Crucial Roles of MicA and RybB as Vital Factors for σ^E -Dependent Cell Lysis in *Escherichia coli* Long-Term Stationary Phase

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Key Words

Escherichia coli · σ^E · Cell lysis · sRNA · Outer membrane protein · Long-term stationary phase

Abstract

σ^E -dependent cell lysis has been proposed to eliminate damaged cells in the stationary phase in *Escherichia coli*. In order to explore the relationship of this process to long-term stationary phase existence, we considered that *micA* and *rybB* could be important small regulatory RNA (sRNA) genes for σ^E -dependent cell lysis. A long-term stationary phase was observed at temperatures of $<37^\circ\text{C}$, but not $>38^\circ\text{C}$, and was found even in an *rpoS* knock-out background. Strains with disrupted *micA* or *rybB* were incapable of long-term stationary phase existence. Both strains drastically lost survivability accompanied by a dramatic accumulation of mutations. These findings allow us to speculate that σ^E -dependent cell lysis plays a key role in the establishment of the long-term stationary phase, presumably by eliminating damaged cells and thus preventing the over-accumulation of mutations.

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Introduction

Under optimal growth conditions, *Escherichia coli* exhibits a growth pattern consisting of five distinctive phases, lag phase, exponential phase, stationary phase, death phase and long-term stationary phase [Navarro Llorens et al., 2010; Zambrano and Kolter, 1996]. When adapted to a given environment, *E. coli* cells start to grow and divide exponentially. As nutrients become exhausted and/or cell density reaches a threshold, the cells enter a stationary phase where no increase in cell number occurs, but waste products gradually accumulate as a consequence of metabolic activities. This may become a stress that eventually leads to the death phase in which the number of viable and culturable (VAC) cells declines. Since the majority of cells in the death phase are viable but non-culturable (VBNC) or die, nutrients from a portion of such cells are released into the medium. The released nutrients support the survival of the remaining culturable cells, and VAC cells survive for months or years in the long-term stationary phase [Desnues et al., 2003].

E. coli possesses seven σ factors, each of which governs the expression of a distinct set of genes to conduct intracellular activities in accordance with the environment [Helmann and Chamberlin, 1988; Ishihama, 1999]. σ^S and σ^E play crucial roles in the stationary phase [Battesti et al., 2011; Nitta et al., 2000], during which cells are exposed to various stresses. Nutritional starvation and cytoplasmic stresses, including oxidative stress, trigger expression of the *rpoS* gene encoding σ^S [Zhang et al., 1998], which controls up to 10% of *E. coli* genes. Expression of these genes prepares the organism for survival in the stationary phase [Hengge-Aronis, 2002]. On the other hand, active σ^E accumulates under extracytoplasmic stress conditions and deals with damage to macromolecules caused by the stress [De Las Peñas et al., 1997; Missiakas et al., 1997; Raina et al., 1995]. There are >100 genes belonging to the σ^E regulon in *E. coli* [Kabir et al., 2005].

In the long-term stationary phase, a stable viable cell count is maintained for a long time by the balance between dying and viable cell populations. This is not in a static situation; rather, a dynamic equilibrium is established due to multiple population shifts [Roszak and Colwell, 1987]. The dynamic change has been proposed to be induced by a mechanism called 'growth advantage in stationary phase' (GASP). That is, the population is changing with a better fitness to scavenge for nutrients in the same culture [Zambrano and Kolter, 1996; Zambrano et al., 1993]. Such mutations, conferring a GASP phenotype, have so far been identified in *rpoS* [Zambrano et al., 1993], *lrp* [Zinser and Kolter, 1999, 2000, 2004], and the *ybeJ-gltJKL* region [Zinser and Kolter, 2000, 2004; Zinser et al., 2003]. All of these mutations result in increased catabolic activity of one or more amino acids as a source of carbon and energy [Fukuda et al., 2001; Stancik et al., 2002]. However, these three mutations alone do not seem to be sufficient to explain continuous population takeover or GASP cycling in the long-term stationary phase. It is thus expected that there are additional mechanisms for the dynamic equilibrium including elimination of viable but non-growth-advantaged cells.

In bacteria, small regulatory RNAs (sRNAs) are involved in the fine-tuning of gene expression by binding to target mRNAs with mediation of the RNA chaperone Hfq [Sauer and Weichenrieder, 2011; Sauer et al., 2012]. It is known that almost all *trans*-encoded sRNAs, genes of which are located separately from their target genes on the genome, are expressed under specific environmental stress conditions. These conditions include oxidative stress, anaerobic conditions, cell envelope homeostasis and glucose starvation [Repoila and Darfeuille, 2009; Vo-

Fig. 1. Survivability of a *rpoS* strain during long-term cultivation. W3110 *rpoS::tet* cells were aerobically grown in LB medium at 37°C under conditions as described in the Experimental Procedures section. At the times indicated, CFU were determined.

gel, 2009]. Of the *trans*-encoded sRNAs, *micA* and *rybB*, which are expressed by σ^E under extracytoplasmic stress conditions, downregulate outer membrane proteins (OMPs), OmpA, OmpC and OmpW, in *E. coli* [Udekwi and Wagner EG, 2007; Johansen et al., 2006]. This, in turn, causes disintegration of the outer membrane and leads to cell lysis [Murata et al., 2012]. The cell lysis cascade appears to relate to oxidative stress in the early stationary phase [Noor et al., 2009a]. This allowed us to speculate that the event contributes to the progression toward the death phase and/or long-term stationary phase.

Elucidation of the molecular basis of population takeovers or GASP cycling is crucial for understanding the survival strategy to establish a bacterial life cycle in the long-term stationary phase. In this study, we examine the contribution of σ^E -dependent cell lysis to cell survival in the long-term stationary phase of *E. coli* by focusing on the sRNAs, MicA and RybB.

Results

Long-Term Stationary Phase in the rpoS Knock-Out Background

The role of *rpoS* in the long-term stationary phase remains to be clarified, though aged cells from *rpoS* knock-out strains have been shown to exhibit a GASP phenotype [Finkel, 2006]. We thus examined the necessity of *rpoS* for the long-term stationary phase. W3110 *rpoS::tet* was aerobically grown in Luria-Bertani (LB) medium at 37°C, which is the optimal growth condition to establish survival in the long-term stationary phase [Navarro Llorens et al., 2010; Zambrano and Kolter, 1996], and the number

of colony-forming units (CFU) was determined (fig. 1). The colony-forming cells were always detected during cultivation, and the CFU level was kept at about 10^6 /ml even after 40 days. Therefore, it is likely that the *rpoS*-defective mutant can survive during the long-term stationary phase.

Growth Temperature as a Crucial Factor for Long-Term Stationary Phase

It has been shown that the magnitude of σ^E -directed cell lysis becomes enhanced as temperature increases, presumably due to oxidative stress caused by a high temperature [Noor et al., 2009a]. The stress may cause damage to cellular macromolecules including OMPs, which in turn triggers a σ^E -induced stress response [Martínez-García et al., 2003]. Stable long-term stationary phase in W3110 was observed at 33, 36 and 37°C but not at a temperature of $>38^\circ\text{C}$ (fig. 2 and data not shown), indicating that temperature is one of the critical factors for survival through the long-term stationary phase.

Using W3110, the first large decline in CFU during the death phase was observed after about 3 and 2 days at 33 and 36°C, respectively (fig. 2). The peaks after the first decline were observed at about 14 and 9 days at the temperatures of 33 and 36°C, respectively. The peak at each temperature might correspond to that of the first population with a GASP phenotype since the timing of appearance of the first peak nearly matched that of the previous report [Navarro Llorens et al., 2010]. The difference in peak position between the two different temperatures might reflect the speed of occurrence of cells bearing a GASP phenotype. On the other hand, at 42°C, only the first large decline was observed.

Necessity of MicA and RybB as Key Factors in σ^E -Dependent Cell Lysis for the Long-Term Stationary Phase

σ^E -dependent cell lysis may contribute to the elimination of damaged cells as a portion of the program giving rise to VBNC cells following stresses such as oxidative stress. Such a lysis event could occur after the beginning of the stationary phase [Murata et al., 2012; Noor et al., 2009b]. MicA and RybB are involved in the cascade of cell lysis under the control of σ^E and hamper the synthesis of OMPs to cause cell lysis. We assumed that such stresses also occur in the long-term stationary phase during which the cascade plays an important physiological role. To examine this assumption, W3110 *micA::kan* and W3110 *rybB::kan* strains were subjected to long cultivation (fig. 2). It was found that the CFU of these *micA::kan* and *rybB::kan* strains became non-detectable near the peak of

Fig. 2. Comparison of *micA*, *rybB* and wild-type strains during long-term cultivation at different temperatures. W3110 (circles), W3110 *micA::kan* (squares) and W3110 *rybB::kan* (triangles) cells were grown as described in the legend of figure 1 except for different temperatures, 33°C (a), 36°C (b) and 42°C (c), and CFU were determined at the times indicated.

the putative first GASP population in the parental strain. Notably, a peak or shoulder in both mutants was observed after about 7 days at the temperatures of 33 and 36°C. The position of the peak or shoulder corresponded to that of the lowest value of CFU following the stationary phase, the end of the putative death phase in the parental strain.

It was expected that the defect of σ^E -dependent cell lysis would give rise to the accumulation of damaged cells and an increase in mutation frequency. We thus determined the CFU on streptomycin- and rifampicin-containing plates with prolonged cultivation (fig. 3). The CFU of the *micA*- and *rybB*-disrupted strains on both drug-containing plates dramatically increased just before the

Fig. 3. Spontaneous drug-resistant mutants from *micA*, *rybB* and wild-type strains during long-term cultivation. W3110 (circles), W3110 *micA::kan* (squares) and W3110 *rybB::kan* (triangles) cells were grown as described in the legend of figure 1. CFU were counted on LB plates (a), streptomycin-containing LB plates (b) or rifampicin-containing LB plates (c) with samples collected at the times indicated.

time when the CFU on the drug-free plates became undetectable. The apparent mutation frequency at the peaks in the *micA*- and *rybB*-disrupted strains was 2–3 orders of magnitude higher than that in the parental strain: 5.8×10^{-2} for the *micA* strain on streptomycin plates, 2.4×10^{-2} for the *rybB* strain on streptomycin plates, 3.7×10^{-2} for *micA* on rifampicin plates and 1.2×10^{-2} for *rybB* on rifampicin plates. Notably, the mutation frequency of the parental strain in the long-term stationary phase seems to be much higher than that in the exponential phase, which is consistent with results of previous studies [Finkel, 2006]. Therefore, it is likely that the accumulation of mutations is linked to the drastic decrease in CFU in the *micA*- and *rybB*-disrupted strains. Taken together, the results suggest that *micA* and *rybB* play a key role in maintenance of survival in the long-term stationary phase.

Discussion

The first GASP mutation, identified in the *rpoS* gene, resulted in extension of the C-terminal region of RpoS [Zambrano and Kolter, 1996], conferring enhanced catabolism of amino acids [Zinser and Kolter, 1999, 2004]. Some *rpoS*-down mutations similarly exhibit a GASP phenotype [Zambrano et al., 1993; Zinser and Kolter, 2000], but *rpoS* null alleles were reported to express no GASP phenotype [Zambrano et al., 1993]. On the other hand, the occurrence of *rpoS*-independent GASP has been observed [Martínez-García et al., 2003]. In this study, we suggest that the long-term stationary phase in *E. coli* is maintained without *rpoS*. Both the *rpoS*-disrupted mutant and parental strains exhibited wave-like curves of CFU after the putative death phase (fig. 1, 2). At least the first and second main peaks after the death phase appeared at a time similar to those in previous studies, which suggests that aged cells at the time around the peak outcompeted unaged cells [Finkel, 2006; Martínez-García et al., 2003]. These results may suggest population takeovers by acquisition of mutations in *rpoS* as well as other genes. Interestingly, when examined at different temperatures, the peak position in CFU was greatly shifted (fig. 2). Considering the evidence that oxidative stress increases as temperature rises [Noor et al., 2009b; unpublished], the leftward shift of the peak position of CFU at a higher temperature might be due to faster mutation accumulation to create advantageous cells for growth.

Disrupted mutants of *micA* and *rybB* exhibited a reduction in the CFU as was observed for the parental strain after the stationary phase, but they lost CFU at the beginning of the long-term stationary phase under cultivation conditions at 33, 36 or 37°C (fig. 2, 3). Notably, no polar effect by the *kan* cassette in the disrupted mutants is expected because the transcription direction of their downstream gene is opposite to that of *micA* or *rybB* in the *E. coli* genome. MicA and RybB, which contribute to the cascade of σ^E -dependent cell lysis [Murata et al., 2012], are involved in the negative regulation of *ompA* and *ompC/ompW* expression [Udekwi and Wagner, 2007; Vogel and Papenfort, 2006], but are not related to the repair response under the control of σ^E . Disruption of *micA* and *rybB* results in hampering of σ^E -dependent cell lysis, and enhanced expression of both genes causes an increase in cell lysis [Murata et al., 2012]. This evidence indicates that σ^E -dependent cell lysis is essential for continuous survival in the long-term stationary phase. In addition, based on the number of spontaneous drug-resistant mu-

tants, the mutation frequency dramatically increased just before the decline of CFU to an undetectable level in the *micA*- and *rybB*-disrupted mutants. These findings and previous evidence allow us to speculate that the σ^E -dependent cell lysis pathway eliminates damaged cells, preventing rapid accumulation of mutations in the population. On the other hand, a number of different mutations in the mutant defective for the cell lysis pathway would accumulate, and eventually VAC cells would catastrophically decrease in the population. Consistently, a high mutation frequency [Finkel and Kolter, 1999] and the existence of a (transient) hypermutable state in the long-term stationary phase have been reported [Finkel, 2006; Torkelson et al., 1997], and these may lead to genetic diversity in the phase. It is thus possible that the σ^E -dependent cell lysis pathway contributes to the maintenance of an appropriate mutation level for sustainable survival. The σ^E -dependent cell lysis pathway would have an additional role in providing nutrients for the next generation.

Our data and previous results from other research groups [Finkel, 2006; Navarro Llorens et al., 2010; Zambrano and Kolter, 1996] suggest that the survival of cells in the long-term stationary phase depends on the appearance of new and advantageous mutations. Cells with advantageous mutations may form a dominant population as a GASP phenotype. Continuous changes by such a population may maintain a certain level of VAC cells to establish the long-term stationary phase. Further analysis is required to elucidate the mechanism of dynamic population takeovers in the long-term stationary phase.

Experimental Procedures

Materials

Streptomycin, rifampicin, kanamycin and tetracycline were purchased from WAKO Co., Ltd. (Japan).

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Bacterial Strains, Medium and Culture Conditions

The bacterial strains used in this study were derivatives of *E. coli* K-12 and included W3110 [Kabir et al., 2005], W3110 *micA::kan* [Murata et al., 2012], W3110 *rybB::kan* [Murata et al., 2012] and W3110 *rpoS::tet* [Kabir et al., 2005]. Cell culture was performed using a modified LB medium [1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl] at the indicated temperature under aerobic conditions by reciprocal shaking (100 rpm min⁻¹). Appropriate drugs were added to the following final concentrations: streptomycin, 5 µg/ml; rifampicin, 15 µg/ml; kanamycin, 25 µg/ml; tetracycline, 5 µg/ml.

Cell Growth Experiments

For cell growth experiments, one colony of W3110 or its derivatives, newly grown on LB plates, was inoculated into 3 ml of medium. After 12 h of preculture at 37°C, the culture was diluted and adjusted to an OD₆₀₀ of 0.1. Of this cell suspension, 20 µl was inoculated into 20 ml of medium in a 100 ml Erlenmeyer flask and subjected to aerobic cultivation at the temperature indicated. Cell growth was monitored by measuring OD₆₀₀ and CFU. CFU were estimated by counting colonies at 24 h after appropriate dilution of the cell culture and spreading on LB plates [Nitta et al., 2000]. Cell growth experiments were repeated at least three times. The timing of the main features of the growth curves was reproduced in repeated experiments within 24 h.

Estimation of Mutation Frequency

Cell cultures were appropriately diluted and spread on LB plates or on LB plates containing streptomycin or rifampicin, and CFU were then estimated after incubation at 37°C. The apparent mutation frequency was calculated as the ratio of CFU in the presence of a drug to that in the absence of the drug.

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