Role of the Two-Component Regulator CpxAR in the Virulence of *Salmonella enterica* Serotype Typhimurium

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The CpxAR (Cpx) two-component regulator controls the expression of genes in response to a variety of environmental cues. The Cpx regulator has been implicated in the virulence of several gram-negative pathogens, although a role for Cpx in vivo has not been demonstrated directly. Here we investigate whether positive or negative control of gene expression by Cpx is important for the pathogenesis of *Salmonella enterica* serotype Typhimurium. The Cpx signal pathway in serotype Typhimurium was disrupted by insertional inactivation of the *cpxA* and *cpxR* genes. We also constitutively activated the Cpx pathway by making an internal in-frame deletion in *cpxA* (a *cpxA mutation). Activation of the Cpx pathway inhibited induction of the envelope stress response pathway controlled by the alternative sigma factor σ^E (encoded by *rpoE*). Conversely, the Cpx pathway was highly up-regulated (>40-fold) in a serotype Typhimurium *rpoE* mutant. The *cpxA** mutation, but not the *cpxA* or the *cpxR* mutation, significantly reduced the capacity of serotype Typhimurium to adhere to and invade eucaryotic cells, although intracellular replication was not affected. The *cpxA* and *cpxA** mutations significantly impaired the ability of serotype Typhimurium to grow in vivo in mice. To our knowledge, this is the first demonstration that the Cpx system is important for a bacterial pathogen in vivo.**

Salmonellae are capable of surviving in a number of environmental niches. A variety of signal transduction systems allow salmonellae to perceive alterations in the external environment or damage to their cellular components and to make appropriate changes in their physiology in order to prolong survival. The response to alterations in the cell envelope is governed by at least three extracytoplasmic stress response (ESR) pathways in *Salmonella* spp. One pathway is controlled by the alternative sigma factor σ^E (RpoE) (12, 22), another by the two-component regulator CpxAR (32, 36), and a third, recently described, by the two-component regulator BaeSR (34).

The alternative sigma factor σ^E is activated in response to the accumulation of misfolded polypeptides, in particular outer membrane proteins, in the periplasm (23). Until recently only a few σ^E -regulated genes were known, but a number of new *Escherichia coli* σ^E -regulated genes have now been described (6, 37). The products of these genes include enzymes involved in the correct folding or degradation of proteins in the periplasm or in the biosynthesis of envelope components. σ^E is critically important for the virulence of *Salmonella enterica* serovar Typhimurium (13, 41) and is also involved in the pathogenesis of other bacteria (2, 19, 44).

The CpxAR two-component regulator system (referred to

below as Cpx) consists of a histidine kinase sensor (HKS), CpxA, and a cognate response regulator (RR), CpxR (5, 36). A third component, CpxP, binds CpxA and inhibits its activity (4). The Cpx pathway is activated in *E. coli* by alterations in the cell envelope composition, particularly changes in the levels of the lipoprotein NlpE (40), changes in pH (4, 28), and overexpression of misfolded envelope proteins (5, 16). The first genes that were shown to be Cpx regulated (*htrA*, *dsbA*, and *ppiA*) are involved in protein folding or degradation in the periplasm (3, 5). More-recent work on the CpxAR regulon has identified a greater range of targets for phosphorylated CpxR in *E. coli* (7, 10), indicating a role for the Cpx regulon beyond the periplasm.

The Cpx regulon has been implicated in the virulence of a number of bacterial pathogens. CpxA has been shown to be involved in the pH-dependent regulation of *Shigella flexneri* VirF, a positive regulator of the invasion genes *ipaBCD* (29). More recently, in serovar Typhimurium, Cpx has been shown to be involved in the pH-dependent regulation of HliA, a positive regulator of invasion genes (27). Also, DsbA is required for the folding and secretion of the IpaBCD proteins by *S. flexneri* and of other virulence factors such as the enterotoxin produced by *Vibrio cholerae* (45, 46). A *TnphoA* insertion in *cpxA* reduces the ability of *Salmonella enterica* serovar Typhi to adhere to and invade epithelial cells in vitro (20).

All the above studies used in vitro assays, and there is no direct in vivo evidence that the Cpx regulon is important for the virulence of salmonellae or other pathogens. We have constructed strains of serovar Typhimurium with separate mutations in *cpxA* and *cpxR* that either inactivate or constitutively

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>Salmonella</i> serovar Typhimurium		
SL1344	<i>his</i> mutant mouse virulent strain	12a
GVB368	SL1344 <i>cpxR</i> ::Km ^r	This study
GVB822	SL1344 <i>cpxA</i> ::Gm ^r	This study
GVB870	SL1344 <i>cpxA</i> *	This study
GVB311	SL1344 <i>rpoE</i> ::Km ^r	13
SMS438	SL1344 <i>rpoS</i> ::Ap ^r	30
<i>E. coli</i> SM10 λ pir	<i>thi-1 thr1 leuB6 tonA21 lacY1 supE44</i> <i>recA</i> ::RP4-2-Tc::Mu Km ^r λ pir	17
Plasmids		
pCRII	PCR cloning vector	Invitrogen
pWSK29	Ap ^r ; low-copy-number vector	43
pRDH10	λ pir-based suicide vector; Tet ^r Cm ^r SacB	R. Haigh (Leicester University, Leicester, United Kingdom)
pUC4K	Source of Km ^r gene (<i>aphI</i>) cassette	42
pBBR1MCS-5	Source of Gm ^r gene (<i>accC1</i>)	18
pSH105	<i>cpxR</i> in pCRII	This study
pSH115	pRDH10 <i>cpxR</i> ::Km ^r	This study
pSH130	pRDH10 <i>cpxA</i> ::Gm ^r	This study
pSH137	pRDH10 Δ <i>cpxA</i> (in-frame deletion)	This study
pcpxRA	<i>cpxRA</i> in pWSK29	This study
pTL61T	<i>lacZ</i> reporter plasmid	21
pcpxR::lacZ	<i>cpxRA</i> promoter in pTL61T	This study
prpoEP3::lacZ	<i>rpoEP3</i> promoter in pTL61T	26

activate the Cpx pathway, and we have investigated the effects of these mutations on the ability of serovar Typhimurium to interact with eucaryotic cells in vitro and to infect mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used or constructed in this study are listed in Table 1. All strains were maintained on Luria-Bertani (LB) or M9 minimal medium, prepared as described elsewhere (38, 39). Where required, the medium was supplemented with 100 μ g of ampicillin/ml, 50 μ g of kanamycin/ml, 100 μ g of streptomycin/ml, 50 μ g of chloramphenicol/ml, 50 μ g of gentamicin/ml, and 50 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal)/ml.

Recombinant DNA methods. Standard methods were used for preparation of plasmid or chromosomal DNA, restriction analysis, and ligation. DNA hybridization was carried out by using the Gene Images labeling and detection kit (Amersham Life Sciences). DNA for sequencing was isolated by using QIAGEN plasmid preparation columns. Sequencing reactions were carried out by using the Thermo Sequenase kit (Amersham) and were run on a LI-COR 4000L automated DNA sequencer. All PCRs were carried out using *Taq* polymerase (Red-dymix; ABgene). The PCR template was generated from a single bacterial colony. Standard methods were used for transformation and conjugation of plasmid DNA from *E. coli* to serovar Typhimurium.

Cloning and mutation of the *cpxR* and *cpxA* genes. The serovar Typhimurium *cpxR* gene was amplified by using primers cpxR1 (5'-GAGCTGACTTCCCTAT ATTAAG-3') and cpxR2 (5'-GTCGAAAGGCGTCAGGCGTT-3'). The serovar Typhimurium *cpxA* gene was amplified by using primers cpxAfw (5'-CCGG AACGCAAGACGGTCA-3') and cpxArv (5'-TCGACGGCGAGATAAAAA AT-3'). PCR products were cloned directly into pCR2.1 (Invitrogen) according to the manufacturer's instructions. The clones were checked by DNA sequencing.

The *cpxR* gene was subcloned from pCR2.1 into pBluescript by digestion with HindIII and XbaI to form pSH105. The *cpxR* gene was mutated by the introduction of a BglII site by inverse PCR at positions 324 to 329 to produce pSH110. The kanamycin resistance gene of pUC4K (*aphI* encoding kanamycin kinase; Km^r) was isolated by digestion with BamHI and ligated with BglII-digested pSH110. The disrupted *cpxR* gene (*cpxR*::Km^r) was subcloned into the suicide vector pRDH10 to generate pSH115. pRDH10 requires the *pir* product for

replication and possesses *sacB* from *Bacillus subtilis*, allowing for positive selection for allelic exchange (13). The use of this suicide vector for introducing mutations into serovar Typhimurium has been described previously (13). The wild-type (WT) SL1344 *cpxR* gene was replaced with *cpxR*::Km^r by allelic exchange following introduction of pSH115 by conjugation.

The *cpxA* gene was inactivated by inserting a gentamicin resistance (Gm^r) gene into a StuI site that had been engineered at bp 1483 of *cpxA* by inverse PCR. The Gm^r gene (*accI*) was amplified by PCR from plasmid pBBR1MCS-5 and cloned into pCR2.1. This construct was then digested with EcoRV and EcoI, generating *accI* with blunt ends, which was cloned into the StuI site of the engineered *cpxA* gene. The *cpxA*::Gm^r construct was then subcloned into pRDH10 and used to produce an SL1344 *cpxA* mutant by allelic exchange.

An in-frame deletion mutation was created in the *cpxA* gene to generate a mutation that rendered the Cpx system constitutive (*cpxA** mutation). The mutation was engineered by overlapping PCR, which created a deletion removing amino acids 92 to 104 of CpxA. The deletion also created a new EcoRI restriction site that was used to screen for the mutation. The PCR product containing the Δ *cpxA* gene was then subcloned into pRDH10 and used to generate a serovar Typhimurium mutant as described above. The genotype of the mutants was analyzed and confirmed to be correct by PCR and Southern hybridization (data not shown).

The complete *cpxRA* operon, including the upstream promoter region, was amplified by PCR using primers cpxR3 (5'-GCTTGTAACAACCTCAGCTGC GTGG-3') and cpxR4 (5'-GCTTGGGCAACATCAGGACCAGCATT-3') and was cloned into the low-copy-number vector pWSK29 (*pcpxRA*). This plasmid was used to complement the *cpx* mutations.

Construction of the *pcpxR*::lacZ reporter plasmid and β -galactosidase assay. The promoter region of *cpxR* was amplified by using primers cpxP1Hind (5'-C CCAAGCTTCAAACATGCGTCAGGGGGTG-3') and cpxP1Xba (5'-GCTCT AGAGTTTACGTACCTCCGAGGCAC-3'), which possessed HindIII and XbaI restriction endonuclease sites, respectively. The PCR product was digested with the appropriate enzymes and then ligated with the *lacZ* reporter vector pTL61T (21), which had been similarly treated. The correct products were selected by overnight growth on plates containing ampicillin and X-Gal. A single blue colony was selected and sequenced to confirm that the promoter had been cloned correctly. The plasmid was then transformed by electroporation into WT and mutant serovar Typhimurium strains. The same vector was used previously

to generate a reporter for monitoring expression from the σ^E -dependent promoter (*spoEP3*) of the *spoE* operon (26).

Overnight cultures of strains containing the appropriate plasmid(s) were diluted 1:1,000 into fresh LB broth with appropriate antibiotics, and the cultures were incubated at 37°C with aeration. Samples were taken every hour and assayed for β -galactosidase activity (24), adapted to a 96-well plate format. A mean activity was calculated from seven samples.

Amikacin sensitivity test. Overnight bacterial cultures were diluted in fresh LB to an optical density of 0.25 at 600 nm and were grown for 2 h at 30 or 37°C. After growth, 100- μ l aliquots were used to inoculate 3 ml of top agar, which was used to form a lawn on LB agar plates. Disks containing 30 U of amikacin (Oxoid) were placed on the lawns, and the plates were incubated aerobically overnight at 37°C. The diameter of the zone of inhibition was determined.

Invasion and persistence of serovar Typhimurium in phagocytic and nonphagocytic cells. The abilities of the different serovar Typhimurium strains to invade and survive in phagocytic and nonphagocytic cells were assessed by using the macrophage-like cell line RAW 264.7 and the epithelial cell lines HEP2 and Caco-2. Cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL) supplemented with 4 mM L-glutamine and 10% (vol/vol) fetal bovine serum. For invasion assays, cells were seeded into 24-well tissue culture plates (Costar) at 10^5 per well and were incubated at 37°C with 5% CO₂ overnight or until confluent (5 to 6 days for Caco-2). Where appropriate, cells were incubated with cytochalasin D (CD) (0.5 μ g/ml) for 1 h prior to infection, and CD was maintained in the medium throughout the assay. Prior to infection, the monolayers were washed twice with phosphate-buffered saline (PBS). Bacteria from overnight cultures in LB medium were diluted in 1 ml of DMEM to give a multiplicity of infection of approximately 1:1. The inocula were added to monolayers and then incubated for 1 to 2 h as above. The monolayers were washed twice with sterile PBS, overlaid with 1 ml of DMEM containing 100 μ g of polymyxin B/ml, and incubated for 1 h as above. Then monolayers were washed twice with PBS, and the cells were either lysed (with sterile water plus 1% Triton X-100) or overlaid with 1 ml of DMEM containing 10 μ g of polymyxin B/ml and incubated for a further 3 or 21 h before being washed and lysed as above. Following lysis of the cells, the number of viable bacteria released from the cells was determined by plating serial dilutions on Luria agar. To investigate adhesion to cells in the absence of invasion, cells were incubated with CD (0.5 μ g/ml) for 1 h prior to infection, and CD was maintained in the medium throughout the assay to prevent bacterial internalization. The assay was then performed as described above except that the monolayer was washed four times instead of two and cells were directly lysed and not incubated in the presence of polymyxin B. Statistical differences in the number of bacteria recovered were analyzed by analysis of variance.

Determination of in vivo CI. Salmonellae grown statically overnight in LB medium were recovered by centrifugation and resuspended in sterile PBS (pH 7.2). Groups of three or four female BALB/c mice (6 to 8 weeks old; Charles River, Margate, United Kingdom) were challenged with an inoculum containing equal numbers ($\sim 10^3$ CFU) of two strains of bacteria by either the intraperitoneal or the intravenous route. The actual number of bacteria present was determined by viable counts. Three or four days later, mice were killed, and the numbers of each strain present in the liver and spleen were determined. The competitive index (CI) is calculated as (output CFU of strain A/output CFU of strain B)/(input CFU of strain A/input CFU of strain B). Each assay was performed at least twice. A CI of ~ 1.0 indicates that the strains compete equally well. To determine whether the difference in CFU recovered versus CFU inoculated for the pair of strains was statistically significant, the ratio of CFU recovered to CFU administered for each strain was calculated for individual mice [i.e., for mouse 1, (output CFU of strain A)/(input CFU of strain A) and (output CFU of strain B)/(input CFU of strain B) were calculated], and results were compared by the Mann-Whitney U test.

RESULTS

Construction of serovar Typhimurium *cpx* mutants. A number of in vitro studies have indicated that the Cpx regulon may be involved in the virulence of several bacterial pathogens, including *Salmonella* spp. We and others have shown that the arm of the ESR controlled by σ^E is critically important for the growth and survival of serovar Typhimurium in mice (13). We therefore wished to see if the Cpx regulon is also involved in serovar Typhimurium pathogenesis. We constructed several

FIG. 1. The *cpxA** mutation renders serovar Typhimurium more resistant to the antibiotic amikacin. Serovar Typhimurium strains were tested for sensitivity to amikacin by a disk diffusion assay. Bars, mean diameters of the zone of inhibition (in millimeters); error bars, standard deviations for six replicates.

serovar Typhimurium strains with mutations in *cpxA* or *cpxR* that inactivate or constitutively activate the Cpx regulon (see Materials and Methods). The most profound *cpx* phenotypes described in *E. coli* are attributed to constitutive activation of the Cpx regulon due to particular mutations in *cpxA* designated *cpxA** (9). These mutations were generally found to map to the region of *cpxA* that codes for the periplasmic domain of CpxA and were thought to make the protein signal blind (36). We created an in-frame deletion in *cpxA* which removed amino acids 92 to 104 from CpxA to create a serovar Typhimurium *cpxA** mutant as described above.

To confirm that the mutants had the expected phenotypes, we examined the activity of the *cpxRA* promoter (see below) in the different mutants as well as the mutants' sensitivity to the antibiotic amikacin. *E. coli cpxA** strains are much less sensitive to amikacin than WT strains (9); we therefore checked whether our putative serovar Typhimurium *cpxA** strain had altered amikacin sensitivity. Like *E. coli cpxA** strains, the serovar Typhimurium *cpxA** strain was found to be significantly ($P < 0.001$) more resistant to amikacin than its WT parent (Fig. 1). This phenotype was complemented by a plasmid (*pcpxRA*) carrying a WT copy of the *cpxRA* genes. Both the *cpxR* and the *cpxA* mutant were more sensitive to amikacin than the WT strain, but only the *cpxR* mutant was significantly so ($P < 0.05$).

Effect of *cpx* mutations on *cpxRA* expression in serovar Typhimurium. The activity of the serovar Typhimurium *cpxRA* promoter in the WT and mutant strains was analyzed by using a *lacZ* reporter plasmid, *pcpxR::lacZ* (Fig. 2). The activity of *pcpxR::lacZ* in the *cpxA** mutant was much higher than those of the WT (~ 5 -fold), *cpxA*, and *cpxR* (~ 20 -fold for both mutants) strains, indicating that the Cpx pathway is constitutively active in this strain. In contrast, the β -galactosidase activities of the *cpxA*- and *cpxR*-null mutants were ~ 2 - to ~ 3.5 -fold lower than that of the WT strain. In *E. coli* the *cpxRA* promoter is reported to be dependent on the alternative sigma factor RpoS for activity (8). There was no difference between the β -galactosidase activity of a serovar Typhimurium *rpoS* mutant and that of the WT strain after 5 h of growth (Fig. 2) or at other

FIG. 2. Effect of mutations in stress response regulators on expression of the *cpx* genes. β -Galactosidase activities were determined for serovar Typhimurium strains harboring the *pcpxR::lacZ* reporter plasmid. Strains were grown for 5 h (late-log phase) in LB broth at 37°C with aeration. Bars represent means for seven replicates; error bars, standard deviations.

stages in the growth curve (data not shown). Therefore, it would appear that in serovar Typhimurium, *cpxRA* expression is not dependent on RpoS.

The *cpxRA* promoter is highly active in a serovar Typhimurium *cpxA** mutant; however, it is even more active (~43-fold more than in the WT strain) in a serovar Typhimurium *rpoE* mutant (Fig. 2). This indicates that the absence of *rpoE* causes a massive activation of the Cpx pathway. Overexpression of σ^E does not reduce expression of *cpxR* or *cpxA* (data not shown), so it seems unlikely that a member of the σ^E regulon is a negative regulator of *cpxRA*. The Cpx pathway is probably highly activated in the *rpoE* mutant as a response to the envelope stress experienced by this strain.

In *E. coli* the Cpx pathway negatively regulates expression of *rpoE* directly (10). Recently it also was shown that the *rpoE* transcript was present in a higher concentration in a serovar Typhimurium *cpxR* mutant (26). The serovar Typhimurium *rpoErseABC* operon possesses three promoters, the first two of which are σ^E independent, while the third, *rpoEP3*, is σ^E dependent (26).

In order to examine the effect of the Cpx pathway on induction of the σ^E regulon in serovar Typhimurium, we examined the activity of *rpoEP3* in the different mutants by using a *lacZ* reporter construct (*prpoEP3::lacZ*). Compared to that of the WT strain, the expression of *prpoEP3::lacZ* is reduced approximately two- to threefold in the *rpoE* and *cpxA** strains, respectively (Fig. 3). Conversely, the activity of *prpoEP3::lacZ* is approximately doubled in the *cpx*-null mutants relative to that in the WT strain. Thus, in serovar Typhimurium, as in *E. coli*, activation of the Cpx pathway has a negative impact on induction of the σ^E regulon. The β -galactosidase activity of the *rpoS* mutant is similar to that of the WT strain, indicating that *rpoS*

FIG. 3. CpxAR suppresses activation of the σ^E regulon. β -Galactosidase activities were determined for serovar Typhimurium strains harboring the *prpoEP3::lacZ* reporter plasmid. Strains were grown for 5 h (late log phase) in LB broth at 37°C with aeration. Bars represent means for seven replicates; error bars, standard deviations.

does not influence expression of the σ^E -dependent *rpoEP3* promoter.

Effect of the *cpx* mutations on the ability of serovar Typhimurium to enter and replicate within eucaryotic cells. A serovar Typhimurium *rpoE* mutant invades macrophages normally but survives poorly intracellularly (13). We analyzed the abilities of the *cpx* mutants to invade and persist in macrophages and epithelial cells. Both *cpx*-null mutants invaded the macrophage-like cell line RAW 264.7 as well as the WT strain did; in fact the *cpxA* strain consistently exhibited a slight but significant ($P < 0.05$) increase in invasiveness over that of its WT parent (Fig. 4A). The serovar Typhimurium *cpxA** strain was significantly less invasive than the WT, *cpxA*, and *cpxR* strains ($P < 0.001$). The reduced invasiveness of the *cpxA** mutant could be complemented with plasmid *pcpxRA* (Fig. 4B). At 24 h postinfection, the intracellular numbers of all bacteria had increased, indicating that none of the *cpx* mutations affect the intracellular survival or replication of serovar Typhimurium. Similar results were obtained when the assay was performed with HEP2 or Caco-2 epithelial cells (data not shown). In order to ascertain whether the *cpxA** strain was defective in initial adhesion or in entry into cells, the invasion assay was repeated in the presence of CD to prevent bacterial internalization. The presence of CD did not affect the results of the invasion assay; significantly fewer CFU of the *cpxA** strain than of the WT strain were recovered (data not shown). Similarly, centrifugation of the *cpxA** mutant onto cells did not overcome the internalization defect of the strain. Collectively these results

FIG. 4. Effects of *cpx* mutations on serovar Typhimurium invasion of and survival within macrophages. Bacteria at a multiplicity of infection of $\sim 1:1$ were incubated with the murine macrophage cell line RAW 264.7. The assay was performed as described in the text. Graphs show the number of viable bacteria (as a percentage of the initial inoculum) inside the macrophages at 3 h (open bars) and 24 h (solid bars) after infection. Each bar represents the mean from triplicate experiments. Error bars, standard deviations. (A) Effects of *cpx* mutations; (B) complementation of the *cpxA** mutation.

suggest that adhesion to eucaryotic cells rather than cell entry is affected in the serovar Typhimurium *cpxA** mutant.

Effects of *cpx* mutations on *Salmonella* virulence. The capacities of the *cpxR*- and *cpxA*-null mutants to compete for growth in murine tissues with the WT strain were compared by a competition assay. The CI is determined by dividing the ratio of the two strains isolated from murine tissues by the ratio of the two strains in the inoculum. The CI for the *cpxR* mutant versus the WT was 0.53; the difference in the recovery of the two strains was not significant. The *cpxA*-null strain was significantly ($P < 0.05$) less able than the WT strain to compete in vivo (CI, 0.198). Because the *cpxA** strain does not possess a selectable marker, it is not possible to directly distinguish it from the WT strain in a competition assay. Instead, the *cpxA** strain was made to compete with the *cpxR* strain, whose virulence is very similar to that of the WT strain. The CI was 0.0358 ($P < 0.05$).

We investigated whether the *cpx* mutations affected the ability of serovar Typhimurium to infect mice by the natural route of infection by infecting mice orally and determining the number of CFU in different tissues postinfection. At a dose of ~ 5

FIG. 5. Effects of the *cpxA* mutations on the ability of serovar Typhimurium to colonize murine organs following oral infection. Mice were infected with either the *cpxA* ($\sim 2 \times 10^8$ CFU) or the *cpxA** ($\sim 3 \times 10^8$ CFU) strain. Numbers of bacteria present in different organs of the mice were determined on days 4 and 7 postinfection. Bars represent means for four mice; error bars, standard deviations. MLN, mesenteric lymph node. Solid bars, *cpxA* strain on day 4; open bars, *cpxA* strain on day 7; shaded bars, *cpxA** strain on day 4; hatched bars, *cpxA** strain on day 7.

$\times 10^6$ CFU, the *cpxR* mutant infected the Peyer's patches (PP), mesenteric lymph nodes, liver, and spleen as well as the WT strain did (data not shown). In contrast, most mice challenged with either the *cpxA* or the *cpxA** strain did not become infected, and for those that did, very few organisms could be recovered.

At a higher dose ($\sim 2 \times 10^8$ CFU to 3×10^8 CFU), both the *cpxA* and *cpxA** strains colonized the PP and translocated to deeper tissues. Mice were culled on days 4 and 7, the organs were removed, and CFU were determined (Fig. 5). The *cpxA** strain was found at lower levels than the *cpxA* strain in all of the organs at day 4; although this difference was not statistically significant, it may possibly indicate a role for *cpxA** in the early stages of serovar Typhimurium infection. By day 7 the difference between the two mutants was less apparent. Also, 2 days after oral challenge, much higher numbers of the *cpxA* strain than of the *cpxA** strain were shed in the feces of mice (data not shown).

DISCUSSION

Our results indicate that the Cpx signal transduction system plays a role in the pathogenesis of salmonellae. Null mutations in the genes that encode either the sensor (*cpxA*) or the acti-

vator (*cpxR*) do not adversely affect the attachment and internalization of serovar Typhimurium into eucaryotic cells under the conditions tested in our assay. However, constitutive activation of the Cpx pathway significantly reduced the ability of serovar Typhimurium to enter host cells. This appeared to be due to a reduced ability of the serovar Typhimurium *cpxA** mutant to adhere to eucaryotic cells rather than to a defect in making contact with eucaryotic cells or in internalization. This is because the same difference in cell association between the *cpxA** mutant and the WT strain was evident if internalization was inhibited or if contact with eucaryotic cells was increased by centrifugation.

In *E. coli*, all of the phenotypes of *cpxA** mutants can be accounted for by elevated levels of phosphorylated CpxR (CpxR-P) (9). Mutations in the periplasmic loop region of CpxA, such as that in our serovar Typhimurium *cpxA** strain, are signal blind and are not subject to negative regulation by the cytoplasmic protein CpxP, which down-regulates the Cpx pathway in the absence of stress (35). The molecular mechanism of the effect of CpxP on CpxA activity is not known but is thought to affect the balance of kinase and phosphatase activities (9, 35). The defect in the serovar Typhimurium *cpxA** mutant was complemented by a WT copy of the *cpxA* gene (*cpxRA*) provided in *trans* on a plasmid. The WT CpxA protein is responsive to CpxP, which presumably represses its kinase activity and enhances phosphatase activity. Presumably this is sufficient to dephosphorylate the CpxR-P present in the cell and accounts for the complementation observed. However, it is possible that the WT CpxA gene affects the activity of the mutant CpxA* protein directly or indirectly to reduce its ability to phosphorylate CpxR. It would appear that the WT *cpxA* allele is dominant over the *cpxA** allele. However, more studies would be needed to determine if this is the case, since the WT gene is present in multiple copies and there is only a single copy of the *cpxA** gene.

A serovar Typhi strain with a *TnphoA* insertion in *cpxA* exhibited reduced adhesion to and invasion of Int407 cells (20). The *TnphoA* insertion in *cpxA* mapped to nucleotide 267, which is within the region we deleted to construct the serovar Typhimurium *cpxA** mutant. Therefore, it is possible that the serovar Typhi mutant is behaving as a *cpxA** strain. Recently, it was reported that *cpxA* (but not *cpxR*) was required by serovar Typhimurium for expression of *hilA* at pH 6.0 (27). Mutation of *cpxA* (or *cpxR*) did not affect *hilA* expression at pH 8.0. *HilA* is a positive regulator of the serovar Typhimurium invasion genes (1). When cultured in a medium at pH 6.0 (but not pH 8.0), the serovar Typhimurium *cpxA* mutant exhibited reduced internalization of eucaryotic cells. Interestingly, at pH 8.0, both the *cpxA* and *cpxR* mutants were internalized slightly more efficiently than the WT strain (27). This is in agreement with our results, where we observed a small but consistent increase in the invasiveness of the *cpxA* and *cpxR* mutants over that of the WT strain. In our experiments, invasion assays were performed with bacteria cultured overnight in LB medium; the pH of these cultures was ~8.0.

We postulate that the *cpxA** mutation affects the adhesiveness of serovar Typhimurium. There are a number of studies demonstrating the involvement of the Cpx system in various adhesive processes in *E. coli*. A functional CpxAR pathway has been shown to be important for surface sensing and adhesion

to inanimate surfaces by *E. coli* (31). It was postulated by the authors that the role of the CpxAR pathway was to regulate genes required to maintain stable contact with the inanimate surface. CpxAR also directly and indirectly affects production of the P pili in *E. coli*. In the absence of glucose in the culture medium, CpxR-P binds upstream of the *pap* genes to activate their expression (15). CpxAR also regulates the expression of proteins such as DsbA and PpiA, which assist in pilin assembly in the periplasm. The P pili produced by a *cpxR* mutant are shorter than those of WT *E. coli* (15). Conversely, the Cpx system negatively regulates expression of curli in *E. coli* (33). Thus, in *E. coli*, the Cpx system can positively or negatively regulate the expression of fimbriae.

Salmonellae, including serovar Typhimurium, have the potential to express a diverse number of fimbriae, most of which have only been shown to be expressed in vivo (14). During infection the appropriate expression of fimbriae is important for the interaction of bacteria with host tissues and other bacteria. If, as in *E. coli*, CpxAR regulates the expression of several fimbriae in serovar Typhimurium, this could account for the reduced adhesion to eucaryotic cells in vitro and the reduced colonization of murine tissues in vivo exhibited by the serovar Typhimurium *cpxA** mutant. CpxAR could be involved in negatively regulating the expression of curli in serovar Typhimurium, as is the case with *E. coli*. Preliminary studies have indicated that this may be the case (unpublished observation).

Signals that activate the Cpx pathway have been studied extensively in *E. coli* and include overexpression of the lipoprotein NlpE (40). Adhesion of *E. coli* to inanimate surfaces induces the Cpx pathway in an NlpE-dependent manner (31). Interestingly, in the serovar Typhimurium LT2 genome sequence, the *nlpE* gene is a pseudogene containing a frameshift mutation 450 bp from the start codon, although the *nlpE* gene is intact in the sequenced serovar Typhi strain CT18. We have cloned and sequenced the *nlpE* gene from SL1344 and found 100% identity with serovar Typhi *nlpE* (data not shown). Therefore, NlpE should be functional in the strains used in this study. Also, we have found that overexpression of the NlpE protein activates the Cpx pathway in serovar Typhimurium, as it does in *E. coli* (data not shown).

Among serovar Typhimurium *cpx* mutants, *cpxR* was more virulent than *cpxA*, which in turn was more virulent than *cpxA**. The *cpxA** mutant would be expected to have excess CpxR-P; therefore, virulence would appear to correlate inversely with CpxR-P levels. If this is the case, then CpxR-P may either negatively regulate genes that are important for the infection of mice or, alternatively, positively regulate genes that interfere with the infection process. Appropriate activation of another two-component regulator has been shown to be critically important for serovar Typhimurium virulence. The PhoPQ two-component regulator controls the expression of genes that are needed for different stages in the pathogenesis of serovar Typhimurium infection. PhoP-activated genes (*pag*) are involved in survival within macrophages, and PhoP-repressed genes (*prg*) are involved in the invasion of epithelial cells. Consequently, serovar Typhimurium strains with null mutations in the RR gene *phoP* (which prevent expression of *pag* and allow constitutive expression of *prg*) and strains with deletions in the periplasmic domain of the sensor kinase PhoQ, which constitutively activate the PhoPQ pathway (constitutive

activation of *pag* and complete repression of *prg*), are both attenuated (25). Therefore, the context of PhoPQ activation is very important for *Salmonella* infection. The same may also apply to CpxAR.

Because of the organization of the *cpxRA* operon, insertions into *cpxA* should not have any effect on *cpxR* expression, whereas mutations in the *cpxR* gene may also affect the expression of *cpxA*. Therefore, CpxR is likely to be expressed in the *cpxA*-null mutant. Although CpxR could not be phosphorylated by CpxA, other phosphate donors, such as acetyl phosphate, or an alternative HKS protein may activate CpxR. It is also possible that CpxA may interact with alternative RRs. Indeed, several studies have indicated that the CpxA and CpxR proteins can act independently; for example, mutation of *cpxA* but not *cpxR* affects the expression of *hilA* at pH 6.0 in serovar Typhimurium (27). Also, in *E. coli*, growth-dependent activation of CpxR is independent of CpxA (11).

The *rpoE* gene is negatively regulated by CpxR-P. The induction of the σ^E -dependent *rpoEP3* promoter in the serovar Typhimurium *cpxA** mutant is similar to that in the serovar Typhimurium *rpoE* mutant. The *rpoEP3* promoter is essential for expression of *rpoE* under conditions that activate the σ^E pathway; therefore, constitutive activation of the Cpx system may render σ^E levels suboptimal in vivo. Since σ^E is critical to serovar Typhimurium pathogenesis, the reduced virulence of the serovar Typhimurium *cpxA** strain may be partly due to *rpoE* repression. However, the *rpoE* and *cpxA** mutations affected the ability of serovar Typhimurium to adhere to, enter, and survive and replicate within eucaryotic cells in vitro in different ways. A serovar Typhimurium *rpoE* mutant could bind to and enter eucaryotic cells almost as well as the isogenic WT strain but could not survive intracellularly (13). In contrast, the serovar Typhimurium *cpxA** mutant appears to be able to grow and survive normally within eucaryotic cells but was defective in the initial interaction with eucaryotic cells. We are currently identifying genes which are positively or negatively regulated by the Cpx system in serovar Typhimurium and investigating their role in the infectious process.

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