

In vivo transcriptome analysis provides insights into host-dependent expression of virulence factors by *Yersinia entomophaga* MH96, during infection of *Galleria mellonella*

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Abstract

The function of microbes can be inferred from knowledge of genes specifically expressed in natural environments. Here, we report the *in vivo* transcriptome of the entomopathogenic bacterium *Yersinia entomophaga* MH96, captured during initial, septicemic, and pre-cadaveric stages of intrahemocoelic infection in *Galleria mellonella*. A total of 1285 genes were significantly upregulated by MH96 during infection; 829 genes responded to *in vivo* conditions during at least one stage of infection, 289 responded during two stages of infection, and 167 transcripts responded throughout all three stages of infection compared to *in vitro* conditions at equivalent cell densities. Genes upregulated during the earliest infection stage included components of the insecticidal toxin complex Yen-TC (*chi1*, *chi2*, and *yenC1*), genes for rearrangement hotspot element containing protein *yenC3*, cytolethal distending toxin *cdtAB*, and vegetative insecticidal toxin *vip2*. Genes more highly expressed throughout the infection cycle included the putative heat-stable enterotoxin *yenT* and three adhesins (usher-chaperone fimbria, filamentous hemagglutinin, and an AidA-like secreted adhesin). Clustering and functional enrichment of gene expression data also revealed expression of genes encoding type III and VI secretion system-associated effectors. Together these data provide insight into the pathobiology of MH96 and serve as an important resource supporting efforts to identify novel insecticidal agents.

Keywords: transcriptome; entomopathogenic bacteria; insecticidal toxins; *Galleria mellonella*; *Yersinia* virulence factors

Introduction

For successful invasion, colonization, and bioconversion of host tissues, bacterial pathogens must acclimate to the host environment via alterations in patterns of gene expression (Crawford *et al.* 2010; Tran *et al.* 2013; Heroven and Dersch 2014; Fang *et al.* 2016; Reniere 2018). Knowledge of those genes activated specifically within host tissues allows insight into the genetic determinants of *in vivo* colonization and pathogenicity. An often used strategy involves transcriptional analysis of pathogen gene expression (transcriptomics) based on patterns of mRNA transcript abundance during infection (Deng *et al.* 2018; Valenzuela-Miranda and Gallardo-Escárate 2018; Nobori *et al.* 2018; Arenas *et al.* 2019; Griesenauer *et al.* 2019; Haueisen *et al.* 2019; Ibberson and Whiteley 2019; Sun *et al.* 2019; Tang *et al.* 2019; Fan *et al.* 2020; Luo *et al.* 2020).

In vivo transcriptomic approaches have been widely applied to human pathogenic bacteria using cell lines and model hosts (Yan *et al.* 2013; Avican *et al.* 2015; Baddal *et al.* 2016; Damron *et al.* 2016; Westermann *et al.* 2016; Nuss *et al.* 2017). Additionally, time-resolved *in vivo* transcriptomics have increased

understanding of the function of bacterial pathogens during natural infection processes (Aprianto *et al.* 2016; Westermann *et al.* 2019; Li *et al.* 2020; Luo *et al.* 2020; Tang *et al.* 2020). However, similar strategies are yet to be applied to pathogens infecting insects, despite the obvious potential to identify new insect-active agents.

Yersinia entomophaga MH96 is an entomopathogenic bacterium that was isolated from *Costelytra giveni* (Coleoptera: Scarabaeidae) (Hurst *et al.* 2011a), an endemic and economically significant pasture pest in New Zealand (Ferguson *et al.* 2019). Development of MH96 as a biopesticide has proven consistent pathogenesis by *per os* challenge against *C. giveni*, as well as a wide range of coleopteran, lepidopteran, and orthopteran species (Hurst *et al.* 2011a, 2014, 2015, 2019; Marshall *et al.* 2012). The key virulence determinant of MH96 is an insecticidal toxin complex (TC) called Yen-TC, which facilitates ingress of MH96 through the insect midgut (Hurst *et al.* 2011b, 2014; Marshall *et al.* 2012) and is constitutively produced and secreted by MH96 when grown in broth culture at temperatures of 25°C or lower (Hurst *et al.* 2011b). In addition to the orally active insecticidal activities of Yen-TC, structural characterization and understanding the molecular mechanisms of

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pore-formation and translocation of cytotoxin has been the focus of much research (Landsberg et al. 2011; Busby et al. 2012, 2013; Piper et al. 2019).

Despite the importance of Yen-TC in MH96's ability to breach the insect midgut, additional investigations have suggested that as yet unknown virulence factors (VFs) are deployed during intrahemocoelic infection of *Galleria mellonella*. As a prime example, Hurst et al. (2015) showed that both wild-type MH96 and Δ TC (a Yen-TC deficient strain) have the same median lethal dose (LD_{50}) of only ~ 3 cells and were equally as effective at stopping the host's phenoloxidase immune response when injected directly into the hemocoel of *G. mellonella* (Hurst et al. 2015). Genes for many other diverse putative VFs, including known insecticidal toxins and effectors, secretion and iron acquisition systems, proteolytic enzymes, and adhesins have also been identified in the MH96 draft genome (Hurst et al. 2016). The extensive repertoire of putative VF-encoding genes represents an opportunity to utilize *in vivo* transcriptomics to further characterize this entomopathogen's mode of action against insects, especially during intrahemocoelic infection of *G. mellonella*.

Larvae of the greater wax moth *G. mellonella* (Lepidoptera) have emerged as a valuable model host for study of microbial infection and innate immunity (Desbois and McMillan 2015; Champion et al. 2016; Tsai et al. 2016). Yet, to date, pathogen *in vivo* transcriptome studies from *G. mellonella* have been confined to fungal pathogens only, including *Candida albicans* (Amorim-Vaz et al. 2015) and entomopathogenic *Beauveria bassiana* (Chen et al. 2018). Since the immune system of *G. mellonella* consists of both cellular (i.e., hemocytes) and humoral components (Wojda 2017; Singkum et al. 2019), the intrahemocoelic infection is useful to characterize host-dependent genome-wide transcriptional modulation by MH96 during three different stages of infection using a time-resolved approach.

Here, we focus on the interaction between MH96 and *G. mellonella*, using an *in vivo* transcriptomic strategy to identify pathogen genes activated under initial insect immunity directly following intrahemocoelic infection, as well as later septicemic and pre-cadaver stages of infection. Differential expression (DE) analysis comparing MH96 transcriptome under *in vivo* and *in vitro* conditions identified pathogen genes, including known and putative VFs, that significantly responded either at specific or multiple timepoints throughout the infection process. To further understand the pathobiology of MH96, time-resolved *in vivo* versus *in vitro* fold-changes were clustered and functionally annotated using a database of known bacterial VFs. Particularly notable was upregulation of multiple genes encoding predicted adhesins, toxins, and type III secretion system (T3SS) and T6SS translocated effectors.

Materials and methods

Intrahemocoelic infection of *G. mellonella* and pathogen RNA enrichment

Larval *G. mellonella* (Biosuppliers, Auckland, New Zealand) was provided with fresh diet of liquid honey, glycerol, Farex® brand baby rice cereal, and granular yeast for up to 1 week. Healthy final instar larvae of similar lengths (>20 mm, between 0.15 and 0.30 g) were selected and immobilized on ice. For intrahemocoelic infection, MH96 was grown overnight (18 h) in 3-ml Luria Broth Base (Invitrogen) with shaking (250 rpm) at 30°C, and then cultures were diluted in phosphate buffer solution (Sigma) and placed on ice prior to injection. Larvae were surface sterilized with 70% ethanol and then injected below the third right leg with

10 μ l of inoculum using a 30 gauge 12-mm needle on a 1-ml tuberculin syringe (Terumo) with a microinjector.

Dilutions of MH96 ranging from ~ 157 up to 2×10^7 colony forming units (CFUs) were injected into *G. mellonella* depending on the stage of infection for each *in vivo* treatment (i.e., early, middle, or late) (Supplementary Table S1). To ensure sufficient quantity of pathogen RNA for *in vivo* transcriptomics, early *in vivo* treatments (i.e., initial insect immunity) required that larvae were injected with a greater amount of pathogen cells than would be expected to naturally occur during early intrahemocoelic infection. Following injection, hemolymph from early *in vivo* treatment larvae was collected within 75- to 90-min post-intrahemocoelic infection, which was considered sufficient time to trigger a measurable host-dependent transcriptional response by MH96. To first define the respective 18–19 and 26–30 h incubation times for the middle (i.e., septicemic) and late infection treatments (i.e., pre-cadaver) a 48 h post-inoculation (hpi) growth curve was obtained following injection of ~ 345 CFUs (Supplementary Figure S1). MH96 CFU/larva were then enumerated on *Yersinia* semi-selective medium from dilutions of homogenized whole infected larvae collected at specific timepoints post-inoculation. Consistent with the 48 hpi growth curve, larvae for middle, and late *in vivo* treatments were injected on separate days with either ~ 157 or 693 CFUs and collected after 18–19 and 26–30 hpi, respectively.

Following injection, the larvae were placed in petri-dishes and incubated at 25°C within sealed plastic bags with moistened paper towel. After incubation, hemolymph was obtained by puncturing the dorsal cuticle near the second segment with a needle. Hemolymph was pooled from five to six individuals, yielding ~ 150 - μ l total volume. Four biological replicate samples were collected from each of the three *in vivo* infection stages (12 *in vivo* samples total). Pooled hemolymph samples were collected directly into microcentrifuge tubes with 300 μ l of RNAprotect Bacterial Reagent (Qiagen), vortexed for 5 s, incubated at room temperature for 5 min, and then centrifuged at $300 \times g$ for 5 min at 4°C to pellet hemocytes and cell debris. The supernatant was carefully separated from the host cell pellet by pipette and bacterial cells were then pelleted by centrifugation (10 min at $5000 \times g$ at ambient temperature of $\sim 22^\circ\text{C}$). Following supernatant removal, the pellets were stored at -80°C . To estimate the CFUs per larva, serial dilutions from *G. mellonella* homogenate were plated on a *Yersinia* semi-selective medium and all *Yersinia*-positive colonies were counted within 24 h (five separate larva per treatment; surface sterilized with 70% ethanol). The weight of each larva was measured enabling determination of CFU per gram larval homogenate.

Control *in vitro* RNA samples were collected separately from broth cultures and two independent biological replicate samples were collected for each of the *in vitro* treatments—lag, exponential, and stationary growth phases (6 *in vitro* samples total). For *in vitro* growth MH96 was grown overnight in 3-ml broth at 30°C with shaking (250 rpm). The next day, flasks containing 50-ml fresh broth were inoculated with 1% overnight culture and grown at 25°C until samples were collected after 4 h 45 min (2 ml), 6 h (1 ml), and 9 h (1 ml) corresponding to specific cell densities of $3.2 \pm 0.1 \times 10^7$, $2.9 \pm 0.9 \times 10^8$, and $4.4 \pm 0.1 \times 10^9$ CFU ml^{-1} (comparable to CFU g^{-1} assessed from *in vivo* *G. mellonella* hemolymph RNA-seq samples), respectively. *In vitro* RNA samples were stabilized in RNAprotect Bacterial Reagent (Qiagen) according to the manufacturer's protocol and also stored at -80°C .

RNA extraction, library preparation, and sequencing

Within 1 week of collection, RNA samples were re-suspended in 100- μ l Tris-EDTA buffer (10-mM Tris-HCl, 1-mM EDTA; pH 8.0) (Ambion) containing 15-mg ml⁻¹ lysozyme (Roche or Sigma-Aldrich) and 10- μ l proteinase K (Roche). The samples were incubated for 10 min, interrupted by 10 s vortex every 2 min to lyse the cells. The RNeasy mini kit (Qiagen) was used for RNA extraction and RNA clean-up, which included two separate DNA digestions (on-column then off-column) with DNase (Qiagen), all according to manufacturer's guidelines. RNA samples were then precipitated at -20°C overnight in three volumes 100% cold isopropanol with 1/10 volumes 3 M sodium acetate (pH 5.2), followed by two 70% cold ethanol washes. Purified RNA was re-suspended in RNase-free water and stored at -80°C.

RNA extract from early infection hemolymph contained approximately half the amount of total RNA required for sequencing, so early infection RNA samples were doubled such that each sample was comprised from a pool of 10–12 larvae, while middle and late infection RNA samples were from five or six pooled individuals. Sufficient removal of genomic DNA from the samples was determined by confirming lack of PCR amplification from *in vivo* RNA template using primers RecA_352F 5'-TCTCAGCCAGATACCGGTGA and RecA_987R 5'-CAGC AACATTTACGCAGCT for the housekeeping gene *recA*. Middle and late infection *in vivo* RNA samples containing ~7.2- μ g total RNA and early infection samples containing <7.2 μ g were stabilized on RNastable 1.5 microcentrifuge tubes (Biomatrix) by SpeedVac (Savant) at ambient temperature (~22°C) for 1.5 h.

Macrogen Korea carried out library preparation and sequencing. Following recovery from the RNastable tubes, RNA quantity and Integrity Number were determined using the 2200 TapeStation (Agilent Technologies). Host and bacterial ribosomal RNA (rRNA) was depleted from the *in vivo* samples using the ScriptSeq Complete Gold Kit (Epidemiology) (Illumina) then strand-specific cDNA libraries were prepared using the ScriptSeq RNA-Seq Library Preparation Kit (Illumina) according to the manufacturer's guidelines. The *in vitro* samples were prepared using the TruSeq stranded mRNA (Illumina) with the Ribo-Zero rRNA removal kit for Bacteria (Epicentre) according to standard protocol. To verify the size of the PCR enriched fragments, the template size distribution was assessed on the 2100 Bioanalyzer using a DNA 1000 chip (Agilent Technologies). The libraries were sequenced on the HiSeq2500 platform (Illumina) (Version HCS v2.2) to generate 101-bp paired-end reads.

Bioinformatic analysis

Adapter and barcode contamination were filtered using *Flexbar* (v2.4) against Illumina adapter and barcode reference sequences (Supplementary Tables S2 and S3). Reads were trimmed in "ANY" mode with minimum overlap of 5, maximum uncalled base of 1, and minimum quality threshold of 20 (Dodt et al. 2012). The *bbsplit.sh* function of the *BBSplit* package was used to quantify and remove contaminating rRNA sequences with minimum ratio of 0.5, minimum hit number of one, and maximum insertion/deletion length of 500 bp (Bushnell 2015). All libraries were screened against full-length 5S, 16S, and 23S rRNA sequence from the MH96 genome (see accession information below). To remove host sequences, *in vivo* libraries were also mapped against the *G. mellonella* partial 18S and 28S rRNA sequence retrieved from GenBank (Accessions: U65198.1, U65138.1, AF286298.1,

AF423921.1, and X89491.1) and the *G. mellonella* immune cDNA library (JG394435–JG406465) (Vogel et al. 2011).

Remaining paired-end reads were aligned to the MH96 genome (Accession: CP010029.1, GI: 1034308998) using *Rockhopper* (v2.03) in verbose mode. Alignment stringencies were increased from default values, such that allowable mismatch threshold was decreased from 15% to 10% of read length and the minimum seed was increased from 33% to 50% of read length. Any residual count data aligning to 5S, 16S, and 23S rRNA genes or any short (<50bp) non-coding RNA or anti-sense RNA were not considered further in the analysis.

Due to low alignment rates to the reference genome, most of the early infection *in vivo* libraries contained less than the minimal threshold of between 2 and 3 million transcripts per library determined by Haas et al. (2012) as necessary for adequate detection of differentially expressed genes in bacteria. Accordingly, the early infection *in vivo* libraries were investigated using the *iNEXT* R package (*nboot* = 50) (v2.0.18) (Hsieh et al. 2016) whether the sequence depth adequately captured the majority of MH96 low-abundance transcripts. The *RUVseq* R package (v1.16.0) was used to generate relative log expression (RLE) plots and conduct principle component analysis (PCA) (Risso et al. 2014). Upper-quartile normalized (Bullard et al. 2010) libraries were filtered for transcripts with greater than five aligned reads in at least two libraries.

Normalized expression data were then converted to log₂-counts-per-million (Log₂CPM) using the R package *limma* (v3.38.3) (Smyth 2004; Ritchie et al. 2015) and the mean-variance relationship was modeled using the "voom" method (Law et al. 2014). Genome-wide host-dependent responses by MH96 to both *in vitro* and *in vivo* conditions were visualized with *Circos* (v0.69) (Krzywinski et al. 2009) and putative genomic islands (GIs) were assigned using the webserver *IslandViewer 4* (Bertelli et al. 2017). To determine MH96 genes that significantly responded to *in vivo* conditions, a DE analysis was undertaken by comparing average transcript abundance between *in vivo* and *in vitro* libraries sharing equivalent cell densities (i.e., early infection vs lag growth in broth, middle infection vs exponential growth in broth, and late infection vs stationary growth in broth). Genes with significantly DE were defined by multiple testing with false discovery rate of 0.05 using the Benjamini and Yekutieli multiple test correction method (Benjamini and Yekutieli 2001) using *limma*.

Time-resolved average *in vivo* vs *in vitro* log₂CPM fold-change values for all genes with significantly DE identified between the *in vivo* and *in vitro* treatments (DE analysis described above) were clustered using the fuzzy c-means (FCM) algorithm (Futschik and Carlisle 2005) with the *MFuzz* R package (v2.42.0) (Kumar and Futschik 2007). A heuristic qualitative assessment was used to identify both the optimal FCM parameter (i.e., "fuzzifier") and number of clusters (Kumar and Futschik, 2007). The assessment considered both predicted operon structure (output from *Rockhopper*) and functional annotations assigned using homology-based search (BLASTx, e-value cutoff = 1 × 10⁻⁸) against the virulence factor database (VFDB) (accessed July 2018) (Chen et al. 2016).

MH96 host-specific factors were identified among clusters containing transcripts with comparably greater median log₂CPM *in vivo* fold-change values ($\geq \sim 2$) for at least one stage of growth during intrahemocoelic infection. Next, these host-specific factors were functionally characterized as putative VFs when significant sequence homology was identified against an already characterized VF found in the VFDB. Finally, functional categories of virulence were assigned to all of these putative VFs

primarily based on the VFDB-derived annotation, in accordance to our definitions found in Supplementary Table S4. When VFDB-derived annotations were not informative, additional information, such as top-scoring BLASTx hits from the GenBank non-redundant protein database, was used to assign a possible function or otherwise these hypotheticals were left unclassified.

Data availability

Sequence data and both raw and normalized count tables were deposited in the GenBank Geo Submission Omnibus under accession: GSE142509. All genes with significantly DE detected between *in vivo* vs *in vitro* time-resolved contrasts (early infection vs lag growth *in vitro*; middle infection vs exponential growth *in vitro*; late infection vs stationary growth *in vitro*) are provided in Supplementary Table S5. All functionally annotated putative VFs identified from host-specific fuzzy clusters are presented in Supplementary Table S6. All Supplementary figures, tables, and datasets (i.e., Supplementary Tables S5 and S6) associated with this study have been deposited in figshare: <https://doi.org/10.25387/g3.13206221>. Open-source R code for statistical analysis is available on github.com/damselflywingz/in_vivo_infection_series_fuzzy_RNA-seq.

Supplementary material is available at figshare DOI: <https://doi.org/10.25387/g3.13206221>.

Results

Experimental infection and RNA sequencing

MH96 was enriched from hemolymph at cell densities of 4.4×10^7 , 4.4×10^8 , and 5.0×10^9 CFU g^{-1} (Table 1). In order to distinguish host-specific gene induction by MH96 from response to general growth conditions, *in vitro* samples were collected across three growth phases at approximately equivalent cell densities to the *in vivo* samples (Table 1).

After quality trimming, adapter removal of host and rRNA sequences, the paired-end reads were aligned to the draft MH96 genome. Only an average of 9% of reads from early infection libraries could be aligned to the reference, which was lower by comparison to the average reads aligned from middle (58%) and late infection (59%), or *in vitro* libraries (84–93%) (Supplementary Figure S2 and Table S7). In order to determine if transcripts expressed in lower abundances were adequately captured within the early infection libraries, these libraries were analyzed by rarefaction, which predicted only a slight improvement in total transcript detection if sequencing depth were increased (Supplementary Figure S3). Based on this analysis, the *in vivo* early infection libraries were determined to have captured most MH96 transcripts produced across a dynamic range of conditions expected under these conditions and were considered saturated.

Preliminary data analysis explored the libraries for unwanted technical variation arising from batch effects and potential

outliers using both RLE plots and PCA (Supplementary Figures S4 and S5), respectively. Variation in sequencing depth between libraries was found to have affected the distribution of RLE values (Supplementary Figure S4A), which was largely controlled for by upper-quartile normalization (Supplementary Figure S4B). Even after normalization, the variation in RLE values from the stationary growth *in vitro* libraries was greater when compared to all other libraries indicating residual unwanted technical variation arose from this treatment. A clear separation between *in vitro* and *in vivo* libraries was determined by PCA of normalized count data (Supplementary Figure S5). Libraries consistently clustered by treatment, except one *in vivo* late infection library, which clustered more closely to the middle infection *in vivo* libraries and was identified as a biological outlier and removed from subsequent analysis.

DE of MH96's transcriptome during early, middle, and late infection stages

Comparison of average transcript abundances across three stages of growth identified 2397 differentially expressed genes that were either upregulated or downregulated between *in vivo* and *in vitro* libraries (Supplementary Figure S6 and Table S5). MH96 upregulated 1285 genes during intrahemocoelic infection of *G. mellonella*; 829 genes responded to *in vivo* conditions during at least one stage of infection, 289 responded during two stages of infection and 167 transcripts responded throughout all three stages of infection. While the majority of MH96 transcripts significantly upregulated during *in vivo* conditions represented protein-coding genes, 72 non-coding and 19 anti-sense RNAs were also more highly expressed *in vivo*. MH96 also upregulated 1243 genes under *in vitro* conditions, of which 131 were also found to be upregulated under *in vivo* conditions at different cell densities.

Regions of interest from the MH96 draft genome either known or suspected to encode VFs (Supplementary Table S8) were explored with respect to time-resolved genome-wide host-dependent transcriptional responses during intrahemocoelic infection (Figure 1). Several predicted GIs partially or fully overlapped with previously identified genomic regions of interest (i.e., unique region 1 overlaps Is.2, Rhs2 overlaps Is.5, T3SSYE1 overlaps Is.6, Rhs5 overlaps Is.11, T3SSYE2 overlaps Is.12, Rhs4 overlaps Is.14, unique region 2 overlaps Is.16, T2SS overlaps Is.18, and Rhs3/T6SS overlaps Is.21).

Wide-spread host-dependent upregulation of multigene clusters located within several regions of interest/predicted GIs, including unique regions 1 and 2, Rhs2/Is.5, T3SSYE2/Is.12, and T2SS/Is.18, was observed in MH96. Significantly higher expression for the entire T2SS island was measured during early and/or late infection stages compared to *in vitro* growth conditions. Significantly higher responses by genes for several putative T3SSYE2 structural components to *in vivo* growth conditions

Table 1 MH96 cell density under *in vivo* and *in vitro* conditions used for DE analysis

<i>In vivo</i> infection stage/ <i>in vitro</i> growth	Mean cell density <i>in vivo</i> (i.e., <i>G. mellonella</i> homogenate) (CFU $g^{-1} \pm$ SEM)	Mean cell density <i>in vitro</i> (i.e., Luria Broth Base broth) (CFU $ml^{-1} \pm$ SEM)
Early infection/ lag-growth	$4.4 \pm 0.5 \times 10^7$	$3.2 \pm 0.1 \times 10^7$
Middle infection/ exponential growth	$4.4 \pm 1.7 \times 10^8$	$2.9 \pm 0.9 \times 10^8$
Late infection/ stationary growth	$5.0 \pm 2.2 \times 10^9$	$4.4 \pm 0.1 \times 10^9$

CFU, colony forming units; SEM, standard error of the mean.

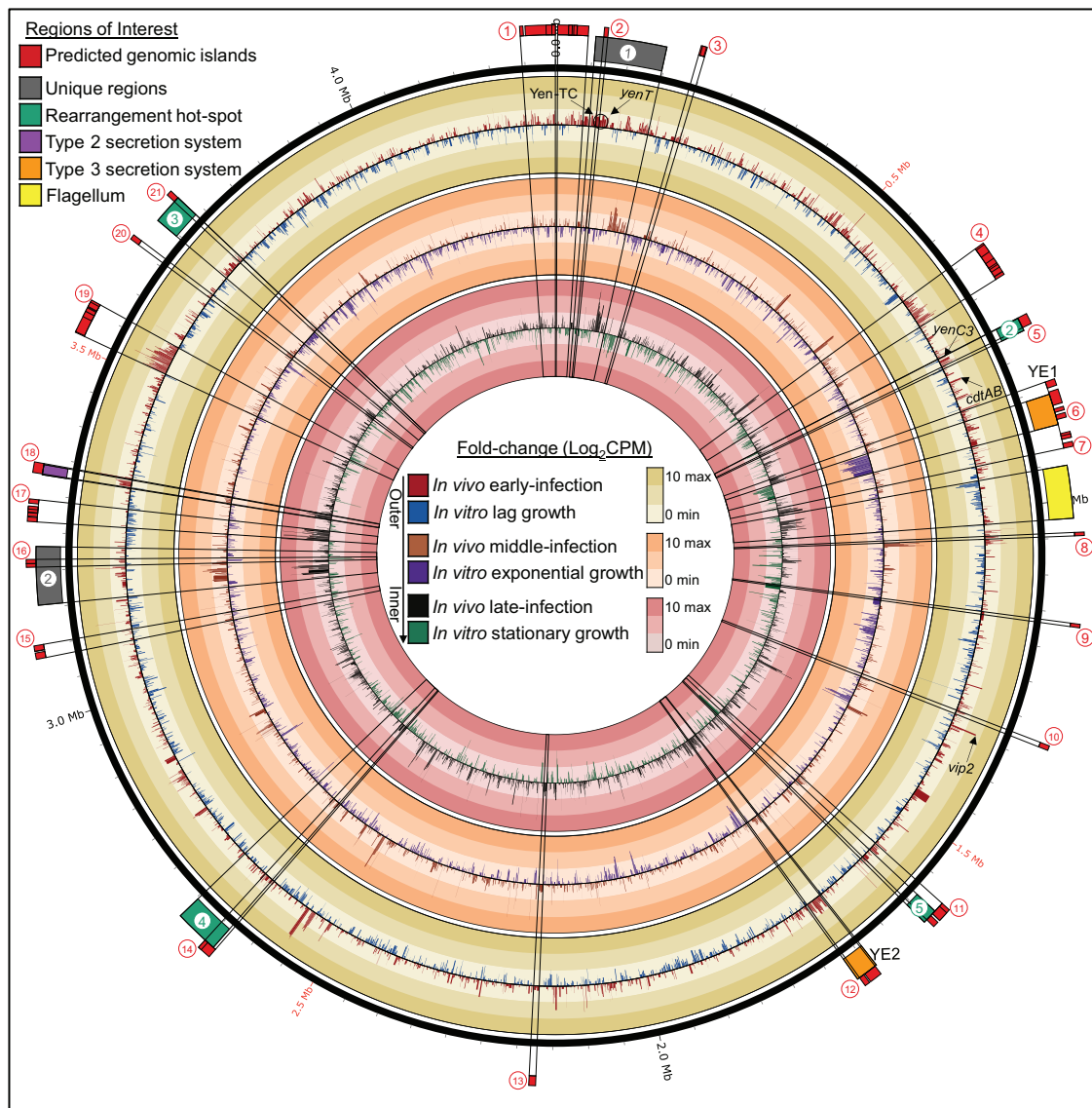


Figure 1 Circos plot of *Y. entomophaga* MH96 genome-wide transcriptional response to *in vivo* and *in vitro* conditions during early, middle, and late intrahemocoelic infection and lag, exponential and stationary growth, respectively. CPM, counts-per-million. Mean fold-change in log counts-per-million (Log_2CPM) (*in vivo* early and middle infection: $n = 4$, *in vivo* late infection: $n = 3$, and all *in vitro*: $n = 2$). Red circles numbered 1–21 represent predicted GIs identified using IslandViewer 4 webserver (refer to Supplementary Table S8). Regions of interest/loci previously identified from the draft genome are numbered or labeled as required (Hurst et al. 2016).

compared to *in vitro* during early and/or late infection were also identified. In contrast, most of the T3SSYE1 structural component genes were significantly upregulated by MH96 either in response to *in vitro* conditions or both *in vitro* and *in vivo* conditions depending on cell density, except putative T3SSYE1-associated chaperone (PL78_18170) that was more highly expressed *in vivo* during early intrahemocoelic infection only. Multiple genes located within a predicted phage-like islands Is.4, Is.8, and Is.19 were also significantly upregulated during early stage of intrahemocoelic infection, compared to growth *in vitro*.

Genes for other putative VFs from MH96 genomic regions of interest were more responsive during late stages of intrahemocoelic infection compared to other time points. Many of the iron acquisition gene clusters located within unique region 2 were found to have higher expression under *in vivo* conditions, especially during later stages of infection. Similarly, many flagellum

genes were also more highly expressed mostly during the later infection stage. Furthermore, phage-related and hypothetical genes encoded within the predicted GI Is.17 were also found to have significant transcriptional response to *in vivo* conditions compared to growth *in vitro* during late infection stages as well.

An important region in the MH96 genome is Unique Region 1 (contains PAI_{YE96}), where genes for hypothetical proteins *yenU* (PL78_03775) and *yenW* (PL78_03790), putative heat-stable enterotoxin *yenT* (PL78_03785), as well as several iron-siderophore biosynthesis/transport and serralsyn genes were significantly upregulated in the host at various or throughout all stages of infection compared to *in vitro* treatments. Also located in Unique region 1, Yen-TC genes *chi1* (PL78_03740), *chi2* (PL78_03755), and *yenC1* (PL78_03765) were found to have significantly higher expression during early intrahemocoelic infection only, while DE was not detected for other Yen-TC component genes.

Functional enrichment of *in vivo* host clusters

Qualitative assessment of fuzzy clustering results showed that 20 clusters with fuzzifier value of 1.5 were optimal for the analysis of fold-change \log_2 CPM values comparing MH96 transcriptome across three different stages of intrahemocoelic infection to *in vitro* growth controls. Among these 20 clusters, 8 clusters (containing 798 transcripts) exhibited the most striking responses to *in vivo* conditions compared to *in vitro* growth (Supplementary Figure S7). These eight “*in vivo* clusters” were further organized into three main “*in vivo* cluster types” based on shared expression profiles across multiple *in vivo* clusters (Figure 2A). *In vivo* clusters containing transcripts with elevated abundances across all three stages of infection were identified as “Host All” type clusters, while “Host Early” and “Host Late” type clusters were characterized by containing mostly transcripts with elevated abundances during early or late stages of infection only, respectively.

To further classify the role of putative MH96 VFs in pathogenesis, those transcripts from the eight *in vivo* clusters were functionally annotated using VFDB. Using this approach, 28.7% of the transcripts from *in vivo* clusters could be further categorized by potential role in pathogenicity (*i.e.*, adherence, defense response, horizontal gene transfer, iron acquisition, metabolic adaptation, mobility, outer membrane, regulation, secretion system, and

toxin/effector or unclassified) (Figures 2B and 3 and Supplementary Table S6). Transcripts related to iron acquisition, secretion systems, regulation, and metabolic adaptation were commonly identified among each of the three different *in vivo* cluster types but an obvious temporal shift in the expression of iron acquisition and flagellar motility vs toxins, effectors, and adhesions were identified when comparing early and late *in vivo* clusters.

Four genes encoding an usher-chaperone fimbrial cluster (PL78_12465, PL78_12470, PL78_12475, and PL78_12480) coexpressed with putative T3SS lipoprotein chaperone YscW-like (PL78_14485) were identified as some of the most host-responsive genes in the MH96 genome, especially at the early stage of intrahemocoelic infection (*in vivo* early infection vs *in vitro* growth fold-change values ranging from greater than 4.6 up to 7.1 \times \log_2 CPM). Genes for filamentous hemagglutinin (PL78_11060, previously annotated as a hemolysin) and its cognate two-partner secretion system (TPS) (PL78_11055) also had significantly higher expression during early and middle stages of intrahemocoelic infection. A predicted fimbrial gene (PL78_08275) and chitin-binding protein (PL78_08295) both associated with the Chi-Fim region (Hurst *et al.* 2016) were also captured from *in vivo* Host all type clusters but the relative expression levels of the Chi-Fim-associated fimbrial and chitin-binding protein genes were orders

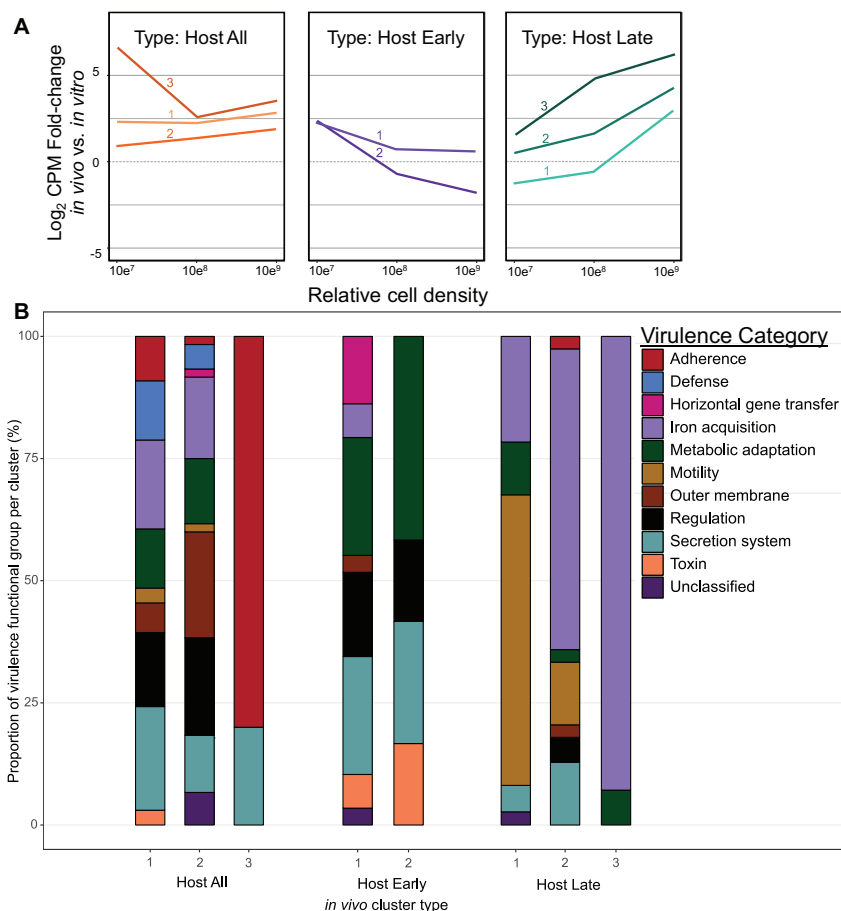


Figure 2 *Yersinia entomophaga* MH96 putative host-specific VFs sharing significant sequence similarity to known VFs from the VF database identified among host-specific *in vivo* clusters. (A) Median \log_2 counts-per-million-fold-change between *in vivo* and *in vitro* libraries organized by *in vivo* cluster type (each line represents a separate host-specific *in vivo* cluster, refer to Supplementary Figure S7). (B) The proportion of genes organized by virulence function classification among *in vivo* cluster types. Refer to Supplementary Table S6.

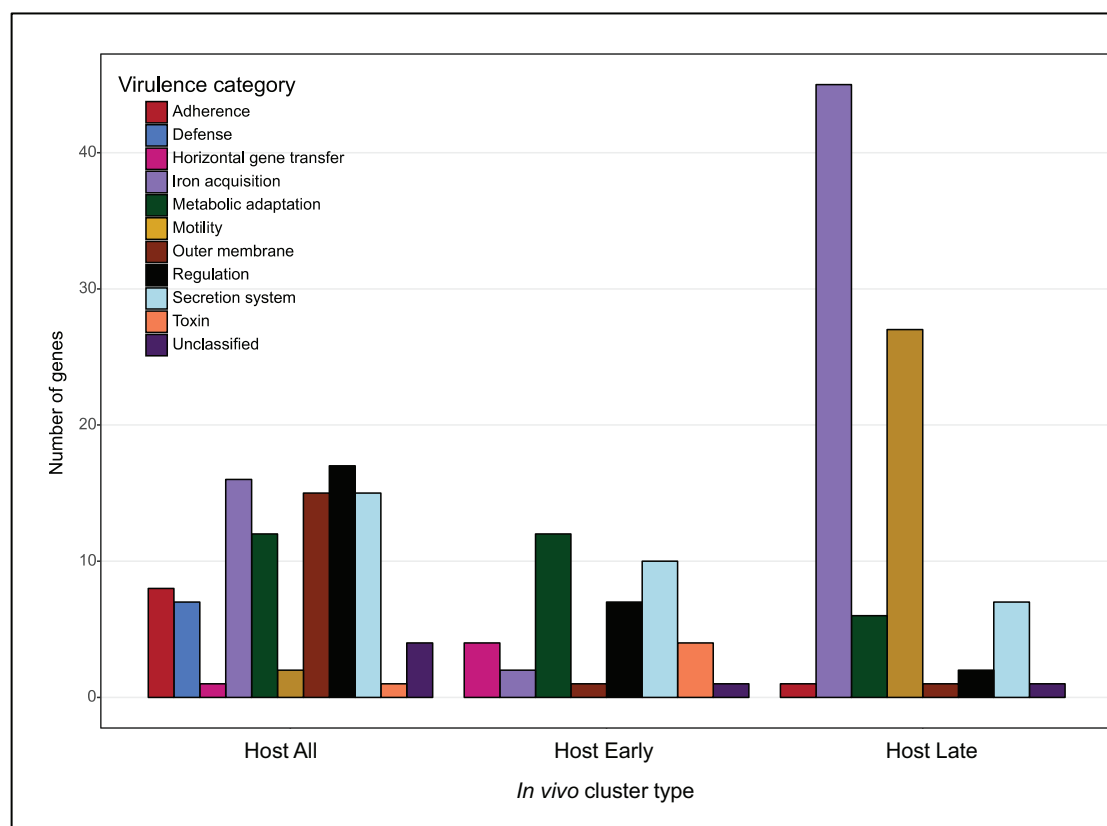


Figure 3 Number of genes assigned to each virulence category for putative VFs identified from *in vivo* cluster types. *In vivo* clusters combined by type: Host All = constitutively higher *in vivo* expression during early, middle, and late infection; Host Early = higher *in vivo* during early infection only; Host Late = higher *in vivo* expression during late infection only. See Figure 2A for Host All, Host Early, and Host Late median expression profiles and Supplementary Table S6 for more details.

Table 2 Putative MH96 toxin and effector genes from host-specific gene expression clusters with significant sequence homology to other known toxins or effectors from VFDB

Toxin/effector type	Locus tag	Designation	Host cluster type	Region of interest/GI/locus ^a
Toxin	PL78_03785	<i>yenT</i>	Host all	Unique region 1-PAI _{YE96}
Toxin	PL78_18780	<i>yenC3</i>	Host early	Rhs-associated region 2/Is. 5
Toxin	PL78_18440	<i>cdtA</i>	Host early	<i>cdtAB</i>
Toxin	PL78_18445	<i>cdtB</i>	Host early	<i>cdtAB</i>
Toxin	PL78_16145	<i>vip2</i>	Host early	<i>vip2</i>
Effector—T3SS	PL78_14610	<i>sicC</i> -like	Host all	T3SSYE2/Is.12
Effector—T3SS	PL78_07345	<i>ipgD/sopB</i> -like	Host all	—
Effector—T3SS	PL78_14615	<i>sicD</i> -like	Host early	T3SSYE2/Is.12
Effector—T3SS	PL78_18085	<i>ipaB/evcA</i> -like	Host early	T3SSYE1/Is.6
Effector—T3SS	PL78_14295	<i>sopD</i> -like	Host early	—
Tube/Effector—T6SS	PL78_02751	<i>hpc</i>	Host all	—
Tube/Effector—T6SS	PL78_00900	<i>hpc</i>	Host early	Rhs-associated region 3
Effector—T6SS	PL78_01040	<i>rhs</i> -containing	Host early	Rhs-associated region 3/Is.21
Tube/Effector—T6SS	PL78_03598	<i>hpc</i>	Host late	Is.1
Tube/Effector—T6SS	PL78_09211	<i>hpc</i>	Host late	Is.17
Effector—T5SS	PL78_10240	<i>aidA</i>	Host all	—

^a Regions of interest (see Figure 1): MH96 pathogenicity-associated island (PAI_{YE96}), rearrangement-hotspot (Rhs), MH96 type 3 secretion systems (T3SSs) T3SSYE1 and T3SSYE2 (Hurst et al. 2016); predicted GIs see Figure 1 and Supplementary Table S8; loci: cyto-lethal distending toxin components (*cdtA*, *cdtB*), vegetative insecticidal protein 2 (*vip2*) (Hurst et al. 2016). T3SS, type III secretion system; T5SS, type V secretion system; T6SS, type VI secretion system.

of magnitude lower than genes for other putative MH96 adhesions that were upregulated *in vivo*.

Transcripts for several putative toxins and effectors were identified among *in vivo* clusters (Table 2). The aforementioned heat-stable enterotoxin *yenT* was the only putative toxin gene highly expressed *in vivo* at two timepoints during infection and

the transcript encoding putative AidA-like type V secretion system (T5SS) secreted adhesin clustered with other MH96 transcripts with higher *in vivo* gene expression throughout infection (Table 2).

Several other transcripts for putative toxins were identified from Host Early *in vivo* clusters, including putative cytolethal

distending toxin subcomponents *cdtA* and *cdtB*, *yenC3* (a YenC-homolog also known as *rhsA* encoded within *Rhs2/Is.5*) and putative vegetative insecticidal protein component, *vip2* (Table 2). Transcripts for known, and putative Yen-TC components, *chi1* and *yenC3*, respectively, were captured within a Host Early *in vivo* cluster, but only *yenC3*, not *chi1*, shared any significant homology against known VFs from the VFDB using BLASTx homology search.

In addition to predicted toxins, transcripts for T2SS and T3SSYE2 structural components were also identified as putative VFs from *in vivo* clusters (Supplementary Table S6). Genes for T3SSYE2 co-located putative effectors were represented from *in vivo* clusters and found to have significantly greater expression under *in vivo* conditions compared to *in vitro* controls, including *sicC*-like and *sicD*-like, with the former being more highly expressed across all cell densities and the latter more highly expressed during early infection only. In addition to T3SSYE2-associated effector genes, two other genes for putative T3SS effectors were also found to be more highly expressed *in vivo* during early infection including T3SSYE1-associated *ipaB/evcA*-like and orphan *sopD*-like. Another gene for putative T3SS orphan effector *IpgD/SopB*-like represents a fifth possible T3SS effector gene, with significantly increased expression throughout all three stages of infection, compared to *in vitro* controls.

With respect to T6SS, genes for structural machinery components were not identified as having significantly greater expression *in vivo* compared to *in vitro* controls. MH96 genes for putative T6SS effectors were however identified from several of the *in vivo* cluster types, including genes for Rhs-containing protein and five different hcp-type structural tube/effector proteins. Also, the gene encoding the putative T6SS effector that shares sequence similarity to the eukaryotic transcription elongation factor domain Spt4 (PL78_00995) was found to have higher *in vivo* gene expression throughout infection but was not assigned a VFDB annotation based on amino acid sequence.

Not all MH96 predicted toxins and effectors were highly expressed under *in vivo* conditions though; genes for putative PirAB binary toxin (PL78_09590 and PL78_09595), RTX toxin (PL78_16910), and adenylate cyclase (PL78_08395) were found to have higher expression during exponential and/or stationary growth *in vitro*, with no significant response to *in vivo* conditions observed. Furthermore, despite having significantly greater abundance during early infection of *G. mellonella* compared to growth *in vitro*, transcripts for some other known or suspected MH96 VFs, including insecticidal TC components *Chi2* and *YenC1*, and putative T3SS effector *LopT* (PL78_18760) were not found to cluster among the eight *in vivo* clusters, instead clustering with other transcripts associated with comparatively lower overall median *in vivo* vs *in vitro* fold-change values.

Discussion

MH96 *in vivo* transcriptome revealed wide-spread and coordinated pathogen response to different stages of intrahemocoelic infection

Our *in vivo* transcriptome analysis has shed light on MH96 genes activated during intrahemocoelic infection of *G. mellonella*. Major shifts in gene expression by MH96 were observed between *in vivo* and *in vitro* conditions across all three stages of growth. The mosaic nature of the MH96 genome was highlighted by widespread coexpression of genes located in horizontally acquired regions that positively responded to host, including genes for Yen-TC components, putative toxins (*YenT*, *YenC3*), translocated

effectors, secretion system component (T2SS and T3SSYE2), iron acquisition systems, and phage-encoded hypotheticals. These findings provide evidence that complex genome-wide responses by MH96 occur throughout different stages of intrahemocoelic infection and reveal important insights into host-dependent transcriptional responses by entomopathogenic bacteria in nature.

Following disassociation of host midgut tissues (Marshall et al. 2012), MH96 gains access to the hemocoel where it encounters the cellular and humoral components of the insect host immune system (Nielsen-LeRoux et al. 2012). Within lepidopteran larvae, the hemolymph contains different hemocytes involved in phagocytosis, nodulation, and encapsulation as well as humoral factors like prophenoloxidase, antimicrobial peptides, reactive nitrogen, and oxidative species or lysozyme (Jiang et al. 2010; Casanova-Torres and Goodrich-Blair 2013; Wojda 2017). In order to occupy the insect hemocoel, MH96 produces VFs that combat the host immune defenses encountered during early intrahemocoelic infection, including anti-hemocytic factors such as toxins or translocated effectors. Upon incapacitation of the host immune system, MH96 produces additional VFs that function to mobilize and acquire essential nutrients, like iron, from insect cells to fuel pathogen metabolism and proliferation, ultimately leading to septicemia and host death (Nielsen-LeRoux et al. 2012).

MH96 upregulated toxins, secretion systems, effectors, and adhesions during intrahemocoelic infection of *G. mellonella*

During early stages of intrahemocoelic infection MH96 upregulates expression of Yen-TC components *chi1*, *chi2*, and *yenC1* as well as putative Yen-TC component *yenC3* and toxins *cdtAB* and *vip2*, which are known or suspected to be anti-hemocytic factors based on functional enrichment. *CdtAB* is a predicted genotoxin, which are known to interfere with normal hemocyte cell cycle (Lara-Tejero and Galán 2000; Heywood et al. 2005), while *Vip2* and *YenC3* may act on host cytoskeleton through (ADP)-ribosyltransferase activity targeting globular (G)-actin (Barth et al. 2004) or Rho GTPases (Lang et al. 2010), respectively. While Yen-TC is established as the primary orally active virulence determinant of MH96 (Hurst et al. 2011b; Marshall et al. 2012), purified Yen-TC was also found to cause ruffling of *G. mellonella* hemocytes (Hurst et al. 2015) and nuclear and actin condensation and apoptosis of *Spodoptera frugiperda* insect cell line Sf9 (Marshall et al. 2012). Taken together, Yen-TC should be considered both an anti-hemocytic factor as well as an orally active insecticidal toxin.

Unlike putative toxin genes that were upregulated only during the initial intrahemocoelic infection, the MH96 heat-stable enterotoxin, *yenT*, was highly expressed during both the septicemic and pre-cadaveric stages of infection compared *in vitro* control treatments. Similar in size *YenT* shares N-terminal amino acid sequence homology to the heat-stable enterotoxins *YstA* and *YstB* (Hurst et al. 2011b), encoded by gastrointestinal-disease causing strains of *Yersinia enterocolitica* (Delor et al. 1990; Ramamurthy et al. 1997; Grant et al. 1998; Singh and Virdi 2004), *Yersinia frederiksenii*, *Yersinia intermedia*, and *Yersinia kristensenii* (Imori et al. 2017). Recently, the heat-stable enterotoxin, *YacT*, produced by *Y. frederiksenii* has been implicated in host immune modulation following intrahemocoelic injection of *G. mellonella*. The *Escherichia coli* recombinant *YacT*, altered the morphology and increased the aggregation of *G. mellonella* hemocytes (Springer et al. 2018). It is plausible that, like *YacT* of *Y. frederiksenii*, MH96 *YenT* also functions as an anti-hemocytic factor given the responsiveness of *yenT* to intrahemocoelic conditions at multiple stages of infection.

Among pathogenic *Yersinia*, T3SSs are known to target host cells through tight attachment and translocate effectors into the cytoplasm to disrupt cytoskeleton, trigger apoptosis, prevent phagocytosis, or interfere with immune pathways (Bliska et al. 2013; Plano and Schesser 2013; Pha and Navarro 2016). The *Y. enterocolitica* T3SS Ysa (encoded by *ysa* found in biotype 1B strains) (Foultier et al. 2002; Thomson et al. 2006) shares the same gene order with the MH96 T3SSYE2 locus (Hurst et al. 2016) of the predicted island Is.12, suggesting a common origin in both pathogens. In addition to interacting with mammalian cells (Matsumoto and Young 2006; Bent et al. 2013), Ysa is essential for replication within *Drosophila melanogaster* S2 cells (Walker et al. 2013) and secretes effectors at temperatures of <26°C in nutrient-rich broth with high salt concentrations (Venecia and Young 2005). Based on the similar gene order to *ysa* and the upregulation during intrahemocoelic infection, T3SSYE2 is likely another anti-hemocytic mechanism used by MH96 to overcome the insect immune system.

Also, similar to Ysa, which secretes effectors encoded by genes within distal regions of the *Y. enterocolitica* genome (Walker and Miller 2009), the MH96 *in vivo* transcriptome revealed evidence for coordinated host-dependent regulation of genes for T3SSYE2 structural components and several predicted effectors, some of which were not co-located within the T3SSYE2 region of the genome. For example, MH96 encodes a gene for a putative LopT-like effector, which is homologous to LopT, a T3SS effector (similar to YopT of *Y. pestis*) with known insecticidal activity in *Photorhabdus luminescens* (Brugirard-Ricaud et al. 2005). Expression of *lopT* was significantly upregulated by MH96 during initial intrahemocoelic infection. Notably, *lopT* and *yenC3* are both co-located within an effector-island (i.e., Rhs-associated region 2/Is.5), and are possibly coopted by respective translocation machineries T3SSYE2 and Yen-TC and reflect the multiple diverse modes of action deployed by MH96 against the host during early stages of intrahemocoelic infection.

In contrast to T3SSYE2 and its predicted effectors that were upregulated under *in vivo* conditions, genes for T3SSYE1 of the predicted island Is.6 responded mainly to *in vitro* conditions. The differing transcriptional responses by the MH96 T3SSs depending on growth under *in vivo* and *in vitro* conditions allude to a specific role of each T3SS in MH96 pathogenicity. It was also noted that MH96 genes encoding the predicted PirAB binary toxin, RTX toxin, and adenylate cyclase had significantly higher expression *in vitro* compared to *in vivo* conditions. Clearly, some yet unknown environmental factor appears to control expression of T3SSYE1 and other insect-active genes. It is plausible that abiotic factors like pH and concentration of oxygen or nutrients occurring during *in vitro* growth could mimic signals that MH96 is likely to encounter in its natural environment, such as intracellular replication within *G. mellonella* hemocytes (Hurst et al. 2015; A. R. Paulson, personal communication), infection of insect midgut or different insect hosts (Hurst et al. 2011b, 2019) or even the occupation of the human urinary tract during asymptomatic catheter infection (Le Guern et al. 2018).

Along with T3SSs, another major class of well-studied VFs known from pathogenic *Yersinia* is adhesins, including T5SS-secreted adhesins and fimbriae (Chauhan et al. 2016). Putative adhesin genes upregulated by MH96 throughout or during specific stages of intrahemocoelic infection included T5SS-secreted AidA-like and filamentous hemagglutinin, and four-gene usher-chaperone fimbrial cluster, the latter representing some of the most responsive genes to *in vivo* conditions in the entire MH96 genome. In addition to host cell attachment or biofilm formation,

in other pathogenic *Yersinia* species, adhesins have also been linked to effector translocation through enhanced attachment to host cells (Maldonado-archo et al. 2013; Keller et al. 2015; Mühlkamp et al. 2015; Deuschle et al. 2016). Perhaps adhesins also contribute to the effective anti-hemocytic activities of MH96 during intrahemocoelic infection of *G. mellonella* by enhancing effector translocation efficiency like other pathogenic *Yersinia*, yet such mechanisms remain unknown among entomopathogenic bacteria.

Transcriptional activation of numerous genes for known and putative VFs supports insecticidal activities of MH96 by “over-kill” strategy

The MH96 *in vivo* transcriptome revealed a high degree of functional redundancy, with multiple toxins, T3SS and T6SS effectors, and adhesins all likely to target hemocytes during early and/or throughout multiple stages of infection. Moreover, greater transcriptional activation of hypothetical genes encoded within several putative phage-related GIs suggests additional factors may further contribute to MH96 highly insecticidal activities. Such functional redundancy has also been observed in another highly entomopathogenic bacterium, *P. luminescens* (Wilkinson et al. 2009), that like MH96, produces many toxins, including insecticidal TC (ffrench-Constant et al. 2007), makes caterpillar floppy (Daborn et al. 2002), PirAB binary toxin (Waterfield et al. 2005; Wu and Yi 2016), and several RTX-like toxins (Tobias et al. 2017). Such abundance of toxins carried within the genomes of obligate entomopathogenic bacteria is thought to have arisen by adaptation for over-kill of a wide variety of hosts (ffrench-Constant et al. 2003). This is likely also true for MH96, which has broad insecticidal activities against diverse insect hosts (Hurst et al., 2011b, 2014, 2015, 2019). Also, MH96 is highly virulent against *G. mellonella* during intrahemocoelic infection but the LD₅₀ of a Yen-TC deficient strain (Δ TC) was comparable to that of wild type (~3 cells). It was also found that both wild type and Δ TC had the same ability to reduce the phenoloxidase activity of *G. mellonella* hemolymph 24 hpi, findings that suggest the presence of additional hemocytic VFs during intrahemocoelic infection by MH96 (Hurst et al. 2015). Transcriptional response by MH96 to *in vivo* conditions, including widespread and significant host-dependent upregulation of genes for Yen-TC components and numerous other predicted toxins, secretion systems, effectors, adhesins, and GI-encoded hypotheticals is another indicator that the highly insecticidal nature of MH96 stems from multiple diverse modes of action.

Beyond primary VFs that can act directly to modulate or destroy host cells, functional enrichment of the MH96 *in vivo* transcriptome also revealed changes in the temporal expression of genes for other putative in-host fitness factors (i.e., motility, metabolism, iron acquisition, response to host-induced stress, etc.) vs toxins, effectors, and adhesins from early to late infection. Notably, during the late stage of infection, MH96 significantly increased expression of genes related to flagellum and iron acquisition, compared to early and middle infection stages, which supports a role for iron acquisition and flagellum-related motility in MH96 in-host fitness during pre-cadaveric stages of insect infection.

Specific to entomopathogenic bacteria, both the flagellum and iron acquisition systems have already been implicated in intrahemocoelic infection of *G. mellonella* by triphasic nematode symbionts *P. luminescens* and closely related *Xenorhabdus nematophila*. Flagellum-driven motility provided a selective fitness advantage during colonization of *G. mellonella* by *P. luminescens* (Easom and

Clarke 2008). Also, iron sensing by *X. nematophila* was shown to regulate the expression of flagellin gene *fliC* and hemolysin genes *xaxAB*, which significantly increased during late stage intrahemocoelic infection of *G. mellonella* and reached maximum levels following host death (Jubelin et al. 2011). Beyond entomopathogenic bacteria, iron homeostasis and hemoglobin utilization genes of *C. albicans* were found to be upregulated during later infection of *G. mellonella* and murine models compared to an earlier infection; however, in this study, the early to late increase in iron-related genes was much greater in *G. mellonella* compared to the mouse model (Amorim-Vaz et al. 2015). Taken together, pathogens infecting *G. mellonella*, whether bacterial or fungal, appear to shift into an iron scavenging mode during late stage (i.e., pre-cadaveric) infection, indicating that pathogen sensing and response to *in vivo* iron fluxes during insect infection are likely an important component of in-host fitness or represent an interspecific competitive strategy.

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