ClpP deletion causes attenuation of *Salmonella Typhimurium* virulence through mis-regulation of RpoS and indirect control of CsrA and the SPI genes

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*Salmonella enterica* serovar Typhimurium requires the type III secretion system encoded by *Salmonella* pathogenicity island 1 (SPI1) and controlled by the master regulator, HilA, to penetrate the intestinal epithelium. Numerous regulators affect virulence through influence on this system, including the proteolytic component ClpP, the stationary phase regulator RpoS and the carbon-storage regulator CsrA. However, the mechanism behind the ClpP regulation is not fully understood. To elucidate this we examined differentially expressed genes in a ΔclpP mutant compared with WT using global transcriptomic analysis. SPI1 and SPI4 virulence genes were significantly downregulated in the ΔclpP mutant, whereas several RpoS-dependent genes and the fliC gene encoding flagellin were upregulated. While the ΔclpP mutant was attenuated in cell invasion, this attenuation was not present in a ΔclpP/rpoS::amp double mutant, suggesting the repression of invasion was directed through RpoS. The expression of the csrA virulence regulator was increased in the ΔclpP mutant and decreased in the rpoS::amp and ΔclpP/rpoS::amp mutants, indicating that ClpP affects the csrA expression level as well. Thus, this study suggests that ClpP affects SPI1 expression and thereby virulence indirectly through its regulation of both RpoS and CsrA.

INTRODUCTION

*Salmonella enterica* serovar Typhimurium (S. Typhimurium) penetrates the intestinal epithelium early in the infection process. The genes required for this penetration are mainly located in the *Salmonella* pathogenicity island 1 (SPI1). This 40 kb region encodes components of a type III secretion system (TTSS) and effector proteins essential for invasion (Lostroh & Lee, 2001; Schlumberger & Hardt, 2006). *Salmonella* expresses the syringe-like TTSS, and within a few seconds of host interaction, *Salmonella* triggers the injection of effector proteins into the host cell, which mediates the uptake by actin rearrangements, membrane ruffling and bacterial engulfment (Pizarro-Cerdá & Cossart, 2006).

The regulation of the SPI1 genes is complex and has been investigated intensively (Altier, 2005; Lostroh & Lee, 2001; Schlumberger & Hardt, 2006). HilA is the key regulator of SPI1 and is encoded within the island (Ahmer et al., 1999; Thijs et al., 2007). In addition to controlling SPI1 expression, HilA induces expression of SPI4 and SPI5, and represses expression of the SPI2 genes and flhD expression (Thijs et al., 2007). The expression of hilA is controlled by several regulators encoded both inside and outside SPI1 (Bajaj et al., 1996; Jones, 2005; Lucas & Lee, 2001). HilC, HilD and RtsA are central activators of hilA expression (Ellermeier et al., 2005; Lucas & Lee, 2001; Schechter et al.,...
strains, and it has been speculated that the attenuation in of rpoS-
et al. mechanism(s) ClpP affects virulence in
et al. shown that lack of
1999). Previous studies with transposon mutants have
proteins, including the stationary phase sigma factor RpoS
stressful conditions (Gur
et al. 1995; Matsui
et al. virulence, including the ClpP protease (Hensel
et al., 2003; Webb et al., 1999). In association
the main function of ClpP is
et al. 1995; Matsui
et al. virulence, including the ClpP protease (Hensel
et al., 2003; Webb et al., 1999). However, it still remains unknown exactly through which
mechanism(s) ClpP affects virulence in Salmonella.
Pearson et al. (1996) suggested that an inappropriate level
of rpoS-dependent gene expression results in avirulent
strains, and it has been speculated that the attenuation in
virulence encountered in the ΔclpP mutant is due to
abnormally high levels of RpoS (Webb et al., 1999). The
eyear studies that RpoS in Salmonella controls expression
of the virulence plasmid spv, which is important
for systemic disease in mice (Gulig & Doyle, 1993). RpoS
is important for long-term survival and persistence
in the host, whereas there is no difference between WT and rpoS
mutant for the initial 4–5 days of infection (Coynault et
al., 1996; Kowarz et al., 1994; Yoon et al., 2009). This
is consistent with results of epithelial cell line experiments
showing either no difference (Krogfelt et al., 2000; Methner
et al., 2004; Wilmes-Riesenber et al., 1997) or even
increased invasion in epithelial cells of rpoS mutants as
compared to WT (O’Cróinín & Dorman, 2007). In
proteobacteria, RpoS is the key stress regulator; however,
the role of RpoS in virulence among the same phylum is
highly variable and depends on species as well as niche
of the bacteria (reviewed by Dong & Schellhorn, 2010).

The central regulators of the flagella regulon, FlhDC,
are also a substrate for the ClpXP protease, and the expressions
of class 2 and class 3 flagella genes are upregulated in a
ΔclpP mutant due to high levels of FlhDC leading to
a hyper-flagellated phenotype (Tomoyasu et al., 2002, 2003).
The FliZ regulator is a class 2 gene encoded in the fliAZ
operon together with the regulator of class 3 flagella genes,
FliA. FliZ is a positive regulator of SPI1 expression through
post-transcriptional control of HiiD (Kage et al., 2008; Lin
et al., 2008). Thus, it was also speculated that FliZ is the
possible link between ClpP and virulence expression in
Salmonella (Kage et al., 2008; Lin et al., 2008).

Given the uncertainty of the exact mechanism by which
ClpP protease causes attenuation in S. Typhimurium,
we aimed to reinvestigate the role of the ClpP protease
in virulence. Initially, we verified that a ΔclpP mutant was
attenuated in virulence and non-motile, and that these
phenotypes were due to lack of the proteolytic component,
ClpP, as opposed to downstream effects on the chaperone
ClpX. We used a transcriptomic approach to identify
differentially expressed genes in the ΔclpP mutant
compared to the WT, and by mutational analysis we
characterized mutants in selected genes that were found to
be differentially expressed. Based on the results, we suggest
that ClpP affects virulence in S. Typhimurium through
indirect regulation of the SPI regulon by controlling RpoS
levels as well as the CsrA system.

METHODS

Bacterial strains and growth conditions. The bacterial strains
used in this study are listed in Table 1. Overnight cultures
were grown aerobically in Lennox broth (Maloy et al., 1996) at 37 ºC for 18 h
with shaking and stored thereafter in Lennox broth containing 15 %
glycerol at −80 ºC. To prepare the cultures, frozen stock cultures
were inoculated on Lennox agar and grown at 37 ºC overnight.
The following concentrations of antibiotics were used when appropriate:
50 µg ampicillin ml−1, 50 µg kanamycin ml−1, 100 µg spectinomycin
ml−1 and 20 µg streptomycin ml−1. To investigate the growth in
liquid culture, overnight cultures were diluted 5000-fold in Lennox
broth and incubated at 37 ºC with shaking. The increase in cell
numbers was measured by optical density at 600 nm (OD600).

Construction of a ΔcsrA mutant and double mutants in clpP and rpoS. The Δ Red
recombination described by Datsenko & Wanner (2000) was used to create an in-frame deletion mutant in the
csrA gene of S. Typhimurium C5 WT. In brief, 60 nt primers, pcsrA-
P1 (5′-GACCGAAGCTTTCGTTTATCGCGTGCGCGTT-ATCTCCCCCTTTA-3′) and pcsrA-P2 (5′-CTCGCTGTTGTCAGGCGAGGTCT-GCTCACCAGAAATGAGGACCCGAAACCCCTTACATATGAAAT-
ATCTAATCCCTTTA-3′), were used to construct a 1.6 kb linear PCR
product encoding a kanamycin resistance with pKD4 as template. The
primers were constructed based on the published sequence of S.
Typhimurium LT2 and contained a 40 nt homology extension and a
20 nt priming sequence for the pKD4 plasmids P1 and P2. The
resulting PCR product was purified with a QIAquick Gel Extraction kit
(Qiagen). Electrocompetent S. Typhimurium C5 carrying the pKD46
recombinase (1 mM L-arabonose) and transformed with approxi-
mately 800 ng of PCR product. Cells were added to 1 ml of Lennox
broth and after 1 h incubation at 37 ºC the cells were plated on
selective media and incubated overnight at 37 ºC. The insertion of the
kanamycin cassette was confirmed by PCR using primer k1, k2, pcsrA-up
and pcsrA-down (5′-GACCCATATCAACAGTGAGG-3′, 5′-CGGGTGGC-
CTGAATGAACTGC, 5′-ACCATAGGTTTGTGCTCAG-3′ and 5′-
GGACCCATATCAACAGTGAGG-3′, respectively). The ΔcsrA::kan
mutant was transferred into LT1100, LT1104 and LT1105 to create
double and triple mutants with clpP and rpoS by transduction with
P22HT105/int+ 201, as described by Maloy et al. (1996).

Cell invasion assays. Two different in vitro virulence assays were
employed. In order to mimic the initial phase of infection, adhesion
and invasion into epithelial cells were determined in Int407 cells (Health Proction Agency Culture Collection 85051004), essentially as described by Aabo et al. (2002). Cells were grown to the stationary phase in Lennox broth, harvested and in the stationary phase were regrown to the exponential phase and used to challenge cells in an m.o.i. of 100 in 24-well microtitre plates. Plates were centrifuged at 1000 g for 3 min and the cells were washed and incubated in essential medium (MEM) provided from Invitrogen/Gibco. Bacteria were grown to the stationary phase in Lennox broth, harvested and plates were centrifuged at 1000 g for 3 min and incubated at 37 °C for 16–18 h. Results were expressed as a percentage of recovered bacteria relative to the WT strain with two biological replicates each having three technical replicates.

Survival in macrophages was mimicked using J774A.1 macrophage cells (Health Proction Agency Culture Collection 91051511), essentially as described by Chadfield et al. (2003). In brief, bacteria were grown to the stationary phase in Lennox broth, harvested and resuspended in saline buffer, opsonized with mouse serum and added to macrophages in an m.o.i. of 10:1 in 24-well microtitre plates. Plates were centrifuged at 1000 g for 3 min and incubated at 37 °C, 5% CO₂ in RPMI (Roswell Park Memorial Institute) medium with 10% heat-inactivated FBS for 30 min. After removing the media and washing the cells, cells were incubated in the RPMI with FBS and 100 µg ml⁻¹ gentamicin for 1 or 2 h. For longer incubations, the medium was removed, and the cells were washed and incubated in RPMI with FBS and 25 µg ml⁻¹ gentamicin. Bacterial counts were determined as described above. The assays were performed in at least two independent replicates, each with three technical replicates.

Results are expressed as log₁₀ c.f.u. per well of each strain recovered from the cell culture.

**Animal experiments.** Female BALB/c or C57/BL6 mice (6–8 weeks old) were used for the animal infection experiments. Two types of experiments were used to compare WT and clpP mutant. BALB/c mice per bacterial strain were infected intragastrically with 5 × 10⁵ c.f.u. Six to ten days post-infection, the mice were sacrificed by cervical dislocation and their spleens were dissected. The spleens were homogenized and 10-fold serial dilutions of the homogenates were plated on Lennox agar. Colonies were counted after overnight incubation at 37 °C. In complementation experiments performed to rule out that differences between WT and mutant bacteria were caused by random mutations, and where the purpose was just to assess that the complemented strain was not statistically less virulent than the WT strain, five C57/BL6 mice were challenged intragastroically with 5 × 10⁵ c.f.u. of a 50:50 mixture of WT and complemented clpP+ mutant, and the ratio of WT strain to the complemented mutant was determined in spleens 5 days post-infection, as previously described (Jelsbak et al., 2012). This assay type was chosen to minimize the use of experimental animals. Mice experiments were conducted with permission from the Animal Experiments Inspectorate (http://www.foedevarestyrelsen.dk) in accordance with Danish law, licence number 2009/561-1675. The initial comparison of virulence was conducted as two biological replicates, each having three technical replicates and invasion into epithelial cells were determined in Int407 cells (Health Proction Agency Culture Collection 85051004), essentially as described by Aabo et al. (2002). Cells were grown to the stationary phase in Lennox broth, harvested and in the stationary phase were regrown to the exponential phase and used to challenge cells in an m.o.i. of 100 in 24-well microtitre plates. Plates were centrifuged at 1000 g for 3 min and incubated at 37 °C for 16–18 h. Results were expressed as a percentage of recovered bacteria relative to the WT strain with two biological replicates each having three technical replicates.

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**DNA microarray.** Global expression profiles were investigated by DNA microarray. Late exponential cells of C5 and the ΔclpP mutant were obtained by diluting the overnight cultures 5000-fold and
incubating at 37 °C with agitation (130 r.p.m.). At an OD$_{600}$ of 0.7 (exponential phase), cells were harvested by centrifugation for 1 min at 14 000 g. Total RNA was extracted immediately afterwards with the RNaseasy Lipid Tissue kit (Qiagen). RNA was reverse transcribed to cDNA and labelled with Cyanine 3 (Cy3)-dCTP or Cyanine 5 (Cy5)-dCTP with the LabelStar Array kit (Qiagen). After labelling, the Cy3- or Cy5-labelled cDNA was purified with the LabelStar purification kit (Qiagen). Equal amounts of Cy3- and Cy5-labelled cDNA were mixed and hybridized to microarray slides (Matisaviciova et al., 2007) at 55 °C overnight using the SlideHyb buffer (Ambion) according to the manufacturer’s instructions. After hybridization, the slides were washed twice in saline sodium citrate (SSC) with 0.5 % SDS for 15 min, then twice in 0.5 % SSC with 0.3 % SDS for 15 min. The slides were dried by centrifugation at 1000 g for 5 min and then scanned using a ScanArray Express Microarray Scanner (Perkin Elmer). Three biological replicates were performed with dye-swap hybridization. Statistical significance for the difference in expression levels between ΔclpP mutant and WT was assessed using the ranking products algorithm, a Perl script implementation (Breitling et al., 2004), using 1000 permutations and a false discovery rate with cut-offs of <20 %, giving a 2.69-fold cut-off as the minimum.

**Northern blotting.** For transcriptional analysis, total RNA was purified from late exponential cells (OD$_{600}$ ≈ 0.65). The cells were harvested by centrifugation at 10 000 g for 2 min and the pellet was stored at −80 °C. Total RNA extraction and Northern blotting were performed as described by Frees et al. (2005). Total RNA (5 μg) was loaded on the gel, and controlled for equal amounts loaded by staining with ethidium bromide. Hybridization probes were generated by PCR from chromosomal DNA of S. Typhimurium C5 using gene specific primers for the clpP (5'-ATGTCATACACGGGCCAGC and 5'-AGATGGACCCGCTGATGTCG-3'), clpP (5'-AACAGGACCTGC- CTGAGAAGA-3' and 5'-TCGTTGACAGGACCTAGC-3'), ctsA (5'-ATGCTGATTCTGACCTGC-3' and 5'-TTAGTAACTGGAC- TGCCTGG-3'), hld (5'-AGACGGATTACCTACCAAAATCTTTATG-3' and 5'-TGAGCGGAGCTAAGGATGATC-3'), pspC (5'-CGTTT- ATATGACCGCGTTAGG-3' and 5'-GCTTCTTTCAATGGCTTTACG- 3'), fbD (5'-AATGCTGATACATCGCCATGTTGCT-3' and 5'-CTTCTCT- TAGCGCGCTGATC-3') and fliC (5'-AGGACAAACCCTGAC- ATC-3' and 5'-AACACCTGCTGTCAAT-3'). The probes were labelled with [α-32P]dCTP, and hybridization was visualized with a STORM 840 Phosphorimager (Molecular Dynamics). Quantification was performed using ImageQuant Version 5.0 (Molecular Dynamics).

**Western blot analysis.** For the analysis of RpoS expression, cell lysates were prepared by centrifugation of 2 ml cultures grown to OD$_{600}$ 0.65 at 37 °C. The cells were then stored at −80 °C until analysed. Cell pellets were resuspended in lysis buffer [50 mM Tris/ HCl (pH 8.0), 100 mM NaCl, 5 mM DTT, 1 mM PMSF] and lysed by a FastPrep FP120 instrument (Bio 101, ThermoSavent) for five rounds of 30 s each at speed 6.5, followed by 2 min on ice. Cell debris was removed by centrifugation, 8500 g for 15 min. The protein concentration was determined by using a Bio-Rad protein assay (Bio-Rad Laboratories), and 5 μg protein was separated on NuPAGE 4–12 % Bis-Tris gels (Invitrogen) using a MOPS buffer (Invitrogen). The gels were stained with Coomassie blue using Safestain (Invitrogen) to check for equal amounts of protein or transferred onto a polyvinylidene difluoride membrane (Invitrogen) using an XCell SureLock Mini-Cell system (Invitrogen), as recommended by the supplier. RpoS was detected using *Escherichia coli* RpoS monoclonal antibodies (NeoClone Biotechnology) at a 1:1000 dilution and the WesternCleave Chemiluminescent Anti-Mouse kit (Invitrogen).

**Swimming assay.** To assess swimming of the strains, the overnight cultures were adjusted to OD$_{600}$=0.2. One microlitre of this dilution was spotted onto Lennox agar with 0.3 % Difco Bacto agar and the plates were incubated at 37 °C for 5 h.

### RESULTS

**In-frame deletion of clpP in S. Typhimurium C5 causes attenuation in virulence**

Previously, *S. Typhimurium* clpP mutants constructed by transposon and insertion mutagenesis were shown to be avirulent in mice (Hensel et al., 1995; Matsui et al., 2003; Webb et al., 1999; Yamamoto et al., 2001). To verify that this attenuation was due to the lack of ClpP, and not caused by a polar effect on the downstream chaperone gene *clpX*, the virulence of an in-frame *clpP* deletion mutant, LT1100, was compared to that of the WT C5 in *in vitro* and *in vivo* assays. Previously published data show that the expression of *clpX* is unaffected in the LT1100 strain, which has a 240 bp in-frame deletion, including the three essential amino acids in the proteolytic site (Thomsen et al., 2002). The ΔclpP mutant was significantly (*P*<0.0001) attenuated in adhesion and invasion of the human-derived epithelial cell line Int407 (Fig. 1a, b). This phenotype was complemented by providing *clpP* into the mutated strain. In contrast, the ΔclpP mutant was not significantly different from the WT strain in uptake by the macrophage cell line J774, and there was no difference in survival after 24 and 48 h in the macrophage cell line between the WT and mutant (data not shown). Uptake in epithelial cells is an active process requiring expression of SPI1 (Schlumberger & Hardt, 2006), and the low invasion suggests a changed function of the SPI1-encoded TTSS. In contrast, uptake in macrophages is a passive process that does not involve expression of SPI1 or SPI2 genes.

When mice were challenged with *S. Typhimurium* C5 intragastrically, all mice had to be sacrificed due to symptoms of illness after 5–6 days, and high levels (>10^8 c.f.u. per spleen) of C5 were recovered from the spleen. In contrast, bacterial counts were below the detection level (100 c.f.u. per spleen) in mice challenged with the ΔclpP mutant. To verify that the attenuation was due to the absence of the *clpP* gene, a competitive challenge experiment was performed. In the *clpP*+ strain, the ΔclpP allele was replaced by *clpP*:·Tn10dTc, giving a functional *clpP* gene. This complemented *clpP*+ mutant was reisolated in a slightly higher level than the WT C5 (competitive index=1.88), demonstrating that the attenuation was caused by the *clpP* mutation. These results confirm that ΔclpP mutants are severely attenuated in virulence and demonstrate that this effect is not caused by secondary mutations. Furthermore, the results suggest that the effect on virulence is related to the initial steps where the bacterium interacts with epithelial cells, most likely during intestinal invasion. A likely explanation could be mis-expression of SPI1-encoded virulence genes.

**Identification of genes affected by the ClpP protease**

To identify genes that are controlled directly or indirectly by the ClpP protease, we performed a transcriptomic...
Based on the transcriptomic analysis and in line with Bearson et al. (1996), we assumed that the attenuation of the ΔclpP mutant was due to an elevated RpoS level, which indirectly caused a decreased virulence gene expression. To test this hypothesis, we measured the hilD expression of ΔclpP, ΔclpP/rpoS::amp and rpoS::amp mutants (Fig. 2a). The hilD expression, in confirmation of the array results, was reduced in the clpP mutant and returned to WT level in the rpoS single and double mutants, indicating that the repression of SPI1 expression is directed through RpoS and potentially caused by the abnormal RpoS level. Next, we aimed to identify the mechanism by which RpoS mis-regulation affects the SPI expression as previously suggested.

The ClpP protease regulates CsrA indirectly through RpoS

In enterohaemorrhagic E. coli, the ClpXP represses the expression of the virulence-associated TTSS through an unidentified repressor, which is controlled by RpoS (Iyoda & Watanabe, 2005; Tomoyasu et al., 2005). A similar regulatory network is found in Dickeya dadantii, where the unknown regulator was identified as RsmA, a homologue of CsrA in Salmonella (Li et al., 2010). Furthermore, RpoS affects the expression of csrA or rsmA in S. Typhimurium, E. coli and Erwinia carotovora (Dong & Schellhorn, 2009; Mukherjee et al., 1998). Hence, we speculated that ClpP affected the virulence expression indirectly through the csrA system.

Initially, we investigated the expression of csrA in ΔclpP and rpoS::amp mutants by Northern blot analysis (Fig. 3a). The expression of csrA was slightly increased in the ΔclpP mutant, but returned to WT level in the complemented clpP+ strain. Consistent with the Northern blot analysis, the csrA expression was 1.7-fold induced in the transcriptomic analysis (Table S1, available in Microbiology Online). Since a 2.69-fold cut-off was used for the transcriptomic data, csrA was not identified as significantly upregulated by that method. However, studying regulator expression, we would expect a less than twofold change can cause a phenotypic effect. In the absence of rpoS, in both the rpoS::amp and ΔclpP/rpoS::amp mutants, the csrA expression was reduced compared to the WT. Finally, the expression levels of csrA correlated well with the protein level of RpoS in the ΔclpP mutant and was restored in the complemented clpP+ strain (Fig. 3d). Together these results indicate that the csrA expression level in Salmonella is controlled by RpoS, consistent with previous observations in S. Typhimurium, E. coli and Er. carotovora (Dong & Schellhorn, 2009; Mukherjee et al., 1998).

Detailing the interactions in the ClpP-RpoS-CsrA network

To investigate the hypothesis that ClpP is involved in virulence regulation through RpoS and CsrA, and to detail the interaction between the major players in this regulatory

Fig. 1. Adhesion and invasion of S. Typhimurium (ST) C5 and mutants in epithelial Int407 at 1 h (a) and 2 h (b). Results are expressed as percentage of recovered bacteria relative to the WT strain with two biological replicates each having three technical replicates. Asterisk denotes P<0.05 when comparing mutant with WT.
network, a ΔcsrA::kan mutant was constructed. The ΔcsrA::kan mutation was transduced into the ΔclpP, rpoS::amp and ΔclpP/rpoS::amp mutants to create double and triple mutants. The ΔcsrA::kan mutant had severe growth defects at 37 °C and suppressor mutants with restored growth arose spontaneously. Similarly to the ΔcsrA::kan mutant, the double mutant rpoS::amp/ΔcsrA::kan and the triple mutant ΔclpP/rpoS::amp/ΔcsrA::kan had severe growth defects and suppressor mutants arose spontaneously. The ΔclpP/ΔcsrA::kan mutant was even further affected and, during the construction of the mutant, only a few transductants were obtained (data not shown). Similar growth defects were reported for the ΔcsrA::cam mutant in S. Typhimurium ATCC14028s and in SL1344 (Altier et al., 2000a; Martinez et al., 2011) and Altier and colleagues showed no difference in the global expression profiles between the ΔcsrA::cam mutant and a spontaneous suppressor mutant using promoter-fusions and DNA microarray (Altier et al., 2000a; Lawhon et al., 2003). To overcome the uncertainty of working with a mixed population of original and spontaneous suppressor mutants, we chose to work with ΔcsrA::kan suppressor mutants, hereafter called ΔcsrA::kan (sup).

Previous work had shown increased expression of rpoS in Pseudomonas fluorescens and Legionella pneumophila csrA mutants (Forsbach-Birk et al., 2004; Heeb et al., 2005). Initially, we investigated the effect of CsrA on the expression of the clpP and rpoS genes in S. Typhimurium C5 to elucidate if regulatory loops are present in Salmonella as well. No major effects were observed on the expression of clpP in the absence of rpoS and csrA (Fig. 3b), but in the rpoS::amp/ΔcsrA::kan (sup) mutant the clpP level was slightly lower than the WT level. In the ΔcsrA::kan (sup) mutant, the level of RpoS protein was increased (Fig. 3d), suggesting both CsrA and ClpP appear to affect the level of RpoS protein.

Table 2. Genes upregulated in a ΔclpP mutant of S. Typhimurium C5 during late exponential growth in Lennox broth at 37 °C with shaking.

<table>
<thead>
<tr>
<th>Name no</th>
<th>Gene name*</th>
<th>Gene function*</th>
<th>Fold change†</th>
<th>RpoS-dependent‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM0384</td>
<td>psiF</td>
<td>Phosphate starvation-inducible protein</td>
<td>3.47</td>
<td>W</td>
</tr>
<tr>
<td>STM0759</td>
<td>ykgS</td>
<td>Hypothetical protein</td>
<td>3.22</td>
<td>PW</td>
</tr>
<tr>
<td>STM0973</td>
<td>pflB</td>
<td>Pyruvate formate lyase I</td>
<td>3.27</td>
<td>D</td>
</tr>
<tr>
<td>STM1120</td>
<td>ycfD</td>
<td>Pseudogene; in-frame stop following codons 5 and 21</td>
<td>3.34</td>
<td></td>
</tr>
<tr>
<td>STM1121</td>
<td>ymdF</td>
<td>Putative cytosolic protein</td>
<td>3.65</td>
<td></td>
</tr>
<tr>
<td>STM1135</td>
<td>ycdW</td>
<td>Putative oxidoreductase</td>
<td>3.63</td>
<td></td>
</tr>
<tr>
<td>STM1261</td>
<td></td>
<td>Putative cytosolic protein</td>
<td>3.51</td>
<td></td>
</tr>
<tr>
<td>STM1290</td>
<td>gapA</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase A</td>
<td>3.20</td>
<td>D</td>
</tr>
<tr>
<td>STM1463</td>
<td>add</td>
<td>Adenosine deaminase</td>
<td>3.84</td>
<td></td>
</tr>
<tr>
<td>STM1513</td>
<td></td>
<td>Putative cytosolic protein</td>
<td>6.63</td>
<td></td>
</tr>
<tr>
<td>STM1563</td>
<td>osmC</td>
<td>Putative resistance protein, osmotically inducible</td>
<td>3.57</td>
<td>DPW</td>
</tr>
<tr>
<td>STM1587</td>
<td>yncD</td>
<td>Paral putative outer membrane receptor, iron complex</td>
<td>5.58</td>
<td></td>
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<tr>
<td>STM1724</td>
<td>trpD</td>
<td>Anthranilate phosphoribosyltransferase</td>
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<tr>
<td>STM1729</td>
<td>yclP</td>
<td>Putative cytosolic protein</td>
<td>3.84</td>
<td>PW</td>
</tr>
<tr>
<td>STM1770</td>
<td>chaB</td>
<td>Cation transport regulator;</td>
<td>3.29</td>
<td>W</td>
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<tr>
<td>STM1804</td>
<td>ycgB</td>
<td>Putative cytosolic protein</td>
<td>5.88</td>
<td>P</td>
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<tr>
<td>STM1881</td>
<td>yebF</td>
<td>Putative periplasmic protein</td>
<td>3.43</td>
<td>W</td>
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<tr>
<td>STM1928</td>
<td>otsA</td>
<td>Trehalose-6-phosphate synthase</td>
<td>3.32</td>
<td>PW</td>
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<tr>
<td>STM1959</td>
<td>fliC</td>
<td>Flagellar biosynthesis, filament structural protein</td>
<td>3.11</td>
<td>D</td>
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<tr>
<td>STM2141</td>
<td>fbaB</td>
<td>Fructose-bisphosphate aldolase, class I</td>
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<td>DW</td>
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<td>STM2407</td>
<td>ypeC</td>
<td>Putative periplasmic protein</td>
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<td>STM2596</td>
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<td>Minor tail-like protein</td>
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<td></td>
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<tr>
<td>STM3118</td>
<td></td>
<td>Putative acetyl-CoA hydrolase</td>
<td>3.36</td>
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<tr>
<td>STM3119</td>
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<td>Putative monoamine oxidase</td>
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<tr>
<td>STM3228</td>
<td>yjaC</td>
<td>Putative periplasmic protein</td>
<td>3.24</td>
<td>P</td>
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<tr>
<td>STM3335</td>
<td>yhcH</td>
<td>Putative cytosolic protein</td>
<td>2.69</td>
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<tr>
<td>STM3585</td>
<td>yhlF</td>
<td>Putative ABC superfamily transport protein, antibiotic transport system permease protein</td>
<td>3.16</td>
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</tbody>
</table>

*Gene name and function according to GenomeNet (http://www.genome.jp).
†Differential gene expression according to rank product. Fold change calculated as clpP mutant/WT.
‡RpoS-dependent genes: genes indicated with D, P or W are identified in Dong et al. (2008), Dong & Schellhorn (2009), Patten et al. (2004) or Weber et al. (2005), respectively.
Table 3. Genes downregulated in a ΔclpP mutant of S. Typhimurium C5 during late exponential growth in Lennox broth at 37 °C with shaking

NADH, nicotinamide adenine dinucleotide.

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene name*</th>
<th>Gene function*</th>
<th>Fold change†</th>
<th>SPI gene§</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM0492</td>
<td>ybaL</td>
<td>Putative CPA2 family transport protein; monovalent cation:H + antipporter-2</td>
<td>2.86</td>
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<tr>
<td>STM1090</td>
<td>pipC (sigE)</td>
<td>Pathogenicity island-encoded protein C</td>
<td>4.76</td>
<td>SPI5</td>
</tr>
<tr>
<td>STM1328</td>
<td></td>
<td>Putative outer membrane protein</td>
<td>4.17</td>
<td></td>
</tr>
<tr>
<td>STM1398</td>
<td>sseB</td>
<td>Translocation machinery component</td>
<td>3.44</td>
<td>SPI2</td>
</tr>
<tr>
<td>STM1458</td>
<td>ydgM</td>
<td>Putative alternative beta subunit of Na + -transporting NADH; ubiquinone oxidoreductase</td>
<td>3.70</td>
<td></td>
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<tr>
<td>STM1629</td>
<td></td>
<td></td>
<td>3.57</td>
<td></td>
</tr>
<tr>
<td>STM2875</td>
<td>hiiD</td>
<td>Regulatory helix–turn–helix proteins, araC family</td>
<td>3.85</td>
<td>SPI1</td>
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<tr>
<td>STM2881</td>
<td>iacP</td>
<td>Putative acyl carrier protein; K02078 acyl carrier protein</td>
<td>3.85</td>
<td>SPI1</td>
</tr>
<tr>
<td>STM2884</td>
<td>sipC</td>
<td>Translocation machinery component</td>
<td>3.57</td>
<td>SPI1</td>
</tr>
<tr>
<td>STM2892</td>
<td>invI</td>
<td>Surface presentation of antigens; secretory proteins</td>
<td>3.85</td>
<td>SPI1</td>
</tr>
<tr>
<td>STM2893</td>
<td>invI</td>
<td>Surface presentation of antigens; secretory proteins</td>
<td>4.00</td>
<td>SPI1</td>
</tr>
<tr>
<td>STM2894</td>
<td>invC</td>
<td>Surface presentation of antigens; ATP synthase in TTSS</td>
<td>4.00</td>
<td>SPI1</td>
</tr>
<tr>
<td>STM4257</td>
<td>siiA</td>
<td>Hypothetical protein</td>
<td>6.25</td>
<td>SPI4</td>
</tr>
<tr>
<td>STM4258</td>
<td>siiB</td>
<td>Putative methyl-accepting chemotaxis protein</td>
<td>6.67</td>
<td>SPI4</td>
</tr>
<tr>
<td>STM4259</td>
<td>siiC</td>
<td>Putative ABC exporter outer membrane component</td>
<td>7.69</td>
<td>SPI4</td>
</tr>
<tr>
<td>STM4260</td>
<td>siiD</td>
<td>Predicted cation efflux pump</td>
<td>5.56</td>
<td>SPI4</td>
</tr>
<tr>
<td>STM4261</td>
<td>siiE</td>
<td>Putative inner membrane protein</td>
<td>7.14</td>
<td>SPI4</td>
</tr>
<tr>
<td>STM4262</td>
<td>siiF</td>
<td>Putative ABC-type bacteriocin/lantibiotic exporter, contains an N-terminal double-glycine peptidase domain</td>
<td>3.45</td>
<td>SPI4</td>
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<tr>
<td>STM4293</td>
<td>yidB</td>
<td>Putative integral membrane protein</td>
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<tr>
<td>STM4314</td>
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<td>Putative regulatory protein</td>
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<tr>
<td>STM4315</td>
<td></td>
<td>Putative DNA-binding protein</td>
<td>4.00</td>
<td></td>
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</tbody>
</table>

*Gene name and function according to GenomeNet (http://www.genome.jp), expect for siiA-F operon, which is taken from Thijs et al. (2007).
†Differential gene expression according to rank product. Fold change calculated as WT/clpP mutant.
§SPI-encoded genes specified with the SPI they are encoded within.

decreased expression of SPI genes in Salmonella by indirectly controlling levels of RpoS and CsrA. To further investigate this hypothesis, we examined the expression of hiiD, a regulator of HilA in SPI1, and pipC, a SPI5 gene, in the different mutants. As mentioned above, the expression of both genes was decreased in the ΔclpP mutant (Fig. 2a, b). However, the expression level of hilD in ΔclpP/rpoS::amp, rpoS::amp, ΔcsrA::kan (sup) and the complemented clpP+ strain was similar to the expression level in the WT, suggesting that the absence of RpoS does not affect the SPI1 expression to the same extent as high RpoS levels do (Fig. 2a, b).

Phenotypic effects of rpoS and csrA deletion

To investigate whether the observed expression levels had a phenotypic effect, we investigated the adhesion and invasion of the mutants in the epithelial cell line Int407. As seen with the ΔclpP mutant, the rpoS::amp/ΔcsrA::kan (sup) mutant was attenuated in adhesion to and invasion of the Int407 epithelial cells (Fig. 1a, b), while the rpoS::amp, ΔcsrA::kan (sup) and ΔclpP/rpoS::amp mutants had similar adhesion and invasion profiles to the WT strain (Fig. 1b), and thus the expression data and the virulence data are consistent.

Similar to their involvement in virulence, ClpP, RpoS and CsrA are all involved in expression of motility (Lawhon et al., 2003; Patten et al., 2004; Tomoyasu et al., 2002, 2003). All mutants showed decreased motility in a swimming assay (Fig. 4), consistent with decreased expression of flhD in all mutants (Fig. 2c). Furthermore, the complemented strains clpP+ and csrA+ recovered motility (Fig. 4) and flhD expression (Fig. 2c). The hyperflagellated ΔclpP also showed reduced motility (Fig. 4).

DISCUSSION

The relevance of controlled proteolysis for control of gene expression and the survival of pathogenic bacteria during interaction with the host has been increasingly recognized since the discovery that several global regulatory proteins are under proteolytic control (Gottesman, 2003). In S. Typhimurium, the Lon protease represses the expression of SPI genes by degradation of the HilD and HilC regulators (Boddicker & Jones, 2004; Takaya et al., 2002, 2005).
Consistent with the results of others (Hensel et al., 1995; Webb et al., 1999), we found that the ClpP protease is also important for virulence in mice and, further, that this attenuation most likely is caused by indirect regulation of the SPI genes through RpoS and CsrA.

Our results support previous observations on the reduced virulence of ΔclpP mutants (Hensel et al., 1995; Webb et al., 1999), but they are inconsistent with Kage et al. (2008). They found that the absence of the clpPX genes in S. Typhimurium Δ3306 caused increased expression of the SPI1 virulence genes and that the ΔclpPX mutant had increased virulence compared to the WT Δ3306. This boost in virulence was suggested to be caused by increased flagellar expression, including the FltZ regulator controlling the SPI1 regulator HilD at post-transcriptional level (Kage et al., 2008). The disagreement may be attributed to the use of different WT strains or the fact that Kage et al. (2008) used a ΔclpPX double mutant, as opposed to the 240 bp in-frame deletion in the ΔclpP mutant used in this study. It would be interesting to compare a clpP mutant with a clpPX double mutant to examine if the differences observed in the studies are due to clpX or caused by strain variations.

To elucidate the role of the ClpP protease in virulence, we compared the transcriptome of C5 WT with the ΔclpP mutant. The transcriptomic analysis showed that both SPI1 and SPI4 virulence genes, which are essential for uptake of S. Typhimurium in epithelial cells (Gerlach et al., 2008;
observed in the absence of clpP cell invasion, indicating that the decreased virulence in candidate. CsrA plays a central role in the expression of virulence in S. Typhimurium (Martínez et al., 2011; Vogel, 2009) and, in agreement with other studies, our data show that RpoS controls the expression level of CsrA (Dong & Schellhorn, 2009; Mukherjee et al., 1998). This is consistent with a recent E. coli study which revealed that the P1 and P3 promoters of csrA transcription are RpoS-dependent (Yakhnin et al., 2011). In the C5 background, the csrA expression correlated with the RpoS level, i.e. increased in the clpP mutant, which has an increased RpoS level, and decreased in the rpoS::amp and ΔclpP/rpoS::amp mutants, indicating that the ClpP protease affects csrA expression through RpoS.

The transcriptomic analysis showed that in the ΔclpP mutant with an increased level of csrA, the expression levels of hilD and other SPI1 genes were low. These results are consistent with previous studies (Altier et al., 2000a; Lawhon et al., 2003; Martinez et al., 2011), showing that overproduction of csrA reduces expression of hilD and hilA. This CsrA-mediated repression of hilD is caused by binding of CsrA to the Shine–Dalgarno sequence of hilD mRNA (Martínez et al., 2011). Further, Martinez et al. (2011) found that deletion of csrA induces SPI gene expression, which correlates well with the increased expression of pipC observed in the current study. We also found that hilD expression was increased in the triple mutant ΔclpP/rpoS::amp/ΔcsrA::kan (sup), but we did not observe differential expression of hilD in the csrA single mutant when compared to the WT. In contrast, Altier et al. (2000a) showed that deletion of csrA causes repression of hilD expression. The differences might be caused by the use of three different strains, i.e. SL1344, ATCC14028 and C5, or by different growth conditions or phases used in the studies. The presence of uncharacterized suppressor mutants might affect the result altogether although this is not expected due to the results of Altier and co-workers (Altier et al., 2000a; Lawhon et al., 2003). However, identification of the suppressor mutations in csrA mutants would be of great value.

Altogether, data obtained in this study and data from previous published papers strongly suggest that a ClpP-RpoS-CsrA-HilD regulatory network is present in S. Typhimurium, as depicted in Fig. 5. Similar regulatory networks have been described in other Enterobacteriaceae, such as enterohaemorrhagic E. coli and D. dadantii (Iyoda et al., 2006; Iyoda & Watanabe, 2005; Li et al., 2010; Tomoyasu et al., 2005), indicating that this is a conserved regulatory network across different phylogenetic groups. We suggest that ClpP influences the virulence expression by balancing the RpoS level during growth and potentially during specific stress conditions, and thus contributes to the optimization of when and where the bacteria should prepare to invade the host.

The absence of clpP, along with rpoS and csrA, caused high expression of the flagellar regulon, which could theoretically induce virulence expression through the FlhZ regulator encoded in the flagella regulon and result in a ClpP-FlhDC-FlhZ-HilD regulatory cascade. Owing to the lack of FlhDC degradation in the clpP mutant, it is likely

![Fig. 4. Investigation of motility in S. Typhimurium C5 and mutants. The swimming phenotype of C5 and the isogenic ΔclpP, rpoS::amp and ΔcsrA::kan mutants when grown in motility agar (Lennox agar with 0.3 % Difco Bacto agar) at 37 °C.](http://mic.sgmjournals.org)
that the expression of FlIZ is high in this strain, consistent with measured level of fliC expression. However, the phenotypes we observed were not consistent with the expected effect of increased FlIZ levels, being increased expression of hilD and virulence expression. Thus, instead of a ClpP-FlhDC-FlIZ-HilD link, we suggest that the ClpP-RpoS-CsrA-HilD link illustrated in Fig. 5 is more plausible.

The model outlined in Fig. 5 does not account for all the possible interactions indicated in our own observations and published data. These interactions may be caused by additional regulatory feedback loops, which complicate the regulatory network. For example, the absence of CsrA caused an increased RpoS protein level in our study, indirectly through Hfq.

The model outlined in Fig. 5 suggests that the virulence effect of ClpP is primarily through the indirect effect on SPI1 regulation. Two other possible mechanisms have previously been discussed. The reduced stress tolerance of a clpP mutant, which is caused by the inability to degrade denatured proteins, may affect the survival in vitro and in vivo and thereby influence virulence (Thomsen et al., 2002). From our data it is not possible to rule this out. Further, the hyper-flagellated phenotype of ΔclpP mutants might affect virulence, as flagella are recognized by Toll-like receptors (Iqbal et al., 2005). In a recent publication, a flic mutant of S. Dublin was provided with flic of S. Typhimurium in trans. This caused a hyper-flagellated phenotype similar to a clpP deletion mutant. This strain is highly attenuated after both oral and intraperitoneal challenge, supporting that hyper-flagellation can be the cause of attenuation (J.E. Olsen, unpublished results).

The expression of the flagella regulon in the ΔcsrA::kan (sup) mutant in S. Typhimurium C5 differed from the reported expression in a csrA mutant in S. Typhimurium ATCC14028, potentially due to differences between the two WT strains. The csrA mutant S. Typhimurium ATCC14028 is non-motile (Lawhon et al., 2003), whereas a ΔcsrA::kan (sup) mutant S. Typhimurium C5 has reduced motility. The expressions of both virulence genes and genes involved in motility are complex and many factors affect these, e.g. Lon, SirA/BarA, csrB and csrC. The non-coding small RNAs CsrB and CsrC control the level of CsrA; thus, deletion of the csrA gene will affect the fine-tuned balance of the csr system.

In conclusion, we found that the ClpP protease is involved in virulence in S. Typhimurium through indirect regulation of SPI1 genes. It directly controls the level of the alternative sigma factor, RpoS, at the post-translational level, and, through this, the ClpP protease controls the level of csrA expression and thereby the expression of hilD, hilA and the virulence gene.

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