ClpP is involved in the stress response and degradation of misfolded proteins in Salmonella enterica serovar Typhimurium

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Components of the ATP-dependent Clp protease complex are found in a wide range of prokaryotic cells and they are often expressed as part of the cellular stress response. To investigate the physiological role of the proteolytic subunit, ClpP, in Salmonella enterica serovar Typhimurium (S. typhimurium) an in-frame deletion of the clpP gene was constructed. Growth experiments revealed that clpP is important for the ability of S. typhimurium to grow under various stressful conditions, such as low pH, elevated temperature and high salt concentrations. Since the stationary-phase sigma factor, RpoS, is a target of the Clp proteolytic complex, the effect of the clpP deletion in the absence of RpoS was examined; it was observed that growth of the S. typhimurium clpP mutant is affected in both an RpoS-dependent and an RpoS-independent manner. Analysis of the degradation of abnormal puromycyl-containing polypeptides showed that ClpP participates in the proteolysis of such proteins in S. typhimurium. These findings prompted an investigation of the growth of an Escherichia coli clpP mutant under various stress conditions. The growth of this E. coli mutant was affected by heat, salt and low pH, although not to the same extent as observed for the S. typhimurium clpP mutant. The results of this study indicate that the S. typhimurium clpP mutant is generally more sensitive to environmental stress than the E. coli clpP mutant and it is proposed that this is due to a reduced ability to degrade misfolded proteins generated under these conditions.

Keywords: Salmonella typhimurium, Clp protease, RpoS

INTRODUCTION

The continuous requirement for adaptation of bacteria to starvation and physical stress has forced the development of very complex regulatory networks that respond to changes in the environment. During stress, abnormal or misfolded proteins will accumulate in the cell due to denaturation and errors in biosynthesis. The cell responds to this accumulation by increasing the synthesis both of molecular chaperones, which assist the proper folding or refolding of proteins, and of proteases, which degrade the proteins that cannot be refolded (Goff & Goldberg, 1985). Energy-dependent protein degradation is important in both prokaryotic and eukaryotic cells and it is carried out by multimeric protein complexes, such as the proteasome of eukaryotic and archaeal cells (Kessel et al., 1995) and the ATP-dependent proteases of bacterial cells (De Mot et al., 1999). In addition to eliminating abnormal proteins, proteolysis also controls the level of naturally short-lived regulatory proteins (Mhammedi-Alaoui et al., 1994; Schweder et al., 1996) and therefore is essential for cell homeostasis and optimal metabolic activity (Gottesman, 1996).

In Escherichia coli, several ATP-dependent proteases have been characterized (for reviews see Gottesman, 1996; Miller, 1996; Porankiewicz et al., 1999). Among these is the Clp protease, which together with Lon accounts for up to 80% of the protein degradation in the cell (Goldberg et al., 1994; Laskowska et al., 1996; Porankiewicz et al., 1999). The Clp protease complex consists of a proteolytic component, ClpP, to which substrate specificity is conferred through association with either of the ATPases, ClpA or ClpX. Beside their functions in proteolysis, both ClpA and ClpX possess
chaperone-like activities (Wawrzynow et al., 1995; Wickner et al., 1994). The Clp protease complex mediates the turnover of specific short-lived regulatory proteins (Mhammedi-Alaoui et al., 1994; Schweder et al., 1996), among them the stationary-phase sigma factor, σ^8 (RpoS). RpoS regulates the expression of more than 50 genes in the response to stress or the entry into stationary phase (Hengge-Aronis, 1996, 2000; Loewen & Hengge-Aronis, 1994; Schweder et al., 1996). During exponential growth the level of RpoS is low, in part due to degradation by ClpXP (Lange & Hengge-Aronis, 1999; Msadek et al., 1999; Yamamoto et al., 2002). In Gram-negative bacteria the role of ClpP during stress is less clear, as indicated by the lack of obvious phenotypes of an E. coli clpP mutant (Maurizi et al., 1990b). In recent studies mutants were generated in the clpP gene of Salmonella enterica serovar Typhimurium (referred to as Salmonella typhimurium) (Hensel et al., 1995; Webb et al., 1999; Yamamoto et al., 2001). S. typhimurium is a facultative intracellular pathogen that upon contact with host cells can promote its own entry (Galán, 1996). In this organism clpP is required for virulence in a mouse assay (Hensel et al., 1995; Webb et al., 1999; Yamamoto et al., 2001) and for growth and survival within peritoneal macrophages (Yamamoto et al., 2001). ClpP has also been found to be involved in the regulation of flagellum biosynthesis in S. typhimurium, and the lack of ClpP leads to a hyper-flagellate cell (Tomoyasu et al., 2002).

Since S. typhimurium encounters various hostile conditions during the infection process (Foster & Spector, 1995), we were prompted to investigate the importance of ClpP for growth in the presence of stress. We find that clpP mutant cells have a reduced ability to grow compared to wild-type cells when exposed to high temperature, low pH or a high salt concentration. Furthermore, we demonstrate that the clpP mutant degrades puromycyl-containing polypeptides to a lesser extent than the wild-type, indicating that S. typhimurium ClpP is important for the degradation of misfolded proteins generated when exposed to stress.

METHODS

**Media and bacterial strains.** The bacterial strains used in this study are shown in Table 1. Luria–Bertani (LB) broth and M63 medium (Miller, 1992) were prepared as liquid or solid (1.5% agar) media. Unless otherwise stated the strains were grown in LB. Antibiotics were used at the following concentrations for both S. typhimurium and E. coli: 50 µg ampicillin ml⁻¹, 30 µg chloramphenicol ml⁻¹ and 20 µg tetracycline ml⁻¹.

**General methods.** P22 transductions were performed with P22HT105/int 201 as described by Maloy et al. (1996). For plasmid constructions, E. coli DH5α was used. S. typhimurium KP1274 was used for transfer of DNA from E. coli to S. typhimurium. Electroporation and plasmid transformations were performed as described by O’Callaghan & Charbit (1990) and Sambrook et al. (1989). Plasmid purification was performed according to the manufacturer’s instructions (Qiagen).

**Construction of a S. typhimurium clpP deletion mutant.** Using a replacement recombination technique, a recombinant strain of S. typhimurium C5 carrying an 80 amino acid in-frame deletion of clpP was constructed. By PCR amplification of chromosomal S. typhimurium DNA a 750 bp fragment carrying part of the upstream region of clpP was obtained using the primers ClpP-B1 (5’-AGTAGATCTGGCTGTTAGAACAGATCC-3’) and ClpP-Ec1 (5’-AGAGAATTCTCTGTCACATACCAAATGGTGC-3’), while a 642 bp fragment carrying the downstream part of clpP was obtained using the primers ClpP-Ec2 (5’-CTCGAATTCCCTGGAAAGCGTAGAATACG-3’) and ClpX-H1 (5’-CTTAAGGTCACCATGCTGTTATCG-3’).

The two fragments were digested with EcoRI/BglII and EcoRI/HindIII, respectively, and cloned into the BamHI–HindIII sites of the thermosensitive vector pTSA29 (Phillips, 1999), resulting in the plasmid pLT11, carrying a 1376 bp insert.

S. typhimurium C5 was transformed with pLT11 by electroporation and integration was promoted by incubation at 42 °C in the presence of ampicillin. To excise the plasmid from the chromosome, the integrants were grown overnight at 30 °C and plated in the presence of ampicillin. The excised plasmid was cured by incubation of the strain in the absence of antibiotics at 42 °C for 40 hr. Ampicillin-sensitive colonies were analyzed by PCR to identify mutants with an internal deletion. One colony gave a single 1376 bp fragment, corresponding to the clpP gene with a 240 bp deletion; the wild-type strain C5 (1616 bp fragment) gave a single 1376 bp fragment (data not shown). The correct construction of the resulting clpP mutant (LT100) was verified by sequencing the clpP gene.

**Growth experiments.** Growth was followed by diluting cultures (grown overnight at 37 °C in LB) 100-fold into LB and incubating either at 37 °C, at 45 °C, at 37 °C with 5% NaCl, or at 37 °C with the pH reduced to pH 4.5. The optical density was measured at 450 nm (OD_{560}).

In plating experiments overnight cultures were diluted 100-fold in LB and allowed to grow to OD_{560} 0.4 at 37 °C. Samples (10 µl) of culture were spotted on plates with or without 5% NaCl. Plates were incubated overnight at either 37 °C or 45 °C.

**Immunoblotting.** Western blot analysis using monoclonal anti-σ^8 antibodies (obtained from Neoclide) was performed essentially as described by Lee et al. (1995). Cells were grown to the mid-exponential growth phase (OD_{560} 0.4) or late stationary phase (15 h growth) in LB. Equal amounts (5 µg) of protein were loaded for each sample.

**Two-dimensional protein gel electrophoresis.** Two-dimensional SDS-PAGE analysis was performed as described by Spector et al. (1986) with minor modifications. Strains were grown in M63 supplemented with 0.05% Casamino acids at
37 °C until the OD_{600} was 0.4. The cultures were then transferred to 45 °C and allowed to grow for 1h. Samples were labelled with 35S-translabel (40 μCi ml⁻¹, 1.48 × 10⁶ Bq ml⁻¹; ICN Pharmaceuticals) for 3 min. In the first dimension proteins were separated using ReadyStrip IPG Strips pH 4–7 (Bio-Rad) and in the second dimension an SDS-5% polyacrylamide gel was used.

**Measurement of degradation of puromycyl-containing peptides.** The experiment was performed essentially as described by Raina & Georgopoulos (1990). *S. typhimurium* wild-type and *clpP* mutant cells were grown at 37 °C in M63 until the OD_{600} reached 0.4. The cells were subsequently incubated with puromycin (200 μg ml⁻¹, Sigma) for 10 min and then labelled with 30 μCi (1.11 × 10⁶ Bq ml⁻¹) [35S]methionine per ml for 10 min. The cells were washed and resuspended in M63 containing 500 μg unlabelled methionine ml⁻¹. Samples (300 μl) were collected at 5 min intervals and precipitated with 6% trichloroacetic acid. The radioactivity of the acid-soluble fraction was measured by liquid-scintillation counting.

**RESULTS AND DISCUSSION**

**ClpP is important for growth under stress conditions in S. typhimurium**

With the aim of investigating the physiological role of ClpP in *S. typhimurium* C5, we constructed a *clpP* mutant (LT1100) by deleting an internal fragment corresponding to 80 amino acids, including the three amino acids known to be required for the proteolytic activity of ClpP in *E. coli* (Maurizi et al., 1990a; Wang et al., 1997). By Western-blot analysis using a ClpX antibody we confirmed that the deletion did not affect the expression of ClpX located downstream of *clpP* (data not shown). When we investigated the growth of LT1100 at 37 °C the growth rate was comparable to that of the wild-type in both enriched (Fig. 1) and minimal broth (data not shown). However, when mutant cells were shifted to 45 °C, the growth was impaired compared to the wild-type, as observed by a reduction in the growth rate and by the inability to reach the same density as the wild-type even after 24 h (Fig. 1). A high salt concentration (5% NaCl) as well as low pH (pH 4.5) also reduced the growth rate of the *S. typhimurium clpP* mutant (Fig. 1). Furthermore, when mutant and wild-type cells were plated either at 45 °C or in the presence of 5% salt the ability of the mutant to form colonies was greatly reduced (data not shown). To verify that these differences were due to the lack of ClpP, we repaired the deletion by transducing the *clpP*+ allele together with Tn10 from JF3717 into LT1100, resulting in LT1102 (*clpP*+). Under all conditions tested LT1102 grew like wild-type cells (data not shown), confirming that it is the lack of functional ClpP that affects the growth during stress. Thus, our results reveal that the growth of the *S. typhimurium clpP* mutant is impaired when exposed to stress.

**ClpP affects growth of S. typhimurium independently of RpoS**

In both *S. typhimurium* and *E. coli*, the ClpXP protease is involved in the regulation of the level of RpoS by degradation and a *clpP* mutation results in increased concentrations of RpoS (Schwedet al., 1996; Webb et al., 1999). Since RpoS regulates or augments the expression of many stress-regulated genes in *S. typhimurium* and *E. coli* (Henge-Aronis, 1996; Ibanez-Ruiz et al., 2000; Loewen & Henge-Aronis, 1994; O’Neal et al., 1994) we addressed whether the stress sensitivity observed for the *clpP* mutant was related to *rpoS* by

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**Table 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>S. typhimurium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JF2690</td>
<td>UK rpoS::Ap</td>
<td>Lee et al. (1995)</td>
</tr>
<tr>
<td>JF3717</td>
<td>UK 1::6014::Tn10dCm (48% linked to <em>clpP</em>)</td>
<td>J. W. Foster, unpublished data</td>
</tr>
<tr>
<td>JF3487</td>
<td>UK 1::cI AclpP::C5</td>
<td>Webb et al. (1999)</td>
</tr>
<tr>
<td>C5</td>
<td>Virulent wild-type</td>
<td>Hormacche (1979)</td>
</tr>
<tr>
<td>LT1100</td>
<td>C5 AcI ClpP::C5</td>
<td>This work</td>
</tr>
<tr>
<td>LT1102</td>
<td>LT1100 with Tn10 linked to <em>clpP</em> (linkage 48%)</td>
<td>JF3717 × LT1100, this work</td>
</tr>
<tr>
<td>LT1103</td>
<td>LT1102 clpP1::Tn10dCt</td>
<td>JF3487 × LT1102</td>
</tr>
<tr>
<td>LT1104</td>
<td>LT1100 rpoS::Ap</td>
<td>JF2690 × LT1100, this work</td>
</tr>
<tr>
<td>LT1108</td>
<td>LT1102 rpoS::Ap</td>
<td>JF2690 × LT1102, this work</td>
</tr>
<tr>
<td>LT1115</td>
<td>C5 clpP1::C5</td>
<td>JF3487 × C5</td>
</tr>
<tr>
<td>KP1274</td>
<td>LT2 metA2 metE55 val galE496 rpsL120 xyl-404 H1-b nml H2-e,n,x hsdL6 hsdSA29 (F–)</td>
<td>Enomoto &amp; Stocker (1974)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
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</tr>
<tr>
<td>DH5α</td>
<td>F− g80d lacZA15Δ(ac2YA–argF)U169 deoR recA1 endA1 hsdR17 (rC mG supE44 thi-1 gyrA96)</td>
<td>Hanahan (1985)</td>
</tr>
<tr>
<td>AMS6</td>
<td>K-12 (F– Δlac)</td>
<td>Schultz et al. (1988)</td>
</tr>
<tr>
<td>AMS6P</td>
<td>AMS6 but clpP, Cam’</td>
<td>Schweder et al. (1996)</td>
</tr>
</tbody>
</table>
The effect of the clpP protein was affected by the absence of RpoS, suggesting that these proteins could be related to the observed phenotype (data not shown).

Recently it was shown that the accumulation of another sigma factor, σ28, encoded by fliA, is also affected by a clpP mutation in S. typhimurium leading to increased levels of FliA and a hyper-flagellate phenotype (Tomoyasu et al., 2002). When we transduced the fliA mutation into C5 and LT1100 and compared growth of the resulting strains under stress we found that fliA did not alter the growth characteristics of the wild-type or the mutant strain, demonstrating that the accumulation of FliA does not account for the observed stress sensitivity of the clpP mutant (data not shown).

During the course of these experiments we observed that LT1100 has a normal colony size. In a recent study, a S. typhimurium clpP mutant displayed a small-colony morphology (Webb et al., 1999), suggesting that LT1100 could carry a secondary mutation. We verified this notion by transducing the transposon-disrupted clpP allele, clpP::Tn10dTc (Webb et al., 1999) into C5, and found that the resulting C5 clpP mutant had a small-colony morphology (data not shown). Interestingly, large-colony revertants arose with high frequency, thus showing that the mutant is unsuitable for growth experiments. When we introduced the clpP::Tn10dTc allele into LT1102, which carries the wild-type clpP allele in the clpP deletion mutant background, the resulting strain, LT1103 (clpP::Tn10dTc), had retained the large-colony morphology, demonstrating that the secondary mutation is unlinked to clpP (data not shown). However, as growth of LT1102 was identical to growth of C5 and, in addition, LT1103 (clpP::Tn10dTc) behaved like LT1100 (clpP) under the various stress conditions tested (Fig. 1 and data not shown), our results show that the impaired growth observed for LT1100 is not a consequence of the secondary mutation but rather it is caused by the lack of ClpP.

The absence of ClpP in E. coli also affects growth during stress

In E. coli the Clp protease degrades intrinsically unfolded protein substrates such as the CRAG protein (Kandror et al., 1999) and a non-secreted alkaline phosphatase mutant protein (Huang et al., 2001), indicating that the proteins formed during stress could be degraded by Clp. The results we obtained with S. typhimurium therefore prompted us to analyse how an E. coli clpP mutant behaved when exposed to stress using the same experimental conditions as for S. typhimurium. In agreement with a previous finding (Maurizi et al., 1999b), we found that growth of the E. coli clpP mutant was identical to that of the wild-type at 37 °C (Fig. 2). However, when the mutant was shifted to 5% NaCl, to 45 °C or from neutral pH to pH 4.5, we reproducibly obtained results showing that the growth was impaired compared to the wild-type (Fig. 2) although not to the same extent as observed for the

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**Fig. 1.** The S. typhimurium clpP mutant LT1100 exhibits slower growth when exposed to stress. Overnight cultures were diluted into fresh medium and growth was followed under the following conditions: at 37 °C; at 45 °C; at pH 4.5 at 37 °C; or in the presence of 5% NaCl at 37 °C. ● S. typhimurium wild-type C5; □, S. typhimurium LT1100 (clpP); ▲, S. typhimurium LT1104 (clpP rpoS); △, S. typhimurium LT1108 (rpoS). The data shown are from one of at least three experiments that gave similar results.
S. typhimurium clpP mutant. Thus, our results indicate that the S. typhimurium clpP mutant is generally more sensitive to stress than the E. coli clpP mutant.

ClpP participates in proteolysis of misfolded protein

In previous studies it has been shown that ClpP is important for degradation of misfolded proteins in the Gram-positive bacteria Bacillus subtilis, Listeria monocytogenes and Lactococcus lactis, whereas degradation of such proteins in E. coli is essentially unaffected by a clpP mutation (Frees & Ingmer, 1999; Gaillot et al., 2000; Kruger et al., 2000; Maurizi et al., 1990b). With the aim of investigating the turnover of misfolded protein in the S. typhimurium clpP mutant, both mutant and wild-type cells were grown in the presence of the tRNA analogue puromycin, which interferes with translation, resulting in the production of misfolded puromycyl-containing peptides. Interestingly, we observed a decrease in colony size of the clpP mutant compared to the wild-type, suggesting that the clpP mutant is more sensitive to puromycin (data not shown). To examine whether a mutation in the clpP gene affects cellular proteolysis in S. typhimurium, the rate of degradation of puromycyl-containing polypeptides in LT1100 and wild-type cells was determined as described by Raina & Georgopoulos (1990). The result, presented in Fig. 3, shows that the clpP mutant degrades these peptides both at a reduced rate and to a lower extent than the wild-type, suggesting that ClpP is involved in the overall degradation of misfolded proteins in S. typhimurium and that the impaired growth of the mutant could be a result of the accumulation of misfolded proteins. In the literature it has furthermore been reported that the phenotype of an E. coli clpP lon double mutant resembles that of a strain carrying a mutation in lon (Maurizi et al., 1990b), the major ATP-dependent protease responsible for degradation of proteins generated during stress in E. coli (Chung & Goldberg, 1981; Maurizi et al., 1985). In contrast, the S. typhimurium clpP lon double mutant grows poorly (Wang et al., 1999) although the phenotype of a S. typhimurium lon mutant is similar to that observed in E. coli (Downs et al., 1986). These observations further support the notion that ClpP has a more prominent role in degrading misfolded proteins accumulating during stress in S. typhimurium than in E. coli.

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Role of *S. typhimurium clpP* in stress response


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