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RETRACTED: The two-component system CpxR/A represses the expression of *Salmonella* virulence genes by affecting the stability of the transcriptional regulator HilD

Miguel A. De la Cruz[1†], *Deyanira Pérez-Morales*[2†], *Irene J. Palacios*[2], *Marcos Fernández-Mora*[2], *Edmundo Calva*[2] and *Víctor H. Bustamante*[2*]

[1] Unidad de Investigación Médica en Enfermedades Infecciosas y Parasitarias, Centro Médico Nacional Siglo XX1-IMSS, México DF, Mexico, [2] Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

Salmonella enterica can cause intestinal or systemic infections in humans and animals mainly by the presence of pathogenicity islands SPI-1 and SPI-2, containing 39 and 44 genes, respectively. The AraC-like regulator HilD positively controls the expression of the SPI-1 genes, as well as many other *Salmonella* virulence genes including those located in SPI-2. A previous report indicates that the two-component system CpxR/A regulates the SPI-1 genes: the absence of the sensor kinase CpxA, but not the absence of its cognate response regulator CpxR, reduces their expression. The presence and absence of cell envelope stress activates kinase and phosphatase activities of CpxA, respectively, which in turn controls the level of phosphorylated CpxR (CpxR-P). In this work, we further define the mechanism for the CpxR/A-mediated regulation of SPI-1 genes. The negative effect exerted by the absence of CpxA on the expression of SPI-1 genes was counteracted by the absence of CpxR or by the absence of the two enzymes, AckA and Pta, which render acetyl-phosphate that phosphorylates CpxR. Furthermore, overexpression of the lipoprotein NlpE, which activates CpxA kinase activity on CpxR, or overexpression of CpxR, repressed the expression of SPI-1 genes. Thus, our results provide several lines of evidence strongly supporting that the absence of CpxA leads to the phosphorylation of CpxR via the AckA/Pta enzymes, which represses both the SPI-1 and SPI-2 genes. Additionally, we show that in the absence of the Lon protease, which degrades HilD, the CpxR-P-mediated repression of the SPI-1 genes is mostly lost; moreover, we demonstrate that CpxR-P negatively affects the stability of HilD and thus decreases the expression of HilD-target genes, such as *hilD* itself and *hilA*, located in SPI-1. Our data further expand the insight on the different regulatory pathways for gene expression involving CpxR/A and on the complex regulatory network governing virulence in *Salmonella*.

Keywords: *Salmonella*, SPI, CpxR/A, HilD, Lon, regulation, RpoH, virulence

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***Correspondence:**
Víctor H. Bustamante, Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Cuernavaca, Mor 62210, Mexico
victor@ibt.unam.mx

[†] *These authors have contributed equally to this work.*

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Introduction

Salmonella enterica groups Gram-negative bacteria comprising around 2500 serotypes, which can infect a wide variety of hosts ranging from humans to birds (Haraga et al., 2008; Sánchez-Vargas et al., 2011). Acquisition of DNA fragments by horizontal transfer and the ensuing adaptation of regulatory mechanisms to control the expression of the newly acquired genes have been pivotal events in the *Salmonella* pathogenicity evolution (Schmidt and Hensel, 2004; Fàbrega and Vila, 2013). Around 30% of the *S. enterica* genome has been shaped by horizontal transfer events; most of the acquired genes are clustered in regions denominated islands (Mcclelland et al., 2001; Porwollik and Mcclelland, 2003). *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2), which are chromosomal regions composed of 39 and 44 genes, respectively, have crucial roles in the pathogenesis of *Salmonella* (Hansen-Wester and Hensel, 2001; Haraga et al., 2008; Fàbrega and Vila, 2013). SPI-1 is conserved in the two *Salmonella* species, *enterica* and *bongori*, whereas SPI-2 is only present in *S. enterica*, suggesting that SPI-1 was acquired earlier than SPI-2 during the evolution of *Salmonella* pathogenicity (Groisman and Ochman, 1997; Porwollik and Mcclelland, 2003). Both SPI-1 and SPI-2 encode a type 3 secretion system (T3SS), different effector proteins, chaperones, and transcriptional regulators that control the expression of the genes within each island (Hansen-Wester and Hensel, 2001; Haraga et al., 2008; Fàbrega and Vila, 2013). The T3SSs are highly complex needle-like nanomachines formed by more than 20 proteins, which span the inner and outer membrane of the bacteria and thus are able to inject effector proteins from the bacterial cytoplasm into the eukaryotic cytosol; once inside the host cell, effector proteins translocated by their cognate T3SS manipulate different signal transduction pathways and induce rearrangement of the host cell cytoskeleton (Moest and Méresse, 2013; Abrusci et al., 2014; Diepold and Wagner, 2014). The T3SS-1 and effector proteins encoded in SPI-1 are necessary for *Salmonella* invasion of intestinal epithelial cells, and thus for the intestinal colonization leading to enteritis; whereas the T3SS-2 and effector proteins encoded in SPI-2 are mainly required for *Salmonella* survival and replication inside macrophages, and hence for the systemic disease (Hansen-Wester and Hensel, 2001; Haraga et al., 2008; Fàbrega and Vila, 2013; Moest and Méresse, 2013). Different studies have shown that the SPI-2 genes also induce a *Salmonella* non-proliferative life style inside phagocytes and non-phagocytic cells (Grant et al., 2012; Núñez-Hernández et al., 2014) and that they contribute to the development of the intestinal inflammatory disease (Bispham et al., 2001; Coburn et al., 2005; Coombes et al., 2005). *S. enterica* serovar Typhimurium (*S.* Typhimurium) can cause self-limiting enteritis in humans, chickens and calves, while in mice it produces a systemic infection similar to the typhoid fever caused by *S.* Typhi in humans (Haraga et al., 2008; Sánchez-Vargas et al., 2011). Since *S.* Typhimurium can cause both intestinal and systemic infections in different hosts, it is widely used as a model to study the molecular virulence mechanisms of *Salmonella*.

The expression of the SPI-1 and SPI-2 genes is induced in different *in vivo* and *in vitro* growth conditions. *In vivo*, the SPI-1 genes are mainly expressed when *Salmonella* is in the intestinal lumen, associated with the epithelium or with extruding enterocytes (Laughlin et al., 2014), and also in a *Salmonella* subpopulation that replicates in the cytosol of epithelial cells (Knodler et al., 2010). In contrast, the SPI-2 genes are mainly expressed when *Salmonella* is inside epithelial cells or macrophages, within vacuoles (Cirillo et al., 1998; Deiwick et al., 1999; Eriksson et al., 2003; Knodler et al., 2010), and also when *Salmonella* is in the intestinal lumen (Brown et al., 2005), in the lamina propria or in the underlying mucosa (Laughlin et al., 2014). *In vitro*, the SPI-1 and SPI-2 genes are both expressed when *Salmonella* is grown in nutrient-rich media, such as the Luria-Bertani (LB) medium, albeit they are differentially regulated by growth phase (Lundberg et al., 1999; Miao and Miller, 2000; Bustamante et al., 2008; Kröger et al., 2013). Moreover, the expression of the SPI-2 genes is also induced when *Salmonella* is grown in acidic minimal media containing low concentrations of phosphate, calcium, and magnesium (Deiwick et al., 1999; Miao and Miller, 2000; Kröger et al., 2013).

SPI-1 encodes the transcriptional regulators HilD, HilA and InvF, which induce the expression of the genes within this island in a cascade fashion (Golubeva et al., 2012; Fàbrega and Vila, 2013). HilD, a member of the AraC family of transcriptional regulators, induces the expression of HilA (Schechter et al., 1999; Schechter and Lee, 2001; Ellermeier et al., 2005), an OmpR/ToxR-like transcriptional regulator, which in turn, activates the expression of InvF (Lostroh et al., 2000; Lostroh and Lee, 2001), another AraC-like regulator. HilA directly activates the expression of genes encoding T3SS-1 components, whereas InvF induces the expression of SPI-1 genes encoding effector proteins (Golubeva et al., 2012; Fàbrega and Vila, 2013). Furthermore, HilD regulates directly, or indirectly, through HilA and InvF, the expression of several other genes located outside SPI-1, including acquired and ancestral genes (Bustamante et al., 2008; Golubeva et al., 2012; Fàbrega and Vila, 2013; Petrone et al., 2014; Singer et al., 2014). Interestingly, when *S.* Typhimurium is grown to late stationary phase in LB medium, HilD directly induces the expression of the *ssrAB* operon that is located in SPI-2 and codes for the SsrA/B two-component system, the central positive regulator of the SPI-2 genes, thus establishing a transcriptional cross talk between SPI-1 and SPI-2 (Bustamante et al., 2008; Martínez et al., 2014).

Many *Salmonella*-specific and global regulators have been involved in the expression of the SPI-1 and SPI-2 genes, which mainly act on the expression of *hilD*, *hilA*, or *ssrAB* (Fass and Groisman, 2009; Martínez et al., 2011; Golubeva et al., 2012; Fàbrega and Vila, 2013). Notably, according to its role as a central regulator of the SPI-1 and several other virulence genes, the expression, concentration and activity of HilD is highly controlled. At the transcriptional level, the expression of *hilD* is positively autoregulated and modulated by a feed-forward regulatory loop involving HilD itself and the AraC-like regulators HilC and RtsA (Olekhnovich and Kadner, 2002; Ellermeier et al., 2005; Golubeva et al., 2012), while post-transcriptionally it is positively controlled by a regulatory cascade integrated by the SirA/BarA and Csr global regulatory systems (Martínez et al., 2011). On the other hand, HilD activity is positively regulated by FliZ and Fur, through still unknown mechanisms (Ellermeier and Slauch, 2008; Chubiz et al., 2010), as well as negatively regulated by HilE, through protein-protein interactions (Baxter et al., 2003). Moreover, the cellular concentration of HilD is controlled by the Lon protease (Takaya et al., 2005).

One of the regulators that have been involved in the expression of the SPI-1 genes is CpxA, the sensor histidine kinase of the Cpx-envelope stress two-component system (Nakayama et al., 2003). CpxA phosphorylates its cognate response regulator CpxR in response to a broad range of stimuli that cause perturbations in the cell envelope, such as pH, salt, metals, lipids and misfolded proteins; whereas in the absence of these activating signals CpxA has phosphatase activity on CpxR (Hunke et al., 2012; Vogt and Raivio, 2012; Raivio, 2014). CpxR can also be phosphorylated independently of CpxA by acetyl phosphate, which is generated *in vivo* from acetyl-CoA by the phosphotransacetylase (Pta) and acetate kinase (AckA) enzymes (Raivio and Silhavy, 1997; Wolfe et al., 2008). Phosphorylated CpxR (CpxR-P) positively or negatively regulates many genes encoding protein folding and degrading factors, peptidoglycan metabolic enzymes, inner membrane proteins, envelope-localized protein complexes, and other cellular regulators (Hunke et al., 2012; Vogt and Raivio, 2012; Raivio, 2014). Additionally, the Cpx system has been involved in the expression of virulence genes in different pathogenic bacteria, such as enteropathogenic and uropathogenic *Escherichia coli*, *Yersinia*, *Shigella*, *Legionella*, *Haemophilus* and *Salmonella* (Hunke et al., 2012; Vogt and Raivio, 2012; Raivio, 2014). In *S.* Typhimurium, deletion of *cpxA*, but not *cpxR*, decreases the expression of the SPI-1 genes and, as a consequence, reduces *Salmonella* invasion into host cells (Nakayama et al., 2003). Therefore, on the basis of these results, it was suggested that CpxA positively regulates the SPI-1 genes through regulator(s) other than CpxR (Nakayama et al., 2003).

In this work, we determined that the absence of CpxA renders activation of CpxR via the AckA-Pta pathway, which represses the expression of the SPI-1 genes. Consistently, it was found that CpxR-P generated by the activation of CpxA, or the overexpression of CpxR, also represses the expression of these genes. Our results indicate that CpxR negatively controls the expression of the SPI-1 genes, as well as genes located in SPI-2, by repressing the autoregulation of HilD, a central positive regulator for the expression of the genes within SPI-1 and SPI-2 and other virulence genes. Furthermore, we found that activation of CpxR decreases the stability of HilD and that, in the absence of the Lon protease, which degrades HilD, the CpxR-mediated repression of the SPI-1 genes is mostly lost. Thus, our data clarify and expand the regulatory role of the two-component system CpxR/A for the expression of *S.* Typhimurium virulence genes.

Materials and Methods

Bacterial Strains, Media, and Culture Conditions

Bacterial strains used in this study are listed in **Table 1**. Bacterial cultures were grown at 37°C in LB medium containing 1% tryptone, 0.5% yeast agar and 1% NaCl, pH 7.5. When necessary, media were supplemented with ampicillin (200 µg ml^{-1}), kanamycin (20 µg ml^{-1}) or chloramphenicol (30 µg ml^{-1}). Cultures for chloramphenicol acetyltransferase (CAT), Western blot and protein secretion assays were performed as we described previously (Bustamante et al., 2008; Martínez et al., 2011).

Construction of Plasmids

Plasmids and primers used in this study are listed in **Tables 1**, **2**, respectively. To construct the plasmids containing the transcriptional fusions *lon-cat*, *clpX-cat, clpP-cat* and *cpxRA-cat*, the regulatory regions of *lon*, *clpX*, *clpP,* and *cpxRA* were amplified by PCR with the primer pairs promlon-Fw1/promlon-Rv1, pClpX-Bam/pClpX-Hind, pClpXP-Bam/pClpXP-Hind and CpxR-Bam-5′/CpxR-Hind-3′, respectively. The PCR products were digested with BamHI and HindIII restriction enzymes and then cloned into the BamHI and HindIII sites of the vector pKK232-8, which carries a promoterless *cat* gene (Amersham Pharmacia LKB Biotechnology), generating plasmids plon-cat, pclpX-cat, pclpP-cat, and pcpxRA-cat. To construct the plasmids pK3-CpxR and pK3-RpoH, the *cpxR* and *rpoH* genes were amplified by PCR using primer pairs CpxR-Fw1/CpxR-Rv1 and RpoH-FwKpn/RpoH-RvBam, respectively. The PCR products were digested with HindIII and BamHI (*cpxR* gene) or KpnI and BamHI (*rpoH* gene) restriction enzymes and then cloned into the vector pMPM-K3 (Mayer, 1995) digested with the respective restriction enzymes. pK3-CpxR and pK3-RpoH constitutively express CpxR and RpoH, respectively, under a *lac* promoter, since *Salmonella* and the vector pMPM-K3 lack the gene encoding LacI, the repressor of *lac*.

Construction of Deletion Mutants and Strains Expressing FLAG-tagged Proteins

Non-polar gene-deletion mutant strains were generated by the λRed recombinase system, as reported previously (Datsenko and Wanner, 2000), using the respective primers described in **Table 2**. The genes *cpxR*, *cpxA*, *cpxRA*, *ackA-pta*, *hilE,* or *lon* were replaced with a selectable kanamycin resistance cassette in the *S.* Typhimurium strain 14028s, generating the Δ*cpxR::kan* (DTM48), Δ*cpxA::kan* (DTM50), Δ*cpxRA::kan* (DTM52), Δ*ackA-pta::kan* (DTM54), Δ*hilE::kan* (DTM56) and Δ*lon::kan* (DTM60) mutants, respectively. The kanamycin resistance cassette was excised from the Δ*cpxR::kan* (DTM48), Δ*cpxA::kan* (DTM50), Δ*cpxRA::kan* (DTM52), Δ*hilE::kan* (DTM56), Δ*cpxA* Δ*hilE::kan* (DTM58), Δ*lon::kan* (DTM60), Δ*cpxA* Δ*lon::kan* (DTM62) and Δ*hilD::kan* (DTM64) mutants, by using helper plasmid pCP20, expressing the FLP recombinase, as described previously (Datsenko and Wanner, 2000), generating the Δ*cpxR* (DTM49), Δ*cpxA* (DTM51), Δ*cpxRA* (DTM53), Δ*hilE* (DTM57), Δ*cpxA* Δ*hilE* (DTM59), Δ*lon* (DTM61), Δ*cpxA* Δ*lon* (DTM63) and Δ*hilD* (DTM65) mutants, respectively. P22 transduction was used to transfer the Δ*hilD::kan* allele from strain JPTM5 into strain 14028s, generating strain DTM64, to transfer the Δ*ackA-pta::kan*, Δ*hilE::kan* or Δ*lon::kan* alleles from strains DTM54, DTM56 and DTM60 into strain DTM51, generating the Δ*cpxA* Δ*ackA-pta::kan* (DTM55), Δ*cpxA* Δ*hilE::kan* (DTM58) and Δ*cpxA* Δ*lon::kan* (DTM62) mutants, respectively, to transfer the Δ*cpxA::kan* or Δ*lon::kan* alleles from

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	References or sources
***S.* TYPHIMURIUM STRAINS**		
14028s	Wild-type	ATCC
DTM48	Δ*cpxR::kan*	This study
DTM49	Δ*cpxR*	This study
DTM50	Δ*cpxA::kan*	This study
DTM51	Δ*cpxA*	This study
DTM52	Δ*cpxRA::kan*	This study
DTM53	Δ*cpxRA*	This study
DTM54	Δ*ackA-pta::kan*	This study
DTM55	Δ*cpxA* Δ*ackA-pta::kan*	This study
DTM56	Δ*hilE::kan*	This study
DTM57	Δ*hilE*	This study
DTM58	Δ*cpxA* Δ*hilE::kan*	This study
DTM59	Δ*cpxA* Δ*hilE*	This study
DTM60	Δ*lon::kan*	This study
DTM61	Δ*lon*	This study
DTM62	Δ*cpxA* Δ*lon::kan*	This study
DTM63	Δ*cpxA* Δ*lon*	This study
JPTM5	SL1344 Δ*hilD::kan*	Bustamante et al., 2008
DTM64	14028s Δ*hilD::kan*	This study
DTM65	Δ*hilD*	This study
DTM66	Δ*hilD* Δ*cpxA::kan*	This study
DTM67	Δ*hilD* Δ*lon::kan*	This study
JPTM7	SL344 *hilA::3XFLAG-kan*	Bustamante et al., 2008
DTM68	14028s *hilA::3XFLAG-kan*	This study
DTM69	Δ*cpxR hilA::3XFLAG-kan*	This study
DTM70	Δ*cpxA hilA::3XFLAG-kan*	This study
DTM71	Δ*cpxRA hilA::3XFLAG-kan*	This study
JPTM30	SL1344 *ssrB::3XFLAG-kan*	Martínez et al., 2011
DTM72	14028s *ssrB::3XFLAG-kan*	This study
DTM73	Δ*cpxR ssrB::3XFLAG-kan*	This study
DTM74	Δ*cpxA ssrB::3XFLAG-kan*	This study
DTM75	Δ*cpxRA ssrB::3XFLAG-kan*	This study
DTM76	14028s *invF::3XFLAG-kan*	This study
DTM77	Δ*cpxR invF::3XFLAG-kan*	This study
DTM78	Δ*cpxA invF::3XFLAG-kan*	This study
DTM79	Δ*cpxRA invF::3XFLAG-kan*	This study
DTM80	Δ*hilE invF::3XFLAG-kan*	This study
DTM81	Δ*cpxA* Δ*hilE invF::3XFLAG-kan*	This study
DTM82	Δ*lon invF::3XFLAG-kan*	This study
DTM83	Δ*cpxA* Δ*lon invF::3XFLAG-kan*	This study
MF100	14028s Δ*Cthns::kan* (lacking codons 99–136 of *hns*)	Fernández-Mora, personal communication
DTM84	Δ*hilD* Δ*Cthns::kan*	This study
***E.COLI* K12 STRAIN**		
DH10β	Laboratory strain	Invitrogen
PLASMIDS		
pKK232-8	pBR322 derivative containing a promoterless chloramphenicol acetyltransferase (*cat*) gene, Ap^R	Brosius, 1984
philD-cat	pKK232-8 derivative containing a *hilD-cat* transcriptional fusion from nucleotides −364 to +88	Bustamante et al., 2008
philA-cat	pKK232-8 derivative containing a *hilA-cat* transcriptional fusion from nucleotides −410 to +446	Bustamante et al., 2008
pinvF-cat	pKK232-8 derivative containing a *invF-cat* transcriptional fusion from nucleotides −306 to +213	Bustamante et al., 2008
psirA-cat	pKK232-8 derivative containing a *sirA-cat* transcriptional fusion from nucleotides −563 to +98	Martínez et al., 2011
plon-cat	pKK232-8 derivative containing a *lon-cat* transcriptional fusion from nucleotides −296 to +61	This study
pclpX-cat	pKK232-8 derivative containing a *clpX-cat* transcriptional fusion from nucleotides −330 to +76	This study
pclpP-cat	pKK232-8 derivative containing a *clpP-cat* transcriptional fusion from nucleotides −335 to +57	This study
pcpxRA-cat	pKK232-8 derivative containing a *cpxRA-cat* transcriptional fusion from nucleotides −544 to +57	This study
pCA24N	High-copy-number cloning vector, *lac* promoter, *lacI*^{q}, Cm^R	Kitagawa et al., 2005
pCA-NlpE	pCA24N derivative expressing *E. coli* K12 NlpE from the *lac* promoter	Kitagawa et al., 2005
pCA-CpxR	pCA24N derivative expressing *E. coli* K12 CpxR from the *lac* promoter	Kitagawa et al., 2005
pMPM-K3	p15A derivative low-copy-number cloning vector, *lac* promoter, Kan^R	Mayer, 1995
pK3-CpxR	pMPM-K3 derivative expressing *S.* Typhimurium 14028s CpxR from the *lac* promoter	This study
pK3-RpoH	pMPM-K3 derivative expressing *S.* Typhimurium 14028s RpoH from the *lac* promoter	This study
pBAD-HilD	pBADMycHis derivative expressing HilD-MycHis from the *ara* promoter	Martínez et al., 2011
pKD46	pINT-ts derivative containing red recombinase system under an arabinose-inducible promoter, Ap^R	Datsenko and Wanner, 2000
pKD4	pANTsγ derivative template plasmid containing the kanamycin cassette for λ Red recombination, Ap^R	Datsenko and Wanner, 2000
pCP20	Plasmid expressing FLP recombinase from a temperature-inducible promoter, Ap^R	Datsenko and Wanner, 2000
pSUB11	pGP704 derivative template plasmid for FLAG epitope tagging	Uzzau et al., 2001

The coordinates for the cat fusions are indicated with respect to the transcriptional start site for each gene. Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Kan^R, kanamycin resistance.

strains DTM50 or DTM60 into strain DTM65, generating the Δ*hilD* Δ*cpxA::kan* (DTM66) and Δ*hilD* Δ*lon::kan* (DTM67) mutants, respectively, and to transfer the Δ*Cthns::kan* allele

TABLE 2 | Primers used in this study.

Primer	Sequence (5′-3′)	Target gene	RE
FOR *CAT* TRANSCRIPTIONAL FUSIONS			
promlon-Fw1	GTC*GGATCC*TGCCGGTCAGAGTAAGCCG	*lon*	BamHI
promlon-Rv1	TAC*AAGCTT*GGGTATGACCATGTGCGG		HindIII
pClpX-Bam	TCA*GGATCC*TGAGCAGATTGAACGTGATAC	*clpX*	BamHI
pClpX-Hind	GA*AAGCTT*CGGATGGACCGGCAATCAG		HindIII
pClpXP-Bam	GAT*GGATCC*TATGCGTAACGTCGCTCTGG	*clpP*	BamHI
pClpXP-Hind	GAG*AAGCTT*TATCAAAAGAGCGCTCACCG		HindIII
CpxR-Bam-5′	TTG*GGATCC*CGCCACGTCGCGC	*cpxRA*	BamHI
CpxR-Hind-3′	ATC*AAGCTT*CTTTAACAGGATTTTATTC		HindIII
FOR GENE CLONING			
CpxR-Fw1	TGA*AAGCTT*TTTCTGCCTCGGAGGTACG	*cpxR*	HindIII
CpxR-Rv1	CAG*GGATCC*CGTCAACCAGAAGATGGCG		BamHI
RpoH-FwKpn	AC*GGTACC*AGGCAATACTGATTGA	*rpoH*	KpnI
RpoH-RvBam	CAT*GGATCC*AACAGATTTGTGTCGGTGGG		BamHI
FOR GENE DELETIONS			
ScpxR-H1Pl	GATGACCGAGAGCTGACTTCCCTGTTAAAAGAGCTCCTCGAATGTGTAGGCTGGAGCTGCTTCG	*cpxR*	
ScpxR-H2P2	TGTTTTAAACCACGGGTGACCGTCTTTGCGTTCCGGCAGTTTCATATGAATATCCTCCTTAG		
ScpxA-H1P1	CTATCTGATGGTTTCCGCTTCATGATAGGAAGTTTAACCGCGTGTAGGCTGGAGCTGCTTCG	*cpxA*	
ScpxA-H2P2	GCATTCGCAGGCCGATGGTTTTTAGGTTCGCTTGTACAGCGGCATATGAATATCCTCCTTAG		
SackA-pta-H1P1	GTATCATAAATAGGTACTTCCATGTCGAGTAAGTTAGTACTGTGTAGGCTGGAGCTGCTTCG	*ackA-pta*	
SackA-pta-H2P2	ATCCGGCATTAGCTTTTACTGTTACTGCTGCTGCTGAGAAGCCATATGAATATCCTCCTTAG		
ShilE-H1P1	TACAGAGACACCAACGAAATGGCTGGAAAATGGAACGTTCTTTGTAGGCTGGAGCTGCTTCG	*hilE*	
ShilE-H2P2	CGCAAGCTTGTTTTGTCCTCATCGCCACAGCGCCTGTCGGTGCATATGAATATCCTCCTTAG		
SlonH1P1	AAACTAAGAGAGAGCTCTATGAATCCTGAGCGTTCTGAACGCTGTAGGCTGGAGCTGCTTCG	*lon*	
SlonH2P2	GTCATTTGCGCGAGGTCACTATTTTGCGGTTACAACCTGCATCATATGAATATCCTCCTTAG		
FOR GENE FLAG TAGGING			
invFflag-F	CCGCGGAAATTATCAAATATTATTCAATTGGCAGACAAAGACTACAAAGACCATGACGGT	*invF*	
invFflag-R	CGGCACATGCCAGCACTCTGGCCAAAAGAATATGTGTCTCATATGAATATCCTCCTTAGTTC		

(Continued)

TABLE 2 | Continued

Italic letters indicate the respective restriction enzyme site in the primer. The sequence corresponding to the template plasmids pKD4 or pSUB11 is underlined. RE, restriction enzyme for which a site was generated in the primer.

from strain MF100 into strain DTM65, generating the Δ*hilD* Δ*Cthns::kan* (DTM84) mutant.

The chromosomal *invF* gene was FLAG-tagged in *S.* Typhimurium strain 14028s, using a modification of the λ Red recombinase system for gene replacement, as described previously (Uzzau et al., 2001), and the respective primers described in **Table 2**, generating strain DTM76. P22 transduction was used to transfer the *invF::3XFLAG-kan* allele from strain DTM76 into strains DTM49, DTM51, DTM53, DTM57, DTM59, DTM61 and DTM63, generating the Δ*cpxR invF::3XFLAG-kan* (DTM77), Δ*cpxA invF::3XFLAG-kan* (DTM78), Δ*cpxRA invF::3XFLAG-kan* (DTM79), Δ*hilE invF::3XFLAG-kan* (DTM80), Δ*cpxA* Δ*hilE invF::3XFLAG-kan* (DTM81), Δ*lon invF::3XFLAG-kan* (DTM82) and Δ*cpxA* Δ*lon invF::3XFLAG-kan* (DTM83) mutants, respectively, to transfer the *hilA::3XFLAG-kan* allele from strain JPTM7 into strains 14028s, DTM49, DTM51 and DTM53, generating the *hilA::3XFLAG-kan* (DTM68), Δ*cpxR hilA::3XFLAG-kan* (DTM69), Δ*cpxA hilA::3XFLAG-kan* (DTM70) and Δ*cpxRA hilA::3XFLAG-kan* (DTM71) mutants, respectively, and to transfer the *ssrB::3XFLAG-kan* allele from strain JPTM30 into strains 14028s, DTM49, DTM51 and DTM53, generating the *ssrB::3XFLAG-kan* (DTM72), Δ*cpxR ssrB::3XFLAG-kan* (DTM73), Δ*cpxA ssrB::3XFLAG-kan* (DTM74) and Δ*cpxRA ssrB::3XFLAG-kan* (DTM75) mutants, respectively. All mutant strains were verified by PCR amplification and sequencing.

CAT Assays

The CAT assays and protein quantification to calculate CAT specific activities were performed as described previously (Puente et al., 1996).

Statistical Analysis

Results from chloramphenicol acetyltransferase (CAT) assays were analyzed using One-Way analysis of variance (ANOVA) with the Dunnett multiple comparison test for **Figures 1A,D**, or *t*-Test with the Mann–Whitney test for **Figures 4A–D**, **7B,C**. A *P*-value of <0.05 was considered significant. This statistical analysis was performed using Prism 5 program version 5.04 (GraphPad Software, San Diego, CA).

Protein Secretion Analysis

Protein secretion assays were performed as we described previously (Martínez et al., 2011). Samples were subjected to SDS-PAGE analysis using 12% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250.

Western Blotting

Whole-cell extracts were prepared from samples collected at the indicated time points of bacterial cultures. Ten micrograms of each extract were subjected to electrophoresis in SDS-12% polyacrylamide gels, and then transferred to 0.45 μm pore size nitrocellulose membranes (Bio-Rad), using a semidry transfer apparatus (Bio-Rad). Membranes were blocked with 5% nonfat milk and then incubated with anti-c-Myc (Sigma), anti-FLAG M2 (Sigma) or anti-DnaK (StressGen) monoclonal antibodies, or anti-SseB polyclonal antibody (Coombes et al., 2004), at 1:3000, 1:4000, 1:20,000 and 1:2000 dilutions, respectively. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Pierce), at a dilution of 1:10,000, were used as the secondary antibodies. Bands on the blotted membranes were developed by incubation with the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer) and exposed to Kodak X-Omat films.

HilD Protein Stability Assays

Bacterial strains were grown in LB medium at 37°C to an OD_{600} equal to 0.8. Then, the expression of HilD-Myc from plasmid pBAD-HilD was induced by adding 0.05% L-arabinose for 45 min. After this time, antibiotics streptomycin, rifampicin and chloramphenicol, at final concentrations of 200, 100 and 200 $\mu g\ ml^{-1}$, respectively, were added to the cultures to prevent transcription and translation. To ensure repression of the *ara* promoter expressing HilD-Myc, 2% glucose was also added. The bacterial cultures were further incubated at 37°C and samples were taken at 0, 15, 30, 60, 90 and 120 min, and analyzed by Western blotting as described above. Intensity of protein bands from the blots was quantified by using ImageJ software (Image Processing and Analysis in Java), version 1.48 (National Institutes of Health, USA). Values for HilD-Myc bands were normalized with those respective of DnaK bands and then the relative percentage of HilD-Myc at each time with respect to time 0 was calculated. The HilD half-life ($t_{1/2}$) was calculated by linear regression.

Results

AckA-Pta-dependent Activation of CpxR Represses the SPI-1 Genes

Intriguingly, the absence of the histidine kinase CpxA, but not its cognate response regulator CpxR, negatively affects the expression of SPI-1 genes, which could suggest that CpxA positively regulates the SPI-1 genes by interacting with regulator(s) other than CpxR (Nakayama et al., 2003). However, there was the possibility that the absence of CpxA, and thus of its phosphatase activity, could lead to the phosphorylation of CpxR, mainly by acetyl phosphate produced by the AckA and Pta enzymes, as described previously (Batchelor et al., 2005; Spinola et al., 2010; Liu et al., 2012). Therefore, an alternative to the proposed positive regulatory role of CpxA on SPI-1 genes, was that CpxR-P generated in the absence of CpxA could actually repress these genes. To investigate this possibility, we tested the expression of a transcriptional fusion of the SPI-1 gene *invF* with the *cat* (chloramphenicol acetyl transferase) reporter gene, in wild-type (WT) *S.* Typhimurium strain 14028s, as well as in different derivative mutants containing single, double or triple deletions of *cpxA*, *cpxR*, *ackA*, or *pta* genes. As a control, the expression of a *cat* transcriptional fusion of *sirA*, which encodes a positive regulator of the SPI-1 genes that is located outside SPI-1, was also assessed. In agreement with the results reported previously (Nakayama et al., 2003), the expression of the *invF-cat* fusion was reduced in the Δ*cpxA* mutant, but was not affected in the Δ*cpxR* mutant (**Figure 1A**). Additionally, the expression of this fusion was not affected in the Δ*cpxRA* or Δ*ackA-pta* double mutants neither in the Δ*cpxA* Δ*ackA-pta* triple mutant, whereas the *sirA-cat* fusion was expressed at a similar level in all strains tested (**Figure 1A**). Consistently, protein secretion analyses showed that the secretion/expression of the SPI-1-encoded proteins SipA, SipB, SipC, and SipD was drastically diminished in the Δ*cpxA* mutant, but not in the Δ*cpxR*, Δ*cpxRA*, Δ*ackA-pta* or Δ*cpxA* Δ*ackA-pta* mutants (**Figure 1B**). Furthermore, the expression of the 3xFLAG-tagged regulators HilA (HilA-FLAG) and InvF (InvF-FLAG), which are encoded in SPI-1, was reduced in the Δ*cpxA* mutant, but not in the Δ*cpxR* or Δ*cpxRA* mutants (**Figure 1C**). These results show that deletion of *cpxR* or the *ackA-pta* genes restores the expression of the SPI-1 genes in the Δ*cpxA* mutant, indicating that the absence of CpxA actually represses these genes through CpxR and the AckA/Pta enzymes.

Previous studies have shown that CpxR-P activates the expression of the *cpxRA* operon (De Wulf et al., 1999; Raivio et al., 1999, 2013; Price and Raivio, 2009). Therefore, to further investigate whether the absence of CpxA turns on CpxR-mediated gene regulation in *S.* Typhimurium, in the growth conditions tested, we determined the expression of a *cpxRA-cat* transcriptional fusion in the WT *S.* Typhimurium strain and its derivative Δ*cpxR*, Δ*cpxA*, and Δ*cpxRA* mutants. As shown in **Figure 1D**, the expression of the *cpxRA-cat* fusion was increased in the Δ*cpxA* mutant, but not in the Δ*cpxR* and Δ*cpxRA* mutants, indicating that the absence of CpxA induces the expression of the *cpxRA* operon through CpxR.

Together, these results strongly support that the absence of CpxA leads to the phoshorylation of CpxR via the AckA-Pta pathway, which in turn represses the expression of the SPI-1 genes and probably induces the positive or negative regulation of the whole CpxR regulon.

CpxA-dependent Activation or Overexpression of CpxR Represses the SPI-1 Genes

Overproduction of the lipoprotein NlpE activates the kinase activity of CpxA and thus the CpxA-dependent phosphorylation of CpxR (Snyder et al., 1995; Hunke et al., 2012; Vogt and Raivio, 2012). Hence, to determine whether the CpxA-mediated activation of CpxR also represses the expression of the SPI-1 genes, we examined the effect of the overexpression of NlpE, from an IPTG-inducible promoter, on the protein secretion profiles

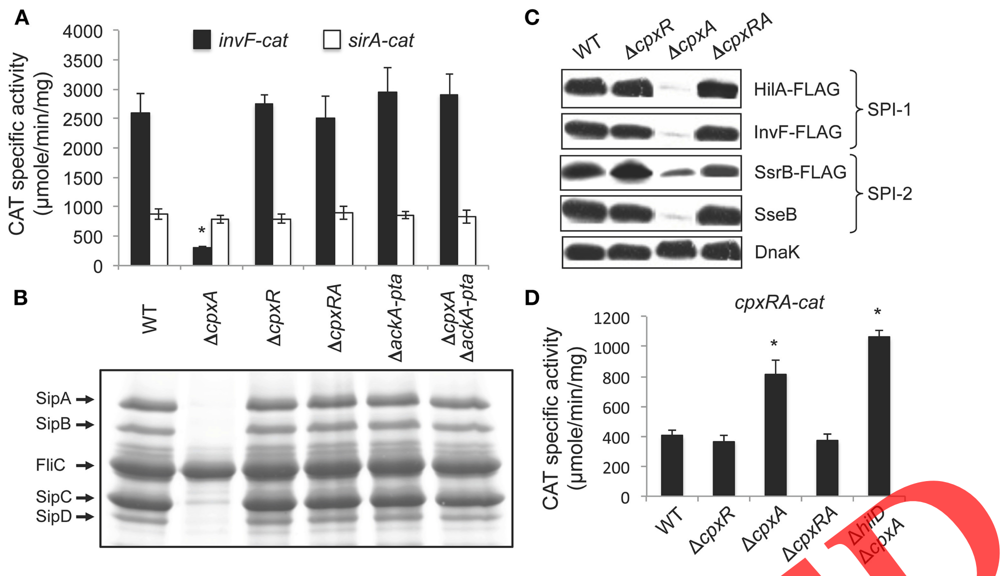

FIGURE 1 | The absence of CpxA represses SPI-1 genes by the activation of CpxR via the AckA and Pta enzymes. (A) Expression of the *invF-cat* and *sirA-cat* transcriptional fusions, carried by plasmids pinvF-cat and psirA-cat, respectively, was tested in the WT *S.* Typhimurium 14028s strain and its isogenic Δ*cpxA*, Δ*cpxR*, Δ*cpxRA*, Δ*ackA-pta*, and Δ*cpxA* Δ*ackA-pta* mutants. **(B)** Secretion analysis of the SPI-1-encoded proteins SipA, SipB, SipC, and SipD was tested in the WT *S.* Typhimurium strain 14028s and its isogenic Δ*cpxA*, Δ*cpxR*, Δ*cpxRA*, Δ*ackA-pta*, and Δ*cpxA* Δ*ackA-pta* mutants grown for 9 h in LB medium at 37°C. FliC is a flagellar protein whose secretion is SPI-1-independent. **(C)** Expression of the SPI-1-encoded HilA-FLAG and InvF-FLAG and the SPI-2-encoded SsrB-FLAG and SseB, in the WT *S.* Typhimurium strain and its isogenic Δ*cpxR*, Δ*cpxA* and Δ*cpxRA* mutants, carrying the respective chromosomal FLAG-tagged gene, was analyzed by Western blotting using monoclonal anti-FLAG or polyclonal anti-SseB antibodies. Whole cell lysates were prepared from samples of bacterial cultures grown in LB medium at 37°C for 5 or 9 h, for detection of SPI-1- and SPI-2-encoded proteins, respectively. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies. **(D)** Expression of the *cpxRA-cat* transcriptional fusion, carried by plasmid pcpxRA-cat, was tested in the WT *S.* Typhimurium 14028s strain and its isogenic Δ*cpxR*, Δ*cpxA*, Δ*cpxRA*, and Δ*hilD* Δ*cpxA* mutants. CAT-specific activity of cat fusions was determined from samples collected of bacterial cultures grown for 5 h in LB medium at 37°C. The data are the averages of three different experiments performed in duplicate. Bars represent the standard deviations. *Expression statistically different with respect to that shown by the same fusion in the WT strain.

of the WT *S.* Typhimurium strain and its derivative Δ*cpxR* mutant. Since *Salmonella* lacks NlpE, the *E. coli* K12 NlpE was used in these assays. As shown in **Figure 2**, the induction of the NlpE expression by the presence of IPTG decreased the secretion/expression of the SipA-D proteins in the WT strain but not in its derivative Δ*cpxR* mutant, indicating that the activation of CpxA represses the secretion/expression of SPI-1-encoded proteins through CpxR. To further confirm the regulatory role of CpxR on the SPI-1 genes, we determined the effect of its overexpression on the protein secretion profile of the WT *S.* Typhimurium strain, since the overexpression can bypass the need for phosphorylation of CpxR to regulate target genes (Macritchie et al., 2008; Acosta et al., 2015; Yun et al., 2015). The *E. coli* K12 CpxR is 97% identical to that of *S.* Typhimurium 14028s; thus, the plasmid pCA-CpxR from the ASKA library (Kitagawa et al., 2005), which expresses the *E. coli* K12 CpxR from an IPTG-inducible promoter, was used in these assays. As shown in **Figure 3A**, the overexpression of CpxR reduced the secretion/expression of the SipA-D proteins. Furthermore, the overexpression of CpxR repressed the expression of HilA-FLAG and InvF-FLAG in the WT *S.* Typhimurium strain (**Figure 3B**). In all, these results indicate that CpxA-mediated phosphorylation of CpxR or the overexpression of CpxR represses the expression of the SPI-1 genes.

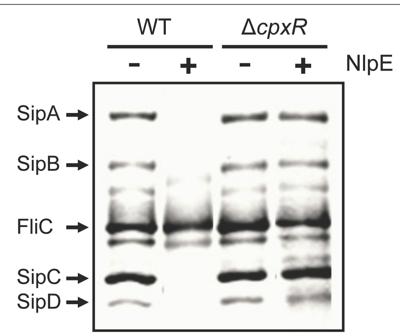

FIGURE 2 | NlpE-mediated activation of CpxA represses SPI-1 through CpxR. Secretion analysis of the SPI-1-encoded proteins SipA, SipB, SipC, and SipD was tested in the WT *S.* Typhimurium strain and its isogenic Δ*cpxR* mutant carrying plasmid pCA-NlpE, grown for 9 h in LB medium at 37°C. FliC is a flagellar protein whose secretion is SPI-1-independent. Expression (+) of NlpE from the *T5-lac* promoter of plasmid pCA-NlpE was induced by adding 50 µM IPTG at the beginning of the bacterial cultures.

CpxR Represses *hilD* and thus Indirectly Affects HilD-regulated Genes

Several global regulators control the expression of the SPI-1 genes by directly affecting the expression, activity or concentration of

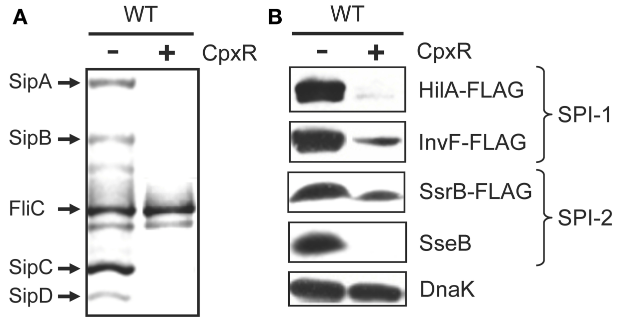

FIGURE 3 | Overexpression of CpxR represses SPI-1 and SPI-2 genes. **(A)** Secretion analysis of the SPI-1-encoded proteins SipA, SipB, SipC, and SipD was tested in the WT *S.* Typhimurium strain carrying plasmid pCA-CpxR, grown for 9 h in LB medium at 37°C. FliC is a flagellar protein whose secretion is SPI-1-independent. **(B)** Expression of the SPI-1-encoded HilA-FLAG and InvF-FLAG and the SPI-2-encoded SsrB-FLAG and SseB, in the WT *S.* Typhimurium strain carrying the respective chromosomal FLAG-tagged gene and containing plasmid pCA-CpxR, was analyzed by Western blotting using monoclonal anti-FLAG or polyclonal anti-SseB antibodies. Whole cell lysates were prepared from samples of bacterial cultures grown in LB medium at 37°C for 5 or 9 h, for detection of SPI-1- and SPI-2-encoded proteins, respectively. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies. Overexpression (+) of CpxR from the *T5-lac* promoter of plasmid pCA-CpxR was induced by adding 50 μM IPTG at the beginning of the bacterial cultures.

HilD or HilA, the central regulators of these genes (Golubeva et al., 2012; Fàbrega and Vila, 2013). Our results indicate that CpxR represses the expression of HilA (**Figure 1C**). To start to define whether CpxR affects *hilA* directly or through HilD, which positively regulates *hilA*, we determined the effect of the overexpression of CpxR on the activity of a *hilD-cat* transcriptional fusion in the WT *S.* Typhimurium strain. Since plasmid pCA-CpxR, expressing the *E. coli* K12 CpxR, is incompatible with the vector carrying the *cat* fusions tested, for the next assays we constructed and used the plasmid pK3-CpxR, which constitutively expresses CpxR of *S.* Typhimurium 14028s. The overexpression of CpxR reduced 50% the expression of the *hilD-cat* fusion (**Figure 4A**), revealing that CpxR represses *hilD*. CpxR could directly repress the transcription of *hilD* or reduce post-transcriptionally the concentration of HilD and thus affect its positive autoregulation. To determine if CpxR affects the autoregulation of *hilD*, the expression of the *hilD-cat* fusion was determined in the WT *S.* Typhimurium strain and its derivatives Δ*cpxA*, Δ*hilD*, and Δ*hilD* Δ*cpxA* mutants. As shown in **Figure 4B**, the expression of the *hilD-cat* fusion was similarly reduced in these three mutants, indicating that the absence of CpxA or HilD has the same effect on the expression of *hilD*, and that, when HilD is not present, the absence of CpxA does not longer repress *hilD*. In contrast, the expression of the *cpxRA-cat* fusion was similarly increased in the Δ*cpxA* and Δ*hilD* Δ*cpxA* mutants (**Figure 1D**), indicating that the absence of CpxA activates the expression of *cpxRA* independently of HilD. These results suggest that CpxR represses *hilA* and thus the other SPI-1 genes by affecting the autoregulation of HilD. To confirm that CpxR regulates *hilA* through HilD and not directly, we analyzed the effect of CpxR on the expression of *hilA* in the presence or not of HilD. Previous studies indicate that HilD induces the expression of *hilA* by counteracting the repression exerted by the nucleoid protein H-NS on the promoter of this gene (Schechter et al., 1999; Schechter and Lee, 2001; Olekhnovich and Kadner, 2006); thus, in the absence of H-NS activity *hilA* can be expressed independently of HilD. Full-length deletion of *hns* produces severe growth defects in *S.* Typhimurium (Lucchini et al., 2006; Navarre et al., 2006). However, deletion of the sequence encoding the C-terminal region of H-NS (Δ*Cthns*), which contains its DNA-binding domain, has only a minor effect on *S.* Typhimurium fitness (Fernández-Mora, personal communication), probably because the N-terminal of H-NS can still repress some of its target genes by interacting with StpA, another nucleoid protein (Free et al., 2001). Therefore, we constructed and tested a *S.* Typhimurium 14028s Δ*hilD* Δ*Cthns* mutant. The overexpression of CpxR, from plasmid pK3-CpxR, reduced five-fold the expression of a *hilA-cat* transcriptional fusion in the WT strain (**Figure 4C**), but did not affect the high levels of expression showed by this fusion in the Δ*hilD* Δ*Cthns* mutant (**Figure 4D**), indicating that CpxR regulates *hilA* through HilD and not directly.

In agreement with our results indicating that CpxR represses the HilD-dependent expression of *hilD* and *hilA*, both the overexpression of CpxR and the absence of CpxA drastically reduced the production of SsrB-FLAG and SseB proteins (**Figures 1C, 3B**), which are encoded in SPI-2 and whose expression is also dependent of HilD in the condition tested.

Taken together, these results show that CpxR represses the autoregulation of HilD, which in turn affects the expression of *hilA* and thus the SPI-1 genes, as well as of other virulence genes regulated by HilD, such as *ssrB* and *sseB* located in SPI-2.

CpxR-mediated Repression of the SPI-1 Genes Is lost in the Absence of the Lon Protease

A previous study shown that the overexpression of the sigma factor RpoH represses the SPI-1 genes through the Lon protease that degrades HilD (Matsui et al., 2008). On the other hand, it was reported that CpxR positively regulates *rpoH* in *E. coli* (Zahrl et al., 2006). Interestingly, we observed a very similar effect with the overexpression of CpxR or RpoH on the activity of *hilD-cat* and *hilA-cat* fusions (**Figures 4A,C,D**), which was initially tested as an expression control of the promoter expressing CpxR from plasmid pMPM-K3. Therefore, we thought that CpxR could repress the SPI-1 genes through RpoH and Lon. To investigate this, we sought to determine the effect of CpxR on the SPI-1 genes in the absence of RpoH or Lon. After several attempts, we were unable to delete *rpoH* in the *S.* Typhimurium 14028s strain by the λRed recombination method (Datsenko and Wanner, 2000), which could suggest that the absence of RpoH affects *Salmonella* fitness; although, a *S.* Typhimurium 14028s Δ*rpoH* mutant was reported previously (Bang et al., 2005). In contrast, a *lon* deletion strain was successful; thus, we constructed and analyzed Δ*lon* and Δ*cpxA* Δ*lon* mutants. Furthermore, since HilE regulates the activity of HilD by protein-protein interaction (Baxter et al., 2003), Δ*hilE* and Δ*cpxA* Δ*hilE* mutants were also constructed and used as controls. Interestingly, the expression

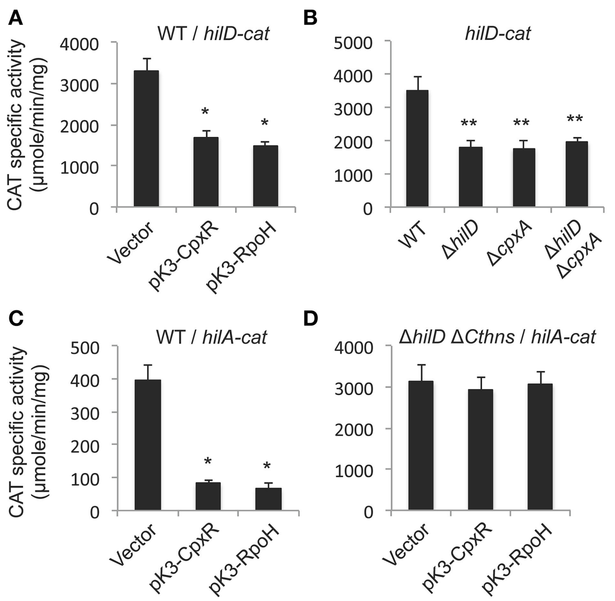

FIGURE 4 | CpxR represses the autoregulation of *hilD* and thus negatively affects the expression of *hilA*. Expression of the *hilD-cat* transcriptional fusion carried by plasmid philD-cat was tested in the WT *S*. Typhimurium strain carrying plasmid pK3-CpxR or pK3-RpoH, or the vector pMPM-K3 **(A)**, as well as in the WT *S*. Typhimurium strain and its isogenic Δ*hilD*, Δ*cpxA*, and Δ*hilD* Δ*cpxA* mutants **(B)**. Expression of the *hilA-cat* transcriptional fusion carried by plasmid philA-cat was tested in the WT *S*. Typhimurium strain **(C)**, or in its isogenic Δ*hilD* Δ*Cthns* mutant **(D)**, containing plasmid pK3-CpxR or pK3-RpoH, or the vector pMPM-K3. Plasmids pK3-CpxR and pK3-RpoH, as well as *Salmonella*, lack the gene encoding the repressor LacI and thus they constitutively express CpxR and RpoH, respectively, from a *lac* promoter. CAT-specific activity was determined from samples collected of bacterial cultures grown for 5 h in LB medium at 37°C. The data are the averages of three different experiments performed in duplicate. Bars represent the standard deviations. *Expression statistically different with respect to that shown by the same fusion in the WT strain containing the vector. **Expression statistically different with respect to that shown by the same fusion in the WT strain.

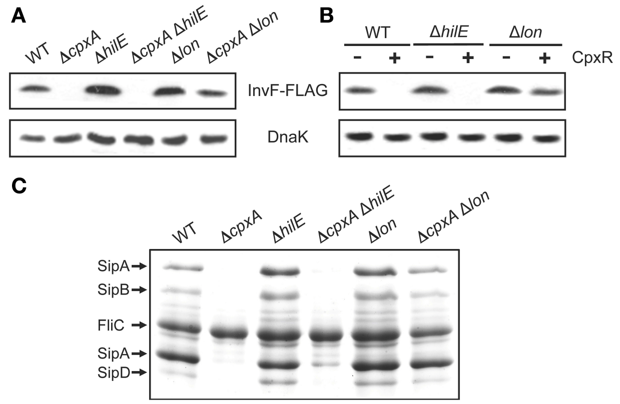

FIGURE 5 | Repression of the SPI-1 genes by CpxR is lost in the absence of the Lon protease. Expression of the SPI-1-encoded InvF-FLAG in the WT *S*. Typhimurium strain and its isogenic Δ*cpxA*, Δ*hilE*, Δ*cpxA* Δ*hilE*, Δ*lon* and Δ*cpxA* Δ*lon* mutants **(A)**, or in the WT *S*. Typhimurium strain and its isogenic Δ*hilE* and Δ*lon* mutants carrying plasmid pCA-CpxR **(C)**, was analyzed by Western blotting using monoclonal anti-FLAG antibodies. Whole cell lysates were prepared from samples of bacterial cultures grown for 5 h in LB medium at 37°C. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies. Overexpression (+) of CpxR from the *T5-lac* promoter of plasmid pCA-CpxR was induced by adding 50 µM IPTG at the beginning of the bacterial cultures. **(B)** Secretion analysis of the SPI-1-encoded proteins SipA, SipB, SipC, and SipD was tested in the WT *S*. Typhimurium strain and its isogenic Δ*cpxA*, Δ*hilE*, Δ*cpxA* Δ*hilE*, Δ*lon*, and Δ*cpxA* Δ*lon* mutants grown for 9 h in LB medium at 37°C. FliC is a flagellar protein whose secretion is SPI-1-independent.

of InvF-FLAG, as well as the secretion/expression of the SipA-D proteins, was drastically reduced in the Δ*cpxA* and Δ*cpxA* Δ*hilE* mutants, but not in the Δ*hilE*, Δ*lon*, and Δ*cpxA* Δ*lon* mutants (**Figures 5A,B**). Consistently, the overexpression of CpxR clearly repressed the expression of InvF-FLAG in the WT strain and the Δ*hilE* mutant, but only slightly in the Δ*lon* mutant (**Figure 5C**). Therefore, these results show that deletion of *lon* counteracts repression exerted by CpxR on the SPI-1 genes, which supports that CpxR acts through the Lon protease to repress these genes. Nevertheless, even in the absence of Lon, either the absence of CpxA or the overexpression of CpxR slightly repressed the SPI-1 genes (**Figure 5**), revealing an additional Lon-independent mechanism for the repression of these genes by CpxR.

CpxR Affects Stability of HilD

On the basis of our results indicating that repression of the SPI-1 genes by CpxR is mostly lost in the absence of the Lon protease, which degrades HilD, we hypothesized that CpxR should reduce the stability of HilD. To investigate this, we determined the *in vivo* half-life of HilD in the presence or absence of CpxA or Lon. The cellular levels of Myc-tagged HilD (HilD-Myc) expressed from plasmid pBAD-HilD, under an arabinose-inducible promoter, were monitored in the Δ*hilD*, Δ*hilD* Δ*cpxA* and Δ*hilD* Δ*lon* mutants, at indicated times after adding a cocktail of transcription and translation inhibitors. As shown in **Figure 6A**, the levels of HilD-Myc were reduced faster in the Δ*hilD* Δ*cpxA* mutant than in the Δ*hilD* mutant, whereas, as expected, the stability of HilD-Myc was drastically increased in the Δ*hilD* Δ*lon* mutant. In these assays, the half-life of HilD-Myc in the presence and absence of CpxA was 38 and 20 min, respectively (**Figure 6B**), supporting the notion that the activation of CpxR by the absence of CpxA decreases the stability of HilD.

CpxR does not Affect the Transcription of *lon*

As most response regulators, CpxR directly controls gene expression at transcriptional level (Hunke et al., 2012; Vogt and Raivio, 2012; Raivio, 2014). Thus, we tested if CpxR affects the transcription of *lon*. In *E. coli*, *lon* seems to be transcribed from promoters located upstream of *lon*, or from those of neighboring genes *clpX* and *clpP* (RegulonDB database, www.regulondb.ccg.unam.mx). Therefore, to monitor the promoters expressing *lon*, we constructed *lon-cat*, *clpX-cat* and *clpP-cat* transcriptional fusions, which contain the full intergenic region upstream of the respective gene (**Figure 7A**). Each of these fusions showed similar levels of expression in the WT strain and its derivative Δ*cpxA* mutant (**Figure 7B**).

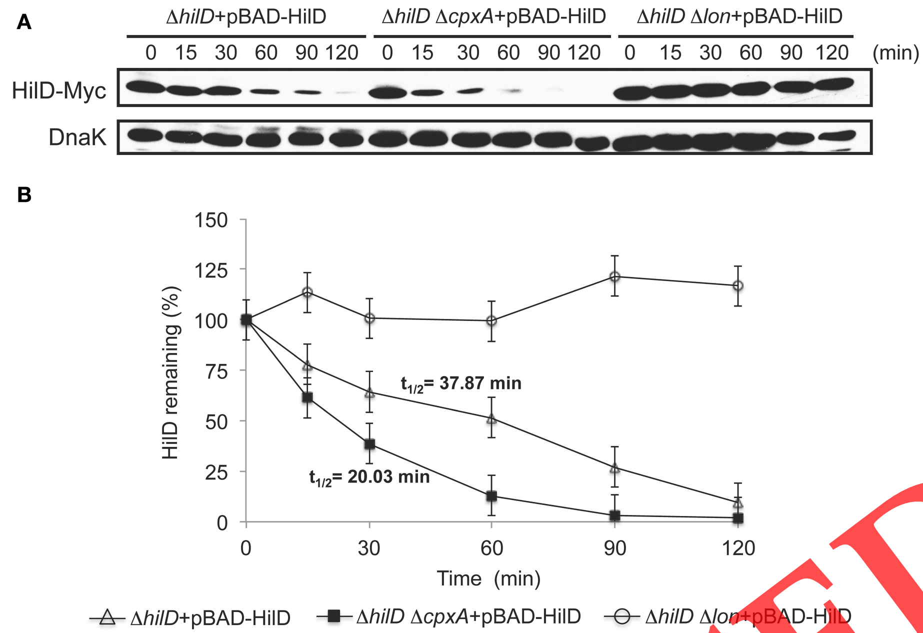

FIGURE 6 | CpxR reduces the stability of HilD. (A) Stability of HilD-Myc was determined in the Δ*hilD*, Δ*hilD* Δ*cpxA*, and Δ*hilD* Δ*lon* mutants carrying plasmid pBAD-HilD, which were grown in LB medium at 37°C. Expression of HilD-Myc, from the arabinose-inducible promoter of plasmid pBAD-HilD, was induced with 0.05% L-arabinose for 45 min; then, transcription and translation were halted by the addition of a cocktail of antibiotics and glucose, and samples of bacterial cultures were taken at indicated times. HilD-Myc was detected from whole cell lysates of the samples by Western blotting using monoclonal anti-Myc antibodies. As a loading control, the expression of DnaK was also determined using monoclonal anti-DnaK antibodies. A representative Western blot of three independent experiments is shown. **(B)** Densitometric analysis of the HilD-Myc bands from the Western blots is indicated as the relative percentage of HilD-Myc at each time with respect to time 0. Intensity values of HilD-Myc bands were normalized with those respective of DnaK bands. The data are the averages of three independent experiments. Bars represent the standard deviations and $t_{1/2}$ indicates the half-life of HilD.

Furthermore, the expression of the *lon-cat*, *clpX-cat*, and *clpP-cat* fusions was not affected in the WT strain by the overexpression of CpxR; in contrast, their expression was increased by the overproduction of RpoH (**Figure 7C**). These results indicate that CpxR does not affect the transcription of *lon* and demonstrate that RpoH positively regulates *lon*, *clpX*, and *clpP* in *S.* Typhimurium.

Discussion

Previous studies have shown that deletion of *cpxA* or mutations in *cpxA* that activate the Cpx system reduce *Salmonella* adherence and invasion to eukaryotic cells, as well as the ability of *Salmonella* to infect mice (Leclerc et al., 1998; Nakayama et al., 2003; Humphreys et al., 2004). Furthermore, it was shown that deletion of *cpxA* decreases the expression of *hilA* and thus the SPI-1 genes (Nakayama et al., 2003), which code for the T3SS-1 and their cognate effector proteins that are required for *Salmonella* invasion into the intestinal epithelium of hosts (Haraga et al., 2008; Fàbrega and Vila, 2013). In this study, we show that deletion of *cpxA* negatively affects the expression of the SPI-1 genes when *S.* Typhimurium is grown in LB medium, but only in the presence of *cpxR*, indicating that the absence of CpxA leads to the repression of the SPI-1 genes through CpxR. Furthermore, we show that deletion of *cpxA* increases the expression of the *cpxRA* operon through CpxR, which is in agreement with previous studies indicating that CpxR-P induces the expression of the *cpxRA* operon (De Wulf et al., 1999; Raivio et al., 1999, 2013; Price and Raivio, 2009). Thus, our results could suggest that the absence of CpxA turns on the regulation of the whole CpxR regulon in *S.* Typhimurium.

Our data provide several lines of evidence strongly supporting that CpxR-P represses the SPI-1 genes. First, the AckA and Pta enzymes, which generate acetyl phosphate that phosphorylates CpxR (Raivio and Silhavy, 1997; Wolfe et al., 2008), are also required for the repression of the SPI-1 genes mediated by deletion of *cpxA*. Second, overexpression of the lipoprotein NlpE, which activates the kinase activity of CpxA on CpxR (Snyder et al., 1995; Hunke et al., 2012; Vogt and Raivio, 2012), represses the SPI-1 genes via CpxR. Third, the overexpression of CpxR, which can bypass the need for phosphorylation of CpxR to regulate target genes (Macritchie et al., 2008; Acosta et al., 2015; Yun et al., 2015), has the same effect on the expression of the SPI-1 genes than the absence of CpxA or the overexpression of NlpE. Furthermore, a previous study showed accumulation of CpxR-P in a *cpxA* deletion mutant of *Yersinia pseudotuberculosis* grown in LB medium, which was generated through the AckA-Pta pathway (Liu et al., 2012). However, since CpxR-P induces its own expression, as mentioned above, both phosphorylation

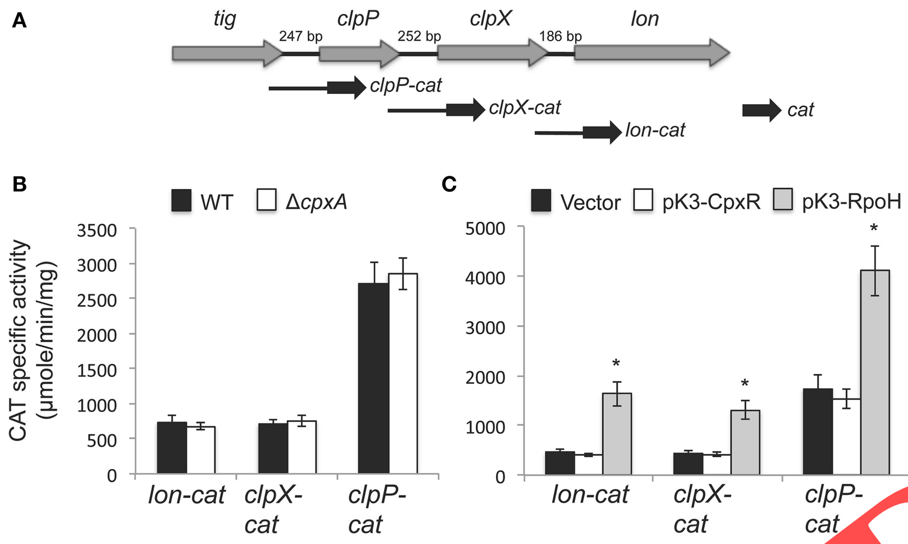

FIGURE 7 | RpoH, but not CpxR, induces the transcription of *lon*, *clpX*, and *clpP*. (A) Genetic organization of the *lon*, *clpX*, and *clpP* genes and schematic representation of the *cat* transcriptional fusions of these genes. Expression of the *lon-cat*, *clpX-cat* and *clpP-cat* transcriptional fusions, carried by plasmids plon-cat, pclpX-cat and pclpP-cat, respectively, was tested in the WT *S.* Typhimurium strain and its isogenic Δ*cpxA* mutant **(B)**, as well as in the WT *S.* Typhimurium strain carrying plasmid pK3-CpxR or pK3-RpoH, which constitutively express CpxR and RpoH, respectively, or carrying the vector pMPM-K3 **(C)**. CAT-specific activity was determined from samples collected of bacterial cultures grown for 5 h in LB medium at 37°C. The data are the averages of three different experiments performed in duplicate. Bars represent the standard deviations. *Expression statistically different with respect to that shown by the same fusion in the WT strain containing the vector.

and a higher concentration of CpxR would be involved in the repression of the SPI-1 genes.

Our data indicate that CpxR-P decreases the stability of HilD, the regulator that is at the apex of a regulatory cascade controlling the expression of the SPI-1 genes, as well as other virulence genes, such as those located in SPI-2 (Bustamante et al., 2008; Golubeva et al., 2012; Fàbrega and Vila, 2013; Martínez et al., 2014). Consistently, we show that CpxR represses the expression of both SPI-1 and SPI-2 (*ssrAB* and *sseB*) virulence genes when *S.* Typhimurium is grown in LB medium. Furthermore, we demonstrate that CpxR-P negatively affects the transcription of the SPI-1 genes *hilD* and *hilA*, but only in the presence of HilD, which would be expected, since the expression of HilD is autoregulated and HilD directly regulates *hilA* (Golubeva et al., 2012; Fàbrega and Vila, 2013). Therefore, the effect of CpxR-P on the expression of the SPI-1 and SPI-2 genes could be the result of its negative control on the stability of HilD and, as a consequence, on the transcription of *hilD*, which, in an additive manner, would decrease the concentration of HilD. In agreement with this conclusion, we did not find any putative CpxR binding-site in the regulatory regions of *hilD*, *hilA*, *ssrAB* and *sseB*, using the Virtual Footprint tool (Munch et al., 2005) (http://prodoric.tu-bs.de/vfp/) with the Position Weight Matrix for the binding-consensus sequence of *E. coli* K12 CpxR, 5′-GTAAA(N)$_5$GTAA(A/G)-3′ (De Wulf et al., 2002), supporting that these genes are not directly regulated by CpxR. In contrast, these analyses revealed CpxR binding-sites in the regulatory regions of the *S.* Typhimurium *cpxR* and *cpxP* genes (data not shown). In *E. coli*, *cpxR* and *cpxP* belong to the CpxR regulon (Raivio et al., 1999, 2013; Price and Raivio, 2009).

CpxR has been shown to directly act as a transcriptional regulator (Hunke et al., 2012; Vogt and Raivio, 2012; Raivio, 2014). However, deletion of *cpxA* represses T3SS genes in *Shigella sonnei* through posttranscriptional processing of the regulator InvE (Mitobe et al., 2005). Furthermore, activation of the CpxR/A system reduces the stability of the *E. coli* F plasmid regulator TraJ via the HsIVU protease-chaperone pair (Gubbins et al., 2002; Lau-Wong et al., 2008). These latter studies indicate that CpxR can indirectly control protein stability by activating proteases. Interestingly, we found that the absence of the Lon protease, which has been shown to degrade HilD (Takaya et al., 2005), severely affects the repression of the SPI-1 genes mediated by CpxR. In contrast, the absence of HilE, a regulator which negatively controls HilD activity by protein-protein interactions (Baxter et al., 2003), does not affect this repression by CpxR. Taken together, our results support that CpxR-P represses the expression of the SPI-1 and SPI-2 genes mainly by reducing the stability of HilD through the Lon protease. However, our data also show that both deletion of *cpxA* and overexpression of CpxR slightly repress the expression of the SPI-1 genes in the absence of the Lon protease, suggesting an additional minor Lon-independent mechanism for the repression of the SPI-1 genes by CpxR-P. Alternatively, this could suggest that CpxR-P actually controls the stability of HilD through another protease, not involving Lon, which could be obfuscated by the extremely high stability of HilD in the absence of Lon. How CpxR-P reduces the stability of HilD or whether there is another mechanism by which CpxR-P represses the SPI-1 and SPI-2 genes is a matter of our current and future studies.

Overexpression of the heat shock sigma factor RpoH represses the SPI-1 genes only in the presence of the Lon protease (Matsui et al., 2008). Our results show that RpoH, but not CpxR, induces the transcription of *lon*, as well as of the *clpX* and *clpP* neighbor genes encoding the ClpXP protease. Accordingly, previous studies indicate that *lon*, *clpX* and *clpP* belong to the RpoH regulon (Nonaka et al., 2006; Wade et al., 2006), but not to the CpxR regulon of *E. coli* (Bury-Mone et al., 2009; Price and Raivio, 2009; Raivio et al., 2013). Therefore, CpxR and RpoH seem to affect HilD concentration and thus repress the SPI-1 genes differentially; RpoH by inducing transcription of *lon* and CpxR by probably affecting the posttranscriptional expression or activity of Lon, or through another protease. Anyway, HilD would integrate the regulation of *Salmonella* virulence genes to the stresses sensed by CpxR/A and RpoH.

The two-component system CpxR/A regulates virulence in many bacteria, mostly by inhibiting the production of secretion systems, pili, flagella, fimbriae and curli, which are required for bacteria interaction with host cells; furthermore, several studies have shown that biogenesis of these envelope-localized multiprotein complexes activates the Cpx response (Hunke et al., 2012; Vogt and Raivio, 2012; Raivio, 2014). In this study, we demonstrate that activation of the CpxR/A system represses the expression of the genes encoding the T3SS-1 and T3SS-2, and their respective effector proteins, in *S.* Typhimurium. The activation of the CpxR/A system also represses the expression of T3SS genes in enteropathogenic *Escherichia coli* (Macritchie et al., 2008), *Yersinia pseudotuberculosis* (Carlsson et al., 2007; Liu et al., 2012) and *Shigella sonnei* (Mitobe et al., 2005, 2011). Therefore, it is tempting to speculate that the CpxR/A system controls biogenesis of T3SSs by sensing misfolded proteins generated during their production.

The insight from this study better explains the mechanism by which the CpxR/A system regulates the expression of the SPI-1 genes and further increases the current knowledge about the complex regulatory network governing virulence in *Salmonella*. Additionally, it reveals that deletion of *cpxA* activates CpxR-mediated gene regulation in *S.* Typhimurium.

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