Chapter 19

Survival Strategy of *Escherichia coli* in Stationary Phase: Involvement of σ^E-Dependent Programmed Cell Death

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http://dx.doi.org/10.5772/67672

Abstract

In a natural habitat, microbes respond to alterations in the amounts of nutrients or to stresses such as osmotic stress and stresses caused by low or high pH, salt, heat, and antibiotics by changing their mode for proliferation or survival. Similarly, *Escherichia coli* cells in a test tube change the growth mode according to environmental conditions when they enter a stationary phase. Until a sufficient supply of nutrients, the organism survives under such stressful and nutrient-limited conditions by altering gene expression to be more protective against such conditions. The definite trigger of the onset of stationary phase is still unclear, but several lines of evidence indicate that the regulation mechanism is very complicated and involves several transcriptional factors including alternative sigma factors, σ^E and σ^S. In addition, *E. coli* cells behave as a community of species and give rise to programmed cell death (PCD) for ensuring survival by controlling the cell number and supplying nutrients to sibling cells in long-term stationary phase (LTSP). The main PCD is probably performed by σ^E in *E. coli*. In this chapter, physiological functions of σ^E and PCD are introduced and reviewed and their possible involvement in survival mechanisms in stationary phase, especially LTSP, is shown.

Keywords: survival mechanism, envelop stress, σ^E, programmed cell death, long-term stationary phase

1. Introduction

1.1. Brief introduction of σ^E

Living *Escherichia coli* cells are constantly suffering from various stresses. The bacterium thus possesses mechanisms to sense stresses and deal with them by changing gene expression

levels and metabolisms. σ^E, one of the seven sigma factors existing in *E. coli* [1], is associated with the core RNA polymerase complex and initiates transcription by directly recognizing a promoter consisting of specific elements. σ^E was found for the time as a heat shock sigma factor due to reduction in the expression level of heat shock proteins at 50 °C in mutants of *rpoE*, encoding σ^E [2] and it is thought to be one of heat shock sigmas like σ^H. σ^E has been recognized as an envelope stress-responsive sigma factor [3] that senses an abnormality of the outer membrane integrity. Under the control of this sigma, several important genes are governed, for example, σ^H and genes for protein folding and degradation [4]. In addition, σ^E represses the synthesis of outer membrane proteins (OMPs). We show ingenious mechanisms of σ^E management of its molecule and activity and functions of genes under the control of σ^E in Section 2.

1.2. Brief introduction of PCD

PCD is conserved for all genetically encoded processes that lead to cell suicide. This conceptual word was first proposed in 1964 [5]. PCD that is observed in development, aging, and pathology in eukaryotic multicellular organisms is classified into three categories based on morphological characteristics such as apoptosis, autophagy, and necrosis. Among these, apoptosis, first described in 1972 [6], is the most well-characterized PCD. The morphological manifestations associated with apoptosis include chromatin condensation, chromosomal DNA fragmentation, membrane blebbing, cell shrinkage, and disassembly of the cell into membrane-enclosed vesicles. Apoptosis is highly regulated, and proteases called caspases play key roles in the induction of DNA fragmentation in the activation cascade [7]. Autophagy is the process by which a vesicle called an autophagosome is constructed for atrophy of the nucleus but with no DNA fragmentation [7]. Necrosis is triggered by activation of various receptors for loss of cell membrane integrity and uncontrollable release of intracellular contents into the extracellular space [7]. The physiological importance of these PCDs in the development of an animal has been well defined. For example, during embryonic development, the earliest form of the human hand resembles a paddle due to the elimination of excess cells by apoptosis. PCD mechanisms are also responsible for the homeostasis of multicellular organisms by the elimination of damaged cells that may become a source of cancer cells in the body.

It was thought that PCD only exists in eukaryotic cells, but several scientists have considered the possibility of the existence of bacterial PCD resembling eukaryotic PCD mechanisms. Indeed, a growing body of evidence has shown that PCD is indispensable for bacterial development and is closely associated with bacterial survival mechanisms [8, 9]. Bacterial communities utilize PCD for survival of their population when suffering from oxidative stress, nutrient deprivation, phage infections, or other problems. The cell survival mechanism is a response to stresses outside cells and inside cells, but excessive damage turns on the PCD mechanism of some cells to help sibling cells. In the development processes of bacteria, PCD provides nutrients to sibling cells, releases components, and promotes special aspects. Indeed, biofilm formation, sporulation, and other multiple cell-like developments have been shown to bear PCD mechanisms in these processes. In biofilm development, cell death and lysis are required for the release of genomic DNA (known as extracellular DNA), which becomes incorporated

into the biofilm matrix and serves as an adherence molecule [10]. For the development of sporulation, sporulating cells produce a killing factor for nonsporulating cells, from which released nutrients support sporulation. Moreover, the mother cell in the sporulating population undergoes PCD to release the mature spore via its autolysis [11]. As other mechanisms, *Fratricide* behavior during its genetic transformation, autolysis in *Myxococcus xanthus* development, developmental cycle in *Streptomyces*, and coccoid formation in *Helicobacter pylori* also include PCD in their processes [9].

Many bacterial PCDs are induced through the toxin-antitoxin (TA) system. Five types of TA systems have been found and characterized [9, 12]. Type I has an antisense RNA that pairs with its corresponding toxin mRNA. The difference in transcription between toxin RNA and antitoxin RNA controls the toxin activity. Type II has a protein antitoxin that detoxifies its corresponding toxin protein by their protein-protein interaction. This type of TA system is most abundant. Type III has an antitoxin RNA that interacts directly with the target toxin protein to form an antitoxin RNA-toxin inactive complex. Type IV has a protein antitoxin that stabilizes the target of the toxin by direct binding. Type V has an endoribonuclease that cleaves the target toxin mRNA. These TA systems play important roles in several cellular processes such as plasmid stabilization, formation of persistent cells, peptidoglycan synthesis, resistance to bacteriophages and antibiotics, and inhibition of macromolecule and biofilm formation [9, 12]. PCD via a TA system is executed by the role of toxin proteins. *mazEF* in the type II TA system in *E. coli* has been the most intensively investigated and it has been shown to play a key role in the PCD process.

It has been suggested that the bacterial strategy for survival against DNA damage resembles the PCD mechanisms in eukaryotes [13]. The PCD mechanisms characterized in both prokaryotic and eukaryotic cells indicate that DNA damage leads to cell death when the damage is irreparable. Bayles reported that the death pathway also leads to apoptosis-like processes or autolysis [13]. The similarity of cell death systems in eukaryotes and bacteria suggests that the common origin of this system is derived from endosymbiotic bacteria [9]. Therefore, PCD is a basic mechanism for organisms in all kingdoms for the maintenance of communities, and this system has been acquired at a very early stage of appearance of life on earth. In Section 3, we summarize PCDs in *E. coli* and show σ^E-dependent cell lysis as one of the PCDs and its physiological roles, which our group has discovered.

1.3. Brief introduction of LTSP

In nutrient-sufficient media in the laboratory, *E. coli* exhibits a typical growth curve consisting of five phases: lag phase, log phase, stationary phase, death phase, and LTSP (**Figure 1**) [14, 15]. Among these phases, characteristics of first three phases have been described in detail elsewhere [15]. It has been thought that cells in the death phase gradually die, and it is known that 99.9% of the cells are not viable in that phase. However, Finkel reported that *E. coli* is able to survive for 5 years in LB medium without any additional nutrients if the volume and osmolarity of the medium are maintained [14]. The transition of viable (colony-forming) cell number from 10^9 cells/ml at the stationary phase to 10^6 CFU/ml at the LTSP [14] is accomplished by a rapid decrease in an exponential fashion in the death phase. Then the viable cell

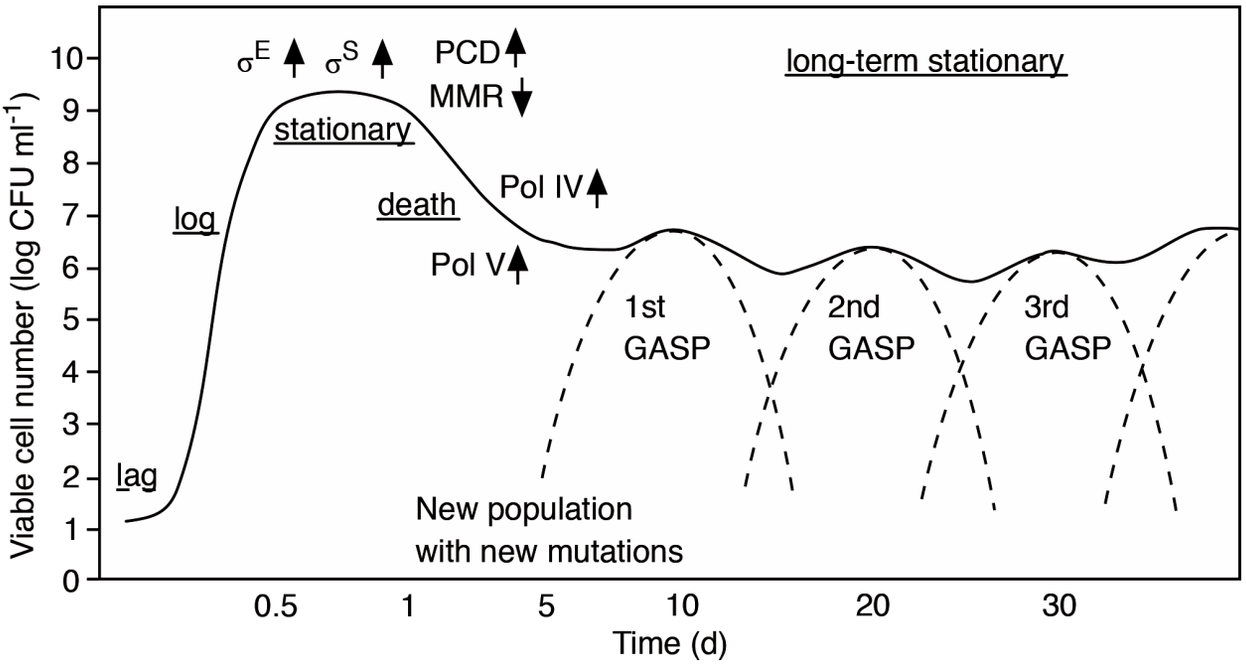

Figure 1. Transition model of viable cells until the LTSP in *E. coli*. The LTSP is created presumably by the sequential alteration of the expression of regulators. The sigma factors σ^E and σ^S are important regulators for onset of the stationary phase and the consequent death phase. PCD and SOS-induced DNA polymerases, Pol II, Pol IV, and Pol V, are thought to be important factors for maintenance of the LTSP. Adapted from Finkel [14].

number is maintained around 10^6 CFU/ml by unknown mechanisms for a very long period. Interestingly, in the LTSP, viable cells show a growth advantage against parent strains [16]. The phenotype of these cells is called growth advantage in stationary phase (GASP). In addition, GASP mutants consecutively occur every 10 days in the same culture [17]. Therefore, cells in the LTSP are not static and a dramatic population change occurs for adapting to the environmental perturbation of nutrients and conditions for survival.

What factors can lead *E. coli* cells from the stationary phase to the LTSP? The onset and course of the stationary phase have been summarized well in other reviews [15, 18]. Briefly, at the beginning of the stationary phase, the abundance of specific sigma factors is known to change: σ^E and σ^S molecules are increased by fivefold and by threefold to fourfold, respectively [19–21]. The physiological roles of σ^E and σ^S suggest that these factors enable cells to adapt to environments in the stationary phase by changing expression of 10% of the genes of *E. coli*. However, it has not been clarified how the death phase and LTSP start. Since protein expression level is kept low for several days in the stationary phase [22], cells may have some activity to accomplish preparation for the coming phases. These activities are probably related to PCD mechanisms [14] as described below. Several factors have been considered for the transition to the LTSP. One of these factors is reactive oxygen species (ROS). Indeed, mutants of genes for NADH dehydrogenase in the respiratory chain, which is a primary source of ROS, exhibited no GASP phenotype [23]. In addition, GASP phenotypes are altered by vessel volume of cultures, probably affecting dissolved oxygen concentrations in the medium [24]. On the other hand, we have revealed that σ^E-dependent PCD is essential for

http://dx.doi.org/10.5772/67672

the GASP phenotype [25], indicating the responsibility of PCDs for the LTSP transition. We describe the possible survival mechanism of *E. coli* in the LTSP and the importance of σ^E-dependent PCD in Section 4.

2. Functions of σ^E

2.1. Mechanisms of membrane stress responses for σ^E activation

Bacteria have mechanisms for rapid responses to environmental stresses, especially on the envelope because cell structure is maintained by integrity of the membrane. There have been many studies on membrane stress responses. In Gram-negative bacteria, such responses are known as envelope stress responses (ESRs). There are five known ESRs, Cpx, σ^E, Bae, Rcs, and Psp ESRs, that are induced by a variety of envelope stresses and alter the expression of adaptive functions to modify the envelope, rid cells of a toxic entity, and/or repair substantial damage [3]. Of these ESRs, σ^E ESR, a subset first found in *E. coli*, is known to respond to stresses such as stresses from heat and alkali due to damage of the outer membrane [26]. The σ^E ESR detects perturbations in biogenesis of the outer membrane or lipopolysaccharide (LPS) due to protein-folding problems in the periplasmic space and outer membrane (**Figure 2**). The key protein in this response is a transmembrane protein of RseA as an anti-σ^E protein capturing σ^E to inactivate it under nonstress conditions. Under stress conditions, σ^E activation is accomplished by the stepped degradation of RseA via three proteases, DegS, RseP (YaeL), and ClpXP. Senescing of the integrity of OMPs, which causes the activation of DegS by binding with unfolded OMPs, is the first key mechanism of σ^E activation [26]. In addition, DegS cleavage of RseA is physiologically inhibited by RseB binding to a conserved region near the C-terminus of the poorly structured RseA domain [27]. Therefore, RseB can negatively regulate the RseA degradation [27, 28]. RseB senses LPS integrity for binding with released LPS, and LPS displaces RseA from RseB due to antagonization of binding [29]. The subsequent intramembrane proteolysis of RseA by RseP is not performed when RseB is bound to RseA due to blockage through the side filtering function of the two PDZ domains of RseP [30]. Under stress conditions, the exposed periplasmic domain of RseA is cleaved by DegS between V148 and S149 [26]. Consequently, specific recognition of cleaved RseA is performed by the PDZ tandem domains of RseP [30], and specific cleavage of the transmembrane region of $RseA^{1–148}$ is also executed at A108 and C109 [31]. Finally, the cleaved cytoplasmic region of $RseA^{1–108}$ is recognized by SspB, and $RseA^{1–108}/\sigma^E$ complex is delivered to cytoplasmic AAA+ proteinases such as ClpXP [32, 33]. Destruction of the RseA fragment allows σ^E liberation and activation to cause the transcription of stress-responsive genes under the control of σ^E [28, 34].

These dual molecular signals (unfolded OMPs and LPS) are key factors for the σ^E ESR to sense outer membrane stresses [29]. For cell formation, OMPs and LPS are transported from the cytoplasm to the outer membrane in *E. coli*. The transport of OMPs as a beta-barrel structure is performed by the Sec-SurA-BAM system [35]. DegS is activated by binding of a peptide bearing a YxF motif at the C-terminus of an OMP, which is exposed by envelope stress,

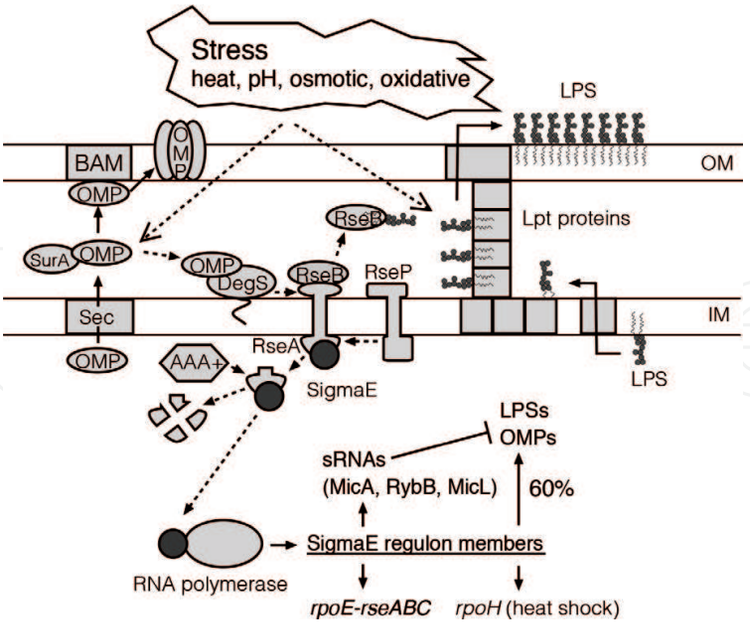

Figure 2. Schematic diagram of the σ^E signaling pathway and the σ^E regulon cascade. RseA is a key protein for σ^E activation. RseA, which is an antisigma factor, captures σ^E and neutralizes its activity. Two types of signaling molecules, OMPs and LPSs, are key activators of the proteinase DegS because the binding of the C-terminus of an OMP is required for DegS activation and the binding of LPS to RseB is required for deblocking of the RseA cleavage by DegS. Consequently, RseA is sequentially digested by RseP, and the RseA-N terminus is degraded by AAA+ proteinase, by which σ^E is released and activated to form a holo-RNA polymerase complex. Expressed σ^E-regulon members consist of LPS- and OMP-related proteins. sRNAs play key roles in the prevention of overproduction of LPSs and OMPs and in elimination of OMPs in σ^E-dependent cell lysis. Adapted from Lima et al. [29].

releasing from SurA or misfolding in BAM [28]. LPS is also transported by the aid of Ltp proteins, and LptA is a key component of the transenvelope complex to shuttle LPS to the outer membrane [36]. LptA less efficiently binds to LPS against RseB at 45°C [29], suggesting that LPS is easily caught by RseB under heat shock conditions. In addition, RseB can sense many mislocalized LPS species [29]. Therefore, both DegS activation and RseB detachment are essential for the initiation of RseA proteolysis for σ^E liberation. However, σ^E activity increases when either OMP or LPS mutations have accumulated [26, 29, 37], suggesting that a crosstalk between OMP and LPS biogeneses might be an additional regulation that can induce σ^E activation [28, 29].

This kind of proteolytic signal transduction and regulator-activating mechanism provides distinctive features for σ^E regulon as a transient expression. In the σ^E ESR, the initial signal-sensing cleavage of RseA is a rate-limiting step but the degradation of cytoplasmically fragmented RseA by AAA+ proteinase is relatively fast. Whereas, RseA is in excess over σ^E under normal conditions and the expression level of *rseA* is higher than that of *rpoE* [38]. Consequently, activated σ^E is rapidly deactivated, resulting in a short-period response to envelope stresses [33].

2.2. σ^E regulon genes

Activated σ^E forms a holo-RNA polymerase with the core RNA polymerase complex to initiate transcription by recognizing consensus sequences located upstream from coding genes

called promoters. Several experiments have been carried out in *E. coli* to find consensus sequences of promoters and σ^E-regulating genes, σ^E regulon genes. Attempts were made to identify consensus sequences for σ^E by several procedures, and genomic information and a search algorithm predicted a conserved −35 motif (GGAACTTTT) and a conserved −10 motif (T/CGGTCAAAA) [39–41]. σ^E regulon members in *E. coli* have been found by proteomics [4, 39], genetic strategies, [39, 40] and microarray analysis [4, 41]. Results of those studies showed that σ^E-holo RNA polymerase transcribes two kinds of RNAs, mRNAs for several genes and antisense sRNAs that repress the expression of several genes. Analysis of σ^E regulon genes showed that the regulon consists of 19 transcription units and 23 proteins. At least 60% of the regulon members are responsible for the synthesis and assembly of LPS and OMPs or regulatory proteins for these two key elements of the outer membrane [41]. The majority of σ^E regulon genes in *E. coli* are genes encoding periplasmic folding factors, periplasmic proteases, OMP assembly proteins, LPS translocation and assembly proteins, proteins for synthesis of phospholipids and lipid A, and a heat shock sigma factor coded by *rpoH* [39, 42]. One of most important operons under the control of σ^E is the *rpoE-rseABC* operon coding σ^E itself, RseA as an anti-σ^E, RseB repressor, and a *soxR*-influencing protein, respectively [43]. This operon is induced by two σ^E promoters, one upstream of *rpoE* and the other upstream of *rseA*. Therefore, σ^E activation causes a negative feedback loop by double transcriptions from the two promoters for rapid repression of σ^E activity. On the other hand, it has been revealed that small RNAs (sRNAs) are controlled by σ^E and work as repressors for gene expression. There are two distinct σ^E-inducible sRNAs, MicA and RybB, that bind to Hfq, an RNA chaperone protein required for the function and/or stabilization of sRNAs, and target mRNAs from 31 genes for major porins, metabolism, ribosome biosynthesis, toxin-antitoxin, and transcriptional factor PhoP [44]. In addition, MicL (SlrA) targets only one mRNA, which encodes the outer membrane lipoprotein Lpp, the most abundant protein of the cell [45]. Taken together, MicA, RybB and MicL allow σ^E to prevent the synthesis of abundant outer membrane proteins in response to stresses.

3. σ^E-dependent PCD

3.1. PCD in *E. coli*

PCD in *E. coli* is also closely associated with the strategy for sensing damage in DNA and the envelope structure. Three PCD mechanisms, a TA system, apoptosis-like death (ALD) and σ^E-dependent cell lysis, have been found in *E. coli* (**Figure 3**). Of these, the most intensively investigated PCD is *mazEF*, a TA system in which *mazF* encodes a stable toxin, sequence-specific endoribonuclease, and *mazE* encodes a labile MazF-antitoxin that is degraded *in vivo* by ATP-dependent ClpPA serine protease [46–48]. Toxicity of MazF is attributable to its endoribonuclease activity, specific for the trinucleotide sequence of ACA in mRNA, including the 3′-end fragment of 16S rRNA, to block protein synthesis and to synthesize specific proteins [49]. Specifically expressed proteins are classified into "survival proteins" and "death proteins" including SlyD, YfiD, YgcR, and ClpX [50]. Death proteins induce the DNA fragmentation and membrane depolarization [48]. In addition, *mazEF*-mediated PCD is regulated by a

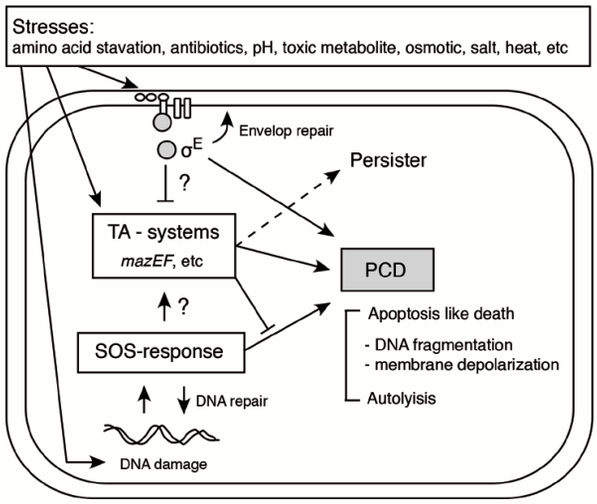

Figure 3. Activation pathways of PCD in *E. coli*. Several stresses affect cellular components including envelopes, DNA, and proteins, and these damaged materials become a signal for each stress response directly or indirectly. If the damage is excessive, PCD is triggered by several mechanisms. In *E. coli*, three mechanisms for PCD including TA systems, SOS-response–dependent cell lysis and σ^E-dependent cell lysis have been reported. SOS mainly responds to DNA damage and σ^E mainly responds to envelope damage. These three responses can directly induce PCD, but they are weakly connected to each other [46, 48].

quorum-sensing factor as a linear pentapeptide Asn-Asn-Trp-Asn-Asn (NNWNN), called an extracellular death factor (EDF) [51]. The EDF directly binds to MazF dimers to release MazF from the MazF–MazE complex, leading to cell death [52]. Moreover, *mazEF*-mediated PCD is activated under various stressful conditions including extreme amino acid starvation, inhibition of transcription and/or translation by antibiotics including rifampicin, chloramphenicol, and spectinomycin, an inhibitor protein of translation, DNA damage caused by thymine starvation as well as by mitomycin C, nalidixic acid and UV irradiation, and oxidative stress [47]. Notably, 28 other putative TA systems including DinJ-DafQ, DinP-YafN, RelB-RelE, and ChpS-ChpB have been identified in the *E. coli* K12 genome [12].

An SOS response-mediated PCD pathway was recently identified in *E. coli* is called apoptosis-like death (ALD) pathway [48]. The ALD pathway is activated by an extreme SOS response under severe DNA damage conditions [53] and follows apoptosis-like characteristics including rRNA degradation by the endoribonuclease YbeY [54], upregulation of a unique set of extensive damage-induced genes, decrease in respiration activity, and formation of high levels of OH^-, resulting in cell death [53]. Analysis of the relationship between *mazEF*-EDF and ALD revealed that the ALD pathway is inhibited by the *mazEF*-EDF–mediated PCD pathway [48].

In addition to DNA damage, envelope damage has been shown to be a trigger of PCD in *E. coli*. Envelope damage is caused by various factors including antibiotics, toxic metabolites, bacteriocins, osmotic, pH, and salt. In Gram-negative bacteria, the damage is sensed and transduced via ESRs. The ESRs alter the expression of specific genes related to functions that modify the envelope, rid cells of the toxic entity and/or repair the envelope damage [3]. σ^E-dependent PCD, which is one of envelope damage related PCDs, was first reported

in 2000 [19]. This PCD occurs as an autolysis mechanism, which is a growth phase-specific cell lysis [19], and removes only viable but nonculturable (VBNC) cells [19]. The molecular mechanism of the PCD is described below in detail. Interestingly, *rpoE* coding σ^E is an essential gene [55] because the absence of σ^E causes a cell death-signaling pathway including *hicB* (*ydcQ*) that encodes for an antitoxin of the HicA toxin proteinase [56, 57].

3.2. Mechanism of σ^E-dependent PCD

At the early stationary phase, *E. coli* cells undergo a decrease in viable cell number and almost all of the cells become VBNC cells [16, 58]. Elevation of the activate intracellular σ^E level, due to disruption of *rseA* for anti-σ^E or *rpoE*-increased expression, causes cell lysis at the beginning of the stationary phase, and this lysis occurs in wild-type cells at a low level [19, 59]. This mechanism may contribute to the removal of VBNC cells that have accumulated at a specific phase probably due to the accumulation of intracellular oxidative stress, and it is called σ^E-dependent PCD. Murata et al. showed that σ^E-dependent PCD is mediated by MicA, RybB, and PpiD [59]. MicA and RybB are transencoded sRNAs, and their expression is positively regulated by σ^E [60–62]. When misfolded OMPs or periplasmic proteins have accumulated, the expression of their sRNAs is induced by active σ^E, and MicA and RybB cause reduction in the levels of mRNAs of *ompA* and both *ompC* and *ompW*, respectively, via interaction between the sRNAs and the corresponding mRNA by assisting Hfq as an RNA chaperon and degradation of the mRNAs by ribonucleases [63]. Some OMPs are known to be physiologically and structurally crucial for cell activity [64]. OmpA as a structure protein is involved in the maintenance of cell shape and the passage of hydrophilic compounds through the outer membrane [65]. OmpC is the major porin protein that functions as a cation-selective porin [66]. However, no physiological function of OmpW has yet been determined [67]. These OMPs are greatly decreased in σ^E-activated cells [4, 19], and *micA*- or *rybB*-disrupted mutants and *micA*- or *rybB*-overexpressed cells repress and induce σ^E-dependent PCD, respectively [59]. Therefore, σ^E-dependent PCD is caused by the reduction of OMPs via posttranscriptional regulation including MicA and RybB. Recently, MicL has been found as the third σ^E-dependent sRNA that targets an mRNA for lipoprotein Lpp [45]. Since Lpp is the most abundant protein in the outer membrane [64], MicL may also be involved in σ^E-dependent PCD.

The level of PpiD is greatly reduced in σ^E-activated cells, though its regulation mechanism is unknown [68]. PpiD is a peptidyl-prolyl *cis-trans* isomerase as a periplasmic folding catalyst that catalyzes the rapid interconversion between the *cis* and *trans* forms of the peptide bond Xaa-Pro [69]. PpiD recognizes the early OMP folding intermediates and suppresses OMP biogenesis defects. Indeed, overexpression of PpiD represses σ^E-dependent cell lysis probably due to the acceleration of OMP folding [68]. Thus, the reduction of PpiD ensures the elimination of OMPs after the degradation of OMP mRNAs by sRNAs.

As shown in the model in **Figure 4**, when cells are exposed to some stresses as signals, mainly oxidative stress [19, 70], unfolded proteins accumulate in the outer membrane or periplasmic space, in turn causing the elevation of active σ^E in the cytoplasm. Active σ^E induces the expression of sRNAs, leading to the reduction of OMPs including Lpp. Furthermore, the reduction of PpiD via active σ^E enhances the disintegration of OMPs, resulting in collapse of the integrity of the outer membrane and finally lysis of cells.

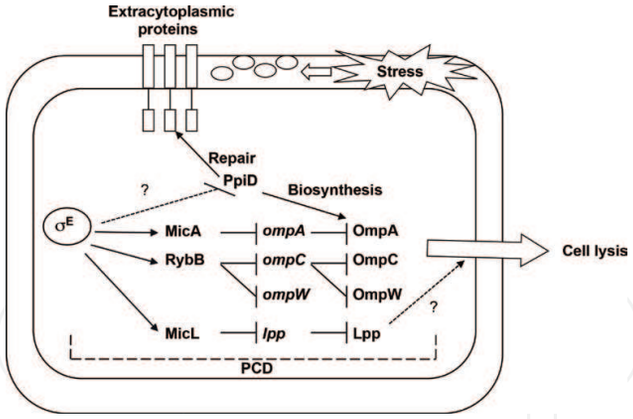

Figure 4. A model of σ^E-dependent PCD. When cells are exposed to stresses such as oxidative stress, σ^E is activated in response to damaged OMPs and increases and decreases the amounts of sRNAs (MicA and RybB) and PpiD as a folding catalyst protein, respectively. The expression of *ppiD* is greatly reduced under the condition of accumulation of active σ^E. The relationship between σ^E and *ppiD*, however, has not been clarified yet. MicA and RybB repress the expression of mRNAs of *ompA* and both *ompC* and *ompW*, respectively. The biosynthesis and repair of damaged OMPs are repressed by reduction in the PpiD level. As a result, the integrity of the outer membrane collapses and cell lysis progresses and finally causes cell death. MicL sRNA, which represses the expression of *lpp* mRNA, may also participate in σ^E-dependent PCD. Adapted from Murata et al. [59].

3.3. Function of σ^E-dependent PCD

Cell lysis in *E. coli* occurs under a general cultivation condition and remarkably increases after the early stationary phase. Most of the lysis seems to be σ^E-dependent because enhanced expression of *rseA* for anti-σ^E diminished the lysis [68]. The lysis level was significantly reduced when plasmid clones of *sodA* and *katE* for superoxide dismutase and catalase, respectively, were introduced [70]. Consistent with the level of lysis, the amounts of ROS are small in the exponential phase and large with a peak at the early stationary phase. The introduction of antioxidative stress genes eliminated about 80% of ROS. These findings suggest that oxidative stress is a trigger for the lysis [70]. The lysis is greatly enhanced in a *katE*-disrupted background, indicating that intracellular oxidative stress is involved in the lysis. Considering the signal transduction cascade to provide active σ^E [28], it is assumed that intracellular oxidative stress causes damage of OMPs by a modification such as carbonylation [71].

The trigger for σ^E-dependent cell lysis seems to be not only oxidative stress but also other stresses. The proposed signal transduction cascade for active σ^E [28] indicates the possibility that extracellular stress evokes σ^E-dependent cell lysis. Indeed, a disrupted mutation of *rpoS* for σ^S enhanced σ^E-dependent cell lysis at the early stationary phase [72]. Consistent with this, extracellular stress like toxic materials increases in a medium at the early stationary phase [14, 18]. Since σ^S functions as a general stress-response sigma factor to protect cells from various stresses [73], *rpoS* mutation results in the elevation of extracellular stress. It is known that σ^E becomes active through the σ^E activation cascade, which is initiated by conformation change of OMPs caused by a high temperature or ethanol as an extracellular stress [26, 29, 61]. Therefore, accumulation of extracellular and/or intracellular stresses beyond the elimination capacity by the stress response mechanism may cause conformation change of outer membrane proteins, which activates σ^E, resulting in σ^E-dependent cell lysis.

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As shown in **Figure 4**, active σ^E determines the direction to either the repair or cell lysis pathway, presumably reflecting the level of damage of OMPs. If only a few OMPs are damaged, the number of active σ^E molecules may be not enough to express sRNAs such as *micA* and *rybB*, which may be insufficient to cause cell lysis but can express genes for the repair pathway. On the other hand, damage of OMPs over a certain threshold evokes the cascade of σ^E-dependent cell lysis. The coincidence of the fact that OMPs are monitoring proteins for cell damage and/or σ^E-dependent cell lysis and the fact that σ^E-dependent cell lysis is induced by reduction in the amount of OMPs is highly notable. OMP-damaged cells may be much more sensitive than undamaged cells to σ^E-dependent cell lysis.

σ^E-dependent cell lysis seems to eliminate some of the VBNC cells that have been damaged by some kinds of stress. The amount of cell lysis increases in parallel with increase in VBNC cells in the stationary phase, and most of the lysis was suppressed by enhanced expression of *rseA* [68]. An *rseA*-disrupted mutant that constitutively expresses active σ^E shows a phenotype that is characterized by decrease in cell density without a significant influence on colony-forming unit (CFU) but with protein accumulation in the medium [19]. This phenotype suggests that VBNC cells or some of the VBNC cells are subjected to σ^E-dependent cell lysis. This limited cell lysis might reflect the existence of a mechanism to distinguish damaged and undamaged cells. It is hypothesized that VBNC cells to be lysed have damaged OMPs to some extent and thus are susceptible to σ^E-dependent cell lysis. Notably, sRNAs such as MicA and RybB play crucial roles in σ^E-dependent cell lysis in the LTSP because mutation rate drastically increases in *micA*- and *rybB*-disrupted mutants [25].

Taken together, the findings have shown that *E. coli* has developed an ingenious mechanism for elimination of damaged cells in order to suppress the accumulation of mutated cells, and this mechanism might contribute to the preservation of the species. Since oxidative stress causes damage to DNA molecules in addition to other macromolecules including RNA, protein and phospholipid, it is assumed that the degree of damage of OMPs is consistent with that of DNA and that an abnormality of OMPs is a signal for removal of cells that have damaged DNA molecules from the cell population.

4. Contribution of PCD for LTSP

4.1. Survival mechanisms in LTSP

In the LTSP, *E. coli* cells can survive for several years [14, 17]. For survival, the cells induce specific sets of genes that support maintenance of their viability and protection against environmental stresses such as an oxidative stress [74]. However, there are potentials for genetic alteration in most cells in the LTSP. It was reported that 10-day-old cells, GASP mutants, were able to compete against 1-day-old cells when they were mixed together [14, 16, 17, 75]. It has been proposed that population exchange continuously occurs in the LTSP (**Figure 1**). Interestingly, the GASP phenotype is mediated by stable genomic mutations that provide benefits to cells for survival. The first mutant exhibiting a GASP phenotype was obtained

from cells cultured for 10 days in LB medium, and its mutation, which was identified in *rpoS* coding σ^S, causes reduction of σ^S activity [16]. In addition, such σ^S activity-attenuated GASP mutants frequently appeared in nonbuffered media and basic media, but not so many appeared in acid and neutral media [76]. The relationship between the attenuation of σ^S activity and the GASP phenotype has not been clarified yet. The effect of the attenuation might be due to the misregulation of members of σ^S regulon. σ^S competes with other sigma factors to bind to the core RNA polymerase complex, and the attenuation of σ^S activity may change the balance in the competition among sigma factors [16].

Using the *rpoS* mutant as a starting strain, subsequent mutants with GASP phenotypes have been isolated. The additional mutations to the *rpoS* mutation have been mapped to *lrp*, coding the leucine-responsive regulator protein as a global regulator [77], or to the *ybeJ–gltJKL* cluster, encoding a high-affinity aspartate, and glutamate transporter [78]. A mutation in the DNA-binding domain of *lrp* has been shown to cause a GASP phenotype by increase in amino acid catabolism during carbon starvation, and mutants having mutation of *ybeJ–gltJKL* also show GASP phenotypes by increase in amino acid utilization [77]. Therefore, although these mutations are involved in different metabolic processes, it is likely that the enhancement of catabolic activity of amino acids for carbon and energy sources is responsible for these GASP phenotypes. Similarly, *sgaA*, *sgaB*, and *sgaC* mutants have been isolated as GASP mutants but have not been characterized yet [77]. Notably, non-*rpoS* mutation-related GASP mutants have also been reported [79].

The mechanism of GASP acquisition has been investigated and two interesting aspects have been shown. One is the reproducibility of GASP mutants and the other is a relatively high mutation rate in the LTSP. Since the speed of cell proliferation is very low in the LTSP, beneficial mutations for the GASP phenotype can appear only under high mutation conditions. It is thus assumed that there are some molecular mechanisms to generate genetic diversity in the LTSP.

Involvement of the methyl-directed mismatch repair (MMR) system and SOS-induced DNA polymerases has been considered for GASP mutations (**Figure 1**). It is known that when *E. coli* enters the stationary phase, the expression of MMR is reduced [80]. On the other hand, SOS DNA polymerases (Pols II, IV, and V) contribute to the generation of GASP mutations. These polymerases work during DNA replication when DNA polymerase III encounters a lesion and cannot proceed further in DNA synthesis. SOS polymerases are error-prone DNA polymerases and are thus responsible for the generation of adaptive mutations. Pol V Mut is a stand-alone DNA polymerase that is able to perform translesion synthesis, and polymerization of the polymerase is regulated by its intrinsic ATP hydrolase activity [81]. The occurrence of the GASP phenotype is highly related to the presence of SOS polymerases. Indeed, when grown in competition with the wild-type strain, mutants lacking one or more of the SOS polymerases suffer from a severe reduction in fitness to the LTSP. These mutants also fail to express the GASP phenotype as do wild-type strains, instead expressing two additional new types of GASP phenotype [82]. In addition, Pol IV and Pol V confer greater relative fitness than does Pol II during the LTSP, but Pol II can express the GASP phenotype faster than can Pol IV or Pol V [83]. Moreover, genes for the SOS polymerases and other SOS genes, especially

genes for Pol IV and Pol V, are induced during the stationary phase [83]. These facts suggest that there are some mechanisms for the expression of these alternative polymerases and that the mechanisms contribute to the relative high mutation rate in the LTSP.

4.2. Importance of σ^E-dependent PCD for survival in the LTSP

E. coli can maintain living cells to some extent for several years (LTSP) in the same medium without supplementation of any nutrients during the cultivation. On the basis of results of recent studies and the discovery of mutants that had gained growth advantages in the beginning of the LTSP [16], it has been proposed that the LTSP consists of a number of distinct populations that continuously appear one after another as shown in **Figure 1** [14]. One of the big questions is how nutrients are supplied to support the formation of each new population in such a closed environment. One possible answer is a simple mechanism by which nutrients are supplied from existing cells. Nagamitsu et al. suggested that σ^E-dependent PCD is involved in the mechanism [25].

σ^E-dependent PCD lyses damaged cells but not undamaged cells or cells with little damage and thus has no influence on viable and culturable (VAC) cells [19]. This PCD is responsible for major cell lysis under general cultivation conditions and is enhanced in the stationary phase due to accumulation of stresses including oxidative stress as described above, and forms ghost cells that discharge cytosolic contents to the outside [59]. This lysis thus appears to be different from explosive cell lysis for the biogenesis of membrane vesicles [84]. As in the stationary phase, it is assumed that cells in the LTSP are exposed to metabolically accumulated stresses including oxidative stress, which trigger σ^E-dependent PCD. Therefore, σ^E-dependent PCD may provide nutrients that are indispensable for the formation and maintenance of new populations in the LTSP.

As mentioned in the previous section, disrupted mutations of *micA* and *rybB*, which are essential factors for σ^E-dependent PCD, caused serious problems such that they were unable to keep VAC cells at the very early period in the LTSP. These mutations give rise to a sudden increase in the mutation rate just before the disappearance of VAC cells [25]. σ^E-dependent PCD thus seems to play an important role in the elimination of DNA-damaged cells in the LTSP in addition to the provision of nutrients. Its role appears to resemble that of PCD, so-called apoptosis in multicellular organisms, by which abnormal cells or DNA-damaged cells are removed.

Although we still have no evidence that dynamic cell population changes continuously occur in the LTSP, results of studies [14, 16, 17] and results of preliminary experiments in its early phase suggest that cells acquiring mutations for GASP become dominant to form a new population and that new GASP mutations constantly appear and displace the preexisting population. σ^E-dependent PCD may contribute to the alteration of populations by the lysis of preexisting populations and the provision of nutrients during the LTSP. For the emergence of GASP mutations, a large number of mutations should be present in addition to them under such nutrient-limited conditions. A hypermutable state might exist in the LTSP as mentioned above [14]. In order for hypermutation and σ^E-dependent PCD to take place,

active metabolisms should be maintained in fractions of the cell population. These active metabolisms are thought to lead to the selection of a dominant mutant and generate genetic diversity.

Further analysis of the LTSP *in vitro* seems to be important for understanding the life cycles of bacterial flora or biofilms and for elucidating the mechanisms of bacterial evolution. In addition, fundamental mechanisms for LTSP formation might be targets for drug design.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science, MEXT/JSPS Kakenhi (25250028) and the Advanced Low Carbon Technology Research and Development Program, Japan Science and Technology Agency (JST). This work was partially performed as collaborative research in the Asian Core Program and in the Core to Core Program, which was supported by the Scientific Cooperation Program agreed by the Japan Society for the Promotion of Science (JSPS), the National Research Council of Thailand (NRCT) and universities involved in the program.

Abbreviations

ALD	apoptosis-like death
CFU	colony-forming unit
ESR	envelope stress response
EDF	extracellular death factor
GASP	growth advantage in stationary phase
LPS	lipopolysaccharide
LTSP	long-term stationary phase
MMR	mismatch repair
OMPs	outer membrane proteins
PCD	programmed cell death
ROS	reactive oxygen species
sRNAs	small RNAs
TA	toxin-antitoxin
VAC	viable and culturable
VBNC	viable but nonculturable

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