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^S (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, 1994). When cells enter stationary phase or encounter various stress conditions, the concentration of RpoS increases as a result of greater resistance to degradation by ClpXP (Schweder *et al.*, 1996; Webb *et al.*, 1999; Zgurskaya *et al.*, 1997).

Components of the Clp complex are highly conserved in prokaryotic cells (Maurizi *et al.*, 1990a; Wawrzynow *et al.*, 1996). In Gram-positive bacteria ClpP is required for survival under various kinds of stress (Frees & Ingmer, 1999; Msadek *et al.*, 1998) and it has been shown that ClpP participates in the degradation of misfolded proteins generated under these conditions (Frees & Ingmer, 1999; Gaillot *et al.*, 2000; Kruger *et al.*, 2000). 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 (Galan, 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 puromycinyl-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 μ g ampicillin ml⁻¹, 30 μ g chloramphenicol ml⁻¹ and 20 μ g 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* DH5 α 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'-AGTAGATCTCGTCTGCTTACGAAGATCC-3') and ClpP-Ec1 (5'-AGAGAATTCCTGTCCCATCAAATGGTGC-3'), while a 642 bp fragment carrying the downstream part of *clpP* was obtained using the primers ClpP-Ec2 (5'-CTCGAATTCCTGAAGCGGTAGAATACG-3') and ClpX-H1 (5'-CCTAAGCTTACGCCATTGCTGTATCG-3').

The two fragments were digested with *Eco*RI/*Bgl*II and *Eco*RI/*Hind*III, respectively, and cloned into the *Bam*HI-*Hind*III 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. Forty ampicillin-sensitive colonies were analysed 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 gave a single 1616 bp fragment (data not shown). The correct construction of the resulting *clpP* mutant (LT1100) 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 (OD₄₅₀).

In plating experiments overnight cultures were diluted 100-fold in LB and allowed to grow to OD₄₅₀ 0.4 at 37 °C. Samples (10 μ l) 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^S antibodies (obtained from Neoclone) was performed essentially as described by Lee *et al.* (1995). Cells were grown to the mid-exponential growth phase (OD₆₀₀ 0.4) or late stationary phase (15 h growth) in LB. Equal amounts (5 μ g) 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

Table 1. Bacterial strains used in this study

Strain	Genotype	Source
<i>S. typhimurium</i>		
JF2690	UK1 <i>rpoS</i> ::Ap	Lee <i>et al.</i> (1995)
JF3717	UK1 <i>xba-6014</i> ::Tn10dCm (48 % linked to <i>clpP</i> ⁺)	J. W. Foster, unpublished data
JF3487	UK1 <i>clpP1</i> ::Tn10dTc	Webb <i>et al.</i> (1999)
C5	Virulent wild-type	Hormaeche (1979)
LT1100	C5 $\Delta clpP$	This work
LT1102	LT1100 with Tn10 linked to <i>clpP</i> ⁺ (linkage 48 %)	JF3717 $\times$ LT1100, this work
LT1103	LT1102 <i>clpP1</i> ::Tn10dTc	JF3487 $\times$ LT1102
LT1104	LT1100 <i>rpoS</i> ::Ap	JF2690 $\times$ LT1100, this work
LT1108	LT1102 <i>rpoS</i> ::Ap	JF2690 $\times$ LT1102, this work
LT1115	C5 <i>clpP1</i> ::Tn10dTc	JF3487 $\times$ C5
KP1274	LT2 (<i>metA22 metE55 val galE496 rpsL120 xyl-404 H1-b nml H2-e,n,x hsdL6 hsdSA29</i> (<i>r</i> ⁻ <i>m</i> ⁺))	Enomoto & Stocker (1974)
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ M15 <i>Δ(lacZYA-argF)</i> U169 <i>deoR recA1 endA1 hsdR17</i> (<i>r</i> _k ⁻ <i>m</i> _k ⁺) <i>supE44 thi-1 gyrA96</i>	Hanahan (1985)
AMS6	K-12 (λ ⁻ F ⁻ Δ <i>lac</i>)	Schultz <i>et al.</i> (1988)
AMS6P	AMS6 but <i>clpP</i> ; Cam ^r	Schweder <i>et al.</i> (1996)

37 °C until the OD₆₀₀ was 0.4. The cultures were then transferred to 45 °C and allowed to grow for 1 h. Samples were labelled with ³⁵S-translabel (40 μ Ci ml⁻¹; 1.48 $\times$ 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-11.5 % polyacrylamide gel was used.

Measurement of degradation of puromycin-containing polypeptides. 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 OD₄₅₀ reached 0.4. The cells were subsequently incubated with puromycin (200 μ g ml⁻¹, Sigma) for 10 min and then labelled with 30 μ Ci (1.11 $\times$ 10⁶ Bq ml⁻¹) [³⁵S]methionine per ml for 10 min. The cells were washed and resuspended in M63 containing 500 μ g unlabelled methionine ml⁻¹. Samples (300 μ l) 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 (Schweder *et al.*, 1996; Webb *et al.*, 1999). Since RpoS regulates or augments the expression of many stress-regulated genes in *S. typhimurium* and *E. coli* (Hengge-Aronis, 1996; Ibanez-Ruiz *et al.*, 2000; Loewen & Hengge-Aronis, 1994; O'Neal *et al.*, 1994) we addressed whether the stress sensitivity observed for the *clpP* mutant was related to *rpoS* by

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.

comparing growth of an *rpoS* mutant (LT1108) with the growth of a *clpP rpoS* double mutant (LT1104) under the same conditions as the *clpP* mutant (Fig. 1). We found that while both strains grew like wild-type cells at 37 °C, an increase in temperature or a downshift in pH resulted in impaired growth of the double mutant as compared to the single mutant or the wild-type. In the presence of a high concentration of salt both of the *rpoS* mutants were unable to grow, as has been observed previously in *E. coli* (Henge-Aronis, 2000). Since the growth of the *clpP rpoS* mutant was less affected than the *clpP* single mutant we conclude that while part of the effect of the *clpP* mutation is dependent on RpoS, ClpP also promotes growth in stress-exposed cells through an RpoS-independent mechanism. By two-dimensional protein gel analysis we observed that expression of at least 11 proteins was affected by the absence of *clpP* in

dependently 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, σ^{28} , 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.*, 1990b), 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

Fig. 2. Growth of the *E. coli* *clpP* mutant is impaired at low pH. 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. ○, *E. coli* AMS6 (wild-type); ●, *E. coli* AMS6P (*clpP*). 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 puromycin-containing peptides. Interestingly, we observed a

Fig. 3. The *S. typhimurium* *clpP* mutant exhibits reduced proteolysis of puromycin-containing polypeptides. LT1100 and wild-type cells were grown exponentially at 37 °C, and following addition of puromycin cellular proteins were pulse-labelled with [³⁵S]methionine. Samples were taken at 5 min intervals and the relative protein breakdown (%) was determined as TCA-soluble counts relative to the TCA-soluble counts obtained for wild-type cells 25 min after the chase. ◆, Wild-type C5; ■, LT1100 (*clpP*). The data shown are from one of three experiments, which gave similar results.

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 puromycin-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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REFERENCES

- Chung, C. H. & Goldberg, A. L. (1981). The product of the *lon* (*capR*) gene in *Escherichia coli* is the ATP-dependent protease, protease La. *Proc Natl Acad Sci U S A* 78, 4931–4935.
- De Mot, R., Nagy, I., Walz, J. & Baumeister, W. (1999). Proteasomes and other self-compartmentalizing proteases in prokaryotes. *Trends Microbiol* 7, 88–92.
- Downs, D., Waxman, L., Goldberg, A. L. & Roth, J. (1986). Isolation and characterization of *lon* mutants in *Salmonella typhimurium*. *J Bacteriol* 165, 193–197.
- Enomoto, M. & Stocker, B. A. (1974). Transduction by phage P1kc in *Salmonella typhimurium*. *Virology* 60, 503–514.
- Foster, J. W. & Spector, M. P. (1995). How *Salmonella* survive against the odds. *Annu Rev Microbiol* 49, 145–174.
- Frees, D. & Ingmer, H. (1999). ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. *Mol Microbiol* 31, 79–87.
- Gaillot, O., Pellegrini, E., Bregenholt, S., Nair, S. & Berche, P. (2000). The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes*. *Mol Microbiol* 35, 1286–1294.
- Galan, J. E. (1996). Molecular genetic bases of *Salmonella* entry into host cells. *Mol Microbiol* 20, 263–271.
- Goff, S. A. & Goldberg, A. L. (1985). Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. *Cell* 41, 587–595.
- Goldberg, A. L., Moerschell, R. P., Chung, C. H. & Maurizi, M. R. (1994). ATP-dependent protease La (*lon*) from *Escherichia coli*. *Methods Enzymol* 244, 350–375.
- Gottesman, S. (1996). Proteases and their targets in *Escherichia coli*. *Annu Rev Genet* 30, 465–506.
- Hanahan, D. (1985). Techniques for transformation of *Escherichia coli*. In *DNA Cloning: a Practical Approach*, pp. 109–135. Edited by D. M. Glover. Oxford, UK: IRL Press.
- Hengge-Aronis, R. (1996). Back to log phase: sigma S as a global regulator in the osmotic control of gene expression in *Escherichia coli*. *Mol Microbiol* 21, 887–893.
- Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*. In *Bacterial Stress Responses*, pp. 161–178. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. *Science* 269, 400–403.
- Hormaeche, C. E. (1979). Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. *Immunology* 37, 311–318.
- Huang, H. C., Sherman, M. Y., Kandror, O. & Goldberg, A. L. (2001). The molecular chaperone DnaJ is required for the degradation of a soluble abnormal protein in *Escherichia coli*. *J Biol Chem* 276, 3920–3928.
- Ibanez-Ruiz, M., Robbe-Saule, V., Hermant, D., Labrude, S. & Norel, F. (2000). Identification of RpoS (σ^S)-regulated genes in *Salmonella enterica* serovar typhimurium. *J Bacteriol* 182, 5749–5756.
- Kandror, O., Sherman, M. & Goldberg, A. (1999). Rapid degradation of an abnormal protein in *Escherichia coli* proceeds through repeated cycles of association with GroEL. *J Biol Chem* 274, 37743–37749.
- Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K. & Steven, A. C. (1995). Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26S proteasomes. *J Mol Biol* 250, 587–594.
- Kruger, E., Witt, E., Ohlmeier, S., Hanschke, R. & Hecker, M. (2000). The *clp* proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. *J Bacteriol* 182, 3259–3265.
- Lange, R. & Hengge-Aronis, R. (1994). The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev* 8, 1600–1612.
- Laskowska, E., Kuczynska-Wisnik, D., Skorko-Glonek, J. & Taylor, A. (1996). Degradation by proteases Lon, Clp and HtrA, of *Escherichia coli* proteins aggregated in vivo by heat shock; HtrA protease action in vivo and in vitro. *Mol Microbiol* 22, 555–571.
- Lee, I. S., Lin, J., Hall, H. K., Bearson, B. & Foster, J. W. (1995). The stationary-phase sigma factor sigma S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol Microbiol* 17, 155–167.
- Loewen, P. C. & Hengge-Aronis, R. (1994). The role of the sigma factor sigma S (KatF) in bacterial global regulation. *Annu Rev Microbiol* 48, 53–80.
- Maloy, S. R., Stewart V. J. & Taylor R. K. (1996). *Genetic Analysis of Pathogenic Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Maurizi, M. R., Trisler, P. & Gottesman, S. (1985). Insertional mutagenesis of the *lon* gene in *Escherichia coli*: *lon* is dispensable. *J Bacteriol* 164, 1124–1135.
- Maurizi, M. R., Clark, W. P., Kim, S. H. & Gottesman, S. (1990a). ClpP represents a unique family of serine proteases. *J Biol Chem* 265, 12546–12552.
- Maurizi, M. R., Clark, W. P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. & Gottesman, S. (1990b). Sequence and structure of ClpP, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli*. *J Biol Chem* 265, 12536–12545.
- Mhammedi-Alaoui, A., Pato, M., Gama, M. J. & Toussaint, A. (1994). A new component of bacteriophage Mu replicative transposition machinery: the *Escherichia coli* ClpX protein. *Mol Microbiol* 11, 1109–1116.
- Miller, C. G. (1996). Protein degradation and proteolytic modification. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 938–954. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Msadek, T., Dartois, V., Kunst, F., Herbaud, M. L., Denizot, F. & Rapoport, G. (1998). ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. *Mol Microbiol* 27, 899–914.
- O'Callaghan, D. & Charbit, A. (1990). High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. *Mol Gen Genet* 223, 156–158.
- O'Neal, C. R., Gabriel, W. M., Turk, A. K., Libby, S. J., Fang, F. C. & Spector, M. P. (1994). RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. *J Bacteriol* 176, 4610–4616.
- Phillips, G. J. (1999). New cloning vectors with temperature-sensitive replication. *Plasmid* 41, 78–81.
- Porankiewicz, J., Wang, J. & Clarke, A. K. (1999). New insights

into the ATP-dependent Clp protease: *Escherichia coli* and beyond. *Mol Microbiol* **32**, 449–458.

Raina, S. & Georgopoulos, C. (1990). A new *Escherichia coli* heat shock gene, *htrC*, whose product is essential for viability only at high temperatures. *J Bacteriol* **172**, 3417–3426.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schultz, J. E., Latter, G. I. & Matin, A. (1988). Differential regulation by cyclic AMP of starvation protein synthesis in *Escherichia coli*. *J Bacteriol* **170**, 3903–3909.

Schweder, T., Lee, K. H., Lomovskaya, O. & Matin, A. (1996). Regulation of *Escherichia coli* starvation sigma factor (σ^S) by ClpXP protease. *J Bacteriol* **178**, 470–476.

Spector, M. P., Aliabadi, Z., Gonzalez, T. & Foster, J. W. (1986). Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. *J Bacteriol* **168**, 420–424.

Tomoyasu, T., Ohkishi, T., Ukyo, Y. & 7 other authors (2002). The ClpXP ATP-dependent protease regulates flagellum synthesis in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **184**, 645–653.

Wang, J., Hartling, J. A. & Flanagan, J. M. (1997). The structure of ClpP at 2.3 Å resolution suggests a model for ATP-dependent proteolysis. *Cell* **91**, 447–456.

Wang, L., Elliott, M. & Elliott, T. (1999). Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in *Salmonella typhimurium*. *J Bacteriol* **181**, 1211–1219.

Wawrzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C. & Zylicz, M. (1995). The ClpX heat-shock protein of *Escherichia coli*, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. *EMBO J* **14**, 1867–1877.

Wawrzynow, A., Banecki, B. & Zylicz, M. (1996). The Clp ATPases define a novel class of molecular chaperones. *Mol Microbiol* **21**, 895–899.

Webb, C., Moreno, M., Wilmes-Riesenberger, M., Curtiss, R., III & Foster, J. W. (1999). Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium*. *Mol Microbiol* **34**, 112–123.

Wickner, S., Gottesman, S., Skowyr, D., Hoskins, J., McKenney, K. & Maurizi, M. R. (1994). A molecular chaperone, ClpA, functions like DnaK and DnaJ. *Proc Natl Acad Sci USA* **91**, 12218–12222.

Yamamoto, T., Sashinami, H., Takaya, A., Tomoyasu, T., Matsui, H., Kikuchi, Y., Hanawa, T., Kamiya, S. & Nakane, A. (2001). Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar Typhimurium results in persistent infection in mice, and development of persistence requires endogenous gamma interferon and tumor necrosis factor alpha. *Infect Immun* **69**, 3164–3174.

Zgurskaya, H. I., Keyhan, M. & Matin, A. (1997). The sigma S level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. *Mol Microbiol* **24**, 643–651.

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