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Transcriptional Analysis of a *Photorhabdus* sp. Variant Reveals Transcriptional Control of Phenotypic Variation and Multifactorial Pathogenicity in Insects▽

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Photorhabdus luminescens **lives in a mutualistic association with entomopathogenic nematodes and is pathogenic for insects. Variants of** ***Photorhabdus*** **frequently arise irreversibly and are studied because they have altered phenotypic traits that are potentially important for the host interaction. VAR* is a colonial and phenotypic variant displaying delayed pathogenicity when directly injected into the insect,** ***Spodoptera littoralis*****. In this study, we evaluated the role of transcriptomic modulation in determining the phenotypic variation and delayed pathogenicity of VAR* with respect to the corresponding wild-type form, TT01α. A** ***P. luminescens*** **microarray identified 148 genes as differentially transcribed between VAR* and TT01α. The net regulator status of VAR* was found to be significantly modified. We also observed in VAR* a decrease in the transcription of genes supporting certain phenotypic traits, such as pigmentation, crystalline inclusion, antibiosis, and protease and lipase activities. Three genes encoding insecticidal toxins (*** ***pit*** **and** ***pirB*****) or putative insecticidal toxins (*** ***xnp2*****) were less transcribed in VAR* than in the TT01α. The overexpression of these genes was not sufficient to restore the virulence of VAR* to the levels of TT01α, which suggests that the lower virulence of VAR* does not result from impaired toxemia in insects. Three loci involved in oxidative stress responses (*** ***sodA*****,** ***katE*****, and the** ***hca*** **operon) were found to be downregulated in VAR*. This is consistent with the greater sensitivity of VAR* to H_2O_2 and may account for the impaired bacteremia in the hemolymph of** ***S. littoralis*** **larvae observed with VAR*. In conclusion, we demonstrate here that some phenotypic traits of VAR* are regulated transcriptionally and highlight the multifactorial nature of pathogenicity in insects.**

Photorhabdus luminescens is a member of the *Enterobacteriaceae* that lives in a mutualistic association with entomopathogenic nematodes and is pathogenic for a wide range of insects. Studies on *Photorhabdus* pathogenicity have shown this bacterium to be highly virulent, due to the production of an array of insecticidal toxins and the induction of host immune depression (6, 18, 25, 28, 41, 67).

Variants frequently occur within the *Photorhabdus* genus. Colonial variants form colonies with different morphotypes and are unstable (31, 32, 38, 39, 65, 69). Some of these morphotypes have modified virulence properties and do not support nematode development and reproduction (31, 38). Phenotypic variation is another phenomenon widely described in the *Photorhabdus* genus. Phenotypic variants generally emerge after prolonged *in vitro* culture of the wild-type strain collected from the nematode (1, 7). The phenotypic variant is characterized by simultaneous changes in many of the traits of the wild-type form (the production of extracellular enzymes, pigments, antibiotics, and crystalline inclusion bodies and the ability to generate bioluminescence). This phenomenon occurs at a low and unpredictable frequency and is rarely reversible. Generally, both wild-type and variant forms are virulent in insect hosts, but only wild-type forms support nematode growth and development (27, 67). Since the two main types of variation in *Photorhabdus* affect interactions with invertebrate hosts and/or phenotypes potentially involved in these interactions, several studies have tried to determine the mechanisms underlying such events.

Comparative genomic, proteomic, and transcriptomic approaches have been used to investigate regulation of the phenotypic switch (22, 31, 40, 53, 61). Comparative genomics studies have provided no useful information about this regulation (31). The generation of phenotypic variants in *Photorhabdus temperata* strain K122 has been shown to be controlled by repressing factors (40, 53), which also play a key role in the regulatory network controlling pathogenicity and mutualism (40). The molecular basis of the phenotypic traits of the wild-type form has also been analyzed. Lipase and protease proteins are present in phenotypic variants, but their activities are inhibited (54, 63). Comparative whole-proteome analysis has been carried out on wild-type and variant forms of *P. luminescens* TT01 and has identified proteomic variations (22, 61). However, few studies have focused on the molecular basis of phenotypic variation traits at the transcriptomic level.

We have previously described different *Photorhabdus* variants (31). One of these variants, the wild-type form TT01α, was collected from a laboratory-maintained symbiotic nematode. From a phenotypic point of view, this variant is not distinguish-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Source or reference
Strains		
P. luminescens		
TT01	Strain isolated from the nematode *Heterorhabditis bacteriophora* THO1 in Trinidad; wild-type form	26
TT01α	Genomic variant of TT01 isolated from the laboratory-maintained nematode *Heterorhabditis bacteriophora* THO1; wild-type form	31
VAR*	Stabilized TT01α phenotypic and colonial variant	31
TT01Δ*sodA*	TT01R *sodA*::*cat*	17
E. coli		
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* F′ [*proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]	Stratagene
S17-1	*pro* r$^-$ n$^-$ Tp^r Sm^r RP4-2-Tc::Mu::Tn*7 recA thi*	60
Plasmids		
pBBR1-MCS1	Broad-host-range vector; Cm^r, *mob*	42
pBB-*pit*	0.5-kb PCR fragment obtained with XhoI-L-plu1537 and PstI-R-plu1537 primers and inserted between the XhoI and PstI sites of pBBR1-MCS1	This study
pBB-*pirAB*	1.7 kb PCR fragment obtained with XhoI-L-plu4093 and PstI-R-plu4092 primers and inserted between the XhoI and PstI sites of pBBR1-MCS1	This study
pBB-*xnp2*	7.3-kb PCR fragment obtained with XhoI-L-plu2444 and PstI-R-plu2441 primers and inserted between the XhoI and PstI sites of pBBR1-MCS1	This study
pBB-UIDK	pBB-*uidA*; Km^r	46
pBB-P$_{lac}$*uidA*	PvuII P$_{lac}$ fragment from pUC19 inserted into pBB-UIDK	This study

[a] Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance.

able from the wild-type form TT01, but it differs in having large-scale deletion events in the flexible genome. The VAR* variant is a derivative of the wild-type form TT01α. This variant is of particular interest because it is both a phenotypic and a colonial variant. Moreover, VAR* displays delayed pathogenicity in the insect *Spodoptera littoralis*. VAR* differs from TT01α principally in the presence of an unusual single-block duplication encompassing 275 kb, corresponding to 4.8% of the TT01 reference genome (31).

The purpose of the present study was to evaluate the role of transcriptomic variation in the phenotypic variation and delayed pathogenicity of the VAR* variant. With this goal in mind, we compared the transcriptomes of TT01α and VAR*. Based on the description of the transcriptomic variation observed, we followed various lines of investigation to account for the observed physiologic variation: phenotypic variation and delayed pathogenicity.

MATERIALS AND METHODS

Bacterial strains and medium. The strains and plasmids used in the present study and their sources are listed in Table 1. Bacteria were routinely grown in Luria-Bertani (LB) broth, on solid LB medium, on 1.5% nutrient agar (GNO) medium, on NBTA (nutrient agar supplemented with 25 mg of bromothymol blue and 40 mg of triphenyl-2,3,4 tetrazolium chloride per liter) medium, or on TreGNO (nutrient agar with 10 g of trehalose and 25 mg of bromothymol blue per liter) medium (31) at 28°C for *P. luminescens* and at 37°C for *Escherichia coli*. When required, the final concentrations of antibiotics used for selection were as follows: 15 μg of erythromycin/ml for *P. luminescens*, 10 mg (liquid culture) and 18 mg (agar plates) of cefoxitin/liter for *P. luminescens* and 20 mg of chloramphenicol/liter for *E. coli*, and 15 mg of chloramphenicol/liter for *P. luminescens.*

Phenotypic analysis of *Photorhabdus* variants. Wild-type and variant traits were assessed as previously described (5) except for the protease assay, which was monitored by a colorimetric method (14). Agglutination assays were assessed as previously described (50) with *Photorhabdus* bacterial suspensions (10^8 cells/ml) prepared from exponential-growth or stationary-phase cultures in LB broth.

RNA preparation. TT01α and VAR* were grown in LB broth for 6 h (5×10^7 CFU/ml; optical density at 540 nm [OD_{540}] of 0.5; exponential-growth phase) or 45 h (5×10^8 CFU/ml; OD_{540} of 13 for TT01α and OD_{540} of 8 for VAR*; stationary-growth phase). Total RNA was extracted with TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and then purified with a High Pure RNA Isolation kit (Roche, Meylan, France), including a DNase I treatment step. The concentration was determined by spectrophotometric measurement of the OD_{260}. For each RNA preparation, DNA contamination was assessed by carrying out PCR.

Microarray experiments and analysis. We synthesized cDNA targets from 10 μg of TT01α and VAR* RNA and labeled them as follows. Each RNA preparation was heated at 70°C for 10 min with random hexamer (0.1 μg/μl; Roche) and cooled at room temperature for 10 min. The RNA was then reverse transcribed by incubation at 42°C for 2 h with SuperScript II (10 U/μl; Invitrogen), dithiothreitol (5 mM; Invitrogen), a mixture of deoxynucleoside triphosphates (1 μl; RPK0147; Amersham/GE Healthcare Europe GmbH, Munich, Germany), and dCTP-Cy3 or dCTP-Cy5 (50 μM; Amersham). The remaining RNA was subjected to alkaline lysis by incubation with 0.2 M NaOH for 10 min at 37°C. The labeled cDNAs were neutralized by adding 625 mM HEPES (pH 8) and immediately purified with a QiaQuick PCR purification kit (Qiagen, Courtaboeuf, France), used according to the manufacturer's recommendations, but with two additional washes in PE buffer (provided in the Qiagen kit). Cy5- and Cy3-labeled cDNAs were mixed, dried under a vacuum, and resuspended in hybridization buffer, as previously described (30).

The *Photorhabdus* DNA microarray (GEO accession number GPL11042) and the prehybridization and hybridization procedures have been described elsewhere (30). Each microarray comparison (TT01α versus VAR* growth in the exponential-growth or stationary phase) included six slides, with three dye-swapping replicates. The intensity of the signal for each microarray spot was determined with ArrayVision software (Amersham).

Microarray data were normalized and statistical analyses were carried out with the Spotfire DecisionSite functional genomic tool (http://spotfire.tibco.com/products/decisionsite-functional-genomics.aspx). The raw intensities were normalized to ensure that the experiments were comparable and to remove sources of systematic variation. For a given hybridization, the median intensity of a given variable is adjusted such that it is equal to the median intensity of the control variable ($\log R - \log G = 0$, where R and G are the summed intensities for each variable) without the designation of a baseline. As such, the intensity levels of the two channels are mutually adjusted (dye normalization). The trimmed median method was used to calculate a median value for which the effect of outliers was minimized. The trim cutoff was set at 10%, resulting in the exclusion of the top 5% and the bottom 5% of the values from the calculation. The

TABLE 2. Oligonucleotides used in this study

Use and oligonucleotide	Sequence (5′–3′)[a]	Location
qRT-PCR		
L-0004	ATACACGAAGAAGAAGGTGTTTCAG	Internal region within plu0004
R-0004	TACCTGTCTGTTCAGTTTCTCCAAC	Internal region within plu0004
L-0157	ATGAAGAGCTGATTGTCGATGATG	Internal region within plu0157
R-0157	CGTAAAAGAGAAAGATAGAGCAGCAC	Internal region within plu0157
L-0183	AGTGTGGAACGTTGGATAGATAGAG	Internal region within plu0183
R-0183	AAGTATAGCTGCACCTACAGAACAAC	Internal region within plu0183
L-1537	ACTTATCCAGGATTCTATTCGTCTG	Internal region within plu1537
R-1537	ACTATAGATTTCGCCTTTGCTATCC	Internal region within plu1537
L-1752	CCTACGATATAGGCAATGGTATCAG	Internal region within plu1752
R-1752	ACAATATCTTGGTAGCCGAAAGAG	Internal region within plu1752
L-1956	GTACCTGTTTGAACAGACTGAAATG	Internal region within plu1956
R-1956	TTGTAGTTACTGAATGCTGACTGTG	Internal region within plu1956
L-1991	GCTAATATTTCCGTAGGCTCTCATC	Internal region within plu1991
R-1991	GGACGTAATCAAAGTGTAATTCTCG	Internal region within plu1991
L-2442	AATAGGCAATATGCTGTACGTCTTC	Internal region within plu2442
R-2442	CAGACCGCAAATACAATAAAGTAGG	Internal region within plu2442
L-3506	TTAAGGGCGATATTGATGTAGAAGG	Internal region within plu3506
R-3506	TTGATAGACATACTGCTGCAACGAC	Internal region within plu3506
L-4092	GACAGTGACCATGAACAGACATAAC	Internal region within plu4092
R-4092	ATATTGGGTTGCTGGTTTAGAGAG	Internal region within plu4092
L-4546	CTGCCGATATAATTGCAGCTTTAC	Internal region within plu4546
R-4546	CCAAATTTCTGAAGGGTGTATTTCC	Internal region within plu4546
Plasmid constructions		
XhoI-L-plu1537	GCGCCTCGAGTTAAAATCAATCACAAGAGG	Upstream of plu1537
PstI-R-plu1537	GCGCCTGCAGAATATCTTCTCACAGGAATGG	Downstream of plu1537
XhoI-L-plu4093	GCGCCTCGAGTTAATGAGGAAAATAAATATGTC	Upstream of plu4093
PstI-R-plu4092	GCGCCTGCAGCTACGTACATAAAATATACTTGTTAAA	Downstream of plu4092
XhoI-L-plu2444	GCGCCTCGAGGAGGCCGATAGCTTCCAAC	Upstream of plu2444
PstI-R-plu2441	GCGCCTGCAGAAAGCGTAGCAATGTTCCTG	Downstream of plu2441

[a] Restriction enzyme sites are underlined.

dye-normalized values were used for a global normalization process in which intensities were rescaled with respect to a baseline intensity control.

After normalization, the fold change (FC) was calculated as follows: FC = R if $R > 1$ and $-1/R$ if $R < 1$, where R = (median of normalized intensity for VAR*)/(median of normalized intensity for TT01α). We used a Student t test to identify statistically significant differences between groups. The original hypothesis (i.e., null hypothesis) was that the median expression levels of a gene did not differ between the two groups. The null hypothesis was then either rejected or accepted for each gene considered. The results are expressed in terms of P values, indicating the significance of the differences observed. These values correspond to the probability of a type I error leading to the conclusion that two median expression values for a given gene are different when there is, in fact, no difference. A significant difference is considered to exist if the P value obtained is below 0.01. The microarray data are accessible under the GEO accession number GSE24627.

qRT-PCR. For the validation of whole-transcriptome data, quantitative reverse transcription-PCR (qRT-PCR) was carried out on the RNA preparations used for *Photorhabdus* microarray hybridization. cDNAs were synthesized with 1 μg of total RNA using the SuperScript II reverse transcriptase (Invitrogen) and random hexamers (100 ng/μl; Roche Diagnostics). qPCR analyses were performed using a LightCycler FastStart DNA Master^{PLUS} SYBR green I kit (Roche Diagnostics) with 1 μl of cDNA synthesis mixture and 1 μM concentrations of specific gene primers (Table 2). The enzyme was activated for 10 min at 95°C. The reactions were performed in triplicate at 95°C for 5 s, 61°C for 5 s, and 72°C for 10 s (45 cycles) and monitored in the LightCycler (Roche). Melting curves were analyzed for each reaction, and each curve contained a single peak. The amount of PCR product was calculated from the standard curves obtained from PCR with serially diluted genomic DNA from *P. luminescens* TT01. All data are presented as a ratio with respect to *gyrB*, which was used as a control gene. Values are means ± the standard deviation and were compared by using a Student t test.

Sequence analysis. The sequence annotations of the TT01 genome were from or updated with PhotoScope, the *Photorhabdus* database of the annotation and comparative analysis platform, MaGe (https://www.genoscope.cns.fr/agc/mage/wwwpkgdb/Login/log.php?pid=13).

Assays of sensitivity to antimicrobial peptides, antibiotics, and toxic compounds. We assessed the susceptibility of the *Photorhabdus* variants to antimicrobial peptides (AMPs) or antibiotics, by determining the MICs of colistin, polymyxin B, cecropin A, and chloramphenicol. Stock solutions of colistin methanesulfonate (Sigma) and polymyxin B (Sigma) were prepared in sterile water, to obtain concentrations of 20 and 0.5 mg/ml, respectively. Stock solutions of cecropin A and B (Sigma synthetic molecules C6830 and C1796, respectively) were prepared in 1% acetic acid to obtain a concentration of 0.25 mg/ml. A stock solution of chloramphenicol (Sigma) was prepared in absolute ethanol to obtain a concentration of 20 mg/ml. The antimicrobial molecules were then added directly into the 96-well microtiter plates in 2-fold serial dilutions. Bacterial cells were grown at 28°C overnight in LB broth and were then washed and resuspended in Mueller-Hinton (MH) broth (Biokar, Marnes-la-Coquette, France). A final inoculum equivalent to 10^3 CFU was dispensed into the wells of 96-well microtiter plates. The MICs were determined after incubation at 28°C for 48 h. The microtiter plates were read by eye. β-Lactam susceptibility assays were carried out by the agar diffusion method in accordance with the instructions of the Antimicrobial Committee of the French Society for Microbiology. The susceptibilities of these cultures to other toxic compounds were assessed on solid media as follows: an inoculum of *Photorhabdus* variants equivalent to 10^3 CFU was evenly spread onto solidified MH medium containing 1.5% agar (BD Difco, Franklin Lakes, NJ) and allowed to dry at room temperature for 20 min. The various toxic compounds (1.5 mM $ZnSO_4$, 30% H_2O_2, crystal violet [4 mg/ml], 20% sodium dodecyl sulfate, 25 mM deoxycholate) were diluted in sterile water and sterilized by passage through a filter with 0.2-μm-pore-size pores. Filter paper discs (bioMérieux S.A, Marcy l'Etoile, France) were impregnated with 5 μl of a solution of the toxic compound to be tested and placed in the center of a MH agar plate. Bacterial growth inhibition was evaluated by measuring the diameter of the zone in which no bacterial growth was observed.

DNA manipulation. Standard DNA manipulations in *Escherichia coli* were carried out as described by Ausubel et al. (2). We evaluated the functional

complementation of VAR* with genes encoding insecticidal toxins (plu1537, *pit*; plu4092, *pirB*; and plu2442, *xnp2*) by constructing low-copy-number plasmids as follows. *pit*, *pirA-pirB*, and the operon containing *xnp2* (plu2444-plu2443-plu2442-plu2441) were amplified by PCR using the primer pairs XhoI-L-plu1537/PstI-R-plu1537, XhoI-L-plu4093/PstI-R-plu4092, and XhoI-L-plu2444/PstI-R-plu2441, respectively (see Table 2). PCR fragments were ligated into pBBR1-MCS hydrolyzed with XhoI and PstI (Roche) to ensure that these fragments were inserted downstream from the P_{lac} promoter. The resulting plasmids were named pBB-*pit*, pBB-*pirAB*, and pBB-*xnp2*, respectively. All constructs were checked by sequencing (Millegen, Labège, France). Plasmids were introduced into VAR* by mating experiments, as previously described (11). We checked that the P_{lac} promoter was functional in the VAR* variant by constructing the pBB-P_{lac} *uidA* plasmid as follows. The P_{lac} fragment from pUC19 was inserted upstream from the promoter-less *uidA* gene in pBB-UIDK (46). The *uidA* gene encodes the β-glucuronidase enzyme, the specific activity of which was measured as previously described (46).

Pathogenicity assays. Pathogenicity experiments were performed as previously described (12). Statistical analysis (Wilcoxon test) was performed as previously described (11, 33) using SPSS version 14.0 (SPSS, Inc., Chicago, IL) to compare the mortality patterns.

Quantification of *P. luminescens* colonization of the *S. littoralis* hemolymph. The bacteria recoverable from the hemolymph were counted as previously described (59) with the following modifications. Insects were injected with 5×10^4 bacteria. Hemolymph was collected by making a lesion in a proleg 0, 3, 6, 9, 24, 30, and 48 h after injection (four larvae per time point). We then plated serial dilution of hemolymph in LB broth on NBTA medium and on TreGNO medium, both of which were supplemented with erythromycin (final concentration, 15 μg ml^{-1}) to prevent the growth of insect-associated bacteria other than *P. luminescens*. The NBTA and TreGNO plates were incubated at 28°C for 48 to 72 h at 28°C, and the CFU were counted. The results are expressed as the number of CFU in the total hemolymph (equivalent to 500 μl of hemolymph).

RESULTS AND DISCUSSION

Comparative analysis of the transcription profiling of TT01α and VAR* by microarray hybridization. For comparison of the transcription profiles of TT01α and VAR* growing in LB broth, we first checked that the two forms had similar growth curves in this medium (data not shown). We then extracted RNA from cells in the exponential growth phase (6-h-old culture, 5×10^7 CFU/ml) and stationary phase (45-h-old culture, 5×10^8 CFU/ml). For each of these growth phases, two independent biological replicates were performed. Genes displaying a significant difference in transcription between TT01α and VAR* in the two biological replicates ($P < 0.01$) and with a fold change in signal intensity (VAR*/TT01α) greater than 1.5 or smaller than −1.5 are listed in Table 3. We found that 148 genes displayed differential patterns of transcription between the two variants: 104 during stationary phase, 28 genes during the exponential growth phase, and 16 in both phases, with the pattern of transcription varying similarly in the two phases.

The transcription data collected by microarray hybridization were checked on the two biological replicates by qRT-PCR on a sample of 10 genes (Table 3). These genes were chosen because they were potentially related to the phenotypic variation and the pathogenicity. As frequently reported, the qRT-PCR fold changes for the expression ratios were greater than the fold changes obtained with microarray data. Nevertheless, 17 on 20 comparisons between TT01α and VAR* gave similar results in microarray hybridization and qRT-PCR (Table 3). We also compared our results with the results of a proteomic approach comparing the TT01 wild-type form and its phenotypic variant (61). As in our study, the difference in expression pattern between the wild-type form and the phenotypic variant was found to be greater in the stationary phase than in the exponential growth phase. These findings validate our microarray analysis.

A high proportion of genes (93/148) displayed higher levels of transcription in TT01α than in VAR*. In order to determine what kind of regulation alteration occurs in VAR*, we compared the qRT-PCR data of six of these genes (Fig. 1). One gene (*pit*) displayed a growth-independent pattern of transcription in both strains, which suggests a repression phenomenon during whole growth in VAR*. Five genes displayed a growth-dependent pattern in TT01α. *cipB*, *xnp2*, and *pirB* transcription was specifically induced in the stationary phase of TT01α, and this induction was diminished or absent in the stationary phase of VAR*. *cpmB* and *ompN* displayed a specific transcriptional induction in the exponential growth phase of TT01α that disappeared in VAR*. Therefore, both a transcriptional repression and an absence of transcriptional induction occurs in VAR*, and the second phenomenon seems more important.

The regions affected by the transcriptional modifications in VAR* were not colocalized. A 275-kb single-block duplication has previously been identified in the VAR* genome but was not observed in the TT01α genome (31). Gene amplification may confer physiological adaptation through the overproduction of transcripts and proteins, including those conferring antibiotic resistance, for example (57). Nevertheless, only 12 of the 148 differentially transcribed genes were located in the duplicated region. Furthermore, four of these genes were less strongly expressed in VAR* than in TT01α. This duplication therefore has no massive effect on transcription as a whole in the VAR* variant. This result confirms that phenotypic variant status is independent of global genomic architecture, as previously suggested (31).

A modified regulator network in VAR*. The transcription of 15 regulators or potential regulators (see regulators in Table 3, column 1) was found to be modified. For 10 of them, the transcription levels were higher in VAR* than in TT01α: the MrfJ repressor, a putative transcriptional regulator from the LuxR family, three of the 13 Ner-like regulatory proteins encoded in the *P. luminescens* TT01 genome, the CspE cold-shock protein, the FliZ regulator, the FimB recombinase, and the PqrA and RcsB regulators. Higher levels of FimB and MrfJ proteins may have affects on fimbrial production. This is validated from a genetic perspective, since four genes encoding fimbrial components—a *mrfA*-like gene (plu0418), *mrfA* (plu0769), *mrfH* (plu0777), and a putative adhesin-encoding gene (plu2156)—are more transcribed in VAR* than in TT01α. Moreover, sheep erythrocytes agglutination was only detected with VAR* bacterial cells (Table 4).

For five molecules, lower levels of transcription were observed in VAR* than in TT01α: the HslJ heat shock protein, three putative transcriptional regulators from the LuxR family, and a putative CI repressor. This large number of regulators with modified transcription patterns suggests that the generation of phenotypic variants in *P. luminescens* TT01 depends on transcription switching mediated by a network of regulators rather than a single regulator.

Previous regulation analyses have deciphered the role of particular regulatory genes in the generation of *Photorhabdus* phenotypic variants (22, 40, 53). The AstR-AstS two-component system controls the timing of the phenotypic switch be-

TABLE 3. Genes differentially transcribed between TT01α and VAR* in exponentially growing or stationary-phase cultures[a]

Category and locus tag	Gene	Protein(s) or similarity	Fold change ratio (VAR*/TT01α)[b]	
			Exp	Stat
Regulators				
plu0778	*mrfJ*	Mobility repressor, *mrfJ*	2.9	NS
plu0945		Putative transcriptional regulator, LuxR family	6.2	NS
plu1015	*ner*-like	Ner-like regulatory protein	3.4	NS
plu1289	*cspE*	Cold shock-like protein, CspE	NS	2.3
plu1956	***fliZ***	**FliZ protein**	**2.5 (NS)**	**NS (NS)**
plu1991	***fimB***	**Type 1 fimbrial regulatory recombinase protein, FimB**	**3.3 (8)**	**2.4 (4)**
plu2144	*hslJ*	Heat shock protein HslJ	–4.7	–33.9
plu2638	*pqrA*	Regulatory protein PqrA	NS	3.6
plu3048	*rcsB*	Capsular synthesis regulator component B, RcsB	NS	2.1
plu3219		Putative transcription regulator, LuxR family	NS	–5.4
plu3220		Putative transcription regulator, LuxR family	NS	–2.6
plu3420	*ner*-like	Ner-like regulatory protein	4.0	NS
plu3721		Putative transcription regulator LuxR family	NS	–2.5
plu4544		Putative repressor protein CI	NS	–2.1
plu4546	***ner*-like**	**Ner-like regulatory protein**	**2.6 (114)**	**NS (22)**
Extracellular proteins or enzymes				
plu0279		Putative secreted protein	3.5	7.8
plu0373		Hcp protein	NS	–2.8
plu0958		Putative acyl-transferase	4.6	2.9
plu1382	*prtS*	Extracellular M4 metalloprotease precursor PrtS	–2.0	–63.3
plu1517		Putative lipase/esterase	NS	–2.2
plu2235		Putative exochitinase	NS	–6.9
plu2337		Hypothetical secreted protein	NS	–4.8
plu2568		Hcp-like protein	–3.3	NS
plu2640		Hypothetical secreted protein	NS	2.3
plu3159		Putative lipase, Pdl	NS	–2.3
plu3369		Putative phospholipase A accessory protein	6.3	NS
plu3794		Some similarity to the hemolysin of *Fusobacterium nucleatum*	NS	–2.2
plu3797		Some similarity to the hemolysin of *Fusobacterium nucleatum*	NS	–3.6
Antibiotic Synthesis				
plu0183	***cpmB***	**CpmB protein involved in carbapenem biosynthesis**	**–4.1 (–27)**	**NS (NS)**
plu0184	*cpmC*	CpmC protein involved in carbapenem biosynthesis	–3.6	NS
plu0884		Putative killer protein of pyocin	NS	–2.2
plu0887		Putative C-terminal region of pyocin S1	–2.2	NS
plu1892		Putative C-terminal region of pyocin S3	–2.5	NS
plu1894		Putative pyocin S2 and S1	–2.0	NS
Resistance				
plu3547	*cat*	Chloramphenicol acetyltransferase	2.2	3.4
plu1369		Putative metal-dependent β-lactamase	NS	–2.2
Pigments				
plu4187		AntH involved in anthraquinone biosynthesis	–1.7	NS
plu0947		Putative anthraquinone monooxygenase	–2.7	NS
Crystalline inclusions				
plu0157	***cipB***	**Crystalline inclusion protein CipB**	**NS (NS)**	**–17.1 (–787)**
plu1575	*cipA*-like	Crystalline inclusion protein type II (CipA-like protein)	–10.5	–2.5
plu1576	*cipA*	Crystalline inclusion protein CipA	NS	–49.5
Envelope				
plu1752	***ompN***	**Outer membrane protein N precursor (porin OmpN)**	**–5.1 (–798)**	**NS (NS)**
plu2559	*mipA*	Scaffolding protein for murein synthesizing machinery	NS	4.7
plu2660	*pbgP1*	UDP-4-amino-4-deoxy-L-arabinose aminotransferase PbgP1	2.9	NS
plu3506	***ccm***	**CcmA1/A2 inner membrane protein**	**2.4 (4)**	**NS (25)**
Iron transport and metabolism				
plu1174		Putative siderophore uptake periplasmic binding protein	NS	–2.0
plu2817		Putative siderophore pyoverdine biosynthesis protein PvcB	NS	–2.6
plu2850	*ireA*	Outer membrane pore protein, putative siderophore receptor IreA	NS	1.9
plu2851		Putative hemin/siderophore ABC transporter, ATP-binding protein	NS	2.0
plu2852		Putative hemin/siderophore ABC transporter, permease protein.	NS	2.7

Continued on following page

TABLE 3—*Continued*

Category and locus tag	Gene	Protein(s) or similarity	Fold change ratio (VAR*/TT01α)[b]	
			Exp	Stat
Metabolism				
Carbohydrate				
plu0055	*rbsD*	High-affinity ribose transport protein RbsD	NS	6.8
plu0913	*eno*	Enolase (2-phosphoglycerate dehydratase)	2.8	NS
plu0955	*epd*	D-Erythrose 4-phosphate dehydrogenase	1.7	NS
plu3606	*rpiA*	Ribose 5-phosphate isomerase A	3.3	2.9
Amino acids				
plu0158	*amt*	L-Arginine:glycine amidinotransferase	NS	–6.8
plu1306	*gltJ*	Glutamate/aspartate transport system permease protein GltJ	NS	1.8
plu1307	*gltI*	Glutamate/aspartate transport system permease protein GltI	NS	7.3
plu1438	*ilvE*	Branched-chain amino acid aminotransferases, IlvE	NS	–4.2
plu1588	*artP*	Arginine transport ATP-binding protein ArtP	NS	1.7
plu2260		Omega-amino acid-pyruvate aminotransferase	NS	–2.4
plu4565		Putative carbamoyltransferase and nodulation protein NodU	NS	–4.6
plu4566		Putative amidinotransferase	NS	–4.6
plu4568	*cysK*	Cysteine synthase, *O*-acetylserine sulfhydrolase subunit	NS	–5.3
Oligopeptides				
plu2494	*oppA2*	OppA2, oligopeptide binding protein	NS	2.5
plu2493	*oppA1*	OppA1, oligopeptide binding protein	NS	2.7
Coenzyme				
plu0935		Putative biotin synthase-related enzyme	NS	2.4
Secondary metabolites				
plu2201	*mhpD*	MhpD involved in cinnamic acid biosynthesis	NS	–1.7
plu2202	*mhpC*	MhpC involved in cinnamic acid biosynthesis	NS	–2.0
plu2204	*hcaE*	HcaE involved in cinnamic acid biosynthesis	NS	–5.8
plu2205	*hcaF*	HcaF involved in cinnamic acid biosynthesis	NS	–1.9
plu2207	*hcaB*	HcaB involved in cinnamic acid biosynthesis	NS	–4.6
plu2209	*hcaD*	HcaD involved in cinnamic acid biosynthesis	NS	–13.2
Energy production and conversion				
plu2349	*ydcW*	Medium chain aldehyde dehydrogenase YdcW	NS	–2.4
plu3739	*aldB*	Aldehyde dehydrogenase B	NS	–7.5
plu4120	*glpQ*	Glycerophosphoryl diester phosphodiesterase,	NS	–2.2
Adhesion				
Fimbrial				
plu0418		Major structural mannose-resistant fimbrial subunit, MrfA-like	2.1	NS
plu0769	*mrfA*	Major structural mannose-resistant fimbrial subunit, MrfA	3.0	NS
plu0777	*mrfH*	Mannose-resistant fimbrial adhesin, MrfH	2.1	NS
plu2156		Putative fimbrial adhesin or pilin precursor	NS	1.8
Nonfimbrial				
plu1561		Putative calcium-dependent cell adhesion molecule-1	–2.0	–4.9
plu4231		Photopexin A/B-like	NS	–3.3
Insecticidal or nematicidal toxins				
plu1537	**pit**	**Insecticidal crystal-related protein Pit**	**–4.0 (–218)**	**–4.1 (–8,250)**
plu2442	***xnp2***	**Putative nematicidal protein 2 of *Xenorhabdus bovienii***	**NS (NS)**	**–2.2 (–13)**
plu2443		Conserved hypothetical protein (second gene of the *xnp2*-containing operon)	NS	–4.4
plu4092	***pirB***	**JHE-like toxin, "*Photorhabdus* insect-related" toxin, PirB**	**NS (NS)**	**–2.7 (–540)**
Stress response				
plu0075	*sodA*	Superoxide dismutase [Mn]	NS	–4.0
plu3068	*katE*	Catalase	NS	–2.0
plu2032	*uspG*	Universal stress protein G	NS	1.8
Other				
plu0318		Putative AidA protein (quorum-sensing and slow killing of *C. elegans*)	NS	–4.6
plu3352		Truncated gene. Putative C-terminal region of ABC transporter	NS	2.1
DNA replication, recombination, and repair				
plu1288	*ogt*	O-6-alkylguanine-DNA:cysteine-protein methyltransferase, Ogt	NS	–1.9
plu1669		Putative transposase	2.3	3.2
plu3546		Transposase, ISNCY family, ISPlu15A	2.2	2.3
plu3915		Putative transposase	2.5	NS

Continued on following page

TABLE 3—*Continued*

Category and locus tag	Gene	Protein(s) or similarity	Fold change ratio (VAR*/TT01α)[b]	
			Exp	Stat
Phages				
plu0014	*pts21*	Major phage tail tube protein	NS	–3.2
plu0442		Putative protein of bacteriophage	NS	–1.9
plu1463		Weakly similar to tail fiber protein (lambdoid prophage)	NS	–4.4
plu1464		Weakly similar to tail fiber protein (lambdoid prophage)	NS	–2.3
plu2022		Putative tail fiber protein	NS	–4.3
plu2024		Putative tail fiber protein from lambdoid prophage	NS	–2.1
plu2034		Putative DNA inversion product	NS	–2.2
plu2035		Putative tail fiber protein of prophage	NS	–3.0
plu2303		Putative tail fiber protein	NS	–3.5
plu2957		Putative bacteriophage protein	NS	–2.9
plu2959		Putative bacteriophage protein	NS	–6.2
plu3026		Putative bacteriophage protein	NS	7.4
plu3030		Putative bacteriophage protein	NS	2.5
plu3031		Putative bacteriophage protein	NS	2.2
plu3034		Putative bacteriophage protein	NS	2.5
plu3035		Putative bacteriophage protein	NS	9.0
plu3036		Putative bacteriophage protein	NS	6.3
plu3329		Putative tail fiber protein GP37	NS	–21.8
plu3331		Putative P2 tail fiber protein H and RNA polymerase beta	–1.9	–5.4
plu3748		Putative phage structural protein P5	NS	–3.5
plu3749		Putative phage lysis protein S (holin)	NS	–4.9
plu4369		Putative bacteriophage tail fiber assembly protein	NS	–6.4
plu4370		Putative N-terminal region of unknown bacteriophage protein	NS	–22.4
Unknown proteins				
plu0257		Conserved hypothetical protein	2.0	31.1
plu0281		Hypothetical transmembrane protein	3.0	5.3
plu0480		Conserved hypothetical protein	–1.9	NS
plu0625		Conserved hypothetical protein	NS	2.4
plu0640		Conserved hypothetical protein	–2.5	NS
plu0786		Putative transmembrane protein with membrane-spanning segments.	NS	–2.5
plu0933		Conserved hypothetical protein	NS	2.3
plu1381		Conserved hypothetical protein	NS	–2.8
plu1574		Conserved hypothetical protein	NS	–5.5
plu1577		Hypothetical protein	NS	–3.2
plu1793		Conserved hypothetical protein; Myb DNA-binding domain	–2.3	NS
plu1840		Conserved hypothetical protein	NS	–21.2
plu1886		Conserved hypothetical protein	NS	–3.3
plu1994		Conserved hypothetical protein; "Winged helix" DNA-binding domain	NS	–2.7
plu1995		Conserved hypothetical protein, PTS-regulatory domain (PRD)	NS	–2.2
plu2046		Conserved hypothetical protein	NS	–2.8
plu2048		Conserved hypothetical protein	NS	–2.4
plu2141		Conserved hypothetical protein	NS	2.5
plu2261		Conserved hypothetical protein	NS	–22.8
plu2534		Conserved hypothetical protein	NS	–8.3
plu2539		Conserved hypothetical protein	NS	–1.8
plu2818		Conserved hypothetical protein	NS	–4.0
plu3023		Conserved hypothetical protein	NS	4.0
plu3039		Conserved hypothetical protein	–1.9	NS
plu3097		Conserved hypothetical protein	NS	–2.6
plu3262		Conserved hypothetical protein	NS	2.0
plu3333		Conserved hypothetical protein	–2.0	–2.9
plu3994		Conserved hypothetical membrane protein	NS	–44.8
plu4238		Conserved hypothetical protein	NS	–5.6
plu4272		Conserved hypothetical protein	NS	3.0
plu4505		Conserved hypothetical protein	NS	–3.1

[a] Genes were differentially transcribed between TT01α and VAR* in exponentially growing (exp) or stationary phase (stat) cultures (fold change VAR*/TT01α >1.5 or <−1.5; $P < 0.01$). Shaded rows indicate genes located in the previously described 275-kb single-block duplication (31). Rows in boldface indicate genes that were chosen for qRT-PCR validation.

[b] Exp, exponentially growing culture; Stat, stationary-phase culture. Values in parentheses indicate the qRT-PCR fold change (VAR*/TT01α) when the validation was undergone. NS, not significant.

tween the wild-type form and phenotypic variants in *P. luminescens* TT01 (22). Activation of the two-component systems is not dependent on the level of transcription of the genes encoding the proteins involved. Instead, it depends on the phosphorylation status of the cytoplasmic receptor (3). Our approach therefore did not allow the detection of RNA variation for the AstR-AstS or any other two-component system. The gene encoding the HexA/LrhA repressor, a LysR-type tran-

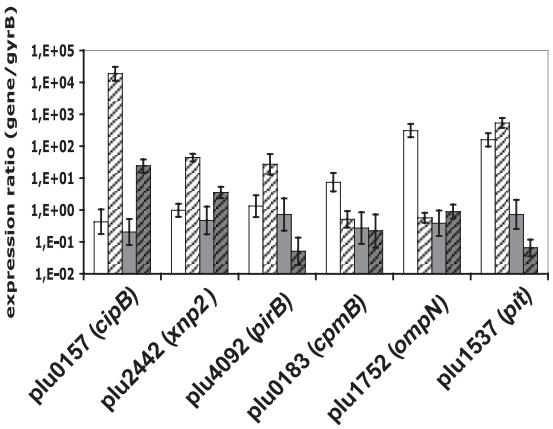

FIG. 1. qRT-PCR data on six genes displaying lower transcription in TT01α than in VAR*. qRT-PCR was carried out with total RNA from TT01α (white bars) and VAR* (gray bars) variants in the growth exponential phase (plain bars) and the stationary phase (hatched bars). The specific internal primers for each gene are listed in Table 2. The data are presented as a ratio, with *gyrB* used as the control gene. Values are means of six assays (three technical replicates on each biological replicates) and were compared using a Student *t* test ($P > 0.05$). The confidence limits are shown.

scriptional regulator, is not transcribed in wild-type forms of *P. temperata* K122 (40). However, our data show no difference in the transcription patterns of the genes encoding HexA/LrhA (Plu3090) or any other LysR-type transcriptional regulator between TT01α and VAR*. Finally, the Ner-like repressor has a more ambiguous role (53). Similar RNA levels for this repressor are found in the wild-type form and phenotypic variants of *P. temperata* K122. Repression of the wild-type form is observed when the *ner* gene is harbored on a plasmid and overexpressed in a phenotypic variant. Our data show that three of the 13 *ner*-like genes present in the *P. luminescens* TT01 genome are more strongly transcribed in VAR* than in TT01α. Therefore, as in *P. temperata* K122, *ner* overexpression is observed in a phenotypic variant of *P. luminescens* TT01. This overexpression may be mediated by both the redundancy of *ner*-like genes in the TT01 genome and the higher level of transcription observed in the VAR* variant. The reason for this higher level of transcription is unknown, but it seems likely to be controlled by an upstream global regulator.

Transcriptomic variations of genes supporting phenotypic variations. The wild-type strain and phenotypic variants of *P. luminescens* can be distinguished by a set of simple bacteriological tests, such as pigmentation on agar medium, trehalose fermentation, luminescence, the production of intracellular protein inclusions, and antimicrobial and extracellular activities (7, 31). TT01α and VAR* harbor the typical wild-type and phenotypic variant traits, respectively (Table 4). We explored the possibility that some phenotypic variant trait-related genes are regulated at the transcriptional level.

(i) Pigmentation. On agar media, wild-type forms generate convex, mucoid, orange colonies, whereas phenotypic variants generate flat, nonmucoid colonies without pigmentation (7, 31). The *antH* gene (plu4187) and plu0947, which are involved in the anthraquinone biosynthesis pathway (10), are less strongly transcribed in VAR* than in TT01α, probably accounting for the loss of pigmentation of VAR*.

(ii) Crystalline inclusion. One of the characteristic phenotypes of the wild-type bacterium is the presence of intracellular protein inclusions, known as crystals, which are absent from phenotypic variants (4). Crystalline inclusion proteins, CipA and CipB, are regulated transcriptionally in VAR*, since the *cipA* gene (plu1576), the *cipB* gene (plu0157), and a *cipA*-like gene (plu1575) displaying some similarity to *cipA* (identity, 36%; E-value, 8.4e−06) are less strongly transcribed in VAR* than in TT01α. The role of the crystalline inclusions remains unclear. They may be involved in nutrient storage, including sulfur storage in particular, due to the high methionine content of CipA (4). Moreover, the inactivation of either *cipA* or *cipB* through insertion results in mutants displaying variation in phenotypic traits and alteration in interactions with nematodes (4). For these reasons, Bintrim and Ensign speculated that Cip proteins might play a role in the global regulation of phenotypic variation. Since VAR* displayed changes to phenotypic traits but not to interactions with nematodes (D. Clarke, unpublished data), these two sets of phenotypes are probably regulated differently. For example, a small number of *cip* transcripts may be sufficient for correct interaction with nematodes but not for intracellular crystal formation and the generation of wild-type form traits.

(iii) Antibiotic synthesis. The phenotypic variant form had lower levels of antimicrobial activity (5). Because classical antibiosis tests are carried out in a Gram-positive bacterium, *Micrococcus luteus* (Table 4), we extended the antibacterial

TABLE 4. Phenotypes of the TT01α and VAR* variants[a]

Phenotype	TT01α	VAR*
Colony morphology	Convex, mucoid	Flat, nonmucoid
Pigmentation	+ (orange)	−
Coloration on TreGNO medium[b]	Green	Yellow
Bioluminescence	+	−
Crystal	+	−
Antibiotic production[c]	+	−
Sheep blood hemolysis[d]	T	−
Lipase activity on:		
Tween 20-60[e]	++	+
Tween 80-85	++	+[v]
Protease activity	++	w
Motility	+	+
Hemagglutination titer[f]		
Rabbit hemagglutination titer	<1	<1
Sheep hemagglutination titer	<1	8

[a] Unless noted otherwise, the results are scored as follows: +, positive; –, negative; v, variable; and w, weak.

[b] On TreGNO medium, phenotypic variant colonies undergoing trehalose fermentation acidify the medium, turning the bromothymol blue yellow, whereas wild-type colonies do not undergo trehalose fermentation and remain green (31).

[c] +, Zone of growth inhibition for *Micrococcus luteus* (laboratory collection); –, no growth inhibition.

[d] T, total hemolysis; –, no hemolysis.

[e] Halo of precipitation (diameter): ++, >20 mm; +, >10 mm.

[f] Agglutination assays were assessed on *Photorhabdus* bacterial suspensions (10^8 cells/ml) prepared from exponential growth or stationary-phase cultures in LB broth.

TABLE 5. Antibacterial activities of the TT01α and VAR* variants against a panel of microorganisms

Strain	Inhibition zone diam (mm)[a]												
	Gram-positive bacteria				Gram-negative bacteria								
	M. luteus	*C. xerosis*	*S. epidermidis*	*S. aureus*	*E. coli*	*P. vulgaris*	*P. aeruginosa*	*O. intermedium*	*O. anthropi*	*E. amylovora*	*Pseudomonas* sp.	*S. enterica*	*Y. enterocolitica*
TT01α	20	20	20	20	10	1	0	0	14	0	10	2	2
VAR*	0	0	0	0	0	13	0	0	0	0	0	0	0

[a] Antibacterial activities are expressed as the diameter of inhibition zones observed in petri plate bioassays. The full strain designations were as follows: *Micrococcus luteus* CIP6821, *Corynebacterium xerosis*, *Staphylococcus epidermidis* CIP6821, *Staphylococcus aureus* CIP7625, *Escherichia coli* CIP7624, *Proteus vulgaris* CIP5860, *Pseudomonas aeruginosa* CIP76.110, *Ochrobactrum intermedium* LMG3301^T, *Ochrobactrum anthropi* ATCC 49188^T, *Erwinia amylovora* CFBP1430, *Pseudomonas* sp. strain BW11M, *Salmonella enterica* 14028s, and *Yersinia enterocolitica* serotype O8.

activity test to other genera of bacteria. The antibacterial activity against the four Gram-positive bacteria observed with TT01α was not observed with VAR* (Table 5). Stilben is the only antimicrobial molecule produced by *P. luminescens* that has been shown to be active against Gram-positive bacteria (68). None of the genes involved in stilben biosynthesis was transcribed to different levels in VAR* and TT01α. The defect in antibiotic production by VAR* is therefore probably due to posttranscriptional modulation of the stilben biosynthesis pathway or antimicrobial molecules that have yet to be identified. Three kinds of antibiosis patterns were observed against Gram-negative bacteria: (i) no antibacterial activity for either form, (ii) antibacterial activity with TT01α but not with VAR* on both closed and more distantly related bacterial species, and (iii) antibacterial activity with VAR* but not with TT01α on *Proteus vulgaris* (Table 5). The pyocin-like bacteriocins of *P. luminescens* can kill related Gram-negative bacteria such as other *Photorhabdus* strains and *E. coli* (58). Two integral and two truncated genes encoding pyocin-like bacteriocins (bacteriocins Plu0884 and Plu1894 and bacteriocins Plu0887 and Plu1892, respectively) are less strongly transcribed in VAR* than in TT01α in the exponential growth or in the stationary phase. *P. luminescens* produces carbapenems of TT01, a class of β-lactam antibiotics with broad-spectrum activity mostly targeting Gram-negative bacteria (21). The *cpmB* and *cpmC* genes (plu0183 and plu0184), involved in the biosynthesis of carbapenems are less strongly transcribed in VAR* than in TT01α. The downregulation of both pyocin-like bacteriocins and carbapenems may therefore account for the lower levels of antimicrobial activity against the related *E. coli*, *S. enterica*, and *Y. enterocolitica* strains observed for VAR*, whereas the downregulation of carbapenems may only be sufficient to account for the lower levels of activity against the more distantly related *O. anthropi* and *Pseudomonas* sp. strains. For *P. vulgaris*, against which VAR* displayed a higher level of antibiosis than TT01α, other antimicrobial molecules are probably involved.

(iv) Extracellular protein activities. Finally, extracellular activities, such as hemolysis and lipase and protease activities, were found to be weaker in the phenotypic variants (5). The activities of two metalloproteases, PrtA and PrtS, were attenuated in the supernatant of the *P. temperata* K122 phenotypic variant (9, 49). Our data suggest that the transcription of *prtA* (plu0655) is not modified but that the *prtS* gene (plu1382) and its downstream gene (plu1381), which probably belongs to the same transcription unit, are less strongly transcribed in VAR* than in TT01α. The regulation of *lip-1* (plu3510), encoding a lipase responsible for Tween 80 degradation, is posttranslational in the K122 phenotypic variant of *P. temperata* (63). Our data confirm this result in *P. luminescens* TT01α, since no change in transcription was detected for the *lip-1* gene. Nevertheless, the uncharacterized lipase genes, plu1517 and plu3159, were less strongly transcribed in VAR*. The low rates of transcription of these two genes may account for poor Tween 20-60 degradation by VAR* (Table 4).

It is widely accepted that posttranscriptional and posttranslational regulation processes are responsible for the differential phenotypes expressed by the two forms of *Photorhabdus* (22, 29, 54, 61, 63, 64). In the present study, we show that genes involved in pigmentation, crystalline inclusion, antibiosis, and protease and lipase activities are controlled at the transcriptional level. Interestingly, for the lipase and protease activities, downregulation by both transcriptional and posttranscriptional mechanisms is probably involved in phenotypic variation.

Toxin overproduction in the VAR* variant does not restore virulence. VAR* is less virulent than TT01α when injected into *Spodoptera littoralis* larvae: 50% mortality (LT_{50}) was reached 25 h after injection with TT01α, but not until 37 h after injection for VAR*, although a larva mortality rate of 100% was nonetheless reached 3 days after infection (Fig. 2).

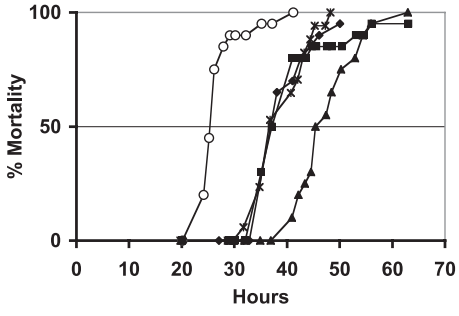

FIG. 2. Mortality in *S. littoralis*. Shown is the mortality in *S. littoralis* infected with the TT01α *P. luminescens* wild-type form (○), the VAR* phenotypic variant (■), and VAR* overexpressing toxins or potential toxin genes: the *pirAB* locus (✳), the *pit* gene (◆), and the *xnp2* gene (▲). Bacteria obtained at the end of the exponential growth phase were injected into fourth-instar larvae. Mortality values are based on data obtained after the injection of 20 larvae. Note that the virulence of the VAR* variant harboring the pBB-*xnp2* plasmid is more attenuated than the VAR* virulence; this is probably due to the energy cost necessary to replicate a plasmid with a large insert (7.3 kb) compared to the 0.5- and 1.7-kb inserts of the pBB-*pit* and pBB-*pirAB* plasmids, respectively.

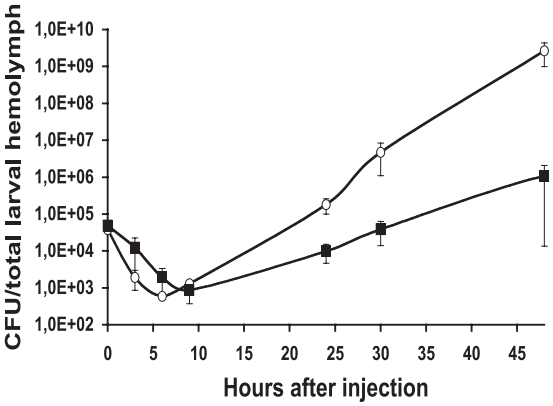

FIG. 3. Bacterial growth after the injection of TT01α (○) and VAR* (■) into *S. littoralis*. The graphs show the mean numbers of CFU recovered from the total hemolymph of single larvae (four larvae per time point). Larvae were each injected with 5×10^4 bacteria at time zero. Errors bars indicate the standard errors of the means.

P. luminescens can kill insects by toxemia. The *P. luminescens* TT01 genome contains a large number of genes thought to encode insecticidal toxins—i.e., the Tc, Mcf, Pir, Txp40, and Pit toxins—and a gene predicted to encode a putative nematicidal protein, the Xnp2 toxin (8, 19, 24, 47, 66). Our transcriptional analyses (microarray hybridization and qRT-PCR) showed that *pit* (plu1537), *pirB* (plu4092), and *xnp2* (plu2442) were less strongly transcribed in VAR* than in TT01α (Table 3 and Fig. 1). Interestingly, plu2443, the second gene of the operon containing *xnp2*, is also less strongly transcribed in VAR* than in TT01α.

We investigated the possible involvement of a defect in toxin gene transcription in the attenuation of virulence in VAR* by constructing strains overproducing the toxins. This was achieved by placing the *pit* gene, the *pirAB* genes and the four genes of the *xnp2*-containing operon under the control of the P_{lac} promoter of the pBBR1-MCS1 plasmid and transferring these constructs into VAR*. We first checked that the P_{lac} promoter was functional in VAR*, by measuring the β-glucuronidase activity of the pBB-P_{lac}*uidA* plasmid in VAR* (data not shown). We then carried out pathological assays, by injecting VAR*/pBB-*pit*, VAR*/pBB-*pirAB*, and VAR*/pBB-*xnp2* into *S. littoralis* larvae. The overproduction of these three proteins did not restore virulence in VAR* (Fig. 2).

Delayed growth of VAR* in *S. littoralis* hemolymph. *P. luminescens* can also kill insects by bacteremia. We evaluated the role of bacteremia in insect death by assessing the growth of VAR* and TT01α bacteria in *S. littoralis* hemolymph by injecting each variant into the hemocoel of larvae (5×10^4 CFU/larva) and counting the CFU produced. As for the closely related bacterium *Xenorhabdus nematophila*, but at a later time point after bacterial injection (59), a partial clearance of circulating bacteria was observed for both TT01α and VAR* (between 6 and 9 h after injection) (Fig. 3). Bacterial growth then increased for both TT01α and VAR*. Nevertheless, in contrast to the results obtained for LB broth, in which TT01α and VAR* grew similarly, TT01α grew more rapidly than VAR* in hemolymph (Fig. 3). The delayed virulence of VAR* may therefore be due to impaired VAR* bacteremia.

Downregulation of genes involved in drug or stress resistance. Our transcription profiling study showed that the expression of genes associated with resistance to antimicrobial compounds or the detoxification of toxic compounds was affected in VAR* (Table 3). Five genes were more strongly transcribed in VAR* than in TT01α: *mipA*, which encodes a protein involved in peptidoglycan synthesis and controlling the growth of the stress-bearing sacculus of *Escherichia coli* (62); *pbgP1*, the first gene of an operon encoding the components of an enzymatic pathway involved in arabinose incorporation in the lipid A moiety of lipopolysaccharide and responsible for polymyxin resistance in *Salmonella enterica* (23, 34, 35); *cat*, which encodes a chloramphenicol acetyltransferase (13); *ccm*, which encodes an internal protein, CcmA, influencing cell shape in *Proteus mirabilis* (37); and *uspG*, which encodes a universal stress protein that may, in some cases, be linked to resistance to DNA-damaging agents and to respiratory uncouplers (45). Eight genes were found to be less strongly transcribed in VAR* than in TT01α: plu1369, which encodes a putative β-lactamase (15); *ompN*, encoding a protein of the porin family responsible for the permeability of Gram-negative bacteria to small, polar molecules (55); *sodA* and *katE* (plu3068), which encode the manganese-dependent superoxide dismutase SodA and the catalase KatE, respectively, both of which are involved in the detoxification of reactive oxygen species in *Escherichia coli* (48); and the *hcaE*, *hcaF*, *hcaB*, and *hcaD* genes, which belong to an operon encoding the components of a pathway involved in the degradation of 3-phenylpropionate and cinnamic acid and participate in oxidative stress resistance in *Photorhabdus* (16, 17).

We investigated the sensitivity of TT01α and VAR* to various antimicrobial molecules, such as antimicrobial peptides (colistin, cecropin A, and polymyxinB) and antibiotics (ampicillin and chloramphenicol), but both forms were found to be equally resistant to the five molecules (data not shown). We then investigated the sensitivity of the variant to toxic compounds. A significant difference in growth between the variant and the wild type was detected only with hydrogen peroxide (H_2O_2), to which VAR* was more susceptible than TT01α. The lower levels of transcription of the *sodA* gene, the *katE* gene and the *hca* operon are probably responsible for this sensitivity. Interestingly, the difference in inhibition diameters between VAR* and TT01α was similar to that between a *P. luminescens* TT01 Δ*sodA* mutant and the corresponding wild-type strain (data not shown). We further investigated the arsenal of genes providing resistance to oxidative stress in the *P. luminescens* TT01 genome. A careful search of the genome showed that *P. luminescens* TT01 does not have the other canonical enzymes involved in resistance to oxidative stress, i.e., those encoding SodB, SodC, and KatG (48). Under oxygen-rich conditions resulting from the Fenton reaction, iron is also a source of dangerous radicals (36), and some iron-transporters protect bacterial cells against lethal doses of hydrogen peroxide (20, 56). Interestingly, the transcription patterns of five genes involved in iron uptake were found to be modified in VAR* (Table 3). Dealing with oxidative stress is a relevant challenge for insect pathogens. Indeed, insect hemocytes engaged in phagocytosis generate reactive oxygen intermediates (51). For instance, during encapsulation of the eggs of the wasp parasitoid *Leptopilina boulardi*, H_2O_2 is one of the principal

generated oxidants (52). Therefore, transcriptional alteration of genic arsenal involved in resistance to oxidative stress may explain the altered pathogenicity of VAR*.

Conclusion. In the present study, we evaluated the extent and impact of transcriptomic modulation in the phenotypes of the VAR* variant, focusing on its phenotypic variant status and lower virulence than the wild-type form TT01α.

In terms of the phenotypic variant status of VAR*, we described the transcriptional regulation of both regulators and genes supporting some of the traits of the wild-type form. Mixed regulation (transcriptional and posttranscriptional) probably occurs for some traits, suggesting that phenotypic variation is a highly complex regulation phenomenon.

Our investigation of the attenuated virulence of VAR* showed in VAR* lower levels of transcription of genes involved in insecticidal toxin production and in the oxidative stress response than in the wild type. Similar effects have already been reported for isogenic mutants displaying impaired production of the regulatory proteins HcaR, SodA, UvrY, and LuxS (17, 43, 44). Like VAR*, none of these four mutants displayed a complete lack of virulence. This is probably due to the high degree of redundancy of toxins and oxidative stress responses in *P. luminescens*. These studies also suggest that there is a close relationship between toxin production, oxidative stress resistance, and virulence, probably mediated by a complex array of regulators. Overall, our transcriptomic data for the VAR* variant displaying delayed pathogenicity for insects, but no defect for nematode reproduction in contrast to other *Photorhabdus* variants, show that different forms and mechanisms of phenotypic variation coexist in the *Photorhabdus* genus. Moreover, our data are consistent with the view that bacterial virulence is both multifactorial and combinatorial.

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REFERENCES

1. **Akhurst, R. J.** 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes Neoaplectana and Heterorhabditis. J. Gen. Microbiol. **121:**303–309.
2. **Ausubel, F. M., et al.** 1993. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, NY.
3. **Beier, D., and R. Gross.** 2006. Regulation of bacterial virulence by two-component systems. Curr. Opin. Microbiol. **9:**143–152.
4. **Bintrim, S. B., and J. C. Ensign.** 1998. Insertional inactivation of genes encoding the crystalline inclusion proteins of *Photorhabdus luminescens* results in mutants with pleiotropic phenotypes. J. Bacteriol. **180:**1261–1269.
5. **Boemare, N., J.-O. Thaler, and A. Lanois.** 1997. Simple bacteriological tests for phenotypic characterization of *Xenorhabdus* and *Photorhabdus* phase variants. Symbiosis **22:**167–175.
6. **Boemare, N. E.** 2002. Biology, taxonomy, and systematics of *Photorhabdus* and *Xenorhabdus*, p. 35–56. *In* R. Gaugler (ed.), Entomopathogenic nematology. CABI Publishing, Wallingford, United Kingdom.
7. **Boemare, N. E., and R. J. Akhurst.** 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (*Enterobacteriaceae*). J. Gen. Microbiol. **134:**751–761.
8. **Bowen, D., et al.** 1998. Insecticidal toxins from the bacterium *Photorhabdus luminescens*. Science **280:**2129–2132.
9. **Bowen, D. J., et al.** 2003. Genetic and biochemical characterization of PrtA, an RTX-like metalloprotease from *Photorhabdus*. Microbiology **149:**1581–1591.
10. **Brachmann, A. O., et al.** 2007. A type II polyketide synthase is responsible for anthraquinone biosynthesis in *Photorhabdus luminescens*. Chembiochem **8:**1721–1728.
11. **Brillard, J., E. Duchaud, N. Boemare, F. Kunst, and A. Givaudan.** 2002. The PhlA hemolysin from the entomopathogenic bacterium *Photorhabdus luminescens* belongs to the two-partner secretion family of hemolysins. J. Bacteriol. **184:**3871–3878.
12. **Brugirard-Ricaud, K., et al.** 2005. Site-specific antiphagocytic function of the *Photorhabdus luminescens* type III secretion system during insect colonization. Cell Microbiol. **7:**363–371.
13. **Bunny, K. L., R. M. Hall, and H. W. Stokes.** 1995. New mobile gene cassettes containing an aminoglycoside resistance gene, *aacA7*, and a chloramphenicol resistance gene, *catB3*, in an integron in pBWH301. Antimicrob. Agents Chemother. **39:**686–693.
14. **Cabral, C. M., A. Cherqui, A. Pereira, and N. Simoes.** 2004. Purification and characterization of two distinct metalloproteases secreted by the entomopathogenic bacterium *Photorhabdus* sp. strain Az29. Appl. Environ. Microbiol. **70:**3831–3838.
15. **Carfi, A., et al.** 1995. The 3-D structure of a zinc metallo-beta-lactamase from *Bacillus cereus* reveals a new type of protein fold. EMBO J. **14:**4914–4921.
16. **Chalabaev, S., et al.** 2008. Cinnamic acid, an autoinducer of its own biosynthesis, is processed via Hca enzymes in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **74:**1717–1725.
17. **Chalabaev, S., et al.** 2007. The HcaR regulatory protein of *Photorhabdus luminescens* affects the production of proteins involved in oxidative stress and toxemia. Proteomics **7:**4499–4510.
18. **Clarke, D. J.** 2008. *Photorhabdus*: a model for the analysis of pathogenicity and mutualism. Cell Microbiol. **10:**2159–2167.
19. **Daborn, P. J., et al.** 2002. A single *Photorhabdus* gene, makes caterpillars floppy (*mcf*), allows *Escherichia coli* to persist within and kill insects. Proc. Natl. Acad. Sci. U. S. A. **99:**10742–10747.
20. **Dellagi, A., M. N. Brisset, J. P. Paulin, and D. Expert.** 1998. Dual role of desferrioxamine in *Erwinia amylovora* pathogenicity. Mol. Plant-Microbe Interact. **11:**734–742.
21. **Derzelle, S., E. Duchaud, F. Kunst, A. Danchin, and P. Bertin.** 2002. Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **68:**3780–3789.
22. **Derzelle, S., et al.** 2004. AstR-AstS, a new two-component signal transduction system, mediates swarming, adaptation to stationary phase and phenotypic variation in *Photorhabdus luminescens*. Microbiology **150:**897–910.
23. **Derzelle, S., et al.** 2004. The PhoP-PhoQ two-component regulatory system of *Photorhabdus luminescens* is essential for virulence in insects. J. Bacteriol. **186:**1270–1279.
24. **Duchaud, E., et al.** 2003. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. Nat. Biotechnol. **21:**1307–1313.
25. **ffrench-Constant, R., et al.** 2003. *Photorhabdus*: toward a functional genomic analysis of a symbiont and pathogen. FEMS Microbiol. Rev. **26:**433–456.
26. **Fischer-Le Saux, M., V. Viallard, B. Brunel, P. Normand, and N. E. Boemare.** 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov., and *P. asymbiotica* sp. nov. Int. J. Syst. Bacteriol. 49 Pt. **4:**1645–1656.
27. **Forst, S., and D. Clarke.** 2002. Bacteria-nematode symbiosis, p. 57–77. *In* R. Gaugler (ed.), Entomopathogenic nematology. CAB International, Wallingford, Oxfordshire, United Kingdom.
28. **Forst, S., B. Dowds, N. Boemare, and E. Stackebrandt.** 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annu. Rev. Microbiol. **51:**47–72.
29. **Frackman, S., M. Anhalt, and K. H. Nealson.** 1990. Cloning, organization, and expression of the bioluminescence genes of *Xenorhabdus luminescens*. J. Bacteriol. **172:**5767–5773.
30. **Gaudriault, S., et al.** 2006. Whole-genome comparison between *Photorhabdus* strains to identify genomic regions involved in the specificity of nematode interaction. J. Bacteriol. **188:**809–814.
31. **Gaudriault, S., et al.** 2008. Plastic architecture of bacterial genome revealed by comparative genomics of *Photorhabdus* variants. Genome Biol. **9:**R117.
32. **Gerritsen, L. J., G. de Raay, and P. H. Smits.** 1992. Characterization of form variants of *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **58:**1975–1979.
33. **Givaudan, A., and A. Lanois.** 2000. *flhDC*, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellular hemolysis, and full virulence in insects. J. Bacteriol. **182:**107–115.
34. **Gunn, J. S., et al.** 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. **27:**1171–1182.
35. **Gunn, J. S., S. S. Ryan, J. C. Van Velkinburgh, R. K. Ernst, and S. I. Miller.** 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. Infect. Immun. **68:**6139–6146.
36. **Hantke, K.** 2001. Iron and metal regulation in bacteria. Curr. Opin. Microbiol. **4:**172–177.
37. **Hay, N. A., D. J. Tipper, D. Gygi, and C. Hughes.** 1999. A novel membrane

protein influencing cell shape and multicellular swarming of *Proteus mirabilis*. J. Bacteriol. **181:**2008–2016.
38. **Hu, K., and J. M. Webster.** 1998. In vitro and in vivo characterization of a small-colony variant of the primary form of *Photorhabdus luminescens* MD (*Enterobacteriaceae*). Appl. Environ. Microbiol. **64:**3214–3219.
39. **Hurlbert, R. E., J. Xu, and C. L. Small.** 1989. Colonial and cellular polymorphism in *Xenorhabdus luminescens*. Appl. Environ. Microbiol. **55:**1136–1143.
40. **Joyce, S. A., and D. J. Clarke.** 2003. A *hexA* homologue from *Photorhabdus* regulates pathogenicity, symbiosis, and phenotypic variation. Mol. Microbiol. **47:**1445–1457.
41. **Joyce, S. A., R. J. Watson, and D. J. Clarke.** 2006. The regulation of pathogenicity and mutualism in *Photorhabdus*. Curr. Opin. Microbiol. **9:**127–132.
42. **Kovach, M. E., et al.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **66:**175–176.
43. **Krin, E., et al.** 2006. Pleiotropic role of quorum-sensing autoinducer 2 in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **72:**6439–6451.
44. **Krin, E., et al.** 2008. Regulatory role of UvrY in adaptation of *Photorhabdus luminescens* growth inside the insect. Environ. Microbiol. **10:**1118–1134.
45. **Kvint, K., L. Nachin, A. Diez, and T. Nyström.** 2003. The bacterial universal stress protein: function and regulation. Curr. Opin. Microbiol. **6:**140–145.
46. **Lanois, A., G. Jubelin, and A. Givaudan.** 2008. FliZ, a flagellar regulator, is at the crossroads between motility, haemolysin expression and virulence in the insect pathogenic bacterium *Xenorhabdus*. Mol. Microbiol. **68:**516–533.
47. **Li, M., et al.** 2008. Expression and activity of a probable toxin from *Photorhabdus luminescens*. Mol. Biol. Rep. **36:**785–790.
48. **Lynch, A. S., and E. C. C. Lin.** 1996. Responses to molecular oxygen, p. 1526–1535. *In* F. C. Neidhart et al. (ed.), *Escherichia coli* and *Salmonella*, vol. 1. ASM Press, Washington, DC.
49. **Marokhazi, J., et al.** 2004. Comparison of proteolytic activities produced by entomopathogenic *Photorhabdus* bacteria: strain- and phase-dependent heterogeneity in composition and activity of four enzymes. Appl. Environ. Microbiol. **70:**7311–7320.
50. **Moureaux, N., T. Karjalainen, A. Givaudan, P. Bourlioux, and N. Boemare.** 1995. Biochemical characterization and agglutinating properties of *Xenorhabdus nematophilus* F1 fimbriae. Appl. Environ. Microbiol. **61:**2707–2712.
51. **Nappi, A. J., and E. Ottaviani.** 2000. Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays **22:**469–480.
52. **Nappi, A. J., and E. Vass.** 1998. Hydrogen peroxide production in immune-reactive *Drosophila melanogaster*. J. Parasitol. **84:**1150–1157.
53. **O'Neill, K. H., D. M. Roche, D. J. Clarke, and B. C. Dowds.** 2002. The *ner* gene of *Photorhabdus*: effects on primary-form-specific phenotypes and outer membrane protein composition. J. Bacteriol. **184:**3096–3105.
54. **Ong, K. L., and F. N. Chang.** 1997. Analysis of proteins from different phase variants of the entomopathogenic bacteria *Photorhabdus luminescens* by two-dimensional zymography. Electrophoresis **18:**834–839.
55. **Prilipov, A., P. S. Phale, R. Koebnik, C. Widmer, and J. P. Rosenbusch.** 1998. Identification and characterization of two quiescent porin genes, *nmpC* and *ompN*, in *Escherichia coli* BE. J. Bacteriol. **180:**3388–3392.
56. **Sabri, M., S. Leveille, and C. M. Dozois.** 2006. A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide. Microbiology **152:**745–758.
57. **Sandegren, L., and D. I. Andersson.** 2009. Bacterial gene amplification: implications for the evolution of antibiotic resistance. Nat. Rev. Microbiol. **7:**578–588.
58. **Sharma, S., et al.** 2002. The lumicins: novel bacteriocins from *Photorhabdus luminescens* with similarity to the uropathogenic-specific protein (USP) from uropathogenic *Escherichia coli*. FEMS Microbiol. Lett. **214:**241–249.
59. **Sicard, M., et al.** 2004. Stages of infection during the tripartite interaction between *Xenorhabdus nematophila*, its nematode vector, and insect hosts. Appl. Environ. Microbiol. **70:**6473–6480.
60. **Simon, R., U. Priefer, and A. Pülher.** 1983. A broad hostrange mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology (NY) **1:**784–791.
61. **Turlin, E., et al.** 2006. Proteome analysis of the phenotypic variation process in *Photorhabdus luminescens*. Proteomics **6:**2705–2725.
62. **Vollmer, W., M. von Rechenberg, and J. V. Holtje.** 1999. Demonstration of molecular interactions between the murein polymerase PBP1B, the lytic transglycosylase MltA, and the scaffolding protein MipA of *Escherichia coli*. J. Biol. Chem. **274:**6726–6734.
63. **Wang, H., and B. C. Dowds.** 1993. Phase variation in *Xenorhabdus luminescens*: cloning and sequencing of the lipase gene and analysis of its expression in primary and secondary phases of the bacterium. J. Bacteriol. **175:**1665–1673.
64. **Wang, H., and B. C. A. Dowds.** 1991. Molecular cloning and characterization of the lux genes from the secondary form of *Xenorhabdus luminescens* K122. Biochem. Soc. Trans. **20:**68S.
65. **Wang, Y., A. L. Bilgrami, D. Shapiro-Ilan, and R. Gaugler.** 2007. Stability of entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus luminescens*, during in vitro culture. J. Ind. Microbiol. Biotechnol. **34:**73–81.
66. **Waterfield, N., S. G. Kamita, B. D. Hammock, and R. ffrench-Constant.** 2005. The *Photorhabdus* Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. FEMS Microbiol. Lett. **245:**47–52.
67. **Waterfield, N. R., T. Ciche, and D. Clarke.** 2009. *Photorhabdus* and a host of hosts. Annu. Rev. Microbiol. **63:**557–574.
68. **Williams, J. S., M. Thomas, and D. J. Clarke.** 2005. The gene *stlA* encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in *Photorhabdus luminescens* TT01. Microbiology **151:**2543–2550.
69. **Wouts, W. M.** 1990. The primary form of *Xenorhabdus* species (*Enterobacteriaceae*, *Eubacteriales*) may consist of more than one bacterial species. Nematologica **36:**313–318.