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CpxRA Influences *Xenorhabdus nematophila* Colonization Initiation and Outgrowth in *Steinernema carpocapsae* Nematodes through Regulation of the *nil* Locus[▽]

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The gammaproteobacterium *Xenorhabdus nematophila* mutualistically colonizes an intestinal region of a soil-dwelling nematode and is a blood pathogen of insects. The *X. nematophila* CpxRA two-component regulatory system is necessary for both of these host interactions (E. Herbert et al., Appl. Environ. Microbiol. 73:7826–7836, 2007). Mutualistic association of *X. nematophila* with its nematode host consists of two stages: initiation, where a small number of bacterial cells establish themselves in the colonization site, and outgrowth, where these cells grow to fill the space. In this study, we show that the Cpx system is necessary for both of these stages. *X. nematophila* Δ*cpxR1* colonized fewer nematodes than its wild-type parent and did not achieve as high a density as did the wild type within a portion of the colonized nematodes. To test whether the Δ*cpxR1* host interaction phenotypes are due to its overexpression of *mrxA*, encoding the type I pilin subunit protein, we assessed the colonization phenotype of a Δ*cpxR1* Δ*mrxA1* double mutant. This mutant displayed the same colonization defect as Δ*cpxR1*, indicating that CpxR negative regulation of *mrxA* does not play a detectable role in *X. nematophila*-host interactions. CpxR positively regulates expression of *nilA*, *nilB*, and *nilC* genes necessary for nematode colonization. Here we show that the nematode colonization defect of the Δ*cpxR1* mutant is rescued by elevating *nil* gene expression through mutation of *nilR*, a negative regulator of *nilA*, *nilB*, and *nilC*. These data suggest that the nematode colonization defect previously observed in Δ*cpxR1* is caused, at least in part, by altered regulation of *nilA*, *nilB*, and *nilC*.

As part of its natural life cycle the gammaproteobacterium *Xenorhabdus nematophila* lives within two animal hosts: it is a mutualistic symbiont of the soil-dwelling nematode, *Steinernema carpocapsae*, and a pathogen toward a number of insect orders (14, 15, 18). *X. nematophila* colonizes a modified intestinal region of a free-living, nonfeeding stage of *S. carpocapsae*, which serves as its vector into the blood system (hemocoel) of susceptible insect hosts (15). *X. nematophila* is released from its nematode vector into the hemocoel and within 1 to 2 days kills the insect (15). The insect cadaver serves as a nutrient source for nematode and bacterium reproduction. Once nutrients inside the insect host are depleted, *X. nematophila* reassociates with the vector stage of the nematode, which migrates into the soil to seek a new insect host and repeat the cycle (16).

The *S. carpocapsae* vector-stage association with *X. nematophila* is species specific: of all *Xenorhabdus* species tested to date, only *X. nematophila* can colonize *S. carpocapsae* nematodes (1, 6). In a fully mature vector-stage nematode, the intestinal colonization site, known as a receptacle (48), or vesicle (2) can contain >100 CFU of *X. nematophila* (33). This bacterial population is founded by one or a few *X. nematophila* cells, and the process of colonization includes distinguishable initiation and outgrowth stages (33). Within the vesicle, *X. nematophila* cells adhere to a nematode-derived structure termed the intravesicular structure that is surrounded by a mucuslike material (32). These data have led to a model in which colonization initiation by *X. nematophila* cells includes binding to a specific sugar associated with the intravesicular structure (32). Newly colonized *S. carpocapsae* nematodes contain very few *X. nematophila* cells that increase in number as the nematodes age, reaching a fully colonized level after ~6 days (33). *X. nematophila* mutants defective in both initiation and outgrowth have been isolated, lending insight into gene products and physiological processes necessary for nematode colonization by this bacterium (6, 23, 32, 49). For example, metabolic mutants defective in the biosynthesis of methionine or threonine can initiate colonization but do not grow within the vesicle (13, 34), while mutants lacking the sigma factor homolog RpoS cannot initiate colonization (23, 49).

Current evidence suggests that, although *X. nematophila* growth occurs in the nematode vesicle, it is a nutrient-limited and stressful environment (7, 19). When released from this environment into insect hemolymph, *X. nematophila* is expected to encounter a relatively nutrient rich environment (7, 17, 20, 38, 41) but also faces insect immune responses. We do not yet understand fully which characteristics of these environments are sensed by *X. nematophila* or how this information is transduced to the expression of symbiotic activities appropriate for each host. However, one regulator involved in this process is the CpxRA two-component signaling system, which is necessary for interactions with both hosts and is hypothesized to help regulate transitions between the nematode and insect environments (21). In other bacteria, the Cpx system senses cell surface-related parameters, such as proximity to a surface

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(39), changes in external conditions (27, 31, 36, 37, 50), or misfolded proteins in the periplasm (11). In response to sensor kinase (CpxA) activation by these signals, the response regulator, CpxR, regulates expression of genes that function in cell envelope integrity (43), motility and chemotaxis (10), biofilm formation (12), adherence and invasion of host cells (24, 31), pilus formation (25), type III secretion (35), and protein folding and degradation (8, 9, 42, 44).

When the *cpxR* gene is deleted in *X. nematophila*, the bacterium is less effective at killing a model insect host, *Manduca sexta*, and colonizes *S. carpocapsae* nematodes at lower levels than its wild-type parent (21). Phenotypic and gene expression studies of a *cpxR* deletion mutant (Δ*cpxR1*) led to the identification of multiple genes in the CpxR regulon, including genes known or thought to be involved in one or both host interactions. One of these genes is *mrxA*, encoding the *X. nematophila* type I pilin subunit (21) reported to be involved in *X. nematophila*-nematode mutualism (3) and virulence toward insects (3, 28–30). Previous studies have shown that the pilin subunit is toxic toward *Helicoverpa armigera* insects in a purified form (28–30) and that these pilin subunits are found in outer membrane vesicle blebs, which, perhaps not coincidentally, are also toxic toward *H. armigera* (28). In the Δ*cpxR1* mutant, *mrxA* transcript is increased >3-fold over that of wild-type cells, indicating that the Δ*cpxR1* mutant cell surface may contain an increased number of pili compared to wild-type cells. This has the potential to severely affect host interactions of a cell due to the potential of pili to stimulate immune systems (either of the nematode or insect) or attach to host ligands or other structures (26, 46).

Three other *X. nematophila* CpxR regulon members—*nilA*, *nilB*, and *nilC* (21)—are each necessary for nematode colonization and are thought to be involved in initiation of colonization, perhaps through signaling or attaching to a nematode surface (4–6). *nilA*, *nilB*, and *nilC* are encoded at the same locus, termed the symbiosis region 1 (23). *nilA* and *nilB* are cotranscribed, while *nilC* is divergently transcribed (23). NilC is a periplasmically oriented outer membrane lipoprotein (4), NilB is an outer membrane protein of unknown function (A. Bhasin and H. Goodrich-Blair, unpublished data), while NilA is predicted to be an inner membrane protein (23).

Both *nil* promoters are repressed by the synergistic activities of Lrp, the leucine-responsive regulatory protein that also plays a role in nematode mutualism and insect virulence, and NilR, a small helix-turn-helix containing protein (4, 5, 7). Maximal expression of the *nil* genes in liquid culture occurs in strains in which both *nilR* and *lrp* are disrupted (5). Derepression of the *nil* genes is thought to be necessary for successful colonization of nematodes, since a *X. nematophila* strain carrying an ectopic copy of NilR showed a 60-fold lower colonization level compared to the control strain (5).

The goal of the present study was to elucidate the mechanism(s) by which the Cpx signal transduction system contributes to *X. nematophila* mutualistic nematode colonization. Specifically, we tested whether CpxR regulation of *mrxA* and *nil* genes influences colonization and whether CpxR contributes to colonization initiation, outgrowth, or both.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in the present study are listed in Table 1. All strains were grown in the dark in LB medium or LB agar plus 0.1% pyruvate at 30°C unless otherwise specified. 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*), gentamicin (25 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and streptomycin (12.5 μg ml^{-1} for *X. nematophila* and 25 μg ml^{-1} for *E. coli*). Sucrose was used at a 5% final concentration.

Molecular biological methods. Standard biological methods were used in the present study (45). Plasmid and PCR purification kits (Zymo Research, Orange, CA) were used according to the manufacturer's recommendations. DNA amplification was performed by using ExTaq (Takara Otsu, Shiga, Japan) according to the manufacturer's directions.

β-Galactosidase assays. β-Galactosidase assays were performed as previously described (4) on overnight cultures inoculated from LB medium plus ampicillin agar *X. nematophila* colonies. *X. nematophila lacZ* fusion strains were created by triparental mating of pTn*7*-*nilA*, pTn*7*-*nilB*, or pTn*7*-*nilC* (5) as previously described (21).

Bacterial mutant construction. The *cpxR* gene was deleted in a Δ*nilR17*::Kan background (5) by allelic exchange using pKRcpxRStr (21). Candidate colonies resistant to ampicillin, kanamycin, and streptomycin, but sensitive to chloramphenicol were chosen. A Δ*cpxR1*/Δ*mrxA1*::ΩKan mutant was created by conjugating pPK13 (3) into the Δ*cpxR1* mutant (21) background (HGB1230). Candidate colonies resistant to ampicillin, kanamycin, streptomycin, and sucrose but sensitive to chloramphenicol and gentamicin were chosen. The presence of an insertion within *mrxA* was confirmed by Southern analysis as described previously (3). Both mutants were tested for the presence of the appropriate insertions by PCR amplification of the loci. Primers EH72 (5′-GAATAGCGGAGTTTGTTATCG-3′) and EH15 (5′-CACTGGTAACAAGGAGTAAGC-3′) amplified the *cpxR* locus. EH193 (5′-GGATCCATGAAACTTAACACAATTGGC-3′) and EH194 (5′-CTCGAGGAGGAAATATGTCACCATCGG-3′) amplified the *mrxA* locus.

Nematode colonization. Colonization assays were performed as previously described (4) using five replicates per bacterial strain per experiment. Day 1 of the assay corresponds to the day infective juvenile (IJ) nematodes were taken from water traps and surface sterilized. IJs were then kept in closed tissue culture flasks at room temperature in the dark for the remainder of the experiment. For all time points, 1,000 IJs were sonicated, dilution plated onto LB plates, and grown overnight at 30°C to determine the average bacterial CFU/IJ for the nematode population. For microscopic analysis, nematodes were paralyzed with levamisole (Sigma, St. Louis, MO) and viewed as previously described (33). Between 100 and 1,000 IJs were viewed per time point per bacterial strain.

Nematode survival and development. Bacterial strains were grown in liquid culture overnight, plated on lipid agar plates, and grown overnight in the dark at 30°C. Nematode eggs were plated in equal volumes onto the bacterial lawns and, at various time points after hatching, the nematodes were washed off the plates with water, and a portion was counted and viewed microscopically to determine the total number of nematodes present, as well as the approximate stage of development.

RESULTS

***cpxR* contributes to initiation and outgrowth during nematode mutualism.** We previously reported that Δ*cpxR1* mutant cells are recovered from a population of colonized nematodes at an average of ~38% that of wild-type cells (21). To determine whether this nematode colonization defect is due to an inability of nematodes to survive or reproduce on lawns of Δ*cpxR1* mutant cells, populations of nematodes growing on either wild-type *X. nematophila*, a Δ*cpxR1* mutant, or the complemented Δ*cpxR1* mutant (Δ*cpxR1* Tn*7*-*cpxRA*) were monitored for 2 weeks to assess differences in nematode viability or development. At various time points after plating nematode eggs on bacterial lawns, the nematodes were rinsed off the lipid agar plates with water, counted microscopically to quantify total numbers, and classified by approximate stage of growth.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	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*::Str	21
HGB1231	HGB800 Δ*cpxR1*::Str Tn*7*	21
HGB1232	HGB800 Δ*cpxR1*::Str Tn*7-cpxRA*	21
HGB1103	HGB800 Δ*nilR17*::Kan	5
HGB1345	HGB800 Δ*nilR17*::Kan Δ*cpxR1*::Str	This study
HGB1117	HGB800 Tn*7-nilA-lacZ*	5
HGB1121	HGB800 Tn*7-nilB-lacZ*	5
HGB1101	HGB800 Tn*7-nilC-lacZ*	4
HGB1239	HGB800 Δ*cpxR1*::Str Tn*7-nilA-lacZ*	21
HGB1240	HGB800 Δ*cpxR1*::Str Tn*7-nilB-lacZ*	21
HGB1241	HGB800 Δ*cpxR1*::Str Tn*7-nilC-lacZ*	21
HGB1119	HGB800 Δ*nilR17*::Kan Tn*7-nilA-lacZ*	5
HGB1123	HGB800 Δ*nilR17*::Kan Tn*7-nilB-lacZ*	5
HGB1346	HGB800 Δ*nilR17*::Kan Tn*7-nilC-lacZ*	This study
HGB1347	HGB800 Δ*nilR17*::Kan Δ*cpxR1*::Str Tn*7-nilA-lacZ*	This study
HGB1348	HGB800 Δ*nilR17*::Kan Δ*cpxR1*::Str Tn*7-nilB-lacZ*	This study
HGB1349	HGB800 Δ*nilR17*::Kan Δ*cpxR1*::Str Tn*7-nilC-lacZ*	This study
HGB1364	HGB800 Δ*mrxA1*::Kan	This study
HGB1365	HGB800 Δ*cpxR1*::Str Δ*mrxA1*::Kan	This study
E. coli S17-1(λ*pir*)	Donor strain for conjugations	47
Plasmids		
pPK13	Allelic-exchange vector containing *mrxA* loci with *aph* cassette insertion	3
pTn*7-nilA-lacZ*	pEVS107+*nilA* 5′-flanking region fused to *lacZ* (Erm^r Kan^r)	5
pTn*7-nilB-lacZ*	pEVS107+*nilB* 5′-flanking region fused to *lacZ* (Erm^r Kan^r)	5
pTn*7-nilC-lacZ*	pEVS107+*nilC* 5′-flanking region fused to *lacZ* (Erm^r Kan^r)	4
pKR*cpxR*Str	Vector for allelic exchange of *cpxR* gene for *aad*, encoding streptomycin resistance	21

[a] Erm^r, erythromycin resistance; Kan^r, kanamycin resistance.

No obvious differences were observed, either in number or growth stage of the nematodes present (data not shown).

One possible explanation for the Δ*cpxR1* mutant colonization defect is that, compared to the number of wild-type *X. nematophila* cells in colonized nematodes, Δ*cpxR1* mutant-colonized nematodes each contain only 38% as many mutant cells, which would indicate an inability of the Δ*cpxR1* mutant to maintain a fully colonized state or to grow past a certain limiting threshold within the nematode. A second possibility is that 38% of the nematodes exposed to the Δ*cpxR1* mutant are fully colonized and that the remaining nematodes are uncolonized, which would indicate a defect in the Δ*cpxR1* mutant's ability to initiate nematode colonization. To distinguish between these two possibilities, we used green fluorescent protein-labeled wild-type *X. nematophila* and Δ*cpxR1* mutant cells to quantify by fluorescence microscopy the number of fully colonized (Fig. 1A), partially colonized (containing fewer than 10 bacterial cells within the vesicle) (Fig. 1B) and uncolonized (Fig. 1C) *S. carpocapsae* nematodes for each bacterial strain. In this experiment, wild-type *X. nematophila* cells fully colonized 89% of the nematodes counted, while the remaining 11% of nematodes counted were either uncolonized or partially colonized (Fig. 2A, day 1). However, of nematodes grown on the Δ*cpxR1* mutant, only 49% were fully colonized, while 40% of nematodes were uncolonized and 11% were partially colonized (Fig. 2B, day 1).

Given the prolonged lag phase of Δ*cpxR1* mutant growth in LB compared to wild-type cells (21), these results raised a third possible scenario, in which the Δ*cpxR1* mutant grows more slowly in the vesicle but, given time, would eventually achieve

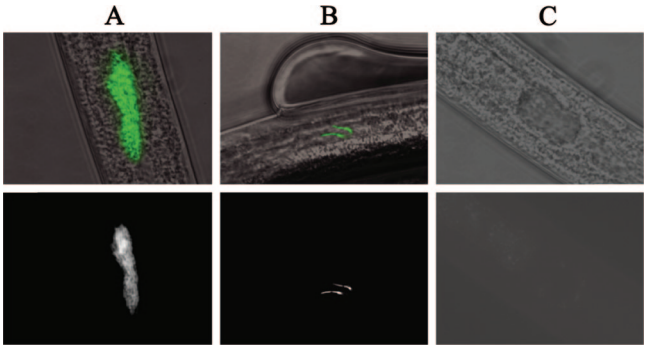

FIG. 1. A Δ*cpxR1* mutant displays variable distribution within nematodes. Fluorescence microscope images of *S. carpocapsae* bacterial receptacles displaying various states of colonization by the Δ*cpxR1* mutant are shown. The bottom row are fluorescence micrographs of nematode receptacles either fully colonized (A), partially colonized (containing 10 bacterial cells or fewer) (B), or uncolonized (C). The top-row images are phase-contrast micrographs of nematodes with false-colored fluorescence micrographs overlaid. All images were taken at a magnification of ×1,000. Bars, 10 μm.

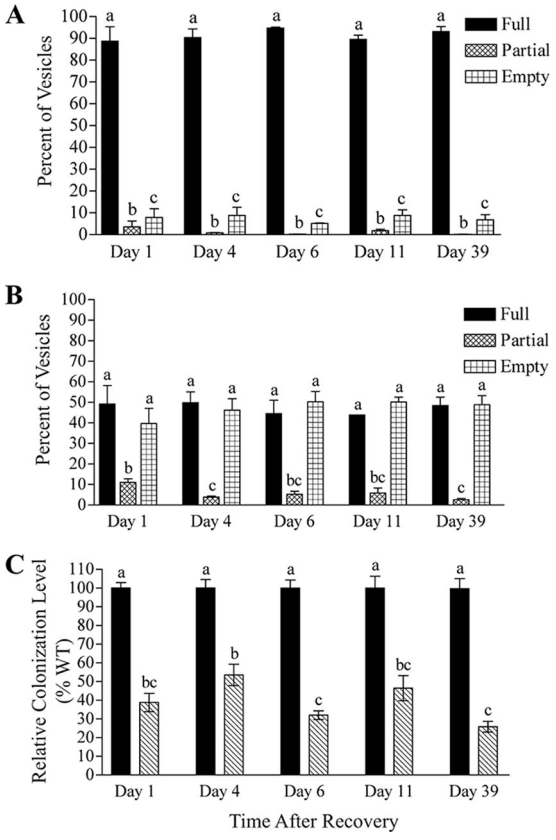

FIG. 2. A Δ*cpxR1* mutant is defective in nematode colonization initiation and outgrowth. The proportion of fully colonized, partially colonized, and uncolonized nematode vesicles in nematodes colonized by wild-type *X. nematophila* (A) or the Δ*cpxR1* mutant (B) over 39 days is shown. (C) The relative colonization level for the nematode population (as determined by dilution plating nematode sonicates) between the Δ*cpxR1* mutant and the wild type was consistent over 39 days. Average values from three experiments are shown. Different letters indicate values that are statistically different from each other. Error bars indicate the standard error. WT, wild type.

full colonization levels in all nematodes in which colonization was initiated. Therefore, we continued our observation of nematode vesicles for 39 days past the initial surface sterilization to determine whether the Δ*cpxR1* mutant showed delayed outgrowth. At days 4, 6, 11, and 39 after the initial surface sterilization, the nematodes were sonicated and plated to determine the average bacterial CFU per nematode, and the proportion of colonized versus uncolonized nematodes was assessed microscopically. Over 39 days, the average colonization of the Δ*cpxR1* mutant (as determined by dilution plating the sonicated nematodes) consistently remained at ca. 26 to 54% that of nematodes colonized by wild-type *X. nematophila* cells (Fig. 2C). When fluorescent nematode vesicles were counted microscopically, wild-type *X. nematophila* cells fully colonized 89 to 95% of nematodes counted over the course of the experiment, as expected (Fig. 2A). The Δ*cpxR1* mutant, however, fully colonized only 44 to 50% of the nematodes counted at all time points, and the number of fully colonized nematodes did not increase significantly over time. The percentage of partially colonized vesicles in both wild-type and Δ*cpxR1*-colonized nematodes decreased slightly during the course of the experiment (Fig. 2B).

Eliminating aberrant *mrxA* expression does not rescue Δ*cpxR1* host interaction defects. CpxR negatively influences the expression of *mrxA* (21), which encodes the *X. nematophila* type I pilin subunit protein. MrxA has been implicated in both mutualism (3) and the pathogenesis (3, 28–30) of *X. nematophila*, and we therefore hypothesized that abnormally high levels of the *mrxA* transcript in the Δ*cpxR1* mutant (21) may contribute to Δ*cpxR1* mutant-host interaction defects. For example, increased pili on the cell surface could mask lectins or receptors necessary for physical interactions within the nematode colonization site. Alternatively, within the insect or nematode host, an increase in surface pili on the Δ*cpxR1* mutant may trigger an immune response (21). To test these ideas, we deleted *mrxA* in a Δ*cpxR1* mutant background. If *mrxA* overexpression is responsible for the nematode colonization defect seen in the Δ*cpxR1* mutant, then colonization may be restored to wild-type levels in the double mutant. One caveat of this approach is that *mrxA* itself was recently implicated as being necessary for nematode colonization (3). However, in contrast to that earlier study (3), we saw no decrease in nematode colonization of our Δ*mrxA1* mutant, which colonized at 121% ± 6% that of the wild type ($P < 0.05$) (Fig. 3A). The absence of *mrxA*-encoded pilin subunit did not rescue the colonization defect of the Δ*cpxR1* mutant (Fig. 3A). In this experiment, the Δ*cpxR1* mutant colonized nematodes at 67% ± 4% that of the wild type ($P < 0.001$), while the Δ*cpxR1* Δ*mrxA1* mutant colonized at 73% ± 6%, a difference statistically different from the wild type ($P < 0.01$) but not different from the Δ*cpxR1* mutant (Fig. 3A).

Since *mrxA* has also been reported to play a role in insect virulence, we assessed whether the absence of *mrxA* rescues the virulence defect of the Δ*cpxR1* mutant. However, virulence of the double mutant was not significantly different from that of the Δ*cpxR1* mutant (Fig. 3B). Wild-type cells and the Δ*mrxA1* mutant killed 100% ± 0% of *M. sexta* insects by 72 h after injection; however, the Δ*cpxR1* and Δ*cpxR1* Δ*mrxA1* mutants each killed significantly fewer insects than did the wild type ($P < 0.01$) at 35% ± 5% and 40% ± 10% of the insects injected, respectively (Fig. 3B). This finding is also in contrast to previously reported data indicating that an *mrxA* mutant has reduced mortality toward *H. armigera* insects (3).

Elevated expression of *nilA*, *nilB*, and *nilC*, rescues the colonization defect of the Δ*cpxR1* mutant. CpxR positively regulates expression of three genes—*nilA*, *nilB*, and *nilC* (21)—that encode colonization factors (4, 23). We hypothesized that the colonization defect of the Δ*cpxR1* mutant is caused by reduced expression of these genes. *nilR* encodes a repressor of *nilA*, *nilB*, and *nilC* expression (5), and an insertion mutation within *nilR* elevates *nilABC* expression above that of wild-type *X. nematophila* levels (5). To test whether elevation of *nilABC* expression in a Δ*cpxR1* mutant would rescue the mutant's nematode colonization defect, we created a Δ*nilR17* Δ*cpxR1* double mutant that expresses greater than wild-type levels of *nilA*, *nilB*, and *nilC* (Fig. 4) and assessed its ability to colonize nematodes (Fig. 5). While a Δ*cpxR1* mutant colonized nematodes at ca. 47% ± 5% that of the wild-type *X. nematophila* in this experiment ($P < 0.001$), the Δ*nilR17* Δ*cpxR1* double mutant colonized at 79% ± 7% that of the wild type ($P > 0.05$)

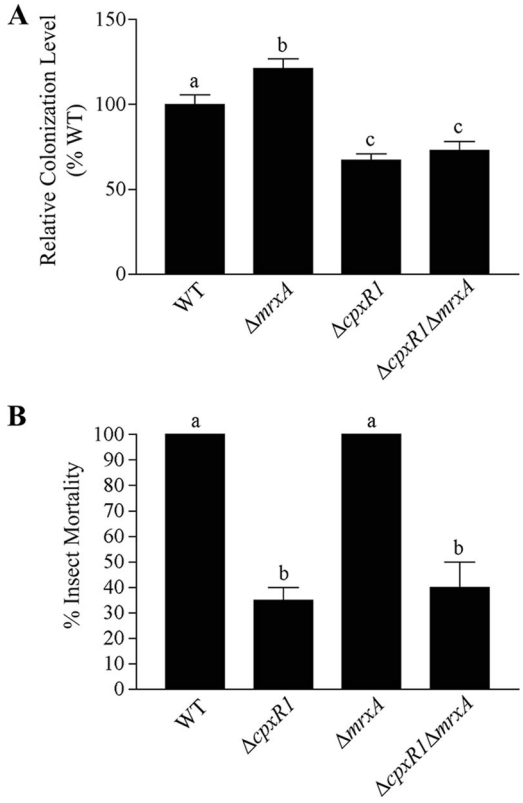

FIG. 3. Eliminating aberrant *mrxA* expression in a Δ*cpxR1* mutant does not rescue nematode colonization or virulence toward insects. For all graphs, error bars represent the standard error, and treatments labeled with different letters are significantly different from each other ($P < 0.05$). (A) The relative nematode colonization of Δ*mrxA1*, Δ*cpxR1*, and Δ*cpxR1* Δ*mrxA1* mutants is expressed as a percentage of the wild-type colonization. The average of two or more experiments is shown. (B) The percent mortalities of insects injected with wild type, Δ*cpxR1*, Δ*mrxA1*, and Δ*cpxR1* Δ*mrxA1* mutants are shown. The data represent the average of two or more experiments. WT, wild type.

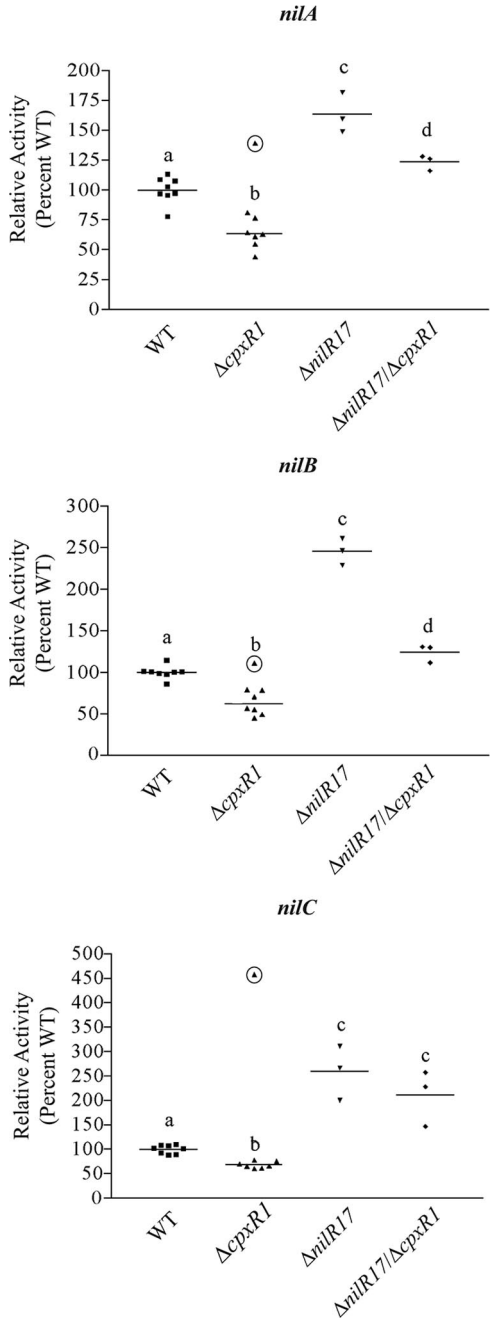

FIG. 4. A mutation in *nilR* elevates *nilABC* expression in a Δ*cpxR1* mutant. The relative expression of *nilA*, *nilB*, and *nilC* (expressed as a percentage of the wild-type activity) was measured by assaying the β-galactosidase activities of reporter fusions in wild-type, Δ*cpxR1*, Δ*nilR17*, and Δ*nilR17* Δ*cpxR1* strain backgrounds. The data points represent individual colonies tested, and bars represent strain averages. Different letters indicate values that are significantly different from the wild type ($P < 0.01$). Outlier Δ*cpxR1* mutant colonies (circled) were left out of strain averages and statistical analysis. WT, wild type.

(Fig. 5). As expected (5), the Δ*nilR17* single mutant colonized nematodes at 117% ± 10%, a level not significantly different from that of the wild type ($P > 0.05$) (Fig. 5).

Interestingly, while a majority of the Δ*cpxR1* single colony isolates tested demonstrated the previously reported reduction in *nil* gene expression, several Δ*cpxR1* clones expressing *lacZ* fusions to one of the *nil* promoters exhibited abnormally high expression of *nilA*, *nilB*, or *nilC* (139, 111, and 458%, respectively, compared to that of the wild type) (Fig. 4). These clones, whose expression varied from the mean by more than twofold the standard deviation value, were not counted toward the average gene expression presented for a given strain. Potential causes of this phenomenon are discussed below.

DISCUSSION

In *X. nematophila*, the Cpx system is necessary for colonization of *S. carpocapsae* nematodes and virulence in *M. sexta* insects (21). In the present study we have shown that *cpxR* impacts two distinct stages of nematode colonization: initiation and outgrowth. Furthermore, we show that the likely cause of the colonization defect is that the Δ*cpxR1* mutant does not express wild-type levels of *nil* genes encoding membrane-localized proteins necessary for nematode colonization.

A Δ*cpxR1* mutant shows a defect in the colonization of *S. carpocapsae* nematodes, where, compared to wild-type cells, between 38 and 67% as many mutant cells are released from

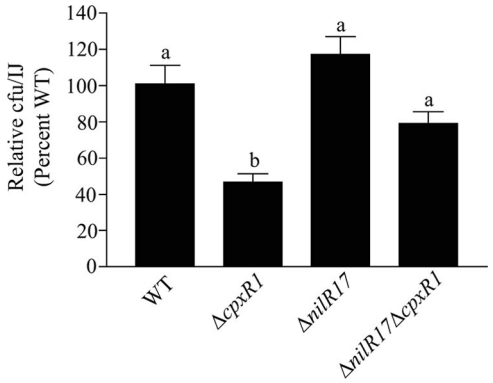

FIG. 5. A mutation in *nilR* rescues the nematode colonization defect of a Δ*cpxR1* mutant. The relative nematode colonization of Δ*cpxR1*, Δ*nilR17*, and Δ*nilR17* Δ*cpxR1* mutants is expressed as a percentage of the wild-type colonization. The average of two or more experiments is shown. Error bars represent the standard error. Different letters indicate values that are significantly different from wild-type colonization levels ($P < 0.001$). WT, wild type.

sonicated nematodes (21). Because these numbers represent an average number of bacterial cells recovered from a nematode population, we assessed whether the Δ*cpxR1* mutant colonized only a portion of the nematode population or whether the entire population of nematodes was colonized to a lower level. We have shown, by fluorescence microscopy of nematodes cultivated with green fluorescent protein-labeled *X. nematophila* strains, that the Δ*cpxR1* mutant failed to colonize 40% of the nematode population (Fig. 2). This indicates that the defect in this mutant is due in large part to an inability to initiate colonization of the nematode host.

The colonization and virulence defects of the Δ*cpxR1* mutant were not rescued by deletion of *mrxA* (Fig. 3), a gene negatively regulated by CpxR (21). This indicates that elevated *mrxA* expression does not cause the host interaction defects of the Δ*cpxR1* mutant. We found that an *mrxA* mutant does not have host interaction phenotypes, in contrast to a recent report stating that an *mrxA* mutant has decreased virulence toward *H. armigera* insects and reduced levels of nematode colonization (3). The authors of that study supplied the *mrxA* mutant construct that was used to create the strain used in the present study. Furthermore, quantitative PCR analysis confirmed that *mrxA* transcript is not expressed in the *mrxA* mutant made for the present study. Therefore, the discrepancies between the two reports cannot be explained by differences in the type of mutation. Both studies used *X. nematophila* ATCC 19061, although the strains were acquired at different times and may have diverged. The strain background used in the present study was the source of genomic DNA for sequencing (https://www.genoscope.cns.fr/agc/mage). The genome sequence contains another type I fimbrial subunit homologue, annotated as *fimA* (XN_978_8275/XN_1735). *fimA* is 48% identical in nucleotide sequence to *mrxA* and shares 36% amino acid identity. Therefore, our Δ*mrxA1* mutant strain may not display defects in host interactions due to functional redundancy with *fimA*. Further experimentation will be required to determine whether this is the case and whether the loss of both type I fimbrial genes causes a decrease in host interactions in *X. nematophila*.

Our findings indicate that the overall colonization defects of the Δ*cpxR1* mutant are due to insufficient levels of Nil colonization factors necessary for initiation. However, our data do not clarify whether CpxR transcriptional regulation of *nil* gene expression is necessary for colonization or whether overexpression of the *nil* genes simply is sufficient to overcome other defects of the Δ*cpxR1* mutant. For example, the Δ*cpxR1* mutant may have general envelope defects that destabilize Nil proteins in the membrane. Elevated expression of *nil* genes by removal of NilR-mediated repression might allow the expression of sufficient levels of Nil proteins to counteract such a general membrane defect.

Of the 60% of nematodes successfully colonized by the Δ*cpxR1* mutant, 18% were only partially colonized (Fig. 2), suggesting that, in some nematodes, the mutant fails to grow. A similar combination of initiation and outgrowth defects has been posited for a *nilA* mutant that, unlike *nilB* and *nilC* mutants, is able to partially colonize a fraction (35%) of nematodes (6, 34). Therefore, the defects of the Δ*cpxR1* mutant in both initiation and outgrowth potentially are due to insufficient expression of *nil* genes. Alternatively, CpxR may be necessary for acquisition of nutrients within the vesicle. Although the vesicle has sufficient levels of many amino acids and vitamins to support bacterial growth (34), it likely is nutrient limited. This idea is based on findings that *X. nematophila* grows slowly in the vesicle (~10 h per doubling compared to 1 h per doubling in LB medium) (33) and that *X. nematophila lrp* mutants show delayed colony formation on LB plates when recovered from nematodes or minimal medium but not when recovered from insect hemolymph or LB medium (7). Two pieces of evidence argue against the idea that the outgrowth defects of the Δ*cpxR1* mutant are due to nutrient limitation. First, the Δ*cpxR1* mutant does not have a growth defect in defined medium containing leucine, glutamate, pyruvate, aspartic acid, and glucose (G. R. Richards, unpublished data), although growth of the Δ*cpxR1* mutant in medium lacking sugars has not been tested. Second, metabolic mutants defective in the synthesis of methionine or threonine do not grow in the nematode vesicle. Such mutants exhibit spheroplast morphology and are eventually cleared from the population (34). Such spheroplast morphology has not been observed in colonizing Δ*cpxR1* mutant cells (Fig. 1), and these mutant cells remain at stable levels over time (Fig. 2).

Unexpectedly, within the Δ*cpxR1* population, several colonies displayed abnormally high levels of *nil* gene expression compared to the majority of other colonies in the population (21) (Fig. 4, circled data points). This phenomenon was seen in both previously stocked (21) and newly constructed *nil* fusion strains (data from both are shown in Fig. 4). There are several possible explanations to explain individual colonies with CpxR-independent expression of *nil* genes, including the presence of suppressor mutations, such as in *nilR*. Alternatively, *nil* gene expression may be affected by an as-yet-undescribed mechanism of phenotypic variation, in which a subpopulation of cells has altered regulation. For example, Lrp is necessary for the repression of *nil* gene expression and is known to control pleiotropic phenotypes in *X. nematophila* (7). Therefore, variation in Lrp activity or levels in subpopulations of the Δ*cpxR1* mutant could cause variations in *nil* expression. In *E. coli*, Lrp and CpxR are known to compete for binding sites upstream of the operon encoding Pap pili (22). Binding of phosphorylated

CpxR prevents the Lrp-mediated transition between phase OFF and phase ON states of *pap* gene expression (22). Although no CpxR consensus binding sites were identified upstream of the *nil* genes in *X. nematophila* (21), a similar regulatory mechanism acting directly or indirectly to influence *nil* gene expression may occur in *X. nematophila*. Consistent with this hypothesis, variant colonies with elevated levels of *nil* gene expression were not detected in the wild-type background, raising the possibility that the absence of *cpxR* increases the appearance of such variants.

X. nematophila was recently reported to undergo virulence modulation, in which a subpopulation of wild-type cells exhibited attenuated virulence and immune suppression in *M. sexta* insects but wild-type colonization levels in nematodes (40). Although the mechanisms controlling virulence modulation are not known, it is possible that *nil* gene expression is affected by this phenomenon. To address this possibility, it will be of interest to assess the colonization and virulence phenotypes of individual Δ*cpxR1* clones exhibiting elevated *nil* gene expression and, conversely, to monitor *nil* gene expression in virulence-modulated strains.

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