Regulation of htrA expression in Yersinia enterocolitica

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Abstract

In Escherichia coli, envelope stress is regulated by the alternative sigma factor σE and the two-component regulatory system CpxRA. Both systems overlap in the transcriptional regulation of the htrA gene encoding a protease that degrades misfolded proteins in the periplasm. In Yersinia enterocolitica, HtrA is important for intracellular survival and full virulence in animal models. Here we show that regulation of htrA expression in Y. enterocolitica is dependent on CpxR and putatively also on RpoE. However, the stimuli inducing both systems were different from E. coli. Most strikingly, we found that overproduction of an outer membrane protein, a typical stimulus for the induction of htrA expression via RpoE in E. coli, led to the induction of htrA expression via the Cpx system in Y. enterocolitica. Interestingly, a Y. enterocolitica CpxR mutant strain was not impaired for virulence in a mouse model of infection.

Keywords: Extracytoplasmic stress; Gene regulation; HtrA

1. Introduction

In Escherichia coli, two partially overlapping systems regulate the stress response to misfolded proteins in the bacterial envelope (inner membrane, periplasm and outer membrane). These include the alternative sigma factor σE, and the two-component regulatory system CpxRA [1]. σE (RpoE) belongs to the family of extracytoplasmic function sigma factors that respond to extracytoplasmic stimuli [2]. In addition to being induced by general stresses such as heat and ethanol, the σE regulon responds to overproduction of outer membrane proteins (OMPs) and misfolded proteins produced in the periplasm [3,4]. The activity of σE is controlled by RseA and RseB which are encoded by the rpoErseABC operon. RseA acts as an anti-sigma factor that binds and thereby inhibits σE under non-inducing conditions [5,6].

The Cpx response to extracytoplasmic stress is mediated by a two-component regulatory system made up of the sensor histidine kinase CpxA and the response regulator CpxR [7]. In the current model for E. coli, sensing of misfolded proteins occurs via CpxA, which is inhibited under non-inducing conditions by the binding of the periplasmic protein CpxP. Under inducing conditions CpxP is titrated away, leading to activation of the Cpx response [8,9]. The two best characterized activation signals of the Cpx system in E. coli are the overproduction of the envelope lipoprotein NlpE and overproduction of P pilus subunits in the absence of their cognate periplasmic chaperone [10–12]. In addition, the Cpx system has been shown to be involved in surface sensing, biofilm formation and invasion into epithelial cells [13–15].

Both the Cpx and the σE system regulate the expression of proteins involved in folding catalysis, catalysis of disulfide bond formation and degradation of misfolded proteins of the bacterial envelope [16,17]. The systems overlap in the regulation of expression of htrA [18]. The HtrA protein, also known as DegP in E. coli, is an envelope-associated serine protease degrading misfolded proteins.

Recent evidence suggests a role for the σE and Cpx systems in virulence of E. coli and other pathogens. In E. coli, Cpx is involved in phase variation of pyelonephritis-associated pilus expression (encoded by pap) and pos-

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sibly regulation of other virulence determinants [12]. Activation of the RpoE ortholog AlgU in *Pseudomonas aeruginosa* is responsible for the conversion to mucoidy, a phenotype which coincides with the establishment of chronic respiratory infections in cystic fibrosis patients [19]. Similarly, a *Salmonella enterica* serovar Typhimurium *rpoE* mutant strain was highly attenuated for virulence in mice [20], further stressing the importance of the *σ^E* regulon in virulence. In addition, HtrA seems to be important for virulence of a variety of microorganisms including *S. enterica* serovar Typhimurium [21,22], *Yersinia enterocolitica* [23] and *Yersinia pestis* [24], and for intracellular survival of *Brucella abortus*, *Bartonella henselae*, *Y. enterocolitica*, *Legionella pneumophila*, *S. enterica* serovar Typhimurium, and *Haemophilus influenzae* [25–30].

*Y. enterocolitica* is a common pathogen for humans causing a variety of gastrointestinal syndromes. During an infection, the bacteria show a tropism for lymphoid tissue, where they survive and replicate extracellularly. This is mediated by a 70-kb virulence plasmid which encodes a type III secretion system that interferes with the immune response of the host [31,32]. In addition to the virulence plasmid multiple chromosomally encoded proteins are important for full virulence of *Y. enterocolitica* [33].

In a recent screen for *Y. enterocolitica* genes expressed in the Peyer’s patches of infected mice we identified the *rpoE* gene encoding the alternative extracytoplasmic function sigma factor *σ^E* [34]. The gene was cloned and its transcriptional regulation was analyzed [35]. In this study, we analyze the role of the Cpx and the RpoE systems in the regulation of *htrA* expression in *Y. enterocolitica*.

### Table 1

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

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<th>Strain or plasmid</th>
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<td><strong>2. Materials and methods</strong></td>
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2.1. **Bacterial strains, plasmids, and media**

Bacterial strains and plasmids used in this study are described in Table 1. All strains were grown in Luria–Bertani (LB) broth or agar plates at 26°C (*Y. enterocolitica*) or 37°C (*E. coli*) unless otherwise indicated. Antibiotics were used in the following concentration where appropriate: ampicillin, 100 μg ml⁻¹; kanamycin, 100 μg ml⁻¹ (*Y. enterocolitica*) or 50 μg ml⁻¹ (*E. coli*); chloramphenicol, 12.5 μg ml⁻¹ (*Y. enterocolitica*) or 25 μg ml⁻¹ (*E. coli*); nalidixic acid, 20 μg ml⁻¹.

2.2. **Construction of mutant strains**

For the construction of the *Y. enterocolitica* *cpxR::pEP185.2* strain, an internal fragment of the *cpxR* gene was amplified by polymerase chain reaction (PCR) using primers cpx2 (5'–CCATCGATCCTATATTACCC–3').
GTGGATGATGACCCG-3′) (ClaI linker is underlined) and cpx3 (5′-GAAGATCTCAAGCACGGCATCCAGCTCAC-3′) (BglII linker is underlined) with chromosomal DNA of JB580v as template. After digestion with Clal and BglII, the fragment was cloned into ClaI/BglII-digested pEP185.2 to result in pEP-cpx2/3. This plasmid was integrated onto the Y. enterocolitica chromosome by homologous recombination with selection for chloramphenicol. The presence of the cpxR::pEP185.2 mutation was confirmed by Southern blot analysis (data not shown).

For the construction of the Y. enterocolitica ΔcpxR strain, the cpxR gene was amplified by PCR using the primers cpx8/2 (5′-CTCGAGTGTTCGGTCAGA-3′) (the XhoI linker is underlined) and cpx9 (5′-GAAGATCTGGCAGATAAAGTTACGCAC-CA-3′) (the BglII linker is underlined) with chromosomal DNA of JB580v as template. The PCR product was digested with XhoI and BglII and cloned into XhoI/BamHI-digested pBluescript II KS+ to result in pGH8. This plasmid was digested with NsiI and religated, leading to the deletion of ~590 nt internal to cpxR, and resulting in plasmid pGH9. After digestion of pGH9 with XhoI and XhoI, an ~800-bp fragment was subcloned into XhoI/ XhoI-digested pEP185.2 to result in pEP-cpxΔ. This plasmid was integrated onto the Y. enterocolitica chromosome by homologous recombination with selection for chloramphenicol resistance, and a merodiploid ΔcpxR-cpxR+ strain was the result. This was confirmed by Southern blot analysis (data not shown). Subsequently, cycloserine enrichment was used to isolate chloramphenicol-sensitive derivatives. The presence of the ΔcpxR mutation was confirmed by PCR and Southern blot analysis (data not shown).

2.3. Construction of the Φ(htrA-lacZYA) and Φ(rpoE-lacZYA) fusion strains

The htrA::lacZYA fusion was constructed by amplifying an approximately 600-bp fragment corresponding to the 5′ end of the htrA gene and its upstream region by PCR using the primers htrAl (5′-GCTCTAGACTGAT- TATTTGTTTCTCTGACTTC-3′) (the XhoI site is underlined) and htrA2 (5′-CAAGCATCAATAATTA- CGCCAG-3′) with chromosomal DNA of JB580v as template. The PCR product was treated with T4 DNA polymerase, phosphorylated with polynucleotide kinase, digested with XhoI, and ligated with XhoI/SmaI-digested pFUSE to result in pFUSE-htrA. The insert of this construct includes the htrA control region. The construct was mated onto Y. enterocolitica and integrated into the chromosome by homologous recombination, and a merodiploid htrA-lacZYA strain was the result. The correct integration of the plasmid was confirmed by Southern blot analysis (data not shown). Construction of the rpoE::lacZYA fusion was previously described [35]. The pFUSE-rpoE construct was mated in Y. enterocolitica GHY102, and correct integration was confirmed by Southern blot analysis (data not shown).

2.4. β-Galactosidase assay experiments

β-Galactosidase assays of the Φ(htrA-lacZYA) fusion strains were performed as previously described [36]. Briefly, overnight cultures grown at 26°C were diluted 1:20 in fresh medium and grown for 3 h with aeration at 26°C or 37°C. The cultures were then collected by centrifugation at 4°C and washed in cold 0.85% (w/v) NaCl before enzyme activity assays.

β-Galactosidase enzyme activities are expressed in arbitrary units, which were determined according to the formula of Miller [36]. Cultures were assayed in triplicate, and reported values are averaged from at least two different experiments. The results obtained were analyzed for significance using an unpaired t-test. Differences were considered significant at P ≤ 0.05.

2.5. Animal experiments

Six- to seven-week-old BALB/c mice were infected intragastrically with wild-type JB580v or with the cpxR:: pEP185.2 mutant strain, and the kinetics of infection was determined as previously described [37].

3. Results

3.1. Growth temperature regulation of htrA expression

By analyzing the 5′ region of the Y. enterocolitica htrA gene in the unfinished Y. enterocolitica genome sequence (http://www.sanger.ac.uk/Projects/Y-enterocolitica/) we identified a typical σE consensus −35 and −10 region (Fig. 1). In an initial attempt to characterize htrA expression in Y. enterocolitica, we constructed a Φ(htrA-lacZYA) fusion strain and monitored for β-galactosidase activity at 26°C and 37°C. Expression of htrA is elevated after growth at 37°C in comparison to growth at 26°C (756 ± 56 Miller units versus 271 ± 6 Miller units). Surpris-
ingly, the change in $\beta$-galactosidase activity in response to either overproduction of $cE$ or the anti-sigma factor RseA from an IPTG-inducible $\text{Ptac}$ promoter was relatively small, but statistically significant ($P = 0.0066$ for RseA overproduction) (Fig. 2). To analyze if the growth temperature regulation of $htrA$ expression is mediated by RpoE, we grew $Y.\text{enterocolitica}$ at $26^\circ\text{C}$ and shifted to $37^\circ\text{C}$ after inducing the overproduction of RseA from a $\text{Ptac}$ promoter (Fig. 3). In the control strain the temperature induction of $htrA$ was not changed, but the expression of $htrA$ was decreased in the RseA-overexpressing strain ($P = 0.0002$), indicating that temperature induction of $htrA$ expression is at least in part mediated by RpoE. The data suggest that $htrA$ might potentially be a member of the $cE$ regulon of $Y.\text{enterocolitica}$. 

3.2. Expression of $htrA$ is induced by osmotic shock

In a recent study we could show that $rpoE$ expression in $Y.\text{enterocolitica}$, in contrast to $rpoE$ expression in $E.\text{coli}$, is not induced by heat shock or ethanol in the growth medium [35]. In accordance with these data and the assumption that $htrA$ expression might be mediated by RpoE, we did not detect an elevated expression of $htrA$ after a heat shock (15 min shift to $42^\circ\text{C}$, $50^\circ\text{C}$, or $10\%$ (v/v) ethanol) (data not shown). However, after shifting the $Y.\text{enterocolitica}$ $\Phi(\text{htrA-lacZYA})$ reporter strain to medium containing $0.49$ M NaCl, $0.49$ M lactose, or $0.49$ M raffinose, expression of the $htrA$ promoter was induced (Fig. 4 and data not shown), indicating that $htrA$ expression is regulated by osmotic shock.

3.3. Ail overproduction leads to increased $htrA$ expression

One stimulus leading to induction of the $cE$ regulon in $E.\text{coli}$ is the overproduction of OMPs [3,38]. Specifically, overproduction of OmpX, which shows homology to the OMP Ail of $Y.\text{enterocolitica}$, increases $cE$ activity. Ail mediates adhesion to mammalian cells and confers serum resistance [39,40]. However, in $Y.\text{enterocolitica}$ the overproduction of Ail led to a reduced expression of $rpoE$ [35]. In contrast, $\beta$-galactosidase activity of the $Y.\text{enterocolitica}$ $\Phi(\text{htrA-lacZYA})$ reporter strain increased after Ail overproduction (Fig. 5). This effect was particularly pronounced at $37^\circ\text{C}$. Together with previous data [35] this indicates that induction of $htrA$ in response to Ail overproduction is not regulated by RpoE, suggesting an alternative mechanism responding to envelope stress induced by OMP overproduction. In $E.\text{coli}$, expression of $htrA$ is

Fig. 2. Regulation of $htrA$ expression by $cE$. To analyze the effects of overproduction of RseA or RpoE on $\beta$-galactosidase activity of the $\Phi(\text{htrA-lacZYA})$ reporter strains grown at $26^\circ\text{C}$ were diluted 1:20 in fresh medium and grown for 1 h at $26^\circ\text{C}$ before addition of IPTG (0.5 mM final concentration), and growth continued for a further 2 h. $\beta$-Galactosidase activity was determined as described in Section 2.

Fig. 3. Temperature dependence of $htrA$ expression in $Y.\text{enterocolitica}$. The $\Phi(\text{htrA-lacZYA})$ reporter strain was grown at $26^\circ\text{C}$ or $37^\circ\text{C}$ and $\beta$-galactosidase activity was determined. To test the effect of RseA overproduction on induction of $htrA$ expression after temperature shift from $26^\circ\text{C}$ to $37^\circ\text{C}$, overnight cultures of $Y.\text{enterocolitica}$ $\text{GHY7}$ and $\text{GHY8}$ were diluted 1:20 in fresh medium and grown at $26^\circ\text{C}$ with aeration for 1 h before addition of IPTG (final concentration of 0.5 mM) and shift to $37^\circ\text{C}$. After 2 h of growth, $\beta$-galactosidase activity was determined as described in Section 2.

Fig. 4. Influence of osmotic stress on $htrA$ expression. The $Y.\text{enterocolitica}$ $\Phi(\text{htrA-lacZYA})$ reporter strain was grown at $26^\circ\text{C}$ or $37^\circ\text{C}$ and stressed by the addition of NaCl (final concentration of 0.49 M) or lactose (final concentration of 0.49 M) for 30 min. $\beta$-Galactosidase activity was determined as described in Section 2.
regulated by RpoE and also by the Cpx system [18]. This prompted us to investigate the role of the Cpx system in regulation of htrA expression in Y. enterocolitica.

3.4. htrA expression is CpxR-dependent

By analysis of the region upstream of the htrA gene we identified a putative binding site for phosphorylated CpxR similar to E. coli (Fig. 1) [16]. The cpxR, cpxA, and cpxP genes of Y. enterocolitica were identified by searching the unfinished Y. enterocolitica genome sequence using the BLAST program [41]. The identities to the corresponding E. coli genes are 81% (cpxR), 74% (cpxA), and 63% (cpxP). As in E. coli, the cpxRA genes are arranged in an operon. The cpxP gene is located immediately upstream and is transcribed divergently. We constructed a Y. enterocolitica ΔcpxR strain and analyzed expression of htrA in this genetic background. The ΔcpxR strain did not show any detectable growth defect under the various stress conditions tested for induction of htrA expression. In addition, the cpxR mutant was not impaired for growth from lag through stationary phase under standard conditions (LB medium, 26°C or 37°C).

Fig. 5. Influence of overproduction of the OMP Ail on htrA expression. The Y. enterocolitica Φ(htrA-lacZYA) reporter strain was grown at 26°C or 37°C and overproduction of Ail was induced by the addition of IPTG (final concentration of 0.5 mM) for 2 h. β-Galactosidase activity was determined as described in Section 2.

The β-galactosidase activity of the Φ(htrA-lacZYA)-ΔcpxR strain was about five-fold reduced in comparison to the wild-type strain (wild-type: 271±5.8 Miller units; ΔcpxR: 58±4 Miller units), whereas the expression of rpoE in the ΔcpxR strain was only negligibly reduced (data not shown), indicating that full expression of htrA requires an intact Cpx system. The Φ(htrA-lacZYA)ΔcpxR strain still responded to the overproduction of RseA and RpoE in the same manner as the wild-type (data not shown). As expected from our previous experiments, elevated htrA expression at 37°C in comparison to 26°C was also observed in the ΔcpxR strain (data not shown), further indicating that this effect is mediated by RpoE and not by Cpx. The effect of various stresses on htrA expression in the ΔcpxR strain background was examined. The response to 10% (v/v) ethanol, 0.49 M NaCl, and 0.49 M lactose showed the same pattern as in the wild-type strain, indicating that response to these stresses does not involve the Cpx system (data not shown). The effect of overproduction of the OMP Ail led to an induction of htrA expression (Fig. 5). β-galactosidase activity of the Φ(htrA-lacZYA)ΔcpxR strain was not affected by Ail overproduction (Fig. 6). These data indicate that htrA expression in response to envelope stress induced by the overproduction of the OMP Ail is regulated in Y. enterocolitica by the Cpx system. As rpoE expression is decreased in response to Ail overproduction and CpxR has a negative regulatory effect on rpoE expression in E. coli [42], we were interested if rpoE down-regulation occurs via CpxR in an Ail-overproducing strain. As shown in Fig. 6, rpoE expression is no longer down-regulated after Ail overproduction in a ΔcpxR background. We conclude from these data that in Y. enterocolitica Ail overproduction may result in down-regulation of rpoE expression via a negative regulatory effect of CpxR, while at the same time htrA expression is up-regulated by CpxR.

3.5. Kinetics of infection of a cpxR mutant strain

We were interested in the role of the extracytoplasmic
stress response in virulence of \textit{Y. enterocolitica}. As \textit{rpoE} is an essential gene in \textit{Y. enterocolitica} \cite{35}, we were not able to analyze an \textit{rpoE} mutant strain in the mouse model of yersiniosis. Therefore, we examined the effect of a \textit{cpxR} mutant on in vivo survival kinetics after oral infection of mice. For this purpose, eight BALB/c mice were infected each with $10^8$ CFU either of strain GHY50 (\textit{cpxR}: \textit{pEP185.2}) or of JB580v (wild-type). Three and five days post infection, four mice per strain were killed. The Peyer’s patches, mesenteric lymph nodes, and spleens were harvested and viable cell counts were assessed for each tissue. Viable counts were similar for GHY50 and JB580v in all tissues at both time points (Fig. 7). This result suggests that the Cpx system was not necessary for full virulence when assayed as kinetics of in vivo survival.

4. Discussion

In \textit{E. coli}, expression of \textit{htrA} is regulated by both the RpoE and the Cpx systems \cite{18}. Our data presented here indicate that in \textit{Y. enterocolitica} \textit{htrA} expression is mediated by CpxRA and possibly also by RpoE; however,
there are considerable differences in comparison to E. coli in which system is activated in response to certain stimuli. Most strikingly, expression of htrA and rpoE in response to overproduction of an OMP is mediated via RpoE in E. coli and via CpxR in Y. enterocolitica (Fig. 8).

Interestingly, overproduction of RpoE or the anti-sigma factor RseA has only minor effects on htrA expression in Y. enterocolitica. This is unexpected, as RpoE and RseA overproduction has a strong effect on htrA expression in E. coli, and a typical consensus sequence of σE-dependent promoters can be identified upstream of the htrA gene of Y. enterocolitica. Data presented in this study show that htrA regulation is complex and putatively involves many factors. It is possible that htrA expression in response to RpoE does overlap with additional inputs that mask the effect of RpoE and RseA overproduction or that htrA expression is fine-tuned by RpoE. Under certain conditions, like change in growth temperature, the effect of RpoE on htrA expression becomes more obvious. Analysis of htrA expression in a ΔcpxR background strengthens the anticipated role of RpoE or another unidentified factor in the regulation of htrA expression in response to certain stimuli like osmotic stress or growth at 37°C in comparison to 26°C, as the magnitude increase in transcription from the htrA: :lacZYA fusion in response to these stresses was not altered in the ΔcpxR strain. However, htrA transcription was reduced in the ΔcpxR strain, indicating that CpxR might modulate σE activity under the conditions tested. This hypothesis is supported by expression analysis of a rpoE::lacZYA reporter in a ΔcpxR background. Although rpoE expression in a ΔcpxR strain is not changed under standard conditions, it is no longer decreased in response to Ail overproduction like the wild-type [35]. Furthermore, a negative regulatory influence of CpxR on rpoE expression has been shown in E. coli [42].

Our data concerning the response of the htrA and rpoE promoters to Ail overproduction are in accordance with the hypothesis of De Wulf et al. [42] that a Cpx response takes precedence over the RpoE response by a negative control of rpoE expression. Our model for regulation of htrA expression in Y. enterocolitica is presented in Fig. 8.

In E. coli, overproduction of OMPs leads to increased transcription from the htrA promoter, an effect that is mediated by σE and not the Cpx system [3,18]. The mechanism underlying the regulation is that the anti-sigma factor RseA is degraded by the membrane-anchored periplasmic protease DegS after overproduction of OMPs, thereby activating σE-mediated transcription [43]. DegS recognizes via a PDZ domain a Tyr-Xxx-Phe motif at the carboxy-terminus of OMPs as an activating signal [44]. In Y. enterocolitica, htrA expression is also induced after Ail overproduction; however, this effect is dependent on the Cpx system, as Ail overproduction does not lead to increased htrA expression in a ΔcpxR background. On the other hand, Ail contains a Tyr-Arg-Phe sequence at its carboxy-terminus, and by analysis of the unfinished Y. enterocolitica genome sequence we identified a putative DegS protein by sequence identity to the corresponding E. coli protein (identity of 64%). However, a putative role of DegS in extracytoplasmic stress regulation in Y. enterocolitica remains to be elucidated. Whether overproduction of OMPs other than Ail also leads to Cpx-mediated expression of htrA needs to be determined; however, the present data suggest that the Cpx system might be able to sense the amount of an OMP in the membrane and in response modulate the transcription of htrA. Alternatively, the Cpx system could be sensing aggregates of misfolded Ail upon overproduction or perturbations in other envelope proteins whose folding is disrupted when Ail is overproduced.

A role of σE in this process cannot be ruled out, although rpoE expression is repressed in response to Ail overproduction in a Y. enterocolitica wild-type strain [35], but not in a ΔcpxR background. Transcription might also be controlled by different sigma factors in conjunction with CpxR under the conditions analyzed. In E. coli the Cpx regulon includes genes transcribed by σ70 and σE [16]. Analysis of the region upstream of htrA of Y. enterocolitica revealed putative σ70 consensus sequences and putative consensus binding sites for CpxR (data not shown); however, activity of these putative promoters needs to be confirmed by primer extension analysis and promoter mapping.

In Y. enterocolitica an htrA mutant strain is impaired in survival in macrophages and pathogenesis in the mouse model of infection [23,26]. In this study, we provide evidence that htrA expression in Y. enterocolitica is regulated by the Cpx system and presumably also by the alternative sigma factor RpoE. Therefore we were interested in the role of extracytoplasmic stress regulation in pathogenesis of Y. enterocolitica. Surprisingly, a cpxR mutant strain was fully virulent when assayed as kinetics of in vivo survival. However, it cannot be excluded that a cpxR mutant might have an in vivo effect that was not detectable in this assay. In this context it is of interest that preliminary data from our laboratory show that the ΔcpxR strain shows an elevated invasion into eukaryotic cells in vitro. This might lead to a higher initial infectivity in mice that could potentially compensate for putative virulence defects during later stages of infection. The phenotype of the ΔcpxR strain in mice might also suggest that extracytoplasmic stress under certain in vivo conditions is preferably regulated by the RpoE system, and that the RpoE system is more important in virulence of Y. enterocolitica than the Cpx system. The role of the RpoE and Cpx systems in Y. enterocolitica pathogenesis remains to be elucidated in further experiments.

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