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Lon Protease Promotes Survival of *Escherichia coli* During Anaerobic Glucose Starvation

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Abstract

In *Escherichia coli*, Lon is an ATP-dependent protease which degrades misfolded proteins and certain rapidly-degraded regulatory proteins. Given that oxidatively damaged proteins are generally degraded rather than repaired, we anticipated that Lon deficient cells would exhibit decreased viability during aerobic, but not anaerobic, carbon starvation. We found that the opposite actually occurs. Wild-type and Lon deficient cells survived equally well under aerobic conditions, but Lon deficient cells died more rapidly than the wild-type under anaerobiosis. Aerobic induction of the Clp family of ATP-dependent proteases could explain these results, but direct quantitation of Clp protein established that its level was not affected by Lon deficiency and overexpression of Clp did not rescue the cells under anaerobic conditions. We conclude that the Lon protease supports survival during anaerobic carbon starvation by a mechanism which does not depend on Clp.

Keywords

Lon protease; Clp protease; anaerobiosis; carbon starvation; stationary phase survival

Introduction

In *Escherichia coli*, proteolysis plays a key role in regulating levels of specific proteins and in degrading damaged or abnormal proteins. The process requires input of substantial

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energy, as over 90% of cytoplasmic protein degradation is energy-dependent (Maurizi, 1992). Proteases are estimated to constitute at least 3% of the enzyme profile of *E. coli*, in which four families of energy-dependent proteases have been identified: Lon, ClpAP/XP, ClpYQ/HslUV, and FtsH (Gottesman, 2003).

The Lon protease is an ATP-dependent protease which has been highly conserved during evolution (Gottesman, 1996). In *E. coli*, Lon is involved in the degradation of abnormal and misfolded proteins (Chung and Goldberg, 1981; Laskowska et al., 1996; Rosen et al., 2002). It also degrades certain regulatory proteins, including the Sula cell division regulator (Mizusawa and Gottesman, 1983; Schoemaker et al., 1984; Higashitani et al., 1997), the positive regulator of capsule synthesis, RcsA (Torres-Cabassa and Gottesman, 1987), and possibly TER components involved in blocking septation sites during the SOS response (Dopazo et al., 1987). Lon is also involved in the turnover of several antitoxin proteins in toxin-antitoxin systems (Gerdes, 2000). For example, Lon degrades RelB, leading to the accumulation of RelE toxin and to global inhibition of translation (Christensen et al., 2001). It has been suggested that this action does not kill cells, but enables them to enter a reversible “bacteriostatic” state (Pedersen et al., 2002). Particularly relevant to survival during stationary phase with amino acid limitation, inorganic polyphosphate accumulates and binds to both free ribosomal proteins and Lon. This facilitates the degradation of the relatively abundant ribosomal proteins by Lon, thus supplying amino acids required for the synthesis of adaptive proteins (Kuroda et al., 2001).

Oxidatively modified proteins are generally not repaired and must be removed by proteolytic degradation (Stadtman and Levine, 2003; Grune et al., 2003). Since production of oxidatively damaged proteins is suppressed by anaerobiosis, we anticipated that Lon deficient cells would die more rapidly under aerobic carbon starvation than the wild type, and that these differences would be minimized under anaerobic starvation conditions. Surprisingly, we found that the opposite occurred. Under aerobic conditions there was no difference in survival of the wild-type and the *lon*⁻ mutant. However, during anaerobic carbon starvation, the Lon-deficient strain lost viability more rapidly than did the wild-type strain.

Materials and methods

E. coli strains, media, and starvation conditions

The *E. coli* K-12 strains used in this study were SG1094 (K12*rel*⁺ (λ), wild-type) and SG1110 (Δlon -510 *zba*-1091:: $\Delta Tn10$, *lon* deficient) (Katayama et al., 1988). The *E. coli* BL21(DE3) competent cells were from Novagen. The K-12 cells were grown aerobically for 24 h at 37°C to stationary phase in minimal M9 medium (Miller, 1992) supplemented with glucose (0.2%), thiamine (1 mg/l), and 1 mM MgSO₄. For carbon starvation studies we used the same medium, omitting glucose (M9-G). The cells were harvested, washed twice with M9-G medium, and resuspended in M9-G at room temperature to a density of $3\text{--}4 \times 10^8$ colony-forming units (CFU) per ml ($OD_{600\text{nm}} = 0.45\text{--}0.5$). For each culture, 100 ml was transferred to each of two 250-ml Erlenmeyer flasks (#431144, Corning Inc.). Cells were then incubated at 37°C in a rotary shaker (225 rpm) for aerobic studies and at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) for the anaerobic studies. This chamber maintained the oxygen concentration at less than 1 part per million. BL21(DE3) cells were grown to stationary phase in Minimal Medium (Budisa et al., 1995), then resuspended in the same medium lacking glucose at a density of $OD_{600\text{nm}} = 2.8$ (4×10^9 CFU/ml) for starvation experiments.

ClpP-ClpX and ClpS-ClpA were overexpressed in SG1110, a *lon*⁻ strain, by transformation with plasmid pWPC9.0 (Maurizi et al., 1990) or pWPC3.1 (Singh and Maurizi, 1994). The

transformed cells were grown and harvested in the same way as the K-12 cells except for the addition of 50 mg/l of ampicillin during aerobic growth.

E. coli K-12 *clpP*⁻ (single mutant, SG1162) and *clpP*⁻*lon*⁻ (double mutant, SG1160) were first grown overnight in rich Luria-Bertani (LB) medium, then diluted 1:5 in M9 medium three times over 6 h to yield an overall 1:125 dilution. This culture was then diluted 1:50 into M9 medium and incubated overnight.

Viability assay

A sample was taken daily from glucose-starved cultures, serially diluted into M9-G medium, and then plated on Luria-Bertani (LB) agar plates supplemented with 1% glucose (Dukan and Nystrom, 1999). Supplementation with 1% glucose supported more rapid formation of colonies under anaerobic conditions. At each time point, four plates were prepared; two were incubated aerobically at 37°C, and the other two were incubated anaerobically in anaerobic pouches (Oxoid Ltd., AnaeroGen Compact). For the anaerobic cultures, all plates and medium were equilibrated in the anaerobic chamber at least 12 h. Plating was performed in the chamber, and then the 4 plates (two in anaerobic pouches) were taken out of the chamber and incubated at 37°C. After growth, colonies were counted manually and the results expressed as CFU/ml of the original growth.

Quantitative immunodetection of ClpP

K-12 cells were harvested on day 1 of glucose starvation. After sonication, 1 µg protein from each extract was electrophoresed on a gradient SDS-PAGE gel. Proteins were transferred to nitrocellulose (LC2001, Invitrogen), and ClpP was detected with 1:10,000 dilutions of rabbit anti-ClpP primary antibody and Alexa Fluor 680 goat anti-rabbit IgG secondary antibody (Molecular Probes, A-21109). ClpP level was quantitated by an Odyssey infrared fluorescence detection system (Li-Cor).

Microarray analysis

Cells were harvested on days 1 and 3 of glucose starvation, with samples taken from the same cultures used for the viability assay. RNA isolation, fluorescent labeling, hybridization, and microarray scanning followed the protocol of the Microarray Research Facility of the National Institute of Allergy and Infectious Diseases. Genes were grouped according to the National Center for Biotechnology Information's Clusters of Orthologous Groups (COG) (Tatusov et al., 1997). As expected from a genome-wide expression analysis, many genes demonstrated substantial changes in expression, although we did not recognize clear foci of changes within the groups. The microarray data have been deposited in the GEO expression database at the NCBI and are freely available (accession number GSE6609).

Results and discussion

Lon protease deficiency decreases the anaerobic survival of glucose-starved cells

Dukan and Nystrom (Dukan and Nystrom, 1999) demonstrated that the life-span of wild-type *E. coli* K-12 MG1655 cells can be extended by omitting oxygen during glucose starvation, consistent with the proposal that aerobically-generated reactive oxygen species decrease the longevity of *E. coli* cells. We initially attempted to replicate their results, but we used *E. coli* BL21(DE3) cells rather than the wild-type MG1655 because BL21(DE3) cells are widely used for protein overexpression. After two days of glucose starvation, 54 ± 5.3% of the cells survived aerobically, while only 4 ± 1.8% cells survived anaerobically (mean ± standard deviation). These results were the reverse of Dukan and Nystrom's with

MG1655 cells, indicating that factors which are essential for anaerobic survival may have been deleted in the BL21(DE3) strain.

Comparing the genotypes of these two strains, MG1655 and BL21(DE3) are both F^- , and BL21(DE3) lacks both the Lon and OmpT proteases because these have been reported to interfere with production of some full-length recombinant proteins. We considered the possibility that the Lon protease may play an essential role in survival under stress conditions (Gottesman and Maurizi, 2001), particularly because Lon degrades ribosomal proteins to supply amino acids required for the synthesis of adaptive proteins (Kuroda et al., 2001). Further, an *E. coli* K-12 mutant lacking four other distinct peptidase activities (N, A, B, and Q) lost viability much more rapidly than the parental strain during aerobic glucose starvation, again suggesting role for protein degradation in the survival during carbon starvation (Reeve et al., 1984).

To directly assess the effect of Lon protease deficiency on stationary-phase survival, we switched from BL21(DE3) to wild-type K-12. (*E. coli* B strains are naturally Lon protease-deficient (Donch and Greenberg, 1968).) To avoid effects of metabolic products and variation in the media on surviving starvation, cells were harvested from 24 h aerobic cultures and resuspended into M9-G medium lacking glucose. To test whether the presence of oxygen had any effect on plating efficiency, samples were plated in duplicate under both aerobic and anaerobic conditions. We found no significant difference in viability between the two plating environments so the results from both were averaged (Fig. 1).

During glucose starvation, the optical density decreased slightly, with no differences between strains nor atmospheric conditions (Fig. 1A). As expected, microscopy confirmed a slow decrease in cell size during starvation, accounting for the observed decrease in optical density (Akerlund et al., 1995). After 4 days of starvation, a few filamentous cells were observed in the Lon deficient cultures, but not in the wild-type. Viabilities of both the wild-type and *lon*⁻ strains during aerobic glucose starvation were similar after 7 days in starvation (Fig. 1B). Wild-type *E. coli* survived better under anaerobic conditions than aerobic (Fig. 1B and 1C), as reported by Dukan and Nystrom (Dukan and Nystrom, 1999). In contrast, the *lon* mutant lost viability faster under anaerobic conditions (Figs. 1B and 1C). Thus Lon protease deficiency decreased the anaerobic survival of *E. coli* without effect on aerobic survival.

Does Clp compensate for Lon deficiency aerobically?

The longer survival of *lon*⁻ cells under aerobic compared to anaerobic conditions might be a consequence of compensatory expression which occurred only in the presence of oxygen. The Clp chaperone-protease complexes constitute the other major ATP-dependent mechanism for degradation of proteins. ClpP is the proteolytic component of both ClpAP and ClpXP and is also highly conserved (Gottesman, 2003). ClpP and ClpX are required for normal adaptation and extended viability in stationary phase (Weichart et al., 2003). ClpS interacts with ClpA, altering the specificity of protein degradation by *ClpAP* (Dougan et al., 2002).

Thus, the compensatory expression of the Clp proteases under aerobic conditions might contribute to the improved survival of Lon deficient, and we tested this possibility experimentally. Firstly, we quantitated the amount of ClpP protein utilizing an antibody tagged with an infrared fluorophore. There was no difference in ClpP content in the *lon*⁺ and *lon*⁻ cells grown aerobically, nor was ClpP content decreased during anaerobic growth (Fig. 2). Secondly, we overexpressed either ClpP-ClpX or ClpA-ClpS in *lon*⁻ cells and tested whether the cells were rescued during anaerobic carbon starvation. We found that they were not (not shown). Conversely, we chose to compare the aerobic and anaerobic survival of a

single mutant lacking *clpP* with that of a double mutant lacking both *clpP* and *lon*. The single mutant, *clpP*⁻, was observed to have a modest decrease in survival after overnight starvation (Weichart et al., 2003), and it was able to grow in our M9 medium. However, the double mutant, *clpP*⁻*lon*⁻ could not grow, consistent with the observations of Kornberg and colleagues (Kuroda et al., 2001). We conclude that the effect of the Lon protease is not mediated through a compensatory increase in the Clp protease and that the mechanism by which Lon supports survival during anaerobic carbon starvation remains to be elucidated.

How are Lon deficient cells protected during aerobic carbon starvation?

Oxidatively damaged proteins increase during aerobic carbon starvation (Nystrom, 2004; Dukan and Nystrom, 1999), and cells which fail to control the burden of oxidized proteins lose viability (Desnues et al., 2003). We thus speculate that the aerobically-driven increase in oxidized proteins may be mechanistically important for induction of the adaptive response in Lon deficient cells.

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Figure 1.

Survival of wild-type and Lon deficient *E. coli* strains starved for glucose. (A) Optical density (B) Viability under aerobic conditions (C) Viability under anaerobic conditions. Each point in panels (B) and (C) shows the average and standard deviation from 4 plates; coefficients of variation were typically less than 15%.

Figure 2.
The effect of *lon* and of atmosphere on ClpP protein levels. ClpP was quantitatively immunodetected as described in Materials and Methods.