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Examination of *Xenorhabdus nematophila* Lipases in Pathogenic and Mutualistic Host Interactions Reveals a Role for *xlpA* in Nematode Progeny Production▽†

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***Xenorhabdus nematophila* is a gammaproteobacterium and broad-host-range insect pathogen. It is also a symbiont of *Steinernema carpocapsae*, the nematode vector that transports the bacterium between insect hosts. *X. nematophila* produces several secreted enzymes, including hemolysins, lipases, and proteases, which are thought to contribute to virulence or nutrient acquisition for the bacterium and its nematode host in vivo. *X. nematophila* has two lipase activities with distinct in vitro specificities for Tween and lecithin. The gene encoding the Tween-specific lipase, *xlpA*, has been identified and is not required for *X. nematophila* virulence in one insect host, the tobacco hornworm *Manduca sexta*. However, the gene encoding the lecithin-specific lipase activity is not currently known. Here, we identify *X. nematophila estA*, a gene encoding a putative lecithinase, and show that an *estA* mutant lacks in vitro lipase activity against lecithin but has wild-type virulence in *Manduca sexta*. *X. nematophila* secondary-form phenotypic variants have higher in vitro lecithinase activity and *estA* transcript levels than do primary-form variants, and *estA* transcription is negatively regulated by NilR, a repressor of nematode colonization factors. We establish a role for *xlpA*, but not *estA*, in supporting production of nematode progeny during growth in *Galleria mellonella* insects. Future research is aimed at characterizing the biological roles of *estA* and *xlpA* in other insect hosts.**

Xenorhabdus nematophila is a gammaproteobacterium that maintains a mutually beneficial symbiosis with the nematode *Steinernema carpocapsae* (19, 28, 56), with which it causes disease and death of a broad range of insects, including lepidopterans (butterflies and moths), coleopterans (beetles and weevils), dipterans (flies), and orthopterans (grasshoppers, crickets, and locusts) (19, 20, 28). Many of these insect hosts, such as the tobacco hornworm, *Manduca sexta*, and the greater wax moth, *Galleria mellonella* (a parasite of beehives), are agricultural pests, and *X. nematophila* is used as a biocontrol agent (15, 17, 35, 47, 55).

The free-living form of the nematode, called the infective juvenile (IJ), serves as a vector to transport the bacterium between insect hosts (19, 28, 56). Once inside the insect, *X. nematophila* manufactures an arsenal of toxins and extracellular degradative enzymes, including hemolysins, lipases, and proteases, which are thought to contribute to its broad host range and may aid in virulence or decomposition of host tissues (7–9, 13, 19, 46, 53). The insect cadaver serves as a nutrient source for the growing bacteria and nematodes, which reproduce within the insect (19, 28). When nematode and bacterial populations exhaust these nutrients, the bacteria reassociate with the nematodes, which enter the IJ form of their life cycle and leave the cadaver in search of a new insect host (19, 28).

Although many of the specific in vivo nutritional requirements of both organisms are unclear, successful nematode reproduction requires lipids (22, 41, 58) and the presence of *X. nematophila*; *S. carpocapsae* nematodes that are not colonized by their bacterial symbiont produce significantly fewer nematodes than those that are colonized (22, 34, 48). The *X. nematophila* nutrients, signals, or other activities necessary for nematode reproduction and IJ development are unknown (31) but may include its many secreted enzymes. *X. nematophila* produces at least three distinct hemolysins, two lipases, and two proteases (7–9, 13, 18, 19, 46, 53). These activities can be distinguished in vitro based on substrate specificity. For example, one lipase has a primary activity against Tween, while the second is active against lecithin (7, 19, 50, 53). Some but not all of these activities have been genetically characterized. The Tween-specific lipase of *X. nematophila* is encoded by *xlpA* (39, 42), while the genetic locus encoding the lecithinase is unknown. However, the lecithinase activity of the F1 strain of *X. nematophila* has been biochemically characterized and shown to preferentially act on the substrate phosphatidylcholine (53). In addition, a role in virulence against particular insect hosts has been assigned for some enzymes but not others. The hemolysin XhlA contributes to virulence against *M. sexta* (13) and the cotton leafworm *Spodoptera littoralis* (30), while the Tween-specific lipase XlpA does not contribute to virulence in *M. sexta* (39, 42).

The expression of many of the secreted enzymes discussed above is affected by *X. nematophila* phenotypic variation. Wild-type *X. nematophila* exhibits two distinct colony forms that can be distinguished in most strains by binding (primary) or not binding (secondary) to bromothymol blue dye (19). In addition, the secondary form of *X. nematophila* strain ATCC 19061 lacks hemolytic activity but exhibits an increase in lipase activity against Tween compared to that exhibited by the primary form (11, 16, 57). These phenotypic forms do not have distin-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S17-1 (λ*pir*)	*E. coli* donor strain for conjugations	51
DH5α (λ*pir*)	*E. coli* general cloning strain	45
SM10 (λ*pir*)	*E. coli* general cloning strain	51
HGB007	*X. nematophila* wild-type ATCC 19061 (acquired in 1996)	ATCC
HGB009	*Bacillus subtilis* AD623	A. Driks
HGB081	*X. nematophila* wild-type AN6/1 Rif^r	S. Forst
HGB800	*X. nematophila* wild-type ATCC 19061 (acquired in 2003)	ATCC
HGB1300	HGB800 Δ*xlpA2*::Km	42
HGB1301	HGB800 Δ*estA1*::Km	This study
HGB1302	HGB1301 Δ*estA1*::Km Δ*xlpA3*::Sm	This study
HGB1320	HGB800 Δ*lrhA2*	This study
HGB1059	HGB800 *lrp-2*::Km	10
HGB1061	HGB800 secondary form	10
HGB1102	Δ*nilR16*::Sm	11
HGB1137	Δ*nilR16*::Sm (Tn*7-nilQR*)	11
HGB1230	Δ*cpxR1*::Sm	23
HGB151	HGB007 Δ*rpoS1*::Km	56
HGB597	HGB081 *flhD4*::Tn*10*; Km	42
Plasmids		
pBluescript II SK+	Amp^r; general cloning vector	Stratagene (La Jolla, CA)
pBluescript II KS+	Amp^r; general cloning vector	Stratagene (La Jolla, CA)
pKNJ102	Source of *aad* (Str^r) cassette (see Table 2 for primers)	12
pEV2	pBluescript KS+ with Km cassette (1.7 kb) in BamHI site	E. I. Vivas
pBlueStr	pBluescript SK+ with Sm cassette from pKNJ102 (1.17 kb) in BamHI site	This study
pBlueEstUp	pBluescript SK+ with EstUp (1,256-bp insert)	This study
pBlueEstUpDn	pBlueEstUp with EstDn (1,161-bp insert)	This study
pBlueEstUpKmDn	pBlueEstUpDn with Km from pEV2	This study
pBlueXlpUpDn	pBluescript SK with XlpUp (1,096-bp insert) and XlpDn (1,086 bp)	42
pBlueXlpUpSmDn	pBlueXlpUpDn with Sm from pBlueStr	This study
pKR100	Cm^r; *oriR6K* suicide vector	K. Visick, Loyola University
pKREstKm	pKR100 with EstUpKmDn (3.9-kb insert)	This study
pKRXlpSm	pKR100 with XlpUpKmDn (5.2-kb insert)	This study
pKNG101	Sm^r; *oriR6K* suicide vector	27
pKNGLrhAUpDn	pKNG101 with LrhAUp (1,212-bp insert) and LrhADn (1,142 bp)	This study

guishing effects on *X. nematophila*-host interactions; both forms are able to kill insects and colonize nematodes (11, 49, 57). However, in competition assays, the primary form is isolated more readily from nematodes and the secondary form more readily from insect cadavers, indicating that phenotypic variation may reflect adaptations to each host environment (12).

Although many *X. nematophila*-secreted enzymes have been proposed to contribute to the degradation and utilization of insect host tissues, in most cases a biological role has not been definitively determined. For example, while *xhlA* and *xlpA* mutants have been tested for insect virulence and colonization of nematodes after in vitro cocultivation, it is unknown if they have a defect compared to wild-type *X. nematophila* in supporting nematode progeny production during insect infection (13, 39, 42). In some other gram-negative pathogens, lipases have been shown to play a role in nutrient acquisition (38, 43). To assess the role of lipolytic activities in the biology of *X. nematophila*, we identified, mutated, and characterized the regulation of *estA*, encoding lecithinase activity, and analyzed the role of *estA* and *xlpA* in supporting nematode production within *G. mellonella* insects.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids utilized in these experiments are listed in Table 1. Luria-Bertani (LB) broth (33) was used to culture bacteria at 30°C. Media used to grow *X. nematophila* strains were either supplemented with 0.1% sodium pyruvate or stored in the dark (59). Unless stated otherwise, plasmids were introduced into *X. nematophila* strains through conjugation with *Escherichia coli* S17-1 (λ*pir*) as described previously (4, 56). Antibiotic resistance markers of plasmids and strains were selectively maintained at the following concentrations: ampicillin (Amp), 150 μg ml^{-1}; chloramphenicol (Cm), 30 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; and streptomycin (Sm), 25 μg ml^{-1}.

Molecular biological methods. This research was performed using standard molecular biological methods (45). DNA was PCR amplified using either Ex*Taq* (Takara, Otsu, Shiga, Japan) or Platinum *Pfx* (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. To verify correct sequence, inserts of all constructs were sequenced at the University of Wisconsin Biotechnology Center by the use of BigDye version 3.1 (Applied Biosystems, Foster City, CA). PCR purification, plasmid preparation, and gel extraction kits (Qiagen, Valencia, CA) were used according to the manufacturers' directions, as were restriction enzymes (Promega, Madison, WI). The primers used in this work (Integrated DNA Technologies, Coralville, IA; University of Wisconsin Biotechnology Center, Madison, WI) are presented in Table 2.

Construction of the *estA1* and *estA1 xlpA3* mutants. The *estA* and *xlpA* genes were found during a BLAST (2) search of the *X. nematophila* genome (https://www.genoscope.cns.fr/agc/mage/) for homologs of genes encoding other bacterial extracellular lipases (see Table S1 in the supplemental material). For *estA* mutant construction, primers with engineered restriction sites (Table 2) were used to PCR amplify, from HGB800 chromosomal DNA, fragments located upstream (primers EstAApaUpF and EstABamUpR; 1,256-nucleotide [nt] product) and downstream (primers EstABamDnF and EstAXbaDnR; 1,161-nt product) of the 1,953-nt region to be deleted (including the entire coding region, except the last 56 nt). PCR amplifications were conducted using Platinum *Pfx* (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, using

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Use
EstAApaUpF	NNNNGGGCCCCCGATTGTTGTTTCTCCAGAC	Mutant construction
EstABamUpR	NNNNGGATCCGGCACTCTCCTTAAAAGTCGT	Mutant construction
EstABamDnF	NNNNGGATCCGCAGCGCTCAAGATCCTAACT	Mutant construction
EstAXbaDnR	NNNNTCTAGACCAGAATTTCGTCCGGTTCGA	Mutant construction
LrhAApaUpF	NNNNGGGCCCCCAGCATTTCGTCACCTGTAT	Mutant construction
LrhAKpnUpR	NNNNGGTACCGCAGATCGAGATCGAGGTTTA	Mutant construction
LrhAKpnDnF	NNNNGGTACCCCTCTGCCGAAAATATAGAC	Mutant construction
LrhABamDnR	NNNNGGATCCCGCTAGGTTTACTGACTTGA	Mutant construction
StrepBamF	NNNNGGATCCCCAGGACAGAAATGCCTCGAC	pBlueStr construction
StrepBamR	NNNNGGATCCGCGTCGGCTTGAACGAATTGT	pBlueStr construction
RecAminFor	TGTCCGTTTGGATATCCGCC	qPCR
RecAminRev	CCCAGAGTATTAATACCTTCCCCAT	qPCR
EstAintF	GCCACCACAGGTAAACAGTG	qPCR
EstAintF	CCCAAAGTGCATAGAGGAGCT	qPCR

[a] Engineered restriction enzyme sites are underlined; N represents G, A, T, or C.

annealing temperatures of 48°C and 52°C, respectively. These fragments were subsequently cloned, using the engineered restriction sites (Table 2), into pBluescript II SK+, and the Km^r cassette (BamHI digested from pEV2) was cloned into the unique engineered BamHI site between them. The Δ*estA1*::Km construct was then cloned, using the KpnI and XbaI restriction sites, into the suicide vector pKR100 to create pKREstKm. This construct was then conjugated from *E. coli* S17-1 (λ*pir*) into HGB800 to create HGB1301, and allelic replacement in Km^r Cm^s exconjugants was verified by PCR amplification. For *estA xlpA* mutant construction, the Sm^r cassette (BamHI digested from pBlueStr) was cloned into the unique BamHI site of pBlueXlpUpDn (42), and the resulting Δ*xlpA3*::Sm construct was then cloned, using the KpnI and XbaI restriction sites, into the suicide vector pKR100 to create pKRXlpSm. To create the 1,052-nt deletion of *xlpA*, this construct was then conjugated from *E. coli* S17-1 (λ*pir*) into HGB1301 (*estA1*::Km) to create HGB1302, and allelic replacement in Km^r Sm^r Cm^s exconjugants was verified by PCR amplification.

Construction of markerless *lrhA* deletion mutant. The markerless Δ*lrhA* 922-nt deletion (of all but the first 45 nt and last 25 nt of the *lrhA* coding region) mutant was constructed using the method of Kaniga et al. (27). Primers with engineered restriction sites (Table 2) were used to PCR amplify, from HGB800 chromosomal DNA, fragments located upstream (primers LrhAApaUpF and LrhAKpnUpR; 1,212-nt product) and downstream (primers LrhAKpnDnF and LrhABamDnR; 1,142-nt product) of the region to be deleted, using Platinum *Pfx* (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and using annealing temperatures of 49°C and 45°C, respectively. These fragments were subsequently digested with KpnI and ligated together, and the resulting fragment was PCR amplified using the LrhAApaUpF and LrhABamDnR primers. The product was cloned into the suicide vector pKNG101 by using the ApaI and BamHI restriction sites to create pKNGLrhAUpDn. This construct was then conjugated from *E. coli* SM-10 (λ*pir*) into HGB800. HGB800 Sm^r exconjugants sensitive to 5% sucrose were then grown on LB agar overnight and subsequently grown on LB agar plus sucrose to select for sucrose-resistant exconjugants that had excised the vector. The Sm^s phenotype was verified, and deletion of the *lrhA* fragment was confirmed by PCR amplification.

In vitro phenotypic assays. In vitro phenotypic plate analyses were performed as described previously to assay motility (56), lipase activity (50), lecithinase activity (6, 53), protease activity (6), and hemolytic activity (24, 44) on agar containing 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) and antibiotic activity (32, 56) against *Bacillus subtilis*. In vitro phenotypic experiments were conducted a minimum of two times, with a minimum of two replicates per experiment.

Insect virulence assays. Tobacco hornworm *M. sexta* eggs (North Carolina State University) were reared to fourth-instar larval stage on an artificial Gypsy moth wheat germ diet (MP Biomedicals, Aurora, OH) as described previously (56). For virulence assays, three independent experimental replicates were performed. Stationary-phase cultures of *X. nematophila* strains were assessed as explained previously (13, 37). For each experimental replicate, overnight cultures grown at 30°C in LB broth were subcultured at 1:100 into fresh LB and grown for 18 to 24 h. The strains were then washed in PBS, diluted, and plated onto LB agar for calculation of CFU. Each treatment was injected into 10 insect larvae at a level of approximately 10^4 CFU per insect by the use of a 30-gauge syringe (Hamilton, Reno, NV), and mortality was monitored until at least 72 h postinjection. Logarithmic-phase *X. nematophila* cultures were assayed with the following modifications, as described previously (42); when subcultured 1:100 in fresh LB broth, cultures were incubated at 30°C until they reached an optical density at 600 nm of 0.8. Cultures were then injected at a level of approximately 10^2 CFU.

Nematode colonization and progeny production assays. Nematode cocultivations were executed as previously explained, with three independent experimental replicates (24). Briefly, for each experimental replicate, each *X. nematophila* strain was grown on three separate lipid agar plates to which sterile *S. carpocapsae* nematode eggs were added. For colonization assays, IJ nematodes reared on these plates were collected in White traps (29), surface sterilized, and sonicated. The sonicated nematode solution was serially diluted and plated for colonies, and this information was used to calculate the average number of CFU per nematode.

For nematode production assays, *S. carpocapsae* IJ nematodes were colonized with each *X. nematophila* strain as described above and subjected to assays similar to those of Mitani et al. (34), with the specified modifications. Uncolonized nematodes were reared on *X. nematophila* HGB151 *rpoS1*::Km mutants as previously described (56). Nematodes colonized by each strain were injected at a level of approximately 50 IJs into each of 12 *G. mellonella* larvae (Grubco, Hamilton, OH) using a 30-gauge syringe (Hamilton, Reno, NV) and subsequently placed in White traps. After injection, traps were monitored daily for IJ emergence, and the first 10 larvae per treatment to show IJ emergence were used to calculate average time postinjection to first emergence. To determine cumulative numbers of IJ progeny after the first day of emergence, IJs were collected from each trap and counted daily for the first 10 days, every 2 days from day 12 to day 20, and finally on days 21 and 28. Colonized nematodes carrying each *X. nematophila* strain were assayed for colonization preinjection and postemergence to verify the identities of strains and confirm wild-type levels of colonization. Any *G. mellonella* replicates showing signs of fungal contamination were excluded from the analysis.

Quantitative PCR detection of transcript levels. Measurement of transcript levels by using quantitative PCR (qPCR) was performed as described previously (13). By the use of the TRIzol extraction procedure (Invitrogen, Carlsbad, CA), complete cellular RNA was isolated from *X. nematophila* logarithmic-phase cultures grown in LB to an optical density at 600 nm of 0.9. Residual DNA was removed from the cellular RNA samples with DNase I (Roche Diagnostics, Mannheim, Germany). cDNA was synthesized using random hexamer primers (Integrated DNA Technologies, Coralville, IA) and AMV reverse transcriptase (Promega, Madison, WI). The cDNA samples were next subjected to qPCR in duplicate 25-μl reaction mixtures containing iQ SYBR green supermix (Bio-Rad, Hercules, CA) and the relevant primers that are listed in Table 2. Water was added in lieu of cDNA template as a negative control. qPCR reactions were performed on a Bio-Rad iCycler machine, with the resulting data analyzed using Bio-Rad iCycler iQ software. Transcript levels of *recA*, detected with primers RecAminFor and RecAminRev, were used to normalize cycle threshold results between strain cDNA samples. The conclusion that *recA* levels were suitable for normalization in these experiments was based on several facts. First, *recA* cycle numbers do not vary dramatically between reactions of primary- and secondary-form DNA template (data not shown). Second, an *lrp* mutant that displays

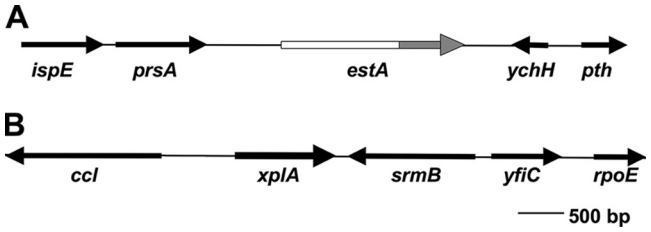

FIG. 1. The *estA* (A) and *xlpA* (B) loci of *X. nematophila*. Arrows indicate genes and their directions of transcription. The regions of *estA* predicted to encode a phospholipase/lecithinase/hemolysin domain and an autotransporter domain are designated by white and gray shadings, respectively. Genes were named based on their similarity to those of *E. coli*, with the exceptions of *estA* and *xlpA*, which were named based on their homologs in *S. liquefaciens* and *Yersinia enterocolitica*, respectively.

secondary-form characteristics (11) has similar levels of *recA* transcript according to whole-genome microarray data (X. Lu and H. Goodrich-Blair, unpublished data). Finally, our previously published data (11) show that most but not all genes, when normalized with *recA*, are expressed at lower levels in the secondary form than in the primary form, while the data presented here show that *estA* is expressed at higher levels in the secondary form. If a change in *recA* levels were occurring in the secondary form relative to those in the primary form, we would expect this change to have a consistent effect on our measurements of RNA levels regardless of the genes being tested.

Statistical analysis was performed on normalized cycle numbers, and data are presented accounting for the twofold change in the amount of product per cycle.

Statistical analysis. Transcript level cycle numbers, antibiotic production, nematode colonization, time to IJ progeny emergence, cumulative IJ progeny numbers, virulence percent mortality, and LT_{50} (time required to kill 50% of injected insects) data were analyzed with either an unpaired *t* test or one-way analysis of variance with Tukey's posttest at a 95% confidence interval by using GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA).

RESULTS

Identification of *estA*, a gene predicted to encode the lecithinase activity of *X. nematophila*. During laboratory growth, *X. nematophila* expresses two lipase activities with in vitro specificities against Tween and lecithin (6, 7, 53). It has been established that the gene *xlpA* (Fig. 1B) is necessary for the Tween-specific activity (39, 42). However, to date, the gene encoding *X. nematophila* lecithinase has not been reported. Using BLAST (2), we searched the *X. nematophila* genome (https://www.genoscope.cns.fr/agc/mage/) for additional genes predicted to encode homologs of other bacterial extracellular lipases implicated in virulence (see Table S1 in the supplemental material). This search revealed an open reading frame (XNC1_2104) that we named *estA* after a homolog encoded by the plant pathogen *Serratia liquefaciens* (Fig. 1A). In *S. liquefaciens*, EstA is a lipase required for the utilization of certain lipids as sole carbon sources (43). *X. nematophila estA* is predicted to encode a 668-amino-acid protein that is 38% identical and 56% similar to the *S. liquefaciens* homolog and also has similarity to the *lip-1* gene of the related entomopathogenic bacterium *Photorhabdus luminescens* (50% identity, 66% similarity) (2). The *X. nematophila estA* genetic locus is syntenic with that of *P. luminescens lip-1* but not with that of *S. liquefaciens estA*, which encodes the *swr* quorum-sensing system downstream. In both *X. nematophila* and *P. luminescens*, the *estA*/*lip-1* homologs are flanked upstream by the tandemly oriented *prsA* (predicted to encode a phosphoribosylpyrophosphate synthetase) and downstream by the divergently oriented *ychH* (predicted to encode a conserved, hypothetical protein) (Fig. 1A). The *X. nematophila* EstA protein has a putative N-terminal phospholipase/lecithinase/hemolysin domain (amino acids 1 to 407) and a C-terminal autotransporter domain (amino acids 426 to 668) predicted to contain integral membrane β barrels involved in autosecretion (Fig. 1A) (2). In addition, EstA is predicted to contain the serine-aspartate-histidine catalytic triad (S34, D350, H353), which is characteristic of lipases (3, 25, 26, 36).

***X. nematophila* mutants deficient in distinct lipase activities.** To determine if *X. nematophila estA* encodes the in vitro lecithinase activity, we created an *estA* deletion mutant, *estA1*::Km, by replacing the gene with a kanamycin-resistant cassette. Unlike the wild-type parent, the *estA1*::Km mutant did not have in vitro lecithinase activity against egg yolk (6, 53) but retained activity against Tweens 20, 40, and 60 (6, 50), indicating that *estA* likely encodes the lecithinase activity of *X. nematophila* (Table 3). *xlpA* lipase mutants exhibit defective in vitro lipase activity against Tweens 20, 40, and 60, as previously reported (39, 42), but have a wild-type level of lecithinase activity (Table 3). As expected, a double mutant lacking both *estA* and *xlpA* (*estA1*::Km *xlpA3*::Sm) lacked activity against Tween or lecithin (Table 3). Thus, the two *X. nematophila* in vitro lipase activities have now been attributed to distinct genetic loci.

TABLE 3. Selected phenotypes of *X. nematophila* lipase mutants

Strain	Activity of[a]:						LT_{50}[g]	
	Lipase[b]	Lecithinase[c]	Protease[d]	Hemolysin[d]	Motility[e]	Antibiotic production[f]	Log[h]	Stat[i]
Wild type	+	+	+	+	+	+	22.4 ± 0.7	23.4 ± 2.7
estA1::Km mutant	+	−	+	+	+	+	24.2 ± 0.9	24.8 ± 0.9
xlpA2::Km mutant	−	+	+	+	+	+	25.5 ± 1.3	25.6 ± 0.3
estA1::Km *xlpA3*::Sm mutant	−	−	+	+	+	+	24.7 ± 2.1	26.7 ± 1.7

[a] +, activity was indistinguishable from wild type; −, activity was not detected.
[b] Qualitative evaluation of halo surrounding the bacterial colony 3 days after inoculation on plates containing Tween 20, 40, or 60.
[c] Qualitative evaluation of halo surrounding the bacterial colony 4 days after inoculation on plates containing egg yolk.
[d] Qualitative evaluation of halo surrounding the bacterial colony 3 days after inoculation on milk or blood agar plates.
[e] Zone of growth 24 h after inoculation on 0.25% agar plates.
[f] Size of halo within lawn of indicator strain (*Bacillus subtilis*) surrounding the *Xenorhabdus* colony after 24 h of incubation.
[g] Data are mean times (h) to 50% mortality ± standard error for *M. sexta* injected with the respective mutants ($n = 3$).
[h] Logarithmic-phase cultures were injected.
[i] Stationary-phase cultures were injected.

To verify that the deletion of the *estA* and *xlpA* genes did not result in additional unexpected defects, *estA1*::Km, *xlpA2*::Km (42), and the *estA1*::Km *xlpA3*::Sm double mutant each were tested for several in vitro phenotypes, including hemolysin and protease activity, motility, and antibiotic production. Each of the three lipase mutants displayed wild-type phenotypes for each of these activities, as expected (Table 3).

***X. nematophila* lipase mutants exhibit wild-type nematode colonization and virulence against *M. sexta* insects.** To examine the potential roles of lipases in the host interactions of *X. nematophila*, *estA1*::Km, *xlpA2*::Km, and *estA1*::Km *xlpA3*::Sm mutants were tested for virulence in *M. sexta* insects and colonization of *S. carpocapsae* nematodes (Fig. 2) (42). Previously we reported that the *X. nematophila xlpA2*::Km mutant is as virulent as its wild-type parent when grown to logarithmic phase and injected into *M. sexta* larvae (42). Similarly, logarithmic-phase cells of *estA1*::Km and *estA1*::Km *xlpA3*::Sm mutants exhibited wild-type virulence, killing 90 to 100% of insects (Fig. 2A). Also, stationary-phase cells of each lipase mutant (the *estA* mutant, the *xlpA* mutant, and the double mutant) killed as many insects as did the wild-type control (stationary-phase cells typically kill fewer insects at higher doses than do logarithmic-phase cultures) (Fig. 2B). None of the mutants were delayed in the time it took them to kill *M. sexta*, as evidenced by their wild-type LT_{50}s (Table 3). Thus, the *estA* and *xlpA* lipase genes do not play a direct role in the ability of *X. nematophila* to cause disease in *M. sexta* insects. In addition, in vitro cultivation assays showed that each of the lipase mutants colonized the IJ stage of *S. carpocapsae* at levels no different from those of their wild-type parent (Fig. 2C), demonstrating that the XlpA and EstA lipases are not required by *X. nematophila* for colonization of the nematode host.

Colonization of *S. carpocapsae* by *xlpA* lipase mutants results in delayed and decreased nematode progeny production during *G. mellonella* insect infection. *X. nematophila* supports the development of *S. carpocapsae* nematodes within insects (22, 28, 34, 48), and *Steinernema* nematodes require host-derived lipids (1) for reproduction (14). However, the role of *Xenorhabdus* lipase activities in providing lipid compounds to their nematode hosts has not been directly tested. If *X. nematophila* lipase mutants are unable to provide specific lipid derivatives as a nutrient source to *S. carpocapsae* in insecta, it could be detrimental to nematode reproduction and lead to a delay in progeny IJ emergence and/or a decrease in the total number of progeny produced. This idea was tested by monitoring the ability of the lipase mutants to support nematode productivity in *G. mellonella* insects. In addition, we tested an *X. nematophila lrhA* mutant lacking the LysR-type regulator LrhA, since an *lrhA* mutant has a defect in Tween lipase activity and decreased levels of *xlpA* transcript (42). Nematodes colonized by wild-type *X. nematophila* or the *estA1*::Km, *xlpA2*::Km, *estA1*::Km *xlpA3*::Sm, or *lrhA2* mutant were injected into *G. mellonella* larvae. Uncolonized nematodes, which do not produce IJs in this insect host (22, 34), were injected as a negative control, and the time postinjection to first progeny IJ emergence, as well as the total number of progeny IJs produced over time, was recorded. All *G. mellonella* larvae died within a few days of injection. This was expected, as *S. carpocapsae* is known to kill *G. mellonella* in the absence of *X. nematophila* (22, 34).

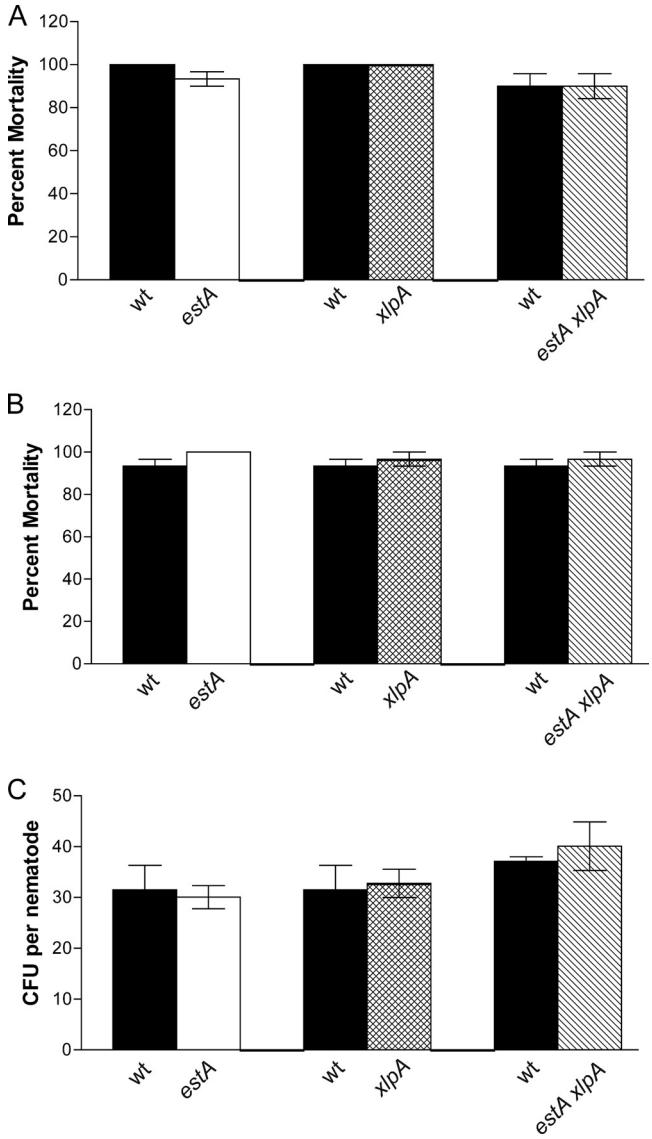

FIG. 2. Host interactions of *X. nematophila* lipase mutants. Ability of logarithmic-phase (A) or stationary-phase (B) *X. nematophila* cultures to kill *M. sexta* insects (note that virulence of logarithmic-phase *xlpA2*::Km cultures are from a study by Richards et al. [42] and are included for comparison). Cultures were injected, and percent mortality at 72 h is shown. (C) *X. nematophila* colonization of *S. carpocapsae* nematodes cultivated on lawns of each strain. The wild type (black bars; wt) and *estA1*::Km (white bars), *xlpA2*::Km (cross-hatched bars), and *estA1*::Km *xlpA3*::Sm (diagonal lines) mutants are shown. Separate wild-type results are presented because independent experiments were performed for each mutant. Error bars represent standard errors ($n = 3$). No significant differences were found ($P > 0.05$).

IJ progeny from nematodes colonized by wild-type *X. nematophila* took an average of 10.9 days postinjection to begin to emerge, and the times to emergence for *estA1*::Km (9.7 days) and *lrhA2* (10.2 days) mutants were not significantly different from that of the wild type ($P > 0.05$) (Fig. 3A). However, both the *xlpA2*::Km mutant and the *estA1*::Km *xlpA3*::Sm double mutant showed a significant increase in the time to emergence (15.9 and 13.9 days, respectively; $P < 0.05$). No progeny IJs emerged from *G. mellonella* injected with uncolonized IJs, as

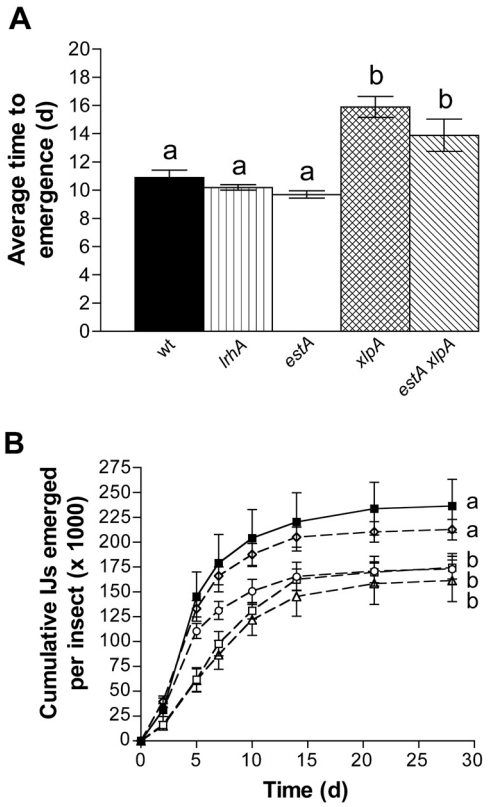

FIG. 3. Progeny production of nematodes colonized by *X. nematophila* lipase mutants. (A) IJ nematodes colonized by *X. nematophila* wild type (black bars; wt) or *lrhA2* (vertical lines) *estA1*::Km (white bars), *xlpA2*::Km (cross-hatched bars), or *estA1*::Km *xlpA3*::Sm (diagonal lines) mutants were injected into *G. mellonella* larvae, and the time to first emergence of progeny IJs was recorded. (B) Larvae were then monitored for total progeny IJ emergence at the indicated days. Nematodes with wild-type (black squares, solid lines) or *lrhA2* (white circles), *estA1*::Km (white diamonds), *xlpA2*::Km (white squares), or *estA1*::Km *xlpA3*::Str (white triangles) mutant treatment are shown. Different letters indicate significantly different values ($P < 0.05$; $n = 10$). In the case of panel B, significant differences refer to cumulative numbers at day 28.

expected. These data indicate that *xlpA*, but not *estA*, plays a role in the timing of nematode emergence from *G. mellonella* cadavers.

The *xlpA2*::Km and *estA1*::Km *xlpA3*::Sm mutants also produced significantly fewer progeny IJs than did the wild type over time. On day 5 of progeny IJ emergence, *xlpA2*::Km and *estA1*::Km *xlpA3*::Sm mutants produced, on average, fewer than half the number of IJs (61,808 and 60,916, respectively) as wild-type *X. nematophila* (145,186) (Fig. 3B). The average number of total progeny IJs emerging from *xlpA2*::Km and *estA1*::Km *xlpA3*::Sm mutant-infected insects remained significantly lower than that from the wild type throughout the experiment ($P < 0.05$) (Fig. 3B). One exception was that on day 14, the number of progeny from *xlpA2*::Km-infected insects, though fewer than those from wild-type-infected insects, was not significantly different ($P > 0.05$) (Fig. 3B). By 28 days, insects infected with the *xlpA2*::Km and *estA1*::Km *xlpA3*::Sm mutants produced, respectively, 74% (174,337) and 68% (161,124) the total number of progeny IJs produced by wild-type-infected insects (236,404) (Fig. 3B).

The number of cumulative progeny from insects infected with *lrhA2* mutant-colonized nematodes was fewer than that from the wild-type treatment throughout the experiment, although this difference was only statistically significant on days 21 and 28 ($P < 0.05$) (Fig. 3B). In contrast, insects infected with *estA1*::Km mutant-colonized nematodes generated levels of progeny IJs similar to those for the wild type throughout the experiment. From these results, we conclude that *xlpA*, but not *estA*, aids in production of nematode progeny within the insect host *G. mellonella*. As expected, the number of progeny IJs from uncolonized nematodes was below the level of detection (data not shown), and the *G. mellonella* larvae injected with these nematodes developed fungal contamination, consistent with the observations of Mitani et al. (34).

***X. nematophila* secondary form variant has increased in vitro lecithinase activity that correlates with an increase in *estA* expression.** In addition to regulation by LrhA, it is known that *xlpA* expression is positively regulated by the global regulator Lrp (leucine-responsive regulator protein) and the master flagellar regulator FlhDC through the action of the flagellar sigma factor FliA (11, 39, 42). In addition, secondary-form *X. nematophila* cells exhibit more Tween lipase activity in vitro than do primary-form cells, even though *xlpA* is expressed at lower levels in the secondary form (11). In contrast, nothing is known about the regulation of the *estA*-encoded lecithinase, although it has been observed that the secondary form exhibits more in vitro lecithinase activity than does the primary form (7). Because the regulation of *estA* may provide insights into the biological function of lecithinase activity, we examined the in vitro lecithinase activities of the primary and secondary forms of wild-type *X. nematophila* on egg yolk agar. The secondary form displayed higher levels of lecithinase activity than did the primary form (data not shown), as previously observed (7). To determine if this difference in activity between the two forms resulted from a difference in *estA* transcription, we monitored transcript levels of *estA* in the primary and secondary forms of *X. nematophila*. The secondary form had over four times the amount of *estA* transcript as the primary (Fig. 4A), thereby demonstrating that the increase in in vitro lecithinase activity for the secondary form correlates with an increase in the expression of *estA*. The *estA1*::Km mutant, which served as a negative control, had no detectable expression of *estA* transcript, as expected.

NilR, a regulator of *X. nematophila* colonization factors, mediates repression of *estA* expression. To further examine the regulation of *estA* expression, we tested a variety of *X. nematophila* transcriptional regulator mutants for differences in in vitro lecithinase activity compared to that of wild-type primary form. These included strains with mutations in *lrhA* (42), *lrp* (11), *flhD* (21, 42), *cpxR* (encodes the response regulator of a two-component system involved in both mutualism and pathogenesis of *X. nematophila*) (23), *rpoS* (encodes the stationary-phase sigma factor σ^S, which is required for nematode colonization) (56), and *nilR* (encodes a repressor of factors required for nematode colonization) (10). Each of these mutants had a level of lecithinase activity qualitatively similar to that of the wild-type primary form, with the exception of the *nilR16*::Sm mutant, which, like the wild-type secondary form, had a noticeable increase in lecithinase activity compared to that of the

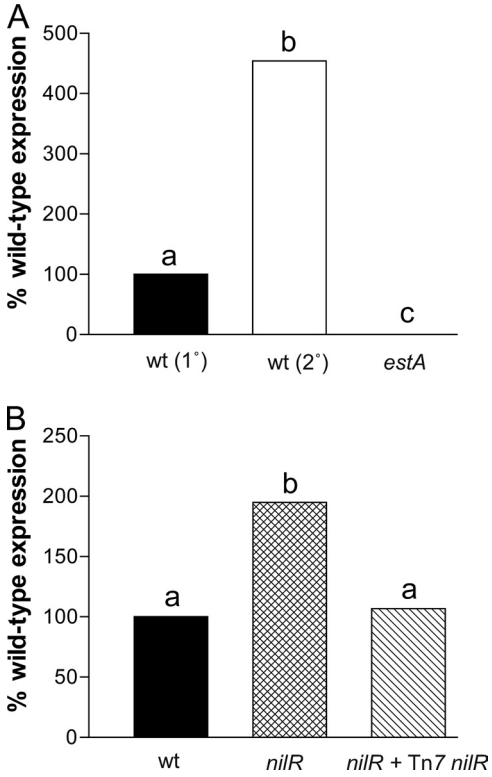

FIG. 4. Transcript levels of the *X. nematophila estA* gene. Total cellular RNA was extracted from logarithmic-phase *X. nematophila* cultures of primary-form wild type (black bar), secondary-form wild type (white bar), and the *estA1*::Km mutant (vertical lines, not visible) (A) or wild-type (black bar), *nilR16*::Sm (cross-hatched bar), and *nilR16*::Sm carrying a wild-type copy of *nilR* (diagonal lines) (B). cDNA was analyzed by qPCR. Levels of transcript are reported as percentages of primary-form wild type. Bars with different letters are significantly different from each other ($P < 0.05$; $n \geq 3$).

wild-type primary form. qPCR revealed that this higher in vitro activity corresponded with an *estA* transcript level approximately twice that of primary-form wild-type X. *nematophila* (Fig. 4B). Introduction of a wild-type copy of *nilR* into the *nilR16*::Sm strain (10) restored *estA* transcript levels to those of the primary-form wild type, confirming that *nilR* was responsible for this difference in transcription (Fig. 4B).

Although the in vitro lecithinase activity of the *nilR16*::Sm mutant was qualitatively indistinguishable from that of the secondary-form wild type, it appears that *estA* expression may be higher in the wild-type secondary form than in the primary-form *nilR16*::Sm mutant (Fig. 4). Thus, in an independent experiment, we directly compared *estA* levels between these two strains. The secondary-form wild type had higher *estA* transcript levels than the *nilR16*::Sm mutant (*estA* level in the secondary-form wild type was 276% that of the primary-form wild type, versus 174% for the *nilR16*::Sm mutant; $n = 3$), although this difference was not statistically significant. Nevertheless, the apparent difference in *estA* expression between the *nilR16*::Sm mutant and the secondary-form wild type implies that one or more additional regulators may contribute to *estA* expression in the secondary form.

DISCUSSION

***estA* is necessary for *X. nematophila* lecithinase activity.** The gene *xlpA*, encoding a Tween-specific lipase activity of *X. nematophila*, was characterized previously (39, 42), but the gene encoding a lecithinase-specific lipase activity remained unknown until this study. Here, we show that deletion of the *estA* gene, predicted to encode a lecithinase/phospholipase C (Fig. 1A), abolishes the in vitro lecithinase activity against egg yolk. The *estA* mutant did not display any differences from the wild type in other enzymatic activities, including protease, hemolysin, and lipase activities against Tween (Table 3). These data support the conclusion that *X. nematophila estA* encodes a lecithinase and, coupled with the previous characterization of the *xlpA*-encoded Tween lipase, establish genes responsible for two phenotypically distinct lipolytic activities produced by *X. nematophila*.

***xlpA*, but not *estA*, contributes to nematode productivity during infection of *G. mellonella*.** The lipase and lecithinase activities of *X. nematophila* are required for neither colonization of *S. carpocapsae* nematodes nor virulence against *M. sexta* insects (39, 42) (Fig. 2). The data presented here demonstrate a role for *xlpA* in supporting *S. carpocapsae* progeny IJ production during infection of *G. mellonella* insects (Fig. 3). Nematodes colonized by the *X. nematophila xlpA2*::Km or *estA1*::Km *xlpA3*::Sm mutants exhibited delayed emergence of progeny IJs (Fig. 3A) and produced significantly fewer total progeny IJs over time than did the nematodes colonized with wild-type *X. nematophila* (Fig. 3B). Based on these data, we conclude that *xlpA* contributes to, but is not essential for, the efficiency of nematode production. We predict that the contribution of *xlpA* to nematode production likely would impact the fitness of *S. carpocapsae* nematodes in nature, where scavengers and competitors threaten the persistence of the infected insect cadaver (for example, see reference 60). Furthermore, it is possible that the IJ progeny derived from insects infected with the *xlpA* mutants are less fit than those cultivated from wild-type-infected insects. Future analysis of other parameters important to the success of *S. carpocapsae* in nature, including size, longevity, infectivity, and lipid content, could reveal additional biological effects of *xlpA* mutants on the nematode life cycle.

Although not as severe at early time points, the *X. nematophila lrhA2* mutant had an overall defect, similar to that of the *xlpA* mutants, supporting the production of fewer progeny IJs than the wild type (Fig. 3B). Like the *lrhA2* mutant, an *lrp* mutant has been shown to exhibit a defect in production of nematode progeny compared to the wild type, although the *lrp* defect was measured during in vitro cultivations of nematodes (11). This is consistent with Lrp as a positive regulator of *lrhA* and LrhA, in turn, positively influencing the expression and secretion of XlpA (11, 42). However, transcription of *xlpA* is not abolished in an *lrhA* mutant (42), and it is likely that some XlpA production in both *lrhA* and *lrp* mutants accounts for these mutants' ability to support the production of higher numbers of IJ progeny than the *xlpA* mutant, either early on (*lrhA*) or overall (*lrp*).

In contrast to the *xlpA2*::Km lipase mutant, the *estA1*::Km lecithinase mutant did not have a defect in supporting nematode productivity in *G. mellonella* larvae (Fig. 3), nor did the

estA1::Km mutant have defects in nematode colonization or virulence against *M. sexta* insects, leaving the biological function of EstA lecithinase activity unclear. It is possible that *estA* does contribute to nematode productivity but that its influence is too subtle to be detected in our assay. Alternatively, the *G. mellonella* and *M. sexta* insects used in these studies may not have substantial levels of a lipid substrate recognized by *X. nematophila* lecithinase (5, 53). Although detailed biochemical characterization has not yet been conducted, *estA* is predicted to encode a phospholipase C activity (cleavage of the phosphoryl group from a phospholipid), while *xlpA* is predicted to encode a phospholipase A (cleaving carboxy-ester bonds) with a potentially broader substrate range (3, 25, 26). Since insect lipid content, including the relative concentrations and types of phospholipids, can vary depending on age, diet, and species (5), it is plausible that the role of *X. nematophila estA* would be more apparent in assays conducted in wild insects or in another insect species.

The regulation of *X. nematophila estA* expression may provide insights into its function. Transcript levels of *estA* are significantly higher in the secondary-form phenotypic variant of wild-type *X. nematophila* than they are in the primary form (Fig. 4A), which is consistent with the qualitatively higher level of in vitro lecithinase activity in secondary-form cells. While phenotypic variation of several activities is known to occur at the level of transcription (11), to our knowledge, *estA* is the first gene demonstrated to have elevated transcript levels in the secondary form. (Although secondary form cells also have increased in vitro lipase activity against Tween relative to primary form cells, *xlpA* transcript levels unexpectedly were lower in the secondary form than in the primary form [11].)

The lower level of *estA* transcript in primary form relative to that of secondary-form cells appears to be due to negative regulation mediated through a putative transcription factor, NilR, since the primary-form *nilR* mutant expresses *estA* at levels similar to those of wild-type secondary-form cells (Fig. 4B). NilR was discovered based on its role in repression of the nematode colonization factors *nilA*, *-B*, and *-C* (10). Although it is not yet known whether NilR regulates *estA* directly or if other regulators act on *estA*, the common regulation of *estA* with genes necessary for nematode colonization initiation may suggest that the lecithinase activity is important at late stages of nematode development, when population levels are high, nutrients are limiting, and IJs are forming. This is consistent with the fact that *estA* is not required for virulence during early stages of insect infection (Fig. 2). Although *estA* is not required for nematode productivity (Fig. 3), perhaps it functions to supply alternative or additional nutrients for the bacteria or nematodes in a nutrient-depleted environment. Indeed, *S. liquefaciens estA* mutants are defective in in vitro growth on lipids as a sole carbon source (43), and the role of phospholipases in phosphate acquisition has been posited for some bacteria (38, 52, 54). In vivo expression studies of *nilR*, *estA*, and *nilA*, *-B*, and *-C* during the course of insect infection could help shed light on when NilR and lecithinase activity are important.

The significant role of *Xenorhabdus* bacteria in supporting *Steinernema* nematode reproduction has long been recognized (40) and routinely has been presumed to be due to bacterial enzymatic activities. The identification of genetic elements responsible for *X. nematophila* lipase activity and the demonstration of a role for one of these, *xlpA*, in nematode productivity represent a first step toward understanding the molecular basis of this aspect of the symbiosis between *X. nematophila* and *S. carpocapsae*.

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