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CpxRA Contributes to *Xenorhabdus nematophila* Virulence through Regulation of *lrhA* and Modulation of Insect Immunity▽

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The gammaproteobacterium *Xenorhabdus nematophila* is a blood pathogen of insects that requires the CpxRA signal transduction system for full virulence (E. E. Herbert et al., Appl. Environ. Microbiol. 73:7826–7836, 2007). We show here that the Δ*cpxR1* mutant has altered localization, growth, and immune suppressive activities relative to its wild-type parent during infection of *Manduca sexta* insects. In contrast to wild-type *X. nematophila*, which were recovered throughout infection, Δ*cpxR1* cells did not accumulate in hemolymph until after insect death. In vivo imaging of fluorescently labeled bacteria within live insects showed that Δ*cpxR1* displayed delayed accumulation and also occasionally were present in isolated nodes rather than systemically throughout the insect as was wild-type *X. nematophila*. In addition, in contrast to its wild-type parent, the Δ*cpxR1* mutant elicited transcription of an insect antimicrobial peptide, cecropin. Relative to phosphate-buffered saline-injected insects, cecropin transcript was induced 21-fold more in insects injected with Δ*cpxR1* and 2-fold more in insects injected with wild-type *X. nematophila*. These data suggest that the Δ*cpxR1* mutant has a defect in immune suppression or has an increased propensity to activate *M. sexta* immunity. CpxR regulates, directly or indirectly, genes known or predicted to be involved in virulence (E. E. Herbert et al., Appl. Environ. Microbiol. 73:7826–7836, 2007), including *lrhA*, encoding a transcription factor necessary for *X. nematophila* virulence, motility, and lipase production (G. R. Richards et al., J. Bacteriol. 190:4870–4879, 2008). CpxR positively regulates *lrhA* transcript, and we have shown that altered regulation of *lrhA* in the Δ*cpxR1* mutant causes this strain's virulence defect. The Δ*cpxR1* mutant expressing *lrhA* from a constitutive *lac* promoter showed wild-type virulence in *M. sexta*. These data suggest that CpxR contributes to *X. nematophila* virulence through the regulation of *lrhA*, immune suppression, and growth in *Insecta*.

Xenorhabdus nematophila is a model organism for the study of microbe-host interactions due to its biphasic life cycle, during which it acts as a mutualistic symbiont of the nematode *Steinernema carpocapsae* and a pathogen of many types of insect (1). In nature, the bacteria are transported by the nematode vector into an insect body, where they rapidly cause disease and death of the insect (16). *Manduca sexta* insects mount both humoral and cellular innate immune responses to microbial invaders (26). In response to microbe-associated molecular patterns, such as lipopolysaccharide and flagella, *M. sexta* fat body and hemocytes produce antimicrobial peptides effective against gram-positive and gram-negative organisms (12, 31). Transcription of some antimicrobial peptides, including cecropin, is induced upon microbial invasion (18). *M. sexta* immune cells also encapsulate parasites and pathogens in nodules (18) that become melanized through the action of a phenoloxidase cascade (26).

X. nematophila produces a variety of exoenzymes and secreted proteins, such as toxins and hemolysins that contribute to pathogenicity in insects (2–4, 10, 25, 30, 36, 37, 42). The bacteria actively suppress both cellular and humoral insect immunity through destruction of insect immune cells (5), inhibition of phagocytosis (38), and suppression of both phenoloxidase activation (11, 14, 17) and transcription of genes encoding insect antimicrobial peptides (24, 33).

Regulators of both mutualism and pathogenicity in *X. nematophila* have been described and linked to genes specifically involved in one or both interactions. Regulatory proteins that contribute to pathogenesis include LrhA (35) and FlhDC (19, 32), while Lrp and CpxR are necessary for both interactions (9, 21).

The CpxRA two-component signaling system regulates both mutualism and pathogenesis in *X. nematophila*, since a *cpxR* deletion mutant (Δ*cpxR1*) is defective in insect killing, as well as nematode colonization (21). A number of genes have been identified as CpxR regulon members through phenotypic analysis and quantitative measurement of transcript levels in a Δ*cpxR1* mutant (21). These genes encode various pathogenicity and mutualism factors (21), including membrane proteins necessary for nematode colonization (*nilABC*) (7, 8, 23), the *X. nematophila* C1 hemolysin (*xaxA*) (42), a protease required for full virulence toward *M. sexta* insects (*prtA*) (C. Lipke, unpublished data), and LrhA, encoding a LysR-type regulator of lipase activity, motility, flagellar export, and toxin expression (35).

Lrp was shown to affect expression of at least 65% of cellular proteins in *X. nematophila* (9) and is thought to help the cell respond to internal stresses, such as metabolic changes, while CpxR, based on the *Escherichia coli* model (34), is expected to respond to changes in the external environment and maintain the integrity of the cell envelope and its components. Therefore, Lrp and CpxR and their regulons are of special interest

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
X. nematophila strains		
HGB800	*X. nematophila* ATCC 19061, wild type	American Type Culture Collection
HGB1227	HGB800 Tn*7*	21
HGB1230	HGB800 Δ*cpxR1*	21
HGB1231	HGB800 Δ*cpxR1* Tn*7*	21
HGB1232	HGB800 Δ*cpxR1* Tn*7-cpxRA*	21
HGB1350	HGB800, recovered from dead insect hemolymph, single-colony isolate 1	This study
HGB1351	HGB800, recovered from dead insect hemolymph, single-colony isolate 2	This study
HGB1352	HGB800, recovered from dead insect hemolymph, single-colony isolate 3	This study
HGB1353	HGB800, recovered from nematode vesicle, single-colony isolate 1	This study
HGB1354	HGB800, recovered from nematode vesicle, single-colony isolate 2	This study
HGB1355	HGB800, recovered from nematode vesicle, single-colony isolate 3	This study
HGB1356	HGB800 Δ*cpxR1*, recovered from dead insect hemolymph, single-colony isolate 1	This study
HGB1357	HGB800 Δ*cpxR1*, recovered from dead insect hemolymph, single-colony isolate 2	This study
HGB1358	HGB800 Δ*cpxR1*, recovered from dead insect hemolymph, single-colony isolate 3	This study
HGB1359	HGB800 Δ*cpxR1*, recovered from nematode vesicle, single-colony isolate 1	This study
HGB1360	HGB800 Δ*cpxR1*, recovered from nematode vesicle, single-colony isolate 2	This study
HGB1361	HGB800 Δ*cpxR1*, recovered from nematode vesicle, single-colony isolate 3	This study
HGB1362	HGB800 Tn*7*-P_{lac}-*lrhA*	This study
HGB1363	HGB800 Δ*cpxR1* Tn*7*-P_{lac}-*lrhA*	This study
S. enterica HGB298	*S. enterica* serovar Typhimurium	D. Downs, University of Wisconsin—Madison
E. coli S17-1(λ*pir*)	Donor strain for conjugations	40
Plasmids		
pEVS107	Mini-Tn*7* transposon vector	29
pVSV209	Source of constitutive P_{lac} promoter	13
pEVS107-P_{lac}	pEVS107 + P_{lac} promoter cloned in using AvrII and ApaI restriction sites	This study
pEVS107-P_{lac}-*lrhA*	pEVS107/P_{lac} + promoterless *lrhA* gene cloned in using ApaI and NdeI sites	This study

due to their likely role in adaptation to diverse environments and nutritional conditions associated with transitions between animal hosts. Partially overlapping regulons comprised of genes linked to either virulence and/or mutualism have been associated with Lrp and CpxR, such as *mrxA*, encoding the *X. nematophila* type I pilin subunit protein, and *nilA*, *nilB*, *nilC*, and *lrhA* (7, 21, 35). Lrp and CpxR both positively regulate *lrhA* (35) and, consistent with this, both *lrp* and *cpxR* mutants display defects in motility and lipase activity similar to, though less severe than, *lrhA* mutants (9, 21).

To provide insights into how CpxR contributes to virulence and immune suppression functions of *X. nematophila*, specific aspects of the disease process were examined in insects injected with wild-type and Δ*cpxR1* mutant strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in the present study are listed in Table 1. All bacterial strains were grown in LB medium or LB agar (20 g liter^{-1}) (28) plus 0.1% pyruvate and incubated in the dark at 30°C, unless specified otherwise. Antibiotics were used at the following concentrations: ampicillin (150 μg ml^{-1}), chloramphenicol (15 μg ml^{-1} for *X. nematophila* and 30 μg ml^{-1} for *E. coli*), kanamycin (50 μg ml^{-1}), and streptomycin (12.5 μg ml^{-1} for *X. nematophila* and 25 μg ml^{-1} for *E. coli*).

Phenotypic assays. *X. nematophila* overnight cultures from individual colonies picked from plates with LB medium plus ampicillin were used in phenotypic assays as previously described (21).

Constitutive *lrhA* expression. The constitutive *lac* promoter was PCR amplified out of pVSV209 (13) by using the primers EH196 (5′-GCCTAGGAACACCCCTTGTATTACTG-3′) and EH197 (5′-GCGGGCCCATGTATATCTCCTTTCTTAG-3′) (the restriction enzyme sites are underlined).

The *lac* promoter fragment was cloned into pEVS107 (containing a mini-Tn*7* transposon) by using AvrII and ApaI restriction sites to create pEVS107/P_{lac}. The *lrhA* gene (without its promoter region) was PCR amplified from HGB800 chromosomal DNA by using the primers EH218 (5′-GGGGGCCCATGATAAATGCAAATCGTCAG-3′) and EH219 (5′-GGGGGCCCTTATTCGTCTATATTTTCGG-3′). The *lrhA* promoter fragment was cloned into pEVS107/P_{lac} by using ApaI restriction sites to create pEVS107/P_{lac}-*lrhA*. pEVS107/P_{lac}-*lrhA* was mated into wild-type *X. nematophila* and the Δ*cpxR1* mutant. Clones were picked based on ampicillin and erythromycin resistance and sensitivity to kanamycin. Successful conjugation of the Tn*7* transposon was verified by PCR, and *lrhA* expression was monitored by quantitative PCR (qPCR) of stationary-phase cells.

Insect assays. All injections were performed on fourth- or fifth-instar *M. sexta* insects that had been reared from eggs as previously described (10). For mortality assessment, bacterial strains were grown overnight in the dark in LB medium, and ~1,000 stationary-phase cells were injected. For assessment of bacterial growth in insects, overnight cultures were subcultured 1:100 and grown to an optical density of ~0.5, and then ~100 log-phase cells were injected. At time points after injection, insects were kept briefly on ice, surface sterilized with 75% ethanol, and poked in the leg with a needle (Becton Dickinson, Franklin Lakes, NJ) to extract hemolymph. Hemolymph was then serially diluted in phosphate-buffered saline (PBS), plated on LB medium plus ampicillin, and grown overnight at 30°C. Colonies were counted to determine bacterial CFU/μl of insect hemolymph.

In vivo imaging of infections. *M. sexta* insects were treated as described above. At various time points after injection, 5 to 10 insects per experiment were visualized over time by using an IVIS Imaging System 200 (Xenogen Corp., Alameda, CA). Fluorescence was quantified by using Living Image software v2.6 (Xenogen).

Cecropin transcript measurement. *M. sexta* insects were treated as described above. At 9 and 16 h after the injection of ~100 log-phase cells, the insects were

frozen at −80°C. Total insect RNA was extracted from the frozen insects as previously described (10). Total RNA was treated with DNase I enzyme to remove contaminating DNA, and the RNA was reverse transcribed into cDNA by using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) as described previously. Cecropin transcript level was quantified by using qPCR as described previously (10).

RESULTS

Accumulation of suppressor mutations does not account for partial virulence and nematode colonization of the Δ*cpxR1* mutant. The virulence defect of a Δ*cpxR1* mutant is variable and, in some cases, although delayed relative to the wild type, the Δ*cpxR1* mutant can kill up to an average of 75% of insects by 96 h (21). Also, on average, only ~50% of nematodes grown on the Δ*cpxR1* mutant are successfully colonized (22a), resulting in a lower average CFU/infective juvenile than that of wild-type *X. nematophila* (21). A possible explanation for both of these phenotypes is the presence and selection within the animal host environments of *X. nematophila* mutations that suppress the Δ*cpxR1* defect. To test this idea, we isolated bacteria from insects and nematodes successfully killed or colonized by the Δ*cpxR1* mutant. If these strains represent suppressors of the Δ*cpxR1* mutant-host interaction defects, then they should display wild-type phenotypes when reintroduced into their hosts. We confirmed, through PCR amplification of the *cpxR* gene locus in single colonies of these insect-recovered (IR) and nematode-recovered (NR) Δ*cpxR1* mutant strains, that the *cpxR* deletion was retained during passage through animals. Δ*cpxR1*-IR and Δ*cpxR1*-NR strains were assayed for their ability to kill insects and colonize nematodes (Fig. 1).

When injected into insects, wild-type cells took an average of 23 ± 2 h to kill 50% of the insects injected (LT_{50}), while the Δ*cpxR1* mutant took longer, as expected, with an average LT_{50} of 34 h ± 0 h (Fig. 1A). Single-colony WT-IR and Δ*cpxR1*-IR isolates recovered from the hemolymph of dead insects were not significantly different from their respective counterparts cultured directly from frozen stocks: three single WT-IR colonies had an average LT_{50} of 21 h ± 1 h, while Δ*cpxR1*-IR colonies had an average LT_{50} of 35 h ± 1 h. Cells recovered from nematodes colonized with the WT-NR and Δ*cpxR1*-NR strains had LT_{50} values not significantly different from their parent strains (Fig. 1A), indicating that a single passage through the vector nematode host does not select for increased rate of killing. Similarly, bacteria recovered from dead insects did not show a significant increase in nematode colonization compared to wild-type *X. nematophila* (Fig. 1B).

When bacterial cells isolated from colonized nematodes were used to colonize new nematodes, the WT-NR strain had an average colonization level of 110% that of wild-type cells, while the Δ*cpxR1*-NR strain showed a colonization level of 54% that of wild-type cells, a level significantly different from those of wild-type and WT-NR strains ($P < 0.05$) but not significantly different from that of the parent Δ*cpxR1* strain (Fig. 1B).

The Δ*cpxR1* mutant is defective in insect humoral immune suppression. We hypothesized that the Δ*cpxR1* delay in insect killing compared to wild-type cells may be due to a reduced ability of the Δ*cpxR1* mutant to suppress *M. sexta* immunity. To test this, we measured transcript levels of an *M. sexta* gene encoding an antimicrobial peptide, cecropin, which is induced upon bacterial infection (18). A recent study revealed that *X. nematophila* can suppress cecropin transcript as early as 4 h after injection, and this suppression is maintained up to 10 h after injection (the last time point measured in the study) (24, 33). We measured the cecropin transcript in *M. sexta* RNA isolated at 9 and 16 h after injection of ~100 log-phase CFU of wild-type or Δ*cpxR1* mutant strains carrying an empty Tn*7* transposon (WT Tn*7* and Δ*cpxR1* Tn*7*, respectively), Δ*cpxR1* mutant expressing the *cpxRA* operon in *trans* (Δ*cpxR1* Tn*7*-*cpxRA*), *Salmonella enterica* serovar Typhimurium, or PBS. At 9 h postinjection, insects injected with Δ*cpxR1* Tn*7* showed cecropin transcript levels not significantly different from those of insects injected with WT Tn*7* (data not shown). However, at 16 h postinjection, insects injected with Δ*cpxR1* Tn*7* induced cecropin transcript an average of 21-fold more than did PBS-injected insects, a level significantly greater than that in insects injected with WT Tn*7* (twofold that of PBS-injected insects) ($P < 0.05$) (Fig. 2A). Insects injected with Δ*cpxR1* Tn*7*-*cpxRA* had cecropin transcript levels 0.5-fold that of PBS-injected insects, a level not significantly different from insects injected with WT Tn*7* (Fig. 2A), indicating that the induction of cecropin transcript observed in Δ*cpxR1* mutant-injected insects is due to the *cpxR* mutation.

In contrast to previous experiments in our laboratory, *S. enterica* did not induce an average cecropin transcript significantly greater than that induced in insects injected with PBS (Fig. 2A). The reason for this lack of induction is not clear but may reflect insect variability in immune responses. In agree-

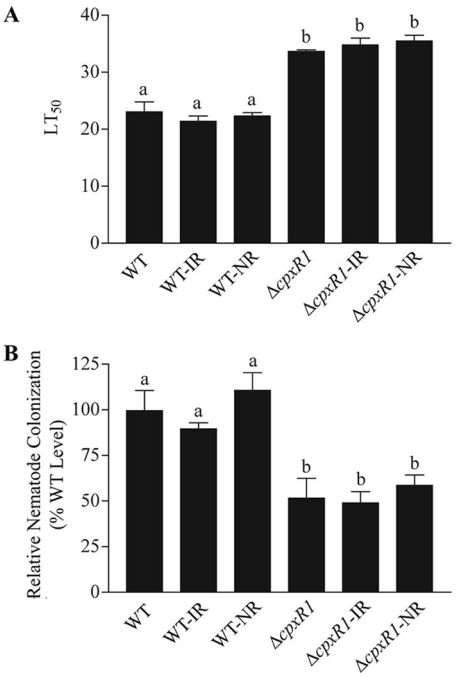

FIG. 1. Partial virulence and nematode colonization of a Δ*cpxR1* mutant are not caused by accumulation of suppressor mutations. Wild-type (WT) or Δ*cpxR1* bacteria isolated from hemolymph of dead insects or from colonized nematodes were used to inject new insects (A) and colonize new nematodes (B). Averages for three colony isolates from two or more experiments are shown. The letters a and b indicate values that are significantly different.

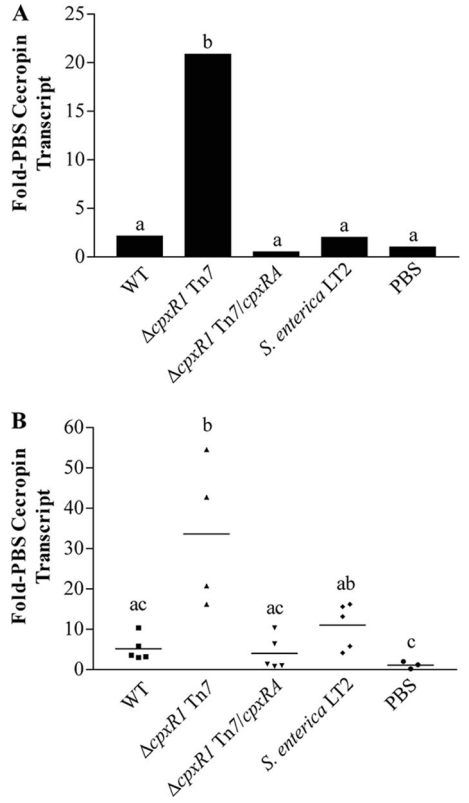

FIG. 2. A Δ*cpxR1* mutant fails to suppress *M. sexta* cecropin transcript. (A) Average cecropin transcript levels in *M. sexta* insects relative to that in insects injected with PBS is shown. Insects were injected with WT Tn*7*, Δ*cpxR1* Tn*7*, and Δ*cpxR1* Tn*7*-*cpxRA X. nematophila* strains; *S. enterica* serovar Typhimurium; or PBS. Total RNA was isolated from three to five insects per bacterial strain 16 h after injection, and RNA from insects injected with the same bacterial strain was pooled. Values are averages of two experiments. (B) Cecropin induction for individual insects is shown relative to insects injected with PBS. Each point represents RNA transcript from one insect. Letters (a, b, and c) indicate values that are significantly different from each other ($P < 0.05$). WT, wild type.

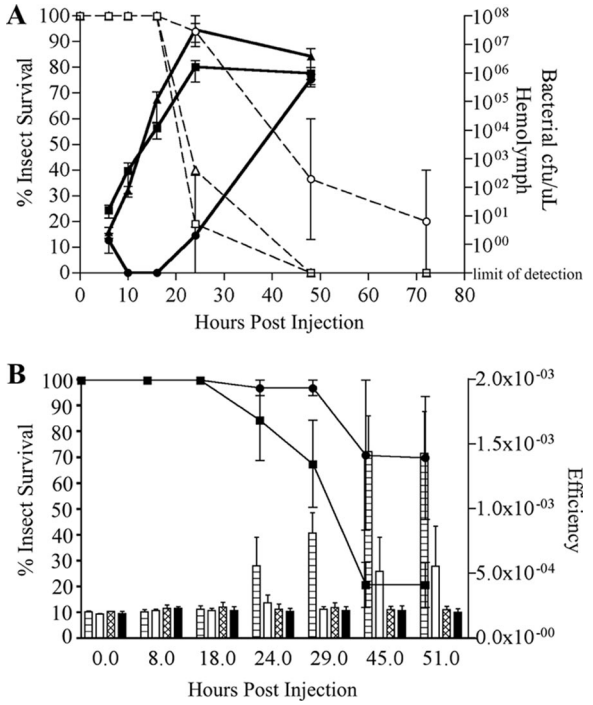

FIG. 3. Systemic Δ*cpxR1* mutant growth is delayed. (A) Bacteria were recovered from insect hemolymph after injection of WT Tn*7* (squares), Δ*cpxR1* Tn*7* (circles), or Δ*cpxR1* Tn*7*-*cpxRA* (triangles) strains. The percent survival of injected insects is measured on the left *y* axis with data points designated by open symbols and dashed lines. Bacterial CFU counts per μl of hemolymph are measured on the right axis with data points designated by closed symbols and solid lines. The averages from three experiments are shown. Error bars indicate the standard error. (B) Columns indicate fluorescence emitted by insects injected with GFP-labeled wild-type (▤) or Δ*cpxR1* (□) *X. nematophila*, *S. enterica* serovar Typhimurium (▨), or PBS (■) quantified over time by efficiency (radiance of the subject/illumination intensity) and is measured on the right *y* axis. A survival curve showing the percent insect survival over time after injection of wild-type (■) or Δ*cpxR1* (●) *X. nematophila* is shown on the left *y* axis.

ment with this hypothesis, variability was observed among individual insects injected within each treatment group (Fig. 2B). However, each of the insects injected with Δ*cpxR1* Tn*7* had higher levels of cecropin transcript than any of the insects injected with WT Tn*7* or Δ*cpxR1* Tn*7*-*cpxRA*. Three of five insects injected with *S. enterica* expressed cecropin levels higher than any of the insects injected with wild-type *X. nematophila* (the expected phenotype) and displayed cecropin transcript at levels similar to previously reported levels of 13- to 16-fold compared to PBS-injected insects (9). The other two insects, however, showed cecropin levels similar to that of wild-type *X. nematophila*-injected insects (Fig. 2B). Therefore, increased variability in cecropin transcript levels among insects injected with bacterial strains in the present study, relative to previous studies, may be a contributing factor in the lack of significant induction of cecropin transcript by *S. enterica* injection measured from pooled insects in the experiments presented here.

Systemic infection by Δ*cpxR1* mutant is delayed. The Δ*cpxR1* mutant is slower to kill *M. sexta* insects upon injection, taking longer than wild-type *X. nematophila* to kill 50% of the insects injected (23 h for wild type versus 34 h for the Δ*cpxR1* mutant). In addition, the Δ*cpxR1* mutant displays an increased lag phase when grown in either LB culture or hemolymph (21). Therefore, it is possible that the delay in Δ*cpxR1* mutant killing is caused by a growth defect within insects. To test this, *M. sexta* insects were injected with ~100 log-phase cells of WT Tn*7*, Δ*cpxR1* Tn*7*, or Δ*cpxR1* Tn*7*-*cpxRA X. nematophila* strains. At various time points after injection, insects were bled, and the resulting hemolymph was dilution plated to determine the bacterial CFU per μl of hemolymph. In this experiment, the Δ*cpxR1* Tn*7* strain showed the expected delay in insect killing, and killed fewer insects overall than the WT Tn*7* strain (Fig. 3A). The WT Tn*7*, Δ*cpxR1* Tn*7*, and Δ*cpxR1* Tn*7*-*cpxRA* strains were all recovered at levels of between 1 and 16 cells/μl hemolymph at 6 h postinjection. However, while the recoverable CFU of WT Tn*7* and Δ*cpxR1* Tn*7*-*cpxRA* steadily increased in number over the course of the experiment (Fig. 3A), Δ*cpxR1* Tn*7* cells were not recovered from hemolymph at 10 and 16 h after injection. By 24 h postinjection, as some Δ*cpxR1* Tn*7*-infected insects were beginning to die, Δ*cpxR1* Tn*7* cells

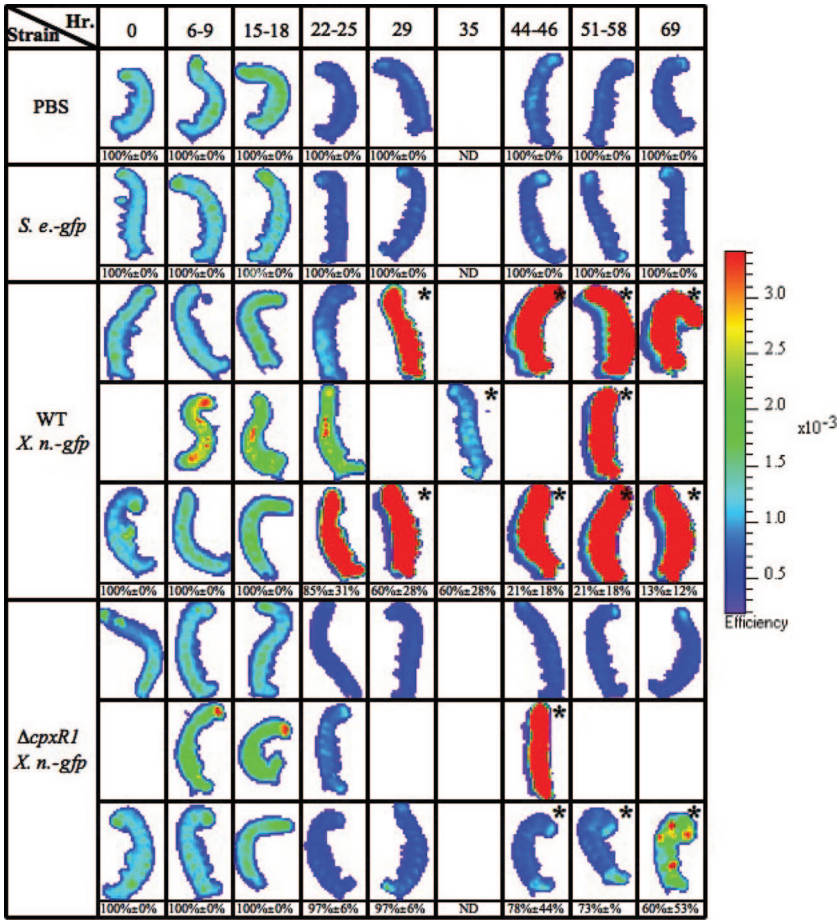

FIG. 4. A Δ*cpxR1* mutant has altered growth within insects. Fluorescence images taken of live and dead (indicated by an asterisk) insects infected with GFP-expressing wild-type or Δ*cpxR1 X. nematophila*, *S. enterica* serovar Typhimurium, or PBS were taken using GFP-appropriate excitation and emission filters. Color differences correspond to various levels of emitted fluorescence. A color spectrum with corresponding fluorescence levels is shown on the right. The efficiency is calculated as the radiance of the subject divided by the illumination intensity. White boxes indicate images that were not taken for a particular time point. One (*S. enterica* or PBS) or three (wild-type or Δ*cpxR1 X. nematophila*) representative insects per strain are shown. Average percent insect survival for each strain is indicated at each time point. The data represent the averages of four experiments.

were recovered at levels similar to that of the 6-h time point. After 24 h, the number of Δ*cpxR1* Tn*7* cells recovered from insect hemolymph was equal to that of the WT Tn*7* strain (close to 10^6 CFU/μl of hemolymph) (Fig. 3A). The numbers of Δ*cpxR1* Tn*7-cpxRA* cells recovered from hemolymph matched or exceeded those of WT Tn*7* cells at all time points examined (Fig. 3A).

Although Δ*cpxR1* cells are not recoverable from insect hemolymph at early time points postinfection, it is possible that they are present in numbers equal to those of the wild type but are localized within a body cavity or adhering to insect tissue rather than in hemolymph. In agreement with this hypothesis, some insects injected with the Δ*cpxR1* Tn*7* strain began to die at the same time that Δ*cpxR1* Tn*7* cells were again recovered from hemolymph, suggesting that these cells were present and causing illness within the insect even though they were not recovered from hemolymph samples.

Due to the relative transparency of the *M. sexta* cuticle, it was possible to use in vivo imaging system (IVIS) technology (6) to visualize green fluorescent protein (GFP)-expressing *X. nematophila* in live insects during infection (Fig. 4). Approximately 100 CFU of GFP-expressing wild type or Δ*cpxR1* mutant *X. nematophila* were injected, and increasing fluorescence (corresponding to bacterial growth) inside infected insects was quantified over time (Fig. 3B). Occasionally, insects injected with either wild-type or Δ*cpxR1 X. nematophila* died before fluorescence was detectable by the IVIS, confirming that insect death can result from the action of cytolysins or toxins produced by *X. nematophila* and not bacterial load (39, 43) (Fig. 4). In these experiments, 32 of 37 insects injected with wild-type *X. nematophila* died by the end of the experiment. Within this group of 32 insects, death occurred before detectable fluorescence in at least 8 insects (25%) (Fig. 4, see the 35-h time point). In 21 insects (66%), the time point at which death was scored was concurrent with observed fluorescence (although the relative timing of the appearance of fluorescence and death in these insects is not known) (Fig. 4, see the 29-h time point). Three insects (9%) showed detectable fluorescence before death (Fig. 4, see 22- to 25-h time point).

As expected, insects injected with the nonpathogen *S. en-*

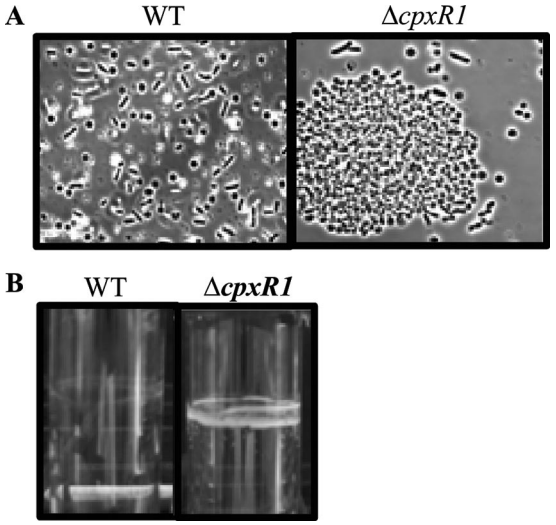

FIG. 5. Δ*cpxR1* mutant cells form aggregates. Δ*cpxR1* mutant cells grown in liquid culture under static (A) or shaking (B) cultures are shown.

terica serovar Typhimurium did not die by the end of the experiment, and fluorescence of these insects was never detected above the level of insects injected with PBS (Fig. 3B and 4). When insects were injected with GFP-expressing wild-type *X. nematophila*, fluorescence greater than that of insects injected with PBS was detected an average of 24 h postinjection (Fig. 3B and 4). However, in Δ*cpxR1* mutant-injected insects, those that eventually showed fluorescence above that of PBS-injected insects had an average time to fluorescence of 45 h postinjection (Fig. 3B and 4), a finding in agreement with both the previously reported LT_{50}s of these strains (21), and the time at which bacterial levels above 1,000 CFU/μl of hemolymph were recovered from insect hemolymph (Fig. 3A). Insects that survived wild-type or Δ*cpxR1* infection did not show fluorescence greater than that of PBS-injected insects during the experiment, suggesting that bacteria are not able to grow within these insects (Fig. 4). Interestingly, two of six insects that died after the injection of Δ*cpxR1* cells showed focal points of fluorescence within the dead insects (see, for example, the 69-h time point in Fig. 4), indicating a lack of systemic infection, perhaps due to aggregation or lack of motility of the Δ*cpxR1* cells in these insects. At later time points, at least one of these insects showed fluorescence corresponding to systemic Δ*cpxR1* mutant growth within the entire insect. In agreement with the idea that the Δ*cpxR1* mutant can form aggregates in insects, the Δ*cpxR1* mutant forms aggregates in vitro under certain growth conditions (Fig. 5). In static liquid culture left overnight at room temperature, the Δ*cpxR1* mutant formed small and large aggregates of cells (Fig. 5A), whereas wild-type *X. nematophila* did not. In addition, in shaking liquid culture grown for 24 to 48 h at 30°C, the Δ*cpxR1* mutant formed substantial aggregates of cells on glass test tubes at the liquid-air interface (Fig. 5B).

CpxR-dependent expression of *lrhA* is necessary for *X. nematophila* virulence. *lrhA*, a regulator of lipase and motility in *X. nematophila*, is necessary for virulence toward *M. sexta* insects (35). A Δ*cpxR1* mutant displays reduced levels of *lrhA* transcript, indicating that CpxR positively regulates this gene (21). The Δ*cpxR1* mutant also shows decreased motility and lipase production compared to wild-type cells (21), an effect potentially due to the subsequent decrease in positive regulation of flagellar genes and lipase production by LrhA (35).

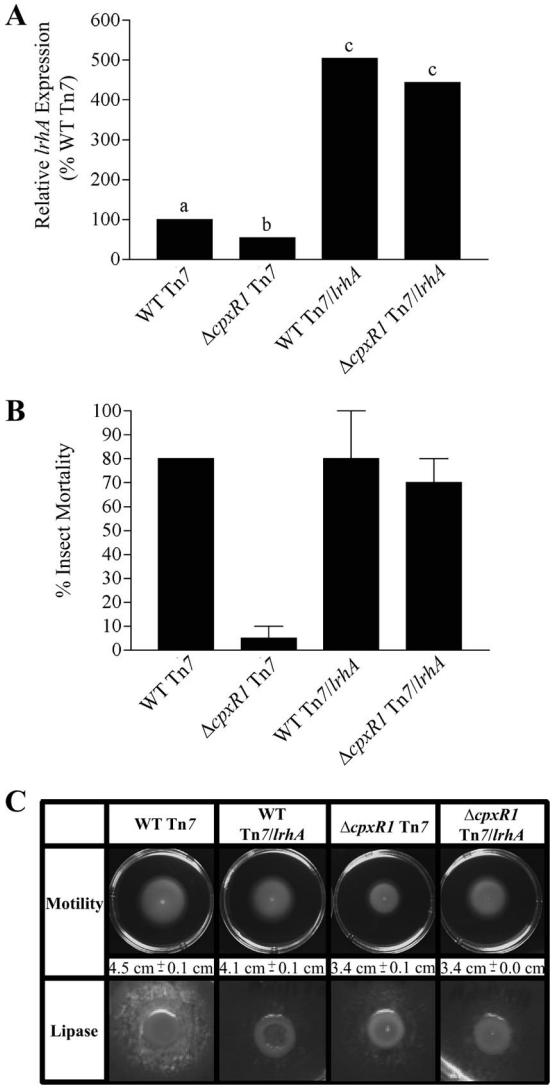

FIG. 6. Constitutive *lrhA* expression restores virulence, but not motility or lipase production, to a Δ*cpxR1* mutant. *lrhA* was constitutively expressed in wild-type (WT) and Δ*cpxR1 X. nematophila*. (A) Strains were analyzed for relative *lrhA* transcript level in stationary-phase cells. The data represent averages of three replicate cultures. (B) The percent insect mortality in *M. sexta* insects was determined. The data represent the average values from two experiments, where 10 insects were injected per strain, per experiment. (C) Motility and lipase phenotypes are shown. For all figures, different letters indicate values that are significantly different from each other ($P < 0.05$).

To determine whether the lack of CpxR-dependent expression of *lrhA* causes the virulence defect of a Δ*cpxR1* mutant, *lrhA* was ectopically expressed from a constitutive promoter in the Δ*cpxR1* Tn*7*-P_{lac}-*lrhA* strain. *lrhA* transcript levels in strains carrying Tn*7*-P_{lac}-*lrhA* were CpxR independent, as measured by qPCR (Fig. 6A). Δ*cpxR1* Tn*7*-P_{lac}-*lrhA* had at least fourfold higher levels of *lrhA* transcript than did wild-type *X. nemato-*

phila compared to the Δ*cpxR1* mutant, which expressed half the levels of *lrhA* transcript compared to the wild type (Fig. 6A). Wild-type *X. nematophila* carrying the Tn*7*-P_{lac}-*lrhA* strain exhibited fivefold higher levels of *lrhA* transcript relative to the wild-type strain lacking Tn*7*-P_{lac}-*lrhA*.

When ~1,000 stationary-phase *X. nematophila* cells were injected into fourth- or fifth-instar *M. sexta* larvae, the WT Tn*7* strain killed 80% ± 0% of the insects by 96 h postinjection (Fig. 6B). As expected, the Δ*cpxR1* Tn*7* strain killed only 5% ± 5% of the insects injected by 96 h after injection, a difference significantly different from that of the WT Tn*7* strain ($P < 0.01$). The WT Tn*7*-P_{lac}-*lrhA* strain killed 80% ± 20% of insects, while the Δ*cpxR1* Tn*7*-P_{lac}-*lrhA* strain killed 70% ± 10% of insects injected by 96 h after injection, differences not statistically different from the findings observed for the WT Tn*7* strain (Fig. 6B).

Constitutive expression of *lrhA* did not rescue the motility defect of the Δ*cpxR1* mutant and in fact caused reduced motility of wild-type cells (Fig. 6C): wild type with or without Tn*7*-P_{lac}-*lrhA* showed zones of migration of 4.1 ± 0.1 cm or 4.5 ± 0.1 cm, respectively ($P < 0.05$). Zones of migration for the Δ*cpxR1* mutant with or without Tn*7*-P_{lac}-*lrhA* were 3.4 ± 0.0 cm or 3.4 ± 0.1 cm, respectively, values that were significantly different from the WT Tn*7* strain ($P < 0.001$) but not significantly different from each other (Fig. 6C). The lipase activity defect of the Δ*cpxR1* strain also was not rescued by constitutive expression of *lrhA*, as determined by a qualitative plate assay (Fig. 6C).

DISCUSSION

In *X. nematophila*, deleting *cpxR* results in reduced virulence toward an insect host, *M. sexta* (21). In the present study, we sought to elucidate the role of CpxR as a regulator of *X. nematophila* virulence through observation of Δ*cpxR1* mutant behavior and growth in *Insecta* and by characterizing the contribution of one specific CpxR regulon member, *lrhA*, to the Δ*cpxR1* mutant virulence defect.

A Δ*cpxR1* mutant showed delayed reproduction in *M. sexta*, as monitored by cell counts in hemolymph (Fig. 3A) and fluorescence in whole insects (Fig. 3B and 4). Possible explanations for this delayed reproduction are that, relative to the wild type, the Δ*cpxR1* mutant does not adapt its metabolism as quickly to the nutrients available in hemolymph, is more sensitive to insect antimicrobial activities, or is more prone to clearance from the hemolymph and sequestration in tissues or nodules. These possibilities are not mutually exclusive. Indeed, the Δ*cpxR1* mutant displays a prolonged lag phase during in vitro growth in hemolymph (21), a finding consistent with the idea that it is defective in adapting to this nutrient source. However, Δ*cpxR1* cell numbers in insect hemolymph fall below the level of detection at early time points postinfection, while wild-type cells exhibit consistent and exponential increase in cell number. This observation suggests that Δ*cpxR1* mutants are being killed or removed from the hemolymph to an alternate location. The fact that the Δ*cpxR1* mutant is not detectable in whole insects using in vivo fluorescence might suggest a killing mechanism. However, we have not ruled out the possibility that Δ*cpxR1* cells are present in hemolymph or tissues at numbers below our level of detection. This is a likely possibility due to the fact that some Δ*cpxR1* mutant-injected insects begin to die at the same time that Δ*cpxR1* cells are again recoverable from insect hemolymph (Fig. 3A), suggesting that cells are present and causing disease even though they are not detectable in hemolymph by our assay.

Expression of *M. sexta* cecropin is transcriptionally induced in response to microbial infection (18) but can be suppressed by *X. nematophila* (24, 33). Previously, suppression had been observed at 9 h postinjection with wild-type *X. nematophila*, and at this time point no significant difference in cecropin transcript was detected in insects injected with WT Tn*7* or Δ*cpxR1* Tn*7* *X. nematophila*. However, at 16 h after injection, insects infected with the Δ*cpxR1* mutant show significantly higher levels of cecropin transcript than insects infected with wild-type *X. nematophila* (Fig. 2). The differences in Δ*cpxR1* immune-suppression phenotypes at these two time points could be explained if cecropin expressed at 9 and 16 h postinjection is induced by distinct signal transduction cascades, and the Δ*cpxR1* mutant is defective only in suppression of the 16-h induction pathway. Regardless, our data indicate Δ*cpxR1* cells either have a defect in suppressing cecropin induction or increased immunogenicity. The 16-h time point coincides with an absence of detectable Δ*cpxR1* cells in the hemolymph (Fig. 3A), suggesting that *X. nematophila* may need to be present in the hemolymph to effectively suppress antimicrobial peptide induction. For example, *X. nematophila* mechanisms of cecropin suppression could include the destruction of insect blood cells involved in microbial detection and response. In turn, the Δ*cpxR1* mutant may be more immunogenic than wild-type *X. nematophila* due to altered regulation of the cell envelope and its components, such as lipopolysaccharide, a known inducer of insect antimicrobial peptides (12). Such immunogenicity could conceivably counteract any suppressive factors.

In two dead Δ*cpxR1* mutant-infected insects, distinct points of fluorescence were observed rather than the systemic fluorescence seen in insects killed by wild-type *X. nematophila*. In vitro, Δ*cpxR1* cells left in static culture at room temperature overnight are clumped together in large aggregates (Fig. 5). This raises the intriguing possibility that these same cell clusters are formed during Δ*cpxR1* mutant infection in insects. A model derived from these data is that a portion of Δ*cpxR1* mutant cells may be able to adhere to insect surfaces early in infection and form aggregates that increase its resistance to the enhanced cecropin response it has elicited. These cells may then be able to cause infection and death of the insect, albeit delayed in comparison to wild-type *X. nematophila* infection.

The CpxR response regulator is expected to respond to environmental signals and regulate appropriate adaptive gene expression. In *X. nematophila* CpxR appears to be involved in responding to both the nematode and insect environments, since it is responsible for the induction of distinct genes necessary for establishing interactions with each of these hosts. We have shown that the insect virulence defect previously ascribed to the Δ*cpxR1* mutant is caused, at least in part, by the decrease in *lrhA* transcript in this mutant (Fig. 6), since constitutive expression of *lrhA* in Δ*cpxR1* rescued the virulence defect (Fig. 6B), measured by insect mortality and LT_{50}. Δ*cpxR1* defects in insect immune suppression are unlikely to be caused by the decrease in *lrhA* expression, since an *lrhA* mutant

does not show a defect in humoral immune suppression (G. R. Richards and H. Goodrich-Blair, unpublished data).

Unexpectedly, the motility and lipase production defects of the Δ*cpxR1* mutant (21) were not rescued by constitutive expression of *lrhA* (Fig. 6C). This may indicate that CpxR regulates motility and lipase activity independently of LrhA, although previous studies showed that transcript levels of the flagellar genes *fliC*, *flhD*, *flgE*, and *fliA*, as well as *xlpA*, encoding the XlpA lipase, were not significantly different in wild-type and Δ*cpxR1* cells (21). The XlpA lipase is secreted through the flagellar export apparatus, so defects in the construction of this apparatus are expected to perturb both motility and lipase activity. Therefore, the Δ*cpxR1* mutant defect in motility and lipase activity may be due to a general disturbance in membrane structure and function rather than to direct regulation of genes involved specifically in motility or lipase production. Alternatively, regulation of motility and lipase activity may be particularly sensitive to levels of LrhA, and inappropriate regulation of *lrhA* transcription may have negative effects on these processes. Consistent with this idea, wild-type cells constitutively expressing *lrhA* exhibited a slight but significant motility defect. Regardless, the fact that constitutive expression of *lrhA* rescues the virulence defect of the Δ*cpxR1* mutant is consistent with the previously posed hypothesis that LrhA regulates virulence factors that are not members of the flagellar pathway (35) since lipase and motility mutants do not have as severe a virulence defect as the *lrhA* mutant (35).

The data presented here indicate that in *X. nematophila*, the Cpx system is necessary for adaptation to the insect environment by regulating efficient growth, appropriate localization, and suppression of an insect antimicrobial peptide within the insect host. Similarly, the broad-host-range, opportunistic pathogen *P. aeruginosa* requires a two-component regulator GacA for virulence (41) and immune suppression (including of antimicrobial protein induction) in an invertebrate host, *Caenorhabditis elegans* (15). Furthermore, in both *P. aeruginosa* and *X. nematophila*, virulence and immune suppression are not completely overlapping since virulence genes (e.g., DsbA in *P. aeruginosa* and LrhA in *X. nematophila*) in the regulons of GacA and CpxR, respectively, are not necessary for immune suppression (15; the present study). Invertebrate models have already proved useful for the elucidation of virulence factor function and host targets (20, 22, 27). Similarly, continued investigations of model interactions between pathogens and invertebrates, such as the one described here, will contribute to the identification of effectors that modulate host immunity and will help reveal common themes in the coordinated regulation of virulence and immune suppressive factors.

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