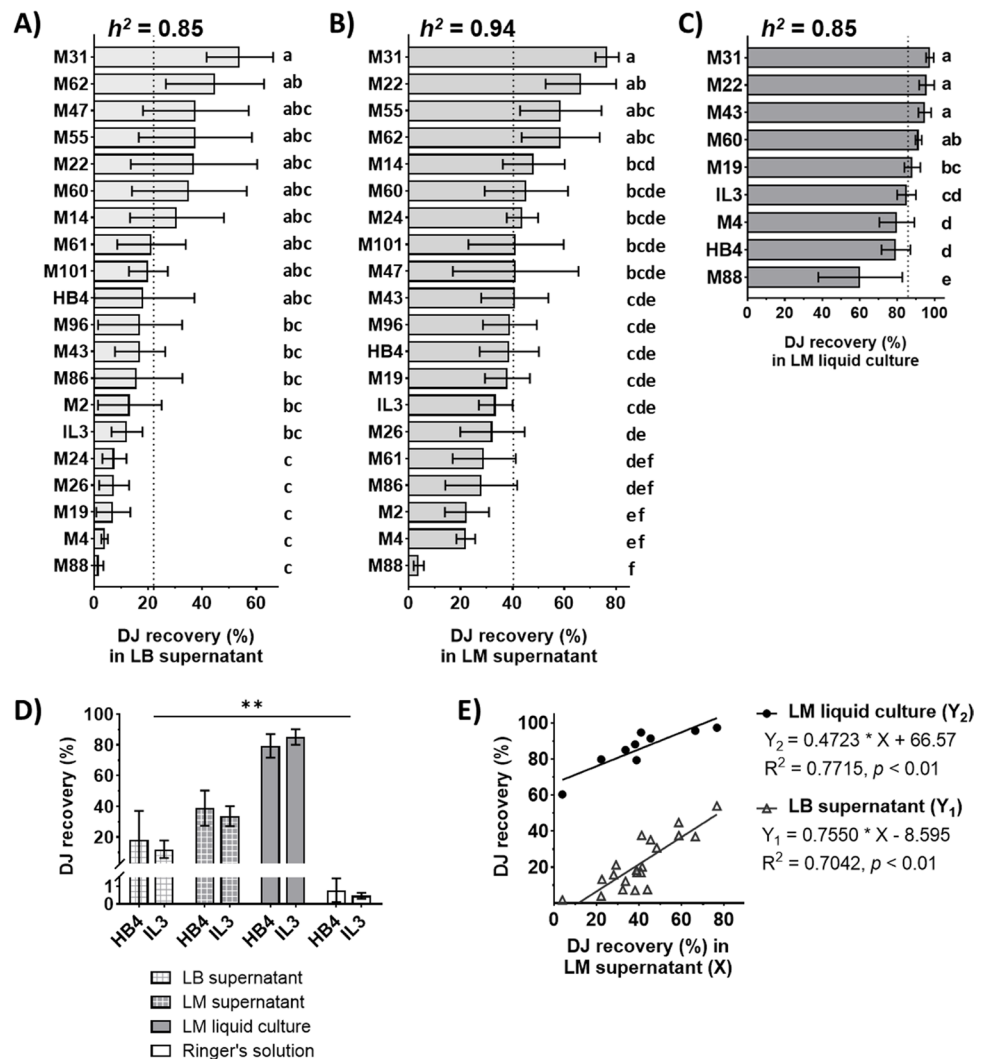
overview of the DJ recovery among the WT lines under the two test conditions is depicted in Fig. 2.

DJ recovery of mutant lines

As alternative to the use of natural variability, we pre-phenotyped a large set of *H. bacteriophora* in 168 mutant inbred lines. Initial experiments were carried out with a subset of 18 mutant lines for comparison of recovery in *P. laumondii* supernatant grown in LB and LM, reaching a bacterial density of 2.6×10^9 and 6.9×10^9 colony-forming-units ml^{-1} , respectively. In parallel, seven of these mutant lines were grown in monoxenic liquid culture using LM as a starting medium (Fig. 3). For the mutant line M31, the highest recovery was determined in both supernatants, and it was significantly higher than that of the donor line IL3 in LB supernatant ($54 \pm 10.7\%$ vs $12.2 \pm 5\%$), LM supernatant ($76.6 \pm 4.1\%$ vs $33.6 \pm 5.9\%$) and LM liquid culture ($97.4 \pm 1.8\%$ vs $85 \pm 4.4\%$). In contrast, DJ recovery of the line M88 was the lowest under all test conditions ($1.7 \pm 1.5\%$ in LB supernatant, $3.9 \pm 1.8\%$ in LM supernatant, and $60.3 \pm 19.4\%$ in LM liquid culture). The heritability of DJ recovery of mutants within each tested materials (LB and LM

supernatant and LM liquid culture) was high ($h^2 \geq 0.84$) indicating that the genetic differences between mutant lines are the main source of variation. The DJ recovery phenotype in LM supernatant was significantly correlated with LB supernatant and LM liquid culture recovery, respectively (Pearson correlation; $R = 0.78, 0.88$; $P < 0.01$; $n = 20$). Recovery in LB supernatant and LM liquid culture were also correlated (Pearson correlation, $R = 0.71$, $P < 0.01$, $n = 9$). In general, DJ recovery in LM liquid culture was the highest, followed by LM and LB bacterial supernatant, and the differences were significant (two-way ANOVA; $F = 344.2$; $\text{df} = 2, 99$; $P < 0.01$) (Fig. 3). These results indicate a robust reproducible recovery in two different media for *P. laumondii* production (LB and LM) and two types of DJ-exposure (bacterial supernatant and immersion of DJs in LM liquid culture). Hence, the use of bacterial supernatant is a fast and accurate predictor of DJ recovery of *H. bacteriophora* in liquid culture. Based on these results, the full collection of 168 mutant *H. bacteriophora* lines was phenotyped for DJ recovery in LM-produced *P. laumondii* supernatant together with the reference lines HB4 and IL3. Differences between the mutant lines were significant (one-way ANOVA, $F = 15.56$; $\text{df} = 167, 593$; $P < 0.01$) and DJ recovery ranged

Fig. 3 Average DJ recovery (%) of a subset of 18 *Heterorhabditis bacteriophora* mutant lines incubated in *Photorhabdus laumondii* (strain DE2) supernatant produced in LB medium (A), supernatant produced in LM medium (B), and for seven lines in liquid culture LM medium (C). The DJ recovery of reference lines HB4 and IL3 incubated in two supernatants, liquid culture and Ringer's solution (D). Linear regression between DJ recovery (%) of mutant lines in LM supernatant and LB supernatant, as well as in the LM supernatant and LM liquid culture (E). The DJ recovery was assessed 72 h after DJ incubation. The experiments were repeated four, six, and four different times in LB supernatant, LM supernatant, and LM liquid culture, respectively, each time with four (LB and LM supernatant) or three (LM liquid culture) replicates. Different letters behind the bars denote significant differences among mutant lines ($P < 0.05$). Asterisks (*) over the bars denote significant differences between four treatments (** $P < 0.01$). Error bars depict the SD. Dash lines (A, B, and C) indicate the average of all lines. The heritability (h^2) is provided on top of the panels A, B, and C



from 4.6 ± 0.8 (M88) up to $67.2 \pm 7\%$ (M31). The recovery of mutant lines from group (a) previously passed through liquid culture (M1-103) has a significantly higher recovery (Mann–Whitney U test, $P < 0.05$) than the lines of group (b), which did not pass through initial liquid culturing (M104-168). An overview of the DJ recovery for all 168 mutant lines in LM supernatant is presented in Fig. 4.

DJ virulence and survival of mutant lines

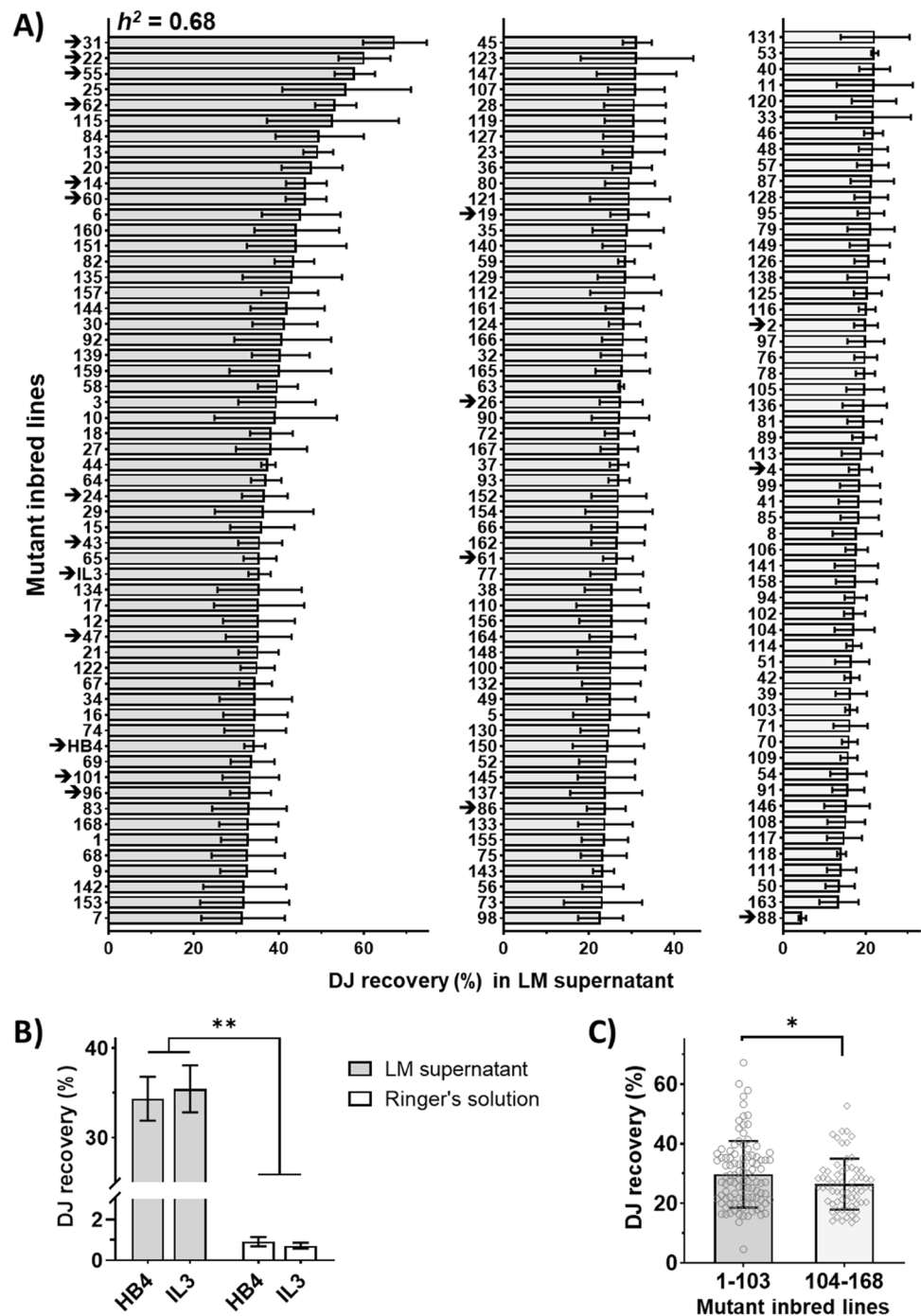
To investigate possible correlations or trade-off effects between the traits DJ recovery, survival, and virulence, the previously used subset of 18 mutant lines was phenotyped together with the reference lines IL3 and HB4. The differences in DJ virulence between mutant lines were significant (two-way ANOVA, $F = 49.59$; $df = 17, 162$; $P < 0.01$). Lines HB4 ($78.3 \pm 4.4\%$), M43 ($78.1 \pm 1.8\%$),

and IL3 ($77.5 \pm 4.8\%$) were the most virulent against mealworm. The most contrasting mutant lines concerning recovery (M31 and M88) were both low in virulence (22 ± 8 and $35.2 \pm 16.3\%$, respectively). Significant differences in virulence were restricted contrasting lines.

Mutant and reference lines were exposed to oxidative stress (70 mM H_2O_2) at 25 °C for calculation of their MTS_{50} . The differences in oxidative stress resistance between mutant lines were significant (Kruskal–Wallis test; $P < 0.01$, $n = 18$). The lines M60 (9.3 ± 3 h), M14 (21.9 ± 9.7 h), M47 (23 ± 8.1 h), and M62 (23.5 ± 9.8 h) had the shortest MTS_{50} and were significantly different to longer living mutants lines as for example M19 (150.8 ± 68.7 h), M4 (146.7 ± 69.1 h), and M86 (144.9 ± 63.7 h).

In both, DJ virulence and DJ oxidative stress resistant experiments, the heritability is high ($h^2 \geq 0.9$). An overview of the experiments is presented in Fig. 5.

Fig. 4 Average DJ recovery (%) of *Heterorhabdus bacteriophora* mutant lines incubated in *Photorhabdus laumondii* (strain DE2) supernatant produced in LM medium and heritability (h^2) of the phenotype (A). DJ recovery in the reference lines HB4 and IL3 incubated in LM supernatant and Ringer's solution (B). Average DJ recovery of liquid culture selected (1–103) and unselected (104–168) lines (C). The DJ recovery was assessed 72 h after supernatant exposure. The assessment was repeated four times, each with four replicates. Asterisks (*) over the bars denote significant differences between treatments (* $P < 0.05$; ** $P < 0.01$). Error bars depict the SE (A and B) or SD (C). The heritability (h^2) is provided on top of panel A. The mutant lines in A marked with an arrow (→) were previously phenotyped for DJ recovery in LB and LM supernatant (Fig. 3) and for the assessment of virulence and longevity (Figs. 5 and 6)



Correlation between phenotypes of beneficial traits

Comparing each independent experiments of mutant *H. bacteriophora* (Fig. 6), the phenotype performance differs between different lines, and the high calculated heritability ($h^2 \geq 0.85$, Figs. 3 and 5) clarified major variation in each phenotypic trait is due to genetic variation between lines. While no mutant line was observed to show a combined high performance in DJ recovery, virulence, and survival.

Throughout the analyzed mutant subset, DJ survival (MTS_{50}) resulted significantly positive correlated with DJ virulence, whereas DJ recovery resulted negatively correlated with virulence and survival, respectively (Fig. 6). A principal component analysis (PCA) was performed with each individual *H. bacteriophora* line to reveal the phenotypic differences among these populations. After *K*-means clustering and PCA plotting, the PC1 resulted to explain most of the phenotypic variance (72.6 %). Plotted results are

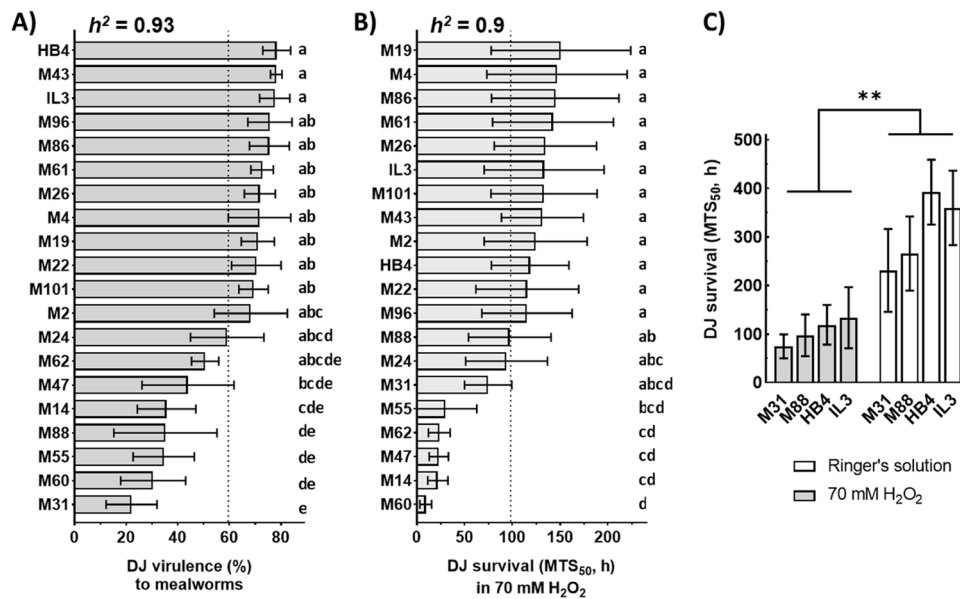


Fig. 5 Phenotypic variability in beneficial traits among subsets of *Heterorhabditis bacteriophora* 18 mutant inbred lines. Average virulence (%) to mealworms (*Tenebrio molitor*) with a dose of 15 DJ per insect (A). Mealworms were incubated at 25 °C for 7 days after nematode inoculation. DJ survival (mean time survived by 50% of the population, MTS₅₀) at 25 °C under oxidative stress (70 mM H₂O₂) (B). Different letters behind the bars (A and B) denote significant dif-

ferences. Reference DJ survival under oxidative stress and in Ringer's solution (C). Asterisks (*) over the bars (C) denote significant differences (**P < 0.01). The DJ virulence and survival experiments were repeated three and four different times, respectively, each with four replicates. Error bars depict the SD. The heritability (h^2) is provided on top of the panels A and B

shown in Fig. 6C, indicating a clear separation influenced by a significantly higher DJ survival (unpaired *t*-test; $t = 10.4$, $df = 18$, $P < 0.01$) and virulence (unpaired *t*-test; $t = 5.1$, $df = 18$, $P < 0.01$) in the depicted lines (blue group). In contrast, a second group (red) is composed by lines that are better in DJ recovery (unpaired *t*-test; LB supernatant, $t = 10.4$, $df = 6$, $P < 0.01$; LM supernatant, $t = 3.1$, $df = 6$, $P < 0.01$). The Eigen vectors from PC1 concerning DJ survival, virulence, and DJ recovery in both, LM and LB media, have clear opposite directions (Fig. 6C).

Single nucleotide polymorphism in mutant lines

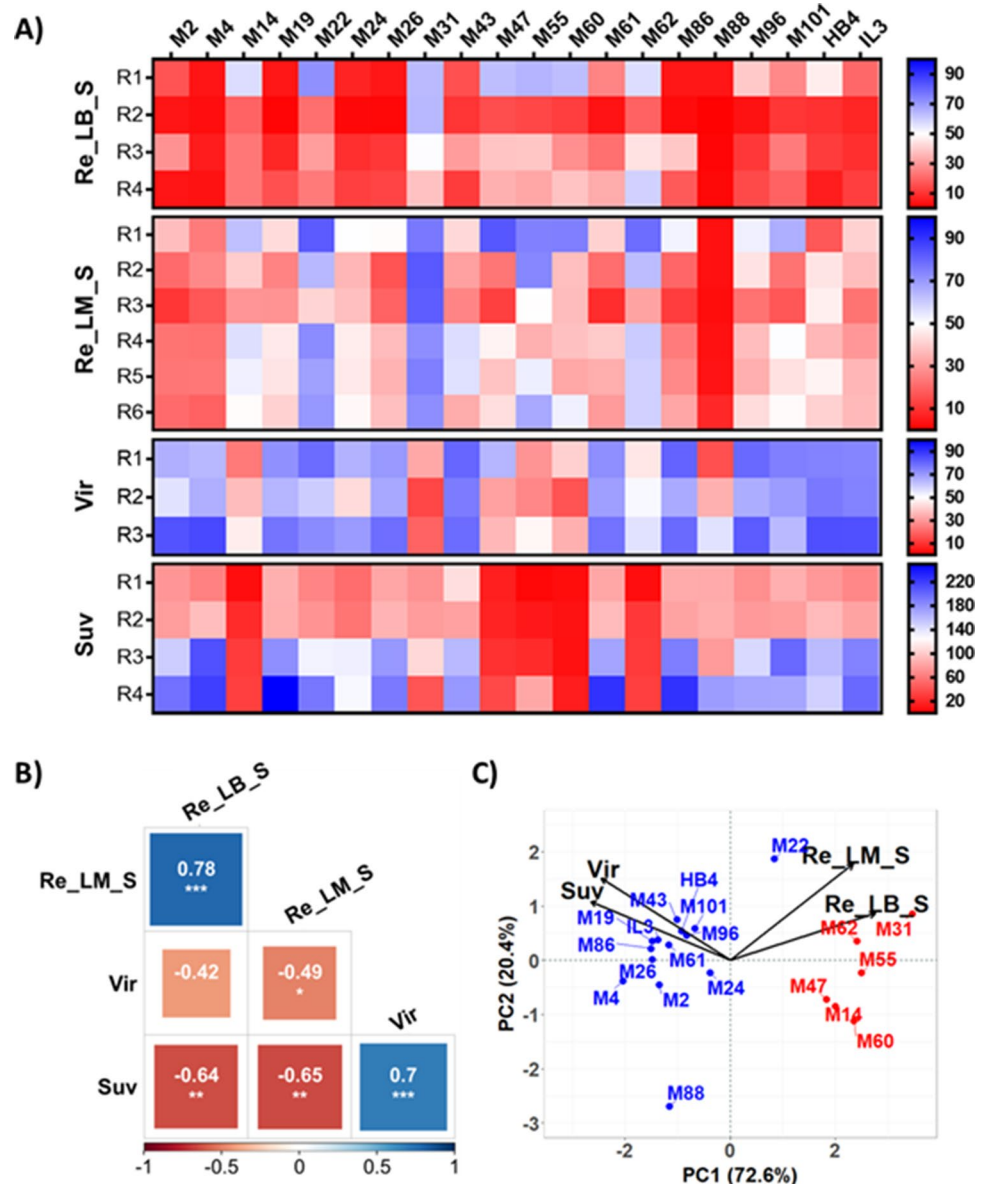
In a recent survey, Godina et al. (2022) identified more than 13,000 SNPs by the GBS approach. To identify SNPs in our mutant lines, the same approach of Godina and co-authors was carried out with a set of 96 mutant lines (M1-96). A total of ~ 0.5 million reads per DNA sample were obtained yielding 1606 SNPs. From these, 822 SNPs had a minimum of 8 read counts per sample and 195 and 157 high quality SNPs had minor allele frequency (MAF) of 5 and 10 %, respectively. An overview of the determined SNPs is shown in Table 1. The observed number of SNPs is fewer than the number found in WT ILs by Godina et al. 2022. Nevertheless, the determined SNPs can be used as markers considering (i) that EMS mutagenesis has been done with the single background of the IL3 line, and (ii)

that the present collection of mutant lines may behave as a segregating population due to the fact that the first generations after EMS mutagenesis were allowed to mate (Sumaya et al. 2018).

Phenotype-genotype association analysis

Association analysis between SNP genotype and DJ recovery phenotype was carried out per separate experiments with: (i) 18 mutant lines exposed to LB supernatant, (ii) 18 mutant lines exposed to LM supernatant, and (iii) 96 mutants exposed to LM supernatant. We assumed that a number of significant SNPs may be overlapping among these three groups. Of 20 SNPs associated ($P < 0.05$) with the DJ recovery trait, the SNPs N1696-4863, N1696-4871, N3144-589, and N3144-606 overlapped throughout all experimental groups (Table 1). Subsequently, we grouped the mutant lines by their alleles for each SNP, compared the recovery level between the allelic groups, and tested the differences for significance ($P < 0.05$). In all independent experiments, alleles in each significant SNP can separate mutant lines into two groups with different DJ recovery. For example, the mutant lines with allele G always had a significantly higher DJ recovery comparing with the allele T at SNP N3144-589 position. The four associated SNPs yielding significant differences between allelic groups are presented in Fig. 7.

Fig. 6 Relationship between DJ recovery (%) in LB and LM supernatant (Re_LB_S and Re_LM_S), survival under oxidative stress (Suv), and DJ virulence (Vir) of *Heterorhabditis bacteriophora* mutant inbred lines. The heatmap (A) indicates consistency of the data between repetitions of experiments. The four phenotyping experiments (Re_LB_S, Re_LM_S, Suv, and Vir) were repeated four, six, four, and three times, respectively, each time with four technical replicates. Correlation between DJ phenotypes (B). Principal component analysis (PCA) of DJ recovery in two supernatants, survival and virulence (C). Asterisks (*) denote significant correlations between phenotypes (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)



Heterorhabditis bacteriophora gene models neighboring significant SNPs

We searched for gene models neighboring the SNPs (N1696-4863, N1696-4871, N3144-589, and N3144-606) with the strong association to DJ recovery within the IL3 genome. We extracted the region 5000 pb up- and downstream the SNPs and screened for gene annotations according to McLean et al. (2018) within the region. Thereafter, we BLASTed the corresponding gene models against the Swissprot database (BLASTx). Two closely located gene models (g1799 and g1800) were found upstream from the SNPs N1696-4863 and N1696-4871, which are located on the *H. bacteriophora* IL3 genome

scaffold N1969 (full length 5214 bp). The two SNPs both have alleles C/G, mapping to positions 4863 and 4871, respectively. Gene model g1799 started from scaffold position 227, covers a genomic region of 2402 bp, and comprises of 11 predicted exons (coding region = 1430 bp). The predicted protein from this gene model has a partial homology (max. protein sequence identity = 100%) to Swissprot protein H2KZ60 (characterized as Contactin). This partial homology is observed along 7 out of the 11 predicted exons. In the model nematode *C. elegans*, this protein is homologous to the protein coded by the *rig-6* gene, which is involved mainly in the regulation of cell migration processes (Katidou et al. 2013). Additionally, gene model g1800 started from

Table 1 Single nucleotide polymorphisms (SNPs) determined in the *Heterorhabditis bacteriophora* mutant-line collection by genotyping by sequencing (GBS) (a) and the SNPs associated with DJ recovery of *Heterorhabditis bacteriophora* mutant lines in bacterial supernatants (b). For each polymorphism, the SNP identifier is provided according to the genome scaffold of the IL3 donor-line genome (unpublished). Additionally, the SNP identifiers are provided according to the position in the *H. bacteriophora* strain TT01 genome version PRJNA13977. WBPS16 deposited at Wormbase (<https://wormbase.org>). Statistical significance for the trait association is shown for three DJ recovery phenotypic traits in parallel. For each SNP, alleles are provided

Total mutant lines sequenced					96
Total reads per sample (millions)					0.5
Total number of SNPs across all samples					1606
Total SNPs with minimum read count of 8					822
Total fully covered SNPs in > 66% of samples, MAF > 5%, min reads > 8					195
Total fully covered SNPs in > 66% of samples, MAF > 10%, min reads > 8					157
SNP identifier (Scaffold location)	Alleles	Significance of associations			
IL3	TT01	18 mutants in LB supernatant	18 mutants in LM supernatant	96 mutants in LM supernatant	
N1532-6740	S115-9798	G/C	*		
N1696-4829	S266-321	T/C	*		
N1696-4837	S266-329	G/C	*		
N1696-4863	S266-355	C/G	*	**	*
N1696-4871	S266-363	G/C	*	**	*
N1816-238	S168-311	G/T			*
N1816-249	S168-322	C/A			**
N2456-741	S175-896	T/G			*
N3-24056	S1358-1862108	G/A			*
N3114-700	S1111-2564	A/T	*	*	
N3144-589	S81-38001	T/G	*	*	**
N3144-606	S81-38018	A/G	*	*	**
N3144-729	S81-38141	G/T			*
N3256-426	S1166-42444	T/C			*
N459-20060	S1298-181082	T/C	*	*	
N522-5922	S958-1117	C/T			*
N522-5935	S958-1130	C/T			*
N6550-49	S1220-11618	T/G			*
N7270-28	S1284-79	T/C	**	*	
N7270-65	S1284-116	G/A		*	**

The asterisks (*) and (**) depict the *P* value of associations or significant differences at 0.05 and 0.01 level, respectively

genome scaffold N1696 position 4553, covers a genomic region of 1768 bp, and comprises of 11 predicted exons (coding region = 743 bp). The predicted protein from this gene model has partial homology (max. protein sequence identity = 61.94%) to Swissprot protein Q21005 (characterized as acetylcholine-gated chloride channel subunit). This partial homology is observed along 3 out of the 7 predicted exons. In *C. elegans*, these proteins are homologous to the proteins coded by the *acc-1* gene, which is involved mainly in signal transduction processes (Putrenko et al. 2005). The other two SNPs N3144-589 and N3144-606 are located on scaffold N3144 position 589 and 606, respectively, but no gene model around these two SNPs over the genome scaffold were found. A visual representation of the SNPs and neighboring gene models is presented in Fig. 8 and main details about the gene models are depicted in Table 2.

Discussion

Entomopathogenic nematodes have already substituted several synthetic chemical insecticide applications in agriculture, horticulture, and forestry, particularly for the control of insect pests in soil environments (Grewal et al. 2005). A wider use of these biological control agents much depends on economically reasonable product costs. Using the economies of scale in mass production is one approach to reach this goal. The production will be even more effective if variable factors influencing DJ yields can be better understood and process conditions adapted accordingly (Ehlers 2001). The key to a successful liquid-culture production is the recovery of the inoculated DJ, which develop to hermaphrodites. The DJ yield consists of offspring produced by the self-fertilizing hermaphrodites. If recovery is low and less synchronous, the hermaphrodite

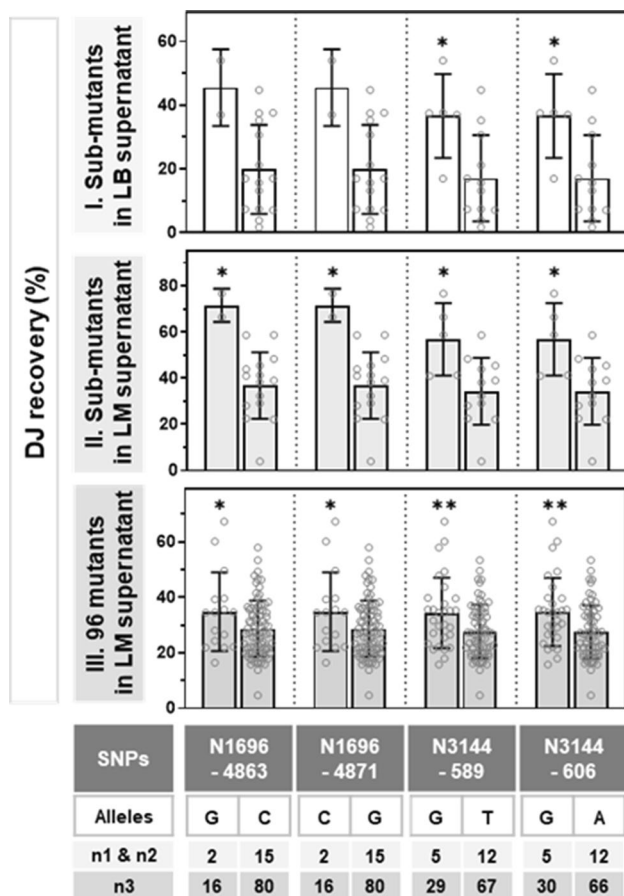


Fig. 7 DJ recovery (%) of single *Heterorhabditis bacteriophora* mutant inbred lines (small circles) and mean (bar with SD) grouped according to alleles associated to high or low recovery for 4 significant SNPs within a subset of 18 mutant lines assessed in LB supernatant (i), LM supernatant (ii), and within an extended set of 96 mutant lines in LM supernatant (iii). Numbers of lines per allele are denoted below each pair of alleles per SNP. The SNP identifiers are provided according to the position in *H. bacteriophora* strain IL3 reference genome. Major correspondence of scaffolds between the IL3 and the published TT01 genome (version PRJNA13977.WBPS16) is deposited in Table 1. Major details for the corresponding SNPs are deposited in Table 1. The asterisks (*) over the bars denote significant differences between different alleles (* $P < 0.05$; ** $P < 0.01$)

population is low, and instead of DJs, a large population of second-generation male and female adults develops, which are unable to copulate in liquid media. They die without reproducing and cause major problems during downstream processing. Hence, process technology would yield higher and more stable results should the recovery of inoculated DJs be more reproducible, higher, and synchronous (Ehlers 2007; Ehlers et al. 1998). This investigation was performed with the objective to contribute to a better understanding of DJ recovery and the relation of recovery with other beneficial traits like virulence and longevity. A large collection of WT and mutant inbred lines were screened for their DJ recovery potential and the heritability of this

trait to provide information for future hybridization and selection programs. The phenotypes were associated with genetic information to identify SNP markers for higher DJ recovery. A comparison with the *H. bacteriophora* genome checked whether the SNPs are related to the described gene models.

Phenotyping inbred lines offers the opportunity to calculate the heritability (h^2). Johnnigk et al. (2002) screened inbred lines of *H. bacteriophora* in liquid culture for DJ recovery and DJ yields. While the heritability for the DJ yield was 0.9, the h^2 for the DJ recovery was only 0.38. In this study, the h^2 of DJ recovery of WT ILs in liquid culture was a little higher (0.44), but the probability for success to improve DJ recovery by selection is not too high. More promising values were obtained (h^2 in LB = 0.85 and in LM = 0.94) using the culture supernatant of the bacterial symbiont (supplemented with an antibiotic) to trigger DJ recovery. This opens the opportunity to select for higher DJ recovery. Thus, the investigation provided a bioassay, which can be used in future approaches.

Opposite to the results of Johnnigk et al. (2002), who reported a heritability of 0.38 for DJ recovery, the h^2 of 0.85 was much higher for mutant inbred lines cultured in liquid culture. This is due to using a single inbred line (IL3) to produce mutant inbred lines, whereas Johnnigk et al. (2002) used a pool of hybrids produced from 12 crosses of 7 WT strains cultured in vivo. The genetic variability of the WT material was much higher than that of a pool of mutant inbred lines originating from a single donor line. The investigation provided evidence that mutagenesis is a feasible approach to improve traits in *H. bacteriophora*; the same has been reported recently by Sumaya et al. (2018) for DJ longevity (survival).

Another indication that selection can result in improved DJ recovery is provided by the comparison of mutant lines 1–103, which had first been cultured for production of inbred lines in liquid culture before continuous inbreeding using single hermaphrodites on NGG medium, with lines 104–168, which immediately were inbred on NGG. The recovery is significantly higher after the selection of lines in liquid culture (Fig. 4C).

Recovery in liquid culture is triggered by “food signals” released by the symbiotic bacteria. Aumann and Ehlers (2001) report that the signal is composed of at least two compounds of less than 20 kDa molecular mass. Whereas hardly any variability of DJ recovery in insects was recorded, a high variability in the duration and percentage is observed when DJs are exposed to the bacterial food signal. Unfortunately, only few individual signals triggering DJ recovery in an insect or the food signals produced by the symbiotic bacteria are characterized. However, the influence of some defined *Photorhabdus* elements on the support of nematode development and growth has been demonstrated by mutation approaches, as exemplarily reported for a *P. luminescens*

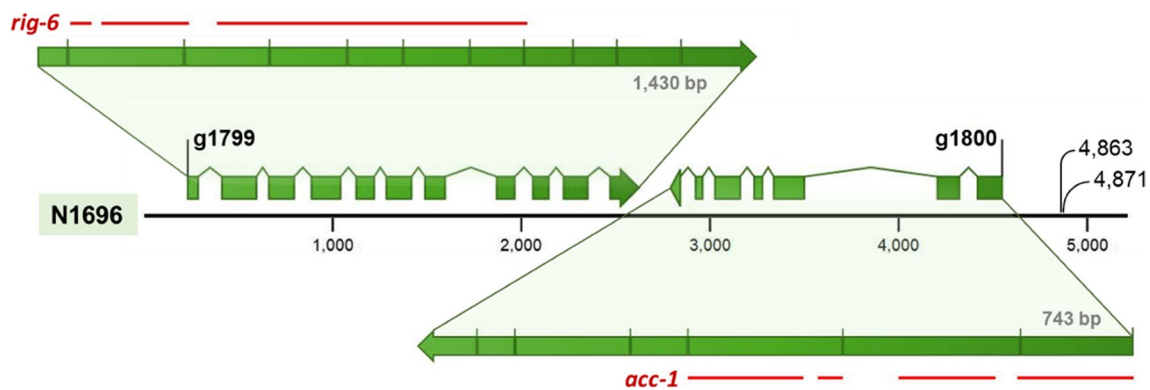


Fig. 8 *Heterorhabditis bacteriophora* gene models neighboring DJ recovery-associated SNPs. For SNP markers N1696-4863 and N1696-4871, significant association with DJ recovery in three independent phenotyping trials has been determined. SNP identifiers are given according to the genome scaffolds of the donor line IL3. Major correspondence of scaffolds between the IL3 and the published TT01 genome (version PRJNA13977.WBPS16) is deposited in Table 1.

The two SNPs are 8 bp from each other on scaffold N1696 (dark line) and are located in the vicinity of two predicted gene models (g1799 and g1800; green blocks and arrows) according to the annotations of McLean et al. (2018). The sequence homology of both gene models (green arrows) to Swissprot- characterized proteins (red lines) is depicted. SNPs and gene model positions are denoted in base pairs (bp) from the origin of the scaffold

Table 2 Gene models in the vicinity of the *Heterorhabditis bacteriophora* SNPs N1696-4863 and N1696-4871. Gene models are based on the annotation of the latest *H. bacteriophora* (strain TT01) genome draft (PRJNA13977.WBPS16) from McLean et al. (2018).

SNP names refer to the designated letter deposited in Fig. 7. Gene names of high-homology genes (after BLASTx at NCBI) from *Caenorhabditis elegans* are provided and the assigned gene ontology biological process

Gene models	Homologous proteins in database Swiss-Prot sequences (Swissprot)				
	Protein name	Protein ID	Gene name	Gene ontology (GO)	Reference
g1799	Contactin rig-6	H2KZ60	<i>rig-6</i>	Cell migration	Katidou et al. (2013)
g1800	Acetylcholine-gated chloride channel subunit acc-1	Q21005	<i>acc-1</i>	Signal transmission	Putrenko et al. (2005)

transposon-induced mutant defective for the *Escherichia coli* EntD homolog gene (Ciche et al. 2001). Also, further works have dissected global pathways for biosynthesis of *Photorhabdus* spp. natural products and detected DJ recovery-influencing compounds like stilbenes (Shi and Bode 2018). To improve DJ recovery in liquid culture, the information on the bacterial food signals would provide important progress to further improve the process stability of liquid-culture production. Knowledge on the insect's food signals could also contribute to a better process technology. Unfortunately, no information is available on the character of the different food signals. The food signal is one source of variability but an even more important reason for variability seems to be the readiness of different DJ batches to react to the bacterial food signal. How large is the influence from the bacterial side and from the nematode side in initiating the DJ recovery process? In the present study, we aimed to dissect the nematode genetic component in DJ recovery, independent from the effects derived from the bacterial cultivation. Two media (LB and LM) were used for the culture of the bacteria and the preparation of the supernatant. The

DJ recovery in both supernatants was significantly positive correlated ($R = 0.78$); however, DJ recovery in LM supernatant was higher than in LB supernatant, indicating that *P. laumondii* growth in LM medium is producing a higher concentration of the food signal or even a different composition. Concerning media components, the LM medium is higher in nitrogen (yeast and soy powder) and carbon sources (vegetable oil), providing more nutrition for bacteria growth. On the other hand, the tested lines had a robust reproducible recovery phenotype. It was possible to distinguish between high- and low-recovering lines, independently from effects derived by the *Photorhabdus* production medium. In addition, a high value for the heritability of DJ recovery indicates that the differences between lines must be associated to genotypic diversity.

Compared to WT ILs DJ recovery ranging from 1.6–23.6%, in LM supernatant, the mutant lines significantly broaden the spectrum of genotypic variability (3.3–76.6%). But what is the reason for an increase in percentage of DJ recovery? The binding to amphid chemical receptors in the model nematode *Caenorhabditis elegans* is the first step

in the dauer entry/exit process inducing a series of major signaling pathways (e.g. TGF- β & IIS) (Bargmann 1998; Fielenbach and Antebi 2008; Hilliard et al. 2002). Also, the binding to amphid chemical receptors may trigger subordinate signaling pathways as for example muscarinic signaling (Tissenbaum et al. 2000). Aumann and Ehlers (2001) observed that n-hexane and chloroform eluates of the bacterial supernatant were biologically inactive, suggesting the presence of a molecule acting antagonistically to the recovery-inducing signal. Such a signal indicates the exhaustion of nutritive resources, induces DJ formation, and inhibits DJ recovery. Mutagenesis, using EMS causes G:C to A:T transitions induced by guanine alkylation (Sega 1984). Considering that certain functions may be destroyed by these point mutations, it can be speculated that a pathway responsible for signal transduction of the antagonistic molecule in the supernatant might have been affected, thus increasing the effect of the food signal.

Besides DJ recovery, two other important beneficial traits were also phenotyped, yielding results on virulence and longevity. No single mutant line combined a good performance in all three traits. DJ survival and virulence were positively correlated, while DJ recovery was negatively correlated with both, virulence and longevity. Undoubtedly, this will cause serious difficulties for EPN breeding if the antagonism is ubiquitously distributed among EPN populations. However, of the four lines that are high in recovery in LM supernatant, two (M22 and M62) are also high in virulence, and M22 is also reasonably high in longevity under oxidative stress. These materials may be further considered for a breeding program to enhance in vitro DJ recovery, but not before they have been further characterized for stability of other beneficial traits.

Single nucleotide polymorphisms (SNP) are the simplest form of DNA variation and have been widely applied on the breeding and characterization of *C. elegans*, other animals, and plants (Davis et al. 2005; Dekkers 2012; Mammadov et al. 2012). Recently, Levy et al. (2020) and Godina et al. (2022) reported the versatility of the GBS technique for the discovery of SNPs in *H. bacteriophora*. Concerning the number of discovered SNPs, our results are modest in comparison to the previous reports (~ 150 vs ~ 4,800 SNPs). These differences may be explained by the nature of the materials used (closely related mutant lines vs WT lines). Apart from C-T substitutions resulting in C/G to T/A transitions, our genotyping results indicate that also less expected polymorphisms such as transversions (C/G–C/G; A/T–T/A) by 7-ethylguanine hydrolysis or A/T–G/C transitions by 3-ethyladenine pairing errors can be also found after EMS mutagenesis, as reported by Serrat et al. (2014) in plants (and references therein). Based on the present set

of polymorphisms, we have detected 80 and 20% transitions and transversions, respectively (data not shown). Since GBS sequencing covers specific regions in the genome (GBS clusters), we cannot provide yet a total estimate of the proportions of the different types of substitutions (beyond the scope of the present work). Future genome re-sequencing approaches can complement this work to determine the overall polymorphism load of the present mutant lines set.

Among the observed genes, homologues to the *C. elegans*, genes *rig-6* and *acc-1* are shown to be related to signal transmission and neuron physiology (Katidou et al. 2013; Putrenko et al. 2005). This small selection of genes deserves focus in upcoming works elucidating their function in EPN. The main factors determining the *C. elegans* dauer formation involve sensory and dietary cues and there are already well-defined regulatory pathways explaining the regulation of dauer entry and exit (Allen et al. 2015; Fielenbach and Antebi 2008). Genes situated close to our associated SNPs are not directly involved in these pathways. These results suggest that (i) our SNPs may not be directly related to functional factors, and therefore, may be regarded merely as molecular markers, and (ii) to date, uncharacterized genes may complement the pathways defined for the model worm *C. elegans*.

A total of 168 *H. bacteriophora* mutant lines were tested and 96 of them were genotyped. The association analysis of geno- and phenotype data found that significant SNP markers can distinguish the DJ recovery by its alleles. These markers have the potential to be genetic-based selection tools for DJ recovery in *H. bacteriophora*. Simultaneously, we phenotyped and analyzed DJ recovery in a smaller sample size (18 mutant and 2 commercial lines) and found that the association results from different sample sizes are not completely the same but overlapping. Furthermore, the strong SNP markers (N1696-4863, N1696-4871, N3144-589, and N3144-606) were consistently found. Two contrasting recovery lines (M31 and M88) sharing the same IL3 background are strong candidates for further studies involving genome-wide expression profiling. The availability of closely related materials with very contrasting phenotypic differences allows ruling out factors related to large genetic differences and helps to focus on the involvement of specific genes. In general, the pheno- and genotyping and the association analysis of *H. bacteriophora* can eventually help EPN beneficial trait improvement, increase the in vitro biotechnical production process, and support the enhancement of the biological control efficiency. Additionally, this generated platform of more than 160 mutant lines is as well a versatile tool for the investigation of many further traits of interest in EPNs.

Author contributions RUE and CM conceived the experiments and the project. CM and ZW designed the experiments. All authors performed the experiments. ZW and CO analyzed the data. ZW wrote the first draft of the manuscript, CM, and RUE read and commented on the previous versions of the manuscript. All authors approved the manuscript.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate This study does not contain any studies with human participants or vertebrate animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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Annex 2: Publication 2

Enhancing mass production of *Heterorhabditis bacteriophora*: Influence of different bacterial symbionts (*Photorhabdus* spp.) and inoculum age on Dauer Juvenile recovery

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Enhancing mass production of *Heterorhabditis bacteriophora*: influence of different bacterial symbionts (*Photorhabdus* spp.) and inoculum age on dauer juvenile recovery

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Abstract

The entomopathogenic nematode *Heterorhabditis bacteriophora* (Nematoda: Rhabditidae) is used in biological insect control. Their dauer juveniles (DJs) are free-living and developmentally arrested, invading host insects. They carry cells of their bacterial symbiont *Photorhabdus* spp. in the intestine. Once inside the insect's hemolymph the DJs perceive a food signal, triggering them to exit the DJ stage and regurgitate the *Photorhabdus* cells into the insect's haemocoel, which kill the host and later provide essential nutrients for nematode reproduction. The exit from the DJ stage is called "recovery". For commercial pest control, nematodes are industrially produced in monoxenic liquid cultures. Artificial media are incubated with *Photorhabdus* before DJs are added. In absence of the insect's food signal, DJs depend on unknown bacterial food signals to trigger exit of the DJ stage. A synchronized and high DJ recovery determines the success of the industrial in vitro production and can significantly vary between nematode strains, inbred lines and mutants. In this study, fourteen bacterial strains from *H. bacteriophora* were isolated and identified as *P. laumondii*, *P. kayaii* and *P. thracensis*. Although the influence of bacterial supernatants on the DJ recovery of three inbred lines and two mutants differed significantly, the bacterial impact on recovery has a subordinate role whereas nematode factors have a superior influence. Recovery of inbred lines decreased with age of the DJs. One mutant (M31) had very high recovery in bacterial supernatant and spontaneous recovery in Ringer solution. Another mutant (M88) was recovery defective.

Keywords Biocontrol nematode · Dauer juvenile · In vitro production · *Photorhabdus* spp. · Influence on dauer recovery · Dauer age

Introduction

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (Nematoda: Rhabditidae) is commercially mass produced in liquid culture industrial scale bioreactors (Ehlers 2001). These safe biocontrol agents (Ehlers 2003) are widely used in pest management to substitute synthetic insecticides, mainly of soil-dwelling insect larvae (Grewal et al. 2005; Lacey et al. 2015). The nematode is symbiotically associated with bacteria of the genus *Photorhabdus* (Gammaproteobacteria: Morganellaceae) (Adeolu et al. 2016). The third nematode stage, the dauer juvenile (DJ) of EPN is free-living, non-feeding and packed with fat reserves, providing it with energy for a long-term survival in the soil. It is developmentally arrested, carrying cells of its bacterial symbiont in the gut. The DJs search for suitable hosts and once inside the hemolymph of an insect, the DJs perceive a

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yet unknown food signal, which induces the exit of the dauer stage and the development to self-fertilizing hermaphrodites. This process is called DJ recovery. During recovery the DJs regurgitate cells of their symbiont into the hemocoel of its host. Both partners overcome the insect's immune response, after 1–2 days the bacteria proliferate and kill the host by septicaemia. The bacterial cells act as nutritional source for the nematodes (Han and Ehlers 2000), which produce offspring. Once the cadaver is consumed, starvation induces the formation of dauers, which leave the host on search for new victims.

The nematode-bacterium interaction is obligatory. The bacteria rely on the nematode for the transmission between insect hosts, and the nematode requires the bacterial symbiont to kill the insect host and to develop and reproduce. Bacteria-free nematodes are non-pathogenic (Han and Ehlers 2000).

Different to the in vivo life cycle, the commercial mass production of EPNs starts with a *Photorhabdus* pre-culture for minimum 24 h. Afterwards DJs are inoculated (Ehlers 2001). Under these conditions, the DJ recovery is triggered by a food signal emitted by their bacteria (Strauch and Ehlers 1998). However, the recovery in bacterial cultures is lower than inside an insect. Whereas almost 100% of DJs recover within a day inside an insect, the recovery in in vitro culture is highly variable and often scattered over a period of 2–5 days resulting in a non-synchronous population development causing lower yields and occurrence of a second generation of males and females, which are unable to mate in liquid culture, thus not producing offspring (Dolan et al. 2002; Han et al. 2000; Strauch and Ehlers 1998). During industrial production the liquid culture can undergo two scenarios. Either the recovery of inoculated DJs is high and synchronized, leading to high DJ yields at the end of the process, with very few non-desired stages such as males, unfertilized females and small juveniles (optimal process) or recovery is low, resulting in many second-generation adults and low DJ yields (suboptimal process). Thus, the rapid, synchronized and high DJ recovery is the key to a successful production of a high DJ yield of good quality (Johnigk et al. 2004).

For many years DJ recovery was less of a problem as the commercial strain recovered well due to selection for high recovery over many years of continuous subculturing. Since *H. bacteriophora* was subjected to intensive breeding activities targeting the improvement of beneficial traits, like virulence, longevity and stress-resistance (e.g. Godina et al. 2023; Mukuka et al. 2010; Sumaya et al. 2017, 2018), superior hybrids are now considered for commercialisation. However, their variable DJ recovery presents a major obstacle during industrial production. Therefore Wang et al. (2023) phenotyped the recovery of mutant inbred lines and wild types (WT) of *H. bacteriophora* by exposing DJs to symbiont culture supernatants of strain *P. laumondii* (DE2).

A large variability of DJ recovery was observed (2% up to 90%) and nematode DNA polymorphisms with potential association to the DJ recovery trait were identified. The results emphasized the relative low DJ recovery in WTs in comparison to the commercial line. Thus, research is crucial to further elucidate factors influencing DJ recovery.

Concerning specificity of *H. bacteriophora* to different *Photorhabdus* spp., the nematode appears to be relatively flexible. Until today, ten *Photorhabdus* species and subspecies have been isolated from *H. bacteriophora*: *P. cinerea* (Kazimierczak et al. 2017), *P. kayaii*, *P. thracensis* (Hazir et al. 2004), *P. laumondii* subsp. *laumondii* and *clarkei* and *P. luminescens* subsp. *luminescens* (Saux et al. 1999), *P. caribbeanensis*, *P. khaini* subsp. *khaini*, *P. stackebrandtii* (Tailliez et al. 2010) and *kleinii* (Machado et al. 2018). Whether this flexibility concerning the bacterial association can bring advantages to the nematode-bacteria complex, has already been demonstrated for nematode virulence. For instance, Machado et al. (2020) improved virulence of *H. bacteriophora* to western corn rootworm (WCR) by increasing benzoxazinoid resistance of *Photorhabdus* through bacterial symbiont engineering. Concerning bacterial natural products, secondary metabolites have been linked to traits like insect pathogenicity, protection of the insect cadaver and support of nematode development (Shi and Bode 2018). Several conservative biosynthetic gene clusters for natural products have been identified from *Photorhabdus* spp. (Shi et al. 2022). Concerning DJ recovery in *H. bacteriophora*, two compounds of less than 20 kDa (non-identified) were reported to influence this recovery (Aumann and Ehlers 2001). Joyce et al. (2008) and Wang et al. (2022) reported that stilbenes produced by *Photorhabdus* trigger *H. bacteriophora* DJ recovery, however, the compounds were not sufficient in absence of bacteria cells or supernatant. Thus, the bacterial food signal appears to be of complex composition and individual elements may not fully trigger DJ recovery. To stabilize the industrial production of novel *H. bacteriophora* strains and lines, it is crucial to determine whether certain WT *Photorhabdus* strains possess a better potential to support DJ recovery.

Up to date there is no report concerning phenotypic variability related to different *Photorhabdus* strains causing DJ recovery. In this study, we (i) isolated fourteen *Photorhabdus* bacterial strains from previously phenotyped nematode WT strains, (ii) we identified the strains to species level based on 16 S rRNA and four housekeeping genes, (iii) we produced sterile bacterial supernatants and tested five previously phenotyped *H. bacteriophora* lines/strains included in the work of Wang et al. (2023) for DJ recovery upon exposure to supernatants, (iv) we tested whether DJ age (storage in 4 °C) influences the DJ recovery and (v) tested whether DJ recovery can be influenced by potential supernatant volatiles.

Materials and methods

Cultivation of *Heterorhabditis bacteriophora* lines

Five *H. bacteriophora* lines were used in this study, including two commercial lines (EN01 and HB4), the inbred line IL3 selected from EN01 (Sumaya et al. 2018) and two mutant inbred lines M31 & M88. The mutant lines derived from donor line IL3 and have been phenotyped for their DJ recovery (Wang et al. 2023). DJs of each line were grown in monoxenic liquid cultures established by egg isolation and surface-sterilization (Lunau et al. 1993), and subsequent incubation in Wouts agar medium (16 g Bacto nutrient broth, 12 g Bacto agar, 5 g corn-oil or sunflower-oil, 1 L distilled water). Wouts agar plates were transferred after ~14 days into 24 h liquid cultures of *P. laumondii* (strain DE2) into LM medium (15 g yeast extract, 20 g soy powder, 4 g NaCl, 0.35 g KCl, 0.15 g CaCl₂, 0.1 g MgSO₄, 6 g lecithin diluted in same volume of rapeseed oil, 40 g rapeseed oil, 1 L distilled water, pH 7 ± 0.1) and cultured on a rotatory shaker (180 rpm, rotation diam. 4 cm) (Ehlers et al. 1998; Hirao and Ehlers 2010). Subcultures were carried out by the same procedure, inoculating DJs at a final density of 4000 DJs mL⁻¹ using cultures of previous generations. To check for monoxenic conditions and excluding contaminated cultures, samples were streaked on NBTA agar (10 g Bacto tryptic soy broth, 0.025 g bromothymolblue sodium salt, 14 g Bacto agar, 1 L distilled water, 4 mL of 1% sterile filtered 2,3,5-triphenyltetrazoliumchloride) (Akhurst 1980). DJs were harvested by pouring cultures on cotton traps and cleaned by vacuum sieved.

Ringer's solution (9 g NaCl, 0.42 g KCl, 0.37 g CaCl₂ × 2 H₂O, 0.2 g NaHCO₃, 1 L distilled water) was used to store DJs at 4 °C.

Isolation of bacteria from WT strains

Symbiotic *Photorhabdus* bacteria were isolated from different *H. bacteriophora* strains (Table 1). DJs from each line/strain were used to infect last instar wax moth larvae (*Galleria mellonella*) at a dose of 50 DJs per insect. After 24 h incubation at 25 °C, the insect larvae were surface sterilized with 70% ethanol for 20 s, and a drop of insect haemolymph was taken by piercing the hind leg of the insect. The protruding drop of hemolymph was streaked on NBTA agar plates, which were then incubated at 25 °C for 48 h. Then single bacterial colonies were selected and re-streaked on NBTA several times until pure colonies were obtained (Ehlers et al. 1991). Subsequently, single colonies were picked from NBTA and grown in LM medium for 24 to 48 h at 25 °C on a rotatory shaker (180 rpm, rotation diam. 4 cm). Thereafter, *Photorhabdus* culture stocks were preserved in glycerol 15% (v/v) at – 80 °C. Aliquots of the bacterial cultures were used for DNA isolation and subsequent species determination. The *Photorhabdus* strains in this study are available at e-nema GmbH upon request and have been submitted to Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

Bacterial supernatant production

Bacterial supernatants were produced from each of the isolated bacteria, as well as from *P. laumondii* strain DE2 as

Table 1 *Heterorhabditis bacteriophora* strains/lines and nematode symbiont *Heterorhabditis bacteriophora* strains/lines used and NCBI accession numbers for bacteria

<i>H. bacteriophora</i>			Symbiotic <i>Photorhabdus</i>		
Strain/line	Geographic origin	Source	Determined species	Abbreviation (strain)	16 S rRNA accession
DE6	Germany	Wild type	<i>P. kayaii</i>	P.k (DE6)	OR350573
HU2	Hungary	Wild type	<i>P. kayaii</i>	P.k (HU2)	OR350579
AU1	Australia	Wild type	<i>P. laumondii</i>	P.l (AU1)	OR350580
DE2	Germany	Wild type	<i>P. laumondii</i>	P.l (DE2)	OR350581
DE8	Germany	Wild type	<i>P. laumondii</i>	P.l (DE8)	OR350582
IL3	–	Lab selected	<i>P. laumondii</i>	P.l (HB1)	OR350583
HY1	–	Lab hybrid	<i>P. laumondii</i>	P.l (HY1)	OR350584
IT4	Italy	Wild type	<i>P. laumondii</i>	P.l (IT4)	OR350585
S-VI-MM14	–	Lab selected	<i>P. laumondii</i>	P.l (MM14)	OR350586
S-VI-MM8	–	Lab selected	<i>P. laumondii</i>	P.l (MM8)	OR350574
PT2	Portugal	Wild type	<i>P. laumondii</i>	P.l (PT2)	OR350575
S-CR1	–	Lab selected	<i>P. laumondii</i>	P.l (S-CR1)	OR350576
XX2	South Africa	Wild type	<i>P. laumondii</i>	P.l (XX2)	OR350577
PT1	Portugal	Wild type	<i>P. thracensis</i>	P.t (PT1)	OR350578

reference. Bacterial cultures were carried out in LM medium (50 mL media in a 250 mL volume flask) for 24 h. Subsequently the cultures were centrifuged at 10,000 rpm at 4 °C for 10 min. Resulting supernatants were sterilized by passing the liquid through 5 µm and then 0.2 µm pore size filters and stored at − 80 °C until used (Wang et al. 2023). The presence of contaminants was tested by inoculation of samples on NBTA plates.

Bacterial species identification

Extraction of DNA was done with the PureLink™ Microbiome DNA purification kit (Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions. DNA quantity and quality was assessed with a Nanodrop™ Lite Spectrophotometer (Thermo Fisher Scientific, MA, USA) and diluted to 5 ng µL^{−1} for PCR amplification. The 16 S rRNA and four housekeeping genes, viz. glutamyl tRNA synthetase (*gluX*), recombinase protein encoding gene (*recA*), beta subunit of DNA polymerase III holoenzyme encoding gene (*dnaN*), and DNA gyrase subunit encoding gene (*gyrB*) were selected for bacterial species identification by sequencing of PCR products. Primers used for the gene amplifications were published by (Sergeant et al. 2006; Tailliez et al. 2010) (Table 2). PCR reactions were carried out in 1× reaction buffer, 400 µM mM dNTPs, 7.5 pmol of each primer (forward and reverse), 25 mM MgCl₂, one unit of *Taq* DNA polymerase and 10 ng of template DNA (final volume 20 µL). PCR conditions were as follow: 94 °C for 2 min, 40 cycles of 94 °C for 20 s followed by 57 °C for 1.5 min and 72 °C for 1 min, and a final extension 72 °C for 3 min in a Gene Touch™ Thermocycler (Bioer, Hangzhou, China). The PCR products were separated by electrophoresis in a 1% TAE-agarose gel stained with Gel-Red™ staining (Biotuim, USA), gel-purified (QIAquick gel purification Kit, Qiagen) and sequenced (StarSeq GmbH, Mainz, Germany). The sequences were BLASTed against NCBI data (<https://www.ncbi.nlm.nih.gov/>) to search for the

highest homology with other *Photorhabdus* species accessions. In parallel, the sequences of the four housekeeping genes were aligned, trimmed and concatenated per separate into a single combined sequence (for each of the fourteen bacterial isolates). A large set of sequences from the same genes was retrieved from NCBI and was used as reference concatemers (Supplementary Table S1). All concatenated sequences were analysed by pairwise alignment using MUSCLE (Edgar 2004), followed by post alignment trimming with G-Blocks as implemented in SeaView 5.0 (Gouy et al. 2020). The TPM2uf + I + G & TIM1 + I + G model was selected as the best-fit model of DNA evolution for 16 S & housekeeping gene setups respectively using jModelTest2 (Darriba et al. 2012) according to the Akaike Information Criterion (AIC). Subsequently, phylogenetic trees were obtained using MrBayes 3.2.7a (Ronquist and Huelsenbeck 2003) at platform CIPRES Science Gateway (<https://www.phylo.org/>) (Miller et al. 2010). The 50% majority rule consensus phylogenetic trees were visualized using FigTree v. 1.4.4 (Rambaut 2018) and Inkscape (Bah 2011).

DJ recovery assay

Recovery of DJs was assessed based on morphological changes of the head region in cell wells under an inverted microscope (Zeiss, Germany) (Strauch and Ehlers 1998). DJs (100–200) of the nematode lines/strains EN01, HB4, IL3, M31 and M88 were inoculated in randomized arrangement into 24-cell-well plates, each cell filled with 200 µL bacterial supernatant containing 0.2% streptomycin sulphate. Controls were carried out in Ringer's solution. Recovery was evaluated three days after incubation at 25 °C by counting recovered and unrecovered DJs in each cell well. DJ recovery assays were carried out in four independent experiments, each with four replicates per each nematode line/strain and supernatant.

Table 2 Primers used for DNA amplification

Amplified fragments	Primers	Orientation	Sequence (5'-3')	References
16 S rRNA	16SP1	Fwd	GAAGAGTTGATCATGGCTC	Tailliez et al. (2010)
	16SP2	Rev	AAGGAGGTGATCCAGCCGCA	
<i>gluX</i>	gX1	Fwd	GCACCAAGTCCTACTGGCTA	
	gX2	Rev	GGCATRCCSACTTTACCCATA	
<i>dnaN</i>	dN1	Fwd	GAAATTYATCATTGAACGWW	
	dN2	Rev	CGCATWGGCATMACRAC	Sergeant et al. (2006)
	dN3	Rev	GTTRTTRCTGCCAATCTG	
<i>recA</i>	rA1	Fwd	CCAATGGGCCGTATTGTTGA	Newly synthesized
	rA2	Rev	TCATACGGATCTGGTTGATGAA	
<i>gyrB</i>	gB1	Rev	TAAGTAGAGTTCAGAGAGTGCC	
	gB2	Fwd	CATGCGGGGGGTAAATTC	

Influence of ageing on DJ recovery

Nematode DJs of the five strains/lines were stored in Ringer's solution at 4 °C for up to 60 days. The DJ recovery was evaluated periodically, and data was adjusted to a regression model. Experiments were repeated three times (in different batches of DJs), each with five evaluation time points and four replications for each time point.

Influence of volatiles from supernatants on DJ recovery

To test whether DJ recovery can be also induced by volatile substances released by the bacterial supernatant of *P. laumondii* (strain DE2), three different conditions were tested: (i) DJs in bacterial supernatant (SN), (ii) DJs in Ringer's solution in neighbouring cell wells with bacterial supernatant (RN), (iii) DJs in Ringer's not neighbouring supernatant cells (RS). The experimental setup is depicted in Fig. 1. DJs were stored less than a week before inoculation. From each of the variants (SN, RN, RS) four replicates were evaluated and four independent experiments were conducted.

Statistical analysis

The Shapiro–Wilk test was used to check for normal distribution and equal-variance of the data before analyses by parametric tests (two- and one-way ANOVA followed by Tukey's HSD test). Non-normally distributed or unequal-variance data were log-transformed or analysed by non-parametric tests (Kruskal–Wallis or Mann–Whitney U). The correlation between phenotypes was assessed by the Pearson correlation coefficient (normally distributed data) or Spearman's rank correlation coefficient (non-normal-distributed data). The DJ recovery was calculated as percentage of recovered DJs in each cell well. The recovery over storage time was modelled by n-parameter logistic regressions according to Commo and Bot (2016). All data

were collated and pre-treated in Microsoft Excel. Data normality & variances test, significance analysis and linear models were performed using GraphPad Prism 12.0 software (San Diego, California, USA). Correlations were assessed with R v4.2.3 in Rstudio v2023.3.0 (Boston, Massachusetts, USA) development environment. The R packages nplr v0.1.7, ggpubr v0.6.0 and ggplot2 v3.3.6 were used to implement the analysis.

Results

Species determination for native bacterial isolates

We isolated and identified the symbiotic bacteria from fourteen *H. bacteriophora* WT strains by analysis of sequences of the 16 S rRNA (1070 bp), *gltX* (1200 bp), *recA* (400 bp), *dnaN* (1065 bp) and *gyrB* (950 bp) genes. Concerning BLAST results, two isolates (DE6 and HU2) showed the highest homology to *P. kayaii* (percent identity (PI) > 96%), eleven isolates (AU1, DE2, DE8, HB1, HY1, IT4, MM14, MM8, PT2, SCR1, XX2) to *P. laumondii*, and one isolate (PT1) to *P. thracensis* NCBI accessions. An overview of the sequence identities of the BLAST hits is deposited in supplementary Table S2. Additionally, we carried out sequence pairwise comparisons of our strains and a set of identified *Photorhabdus* accessions for the given housekeeping genes. The local alignment results were congruent with our BLAST results. The eleven isolates showing homology to *P. laumondii* grouped in the same clade with accessions of this species. The expected grouping was also confirmed for the isolates with high BLAST homology to *P. kayaii* and *P. thracensis*. Phylogenetic trees showing the major groupings are provided in Fig. 2 (16 S rRNA) and Fig. 3 (*gltX*, *dnaN*, *recA* and *gyrB*). Each nematode strain carried a single *Photorhabdus* sp.

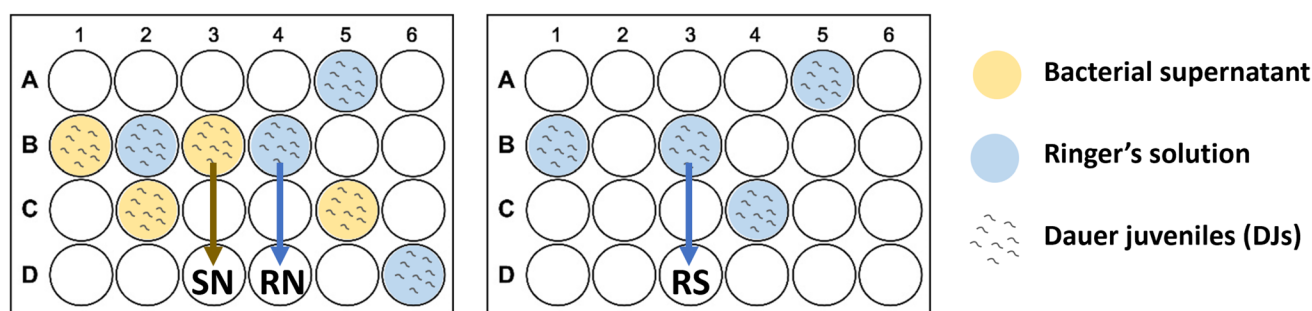


Fig. 1 Experimental setup for testing the influence of volatiles from bacterial supernatants of *Photorhabdus laumondii* (strain DE2) on *Heterorhabditis bacteriophora* DJ recovery. DJs were incubated in

bacterial supernatant (SN), Ringer's solution neighbouring wells with supernatant (RN) and in Ringer's solution separate from wells with supernatant (RS)

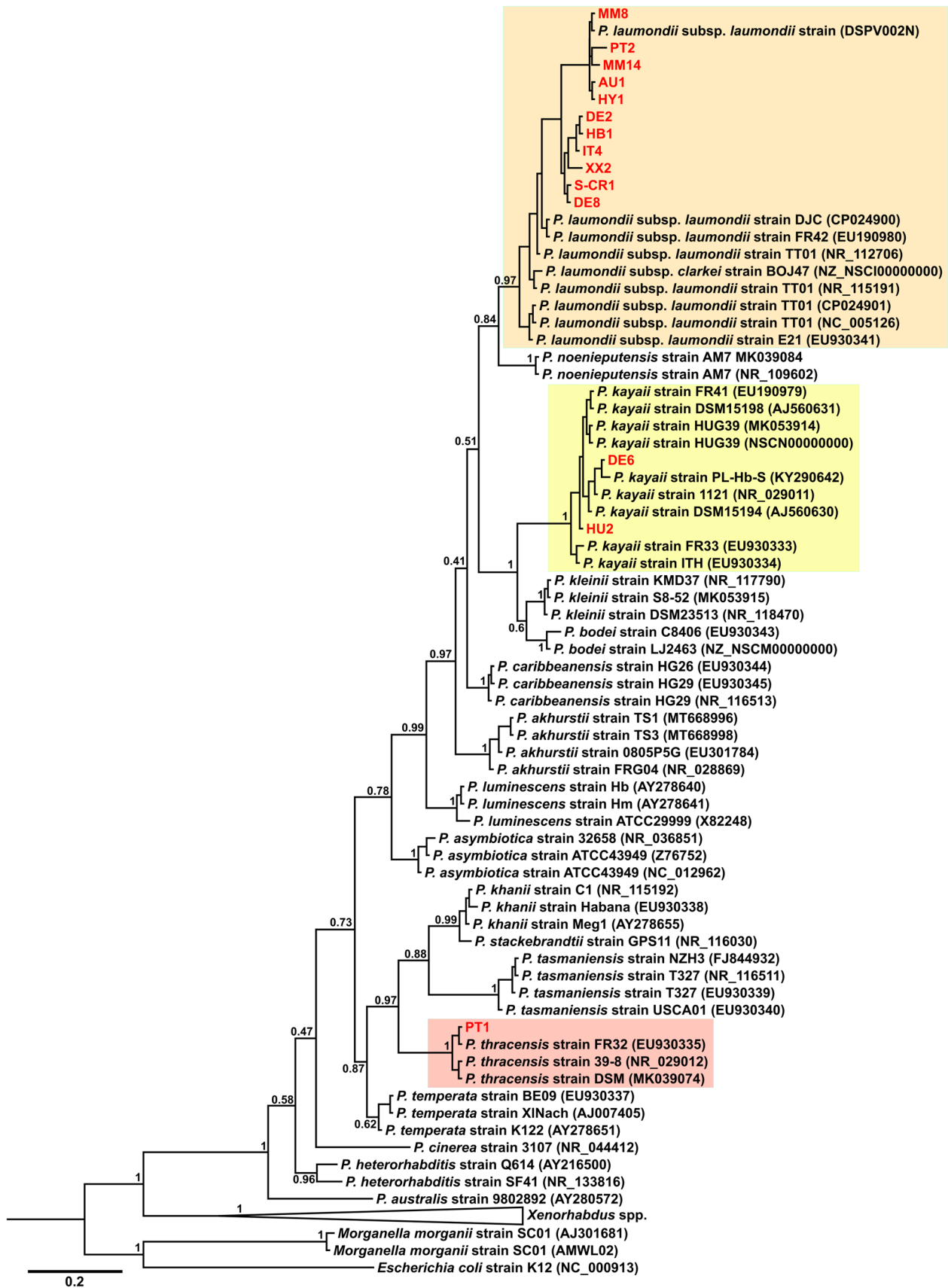


Fig. 2 Phylogenetic relationship of *Photorhabdus* spp. strains built on 16 S rRNA sequences. The sequences of nearly 1000 bp length were considered for the analysis. Posterior probability values indicate branch support based on Bayesian inference (BI) analysis. Bar: 0.2 nucleotide substitutions per sequence position. *Morganella morganii* strain SC01 and *Escherichia coli* strain K12 were used as outgroup. Fourteen new sequences generated in this study are highlighted in red. (Color figure online)

Influence of different bacterial supernatants on DJ recovery

Five *H. bacteriophora* strains/lines were exposed to supernatants of fourteen identified *Photorhabdus* sp. isolates to determine their potential to support DJ recovery. Significant differences were observed between the recovery of the nematode materials (Two-way ANOVA; $F = 535.1$; $df = 4, 210$; $P < 0.01$). The highest mean DJ recovery was recorded for the hybrid line EN01 with $86.6 \pm 7.7\%$ ranging between 69 and 97.7% and for the mutant M31 $82.1 \pm 6.7\%$ ranging between 69.8 and 92.4%. Mutant line M88 had the lowest mean recovery of $5.1 \pm 2.7\%$ ranging between 2.6 and 12.2% (Fig. 4). Within each *H. bacteriophora* material, significant differences were also determined for the recovery in different bacterial strain supernatants (Two-way ANOVA; $F_{\text{EN01}} = 46.4$, $F_{\text{HB4}} = 106.2$, $F_{\text{IL3}} = 61.2$, $F_{\text{M31}} = 25.9$, $F_{\text{M88}} = 28.6$; $df = 13, 168$; $P < 0.01$). The *P. laumondii* strain HY1 induced the highest DJ recovery in average throughout all nematode lines: EN01 ($97.7 \pm 2.5\%$), HB4 ($75.3 \pm 16.9\%$), IL3 ($60.8 \pm 17\%$), M31 ($92.4 \pm 5.4\%$) & M88 ($12.2 \pm 8.1\%$) (Fig. 4). *P. laumondii* strain S-CR1 supernatant was often the lowest in support of DJ recovery, e.g. in EN01 with $69 \pm 19.1\%$, HB4 with $30.2 \pm 8.9\%$ and M88 with $2.6 \pm 1.7\%$. Recovery in *P. laumondii* strain supernatants were generally higher than in supernatants of *P. kayaii* and *P. thracensis*. For the nematode lines IL3 and M31, the lowest recovery was observed upon exposure to *P. kayaii* strain HU2 supernatant (17.1 ± 17.5 and 69.8 ± 16 , respectively). The heritability of the trait DJ recovery in bacterial supernatant was $h^2 < 0.6$ despite the differences induced by the different bacterial supernatants. This indicates that the nematode-derived genetic background has a larger influence on DJ recovery, than factors originating from the symbiotic bacterium. However, the bacterial factor(s) can amplify or suppress the nematode intrinsic DJ recovery. An overview of the DJ recovery of different nematode materials in fourteen bacterial supernatants, as well as the correlation between the nematode phenotypes and variation between four independent experimental repeats is presented in Fig. 4.

A two-way ANOVA was performed to analyse the effect of *H. bacteriophora* lines and *Photorhabdus* strains on DJ recovery. As the ethyl-methane-sulfonate (EMS) mutant lines M31 and M88 represent two extremes in the recovery in bacterial supernatants, another analysis was performed

excluding the two mutant lines. Both analyses provide the same results. Significant differences were recorded between nematode strains, as well as between bacteria strains ($P < 0.01$), but a statistically significant interaction between the nematode and bacteria ($P > 0.05$) was not recorded. The main source of variation originates from *H. bacteriophora* whether the mutants were included or not (Table 3).

Influence of aging on DJ recovery

To find out whether the age (storage time) of the DJ inoculum has effects on their recovery, DJs of five *H. bacteriophora* materials were exposed to supernatant of *P. laumondii* strain DE2. The dynamics of recovery over the storage time followed different patterns. For the three lines EN01, HB4 and IL3, the highest percent recovery was reported at the early observation points (week 1 and 2), whereas the proportion of recovered DJs decreased with increasing storage time. For instance, DJ recovery of EN01 was $> 85\%$ within week 1 and 2, while it decreased down to $18.7 \pm 12.9\%$ after two months (week 8). Differences in DJ recovery over DJ storage time were significant (Two-way ANOVA; $df = 4, 45$; $P < 0.01$) for each of the lines EN01 ($F = 618.5$), HB4 ($F = 218.9$) and IL3 ($F = 470.1$) (Table 4). The DJ recovery of the mutant line M31 was stable over the observation time (79.6 ± 7.5 to $85.9 \pm 9.1\%$), while DJ recovery of line M88 was low over the complete experiment. While four of the nematode lines hardly recovery in Ringer's solution, the DJ recovery of line M31 increased over the DJs storage time and this difference in DJ recovery resulted significant (Two-way ANOVA; $F = 419.2$; $df = 4, 45$; $P < 0.01$). Both mutants have an abnormal reaction on bacterial supernatant. Whereas M31 seems to be recovery-constitutive, exiting the dauer stage even in Ringer's solution, mutant line M88 seems to be recovery-defective with recovery $< 5\%$ in supernatant.

Influence of volatiles from supernatants on DJ recovery

No evidence was found for an influence of volatiles from supernatants on DJ recovery, except for mutant M31. Whereas bacterial supernatant caused all nematode strains to recovery (except for recovery-defective mutant line M88), DJs in Ringer's solution neighbouring cell wells with supernatant did not recovery, and the differences resulted significant (Two-way ANOVA; $F_{\text{EN01}} = 3385$, $F_{\text{HB4}} = 2002$, $F_{\text{IL3}} = 1587$, $F_{\text{M31}} = 715.3$, $F_{\text{M88}} = 95.84$; $df = 2, 36$; $P < 0.01$). Comparing the recovery of DJs in two treatments without direct exposure to bacterial supernatant, no differences were determined between cell wells with or without adjacent wells containing supernatant, except for the recovery-constitutive mutant line M31 which was significantly higher in wells with neighbouring supernatant ($26.8 \pm 16.7\%$) compared to wells without adjacent wells containing supernatant



Fig. 3 Phylogenetic relationship of *Photorhabdus* spp. strains built from concatenated nucleotide sequences of four protein-coding genes (*gltX*, *dnaN*, *recA* and *gyrB*). The concatenated sequences of ~3000 bp length were considered for the analysis. Posterior probability values indicate branch support based on Bayesian inference

(BI) analysis. Bar: 0.1 nucleotide substitutions per sequence position. *Morganella morganii* strain SC01 and *Escherichia coli* strain K12 were used as outgroup. Fourteen new sequences generated in this study are highlighted in red. (Color figure online)

($18.4 \pm 10.7\%$) (Tukey's HSD test, $P < 0.01$) (Fig. 5). The present results confirm that the food signal is non-volatile.

Discussion

Prior to testing the impact of bacterial symbionts on DJ recovery, the strains isolated from the different *H. bacteriophora* nematodes were identified. Initially the 16 S rRNA gene sequence was considered reliable for species identification and phylogenetic analysis, however in some cases species discrimination was impaired. The multi-locus sequence analysis has therefore been proposed to enhance the resolution of sequence analysis for species identification (Gevers et al. 2005; Tailliez et al. 2010). The nucleotide sequences of the fourteen bacterial isolates resulted highly homologous ($> 96\%$) with both, 16 S rRNA and concatenated housekeeping gene sequences (*gltX*, *recA*, *dnaN* & *gyrB*) of *P. laumondii*, *P. kayaii* and *P. thracensis*. The resulting phylogenetic tree topology (Figs. 2 and 3) agrees with previous reports (Kazimierczak et al. 2017; Machado et al. 2018; Orozco et al. 2013; Sajnaga and Kazimierczak 2020). Of the ten *Photorhabdus* described species and subspecies isolated from the nematode *H. bacteriophora* we found three species.

In this study, *H. bacteriophora* strains/lines reacted differentially to diverse *Photorhabdus* supernatants. *P. laumondii* strains from HY1 & MM14 nematodes showed the highest DJ recovery along all lines contrasting with *P. thracensis* strain PT1, *P. laumondii* strain S-CR1 and *P. kayaii* strain HU2. Kazimierczak et al. (2017) reported that *Heterorhabditis* nematodes can swap their symbionts at intra- and interspecies level within the genus *Photorhabdus*. Variations along these nematode-bacterial complexes show that nematode performance can be also affected (Chapuis et al. 2009; Kazimierczak et al. 2017). Instead of swapping the bacteria, Machado et al. (2020) increased *Photorhabdus* benzoxazinoid resistance through experimental evolution, reintroduced engineered *Photorhabdus* into the native *H. bacteriophora* and finally enhanced nematode-symbiont virulence to western corn rootworm. We observed that even very related nematode strains can harbour symbionts conferring different phenotypes regarding DJ recovery. For instance, *P. laumondii* strains MM8 & MM14 were isolated from *H. bacteriophora* undergoing several passages through *Melolontha melolontha* (cockchafer white grub) for 8 and 14 generations, respectively (Berner et al. 2001). We found that DJ recovery upon exposure to bacterial supernatant of MM14 was always higher than in MM8 ($P < 0.05$, Fig. 4), regardless of the nematode line. Thus, whether nematode strains can adapt over generations to the natural product environment provided by the symbiont and modify their DJ recovery and reproductive programs is therefore a question that arises for future attempts for improvement. Considering

that until today we find 10 phylogenetically separated taxa of *Photorhabdus* associated with *H. bacteriophora*, the question arises about the major selection pressure causing the evolution of so many different bacterial symbionts. The support of DJ recovery is certainly not linked to specification in *Photorhabdus* spp. and subspecies, since the bacterial food signal is not involved in DJ recovery inside the insect upon arrival of the nematode in the haemolymph, stage in which the bacteria are not yet released (Ciche and Ensign 2003; Ebrahimi et al. 2011). For industrial mass production it is advisable to select for those symbionts that best trigger DJ recovery and promote high DJ yields.

Knowledge on the nature of the bacterial food signal might help to improve process conditions in biotechnology mass production. Several proteins and biosynthetic compounds have been reported to play an active role in regulating bacteria-nematode-insect interactions (Duchaud et al. 2003; Joyce et al. 2008; Shi and Bode 2018; Shi et al. 2022). Besides conserved core genes that are essential for basic biological functions, unique biosynthetic gene clusters within species also may play an important role for the association with the nematode. Six of the *Photorhabdus* strains analysed in the present study (DE6, HU2, DE2, IT4, HB1 & PT1) were also included in the large analysis reported by Shi et al. (2022). According to the authors, the strains differently synthesized natural products like saccharides and terpenes. Thus, the large contrasts in DJ recovery observed between *P. laumondii* isolates DE2, IT4, and HB1 may be related to strain-specific features in secondary metabolite clusters. However, whether any of these natural products contribute to DJ recovery needs further investigation.

For the same *H. bacteriophora* strain/line, the support of DJ recovery by different *Photorhabdus* strains is variable. But these differences are less pronounced than the differences between the three nematode lines EN01, HB4 and IL3. The analysis for variation clearly indicates that the instability is on the side of the nematode material. So once a supportive bacterial symbiont has been selected, further improvements must be made on the nematode side. This variability is also obvious when the results between the experiments one to four are compared. In EN01 and HB4 the results vary within experiments 1 and 2 and of the first two experiments compared to the last two. The future challenge and focus will be on the improvement of the nematode material. One approach, which already resulted in an improvement in DJ in vitro recovery, is manifested in strain EN01, which has been subcultured over several years leading to enhancement of recovery. The DJ phenotype of this strain may have adapted to the industrial production environment. Another solution is to cross strains with improved traits with EN01 to afterwards select lines which carry the DJ recovery traits of EN01. Single Nucleotide Polymorphisms (SNPs) associated to high DJ recovery have already been identified (Wang

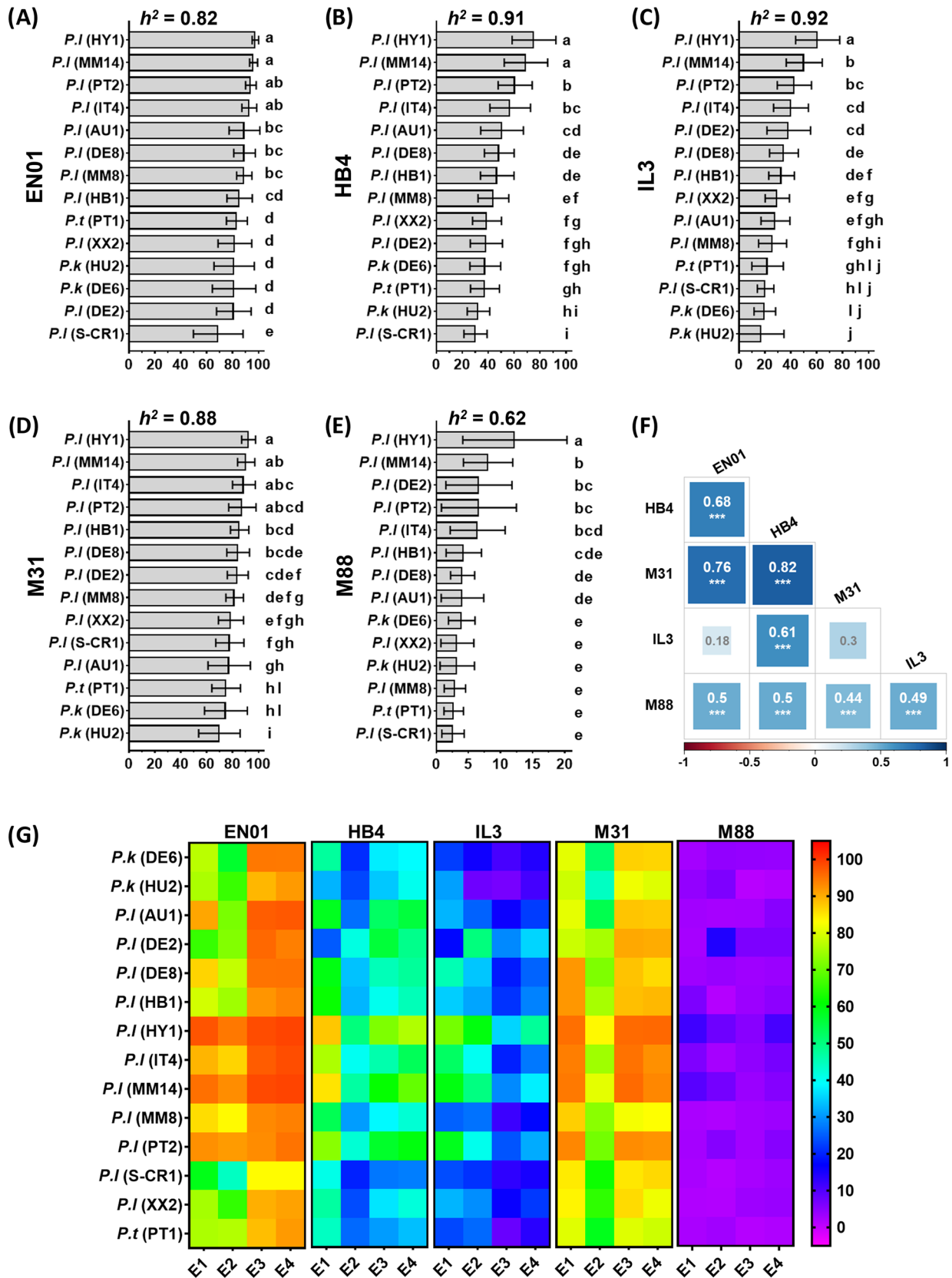


Fig. 4 *Heterorhabditis bacteriophora* DJ recovery (%) of hybrid lines EN01 (A), HB4 (B) & IL3 (C) and mutant lines M31 (D) & M88 (E) in fourteen different *Photorhabdus* spp. supernatants, and the correlation (F) between DJ recovery level of the nematode lines. The heatmap (G) indicates consistency of the data between repetitions of experiments and allows a side-by-side comparison for all nematode strains/lines. The x-axis scales (A–E) and the colored bar (G) depict DJ recovery (%). The DJ recovery was assessed after 72 h incubation at 25 °C. The assessment was repeated four times, each with four replicates. Different letters behind the bars denote significant differences between nematode lines ($p < 0.05$). Error bars depict the SD. Stars denote significant differences between treatments ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). The heritability (h^2) is shown for DJ recovery (A–E) on top of the figure

et al. 2023) and can support such breeding programmes. Possibly, both approaches will contribute to higher DJ recovery in the future together with the identification of a bacterial

symbiont strain with major support of the DJ recovery. We can conclude that different *H. bacteriophora* DJ batches possess a predetermined DJ recovery level, and this level can be increased using a supportive bacterial symbiont in in vitro mass production. Thus, the switch of *Heterorhabditis*–*Photorhabdus* partners can be a good complement to optimize the DJ recovery in *H. bacteriophora*, provide strains with optimal pre-disposition for recovery and reproduction can be selected.

The DJ recovery of lines EN01, HB4 and IL3 was found to decrease along storage time. In *C. elegans*, worm aging is reported to implicate muscle deterioration, metabolic disorder, accumulation of molecular and cellular damage (Golden and Melov 2007). In EPN, the lipid reserves of *Heterorhabditis* DJs are depleting during storage, and their mobility also decreases (Fitters and Griffin 2004; Menti et al. 2000).

Table 3 Two-way ANOVA analysis for the effect of fourteen different *Photorhabdus* isolates and five *Heterorhabditis bacteriophora* strains/lines on DJ recovery in bacterial supernatants

Source of variance	SS (sum of squares)	df (degree of freedom)	MS (mean square)	% of total variation	F	P value
A						
Bacteria	17,326	13	1333	5.58	10.91	< 0.0001
Nematodes	261,371	4	65,343	84.18	535.1	< 0.0001
Interaction	6135	52	118	1.98	0.97	0.54
Residual	25,644	210	122.1			
B						
Bacteria	18,214	13	1401	14.42	8.81	< 0.0001
Nematodes	85,439	2	42,720	67.66	268.5	< 0.0001
Interaction	2573	26	98.98	2.04	0.62	0.92
Residual	20,044	126	159.1			

DJ recovery was assessed after 72 h incubation at 25 °C

The analysis was performed including EN01, HB4 & IL3 and the mutant lines M31 & M88 (A) and without the mutant lines (B)

Table 4 DJ recovery (%) in stored DJs of *Heterorhabditis bacteriophora* lines EN01, HB4 & IL3 and mutants M31 & M88 upon exposure to *Photorhabdus laumondii* (strain DE2) supernatant or Ringer's solution determined after 72 h incubation at 25 °C. Three independent

experiments were carried out each with four technical replications. Data (%) are mean \pm SD of four experiments. For visualization of results: blue intensities: high DJ recovery; red intensities: low DJ recovery

Treatment	<i>H. bacteriophora</i> strain	DJ recovery (%)				
		T1 (Week 1)	T2 (Week 2)	T3 (Week 4)	T4 (Week 5)	T5 (Week 8)
Bacterial supernatant	EN01	86.1 \pm 12.4	96.1 \pm 4.6	65.4 \pm 19.7	39.5 \pm 22.9	18.7 \pm 12.9
	HB4	43.2 \pm 12	26.3 \pm 15.3	20.4 \pm 17.4	6.3 \pm 5	4.4 \pm 3.1
	IL3	45.4 \pm 14.8	28.2 \pm 9.8	22.1 \pm 15.4	11.2 \pm 8.2	5.3 \pm 3.2
	M31	85.9 \pm 9.1	89.1 \pm 5.4	88.2 \pm 7.4	84 \pm 12.3	79.6 \pm 7.5
	M88	8 \pm 5.6	5.9 \pm 4.7	3.9 \pm 3.8	3.3 \pm 4.7	1.4 \pm 1.3
Ringer's Solution	EN01	10.6 \pm 9.4	6.1 \pm 5.2	4.8 \pm 4.3	5.5 \pm 1.8	4.7 \pm 2.4
	HB4	0.5 \pm 0.5	1.3 \pm 1.6	0.7 \pm 0.6	0.7 \pm 0.3	0.3 \pm 0.2
	IL3	0.3 \pm 0.1	0.8 \pm 1.2	0.4 \pm 0.5	0.2 \pm 0.2	0.2 \pm 0
	M31	13 \pm 7.9	26.9 \pm 9.6	63.5 \pm 25.4	71.9 \pm 16.1	78.5 \pm 12.8
	M88	0.4 \pm 0.7	0 \pm 0	0 \pm 0	0.2 \pm 0	0.3 \pm 0.3

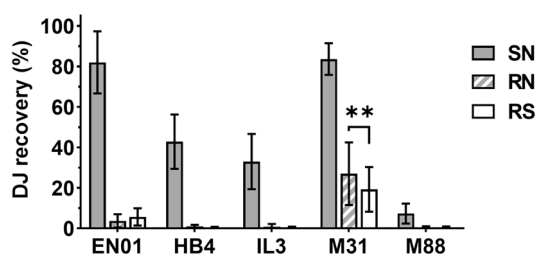


Fig. 5 Influence of volatiles emitted from neighbouring *Photorhabdus laumondii* (strain DE2) supernatants on DJ recovery of *Heterorhabditis bacteriophora* lines. The setup of treatments SN, RN and RS were provided in Fig. 1. DJ recovery was accessed after 72 h incubation in 25 °C. The experiments were repeated four times, each time with four technical replications. Stars over the bars denote significant differences between two treatments (** $p < 0.01$). Error bars depict the SD

This limitation can be overcome by preventing too long storage periods of the nematode inoculum. Given the increasing demand for heterorhabditid nematodes in biological control also during winter periods (Lacey and Georgis 2012), the problem of inoculum storage might be less important in the future, or early precultures should produce inoculum with more predictable DJ recovery percent before the season starts.

The use of the mutant M31 is certainly not a solution to the problems with unpredictable and low DJ recovery as this line recovers even without bacterial triggers, which would cause problems during storage of the DJ material prior to and during transportation to the users. However, both mutant lines can be used for future studies investigating the genetic background for DJ recovery in *H. bacteriophora*.

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Author contributions RUE, CM and BV conceived the experiments and the project. CM, BV and ZW designed the experiments. All authors performed the experiments. ZW and MD analysed the data. ZW wrote the first draft of the manuscript, CM and RUE read and commented on previous versions of the manuscript. All authors approved the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. The *Photorhabdus* sequences in this study are available at the National Center for Biotechnology Information (NCBI). The 16 S rDNA sequences can be found at NCBI under accessions

OR350573 to OR350586. For the gene sequences of *gltX*, *recA*, *dnaN*, *gyrB* homologs, accession numbers from OR364832 to OR364873 and OR393436 to OR393449 have been assigned.

Declarations

Competing interest The authors declare no competing interests.

Ethical approval This study does not contain any studies with human participants or vertebrate animals performed by any of the authors.

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Annex 3: Publication 3

Dauer juvenile recovery transcriptome of two contrasting EMS mutants of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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Dauer juvenile recovery transcriptome of two contrasting EMS mutants of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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Abstract

The entomopathogenic nematode *Heterorhabditis bacteriophora*, symbiotically associated with enterobacteria of the genus *Photobacterium*, is a biological control agent against many insect pests. Dauer Juveniles (DJ) of this nematode are produced in industrial-scale bioreactors up to 100 m³ in liquid culture processes lasting approximately 11 days. A high DJ yield (> 200,000 DJ·mL⁻¹) determines the success of the process. To start the mass production, a DJ inoculum proceeding from a previous monoxenic culture is added to pre-cultured (24 h) *Photobacterium* bacteria. Within minutes after contact with the bacteria, DJ are expected to perceive signals that trigger their further development (DJ recovery) to reproductive hermaphrodites. A rapid, synchronized, and high DJ recovery is a key factor for an efficient culture process. In case of low percentage of DJ recovery, the final DJ yield is drastically reduced, and the amount of non-desired stages (males and non-fertilized females) hinders the DJ harvest. In a preliminary work, a huge DJ recovery phenotypic variability in *H. bacteriophora* ethyl methanesulphonate (EMS) mutants was determined. In the present study, two EMS-mutant lines (M31 and M88) with high and low recovery phenotypes were analyzed concerning their differences in gene expression during the first hours of contact with *Photobacterium* supernatant containing food signals triggering recovery. A snapshot (RNA-seq analysis) of their transcriptome was captured at 0.5, 1, 3 and 6 h after exposure. Transcripts (3060) with significant regulation changes were identified in the two lines. To analyze the RNA-seq data over time, we (1) divided the expression profiles into clusters of similar regulation, (2) identified over and under-represented gene ontology categories for each cluster, (3) identified *Caenorhabditis elegans* homologous genes with recovery-related function, and (4) combined the information with available single nucleotide polymorphism (SNP) data. We observed that the expression dynamics of the contrasting mutants (M31 and M88) differ the most within the first 3 h after *Photobacterium* supernatant exposure, and during this time, genes related to changes in the DJ cuticle and molting are more active in the high-recovery line (M31). Comparing the gene expression of DJ exposed to the insect food signal in the haemolymph, genes related to host immunosuppressive factors were not found in DJ upon bacterial supernatant exposure. No link between the position of SNPs associated with high recovery and changes in gene expression was determined for genes with high differential expression. Concerning specific transcripts, nine *H. bacteriophora* gene models with differential expression are provided as candidate genes for further studies.

Keywords *Heterorhabditis bacteriophora* DJ recovery · *Photobacterium* bacterial supernatant · EMS-mutants; RNA-seq · Time-course transcript expression

Introduction

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (Nematoda: Rhabditida) is used for the biological control of several insect pests (Grewal et al. 2005). The third stage dauer juvenile (DJ; also known as Infective Juvenile, IJ) is the developmentally arrested, non-feeding form that seeks and infects host larvae. When DJ penetrate a new host, the pre-dauer J2 cuticle is shed (Bedding and

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Molyneux 1982). Once inside the insect's haemolymph, the DJ release cells of their symbiotic bacteria *Photorhabdus* (Gammaproteobacteria: Morganellaceae) as a response to so-called “food signals” in the haemolymph. After overcoming the immune response of the host insect, the bacteria proliferate exponentially, killing the insect while acting as a nutritional source for the nematode (Han and Ehlers 2000). The process during which the nematodes exit the DJ stage, grow and develop into hermaphrodites is called DJ recovery (Golden and Riddle 1982).

For commercial use, *H. bacteriophora* DJ are reproduced in bioreactors of up to 100 m³ capacity within an 11-day process. At the start of each production cycle, *H. bacteriophora* DJ are inoculated into the 24 h pre-cultured symbiotic *Photorhabdus* bacteria (Ehlers 2001). In this environment, the DJ recover as a response to food signal excreted by the symbionts into the supernatant (Aumann and Ehlers 2001). After completion of the cycle, a high and homogeneous DJ yield is expected (> 200,000 DJ mL⁻¹, less than 1% other stages). The final DJ yield much depends on a rapid, synchronized, and high recovery of inoculated DJs. A low percentage of DJ recovery leads to low DJ yields and an inefficient final DJ harvest due to the presence of other juvenile and adult stages (Johnigk et al. 2004). Thus, understanding the mechanisms behind DJ recovery is essential for further optimization of the EPN production. Previous works on in vitro culturing showed that the optimal timing for DJ inoculation is dictated by a characteristic pH dynamic in the *Photorhabdus* culture (Johnigk et al. 2004). Joyce et al (2008) determined that *P. laumondii* biosynthesizes stilbenes that have significant effects on the induction of DJ recovery. Wang et al (2022) found isopropylstilbene supporting DJ recovery. Different *Photorhabdus* species/strains can vary significantly in their support of DJ recovery and the reproductive potential of *H. bacteriophora*. However, the bacterial signal plays a subordinate role, and the intrinsic pre-disposition of the DJ to recover is of superior influence (Wang et al. 2024).

The ability of nematodes to respond to the so-called “food signals” triggering the DJ recovery can be influenced by genetic factors in combination with environmental stimuli. Wang et al (2023) determined that there is a large genetic variability within sets of *H. bacteriophora* ethyl methane-sulphonate (EMS) mutant and wild type lines concerning DJ recovery under uniform conditions and that this phenotypic variability is highly reproducible. Sets of lines with robust contrasting phenotypes were identified (Wang et al. 2023, 2024).

Concerning general genomic approaches, Sandhu et al (2006) first reported cDNA-sequencing of the DJ stage of *H. bacteriophora*. Approximately 40% of the expressed sequence tags (ESTs) resulted similar to *Caenorhabditis elegans* proteins related to metabolism and environmental information processing pathways. Later, Bai et al (2007)

found that ESTs in *H. bacteriophora* have homologs in animal parasitic nematodes, which were assigned to putative functions in transcription, signal transduction, cell cycle control, metabolism, information processing, and cellular processes. Related to the genome sequence, Bai et al (2013) and McLean et al (2018) re-visited the *H. bacteriophora* genome and predicted more than 10,000 protein-coding genes including a high proportion of proteins exclusive for EPN. Bai and Grewal (2007) first analyzed the gene expression of *H. bacteriophora* DJ upon insect hemolymph contact (1 h) and identified two down-regulated gene clones encoding for a phosphofructokinase (PFK) and a transialidase homologous to a *Trypanosoma cruzi* gene model. Later, Moshayov et al (2013) treated *H. bacteriophora* DJ with *Galleria mellonella* (Lepidoptera, Pyralidae) hemolymph, compared the expression after two time points (3 and 6 h), and identified eight candidate genes involved in the DJ recovery process. Two of the genes (*akt-1* and *glb-1*) were associated with the insulin/insulin-like growth factor-1 (insulin/IGF-1) signaling pathway in *C. elegans*. Vadnal et al (2017) sequenced RNA from *H. bacteriophora* DJ which had been exposed to *Manduca sexta* (Lepidoptera: Sphinginae) haemolymph for 9 h and found that a large proportion of the differentially expressed genes are unique to parasitic nematodes.

For the model nematode *C. elegans* the pheromones (ascarosides) are the major physiologic signal promoting dauer formation, and a heat-stable or hydrophilic “food signal” promotes dauer recovery (Hu 2018). A wide array of dauer-constitutive (*daf-c*) and dauer-defective (*daf-d*) *C. elegans* mutants has been analyzed and several signaling pathways were identified. These pathways include the guanylyl cyclase pathway, transforming growth factor beta (TGF- β) signaling pathway, insulin/IGF-1 signaling (IIS) pathway and the DAF-12 nuclear hormone receptor pathway (Fielenbach and Antebi 2008; Hu 2018). Several studies have focused on characterizing the recovery-related function of diverse gene/protein types (Ouellet et al. 2008; Gelmedin et al. 2011; Chou et al. 2015; Fernández et al. 2021; Martinez and Gill 2023). The search for genes regulating the DJ recovery process in EPN can use the knowledge gathered in *C. elegans* as a reference. Along the predicted protein-coding genes of *H. bacteriophora*, nearly half of the genes are *C. elegans* homologs (Bai et al. 2013; McLean et al. 2018). *H. bacteriophora* has most (19 of 23) of the insulin/IGF-1 signaling pathway genes also found in *C. elegans*, which are crucial for dauer formation, longevity regulation, stress resistance, and innate immunity (Bai et al. 2013). Moreover, Moshayov et al (2013) determined that two genes *akt-1* and *glb-1* regulating DJ recovery of *H. bacteriophora* are associated with IIS pathways.

Despite the valuable information yielded by the previous reports, the search for further potential DJ recovery

regulators in *H. bacteriophora* must be complemented to find factors that can be transferred to practical use in mass production. This knowledge is crucial for finding predictors for successful DJ recovery in running processes and identifying the reproductive potential of new nematode and bacterial strains. With the mutant lines M31 and M88 as starting point, we (1) conducted a time series of RNA-seq analysis with both mutants upon 0.5, 1, 3 and 6 h after exposure to *Photorhabdus* supernatant, (2) evaluated the transcription dynamics by clustering the expression profile into similar patterns, (3) identified over and under-represented gene ontology (GO) categories cluster-exclusive genes, (4) identified *C. elegans* homologous genes with recovery-related function, and (5) combined the information with available single nucleotide polymorphism (SNP) data. As a final output we propose a set of nine *H. bacteriophora* gene models as candidate genes for further studies. Additionally, we provide a large set of supplementary data that can be further mined to find potential interactions between expression patterns and functional information.

Materials and methods

Biological materials

Two EMS-mutant lines were selected for their robust contrasting DJ recovery phenotypes (Wang et al. 2023). Mutant line M31 is a recovery-constitutive mutant with high recovery even in the absence of food signals and M88 is a recovery defective mutant with recovery < 5% in supernatant. The mutant lines originated from the EMS mutagenesis of the donor line IL3. The symbiotic

Photorhabdus laumondii (strain DE2) was used as a source for bacterial supernatant. An overview of the materials in this study and the analyzed time points for subsequent RNA-seq is presented in Table 1.

Heterorhabditis bacteriophora cultivation

Nematodes mutant lines M31, M88 and the donor line IL3 were grown on solid NGG (nematode growth gelrite) medium (3 g·L⁻¹ Gelrite, 2.5 g·L⁻¹ peptone from casein, 51 mM NaCl, 1 mM CaCl₂·2 H₂O, 1 mM MgSO₄·7 H₂O, 1 mM KH₂PO₄, 12 µM filter-sterilized cholesterol in 99% ethanol) in Petri dishes according to Sumaya et al (2018). A sterilized semi-solid NGG (similar to solid but containing only 1.5 g·L⁻¹ gelrite and 2 g·L⁻¹ peptone) was mixed with a *P. laumondii* (strain DE2) suspension cultured in liquid medium (LM) (15 g yeast extract, 20 g soy powder, 4 g NaCl, 0.35 g KCl, 0.15 g CaCl₂, 0.1 g MgSO₄, 6 g lecithin diluted in same volume of rapeseed oil, 40 g rapeseed oil, 1 L distilled water, pH = 7 ± 0.1) for 24 h at 25 °C and adjusted to a final cell density of 2 × 10¹⁰ cells·mL⁻¹. The bacterial loan (bacteria in semi-solid NGG) was distributed over the solid NGG covering the complete Petri dishes. After 24 h incubation at 25 °C, DJ were inoculated into NGG plates and incubated for 6–7 days at 25 °C until hermaphrodites in the late *endotokia matricida* stage (Johnigk and Ehlers 1999) were obtained. Mature hermaphrodites were washed off with Ringer's solution (9 g NaCl, 0.42 g KCl, 0.37 g CaCl₂·2 H₂O, 0.2 g NaHCO₃, 1 L distilled water) and stored until DJ emigrated from the maternal carcass. Harvested DJ were stored for maximum of two weeks in Ringer's solution at 4 to 6 °C before use.

Table 1 Nematode materials for RNA-seq libraries used for transcriptome analysis of *Heterorhabditis bacteriophora* mutants M31 and M88 exposed to *Photorhabdus laumondii* (strain DE2) supernatant for different periods of time

EMS mutant line	DJ recovery phenotype	Treatments	Time (h) of supernatant exposure	Library code	Reference sample for fold-change comparison	Read counts (Million)
M31	High	Control	–	M31_C	–	23.1
		Supernatant	0.5	M31_0.5 h	M31_C	21.8
		Supernatant	1	M31_1h	M31_C	28.6
		Supernatant	3	M31_3h	M31_C	28.7
		Supernatant	6	M31_6h	M31_C	23.5
M88	Low	Control	–	M88_C	–	25.1
		Supernatant	0.5	M88_0.5 h	M88_C	17.6
		Supernatant	1	M88_1h	M88_C	20.7
		Supernatant	3	M88_3h	M88_C	23.5
		Supernatant	6	M88_6h	M88_C	19.8
Total read counts (Million)						232.4

Control libraries were done with DJ in Ringer's solution

Photorhabdus supernatant production

Production of *Photorhabdus* bacterial LM supernatant was carried out following Wang et al (2023, 2024). Briefly, *P. laumondii* (strain DE2) was cultured in LM medium at 25 °C on a shaker (180 rpm, rotation diam. 4 cm) for 24 h (Ehlers et al. 1998) and then centrifuged at 10,000 rpm (4 °C) for 10 min. Resulting supernatants were passed through 5 and subsequent 0.2 µm pore size filters and stored at – 80 °C until used.

DJ recovery induction and RNA-sequencing analysis

For nematode expression analysis in bacterial supernatant, RNA-sequencing (RNA-seq) was carried out. Dauer populations of the two mutant lines were incubated in 12 cell-well plates in triplicate positions, each with 500 µL supernatant and 4000 DJ per cell. Control wells received Ringer's solution only. Treated DJ and controls were harvested after exposure times of 0.5, 1, 3 and 6 h, immediately washed with a vacuum pump, resuspended in RNA protect (Qiagen, Germany), and frozen at – 20 °C until RNA extraction. A parallel experiment was carried out for observation and quantification of the DJ recovery of each line until three days after supernatant exposure. The DJ morphological change during the recovery process was visualized via a microscope (Zeiss, Germany) with a camera (Di-Li, Germany). Dauer recovery induction in bacterial supernatant was assessed quantifying morphological changes of the DJ head- and esophagus-region following Strauch and Ehlers (1998). RNA-seq samples from each treatment and mutant line consisted of three replicates (cells). All supernatant incubation steps were carried out at 25 °C. Subsequently, harvested DJ samples were sent to LGC Genomics GmbH (Berlin, Germany) for RNA

extraction, cDNA synthesis, amplification, sequence quantification, and differential expression analysis. The sequencing was carried out by an Illumina® Nextseq500/550 system (1 × 75 bp) with 200 million reads. After clipping and filtering of the raw sequencing data, RNA-seq reads were mapped against the *H. bacteriophora* TT01 reference genome deposited at wormbase.org (PRJNA13977.WBPS16), masked with the gene models predicted by McLean et al (2018).

Differential expression analysis between supernatant-exposed with non-exposed DJ (control) was applied with edgeR v3.8.6 (Robinson et al. 2010), DESeq v1.18.0 (Anders and Huber 2010) and cufflinks v2.2.1 (Trapnell et al. 2012), the overlap between the different software packages were determined. The results obtained with the method edgeR have the highest number of differently expressed gene models and were used for the following analysis. The raw *p*-values of the statistical tests were adjusted for multiple testing by the Benjamini–Hochberg false discovery rate (FDR) method. The expression level (fold change) of the gene models was log₂ transformed (hereafter referred to as log2FC). The gene models with log2FC, *p*-value, and FDR were subjected to further analysis.

qPCR on selected gene models

For validation of the gene expression via qPCR, independent DJ recovery induction assays were carried out with M31 and M88 DJ populations under the same conditions as described above. Randomly selected gene models with up- or down-regulation upon *Photorhabdus* supernatant exposure were chosen for validation. The primer design for specific gene models was carried out with the software package CLC-Bio main workbench (Qiagen, Aarhus, Denmark). Primer sequences are presented in Table 2. Total RNA was isolated

Table 2 Gene models and primers used for transcript profiling validation via qPCR

Amplified fragments		Primers		
Gene model in <i>H. bacteriophora</i>	Homologous genes in <i>C. elegans</i>	Orientation	Sequence (5′–3′)	Product length (bp)
g5312	<i>zzz-1</i>	Fwd	TAGTATGGGCAAAGTGGAGT	321
		Rev	GCAGACACAAAACAACAAGA	
g11146	<i>srd-13</i>	Fwd	ACACCTGAAATCTGCAAC	382
		Rev	CAAACCTGAACTGCATAGGAA	
g5555	<i>npr-31</i>	Fwd	GCAGAAGACAAAACGGAA	267
		Rev	TGCTTGATCACCGATTTAC	
g8239	F39B2.8	Fwd	TGTTCACTCTAACTCTC	251
		Rev	ATGTCCTGTTCTCTTACG	
g9082	F35H10.10	Fwd	ATCCATTCAAAGTCCTTCC	215
		Rev	ATCACCCGCATATAGTCC	
g4070	<i>frpr-7</i>	Fwd	CACAATCCACACTGATCCC	231
		Rev	CCACATCTATCCTCAGTCC	

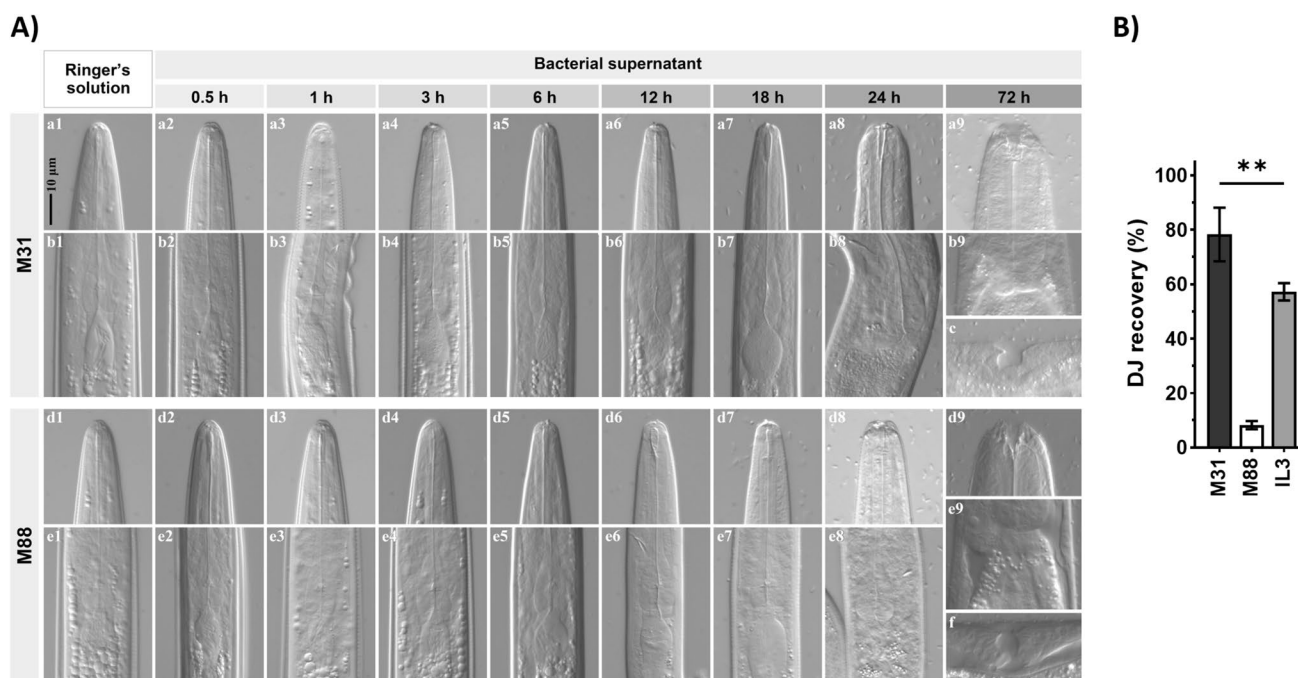


Fig. 1 Morphologic changes during dauer juvenile (DJ) recovery in the *Heterorhabditis bacteriophora* recovery-constitutive mutant M31 and the recovery-defective mutant M88 over a period of 0.5–72 h after exposure to *Photorhabdus laumondii* (strain DE2) supernatant (A). Control DJ were kept in Ringer's solution. Pictures a1–9 (M31) and d1–9 (M88): head region and anterior part of oesophagus; b1–9 (M31) and e1–9 (M88): basal bulb, cardia and anterior part

of intestine; c (M31) and f (M88): vulva of young hermaphrodites. All treatments were incubated at 25 °C. The scale bar for all graphs is presented in picture a1. Average DJ recovery (%) of *H. bacteriophora* lines M31, M88 and IL3 exposed to *P. laumondii* (strain DE2) supernatant (B). DJ recovery (%) was determined 72 h after exposure. Error bars indicate ±SD. Stars over the bars denote significant differences between each line (**: $P < 0.01$)

with the peqGOLD Total RNA Kit (VWR, Radnor, Pennsylvania) according to the manufacturer's instructions. The qPCR amplifications were carried out in the presence of 1 × GoTaq® qPCR Master Mix (Promega, Madison, Wisconsin) in 15 μL reactions. Thermal cycling was done in a StepOne Plus RT-qPCR device (ThermoFisher, Waltham, Massachusetts) with the following profile: initial denaturation of 95 °C for 10 min; 5 cycles of 95 °C for 15 s; 62 °C for 45 s ($-0.5\text{ °C} \times \text{cycle}^{-1}$); 40 cycles of 95 °C for 15 s and 60 °C for 45 s. A melting curve analysis was done by increasing the final amplicon temperature from 60 to 95 °C, by 0.3 °C every 10 s. Expression of the target genes was normalized against the 18S rRNA gene using the primers reported by Yaari et al (2016). Target quantification was done following Pfaffl (2004), using the non-induced DJ samples from each mutant line as control, according to the formula:

$$\text{Relative quantity} = \frac{(E_{\text{Target}})^{\Delta C_{p\text{Target}}(\text{Control-Sample})}}{(E_{\text{Ref}})^{\Delta C_{p\text{Ref}}(\text{Control-Sample})}}$$

where E is the calculated efficiency for the target and house-keeping (Ref) genes and C_p is the cycle at which the amplification reached the exponential phase.

Further transcriptome downstream analyses

The expression of more than 14,000 gene models was

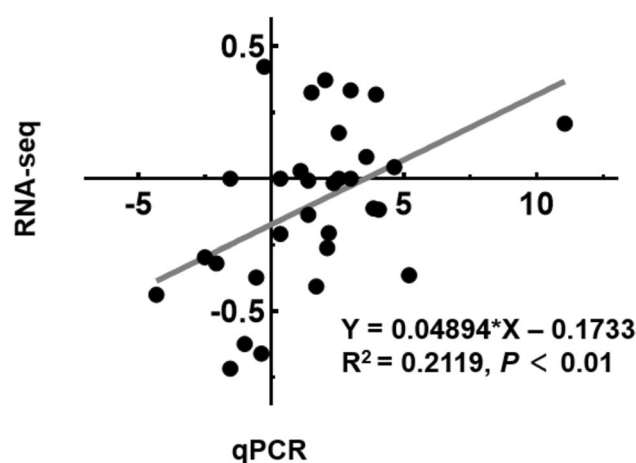


Fig. 2 Linear regression between qPCR and RNA-seq expression data based on the log2FC of six gene models (g11146, g11147, g4070, g5312, g8239 and g9082). The correlation between qPCR and RNA-seq expression resulted significant (Pearson correlation, $R = 0.46$, $P < 0.01$). Further gene information is presented in Table 2

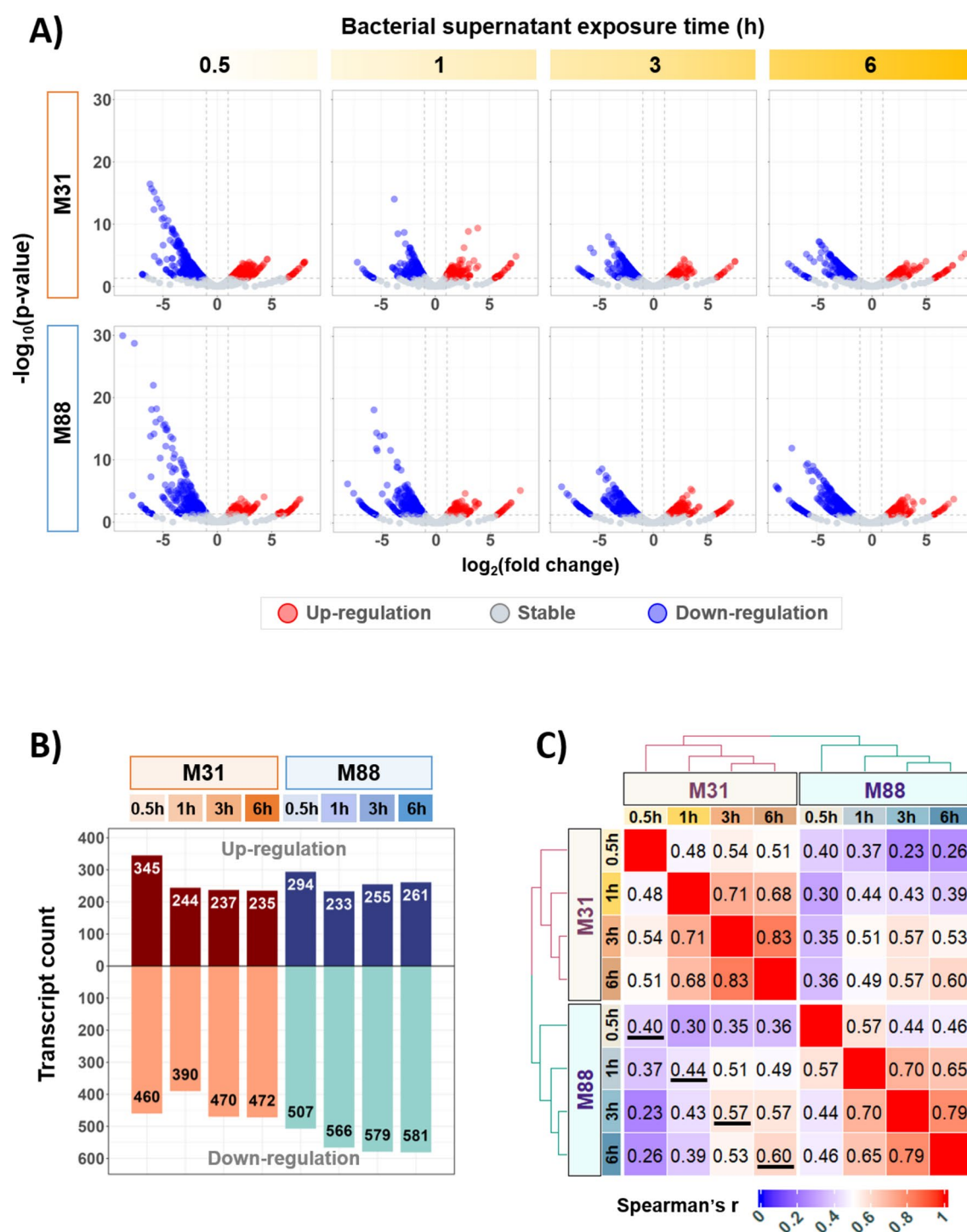


Fig. 3 Differentially up- and down-regulated *Heterorhabditis bacteriophora* gene models (transcripts) in M31 and M88 mutants within 0.5 to 6 h after *Photorhabdus laumindii* supernatant exposure. Expression changes are derived from the comparison with the respective reference RNA-seq control libraries. Volcano plots for each comparison (A). Number of differentially expressed transcripts per comparison

(B) (Threshold: $|\log_2\text{FC}| \geq 1$, $P < 0.05$). Correlation between expression profiles in M31 and M88 mutant lines (C). The gene expression in early time points (0.5 and 1.0 h) is less correlated than expression in late time points (3.0 and 6.0 h). The colored scale bar (C) presents Spearman's correlation coefficient r of transcript expression between different treatments

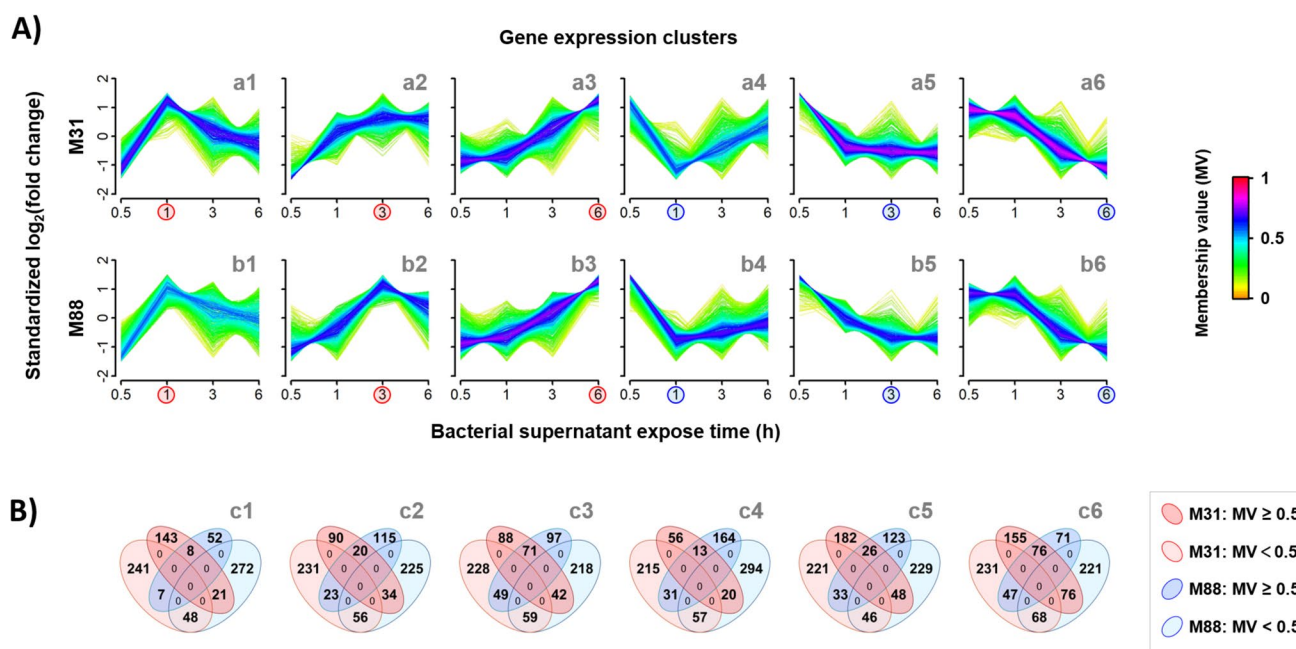


Fig. 4 Gene expression clusters including *Heterorhabdus bacteriophora* gene models with similar expression patterns in mutants M31 and M88 after 0.5 to 6.0 h *Photorhabdus laumondii* (strain DE2) supernatant exposure. The clustering was implemented by using the fuzzy c-means algorithm on 3,060 differentially regulated gene models within M31 and M88 RNA-seq libraries. Gene models with similar expression tendency were grouped into six distinct clusters (M31: a1–a6; M88: b1–b6) (A). Clusters 1 to 3 cover upregulated–,

whereas clusters 4 to 6 cover downregulated gene models. Circles below the scale indicate the time (h) for the highest (red) or lowest (blue) expression peak (or valley). The rainbow-colored scale bar (upper right) presents membership value (MV) of the gene models included in each cluster. (B) The Venn diagrams (c1–c6) depict the number of cluster common- and exclusive gene models from the two mutant lines according to their MV-value. The MV-value thresholds are depicted in different red and blue shades (scale: bottom right)

determined from RNA-seq data. For visualization, a dendrogram and a principal component analysis (PCA) were performed based on the $\log_2\text{FC}$ values of all treatments. The data were filtered by removing gene models with constitutive expression profile. Volcano plots were generated by plotting negative \lg -transformed P -values on the y-axis and $\log_2\text{FC}$ differences on the x-axis for each time point of supernatant exposure in the two mutant lines. Statically significant ($P < 0.05$) and differentially abundant (up-regulated: $\log_2\text{FC} \geq 1$; down-regulated: $\log_2\text{FC} \leq -1$) transcripts were kept for further downstream analyses. All *H. bacteriophora* gene models (McLean et al. 2018) were annotated by BLASTx search against protein sequences from SwissProt/UniProt (<https://www.uniprot.org>) and National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>) databases. Filtration criteria used for BLASTx were: E-value $\leq 1.0 \times 10^{-5}$, query coverage ≥ 80 , and identity percentage ≥ 60 .

The selected significantly expressed gene models from the different supernatant exposure time points were standardized and grouped into different dynamic regulation clusters using Fuzzy clustering (Kumar and Futschik 2007). Subsequently, Venn diagrams for each cluster were produced to determine common and exclusive gene models between mutant lines

M31 and M88. The exclusive and common gene models in each Fuzzy cluster of two mutant lines were used to conduct a GO enrichment analysis. Additionally, a gene-concept network from the five most enriched GO for biological processes (BP) was carried out for each regulation cluster.

Correlations, Volcano plots, bar plots, dendrograms, PCA, fuzzy c-means algorithm, Venn diagrams, GO enrichment, and gene-concept network were assessed with R v4.3.1 in Rstudio v2022.07.1 + 554 (Boston, Massachusetts) development environment. The R packages dplyr v1.1.0, ggplot2 v3.4.1, BiocManager v 1.30.22, cluster v2.1.3, factoextra v1.0.7, FactoMineR v2.4, Mfuzz v 2.58.0, VennDiagram v 1.7.3, org.Ce.eg.db v 3.17.0, clusterProfiler v 4.6.2, enrichplot v 1.18.3 and pathview v.1.40.0 were used to implement the analysis.

General data analysis

For statistical analyses, the data normality and variance equality were tested by the Shapiro–Wilk test and the F-test. The normally distributed and equal-variance data were analyzed by parametric tests (one-way ANOVA). Non-normal-distributed or unequal-variance data were log-transformed or analyzed by non-parametric tests (Kruskal–Wallis or

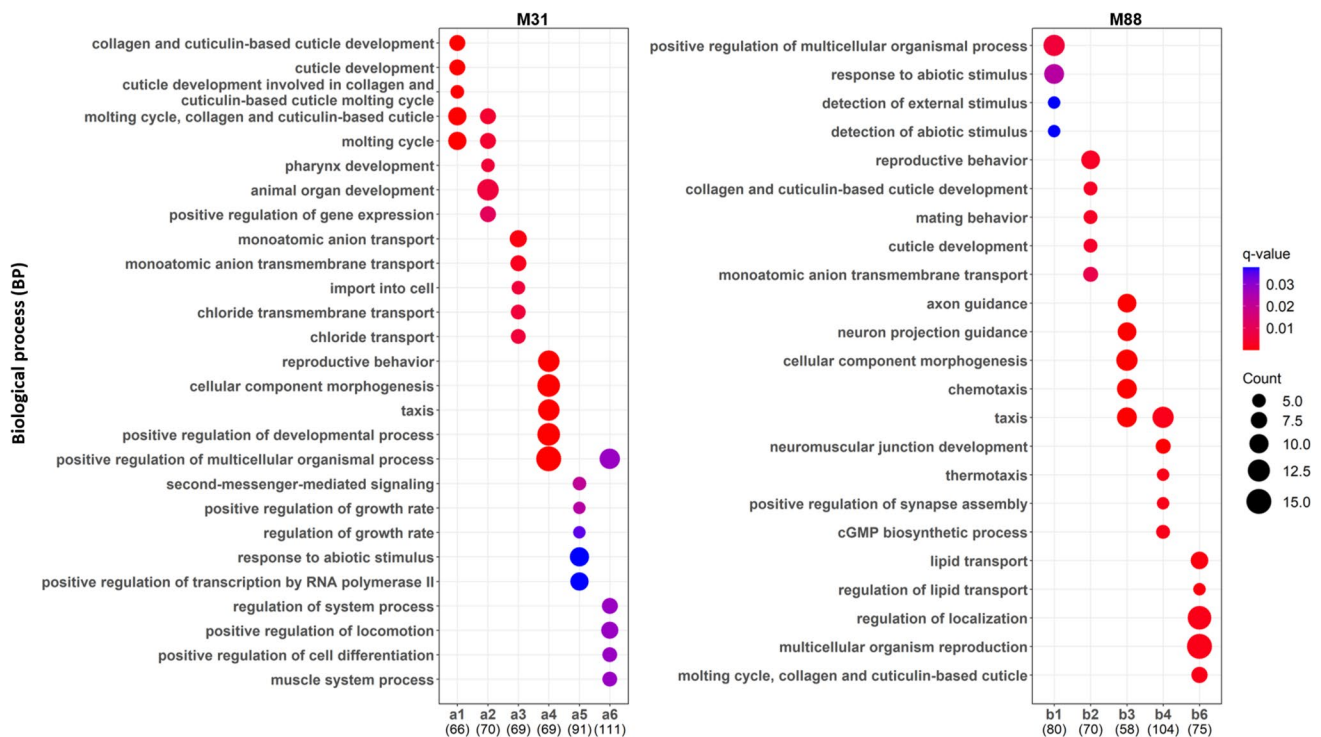


Fig. 5 Parallel gene ontology (GO) enrichment analysis for biological processes (BP) based on the cluster-exclusive *Heterorhabditis bacteriophora* gene models for M31 and M88 mutants. Exclusive gene models belonging to each regulation cluster (Fig. 4B) were analyzed for GO categories enrichment. The five most represented BP

GO terms ($q\text{-value} < 0.05$) for each cluster in M31 (a1–a6) and M88 (b1–a6) are depicted for both mutants side by side. Colored scale bar: $q\text{-values}$ of enriched GO terms. The size of the dots (far right) depicts the counts of clustered genes per GO category. The number in brackets (down) indicate the count of genes included in each cluster

Mann–Whitney U test). The correlation between phenotypes was assessed by Pearson's correlation coefficient (normal-distributed data) or Spearman's rank correlation coefficient test (non-normal-distributed data). A general linear model between phenotypes was built with linear regression.

All data were collated and pre-treated in Microsoft Excel. Data normality and variances test, significance analysis, linear models, and line charts were performed using GraphPad Prism 12.0 software (San Diego, California).

Results

Contrasting DJ recovery upon supernatant exposure

We evaluated the morphological change of DJ exposed to *P. laumondii* supernatant over 72 h (Fig. 1A) and quantified the recovery phenotype of the lines M31 and M88 along the donor line IL3 (Fig. 1B) to confirm the contrasting DJ recovery phenotypes reported by Wang et al (2023). Unrecovered DJ were characterized by a closed mouth and the presence of the pre-dauer J2d cuticle additional to the dauer cuticle. Upon bacterial supernatant exposure, the secondary cuticle was shed within 1–3 h. Thereafter, the developing

mouth and intestine were observed. The major morphologic changes in the anterior part were determined after 1-day of supernatant exposure. After ~72 h, the vulva of young hermaphrodites was differentiated. We determined significant differences in DJ recovery between the lines M31, M88 and IL3 (Kruskal–Wallis test, $P < 0.01$). The recovery rate of M31 reached $78.3 \pm 8.5\%$, in contrast to less than 10% seen in M88. The donor line IL3 showed a recovery rate of $57.2 \pm 2.8\%$.

RNA-seq libraries sequencing and data validation

A total of 10 RNA-seq libraries including two controls (M31_C, M88_C) and four supernatant exposure treatments per line (M31_0.5 h, M31_1h, M31_3h, M31_6h, M88_0.5 h, M88_1h, M88_3h, M88_6h) yielded an average of 23.24 Mio. reads per sample (17 Gbp of sequence information). General details about the RNA-seq libraries are presented in Table 1. The sequencing reads from each library were mapped to the *H. bacteriophora* strain TT01 reference genome comprising 14,960 gene models according to the annotations of McLean et al (2018). An average of 66.8 transcripts per million reads (TPM) and 88 reads per kilobase of transcript per million reads (RPKM) was

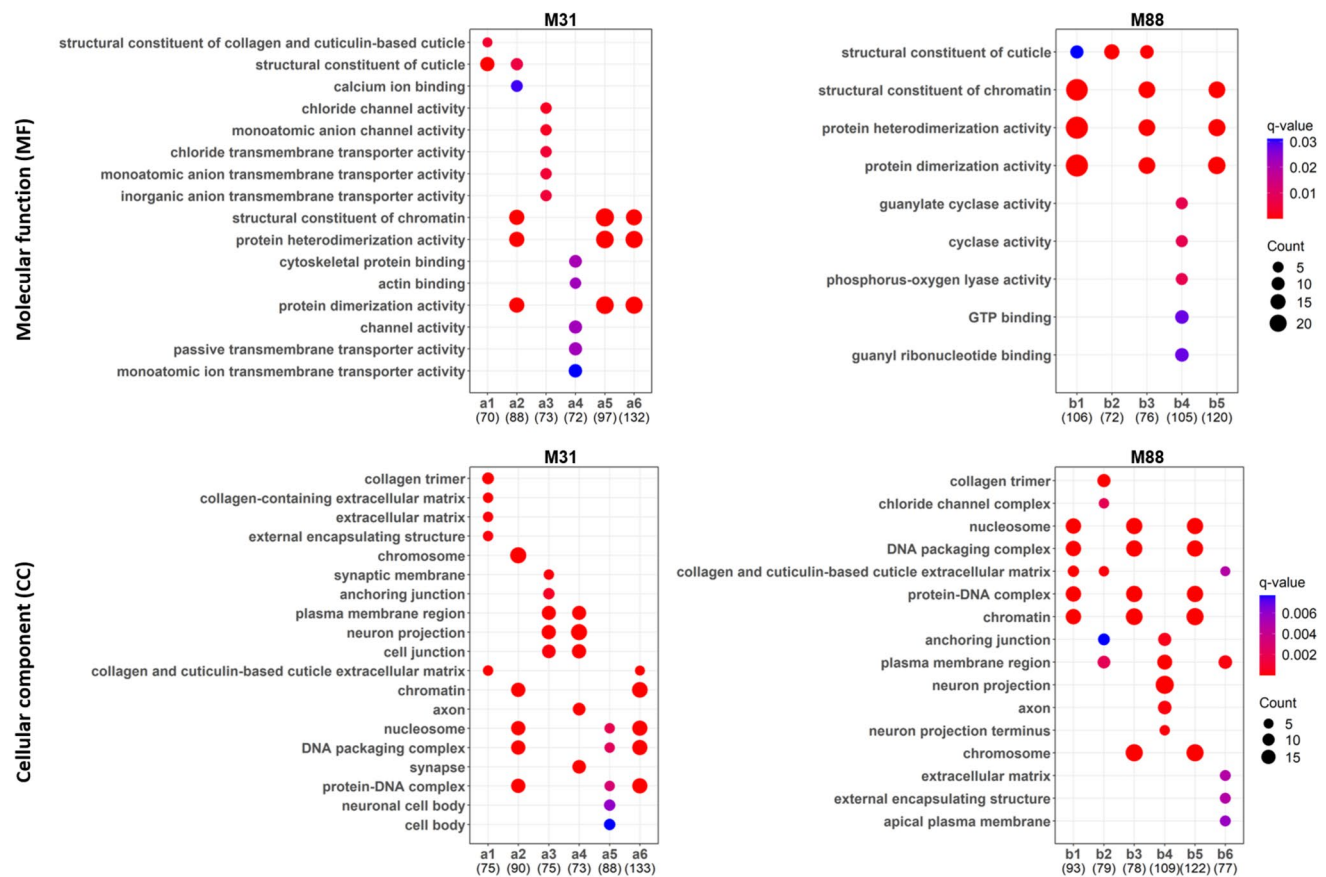


Fig. 6 Parallel gene ontology (GO) enrichment analysis for molecular function (MF) and cellular component (CC) based on the cluster-exclusive *Heterorhabditis bacteriophora* gene models for M31 and M88 mutants. Exclusive gene models belonging to each regulation cluster (Fig. 4B) were analyzed for GO categories enrichment. The five most represented BP GO terms (q-value < 0.05) for each cluster

in M31 (a1–a6) and M88 (b1–a6) are depicted for both mutants side by side. Colored scale bar: q-values of enriched GO terms. The size of the dots (far right) depicts the counts of clustered genes per GO category. The number in brackets (down) indicate the count of genes included in each cluster

determined. The expression data from supernatant-exposed treatments was compared against the respective control in Ringer's solution, and the log2FC for each gene model and time point in both mutants was determined (supplementary Table S1). For validation, six gene models (g5312, g11146, g5555, g8239, g9082, and g4070) were selected for amplification via qPCR in each sample. The RNA-seq and qPCR expression levels were positive and significantly correlated ($R = 0.46$, $P < 0.01$) (Fig. 2).

General transcriptome overview

A principal component analysis (PCA) of the log2FC expression of M31 and M88 lines revealed that the genetic differences between both mutants have a higher effect on the observed expression patterns than the bacterial supernatant exposure time (supplementary Fig. S1). To eliminate gene models with constitutive expression, two thresholds were applied: (1) $|\log_2FC| \geq 1$, $P \leq 0.05$; (2) $|\log_2FC| \geq 1$,

$FDR \leq 0.05$. Thereafter 3,060 and 269 differentially expressed gene models were determined for threshold (1) and (2), respectively. The 3,060 gene models from threshold (1) were used for further bioinformatic analysis. For 1074 (35.1%) of these gene models, homology to *C. elegans* was detected followed by homology to the parasitic nematode *Ancylostoma ceylanicum* (288, 9.4%). The regulation and annotation information of these gene models is deposited in supplementary Tables S2 to S4. An overview of the global up- and down-regulation levels is shown in Fig. 3. In general, the proportion of down-regulated was higher than the proportion of up-regulated gene models. For instance, 0.5 h after supernatant exposure, 460 gene models were down- vs 345 up-regulated in M31, whereas 507 were down- vs 294 up-regulated in M88. Concerning up-regulation along the time series, the highest proportion was detected after 0.5 h of supernatant exposure in both mutant lines. Concerning the correlation between M31 and M88 expression levels along the time points, the similarity between both lines increased

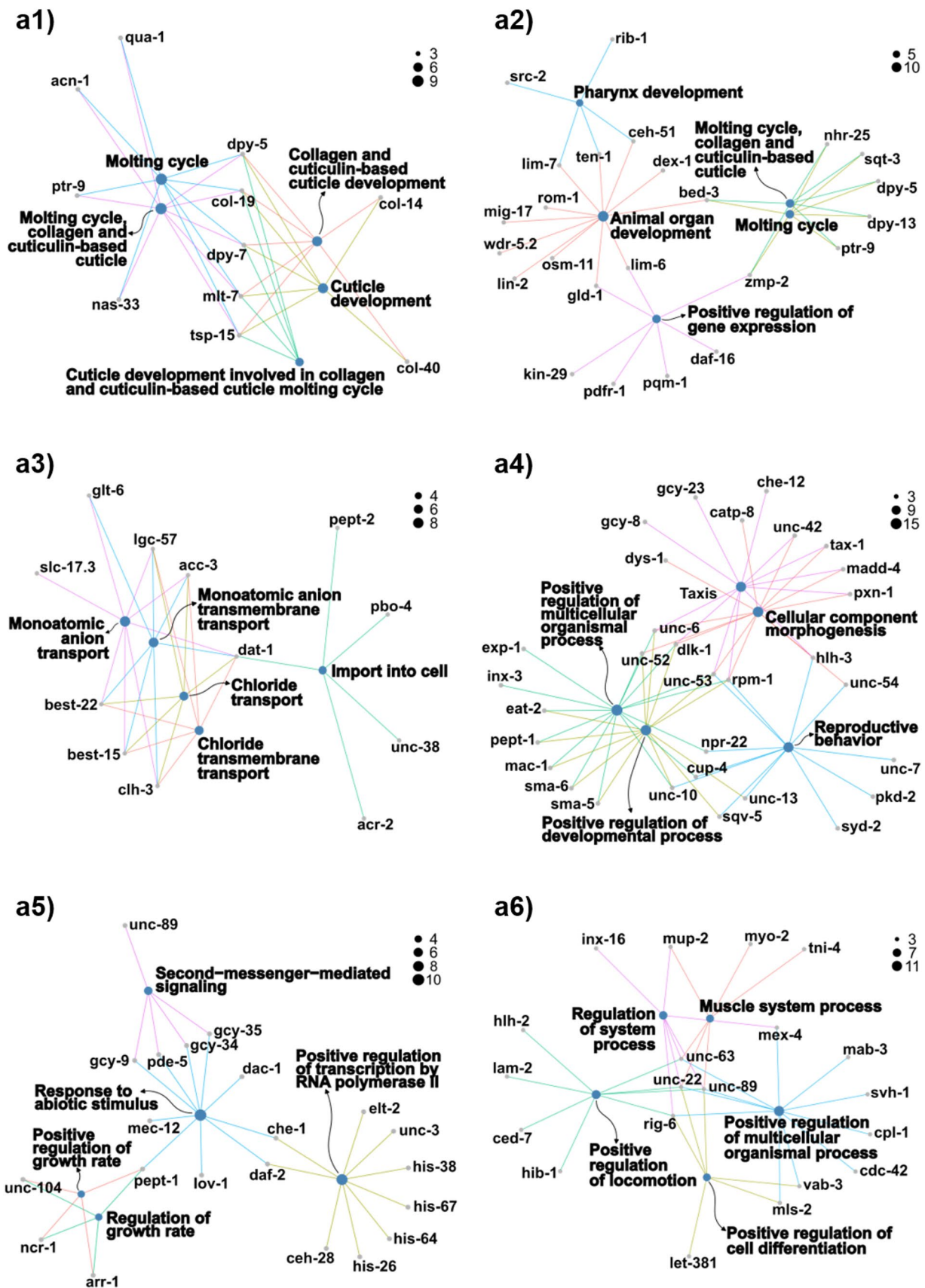


Fig. 7 Gene-concept network of enriched gene ontology (GO) biological process (BP) categories based on M31 exclusive gene models for each regulation cluster (Fig. 3). The five most enriched BP GO terms are depicted for each regulation cluster along the *Caenorhabditis elegans* homologous gene models related to the given categories. The dots indicate the enriched count of clustered genes in each GO term. Clusters designation: top left for each network

with the time of supernatant exposure. Early time points (0.5 and 1 h) showed the highest differences in expression patterns between both lines (Fig. 3C).

Considering all gene models, 10,141 (67.8%) resulted homologous to nematode accessions at NCBI. The expression and annotation information of all gene models are deposited in supplementary Table S1. Additionally, homologous proteins and feeding habits of the characterized nematode for each time point are deposited in supplementary Tables S5 and S6. Interestingly, differentially expressed gene models with homology to vertebrate parasitic nematodes were found more often at early time points.

Transcriptome remodeling along supernatant-exposure time

We determined six distinct clusters of temporal expression patterns on the 3,060 differentially regulated gene models (Fig. 4). Clusters 1–3 contain up-regulated gene models having the highest expression peak 1, 3 and 6 h after supernatant exposure, respectively. Clusters 4–6 contain down-regulated transcripts with the lowest expression valleys 1, 3 and 6 h after supernatant exposure. We observed that the number of shared transcripts per cluster between M31 and M88 varies along the different clusters. The clusters with the latest expression peaks or valleys showed the highest number of common gene models, whereas in clusters with early expression peaks, this proportion was lower. For instance, concerning up-regulation, only 8 gene models appear to be similarly induced in both mutants with an expression peak 1 h after supernatant exposure (clusters a1 and b1, Fig. 4), whereas 6 h after exposure the number of shared transcripts is 71 (clusters a3 and b3, Fig. 4) ($MV \geq 0.5$). Similarly, expression patterns with early down-regulation valleys have 13 common gene models between both lines (clusters a4 and b4, Fig. 4), whereas 6 h after supernatant exposure, 76 gene models are shared (clusters a6 and b6, Fig. 4). Additional information about expression patterns and cluster pairwise comparisons is deposited in supplementary Tables S7 and S8.

Differential representation of gene ontology terms along different clusters

We searched for differential representation of GO biological processes (BPs) along the previously determined clusters, and we analyzed whether the GO representation differs

between both mutants (M31 vs M88). In each mutant line, the five most enriched (q -value ≤ 0.05) GO categories for each cluster are described independently. Clusters a1–a6 describe M31 whereas clusters b1–b6 describe M88 processes (Fig. 5). Concerning the mutant line showing a high DJ recovery rate (M31), cuticle development and molting-related BPs are prevalent in the early time points (cluster a1, peaking 1 h after supernatant exposure). Also cluster a2 (3 h after supernatant exposure) shows two GO categories (0018996, 0042303) related to the molting function. In this cluster, BP related to pharynx and organ development were also represented. Towards 6 h after supernatant exposure (cluster a3), general signaling-related BPs are prevalent (e.g. GO: 0006820, monoatomic anion transport).

The pattern of represented BPs in the mutant with low DJ recovery (M88) seems to show a time frameshift in comparison to M31. For this mutant, cluster b1 contained BPs that are mainly related to stimulus–response, whereas processes related to cuticle development appear only 3 h after supernatant exposure (cluster b2) (Fig. 5). Enrichment of gene models related to molecular function (MF), and cellular components (CC) within each cluster is provided in Fig. 6 and supplementary Fig. S2. Further details on the results of the GO enrichment analysis is deposited in supplementary Table S9.

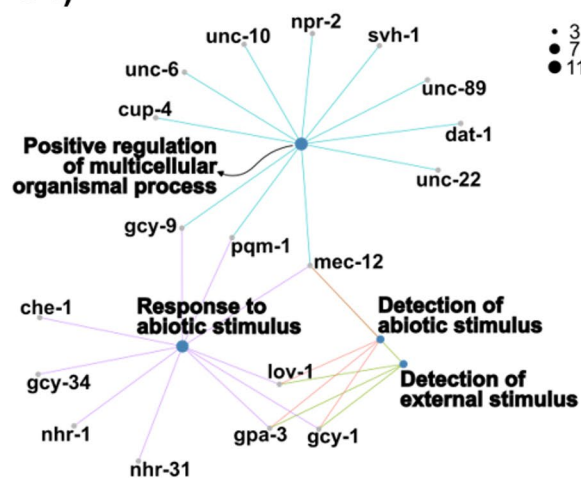
Gene-concept network analysis

We determined the gene-concept networks of the most represented GO categories for each cluster to evidence the relation between the GO categories and the link to characterized *C. elegans* genes. Thereafter, we identified the most interactive genes within each network. The gene-concept network of differentially enriched BPs for M31- and M88-derived clusters is provided in Figs. 7 and 8, respectively. Information about differentially regulated ($|\log_2FC| \geq 1$, $P \leq 0.05$) core gene models belonging to different clusters is available in Table 3. Additionally, details on the gene-concept network of commonly enriched BPs is deposited in supplementary Fig. S3 and S4 as well as supplementary tables S10 and S11.

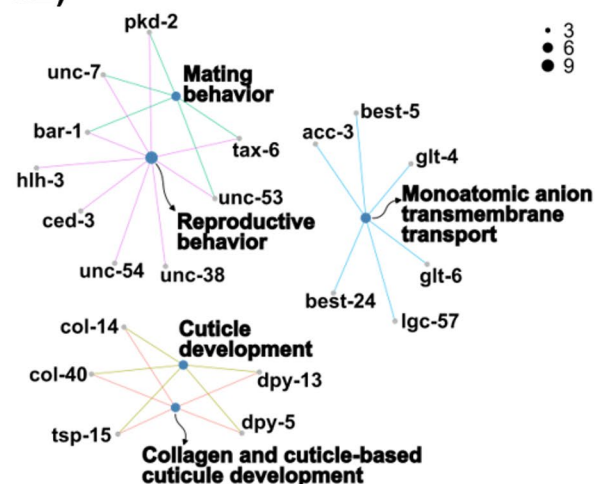
Genotypic differences between M31 and M88 mutants and expression of polymorphic gene models

We compared the SNPs identified in mutants M31 and M88 with their donor line IL3 and found 22 and 24 exclusive SNPs in the two mutants, respectively (Fig. 10A). SNPs information has been included in the work of Wang et al. 2023. For each polymorphism, the position of the SNP on the genome scaffolds of the *H. bacteriophora* TT01 sequence was determined to screen for neighboring gene models. Within the evidenced gene models, 23 show

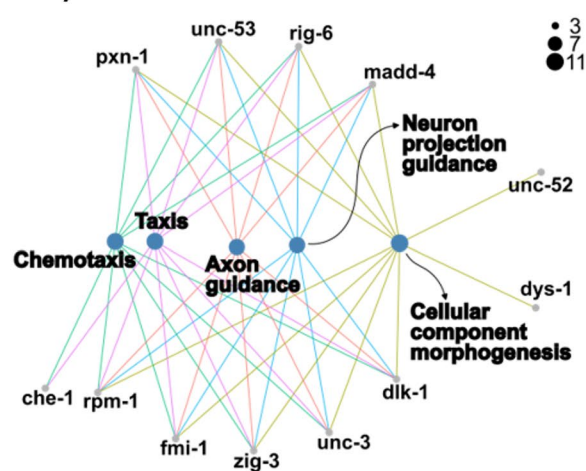
b1)



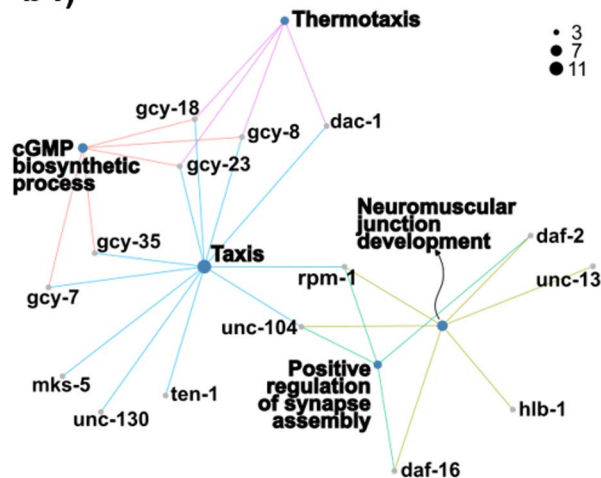
b2)



b3)



b4)



b6)

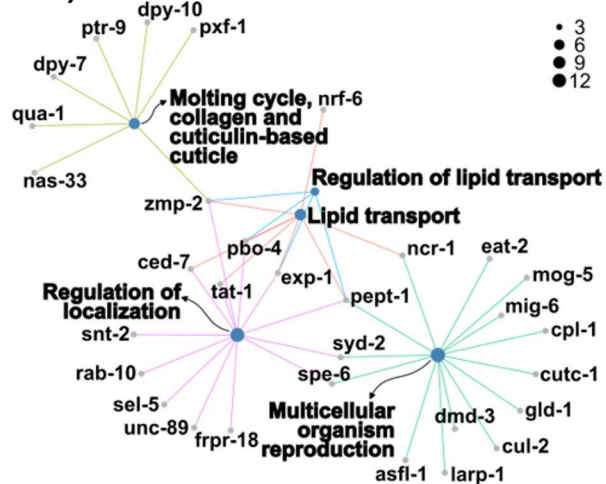


Fig. 8 Gene-concept network of enriched gene ontology (GO) biological process (BP) categories based on M88 exclusive gene models for each regulation cluster (Fig. 3). The five most enriched BP GO terms are depicted for each regulation cluster along the *Caenorhabditis elegans* homologous gene models related to the given categories. The dots indicate the enriched count of clustered genes in each GO term. Clusters designation: top left for each network

differential regulation patterns ($|\log_2\text{FC}| \geq 1$, $P \leq 0.05$) in M31 or M88. These gene models include candidate genes that have been reported to regulate the DJ recovery process (e.g., g3561, Fig. 10B). The overview of exclusive SNPs identified in M31 and M88 is deposited in supplementary Tables S12 and S13, respectively. The differentially regulated transcripts neighboring exclusive SNP in M31 or M88 are deposited in supplementary Table S14.

Discussion

In this study, we selected two *H. bacteriophora* mutant lines, M31 and M88, with opposite DJ recovery phenotype (Wang et al. 2023) as experimental material. We observed morphologic and expression responses upon *Photorhabdus* supernatant exposure leading to DJ recovery within an early narrow timeframe (0.5–6 h). We clustered the observed gene expression in six different patterns and carried out GO enrichment and network analyses based on information derived from the model nematode *C. elegans*. Additionally, we cross-linked information between SNPs and expression levels. In the coming sections, we comment in detail on specific results and link them to available reports. Throughout the sections, links to supplementary data are also provided.

Early responses and morphological changes

As the first morphological reaction upon supernatant exposure, we determined the shedding of the pre-dauer J2d cuticle in both, M31 and M88 mutants, despite the later differences in DJ recovery observed. During the host infection process, *H. bacteriophora* DJ also lose their secondary cuticle while entering the insect. Within 15 min after invasion, DJ react to immunosuppressive factors in the hemolymph and the symbiotic bacteria are released within the first 30 min after invasion (Ciche and Ensign 2003; Ebrahimi et al. 2011). Antagonistic proteins involved in the immune response of insects infected with *H. bacteriophora* have been also detected at very early stages after infection (Arefin et al. 2014; Castillo et al. 2015; Kenney et al. 2019, 2020). Thus, *H. bacteriophora* DJ need to react fast upon contact with the insect food signals leading to DJ recovery and bacterial release, to repress the host's immune response. In our experiments, the DJ were exposed to bacterial supernatant instead

of insect hemolymph. Both mutant lines shed their J2d cuticle within 1–3 h after supernatant exposure, and nematode's oesophagus underwent further activation (Fig. 1A). Despite the different conditions (bacterial supernatant vs in vivo infection and haemolymph contact), the shedding of the J2d cuticle appears to be tightly associated with the triggering the DJ recovery during the infection process. However, M31 showed a significantly higher recovery after 72 h than M88 (Fig. 1B). Thus, we can postulate that the secondary cuticle shedding and further development appear to be non-coupled events in the recovery-deficient M88 mutant.

Use of the knowledge from characterized genes

We detected a large array of expression changes within more than 14,600 *H. bacteriophora* gene models. Previous research has already opened the door to identify putative DJ recovery related genes in *Heterorhabditis*. Several genes reported to regulate dauer recovery and formation in *C. elegans* were detected in *H. bacteriophora* (Bai et al. 2013; McLean et al. 2018). On the other side, as seen in *H. bacteriophora* works analyzing DJ incubated in insect hemocoel, a large proportion of gene models homologous to parasitic nematodes are reported to be still non-characterized (Bai and Grewal 2007; Moshayov et al. 2013; Vadnal et al. 2017). This fact can represent a disadvantage since these uncharacterized gene models are not linked to functional databases. However, in the present study, we have determined the expression dynamics of 1074 *C. elegans* homologs, and linked many of them to predicted biological functions. However, as reported by Gilabert et al. (2016) by analyzing the conservation of the Dauer-related pathway across 24 nematode species, the knowledge transfer from the model nematode may be restricted by the sequence diversity between the different clades of the phylum Nematoda.

Expression clusters and time frameshifts between M31 and M88

How biological processes are arranged over time, was one of the main questions addressed in the present study. Dissimilar to studies in which only two conditions are analyzed, the present study reports a dynamic process of the expression in the two analyzed *H. bacteriophora* mutants. The expression data for M31 and M88 were grouped into six different regulation clusters via fuzzy c-means algorithm (Kumar and Futschik 2007). In agreement with the morphological results, the cuticle development and molting-related GO BPs were enriched in both, M31 and M88. However, this enrichment presents a time frameshift in M88 compared to M31 (cluster a1 vs b2, Fig. 4). Thus, fine tuning expression arrangements cannot be excluded from playing a major role

Table 3 Core genes in *Heterorhabditis bacteriophora* mutant lines M31 and M88 for different regulation clusters

Gene model	Homologous protein information			Cluster	
	Uniprot AC	Gene name	Protein name	M31	M88
g3182^a	P18834	<i>col-14</i>	Cuticle collagen 14	a1	b2
g8815	Q9XVM9	<i>tsp-15</i>	Tetraspanin-15	a1	b2
g13157^b	P34804	<i>col-40</i>	Cuticle collagen 40	a1	b2
g12219	Q23490	<i>mlt-7</i>	Peroxidase mlt-7 precursor	a1	–
g1034^b	G5EFY7	<i>pqm-1</i>	Zinc finger transcription factor pqm-1	a2	b1
g200^{a,b}	P08124	<i>sqt-3</i>	Cuticle collagen 1 precursor	a2	–
g309	Q20930	<i>mig-17</i>	Metalloprotease mig-17 precursor	a2	–
g8904^b	G5EC36	<i>lim-7</i>	LIM/homeobox protein lim-7	a2	–
g10031^b	Q19787	<i>bed-3</i>	Zinc finger BED domain-containing protein 3	a2	–
g10459	Q93847	<i>wdr-5.2</i>	WD repeat-containing protein wdr-5.2	a2	–
g10830	Q21192	<i>lim-6</i>	LIM/homeobox protein lim-6	a2	–
g5100	Q23369	<i>best-22</i>	Bestrophin homolog 22	a3	–
g8889	P48182	<i>acr-2</i>	Acetylcholine receptor subunit beta-type acr-2 precursor	a3	–
g11941	Q9BMK9	<i>clh-3</i>	Chloride channel protein clh-3	a3	–
g2136	Q59E83	<i>npr-22</i>	Neuropeptide receptor 22	a4	b1
g5299	Q17423	<i>che-12</i>	Crescerin-like protein che-12	a4	–
g11697	Q09624	<i>lov-1</i>	Location of vulva defective 1 precursor	a5	b1
g12230	P92006	<i>gcy-34</i>	Soluble guanylate cyclase gcy-34	a5	b1
g7675^a	O02298	<i>gcy-35</i>	Soluble guanylate cyclase gcy-35	a5	b4
g11675	Q966L8	<i>che-1</i>	Transcription factor che-1	a5	b1/b3
g5914	Q23551	<i>unc-22</i>	Twitchin	a6	b1
g2512^b	Q05062	<i>cdc-42</i>	Cell division control protein 42 homolog precursor	a6	–
g8651^b	O61787	<i>inx-16</i>	Innexin-16	a6	–
g10410	O18214	<i>mab-3</i>	Protein male abnormal 3	a6	–
g12035	P12845	<i>myo-2</i>	Myosin-2	a6	–
g339	P28052	<i>gpa-3</i>	Guanine nucleotide-binding protein alpha-3 subunit	–	b1
g3119	G5EDK5	<i>fmi-1</i>	Cadherin EGF LAG seven-pass G-type receptor fmi-1 precursor	–	b3
g118	Q9XWN9	<i>dmd-3</i>	Doublesex- and mab-3-related transcription factor dmd-3	–	b6
g225	G5EEB1	<i>frpr-18</i>	FMRFamide peptide receptor frpr-18	–	b6
g7018	O76840	<i>mig-6</i>	Papilin precursor	–	b6
g8189	P35800	<i>dpy-10</i>	Cuticle collagen dpy-10 precursor	–	b6
g8463	Q17603	<i>asf1-1</i>	Probable histone chaperone asf-1-like protein	–	b6
g12319	Q95PZ9	<i>spe-6</i>	Serine/threonine-protein kinase spe-6	–	b6
g12533	Q09225	<i>nrf-6</i>	Nose resistant to fluoxetine protein 6 precursor	–	b6

The gene models and respective *C. elegans* homologs are denoted. Gene models are based on the annotation of the *H. bacteriophora* (strain TT01) genome draft (PRJNA13977.WBPS16) from McLean et al (2018). These gene models resulted differentially regulated ($|\log_2\text{FC}| \geq 1$, $P \leq 0.05$) in both mutant lines. The gene models with the highest differences in regulation are highlighted in bold and are postulated as candidate genes that may underly the phenotype differences between M31 and M88. The expression dynamic changes are presented in Fig. 9

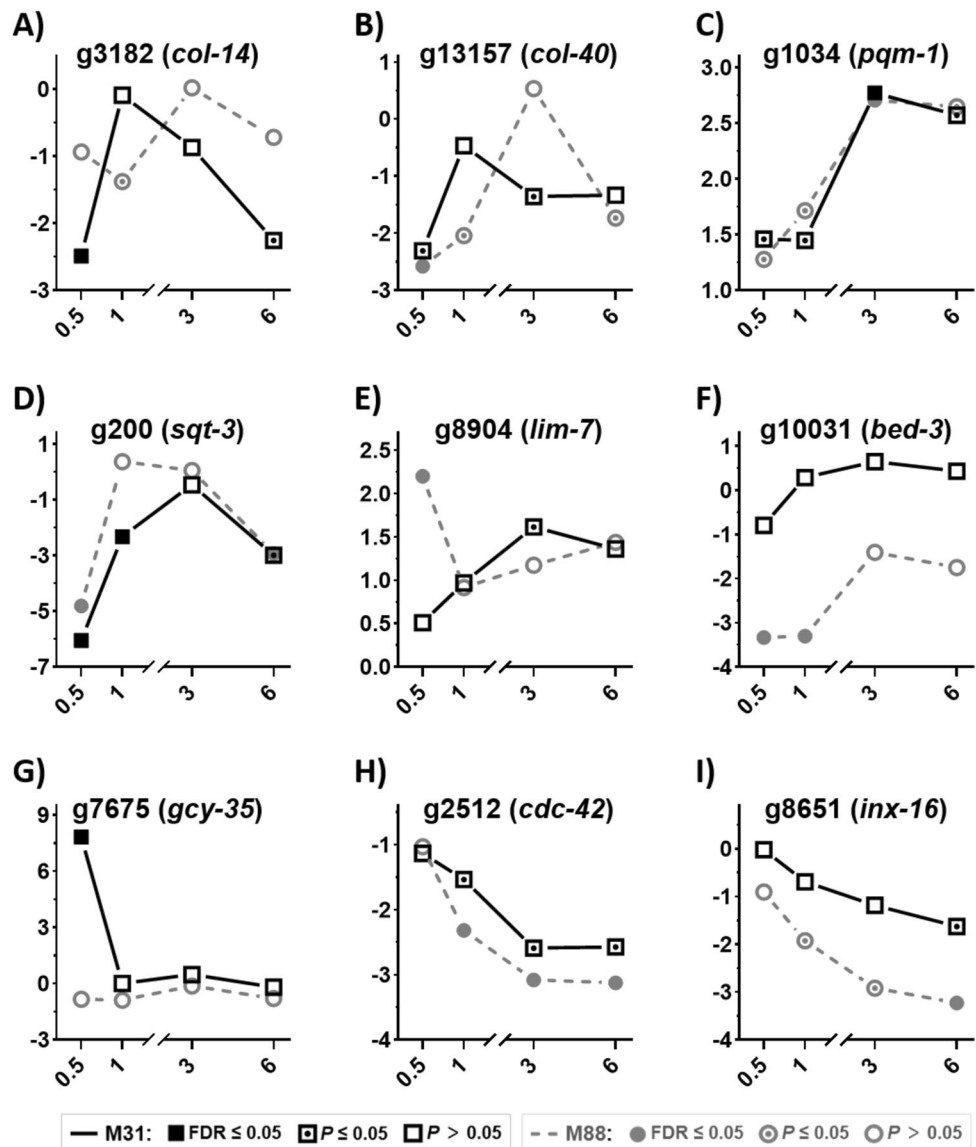
^{a,b}Depict the FDR value of highly regulated ($|\log_2\text{FC}| \geq 1$) gene model is at 0.05 level in *H. bacteriophora* mutant line M31 and M88, respectively

in DJ recovery regulation in *H. bacteriophora*. As example, two gene models, g3182 and g13157, were differentially regulated in both mutant lines (Fig. 9: A and B). These gene models are homologous to the cuticle-collagen related genes *col-14* and *col-40*, respectively, which are expressed during *C. elegans* juvenile molting (Johnstone and Barry 1996; Lažetić and Fay 2017). Further on, gene models that appear

to be early induced in M88 (Fig. 4: b1) are rather downregulated in M31 (cluster a4–a6; Fig. 5), as exemplified by the *C. elegans* homologs *npr-22* (g2036), *lov-1* (g11697) and *unc-22* (g5914) (Table 3).

Cuticle-involved gene models remained less represented in the M88 mutant. Only few of them were found in cluster b6 (6 h downregulation valley) in M88 (Fig. 8). For example,

Fig. 9 Example of core gene models included in Fuzzy clusters along their regulation patterns. Panels A to I depict *Heterorhabditis bacteriophora* gene models based on the nomenclature of McLean et al. (2018) complemented by the *Caenorhabditis elegans* homologous gene (in brackets). Abscissa: exposure time (h) to *Photorhabdus laumondii* (strain DE2) supernatant, ordinate: log₂FC. The name of *C. elegans* homologous genes are included in brackets



dyp-10 (g8189), which is required for maintaining cuticle structure and barrier function in *C. elegans* (Sandhu et al. 2021). Our results allow to postulate that the *H. bacteriophora* DJ detect the “food signal” stimulus from *Photorhabdus* supernatant within 0–1 h, and immediately switch on the molting process.

In later stages after bacterial supernatant exposure (Cluster 3, upregulation peak at 6 h), an enrichment of signal transmission related GO processes was detected in the recovery constitutive line M31. Among the genes found within this category, the gene model homologous to *C. elegans* *acr-2* (g8889) encodes a neuronal acetylcholine receptor that regulates the muscle excitation and inhibition in the model nematode (Jospin et al. 2009). Sensory neurons have been reported to be critical for dauer exit in *C. elegans* (Pandey et al. 2021), and their importance for host seeking in EPN has as well been proven (Hallem et al. 2011).

Concerning genes included in clusters showing either early- or later downregulation (clusters 4–6), both mutant lines are enriched for a wide array of regulation and signaling GO BPs. For example, the gene model homologous to *inx-16* of *C. elegans* is reported to be responsible for intestinal muscle contraction and is expressed at high levels in the intestine throughout the lifespan of the model nematode (Altun et al. 2009).

Expression of *C. elegans* insulin/IGF-1 signalling related genes

Concerning characterized pathways in *C. elegans*, insulin/IGF-1-like signalling (IIS) pathway has been identified as one of the main regulators downstream the activation of the FOXO transcription factor DAF-16 (Hu 2018). Bai et al. (2013) analysed the *H. bacteriophora* genome and found 19

Table 4 Gene models in the vicinity of the exclusive SNPs from *Heterorhabditis bacteriophora* mutant lines M31 and M88

Source of allele	Gene model	Homologous protein information			Organism
		Uniprot AC	Gene name	Protein name	
M31	g841	G5EEE9	<i>gcy-23</i>	Receptor-type guanylate cyclase gcy-23 precursor	<i>C. elegans</i>
	g3030	Q09225	<i>nrf-6</i>	Nose resistant to fluoxetine protein 6 precursor	<i>C. elegans</i>
	g7809	Q18248	<i>dmd-4</i>	Doublesex- and mab-3-related transcription factor dmd-4	<i>C. elegans</i>
	g8757	Q22127	<i>nhr-40</i>	Nuclear hormone receptor family member nhr-40	<i>C. elegans</i>
M88	g14604	Q2HJN8	<i>eft-2</i>	Elongation factor 1-alpha 2	<i>Oscheius tipulae</i>
	g1974	A0A2A2KBF5	WR25_03063	SERPIN domain-containing protein	<i>Diploscapter pachys</i>
	g3561^b	A0A3P7JFX2	SVUK_LOCUS12072	Uncharacterized	<i>Strongylus vulgaris</i>
	g8801	A0A368G582	ANCCAN_15632	MULE domain-containing protein	<i>Ancylostoma caninum</i>
	g9641	A0A368G582	ANCCAN_15632	MULE domain-containing protein	<i>A. caninum</i>

Gene models predicted by the annotation of the *H. bacteriophora* (strain TT01) genome draft (PRJNA13977.WBPS16) from McLean et al (2018). Gene model names of highly homologous proteins from different organisms are provided. The gene models differentially regulated in both mutant lines are included ($\log_2\text{FC} \geq 1$, $P \leq 0.05$)

^bDepicts the FDR value of highly regulated ($\log_2\text{FC} \geq 1$) gene model is at 0.05 level in *H. bacteriophora* mutant line M88

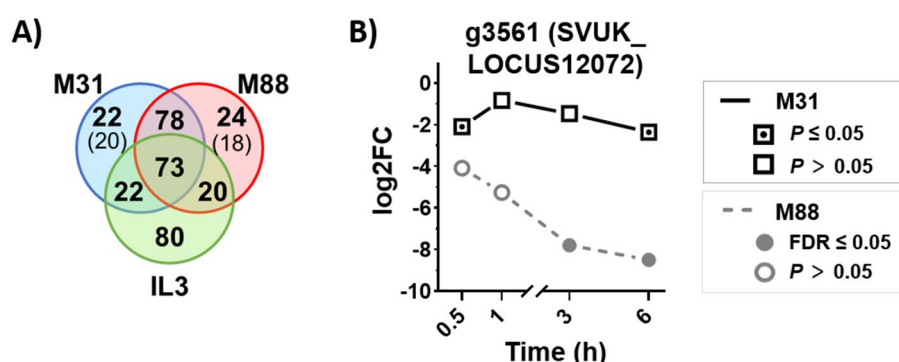


Fig. 10 Common and exclusive EMS-SNPs alleles determined in the *Heterorhabditis bacteriophora* mutant lines (M31 and M88) and the donor line IL3 (A). Numbers in brackets depict SNPs found inside or neighboring (within 2,500 bp up- or down- stream of the

SNP) predicted gene models. **B** Example of distinct regulation pattern observed in the gene model g3561 harboring a SNP with an M31 exclusive allele. Differential expression thresholds are denoted for M31 and M88 per separate

of 23 *C. elegans* IIS pathway genes. Moshayov et al (2013) verified two IIS-associated genes that were activated in *H. bacteriophora* during DJ recovery. Genes related to the *C. elegans* IIS pathway regulation were also found in our GO network analysis (Fig. 7 and 8; supplementary Fig S3). From them, five gene models were differentially regulated, and three are at FDR < 0.05 level. Among the highest differentially regulated gene models, g1034 (*pqm-1*) was found to be upregulated within 0.5 to 3 h after supernatant exposure (Fig. 9: C). The PQM-1 protein in *C. elegans*, required for longevity, development and dauer recovery, controls development genes by binding to the DAF-16-associated elements (Tepper et al. 2013). Several other IIS-related gene models also show downregulation upon supernatant exposure. For instance, *hil-1*, one of the six DAF-16 target genes (Honjoh

et al. 2009), was only found expressed in dauers (Hall et al. 2010) and fasting *C. elegans* adults (Uno et al. 2013). Interestingly, the homologous gene model g10242 was keeping almost constitutive level in M31 and was down-regulated through all the time series in M88 (supplementary Fig S4: B). Somogyvári et al (2018) found that heat-shock protein (Hsp90) coded by gene *daf-21* is required for *C. elegans* fertility, development and nematode longevity by regulation of the DAF-16/FOXO interaction. The *daf-21* was characterized in *H. bacteriophora* by Ciche and Sternber (2007). The authors silenced *daf-21* by RNA interference (RNAi) and produced sterile animals with abnormal gonad morphology. Adhikari et al (2009) found that DJ with higher virulence against *G. mellonella* have higher *Hb-daf-21* expression. Within our observed timeframe the expression of the *daf-21*

homolog, g24777, decreased steadily. The observed expression patterns suggest that the physiological importance of several of the genes mentioned above trespasses the species and habit borders in nematodes.

Expression of genes reported as responsive upon the insect immune responses

Concerning genes that are responsive to the insect immune system, previous transcriptome analyses of *H. bacteriophora* report induction upon recovery. Those genes have been also found in other parasitic nematodes, for example genes coding for C-type lectin in *Ancylostoma ceylanicum* (dog hookworm), *Ascaris suum* (pig roundworm) and *Meloidogyne javanica* (plant-parasitic nematode) (Moshayov et al. 2013). Interestingly, no gene related to the host immunosuppressive factors has been found to react upon supernatant exposure. This finding allows us to postulate that already at this early stage, the insect's and the bacterial food-signals cause distinct reactions in *H. bacteriophora*.

Expression changes and DNA polymorphisms

Concerning polymorphisms inside or in the vicinity of differentially regulated genes, M31 and M88 mutants were derived from the same donor line IL3, and a large number of SNPs was determined for these materials (Wang et al. 2023). We compared the common and exclusive SNP between mutants and their donor line and found annotated genes near each SNP (supplementary Table S14). For most of the SNPs, *C. elegans* or parasitic nematode homologous genes were found in the vicinity. By analyzing SNPs from which the alleles were exclusive to either M31 or M88, we determined a group of genes that can be candidates for further analyses (Table 4).

Candidate genes for further studies and conclusion

By combining the cluster and the gene-concept network analyses, a set of nine gene models (Fig. 9) with distinct regulation profiles (cluster and gene-concept network) in both mutants is proposed to be further characterized. Nowadays, robust techniques for the targeted functional characterization of genes, such as CRISPR-Cas9, are well established in *C. elegans* (Kim and Colaiácovo 2019), and deserve to be optimized in EPN. The homologous genes in *C. elegans* include collagen and cuticle function related genes *col-14* (g3182), *col-40* (g13157) and *sqt-3* (g200) (Johnstone and Barry 1996; Lažetić and Fay 2017; Lang and Lundquist 2021), the dauer formation IIS pathway control factor *pqm-1* (g1034) (Tepper et al. 2013), the LIM-homeodomain (LIM-HD)-encoding gene *lim-7*

(g8904) (Voutev et al. 2008), *bed-3* (g10031), which is involved in organogenesis (Inoue and Sternberg 2010; Goh and Inoue 2018), the sensory neuron control factor *gcy-35* (g7675) (Gray et al. 2004), the cellular and embryonic polarity regulator *cdc-42* (g2512) (Wang et al. 2017), and *inx-16* (g8651), related to intestinal muscle contractions (Peters et al. 2007). We assume that their differential regulation can reflect processes underneath the phenotypic differences between M31 (high mutant rate) and M88 (recovery deficient) mutants.

Seven gene models (supplementary Fig. S4) with similar expression patterns between M31 and M88 deserve also special attention, although they may not be totally crucial to underly the phenotypic differences between M31 and M88. These include two genes, *hil-1* (g10242) and *daf-21* (g2477), both involved in the IIS pathway (Uno et al. 2013; Somogyvári et al. 2018). The collagen synthesis gene *col-19* (g12756) (Thein et al. 2003), and *dpy-22* (g6976) are reported to regulate transcription factors during development (Yoda et al. 2005). The *mup-2* gene (g112) is essential to striated body wall muscle cell positioning (Myers et al. 1996). The *asp-6* homologous gene (g2100) codes for a phosphorylcholine-substituted protein (Lochnit et al. 2006). The gene *pbo-5* (g2542) is reported to be required the muscle acidification response in *C. elegans* (Beg et al. 2008). In addition, the SNP mutations neighboring gene model g1974 (Fig. 10B), which is homologous to WR25_03063 from *Diploscapter pachys*, can be considered targets for further research.

Conclusion

We can confirm that *H. bacteriophora* DJ can rapidly detect and react upon the “food signal” to initiate DJ recovery. Moreover, we provide evidence that there is already at this early stage a differentiation between the insect- and the bacterial- “food signal”. Thus, future phenotypic selections for industrial reproductive potential in this EPN should contemplate this fact. The present results open doors for the improvement of the industrial production of EPN. DJ recovery in large bioreactors may be improved by breeding and selection of specific nematode genotypes and expression-phenotypes favouring the early signal detection and early morphologic responses. Future approaches using the present results should aim to characterize the function of relevant genes and find the compounds activating the early expression induction in crucial regulators.

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Author contributions RUE and CM conceived the experiments and the project. ZW, FG, and CM performed the experiments. ZW and CM analyzed the data. ZW wrote the first draft of the manuscript, CM and RUE read and commented on previous versions of the manuscript. All authors approved the manuscript.

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Data availability The RNA-sequencing data reported in this paper are available in Sequence Read Archive (SRA) data of the National Center for Biotechnology Information (NCBI) database under the submission number SUB13935184. Biosample accessions: SAMN38034906, SAMN38034907, SAMN38034908, SAMN38034909, SAMN38034910, SAMN38034911, SAMN38034912, SAMN38034913, SAMN38034914, SAMN38034915. The *H. bacteriophora* TT01 reference genome is available at wormbase.org (PRJNA13977.WBPS16). This assembly has been masked with the gene models predicted by McLean et al (2018). The gene models and annotations were deposited in supplementary Table S1.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval This study does not contain any studies with human participants or vertebrate animals performed by any of the authors.

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