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# The CpxRA Two-Component System Is Essential for *Citrobacter rodentium* Virulence

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***Citrobacter rodentium* is a murine intestinal pathogen used as a model for the foodborne human pathogens enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli*. During infection, these pathogens use two-component signal transduction systems to detect and adapt to changing environmental conditions. In *E. coli*, the CpxRA two-component signal transduction system responds to envelope stress by modulating the expression of a myriad of genes. Quantitative real-time PCR showed that *cpxRA* was expressed in the colon of C57BL/6J mice infected with *C. rodentium*. To determine whether CpxRA plays a role during *C. rodentium* infection, a *cpxRA* deletion strain was generated and found to have a colonization defect during infection. This defect was independent of an altered growth rate or a defective type III secretion system, and single-copy chromosomal complementation of *cpxRA* restored virulence. The *C. rodentium* strains were then tested in C3H/HeJ mice, a lethal intestinal infection model. Mice infected with the Δ*cpxRA* strain survived infection, whereas mice infected with the wild-type or complemented strains succumbed to infection. Furthermore, we found that the *cpxRA* expression level was higher during early infection than at a later time point. Taken together, these data demonstrate that the CpxRA two-component signal transduction system is essential for the *in vivo* virulence of *C. rodentium*. In addition, these data suggest that fine-tuned *cpxRA* expression is important for infection. This is the first study that identifies a *C. rodentium* two-component transduction system required for pathogenesis. This study further indicates that CpxRA is an interesting target for therapeutics against enteric pathogens.**

Bacteria must be able to detect changes in their environment and adapt accordingly to survive. One way in which bacteria detect changes is through the use of two-component signal transduction systems (TCSs) (1, 2). The typical TCS consists of a histidine kinase (HK) sensor in the bacterial plasma membrane and a cytoplasmic response regulator (RR) protein that usually acts as a transcriptional regulator (2). Changes in the environment are detected by the HK, which results in autophosphorylation of the HK cytoplasmic domain. The subsequent transfer of the phosphoryl group to the RR affects its DNA-binding properties, resulting in changes in the expression of specific genes. The CpxRA TCS is mainly involved in the detection of and adaptation to cell envelope stress (3–6). In the absence of envelope stress, the CpxA HK exhibits phosphatase activity and maintains the CpxR RR in an inactive state (7). In some bacteria, such as *Escherichia coli*, the small periplasmic protein CpxP maintains the CpxA HK in an inactive state through a negative feedback loop (8, 9). The exact *in vivo* signal leading to CpxRA activation is unknown; however, some *in vitro* conditions that activate this TCS have been identified, including the overexpression of pilus components, alkaline pH, and alterations in membrane composition (3, 10, 11). Other studies have shown that CpxRA is activated in *E. coli* cells that overexpress the outer membrane lipoprotein NlpE when they adhere to hydrophobic surfaces (12). Altogether, these studies provide evidence that CpxRA may detect adherence to specific surfaces in addition to its role in monitoring protein folding in the periplasm. More recent studies have shown that the CpxRA signal transduction network affects the expression of hundreds of genes with a plethora of functions, indicating that CpxRA activation affects multiple functions within the cell (13–15).

*Citrobacter rodentium* is a natural murine pathogen used to model infections caused by the human pathogens enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) (16). These pathogens share many virulence factors, including those required for the formation of attaching and effacing (A/E) lesions characterized by intimate attachment to intestinal epithelial cells, localized effacement of microvilli, and the formation of actin-rich pedestals beneath sites of bacterial adherence (16). To date, the TCS involved in fucose sensing has been implicated in the *in vivo* virulence of EHEC (17). Less is known about the *in vivo* role of the other TCSs, including CpxRA.

The CpxRA TCS appears to have contrasting effects on the pathogenesis of different bacterial species. For example, activation of CpxRA in *Shigella sonnei* results in increased expression of virulence genes (18). Further supporting a role for the CpxRA TCS during infection, the insect pathogen *Xenorhabdus nematophila* and the human pathogens *Yersinia pestis* and uropathogenic *E. coli* require the CpxRA TCS for full virulence (19–21). These results

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**TABLE 1** Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| Strains | | |
| *C. rodentium* | | |
| DBS100 | Wild-type *C. rodentium* DBS100 | 48 |
| Δ*cpxRA* | DBS100 Δ*cpxRA* | This study |
| Δ*cpxRA*::*cpxRA* | DBS100 Δ*cpxRA* with a single copy of *cpxRA* integrated at the *att* Tn*7* site; Gent$^r$ | This study |
| Δ*cpxRA*(pSTNSK) | DBS100 Δ*cpxRA* + pSTNSK; Kan$^r$ | This study |
| *spy*::*lacZ* | DBS100 with a chromosomal insertion of *spy*::*lacZ* | This study |
| Δ*cpxRA*::*spy*::*lacZ* | Δ*cpxRA* strain with a chromosomal insertion of *spy*::*lacZ* | This study |
| Δ*cpxRA*::*cpxRA*::*spy*::*lacZ* | Δ*cpxRA*::*cpxRA* strain with a chromosomal insertion of *spy*::*lacZ* | This study |
| *E. coli* | | |
| DH5α | HuA2 Δ(*lac*)*U169 phoA glnV44* ϕ80*lacZ*ΔM15 *endA recA hsdR17* ($r_M^-$ $m_K^+$) *thi-1 gyrA96 relA1* | Invitrogen |
| DH5α(pBSΔ*cpxRA*) | DH5α containing pBSΔ*cpxRA* | This study |
| DH5αλpir | K-12 F$^-$ ϕ80*lacZ*ΔM15 *endA recA hsdR17* ($r_M^-$ $m_K^+$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* λ*pir* | |
| DH5αλpir(pΔ*cpxRA*) | DH5αλpir containing pΔ*cpxRA* | This study |
| DH5αλpir(p*cpxRA*) | DH5αλpir containing p*cpxRA* | This study |
| DH5αλpir(p*spylacZ*) | DH5αλpir containing p*spylacZ* | This study |
| χ7213 | *thr-1 leuB6 fhuA21 lacY1 glnV44 recA1 asdA4 thi-1* RP4-2-Tc::Mu [-*pir*] Kan$^r$ | 49 |
| χ7213(pΔ*cpxRA*) | χ7213 containing pΔ*cpxRA* | This study |
| χ7213(p*cpxRA*) | χ7213 containing p*cpxRA* | This study |
| χ7213(p*spy*::*lacZ*) | χ7213 containing p*spy*::*lacZ* | This study |
| Plasmids | | |
| pSTNSK | pST76-K::tnsABCD; Kan$^r$ | 33 |
| pBluescript SK+ | High-copy-no. cloning vector; Amp$^r$ | Stratagene |
| pBSΔ*cpxRA* | Δ*cpxRA* deletion construct in pBluescript SK+ | This study |
| pRE112 | Sucrose-sensitive (*sacB1*) suicide vector; Cm$^r$ | 50 |
| pΔ*cpxRA* | Δ*cpxRA* deletion construct in pRE112 | This study |
| pGP-Tn7-Gm | pGP704::Tn*7*-Gm; Amp$^r$ Gent$^r$ | 33 |
| p*cpxRA* | *C. rodentium* DBS100 *cpxRA* cloned into pGP-Tn*7*-Gm | This study |
| pFUSE | Suicide vector, *lacZYA*, *mob*$^+$ (RP4), *ori* R6K, Cm$^r$ | 34 |
| p*spy*::*lacZ* | *spy* promoter fusion to *lacZ* in pFUSE | This study |

are in direct contrast to what was observed for *Haemophilus ducreyi*, where activation of the CpxRA TCS results in the downregulation of known virulence genes (14). More importantly, in *H. ducreyi*, the constitutive activation of CpxRA leads to *in vivo* virulence defects, and the deletion of this TCS has no effect on infection (22, 23). These studies are consistent with what was observed during experimental infection with *Salmonella enterica* serovar Typhimurium and *Vibrio cholerae* (24, 25). Therefore, the *in vivo* contribution of the CpxRA TCS to virulence appears to vary among species.

In the A/E pathogen EPEC, CpxRA has been implicated in the *in vitro* regulation of virulence genes (26). The bundle-forming pilus, a structure unique to EPEC, is downregulated following the activation of CpxRA (27). This pilus is required for infection and is involved in early attachment to intestinal cells (28). Other evidence supports a role for CpxRA in the indirect downregulation of type III secretion system (TTSS) genes common to all A/E pathogens (29). Therefore, the constitutive activation of CpxRA negatively impacts virulence gene expression *in vitro*. In addition to these findings, the deletion of *cpxR* and subsequent inactivation of CpxRA leads to decreases in cell adherence and *in vitro* infectivity of EPEC (30). These studies provide compelling evidence that CpxRA is involved in the regulation of essential colonization and virulence factors of EPEC and that the activation of this TCS must be carefully fine-tuned to protect cells against cell envelope stress while allowing for virulence gene expression. The CpxRA proteins in *C. rodentium* are highly homologous to those found in *E. coli* K-12 and EPEC, suggesting that they may have some shared functions (see Table S1 in the supplemental material). Furthermore, the *cpxRA* gene organization is similar, with *cpxR* and *cpxA* forming an operon and the *cpxP* gene located next to *cpxR* in the opposite orientation. To date, there have been no studies investigating the role of CpxRA in the A/E pathogen *C. rodentium*. In this study, we examine the *in vivo* contribution of CpxRA to *C. rodentium* virulence. We show that the *C. rodentium* CpxRA TCS is essential for *in vivo* infection and provide evidence that this virulence defect is independent of a major defect in type III secretion (TTS).

## MATERIALS AND METHODS

**Media and reagents.** Bacteria were routinely cultured at 37°C with aeration (200 rpm) in Luria-Bertani (LB) broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl). When appropriate, LB broth was supplemented with chloramphenicol (Cm) (30 μg/ml), ampicillin (Amp) (300 μg/ml), gentamicin (Gent) (30 μg/ml), kanamycin (Kan) (50 μg/ml), or DL-diaminopimelic acid (DAP) (50 μg/ml).

**Construction of the *C. rodentium cpxRA* deletion strain.** The bacterial strains and plasmids used in this study are listed in Table 1. DH5α and DH5αλpir were routinely used for genetic manipulations. DNA purification, cloning, and transformation were performed according to standard procedures (31). The *C. rodentium* Δ*cpxRA* strain was generated by *sacB*

**TABLE 2** Primers used in this study[a]

| Primer | Sequence | Usage |
|---|---|---|
| cpxRA1 | GCTCTAGAGCTGTTAAGGCGAGAGTGGATGA | Δ*cpxRA* 5′ F XbaI |
| cpxRA2 | CCGCTCGAGCTCTCGGTCATCATCAACTAA | Δ*cpxRA* 5′ R XhoI |
| cpxRA3 | CCGCTCGAGTAATAACGCTGAGTTGCCGGATGG | Δ*cpxRA* 3′ F XhoI |
| cpxRA4 | GGGGTACCGAATGTCTTTATCGGTGATATCC | Δ*cpxRA* 3′ R KpnI |
| cpxRA5 | CCGCTCGAGCACTTGCTCCCAAAATCTTTTCTG | p*cpxRA* F XhoI |
| cpxRA6 | GAGGTACCCGTTTACGCCGCCATCCGGCAA | p*cpxRA* R KpnI |
| spy_F | CCGTCTAGAGCTGGCATCTGGATCTTCACAC | p*spy*::*lacZ* F XbaI |
| spy_R | CCGCCCGGGCGGTTTTGCGTCTGCCGGC | p*spy*::*lacZ* R SmaI |
| qσ$^{70}$1 | ATCAAAGCGAAAGGTCGTAGCCAC | qPCR σ$^{70}$ F |
| qσ$^{70}$2 | CCATCATCACGCGCATACTGTTCA | qPCR σ$^{70}$ R |
| qcpxR1 | GGAACAGGCGCTGGAGCTTC | qPCR *cpxR* 5′ F |
| qcpxR2 | ACGGGGGTCTGGTGTGTCTG | qPCR *cpxR* 3′ R |
| qcpxA1 | AACGCCGTTGACCCGCTTAC | qPCR *cpxA* 5′ F |
| qcpxA2 | GCGCGACATCACCAACAGGT | qPCR *cpxA* 3′ R |
| qcpxP1 | AAGCCATGCTGCTGAAGTCGATAC | qPCR *cpxP* 5′ F |
| qcpxP2 | CGCATCTGTTGACGTTGATGTTCG | qPCR *cpxP* 3′ R |

[a] Restriction sites are underlined. F indicates forward, and R indicates reverse.

gene-based allelic exchange (32). Genomic DNA from *C. rodentium* was used as a template to PCR amplify the upstream (primer pair cpxRA1/cpxRA2) (Table 2) sequence of the *cpxRA* operon. The resultant PCR product was treated with XbaI and XhoI and ligated into pBluescript SK+ that had been treated with the same restriction enzymes, generating pBS*cpxRA*. In a similar manner, the downstream sequence of the *cpxRA* operon was PCR amplified by using primer pair cpxRA3/cpxRA4 (Table 2). The resultant PCR product was treated with XhoI and KpnI and ligated into pBS*cpxRA* that had been treated with the same restriction enzymes, generating pBSΔ*cpxRA*. Plasmid pΔ*cpxRA* was generated by subcloning the DNA fragment from pBSΔ*cpxRA* into the XbaI and KpnI sites of pRE112. The sequence of pΔ*cpxRA* was verified by sequencing (Genome Quebec). Plasmid pΔ*cpxRA* was conjugated into wild-type *C. rodentium* by using *E. coli* χ7213 as the donor strain. Integration of the plasmid into the chromosome was selected for by plating bacteria onto LB agar supplemented with Cm. Cm-resistant transformants of *C. rodentium* were then plated onto peptone agar containing 5% sucrose to isolate colonies that were sucrose resistant. The resultant colonies were also tested for Cm sensitivity. Gene deletions were verified by PCR using primers cpxRA1 and cpxRA4.

**Single-copy chromosomal complementation of the Δ*cpxRA* strain.** The Δ*cpxRA*::*cpxRA* strain with *cpxRA* integrated at the *att* Tn*7* site was generated as previously described (33). Briefly, genomic DNA from *C. rodentium* was used as a template to PCR amplify both the promoter and open reading frame of *cpxRA* (primer pair cpxRA5/cpxRA6) (Table 2). The resultant PCR product was treated with XhoI and KpnI and then ligated into pGP-Tn*7*-Gm that had been treated with the same restriction enzymes, generating p*cpxRA*. The sequence of plasmid p*cpxRA* was verified by sequencing (Genome Quebec). The complementation plasmid, p*cpxRA*, was conjugated into the Δ*cpxRA*(pSTNSK) strain at 30°C by using *E. coli* χ7213 as the donor strain. Integration of the *cpxRA* DNA fragment into the chromosome at the *att* Tn*7* site and loss of pSTNSK were selected for by plating bacteria onto LB agar supplemented with Gent and incubating the cells for 4 h at 42°C, followed by 18 h at 37°C. Gent-resistant transformants of *C. rodentium* were then patched onto LB agar supplemented with either Gent, Amp, or Kan. Colonies that were resistant to Gent but sensitive to Amp and Kan were screened by PCR to ensure the integration of the *cpxRA* fragment at the *att* Tn*7* site.

**Construction of *C. rodentium* strains with chromosomal transcriptional *spy*::*lacZ* fusions.** Chromosomal transcriptional fusions between the *spy* promoter and the *lacZ* reporter gene were generated in *C. rodentium* strains by using the suicide vector pFUSE (34). The *spy*::*lacZ* fusion was constructed by PCR amplifying the *spy* promoter using *C. rodentium* genomic DNA and primers *spy*F and *spy*R. The PCR product was digested with XbaI and SmaI and cloned into the corresponding sites of pFUSE. The resulting construct was transferred into the *C. rodentium* wild-type, Δ*cpxRA*, and Δ*cpxRA*::*cpxRA* strains by conjugation and integrated by homologous recombination as described above.

**β-Galactosidase assays.** β-Galactosidase assays were performed as previously described, with minor modifications (35). Briefly, cultures of *C. rodentium* strains grown overnight were diluted 1:100 into 3 ml of LB medium buffered with 100 mM sodium phosphate at pH 5.5 or 8.5 and grown for 3 h at 37°C with aeration. Cells (2 ml) were recovered by centrifugation and resuspended in Z-buffer (2 ml) (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $Na_2H_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). A volume (500 μl) of resuspended cells was combined with reaction buffer (650 μl) (Z-buffer with 0.008% [wt/vol] SDS and 15% [vol/vol] chloroform), vortexed, and incubated for 5 min at 28°C. The reaction was started by the addition of *ortho*-nitrophenyl-β-galactoside (200 μl; 4 mg/ml) to each reaction tube and allowed to develop at 28°C. Reactions were stopped by the addition of $Na_2CO_3$ (500 μl; 1 M) to the mixture, cell debris was removed by centrifugation, and the optical densities at 420, 540, and 595 nm were measured. All experiments were performed at least three times in triplicate. Miller units were calculated as previously described (36).

**Secretion assays.** Bacterial secretion assays were performed as previously described (37). Briefly, cultures of bacteria grown overnight were subcultured 1:50 into 6-well tissue culture plates containing Dulbecco's modified Eagle medium (DMEM; Gibco). After a 6-h incubation at 37°C with 5% $CO_2$, whole cells were separated from the culture medium by centrifugation. The supernatant was transferred into a new tube, and the bacterial pellets, now referred to as whole-cell lysates, were resuspended in electrophoresis sample buffer (ESB) (0.0625 M Tris-HCl [pH 6.8], 1% [wt/vol] SDS, 10% glycerol, 2% [vol/vol] 2-mercaptoethanol, 0.001% [wt/vol] bromphenol blue), boiled, and stored at −20°C. Contaminating cells in the supernatant were removed by centrifugation (13,000 × *g* for 2 min). The proteins in the supernatant, now referred to as secreted proteins, were precipitated in sterile glass tubes with 10% (vol/vol) 6 M trichloroacetic acid and incubated for 1 h on ice. Secreted proteins were separated by centrifugation (13,000 × *g* for 30 min at 4°C) and washed overnight with acetone at −80°C. The next day, secreted proteins were collected by centrifugation (13,000 × *g* for 30 min at 4°C), supernatants were removed, and the pellets were air dried. Secreted-protein pellets were resuspended in ESB, boiled, separated on 10% SDS-PAGE gels, and visualized by Coomassie blue staining. Aliquots (2 μl) of whole-cell lysate proteins were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes, and DnaK and EspB were

detected by Western blotting using anti-DnaK (Stressgen) and anti-EspB (clone 2A11) antibodies (38).

**qPCR.** Quantitative PCR (qPCR) was performed as previously described (39). Briefly, the terminal centimeter of colon from infected C3H/HeJ or C57BL/6J mice was collected, homogenized by using a Polytron homogenizer, and stored at −80°C in TRIzol. Total RNA was isolated by using TRIzol reagents (Invitrogen) and treated with a DNA-free kit (Ambion) to remove any remaining DNA. The absence of contaminating DNA was confirmed by qPCR using primers qCR16SF and qCR16SR (40). RNA was reverse transcribed by using Superscript III (Invitrogen). As a negative control, a reaction mixture without Superscript III was also included (NRT). qPCRs were performed with a Rotor-Gene 3000 thermal cycler (Corbett Research), using the Maxima SYBR green PCR kit (Fermentas), according to the manufacturer's instructions. Primers used are listed in Table 2. The level of gene transcript was normalized to $\sigma^{70}$ values and analyzed by using the $2^{-\Delta CT}$ (where $C_T$ is threshold cycle) method (41). Reverse transcription (RT) was performed three times independently, and the NRT sample was used as a negative control.

***In vivo C. rodentium* infections.** All animal experiments were performed under conditions specified by the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee. C57BL/6J and C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a specific-pathogen-free facility at McGill University. Four-week-old mice were orally inoculated with *C. rodentium* strains. For oral inoculations, bacteria were grown overnight in 3 ml of LB broth with aeration. Mice were infected by oral gavage of 0.1 ml of LB broth containing $2 \times 10^8$ to $3 \times 10^8$ CFU of *C. rodentium*. The infectious dose was verified by plating serial dilutions of the inoculum onto MacConkey agar (Difco). For survival analysis of C3H/HeJ mice, the mice were monitored daily and were killed if they met any of the following clinical endpoints: 20% body weight loss, hunching and shaking, inactivity, or body condition score of <2. To monitor bacterial colonization in C3H/HeJ and C57BL/6J mice, fecal pellets or the terminal centimeter of colon was collected and homogenized in phosphate-buffered saline by using a Polytron homogenizer. Homogenates were serially diluted in sterile phosphate-buffered saline, and 0.1-ml aliquots of each serial dilution were plated onto MacConkey agar. *C. rodentium* was distinguished by its characteristic colony morphology on this medium, as previously described (42). Plates containing between 30 and 300 colonies were counted. When bacterial loads were low, leading to the undiluted-sample plate having <30 colonies, the number of colonies on this plate was counted. Spleens were removed and weighed, and splenic indexes were calculated [$\sqrt{}$(weight of spleen × 100/weight of mouse)]. For histological analysis, the last 0.5 cm of the colon of infected mice was fixed in 10% neutral buffered formalin, processed, cut into 3-μm sections, and stained with hematoxylin and eosin. Pathological scoring was performed in a blind fashion by a board-certified veterinary pathologist using the scoring matrix provided in Table S2 in the supplemental material. Crypt heights were measured by using Infinity Analyze software on a Zeiss A1 microscope, with 10 well-oriented crypts being measured per mouse.

## RESULTS

**Expression of *cpxRA* during intestinal infection of C57BL/6J mice.** To assess the role of the CpxRA TCS during intestinal infection with *C. rodentium*, we first performed qPCR on cDNA samples prepared from the distal colon of C57BL/6J mice on day 9 of infection. At this time point, bacterial loads in the colon were high enough to obtain reproducible signals for bacterial gene expression. As shown in Fig. 1, both *cpxA* and *cpxR* were expressed in infected colons. To determine if CpxRA is active under these conditions, we also examined the expression of *cpxP*, which has previously been shown to be strongly upregulated by CpxRA as part of a negative feedback loop (10). We found that *cpxP* was highly expressed, suggesting that CpxRA was activated (Fig. 1). To ensure

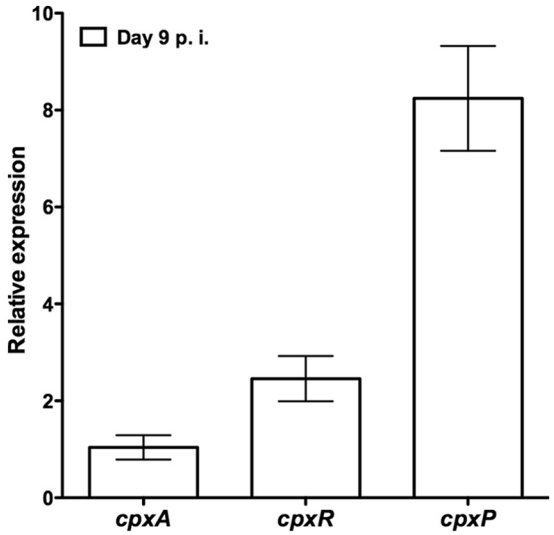


**FIG 1** qPCR analysis of *cpx* gene expression during infection of C57BL/6J mice. Data were normalized to *rpoD* expression levels. Relative expression is indicative of $2^{-\Delta CT}$. Data shown are the means ± standard errors of the means from 5 biological samples, in triplicate. p.i., postinfection.

that the signal detected was not the result of nonspecific amplification of bacterial or murine cDNA found in the colon of mice, we performed control qPCR experiments on uninfected mice with our *rpoD*, *cpxA*, *cpxR*, and *cpxP* primers. These primers did not amplify any products in the absence of *C. rodentium* (see Fig. S1 in the supplemental material). These data provide evidence that *cpxRA* is expressed during intestinal infection.

**The *C. rodentium* CpxRA TCS is activated under *in vitro* conditions similar to those of its *E. coli* homologue.** To further analyze CpxRA in *C. rodentium*, an in-frame *cpxRA* deletion strain (Δ*cpxRA*) and the Δ*cpxRA*::*cpxRA* strain, consisting of Δ*cpxRA* with a chromosomal insertion of *cpxRA*, were generated (Table 1). For *E. coli*, several *in vitro* growth conditions, including alkaline pH, have been shown to activate the CpxRA TCS (35). Under this condition, the *spy* promoter was the second most activated promoter by the CpxRA TCS (35). To determine whether the *C. rodentium* CpxRA TCS is activated by alkaline pH, we generated chromosomal *spy*::*lacZ* transcriptional fusions within the wild-type, Δ*cpxRA*, and Δ*cpxRA*::*cpxRA* strains and grew these strains under both CpxRA-repressing (pH 5.5) and -activating (pH 8.5) conditions. As shown in Fig. 2, there was an increase in β-galactosidase activity in the wild-type strain at pH 8.5 compared to bacteria grown at pH 5.5. These data indicate that the *spy* promoter is activated in wild-type *C. rodentium* at alkaline pH, which is similar to what had been previously observed for *E. coli*. In contrast, there was no difference in the β-galactosidase activity of the Δ*cpxRA* strain when grown at either pH. Complementation of the Δ*cpxRA* strain restored β-galactosidase activity to wild-type levels. Altogether, these data show that the *C. rodentium* CpxRA TCS is activated by growth at alkaline pH; they also suggest that the *C. rodentium* and *E. coli* CpxRA TCSs fulfill similar functions.

**CpxRA impacts colonization and disease in C57BL/6J mice.** We next assessed whether *cpxRA* is required for bacterial fitness and/or virulence by comparing infections of C57BL/6J mice with the wild-type, Δ*cpxRA*, and Δ*cpxRA*::*cpxRA* strains. As shown in Fig. 3A, the Δ*cpxRA* strain displayed a significant defect in intes-

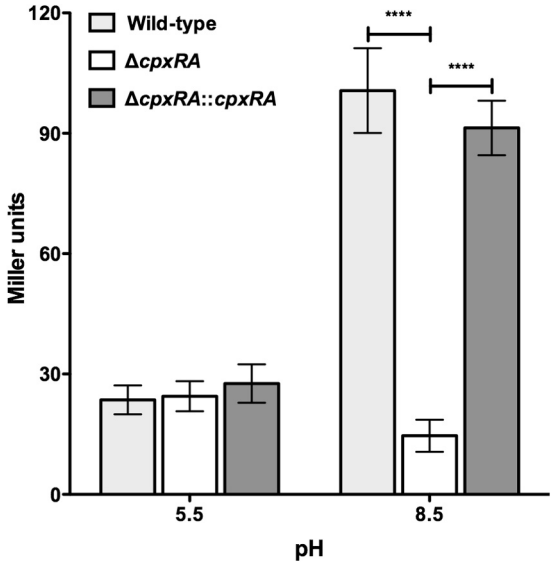


**FIG 2** CpxRA is activated by alkaline pH. The indicated bacterial strains containing a chromosomal *spy*::*lacZ* fusion were grown for 2.5 h in LB medium buffered with sodium phosphate to pH 5.5 or 8.5, followed by determination of β-galactosidase activity, expressed as Miller units. Data are from 5 different experiments performed in triplicate. Data shown are means ± standard errors of the means. Asterisks indicate statistical significance ($P < 0.0001$), as determined by analysis of variance followed by Bonferroni's multiple-comparison *post hoc* analysis.

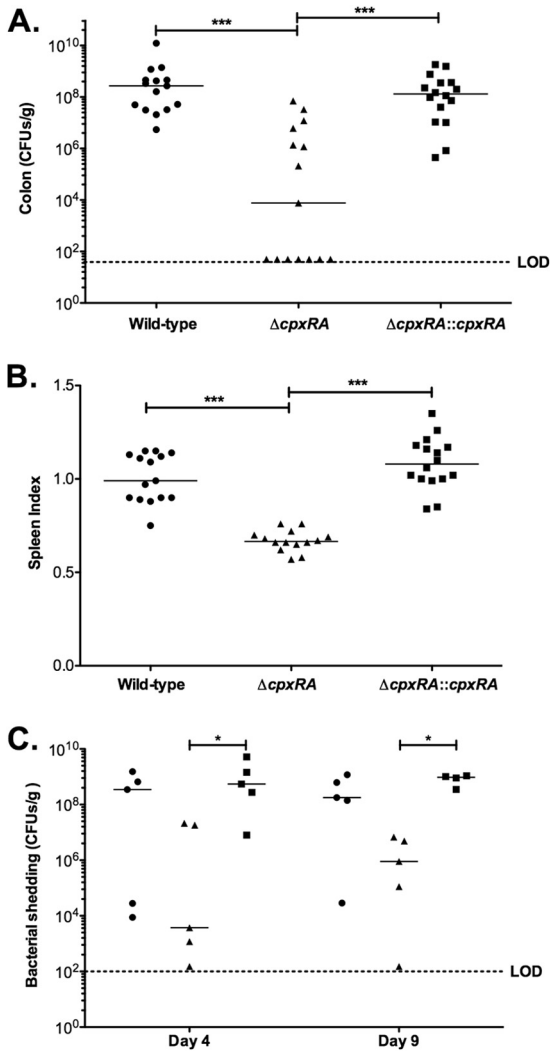


**FIG 3** CpxRA is essential for efficient colonization and infection of C57BL/6J mice. C57BL/6J mice were infected with the indicated *C. rodentium* strains. Mice were sacrificed at 12 days postinfection. Each mouse in the experiment is represented by a single point. The median for each group is indicated on the graph by horizontal bars. Asterisks indicate statistical significance (*, $P < 0.05$; ***, $P < 0.001$), as determined by Kruskall-Wallis analysis and Dunn's multiple-comparison *post hoc* analysis. (A) Numbers of CFU per gram of colon from each animal were determined by serial dilution plating. (B) Prior to dissection, each animal was weighed, the spleens were then removed and weighed individually, and spleen indexes were calculated [√(weight of spleen × 100/weight of mouse)]. (C) Numbers of CFU per gram of feces were determined for mice infected with the wild-type (circles), Δ*cpxRA* (triangles), or Δ*cpxRA*::*cpxRA* (squares) strain by serial dilution plating. LOD indicates the limit of detection.

tinal colonization ($P < 0.001$) compared to wild-type *C. rodentium*. There was a significant increase in colonization with the complemented strain compared to the Δ*cpxRA* strain ($P < 0.001$). A large spread in the bacterial loads of mice infected with the Δ*cpxRA* strain compared to mice infected with the wild-type and Δ*cpxRA*::*cpxRA* strains was observed, with 7 out of 15 Δ*cpxRA*-infected mice having no detectable *C. rodentium* present in the colon (Fig. 3A). These data indicate that, at the peak of infection, intestinal colonization levels of the Δ*cpxRA* strain are significantly decreased and that complementation restores colonization to wild-type levels. We next determined the splenic indexes for these mice. As shown in Fig. 3B, the wild-type-infected mice had significantly larger spleens than did the Δ*cpxRA*-infected mice at day 12 postinfection, indicating that there is less inflammation during infection with the Δ*cpxRA* strain ($P < 0.001$). The spleens of mice infected with the complemented strain were significantly larger than those infected with the Δ*cpxRA* strain and were similar to those of wild-type-infected mice (Fig. 3B).

To investigate the origin of the lower colonization levels at the peak of infection with the Δ*cpxRA* strain, we monitored bacterial colonization with *C. rodentium* at earlier time points by measuring its shedding into the feces of mice (Fig. 3C). These experiments indicated that whereas both the wild-type and Δ*cpxRA*::*cpxRA* strains had already colonized to very high levels by day 4 postinfection, the Δ*cpxRA* strain was already displaying a comparative attenuation in colonization at this time point. Decreased colonization by the Δ*cpxRA* strain was also evident at day 9 postinfection (Fig. 3C). Altogether, these data indicate that *C. rodentium* Δ*cpxRA* cells have an early colonization defect.

Histological analyses of biopsy specimens taken from the distal colon of mice revealed that all animals infected with the Δ*cpxRA* strain had significantly lower pathology scores than did mice infected with either the wild-type or Δ*cpxRA*::*cpxRA* strain, whereas the pathology scores for the wild-type- and Δ*cpxRA*::*cpxRA*-infected mice were similar (Fig. 4E). Colon sections from wild-type- and Δ*cpxRA*::*cpxRA*-infected mice displayed a loss of normal tissue architecture, with signs of epithelial hyperplasia, inflammation, goblet cell loss, and shedding of cells in the intestinal lumen, whereas all Δ*cpxRA*-infected mice showed well-preserved tissue morphology, with few signs of inflammation (Fig. 4A to C). Additionally, mice infected with the wild-type and Δ*cpxRA*::*cpxRA*

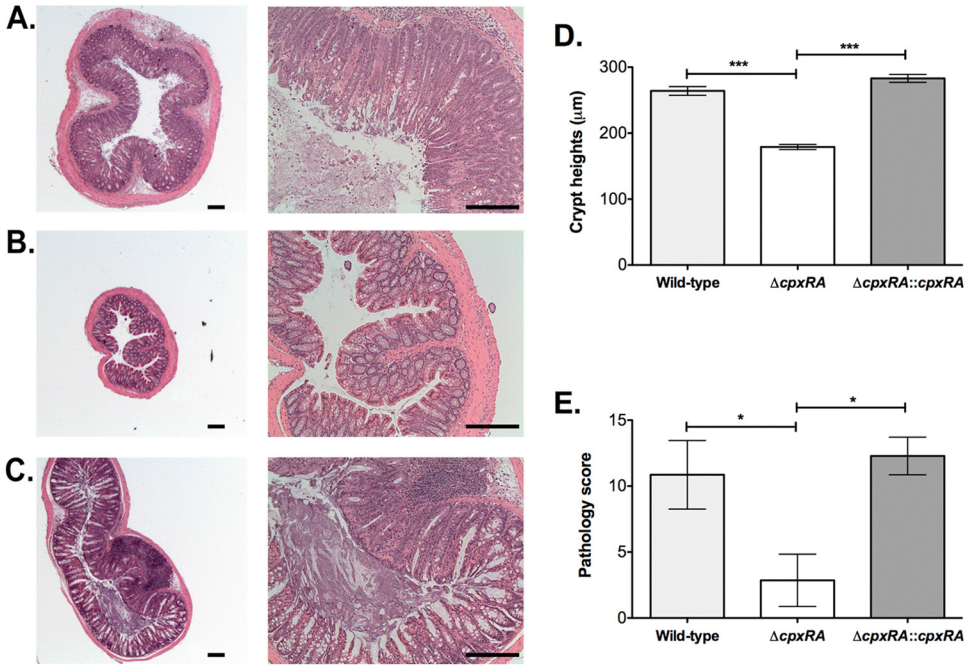


**FIG 4** Histological analysis of infected C57BL/6J mouse colons. (A to C) Mice were infected with either the wild-type (A), Δ*cpxRA* (B), or Δ*cpxRA*::*cpxRA* (C) *C. rodentium* strain and sacrificed at 12 days postinfection. Hematoxylin- and eosin-stained tissue sections for all mice were observed and photographed by using 2.5× (left) and 10× (right) objectives. Typical results are shown. Bar, 200 μm. (D) Crypt heights in mice infected with the indicated *C. rodentium* strains ($n = 7$) were measured. Data shown are the means ± standard errors of the means. Asterisks indicate statistical significance (***, $P < 0.001$), as determined by a Kruskal-Wallis test followed by Dunn's *post hoc* analysis. (E) A pathology score was determined for mice infected with the indicated *C. rodentium* strains ($n = 7$). Asterisks indicate statistical significance (*, $P < 0.05$), as determined by analysis of variance followed by Bonferroni's multiple-comparison *post hoc* analysis.

strains had colonic crypt heights that were approximately double those of mice infected with the Δ*cpxRA* strain, indicating that mice infected with the latter strain suffer less hyperplasia (Fig. 4D).

**The colonization defect of the Δ*cpxRA* strain is due to neither deficient TTS nor an altered growth rate.** Since CpxRA has been implicated in the regulation of TTS in other bacterial species (26), and since TTS is a key virulence mechanism of *C. rodentium* (16), we next asked whether the Δ*cpxRA* strain was deficient in TTS, which would provide an underlying mechanism for its colonization defect. The wild-type, Δ*cpxRA*, and Δ*cpxRA*::*cpxRA* strains were grown under TTS-inducing conditions (37), and the secreted protein and whole-cell fractions were analyzed by Coomassie staining and Western blotting. As shown in Fig. 5A and B, the TTSS was functional in all three strains. Western blotting for the abundant cytoplasmic, nonsecreted bacterial protein DnaK was used to monitor the amount of bacterial lysis or leakage of cytoplasmic components into the secreted protein fractions. This revealed small amounts of DnaK in the secreted protein fractions of all strains, suggesting that small amounts of bacterial lysis or leakage had occurred. The levels of the type III secreted protein EspB detected in the secreted protein fractions were equivalent for all strains (Fig. 5B). There was a slightly elevated amount of EspB detected in the whole-cell fraction of the Δ*cpxRA* strain, but this is unlikely to indicate an increased amount of EspB produced, as it correlates with an increase in DnaK levels, suggesting that slightly more whole-cell protein extract was loaded into this well (Fig. 5B). Growth curve experiments with the wild-type, Δ*cpxRA*, and Δ*cpxRA*::*cpxRA* strains also indicated that the Δ*cpxRA* strain does not have a noticeable growth defect in LB broth (Fig. 5C). In DMEM, with the exception of a longer lag phase for the Δ*cpxRA* strain, there were no significant differences in exponential growth rates and final cell densities between strains (Fig. 5D). Taken together, these results provide evidence that the colonization defect of the Δ*cpxRA* strain is not due to deficient TTS or a change in the bacterial growth rate.

**CpxRA is required for virulence in a fatal infection in susceptible mice.** To gain further insight into the role of CpxRA during intestinal *C. rodentium* infection, we tested the wild-type, Δ*cpxRA*, and Δ*cpxRA*::*cpxRA* strains in a lethal intestinal infection model in susceptible mice (16). Many inbred mouse strains suffer self-limiting disease during infection with *C. rodentium*, whereas some inbred strains such as C3H/HeJ, AKR/J, and FVB/J suffer more severe disease and high mortality rates (43). In C3H/HeJ mice, 100% of animals infected with either the wild-type or *cpxRA*::*cpxRA* strain succumbed to the infection. In contrast, animals infected with the Δ*cpxRA* strain did not develop overt signs of disease, and 100% of them survived up to 30 days postinfection, when the experiment was terminated (Fig. 6A). To determine if the decreased mortality was associated with altered levels of bacterial colonization in C3H/HeJ mice, we assessed shedding of *C. rodentium* strains into the feces of infected mice. As shown in Fig. 6B, at day 3 postinfection, there was significantly ($P < 0.05$ and 0.01) higher levels of colonization by wild-type and Δ*cpxRA*::*cpxRA* bacteria than by Δ*cpxRA* bacteria. Furthermore, whereas the levels of wild-type and Δ*cpxRA*::*cpxRA C. rodentium* strains increased >10-fold between days 3 and 6 postinfection, the amount of bacteria shed by mice infected with the Δ*cpxRA* strain remained stable. The lower level of colonization by the Δ*cpxRA* strain was also evident in the colons of infected C3H/HeJ mice at

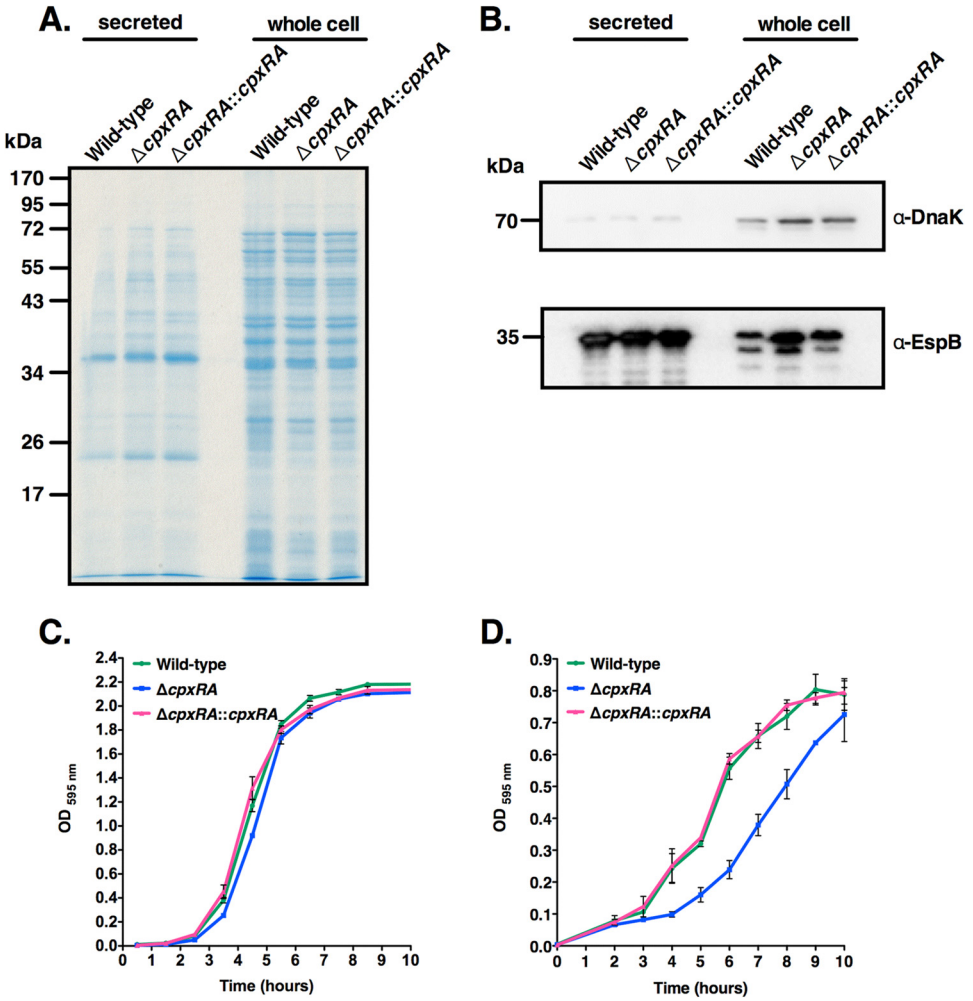


**FIG 5** Deletion of the *cpxRA* genes does not affect TTS or bacterial growth. (A) Coomassie blue-stained SDS-PAGE gel showing secreted and whole-cell proteins from the indicated *C. rodentium* strains. (B) Western blot analyses of secreted and whole-cell proteins from the indicated *C. rodentium* strains, using DnaK and EspB antibodies. (C and D) Growth curves of the indicated *C. rodentium* strains in LB medium (C) or DMEM (D). This experiment was repeated at least twice in triplicate. OD, optical density.

day 9 postinfection (Fig. 6B). Lower levels of colonization by the Δ*cpxRA* strain were not attributed to a delay in colonization, since C3H/HeJ mice infected with the Δ*cpxRA* strain remained healthy up to day 28 postinfection and displayed complete clearance of *C. rodentium* at this time point (see Fig. S2 in the supplemental material). These data clearly show that CpxRA is required for *C. rodentium* virulence in both the lethal and nonlethal infection models.

**Dynamic CpxRA expression during infection of susceptible mice.** To examine when *cpxRA* is expressed during intestinal infection of susceptible mice with *C. rodentium*, we performed qPCR on cDNA prepared from the colon of C3H/HeJ mice on days 4 and 9 of infection. At these time points, bacterial loads in the distal colon of C3H/HeJ mice were high enough to obtain reproducible signals for expression of bacterial genes. As shown in Fig. 7, *cpxA*, *cpxR*, and *cpxP* were significantly more expressed in infected colons on day 4 postinfection than on day 9 postinfection. These data indicate that *cpxRA* expression is temporally regulated, with higher expression levels occurring during the initial stages of *C. rodentium* infection of susceptible mice.

## DISCUSSION

TCSs are activated in response to changing environmental cues, resulting in the transcriptional regulation of gene expression that promotes bacterial adaptation (1). The CpxRA TCS responds to cell envelope stress and is thought to modulate virulence gene expression (3, 4, 26). In this study, we show that the *C. rodentium* CpxRA TCS plays an important role during intestinal infection. Using two different murine infection models, we consistently observed striking defects in colonization and virulence in the Δ*cpxRA* strain, which were restored to wild-type levels when the strain was complemented with a chromosomal insertion of *cpxRA*.

Our results are in good agreement with data from previous studies demonstrating a role for CpxRA in the *in vivo* virulence of some Gram-negative pathogens, including uropathogenic *E. coli* (19) and *X. nematophila* (44). Specifically, our results are consistent with studies that have shown that CpxRA activation has a beneficial effect on bacteria in times of envelope stress. In EPEC, CpxRA is required to adapt to envelope stress; however, CpxRA

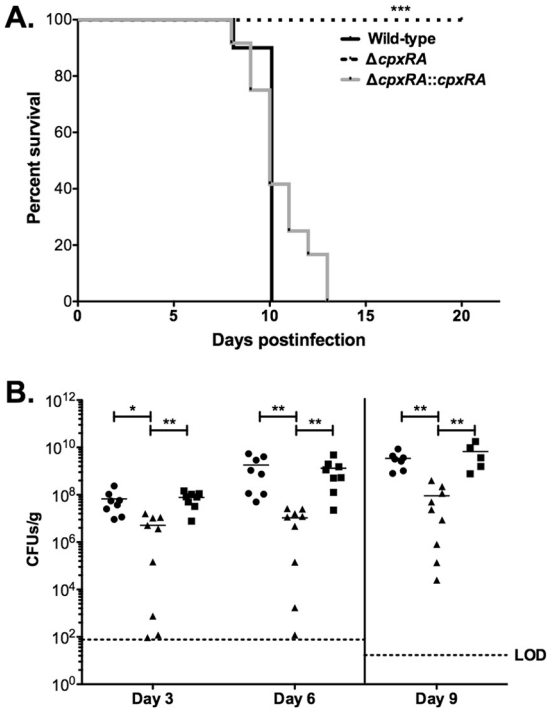


**FIG 6** CpxRA is required for efficient infection of C3H/HeJ mice. C3H/HeJ mice were infected by oral gavage with $2 \times 10^8$ CFU of the wild-type, Δ*cpxRA*, or Δ*cpxRA*::*cpxRA C. rodentium* strain. (A) Each data point represents the percentage of mice still surviving from an initial population of 9 to 12 mice. Statistical analyses were performed by using a Gehan-Breslow-Wilcoxon test, and asterisks indicate statistical significance (***, $P < 0.001$). (B) Numbers of CFU per gram of feces (day 3 and day 6) or colon (day 9) were determined for mice infected with the wild-type (circles), Δ*cpxRA* (triangles), or Δ*cpxRA*::*cpxRA* (squares) strain by serial dilution plating. LOD indicates the limit of detection. The median for each group is indicated on the graph by horizontal bars. Asterisks indicate statistical significance (**, $P < 0.05$; **, $P < 0.01$), as determined by a Kruskall-Wallis test and Dunn's multiple-comparison *post hoc* analysis.

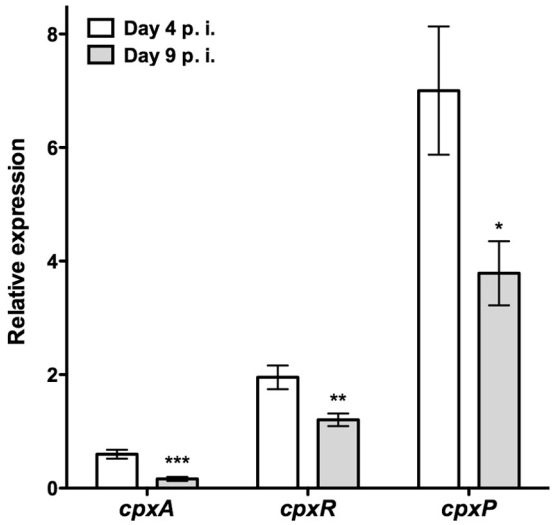


**FIG 7** qPCR analysis of *cpx* gene expression during infection of C3H/HeJ mice. Data were normalized to *rpoD* expression levels. Relative expression is indicative of $2^{-\Delta CT}$. Data shown are the means ± standard errors of the means from 5 biological samples, in triplicate. p.i., postinfection. Asterisks indicate statistical significance between data obtained on days 4 and 9 postinfection (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$), as determined by using an unpaired *t* test.

activation leads to decreased TTS gene expression; these apparently contrasting roles suggest that *cpxRA* expression is under tight control *in vivo* (13). In support of this, it was previously shown that both inhibition and constitutive activation of CpxRA in EPEC negatively affect virulence in the wax worm larva model (30). Consistent with these data, attempts to complement the *C. rodentium* CpxRA TCS by using low-copy-number plasmids were successful *in vitro* but resulted in only inconsistent complementation *in vivo*, further suggesting that fine-tuning of *cpxRA* expression is important for infection (data not shown). In addition, the consistent decreases in *cpxA*, *cpxR*, and *cpxP* expression levels over the course of infection in susceptible mice suggest that the temporal expression of this TCS is critical for *C. rodentium* virulence (Fig. 7).

The CpxRA regulon affects the expression of hundreds of genes, which results in the modulation of a myriad of functions (13). In various bacterial species, CpxRA has been implicated in the modulation of protein folding and degradation, TTS, adhesion and biofilm formation, motility, and chemotaxis. Since *C. rodentium* is nonmotile (45), the loss of virulence of the Δ*cpxRA* strain is not due to a disruption of motility. Additionally, we showed that the virulence defect of the *C. rodentium* Δ*cpxRA* strain is not due to a major defect in TTS or an altered growth rate (Fig. 5). The Δ*cpxRA* strain colonized the intestines of both C57BL/6J and C3H/HeJ mice to a significantly lesser extent than did the wild-type strain (Fig. 3A and C and 6B). In agreement with this observation, the murine commensal *E. coli* MP1 strain lacking *cpxR* was shown to have a marked colonization defect (46). Levels of all indicators of inflammation, pathology, and hyperplasia were significantly lower in mice infected with the Δ*cpxRA* strain than in mice infected with the wild-type and complemented strains (Fig. 3B and 4). We hypothesize that these altered levels of infection severity are directly related to the lower levels of colonization by the Δ*cpxRA* strain.

Collectively, our data suggest that *C. rodentium* CpxRA is required to respond to envelope stress encountered in the intestine. Possible sources of envelope stress during intestinal infection are host-derived antimicrobial peptides and proteins, complement, or pH changes. Furthermore, microbe-derived factors such as bacteriocins or other microbiota-associated antagonistic factors might also contribute to stress (47). Future experiments examining the virulence of *C. rodentium* Δ*cpxRA* strains in mutant mice lacking some of these specific host components or germfree mice that lack microbiota might lead to further insights into the specific stimuli leading to the activation of CpxRA during intestinal infection. Moreover, CpxRA may play a role in *C. rodentium* by modulating the expression of pili required for intestinal colonization. More work is required to identify the *C. rodentium* genes downstream of CpxRA that affect colonization and virulence *in vivo*.

In conclusion, this is the first study to identify a TCS that is essential for *C. rodentium* virulence. This TCS, CpxRA, appears to be involved in the early stages of intestinal colonization, as evidenced by temporal *cpxRA* expression during *in vivo* intestinal infection. In addition, the deletion of *cpxRA* nullified the pathogenic potential of *C. rodentium* in its natural host. By using single-copy chromosomal complementation, we restored the virulence

of the Δ*cpxRA* strain. Future studies should focus on confirming the importance of fine-tuned *cpxRA* expression during infection by EPEC and EHEC, as this TCS presents a promising potential target for therapeutics.

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