Molecular mechanism of σ^E-dependent cell lysis and its physiological role at long-term stationary phase in *Escherichia coli*

（大腸菌におけるσ^E依存性溶菌の分子機構と長期定常期での生理学的役割）

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LIST OF ABBREVIATIONS

Amp	Ampicillin
CFU	Colony forming units
Cml	Chloramphenicol
GASP	Growth advantage in stationary phase
Kan	Kanamycin
OD	Optical density
OMP	Outer membrane protein+
PAGE	Poly acrylamide gel electrophoresis
PCD	Programmed Cell Death
PCR	Polymerase chain reaction
Rif	Rifampicin
RT-PCR	Reverse transcription-PCR
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SM	Streptomycin
sRNA	Small noncoding RNA
TCA	Tricarboxylic acid
TET	Tetracycline
VAC	Viable and culturable
VBNC	Viable but non-culturable

CHAPTER 1

General Introduction and Review of Literatures

1.1 Stationary phase in bacteria

When exposed to rich nutrients, the viable cell number of *Escherichia coli* cells is known to be changing with the following phases, lag, exponential, stationary, death and long-term stationary phases (Fig.1.1). In stationary phase, cells develop increased resistance to environmental stresses by changing metabolism (Siegel and Kolter, 1992; Ishihama, 1999). Lack of nutrition or accumulation of waste products causes death phase, in which only 0.1-1.0% of cells remain survive and the viable cell number is kept for a long time at long-term stationary phase. In this stage, continuous take-over of cell populations is proposed, which is caused by stable mutations that confer an advantageous ability to grow in given environment. This phenomenon is called "growth advantage in stationary phase" (GASP).

1.2 Role of σ^E in stationary phase

To adapt to given environments, *E. coli* cells change metabolism by induction of a large set of genes, which is governed by the coordination of different species of σ subunits of RNA polymerase (Ishihama, 1999). In prokaryote, σ factors that are one of the subunits of RNA polymerase confer ability of promoter recognition, allowing it to express genes suitable for given environments (Kazmierczak *et al.*, 2005; Mooney *et al.*, 2005). σ^S, encoded by *rpoS* is induced by nutritional starvation or cytoplasmic

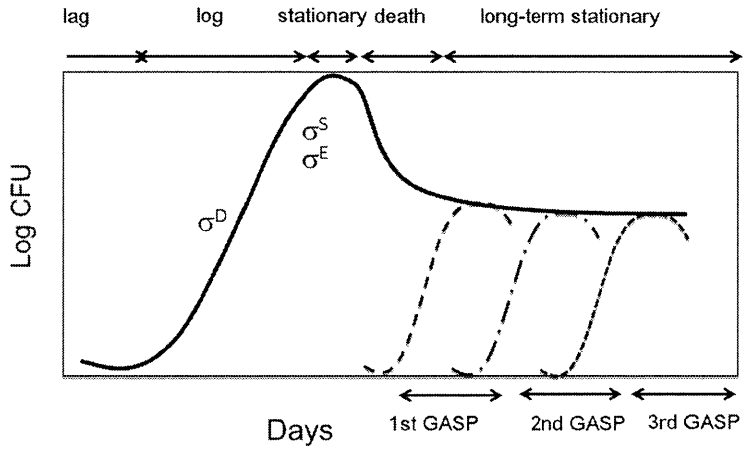


Fig. 1.1 Stages of bacterial growth (adapted from work of Navarro, 2010).
CFU in long-term stationary phase is maintained by continuous take-over of GASP mutants (dashed lines). Major σ factors at corresponding phases are indicated besides the growth curve.

stresses like heat and oxidative stresses. On the other hand, σ^E, which is encoded by *rpoE*, was originally identified as a transcription factor for *rpoH*, the main heat shock σ factor (Erickson and Gross 1989; Wang and Kaguni 1989) and regulate more than 100 genes belonging to σ^E regulon in *E. coli*. In the absence of periplasmic stresses, σ^E is sequestered by RseA, an anti-σ^E, membrane-spanning protein (Fig. 1.2). When aberrant proteins are accumulated in periplasmic space, the PDZ domain of DegS recognizes C-terminal motifs of outer membrane porins, and the resultant active DegS cleaves RseA, which is followed by the second cleavage of RseA by YaeL (RseP, EcfE), inner membrane-bound Zn^{2+}-metalloprotease, to release active σ^E in cytoplasm. The active σ^E triggers induction of genes under the control of σ^E.

1.3 σ^E involvement in cell lysis

In addition to the role of σ^E in repair of aberrant proteins, quite intriguing phenomenon has been discovered. The active form of σ^E caused by *rseA* disruption or *rpoE* increased expression cause cell lysis at early stationary phase without affecting viable cell number (Nitta *et al.*, 2000). Similar effect was observed when *rpoE* was transiently expressed (Kabir *et al.*, 2005). Furthermore, over-expression of anti-σ^E or co-anti-σ^E affected the degree of cell lysis. Even in the wild-type strain, cell lysis is caused by *rpoE* in stationary phase (Noor *et al.*, 2009a). This lysis mechanism appears to remove damaged cells as viable but nonculture ble (VBNC) cells. The molecular mechanism of the lysis process, however, has not been investigated.

1.4 Outer membrane proteins and its regulation

Since the expression of outer membrane protein (OMP) was diminished in *rseA*-

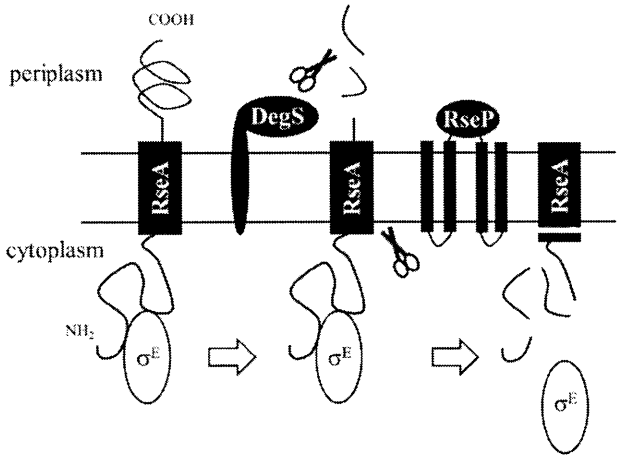


Fig. 1.2 Two-step proteolytic processing of RseA (adapted from work of Kanehara *et al.*, 2002).

disrupted mutation (Kabir *et al.*, 2005), it was assumed that cell lysis process could be attributed to the reduced expression of OMPs in stationary phase. OMPs called porins (Table.1.1) are able to form channels allowing the transport of small molecules like nutrients, toxic salts, and antibiotics across lipid bilayer membranes.

OmpA is a 35 k-Da monomeric 8-stranded β-barrel protein which functions as an anchor for outer membrane to the bacterial cell wall and is involved in passage of hydrophilic compounds (Saint *et al.*, 2000; Guillier *et al.*, 2006). Its expression is subject to various stimuli (Table 1.2). OmpC is a major OMP composed of 16-stranded β-barrels with narrow channels that exclude molecules > 600 Da. As well as OmpF, it is involved in osmo-sensing and their expressions are regulated by a classical two-component signal transduction via OmpR and EnvZ. OmpF is preferentially synthesized in low osmolarity, whereas OmpC is preferentially synthesized in high osmolarity. Physiological role of OmpW (YciD) has not been clarified (Pilsl *et al.*, 1999).

Small untranslated regulatory RNAs called noncoding RNAs (ncRNAs) in eukaryotes and sRNAs in bacteria regulate RNA molecules which prevent translation by base pairing with their target mRNAs in the region encompassing the translation start site. Two sRNAs MicA and RybB have been found to be members of the σ^E regulon family (Thompson *et al.*, 2007; Udekwu *et al.*, 2007). Their products cause reduction in the level of mRNAs of *ompA* and both *ompC* and *ompW*, respectively, via interaction between the RNAs and cognate mRNAs (Fig.1.3) and degradation by ribonucleases (Valentin *et al.*, 2007).

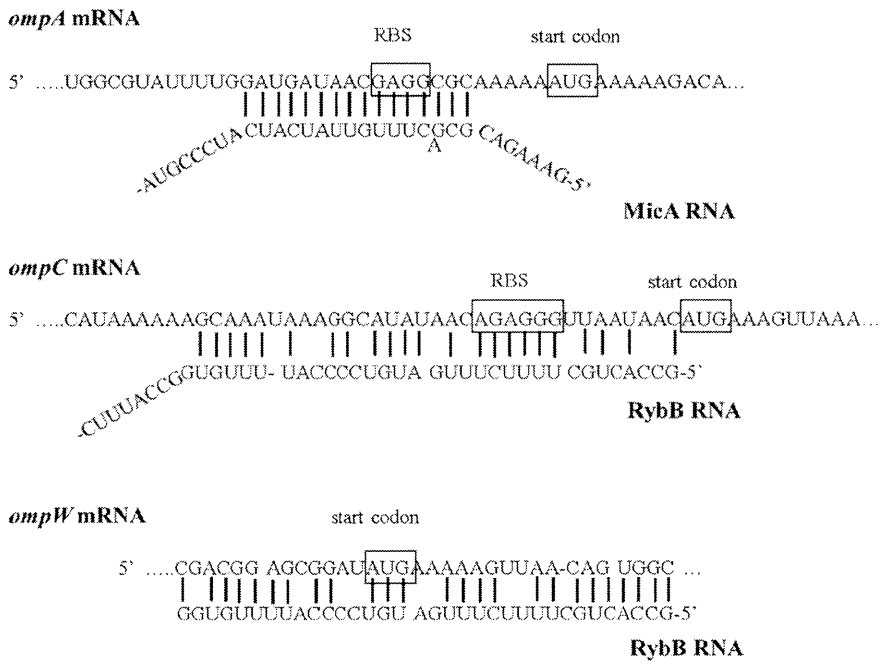


Fig. 1.3 Interaction of *omp* mRNA and sRNA (adapted from work of Guillier *et al.*, 2006)

1.5 GASP

During long term stationary phase, viable cells (VAC) are maintained for a long time, which are kept by equilibrium between dying cell population and occurrence of new one (Fig.1.1, dashed lines). It is considered that continuous stable mutations conferring an GASP phenotype occur to take over the previously existing population. Up to now, the following GASP mutants in *E. coli* have been reported.

First, a down mutation in RpoS, called *rpoS819*, is characterized by 46 base pair duplication at the 3' end, resulting in a protein substitution of 4 amino acid residues at the C-terminal with additional 39 amino acid residues (Fig 1.4) and shows a GASP phenotype (Zambrano *et al.*, 1993). In the strain, σ^D and σ^N are favored over σ^{S819} (Farrel and Finkel, 2003). Furthermore, the three following *sga* (stationary-phase growth advantage) alleles are involved in GASP phenotype of aged culture of *rpoS819*.(Zinser and Kolter, 1999).

sgaA, characterized by genomic rearrangement in the *ybeJ-gltJKL* operon was due to inversion of the operon, which was subjected to the control of *cstA*, encoding oligopeptide permease (Zinser *et al.*, 2003). Enhanced expression of the operon leads to transport ability of glutamate, asparagine and proline to confer the new ability to grow on aspartate as a sole carbon source (Zinser *et al.*, 2003).

sgaB, in which mutation has been mapped to the *lrp*, encoding the leucine-responsive regulatory protein, also shows a GASP phenotype. Lrp belongs to feast/famine regulatory protein (FFRPs) and is a dimeric DNA-binding protein functioning as an activator or repressor for amino acid metabolism, pili synthesis by bending or loop formation of target DNA. In the mutant, in-frame 3-base pair deletion causes lacking glycine residue (G39) in helix-turn-helix domain and shows a GASP phenotype due to ability to scavenge amino acids released by dead cells in particular serine, threonine and alanine (Zambrano *et al.* 1993).

(a)

MAEEELLSQGATQRVLDATQLYLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMI
ESNLRLVVKIARRYGNRGLALLDLIEEGNLGLIRAVEKFDPERGFRFSTYATWWIR
QTIERAIMNQTRTIRLPIHIVKELNVYLRTARELSHKLDHEPSAEEIAEQLDKPVDDV
SRMLRLNERITSVDTPLGGDSEKALLDILADEKENGPEDTTQDDDMKQSIVKWLFE
LNAKQREVLARRFGLLGYEAATLEDVGREIGLTRERVRQIQVEGLRRLREILQTQG
LNIEALFRE

(b)

σ^S (parent)	IEA	LFRE
σ^{S819} (*rpoS819*)	IEA	PFARNPANAGAEYRSAVPRVSKHLSERPVSSEAGLFCA

Fig. 1.4 GASP mutants in *rpoS819* (adapted from work of Zambrano *et al.*, 1993)
(a) Amino acid sequence of σ^S in wild type strain. (b) Comparison of C-terminal portion between parent and *rpoS819* strains.

Table. 1.1 Summary of OMPs in bacteria (adapted from work of Fairman *et al.*, 2011)

No. of Strands	Protein Name	Organism	Function
8	Ail	*Yersinia pestis*	adhesin/invasin
	NspA	*Neisseria meningitidis*	adhesin
	OmpA	*Escherichia coli*	adhesin/invasin/evasin
	OmpA	*Klebsiella pneumoniae*	adhesin/invasin/evasin
	OmpA	*Legionella pneumophila*	adhesin/invasin/evasin
	OmpW	*Escherichia coli*	putative channel
	OmpX	*Escherichia coli*	adhesin/invasin
	OprG	*Pseudomonas aeruginosa*	channel
	PagP	*Escherichia coli*	palmitoyltransferase
	PagL	*Pseudomonas aeruginosa*	deacylase
	TtoA	*Thermus thermophilus*	unknown
10	OmpT	*Escherichia coli*	omptin (protease)
	OpcA	*Neisseria meningitidis*	adhesin/invasin
	Pla	*Yersinia pestis*	plasminogen activator
12	EspP	*Escherichia coli*	autotransporter
	EstA	*Pseudomonas aeruginosa*	autotransporter
	Hbp	*Escherichia coli*	autotransporter
	Hia	*Haemophilus influenzae*	autotransporter
	LpxR	*Salmonella typhimurium*	deacylase
	IcsA	*Shigella flexneri*	autotransporter
	NalP	*Neisseria meningitidis*	autotransporter
	NanC	*Escherichia coli*	porin
	OMPLA	*Escherichia coli*	phospholipase
	OprM	*Pseudomonas aeruginosa*	transporter
	TolC	*Escherichia coli*	transporter
	Tsx	*Escherichia coli*	transporter
	VceC	*Vibrio cholerae*	channel
14	α-HL	*Staphylococcus aureus*	protein pore
	FadL	*Escherichia coli*	transporter
	FadL	*Pseudomonas aeruginosa*	transporter
	OmpG	*Escherichia coli*	channel
	TbuX	*Ralstonia pickettii*	transporter
	TodX	*Pseudomonas putida*	transporter
16	FhaC	*Bordetella pertussis*	transporter
	MspA		
	Omp32	*Comamonas acidovorans*	porin

16	Omp32	*Delftia acidovorans*	porin
	OmpK36	*Klebsiella pneumoniae*	porin
	OmpC	*Escherichia coli*	porin
	OmpF	*Escherichia coli*	porin
	OprP	*Pseudomonas aeruginosa*	porin
	PhoE	*Escherichia coli*	porin
	Gdpa	*Rhodobacter capsulatus*	porin
	Gdpa	*Rhodopseudomonas blastica*	porin
	PorB	*Neisseria meningitidis*	porin
18	BenF	*Pseudomonas fluorescens pf-5*	transporter
	LamB	*Escherichia coli*	porin
	LamB	*Salmonella typhimurium*	Porin
	OpdK	*Pseudomonas aeruginosa*	Porin
	OprD	*Pseudomonas aeruginosa*	porin
	ScrY	*Salmonella typhimurium*	porin
19	VDAC1	*Mus musculus*	channel
	VDAC1	*Homo sapiens*	channel
22	BtuB	*Escherichia coli*	transporter
	Cir	*Escherichia coli*	transporter
	FauA	*Bordetella pertussis*	transporter
	FecA	*Escherichia coli*	transporter
	FepA	*Escherichia coli*	transporter
	FhuA	*Escherichia coli*	transporter
	FptA	*Pseudomonas aeruginosa*	transporter
	FpvA	*Pseudomonas aeruginosa*	transporter
	HasR	*Serratia marcescens*	transporter
	ShuA	*Shigella dysenteriae*	transporter
24	PapC	*Escherichia coli*	transporter

Table. 1.1 Continued.

sgaC allele has not been investigated yet (Zinser and Kolter, 2000). In general, GASP mutant seems to be enhanced uptake of nutrition from dead cells (Fig. 1.5). However, no GASP mutants can survive in environments without nutrition, hence, it is assumed that the mechanism supplying nutrients from the dead or VBNC cells exists. σ^E-directed cell lysis could be accounted for the provision of nutrients, contributing the maintenance of the following populations.

Table 1.2 Regulation of OmpA expression in *E. coli* (adapted from work of Smith *et al.*, 2007)

Stimulus or regulator	Effect on expression	Reference
Acid challenge of nonacid adapted strains	Decreased	Sainz *et al.* (2005)
Adhesion to abiotic surfaces	Decreased	Otto *et al.* (2001)
Anaerobic culture in acidified media	Increased	Yohannes *et al.* (2004)
Antimicrobial peptide-attacin	Decreased	Carlsson *et al.* (1991,1998)
Bacteriophage T2, T4 or T7 infection	Decreased	Ueno and Yonesaki (2004)
Chromate stress	Reduces	Ackerley *et al.* (2006)
Cyclic AMP	Catabolite repression	Gilbert and Barbe (1990)
Defective lipopolysaccharide	Decreased	Ried *et al.* (1990)
Growth in urine	Decreased	Snder *et al.* (2004)
Growth rate	Decreases with growth rate	Lugtenberg *et al.* (1976)
Growth phase	Decreased in stationary phase	Lugtenberg *et al.* (1976)
Nitrogen shortage	Increased	Baev *et al.* (2006)
Starvation in lakewater	Decreased	Ozkanca and Flint (2002)
Polyamines	Increased	Yohannes *et al.* (2005)
MicA /Hfq /RNaseE	Decreased	Udekwu *et al.* (2007)
RNaseR /SigmaR	Decreased	Andrade *et al.* (2006)
Hha	Repressed	Balsalobre *et al.* (1999)

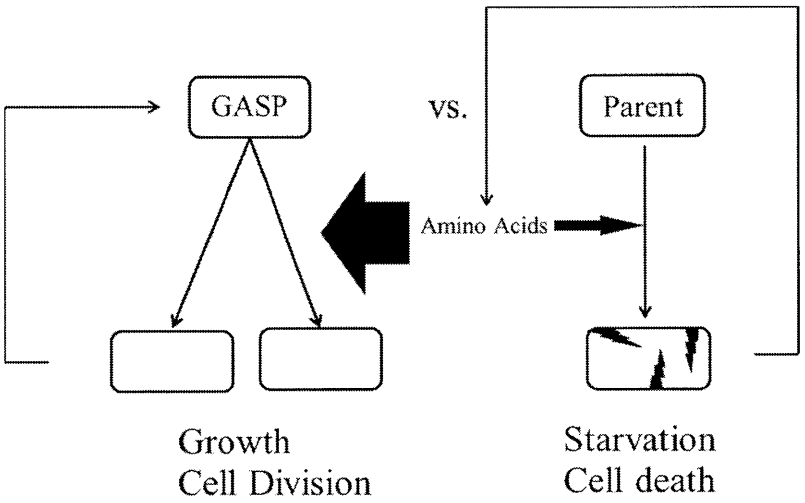


Fig. 1.5 Model of fitness in GASP mutant (adapted from work of Zinser and Kolter, 1999)

CHAPTER 2

Novel pathway directed by σ^E to cause cell lysis in *Escherichia coli*

ABSTRACT

A large number of *Escherichia coli* cells become viable but nonculturable at early stationary phase, most of which are directed to lysis in cells with an enhanced active σ^E level. In this study, we examined the effect of small noncoding RNAs, MicA and RybB, as σ^E regulon as well as regulators of outer membrane protein (Omp) genes, on the lysis process. *micA*- and *rybB*-disrupted mutations almost completely suppressed the cell lysis. Increased expression of *micA* and *rybB* or disrupted mutation of *ompA*, *ompC* and *ompW* led to a significant level of cell lysis. The suppression by Mg^{2+} was found to maintain the integrity of the Omp-repressed outer membrane. Taken together, the results suggest that the cell lysis proceeds in the cascade of σ^E → expression of *micA* and *rybB* → reduction in Omp proteins → disintegration of the outer membrane.

2.1 INTRODUCTION

The natural environment usually provides limited amounts of nutrients for microorganisms, which thereby are mostly in dormant states or sustain only sporadic growth (Hengge-Aronis 1993; Hecker and Völker 2001; Aertsen and Michiel 2004). A number of factors are considered to be involved in the maintenance of a culturable cell population, which ultimately leads to the induction of expression of genes required for entering the dormant state (Ishihama 1999). In *Escherichia coli*, on entry of the culture into stationary phase, most of the genes that are highly expressed in growing cells are turned off and instead a group of genes, which are mostly inactive in growing cells, begin to be expressed. This switching is mainly controlled by the coordination of different species of σ subunits of RNA polymerase, each of which participates in the transcription of a specific set of genes (Helmann and Chamberlin 1988; Ishihama 1999).

σ^E, which is encoded by *rpoE* and was first identified as a transcription factor for *rpoH* encoding a main heat shock σ factor, is involved in the expression of several genes whose products deal with unfolded periplasmic or membrane proteins, caused by heat shock (Erickson and Gross 1989; Raina *et al.*, 1995) or other stresses (Kabir *et al.*, 2004) in *E. coli*. σ^E is inactive under nonstress conditions by the formation of a complex with RseA as an anti-σ^E and RseB as a co-anti-σ^E, which are encoded by a unique operon, *rpoE*-rseABC (Raina *et al.* 1995). On exposure of cells to stresses to accumulate unfolded extracytoplasmic proteins, σ^E is released as an active form from the complex by either detachment of RseB (Missiakas *et al.*, 1997) or degradation of RseA by DegS and YaeL proteinases (Alba *et al.*, 2002; Kanehara *et al.*, 2002; Walsh *et al.*, 2003). The active σ^E

then induces transcription from the *rpoE* P2 promoter to allow its autoinduction and the expression of σ^E regulon genes (Raina *et al.*, 1995; Rouviére *et al.*, 1995).

At the early stationary phase, *E. coli* undergoes a decrease in viable cell number (Zambrano *et al.*, 1995) and more than 60% of cells become viable but nonculturable (VBNC) (Desnues *et al.*, 2003). The elevation of active σ^E, by *rseA* disruption or *rpoE* increased expression, causes a growth phase-specific cell lysis at the beginning of the stationary phase (Nitta *et al.*, 2000). This lysis mechanism appears to remove VBNC cells accumulated at the specific phase. Such a lysis at a low level occurs in the wild type background under general growth conditions, and the transcription level of *rpoE* is consistently increased at the early stationary phase (Nitta *et al.*, 2000; Noor *et al.*, 2009a). These results together with the finding that the transient expression of *rpoE* also induces cell lysis (Kabir *et al.*, 2005) and the lysis level is controlled by anti-σ^E and co-anti-σ^E (Noor *et al.*, 2009a) suggest the occurrence of σ^E-directed cell lysis. Some stresses, including oxidative stress (Desnues *et al.*, 2003), would be accumulated at the specific growth phase to lead to the elevation of active σ^E and to the expression of its regulon, which in turn causes cell lysis in the wild-type culture (Kabir *et al.*, 2004). Recent experiments allow us to assume that oxidative stress that has accumulated in the transition period between exponential and stationary phases gives rise to VBNC cells, which are in turn lysed by a σ^E-dependent process (Noor *et al.*, 2009b). Extensive analyses, including DNA microarray analysis (Kabir *et al.*, 2005), could not clarify the process and molecular mechanism of cell lyses. Some clues, however, have been found: it has been shown that outer membrane proteins, OmpA, OmpC and OmpW, as well as their transcripts are largely decreased by the elevation of active σ^E and that Mg^{2+} suppresses the cell lyses (Kabir *et al.*, 2005). At least two of the Omp proteins are known to be physiologically and structurally crucial for cell activity (Nikaido 2003). OmpA as a structure protein is involved in the maintenance of cell shape and passage of hydrophilic compounds through the outer membrane (Saint *et al.*, 2000), and OmpC is the major porin protein to function as a cation-selective porin (Apirakaramwong *et al.*, 1998). However, no physiological function of OmpW has yet been determined (Pilsl *et al.*, 1999).

Recently, small noncoding RNAs (sRNAs) have become attractive as ubiquitous regulators in all kingdoms of life (Vogel and Sharma 2005), and they are collectively referred to as sRNA in bacteria. Eubacterial sRNA is known to exhibit dramatic heterogeneity in size, 50-250 nucleotides in length, and structure (Urban and Vogel 2007). More than two dozen sRNAs have been assigned in respect to their cellular functions and action modes in *E. coli* (Gottesman, 2005; Marles-Wright and Lewis, 2007). Of those sRNAs, *micA* and *rybB* genes have been found to be members of the σ^E regulon family (Thompson *et al.*, 2007; Udekwu and Wagner 2007) and are expressed when misfolded periplasmic proteins are accumulated (Johansen *et al.*, 2006). Their products cause reduction in the level of mRNAs of *ompA* and both *ompC* and *ompW*, respectively, via interaction between the sRNA and cognate mRNAs and degradation by ribonucleases (Valentin-Hansen *et al.*, 2007).

σ^E is known to be involved in an extracytoplsmic repairing pathway by chaperons or proteases as its regulon (Alba *et al.*, 2002; Walsh *et al.*, 2003). We provided here an

alternative pathway directed by σ^E, which causes lysis of VBNC cells. The latter pathway may contribute elimination of damaged cells in the population, especially at the early stationary phase. In this study, we deciphered for the first time the process of σ^E-directed cell lysis after the accumulation of active σ^E. A molecular biological approach showed that the knock-out mutation of *micA* or *rybB* suppresses the lysis and that of *ompA*, *ompC* or *ompW* causes cell lysis at a significant level. These results with others presented here show a novel function of sRNA to control the cell lysis. On the basis of these results, we propose the entire cascade of σ^E-directed cell lysis and discuss the physiological function of sRNA in *E. coli*.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (MA, USA). A DNA sequencing kit was obtained from Applied Biosystems (CA, USA). DNA primers were synthesized by Proligo Japan K. K. (Tokyo, Japan). Other chemicals were of analytical grade. A gene-disrupted mutant library (Keio collection) was supplied by National Institute of Genetics [National BioResource Project (NIG, Japan)] (Baba *et al.*, 2006). Other *E. coli* strains used in this study are shown in Table 2-1.

2.2.2 Bacterial growth condition and medium

Escherichia coli cells were grown under a shaking condition (100 rpm / min) at 37°C in LB medium (1% Bactotryptone, 0.5% yeast extract and 0.5% NaCl). For growth experiments, one colony was inoculated into 3 mL of medium. After 12-h preculture, the culture was diluted and adjusted to OD600 of 0.1. Of this cell suspension, 20 μL was inoculated into 20 mL of medium in a 100 mL Erlenmeyer flask, and cell growth was monitored under conditions described. CFU were estimated by counting colonies at 24 h after appropriate dilution of cell culture and spreading on LB plates.

2.2.3 Disruption of genomic genes

Gene disruption of *micA* and *rybB* was carried out by the one-step disruption method established previously (Datsenko and Wanner, 2000). The primers are basically a 50-nt sequence homologous to the adjacent upstream or downstream flanking region of a target gene followed by a 20-nt sequence from the upstream or downstream region of kanamycin resistance gene (*kan*). The N-terminal primer consists of the 50-nt upstream sequence of the target gene including its initiation codon (H1) and the 20-nt upstream sequence of kan, 5'-ATTCCGGGGATCCGTCGACC-3' (P1), whereas the C-terminal primer consists of the 50-nt sequence of the 29-nt adjacent downstream sequence plus the C-terminal 21-nt sequence of the target gene including its termination codon (H2) and the

Table 2.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
***Escherichia coli* strains**		
W3110	IN(*rrnD-rrnE*) *rph*-1	Laboratory stock
BW25113	*rrnB3 ΔlacZ4748 hsdR514* Δ (*araBAD*)*567* Δ (*rhaBAD*)*568 rph-1*	Datsenko & Wanner (2000)
YU698	BW25113 *rybB*::*kan*	This study
YU670	BW25113 *micA*::*kan*	This study
YU671	BW25113 Δ*rybB*	This study
YU672	BW25113 Δ*micA*	This study
JW0940	BW25113 *ompA*::*kan*	Baba *et al.* (2006)
JW2203	BW25113 *ompC*::*kan*	Baba *et al.* (2006)
JW1248	BW25113 *ompW*::*kan*	Baba *et al.* (2006)
JW0554	BW25113 *ompT*::*kan*	Baba *et al.* (2006)
YU673	BW25113 Δ*rybB ompC*::*kan*	This study
YU674	BW25113 Δ*rybB ompW*::*kan*	This study
YU675	BW25113 Δ*micA ompA*::*kan*	This study
Plasmids		
pBAD24	Amp^r	Guzman et al. (1995)
pACYC177	Amp^r, Kan^r	Chang & Cohen (1979)
pUC118	lacZα, Amp^r	Vieira & Messing (1982)
pMBL18	lacZα, Amp^r	Nakano et al. (1995)
pKD20	γ, β, and *exo* from r phage, *araC*-P_{araB}, $RepA101^{ts}$, Amp^r	Datsenko & Wanner (2000)
pCP20	FLP^+,λ $cI857^+$, Rep^{ts}, Amp^r, Cml^r r p_R	Datsenko & Wanner (2000)
pBADRPOE	pBAD24 with 660-base PCR fragment bearing *rpoE*	Kabir *et al.* (2005)
pACYCRPOE	pACYC177-322 with 2.8-kb PCR fragment bearing *rpoE* and *rseA*	Nitta *et al.* (2000)
pACYCMICA	pACYC177 with 218-base PCR fragment bearing *micA* and its promoter region	This study
pACYCRYBB	pACYC177 with 213-base PCR fragment bearing *rybB* and its promoter region	This study
pUCMICA	pUC118 with 218-base PCR fragment bearing *micA* and its promoter region	This study
pUCRYBB	pUC118 with 213-base PCR fragment-bearing *rybB* and its promoter region	This study
pUCOMPA	pUC118 with 1.4-kb PCR fragment bearing *ompA* and its promoter region	This study
pMBLOMPC	pMBL18 with 1.1-kb PCR fragment bearing *ompC*	This study

20-nt downstream sequence of *kan*, 5'- TGTAGGCTGGAGCTGCTTCG-3' (P2). PCR was carried out as described previously (Yamada *et al.*, 1993) with the genomic DNA of W3110 as a template. PCR products were recovered by ethanol precipitation, and 50-400 ng of the products was introduced by electroporation into BW25113 carrying pKD20, a Red helper plasmid, which had been grown in LB medium containing ampicillin (50 μg / mL) and 1 mM arabinose at 30°C until OD_{600} of 0.3. The cells electroporated were mixed with a small amount of LB medium, incubated for 2 h at 37 °C and spread onto agar plates containing kanamycin (25 μg/ mL) followed by incubation at 37°C. Homologous recombination proceeded between the constructed *kan*-inserted DNA fragment and the genomic gene of BW25113. The performance of gene disruption was checked by comparison of PCR products of the targeted gene from the genomic DNA of disrupted strains with those from the parental strain using the primers, 5'-GATTTTGAGGATGGTTGAGAGGGTTGCAGGGTAGTAGATAAGTTTTAGATAT TCCGGGGATCCGTCGACC-3' and 5'-GGCACAACCGCAGAACTTTTCCGCAGGGC ATCAGTCTTAATTAGTGCCACTGTGTAGGCTGGAGCTGCTTC-3' for *rybB* and 5'-TGATACCGAACCGTTTGCGGTGTGGTGGAAAAACACGCCTGACAGAAAAATT CCGGGGATCCGTCGACC-3' and 5'-TTTTAAAAATTTTCTGAACTCTTTCTTCCCAG GCGAGTCTGAGTATATGAGTGTAGGCTGGAGCTGCTTC-3' for *micA*. PCP20 is Amp^r and Cml^r plasmid with a temperature-sensitive replication and thermal induction of

FLP synthesis (Datsenko and Wanner, 2000). BW25113 *rybB*::*kan* or BW25113 *micA*::*kan* was transformed with PCP20, and the resultant ampicillin-resistant transformants were obtained at 30°C, from which a few were colony-purified once nonselectively at 43°C. Resultant derivatives were tested by PCR in addition to checking for the loss of all antibiotic resistance. The majority of deletion mutants lost simultaneously the FRT (FLP recombination target)-franked resistance gene and the FLP helper plasmid as described previously (Datsenko and Wanner, 2000). Resultantly, BW25113 ΔrybB and BW25113 ΔmicA were obtained. Furthermore, BW25113 Δ*rybB ompC*::*kan*, BW25113 Δ*rybB ompW*::*kan* and BW25113 Δ*micA ompA*::*kan* were constructed by P1 transduction (Miller,1992).

2.2.4 DNA manipulation

Conventional recombinant DNA techniques were applied (Sambrook *et al.*, 1989). *micA*, *rybB* and *ompA* plasmid clones were constructed by gene amplification and insertion into a plasmid vector. Both genes were amplified by PCR using specific primer sets, 5'-GGGTCTAGATCGATCGACTGTGAAGCTATCTAA-3' and 5'-GGGTCTAGAGTCA TGATGGCCAAGGATT-3' for *micA*, 5'-GGGTCTAGAGTCATGGTATGGCCAAGGAT T-3' and 5'-GGGAAGCTTGTTGCAGGGTAGTAGATAAG-3' for *rybB*, 5'-GGGGAATT CGTCGCCCAGCCAATGCT-3' and 5'-GGGCTGCAGTCTGCAGGCATTGCTGG-3' for *ompA*, and 5'-GGGGGATCCAATAAAGGCATATAACA-3' and 5'-GGGAAGCTTCTGA GTTTGTACGCTGA-3' for *ompC*, those of which are designed according to the genomic sequence (Yamamoto *et al.*,1997) and the genomic DNA of W3110 as a template. The primers for *micA* and *rybB* have *Hin*dIII and *Xba*I sites at the 5'- and 3'-ends, respectively. The primers for *ompA* have *Eco*RI and *Pst*I sites at the 5'- and 3'-ends, respectively. The primers for *ompC* have *Bam*HI and *Hin*dIII sites at the 5'- and 3'-ends, respectively. The PCR products for *micA* and *rybB* were digested both with *Sca*I and *Pst*I and inserted into the *Sca*I-*Pst*I site of pACYC177 (Chang and Cohen, 1979), generating pACYCMICA and pACYCRYBB, respectively. The PCR products for *ompA* were digested with both *Eco*RI and *Pst*I and inserted into the corresponding site of pUC118, generating pUCOMPA. The PCR products for *ompC* were digested with both *Bam*HI and *Hin*dIII and inserted into the corresponding site of pMBL18 (Nakano *et al.*, 1995), generating pMBLOMPC.

2.2.5 Analysis of proteins and β-galactosidase activity in culture fractions

W3110 and its derivatives were grown in 20 mL of LB medium at 37°C. At the times indicated, 2 mL of each culture was subjected to centrifugation at 800 g for 5 min and the resultant supernatant and pellet were collected as a medium fraction and remaining fraction, respectively, as described previously (Nitta *et al.*, 2000). The expression of *rpoE* as a control experiment, when used the plasmid clone of *rpoE*, enhanced expression of *rpoE* was confirmed by RT-PCR. Proteins in the supernatant were recovered as a pellet by the addition of trichloroacetic acid at the final concentration of 5%. The pellet was treated with diethyl ether and resolved with 0.1 mL of 20 mM Tris-HCl, pH 7.0. The remaining

fractions were resuspended in 0.1 mL of the same buffer and subjected to sonic oscillation. Protein amount of both fractions was determined as described previously (Dulley & Grieve, 1975). Medium fractions and both fractions for some cases were then subjected to SDS-12% PAGE, and proteins in gels were stained with Coomassie brilliant blue 250R. In some experiments, at the times indicated, 1 mL of each culture was subjected to centrifugation at 800 g for 5 min to separate medium and remaining fractions. The latter fraction was suspended in 1 mL of Z buffer. β-galactosidase activity of both fractions was measured as described (Baba *et al.*, 2006).

2.2.6 Cell morphology

W3110 cells harboring pBADRPOE or an empty vector, pBAD24, were grown in LB medium with or without 20 mM $MgSO_4$ at 37 °C for the times indicated. Cells were then harvested from culture by centrifugation at 215 g for 20 min and fixed with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd., Berk, UK) in 0.05 M sodium phosphate buffer (pH 7.4). After washing with the same buffer, the cells were dehydrated by washing with a 50%-100 ascending ethanol series, treated with 100% t-butyl alcohol and then dried using a JEOL JFD-300 Freeze Drying Device. The dried cell samples were coated with gold and observed with a JEOL JSM-6100 scanning electron microscope at 15 kV.

2.2.7 EDTA treatment of cells

W3110 cells harboring pBADRPOE or an empty vector, pBAD24, were grown in 20 mL of LB medium with or without 20 mM $MgSO_4$. After the addition of arabinose to induce *rpoE*, cells were further incubated and harvested at the times indicated followed by washing once with saline. The cells were resolved in 1 mL of 0 mM EDTA or 5 mM EDTA solution, and then the change in optical density at 600 nm of the resolved solution was followed by using a photometer (Hitachi U2000). For each sample, optical density at 3 min was compared with that at 0 min. After 3 min, there was almost no change in optical density.

2.3 RESULTS

2.3.1 Effect of *micA*- and *rybB*-deletion derivatives on σ^E-directed cell lysis

In an *E. coli rseA*-disrupted mutant strain that exhibits an increased level of active σ^E, amounts of OmpA, OmpC and OmpW of outer membrane proteins are reduced or diminished (Kabir *et al.*, 2005). Expressional control of these omp genes is achieved by sRNAs of MicA and RybB, of which genes are strictly under the control of σ^E (Johansen *et al.*, 2006; Thompson *et al.*, 2007; Udekwu and Wagner, 2007; Valentin-Hansen *et al.*, 2007). Evidence that elevation of active σ^E causes cell lysis prompted us to examine the linkage of cell lysis to the function of the two sRNAs. The plasmid pBADRPOE bearing *rpoE*, encoding σ^E, under the control of the araBAD promoter or pBAD24 (Guzman *et al.*, 1995)as an empty vector was introduced into disrupted mutant strains, BW25113 *micA*::*kan*

or BW25113 *rybB*::*kan*, or the parental strain, BW25113, and their growth [optical density (OD)] curves were compared (Fig. 2.1 A-C). It was found that the density of the parental strain harboring pBADRPOE was significantly decreased after induction by the addition ofarabinose as observed previously (Kabir *et al.*, 2005). In contrast, the *micA* strain harboring pBADRPOE and the *rybB* strain harboring pBADRPOE as well as all strains harboring pBAD24 showed nearly a constant level of cell density around the early stationary phase. However, colony-forming units (CFU) of all strains were almost the same, which is consistent with the previous results, indicating that only nonculturable cells were lysed by σ^E-directed pathway (Nitta et *al.*, 2000; Kabir *et al.*, 2005). To further examine *micA* and *rybB*, we introduce pACYCMICA and pACYCRYBB into BW25113 *micA*::*kan* harboring pBADRPOE and BW25113 *rybB*::*kan* harboring pBADRPOE, respectively. A similar level of cell lysis in the transformants to that of parental strain harboring pBADRPOE was observed (data not shown).

A specific phenotype of the σ^E-dependent cell death is accompanied with cell lysis, which can be distinguished as the accumulation of a significant amount of proteins in culture medium (Kabir *et al.*, 2005). Protein accumulation levels in their culture medium were thus compared by SDS-polyacrylamide gel electrophoresis (PAGE) among the strains tested (Fig.2.2 D-F). Such a typical phenotype, showing significant protein bands in medium fractions at 36 and 60 h, was observed in BW25113 harboring pBADRPOE, which was consistent with the previous report (Nitta *et al.*, 2000; Kabir *et al.*, 2005). In contrast, the protein accumulation was very low in both sRNA gene-disrupted mutants harboring pBADRPOE. Similar experiments showed that a *micA rybB* double gene-disrupted mutant hardly showed protein accumulation, similarly to that in *rybB* single genedisrupted mutant (data not shown). These results clearly suggest that the *micA* or *rybB* mutation suppresses the σ^E-directed cell lysis.

2.3.2 Contribution of MicA and RybB to σ^E-directed cell lysis in the wild-type background

The expression of *rpoE* dramatically increases at the early stationary phase with protein accumulation to some extent in the culture medium even in the wildtype background, suggesting the occurrence of σ^E-directed cell lysis under ordinary growth conditions (Nitta *et al.*, 2000; Noor *et al.*, 2009a). To address the contribution of MicA and RybB to cell lysis, we further examined the effect of disrupted mutations of sRNA genes on cell growth and lysis as the phenotype of the lysis process in the wild-type strain, where samples from a volume of culture 10-times larger than those used in other figures were applied to SDS-PAGE (Fig. 2.2-K). Both disrupted mutant strains showed a slightly higher optical density than that of the parental strain at the early stationary phase and a significantly lower level of protein accumulation in culture medium, which was obvious at 60 h. These results

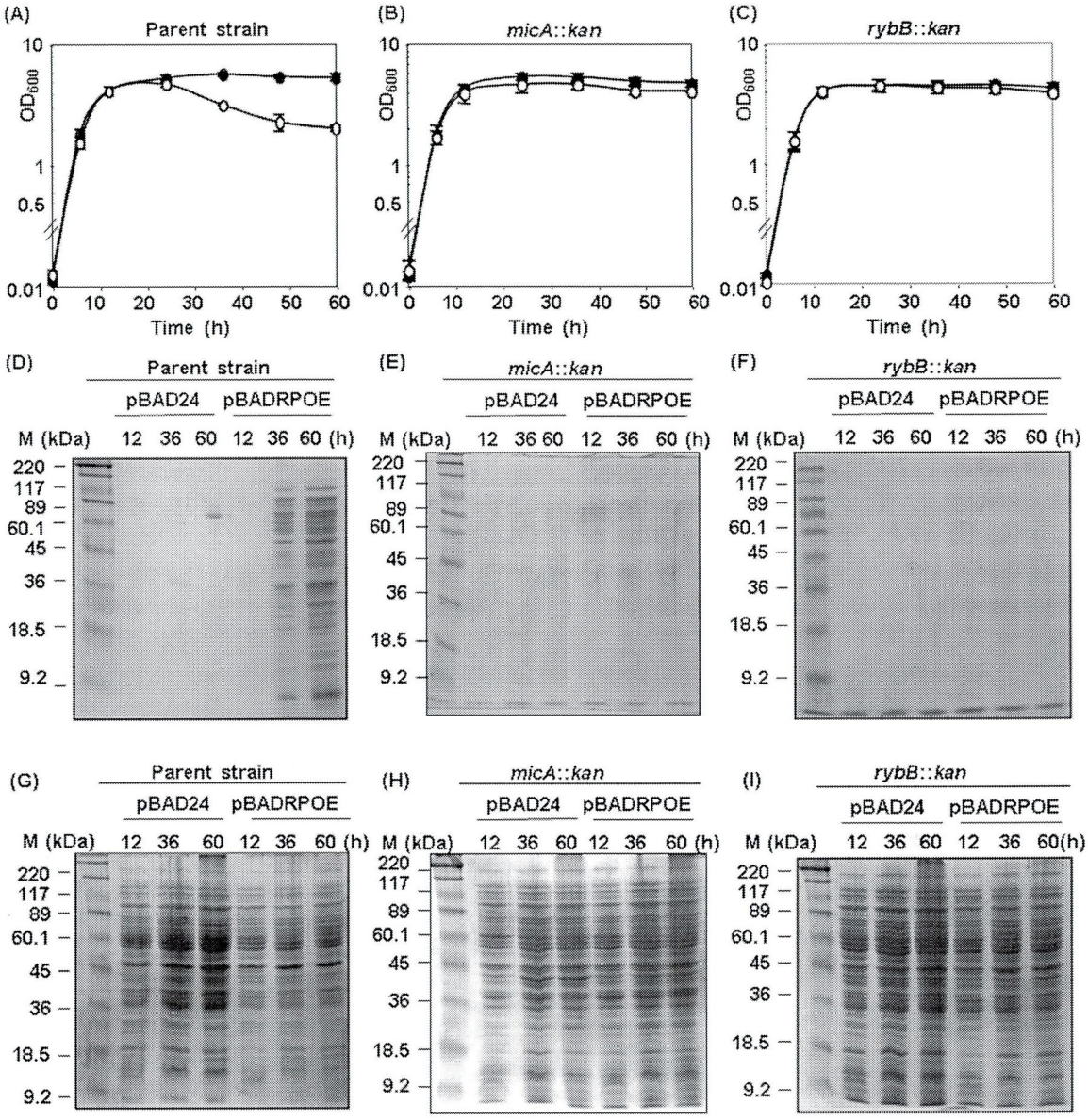
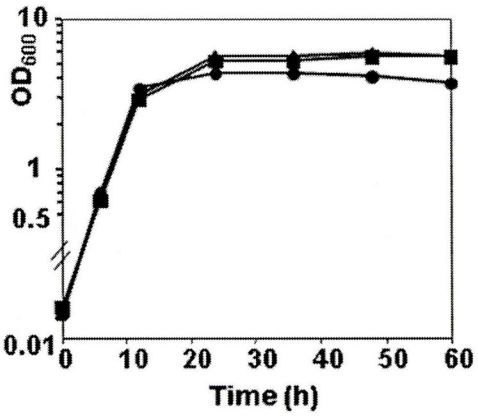


Fig. 2.1 Effects of *micA*- and *rybB*-deletion derivatives on cell lysis directed by σ^E
BW25113 (A, D, G), BW25113 *micA*::*kan* (B, E, H) and BW25113 *rybB*::*kan* (C, F, I) cells harboring pBADRPOE or an empty vector, pBAD24, were grown in LB medium containing ampicillin (50 μg/ ml) at 37 °C. Arabinose was added at 12 h at the concentration of 0.1% to induce *rpoE* encoding σ^E under the control of the araBAD promoter. At the time indicted, turbidity at OD_{600} (A-C) was determined. Cells harboring pBADRPOE and cells harboring pBAD24 are shown by open circles and closed circles, respectively. Medium fractions (D-F) and the remaining fractions (G-I) from cultures at 12, 36 and 60 h were prepared as described and subjected to SDS-12% PAGE. Samples of medium fractions and remaining fractions were applied at equivalent amount to 0.1 and 0.05 mL of culture, respectively.

(J)

	Parent strain		*micA*::*kan*		*rybB*::*kan*	
	pBAD24	pBADRPOE	pBAD24	pBADRPOE	pBAD24	pBADRPOE
Medium fraction (mg protein/ml culture)	<0.1	1.7 ± 0.1	<0.1	0.1 ± 0.05	<0.1	<0.1
Remaining fraction (mg protein/ml culture)	5.8 ± 0.8	4.1 ± 0.7	5.2 ± 0.6	5.3 ± 0.4	5.9 ± 0.8	5.0 ± 0.3

(K)



(L)

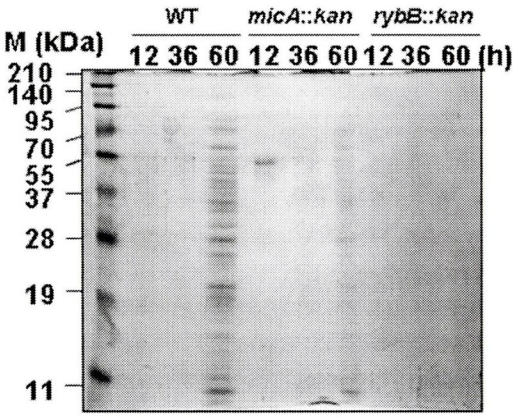
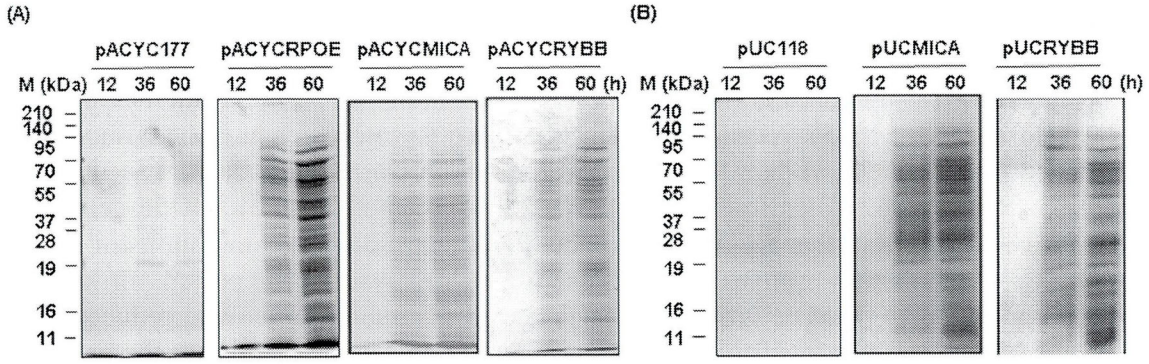


Fig 2.1 continued.
Protein recovered in both fractions from 60 h cultures are shown (J). For analysis of involvement of sRNA in the lysis of wild-type cells, W3110 (circles) and its derivatives, *micA*::*kan* (squares) and *rybB*::*kan* (triangles) were grown in 20 mL of LB or LB containing kanamycin (50 μg/ mL) at 37 °C. At the times indicated, changes in turbidity at OD_{600} (K) and in protein accumulation in the culture medium (L) were analyzed. The values are mean protein amounts with standard deviation. The experiments were repeated at least three times and the patterns were confirmed to be reproducible.

suggest that MicA and RybB are directly involved in cell lysis under ordinary growth conditions.

2.3.3 Induction of cell lysis by over-expression of *micA* or *rybB*

The suppression by mutations defective of both sRNA genes allowed us to assume that MicA and RybB are functionally located downstream from σ^E in the process of cell lysis. To test this assumption, we introduced a multi-copy plasmid clone of the sRNA genes into the BW25113 strain to examine whether cell lysis was induced by the sRNAs under the condition without enhanced expression of *rpoE* (Fig. 2.2). The transformant with the *micA* clone, pACYCMICA, or with the *rybB* clone, pACYCRYBB, exhibited a significant



(C)

	pACYC177	pACYCRPOE	pACYCMICA	pACYCRYBB	pUC118	pUCMICA	pUCRYBB
Medium fraction (mg/ml of culture)	0.1 ± 0.1	1.9 ± 0.4	0.5 ± 0.1	0.5 ±0.05	0.1 ± 0.1	1.7 ± 0.1	1.7 ± 0.2
Remaining fraction (mg/ml of culture)	6.2 ± 1.0	5.7 ± 0.8	5.8 ± 1.0	5.9 ± 1.0	5.5 ± 0.9	5.8 ± 1.4	5.7 ± 1.7

Fig. 2.2 Effects of over-expression of *micA* and *rybB* on cell lysis
BW25113 cells harboring pACYCRPOE, pACYCMICA, pACYCRYBB or an empty vector, pACYC177 (A) and BW25113 cells harboring pUCMICA, pUCRYBB or an empty vector pUC118 (B) were grown and the cultures were fractionated as described in the legend to Fig2.1 except that arabinose was not added. Proteins in medium fractions were analyzed (A, B) and proteins in both fractions were determined (C) as shown in Fig.2.1. The experiments were repeated at least three times and the pateers were confirmed to be reproducible. Data from one representative experiment are shown for A and B.

decrease in cell density at the early stationary phase (not shown), although the decreased level was about 40% of that of the transformant with the *rpoE* clone, pACYCRPOE. The influence of plasmid clones of the sRNAs on cell lysis was then investigated by observing the accumulation of proteins in the culture medium (Fig. 2.2A,C). Consistent with the decrease in cell density, the pronounced protein bands were observed at 36 and 60 h, resembling the phenotypes observed when active σ^E is increased. For further increase in expression of *micA* and *rybB*, pUCMICA and pUCRYBB, both of which have a copy number much higher than those of pACYCMICA and pACYCRYBB, were introduced and examined protein accumulation in the culture medium (Fig. 2.2B,C). The results suggest that both transformants cause protein accumulation at nearly the similar level to that of transformant with pACYCRPOE. To confirm the effect of over-expression of *micA* and *rybB* on the *omp* expression, RT-PCR and Urea-SDS-PAGE experiments were carried out, suggesting that introduction of pACYCMICA and pACYCRYBB clones caused reduction in the quantity of *ompA* and *ompC* mRNAs, respectively, and introduction of pACYCMICA clone largely diminished OmpA protein as in the case of *ompA*::*kan* cells (Fig.2.4, 2.5). Therefore, it is considered that the two sRNAs play vital functions in the

cell-lysis process at the position downstream form σ^E.

2.3.4 Effect of reduction in outer membrane proteins

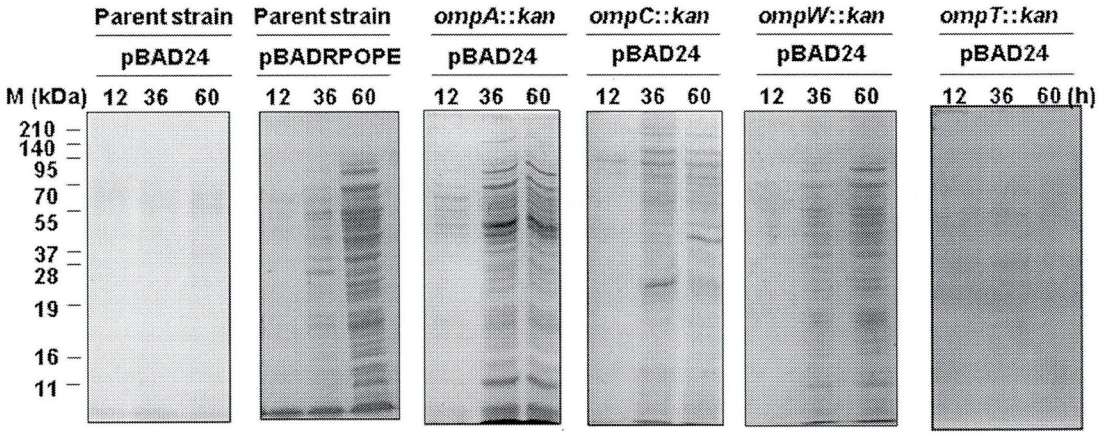
In the strains with a high level of active σ^E, OmpA, OmpC and OmpW proteins become decreased to almost an undetectable level and their gene transcription is greatly declined (Kabir *et al.*, 2005). As stated earlier, MicA and RybB, which have been shown to be involved in the control of expression of *ompA* and both *ompC* and *ompW*, respectively, (Johansen *et al.*, 2006; Valentin-Hansen *et al.*, 2007), seemed to promote the cell-lysis process downstream from σ^E. We thus examined the effect of disrupted mutations of genes encoding outer membrane proteins on cell density and cell lysis. As expected, all mutant strains harboring pBADRPOE grown in the presence of arabinose showed reduced cell density at 36 and 60 h, the levels of which were found to be similar to those of the parental strain harboring pBADRPOE (not shown). However, a significant reduction in cell density was observed in all mutant strains transformed with pBAD24, but the reduction level was lower than those transformed with pBADRPOE. Consistently, the protein accumulation test showed that the disrupted mutants harboring pBAD24 exhibited significant protein bands compared with those of the parental strain harboring pBAD24 (Fig. 2.3 A), although protein density of most bands was lower than those of corresponding mutants harboring pBADRPOE.

Notably, *micA ompA*, *rybB ompC* and *rybB ompW* double mutants showed a slight increase in protein accumulation level in culture medium compared with those in *ompA*, *ompC* and *ompW* single mutants, respectively (not shown). Moreover, a mutant lacking most of the coding region for OmpT, one of outer membrane proteins, exhibited no such phenotype of increased lysis, suggesting that reduction in specific Omp proteins is responsible for cell lysis. We also examined the protective effect of Omp proteins on σ^E-dependent cell lysis. When pMBLOMPC and pUCOMPA were introduced into the *rseA* mutant strain, suppression of protein accumulation in culture medium was observed at about 10% of that of the *rseA* mutant transformed with an empty vector. The low suppression may be due to the effect of cognate sRNAs presented endogenously. Taken together, these results suggest that the reduction in these three outer membrane proteins is responsible for cell lysis in the σ^E-directed process.

2.3.5 Morphological observation of cells in σ^E-directed cell-lysis process

Morphology of cells with a high level of active σ^E was observed under electron microscope (Fig. 2. 4). W3110 cells harboring pBADRPOE or an empty vector pBAD24 were grown in the presence or absence of 20 mM Mg^{2+}, which is efficient concentration to protect cell lysis (Kabir *et al.*, 2005), for 13 and 36 h, corresponding to for 1 and 24 h, respectively, after the addition of arabinose, and subjected to cell morphological observation. Many ghost cells, apparently losing inside materials, were observed at 36 h but

(A)



(B)

	Parent strain		*ompA*::*kan*	*ompC*::*kan*	*ompW*::*kan*	*ompT*::*kan*
	pBAD24	pBADRPOE	pBAD24	pBAD24	pBAD24	pBAD24
Medium fraction (mg protein/ml culture)	<0.1	1.9 ± 0.3	0.5 ± 0.3	0.4± 0.1	0.5 ± 0.1	<0.1
Remaining fraction (mg protein/ml culture)	6.0 ± 0.8	3.8 ± 0.2	5.8 ± 0.8	5.5 ± 0.2	5.3 ± 0.8	6.0 ± 0.8

Fig. 2.3 Effects of *ompA*-, *ompC*- and *ompW*-disrupted mutations on cell lysis
Parent strain, *ompA*::*kan*, *ompC*::*kan* and *ompW*::*kan* cells harboring pBADRPOE or pBAD24 were grown as described in the legend to Fig. 2-1. Analysis of proteins in medium fractions (A) and determination of proteins in both fractions (B) were carried out as shown in Fig.2.1. The experiments were repeated at least three times, and the patterns were confirmed to be reproducible. Data from one representative experiment are shown for A.

not at 13 h in the case of cells harboring pBADRPOE (Fig. 2.6 A, B). No ghost cells were observed even at 36 h in cells harboring pBAD24. When 20 mM Mg^{2+} was added, such ghost cells were not seen in cells harboring pBADRPOE. The period of appearance of ghost cells well agreed with that of a significant protein accumulation in culture medium (Figs 2.1D, 2.3A). Furthermore, to examine release of cytoplasmic materials into culture medium, activity of β-galactosidase as one of cytoplasmic enzymes was measured in medium and remaining fractions from W3110 cells over-expressing *rpoE*, grown under the same conditions as used in Fig. 2.6.About 13%, 37% and 53% of the sum of activities from both fractions were detected in medium fractions at 12-, 36- and 60-h incubation, respectively. Whereas, about 0%, 4% and 9% were detected in medium fractions from control cells at 12-, 36- and 60-h incubation, respectively. These results suggest that cytoplasmic materials are released into culture medium as σ^E-dependent lysis process

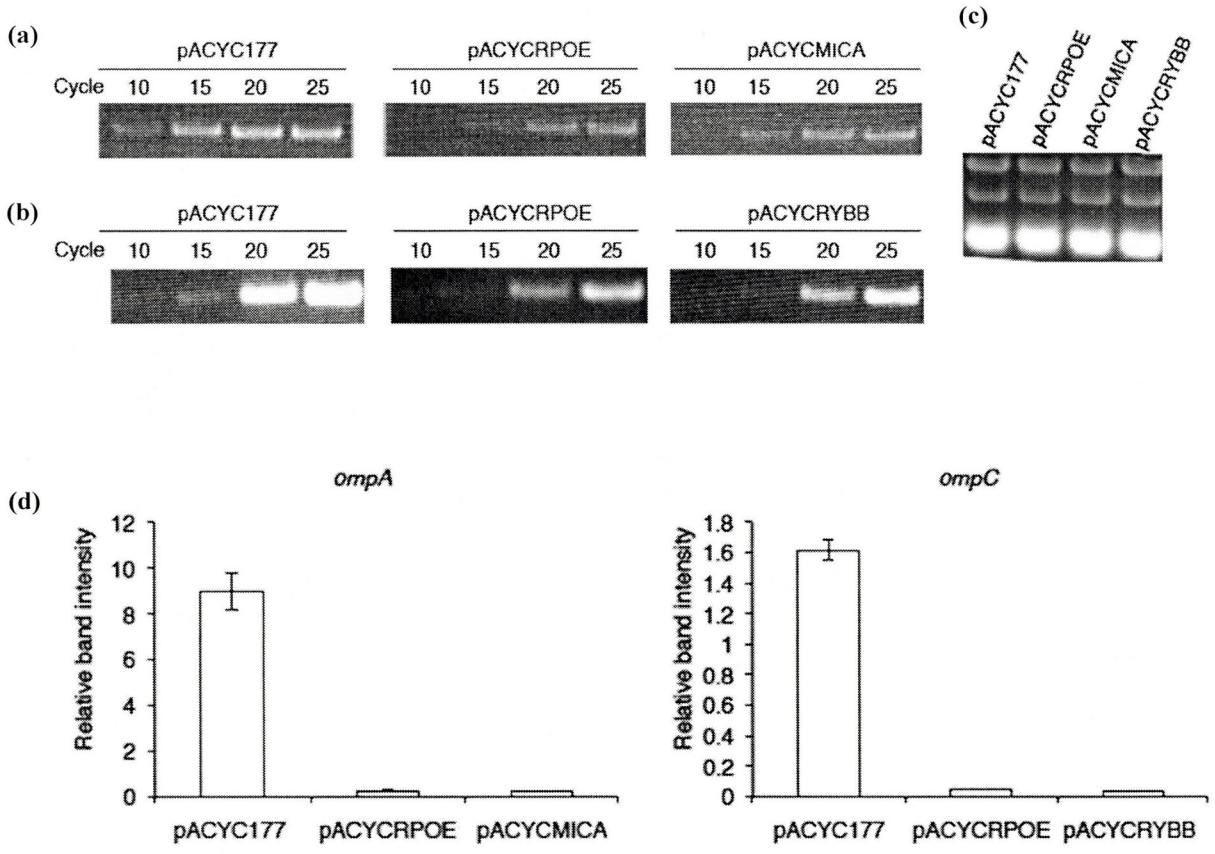


Fig. 2.4 RT-PCR analysis of the *omp* gene expression in BW25113
Cells harboring pACYCRPOE, pACYCMICA and pACYCRYBB or an empty vector, pACYC177. Total RNAs prepared from cells grown at 37°C for 9 h were subjected to RT-PCR with primers for *ompA* (A) and *ompC* (B). Cycles show the number of PCRs. The panel to the right represents rRNAs as a control (C). Band intensity was analyzed by using UNSCAN-IT gel™ automated digitizing system (D). Relative values of intensity of bands in A and B were determined as a ratio of intensity of each band for each gene to that for each rRNA. Bars represent the ±SD for three independent experiments.

proceeds.

2.3.6 Mg^{2+} protection at cell burst step in σ^E-directed cell-lysis process

Mg^{2+} has been shown to suppress the decline in cell density and cell lysis along with the increased expression of *rpoE* (Kabir *et al.*, 2005). However, the step suppressed by Mg^{2+} in σ^E-directed cell-lysis process has not been defined. Considering the effect of

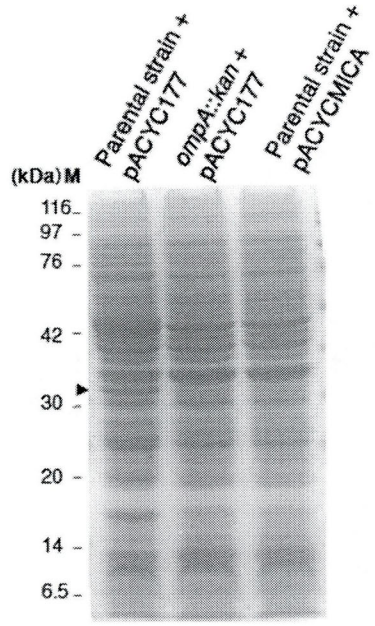


Fig. 2.5 Urea SDS-PAGE analysis of the *omp* gene exression in BW25113
Cells harboring pACYC177 or pACYCMICA and *ompA*::*kan* cells harboring pACYC177. Cell cultivation and cell fractionation were performed as described in the Experimental procedure. The cell fractions subjected to Urea-SDS-PAGE. The experiments were repeated at least three times and the patterns were confirmed to be reproducible. An arrowhead indicates the position of OmpA protein (37.5 kDa).

σ^E-directed deficiency of the three Omp proteins (Kabir *et al.*, 2005), it is possible that the ion interacts with the outer membrane to prevent disruption of the membrane in the cell-lysis process.

From experiments in Fig. 2.6, we assumed that Mg^{2+} extracellularly associates with the outer membrane to protect cell burst. This assumption was thus tested by treatment with EDTA. Cells harboring pBADRPOE or an empty vector, which were cultured for appropriate times after the addition of arabinose in the presence or absence of Mg^{2+}, were exposed to 0 or 5 mM EDTA solution (Fig. 2.7). Consequently, only cells harboring pBADRPOE showed 25%-30% decrease in optical density when exposed to 5 mM EDTA (Fig. 2.7 B). We also examined the effect of the reagent at the range of 1-20 mM EDTA, and nearly, the same results were obtained at more than 5 mM. As 5-20 mM EDTA showed the similar effect, some amount of Mg^{2+} supplemented may be used by cells or accumulated in cells. Control cells without the addition of arabinose showed almost no reduction in optical density by the treatment of EDTA. These results suggest that Mg^{2+} maintains outer membrane integrity by its external interaction and that disintegration of the outer membrane is the final trigger toward cell lysis in the σ^E-directed cell-lysis cascade.

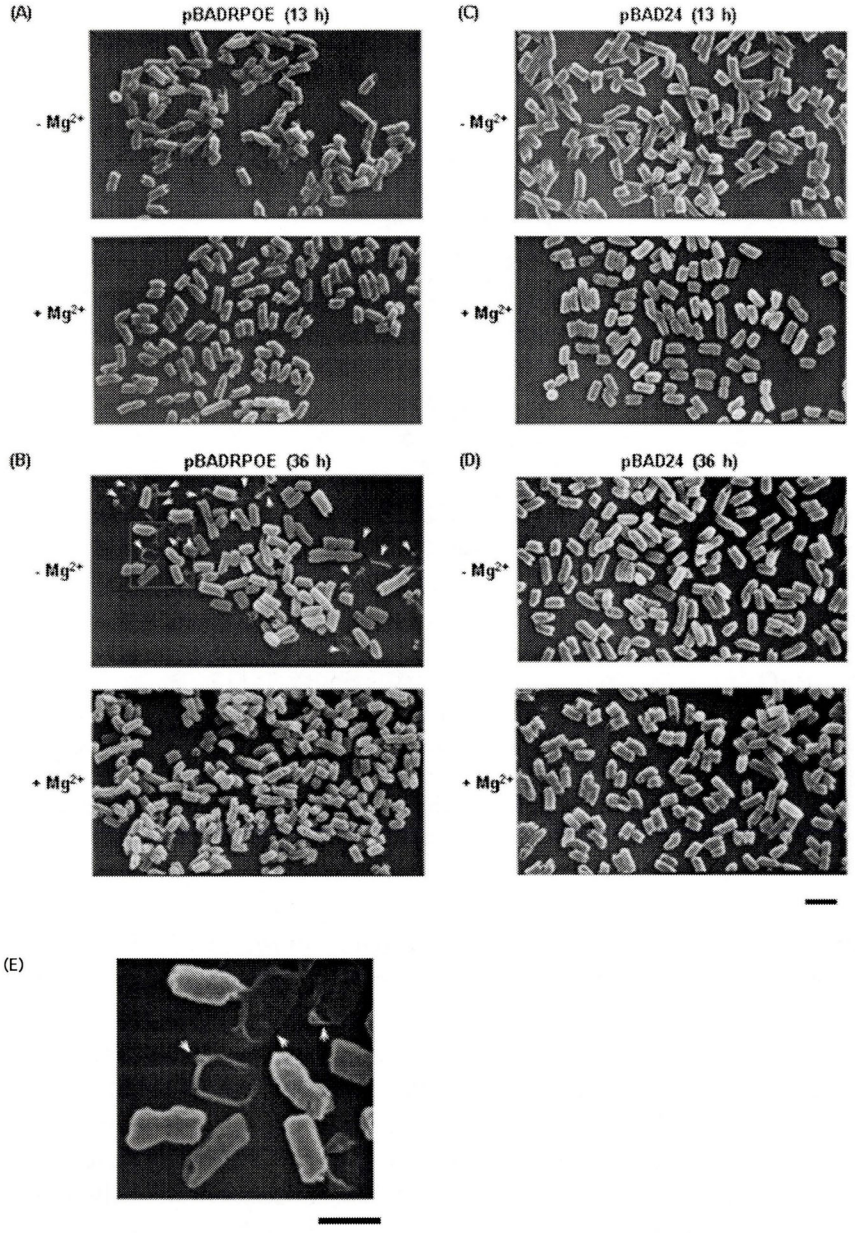


Fig. 2.6 Morphological observation of cells over-expressing *rpoE* and effect of Mg^{2+}
W3110 cells harboring pBADRPOE (A, B) or pBAD24 (C, D) were grown as described in the legend to Fig. 2.1, but in some cases, 20 mM $MgSO_4$ was added into culture ($+Mg^{2+}$). After 13 h (1 h after addition of arabinose) (A, C) or 36 h (24 h after addition of arabinose) (B, D) of cultivation, cells were harvested and prepared for morphological observation as described. An enlarged picture of a dotted square in B is shown in E. Arrows indicate the ghost cells. Scale bars represent 1 μm.

2.4 DISCUSSION

Growth phase-specific σ^E-directed cell lysis in *E. coli* would be one of intrinsic strategies for its survival under nutrient-limited or stressful conditions as in prolonged stationary phase. The lytic mechanism may selectively remove VBNC cells that dramatically arise at the beginning of the stationary phase (Nitta *et al.*, 2000; Noor *et al.*, 2009b). In spite of such an intriguing mechanism, the process toward cell lysis, however, had remained to be resolved. We attempted for the first time to clarify the cascade of σ^E-directed cell lysis. A vital factor in the cascade was found to be sRNA, which has recently been highlighted and shown to generally act as negative or positive translational regulators in bacteria (Gottesman, 2005; Marles-Wright and Lewis, 2007). Such a regulation is highly specific and effective because it is achieved by the interaction of sRNA with the targeted mRNA via an aide of an RNA chaperone, Hfq, to turn off or turn on translation. The sRNAs involved in the cascade are MicA and RybB, whose genes are under the control of σ^E. The involvement of both sRNAs was shown by experiments with disruption and enhanced expression of both sRNA genes, which clearly exhibited diminishment and induction, respectively, of σ^E-directed cell lysis. Both sRNAs control the level of mRNAs of *ompA* and both *ompC* and *ompW*, respectively, (Udekwu and Wagner, 2007; Valentin-Hansen *et al.*, 2007), which is supported by evidence that the amounts of these gene products become trace amounts in *rseA* mutant cells with an increased active σ^E level (Kabir *et al.*, 2005). Consistent with these, the knockout mutant of *ompA*, *ompC* or *ompW* was shown in this study to give rise to a significant level of protein accumulation in culture medium, indicating cell lysis. This lysis may not be due to an envelope stress caused by the deletion of Omp proteins because two kinds of *ompC*- disrupted mutants showed no influence on or large reduction in the *rpoE* expression (McBroom and Kuehn, 2007).

Although σ^E-directed cell lysis is emphasized in strains defective in *rseA* or harboring the *rpoE* plasmid clone, both of which have an increased level of active σ^E in cells (Nitta *et al.*, 2000), it physiologically occurs in wild-type under ordinary growth conditions, which is suggested by the following findings: (i) *rpoE* expression is dramatically increased at the beginning of the stationary phase (Nitta *et al.*, 2000), (ii) the pattern in SDS-PAGE of proteins accumulated in culture medium at the early stationary phase in wild-type is similar to that in cells with an increased level of active σ^E (Nitta *et al.*, 2000), (iii) protein accumulation in the culture medium is almost repressed by the introduction of the *rseA* plasmid clone into the wild-type to enhance the expression of anti-σ^E (Noor *et al.*, 2009a), and (iv) the *micA*- or *rybB*-disrupted mutation strongly suppressed protein accumulation in the culture medium (this study). Considering the finding that most of cells become VBNC in the early stationary phase, the mechanism of σ^E-directed cell lysis would thus contribute to the removal of damaged cells including VBNC cells in cell population in the stationary phase and supply the resultant nutrients to remaining living cells. The signal for conversion of colony-formable cells to VBNC cells is not clear but might be physiologically crucial because of a growth phase-specific event observed at the early stationary phase (Nitta *et al.*, 2000). One of the possible signals may

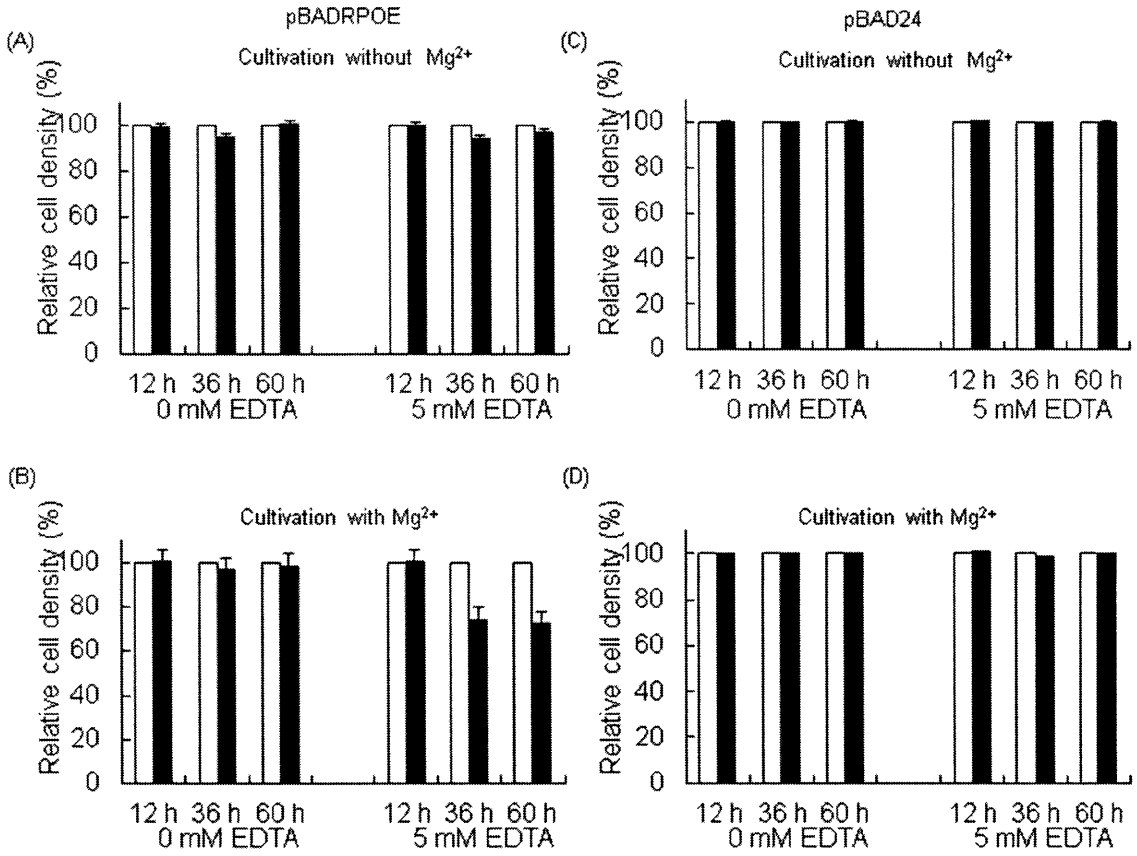


Fig. 2.7 Effects of EDTA on cell growth in the presence or absence of Mg^{2+}
W3110 cells harboring pBADRPOE (A, B) or an empty vector, pBAD24 (C, D) were grown as described in the legend to Fig. 2.1 with or without 20 mM $MgSO_4$. After 13 h (1 h after addition of arabinose), 36 h (24 h after addition of arabinose) and 60 h (48 h after addition of arabinose) of cultivation, cells were harvested and effect of EDTA was then observed by measuring optical density as described. The value at 3 min (black columns) after cells were resolved in 5 mM EDTA in each sample is expressed as a percentage of that at 0 min (white columns) in the same sample.

be oxidative stress. The mutation of *rpoS* encoding σ^S causes an increase in VBNC cells (Kabir *et al.*, 2004), and a similar effect was observed in cells defective in *katE*, encoding catalase, that is under the control of σ^S (Noor *et al.*, 2009b). Both mutations greatly enhance cell lysis directed by σ^E, and the mutation of *katE* or *sodA*, encoding superoxide dismutase, significantly increases expression of *rpoE* (Kabir *et al.*, 2004; Noor *et al.*,

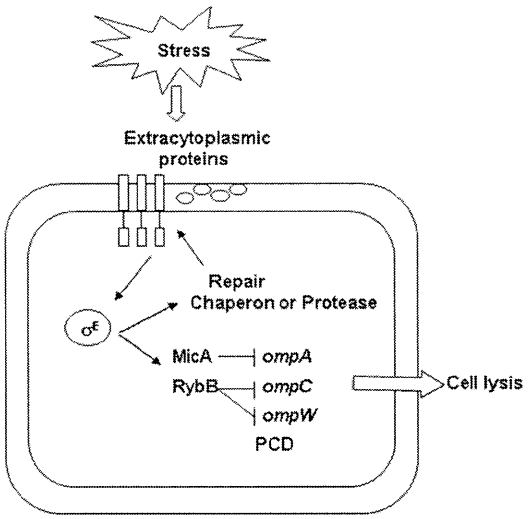


Fig. 2.8 A model of σ^E-directed cell lysis, processed as PCD, in *E. coli*
Depending on the extent of damage of cells, the σ^E-directed response leads to either repair of damaged cells or PCD cascade. Details are given in the text.

2009b). Moreover, intracellular oxidative stress is accumulated around the beginning of stationary phase (Noor *et al.*, 2009b). Meanwhile, as guanosine 3', 5'-bispyrophosphate as a signal for stringent control, which increases during amino acid starvation, including at the transition into stationary phase, has been shown to induce the transcription of *rpoE* (Costanzo and Ades, 2006), amino acid starvation may become another signal for the conversion.

The results of this study together with results of previous studies have showed almost the entire cascade of the σ^E-directed cell lysis as depicted in Fig. 2.8. As the pathway is regulated by the general transcriptional factor, we have proposed it as a programmed cell death. When cells are exposed to some stresses as a signal, mainly an intracellular oxidative stress (Noor *et al.*, 2009b), unfolded proteins are accumulated in the outer membrane or periplasmic space, which in turn causes the elevation of active σ^E in the cytoplasm. In the case of a small extent of damage of proteins, the level of intracellular active σ^E may also be low, which would direct the transcription of proteins required for repair of damaged proteins. In contrast, in the case of a high level, σ^E directs cell lysis by the expression of *micA* and *rybB*, followed by the reduction in Omp proteins and disintegration of the outer membrane. As σ^E is found in beta and gamma proteobacteria, similar pathways would thus operate in other bacteria.

Although no evidence indicating how cells measure the extent of protein damage has so far been presented, it is possible that sRNA species play a key role in this task, based on the general concept that RNA is relatively unstable. As damaged Omp proteins are accumulated and active σ^E level increases, the expression of *micA* and *rybB* is induced. The

production of MicA and RybB appears to provide cells time to deal with the damaged proteins by ceasing the synthesis of Omp proteins as showed previously (Valentin-Hansen *et al.*, 2007). After the task of dealing with the damaged proteins has been completed and σ^E becomes inactive, the cellular level of MicA and RybB molecules may sharply drop. Such a transient elevation of MicA and RybB may thus be physiologically crucial for cell survival. If the extent of damage becomes over a critical point, a certain cellular level of MicA and RybB may be retained to diminish the corresponding Omp proteins, resulting in cell lysis.

It has been shown that σ^E pathway stimulation is sufficient to increase outer membrane vesiculation (McBroom *et al.*, 2006). However, no such vesicles were detected in our experiments (Fig. 2.6 E), and no cell burst is seen in the vesiculation process (McBroom and Kuehn, 2007). Therefore, we speculate that downstream events from σ^E activation in the vesiculation process are different from those in the cell-lysis process. Physiologically, σ^E-directed cell lysis would be involved in removal of damaged cells, whereas outer membrane vesiculation contributes to competition for survival with other bacteria or interaction with eukaryotic cells (McBroom *et al.*, 2006).

Further study is required to clarify whether the σ^E-dependent PCD is related to a stress response directed by misfolded proteins or whether nutrients from lysed cells indeed support prolonged survival of the rest of cell population.

CHAPTER 3

Crucial Roles of MicA and RybB as Vital Factors for σ^E-Dependent Cell Lysis in *Escherichia coli* 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 its relationship to the long-term stationary phase, we focused on *micA* and *rybB* as vital sRNA genes for σ^E-dependent cell lysis but not on σ^E-encoding gene, *rpoE*, as an essential gene. Long-term stationary phase was observed at temperatures of less than 37°C but not more than 38°C and was found even in the *rpoS* knock-out background. Disrupted strains of *micA* and *rybB* were incapable of exhibiting such a long-term stationary phase. Both strains drastically lost survivability following dramatic accumulation of mutations. These findings allow us to speculate that σ^E-dependent cell lysis plays a key role in establishment of a long-term stationary phase presumably by elimination of damaged cells to prevent over-accumulation of mutations.

3.1 INTRODUCTION

Under optimum 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 (Zambrano and Kolter, 1996; Navarro *et al.*, 2010). 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 as a consequence of metabolic activities gradually accumulate, which 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 nuteirnts support the survival of the remaining culturable cells and the level of VAC cells is retained to some extent for months or years in a long-term stationary phase (Desnues *et al.*, 2003).

E. coli possesses seven σ factors, each of which governs the expression of a set of genes to conduct intracellular activities in accordance with their environment (Helmann and Chamberlin, 1998; Ishihama, 1999). σ^S and σ^E play crucial roles in the stationary phase (Battesti *et al.*, 2011; Nitta *et al.*, 2000], in which cells are exposed to various stresses. Nutritional starvation or 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 to prepare the organism for survival in the stationary phase (Hengge-Aronis, 2002). On the other hand, active σ^E is accumulated under extracytoplasmic stress and deals with damages of macromolecules caused by the stress (Missiakas *et al.*, 1997; Raina *et al.*, 1995; De Las Peñas A et al., 1997). There are over 100 genes belonging to 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, which is not in a static but rather in a dynamic equilibrium by 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 one after another 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, 2004; Zinser and Kolter, 1999; Zinser and Kolter, 2000), and *ybeJ-gltJKL* region (Zinser and Kolter, 2004; Zinser and Kolter, 2000; 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 (Stancik *et al.*, 2002; Fukuda *et al.*, 2001). However, only these three mutations do not seem to be sufficient to explain for the continuous population takeovers 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-advantage 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 *et al.*, 2012; Sauer and Weichenrieder, 2011). It is known that almost all *trans*-encoded sRNAs, of which genes are separately located from their target genes on the genome, are expressed under specific environmental stress conditions including oxidative stress, anaerobic condition, cell envelope homeostasis and glucose starvation (Vogel, 2009; Repoila and Darfeuille, 2009). Of the *trans*-encoded sRNAs, *micA* and *rybB*, which are expressed by σ^E under a extracytoplasmic stress condition, down-regulate outer membrane proteins of OmpA and both OmpC and OmpW, respectively, in *E. coli*, which in turn disintegrates the outer membrane to lead to cell lysis (Murata *et al.*, 2012). The event of the cell lysis cascade appears to be related to accumulation of oxidative stress at the early stationary phase (Noor *et al.*, 2009b), which allows us to speculate that the event contributes to the progress to death phase or/and 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 examined the contribution of the σ^E-dependent cell lysis, by focusing on sRNA MicA and RybB essential for the cell lysis, to cell survival in the long-term stationary phase of *E. coli*.

3.2 MATERIALS AND METHODS

3.2.1 Materials

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

3.2.2 Bacterial strains, medium and culture conditions

The bacterial strains used in this study were derivatives of *E. coli* K-12, 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 modified Luria-Bertani (LB) medium [1% (w/v) Bactotryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl] at the temperature indicated under aerobic conditions by reciprocal shaking (100 rpm min^{-1}). Appropriate drugs were added at the following final concentrations: streptomycin, 5 μg/ml; rifampicin, 15 μg/ml; kanamycin, 25 μg/ml; tetracycline, 5 μg/ml.

3.2.3 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 preculture at 37°C, the culture was diluted and adjusted to OD_{600} 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 OD_{600} and colony-forming units (CFU). CFU were estimated by counting colonies at 24 h after appropriate dilution of cell culture and spreading on LB plates (Nitta *et al.*, 2000). Cell growth experiments were repeated at least three times. The appearance timing of main peaks in the growth curves from repeated experiments was reproduced within a day and vertical fluctuations as CFU in the curves were within one order.

3.2.4 Estimation of mutation frequency

Cell culture was 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.

3.3 RESULTS

3.3.1 Long-term stationary phase in the *rpoS* knock-out background

The role of *rpoS* in the a long-term stationary phase remains to be clarified, though aged cells from *rpoS* knock-out strains have been proven 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 LB medium at 37°C, which is the optimum growth condition to establish survival in the long-term stationary phase (Zambrano and Kolter, 1996; Navarro *et al.*, 2010), and number of colony forming units (CFU) was determined (Fig. 3.1). The colony forming cells were always detected during the 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 in the long-term stationary phase.

3.3.2 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.*, 2009b). 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).

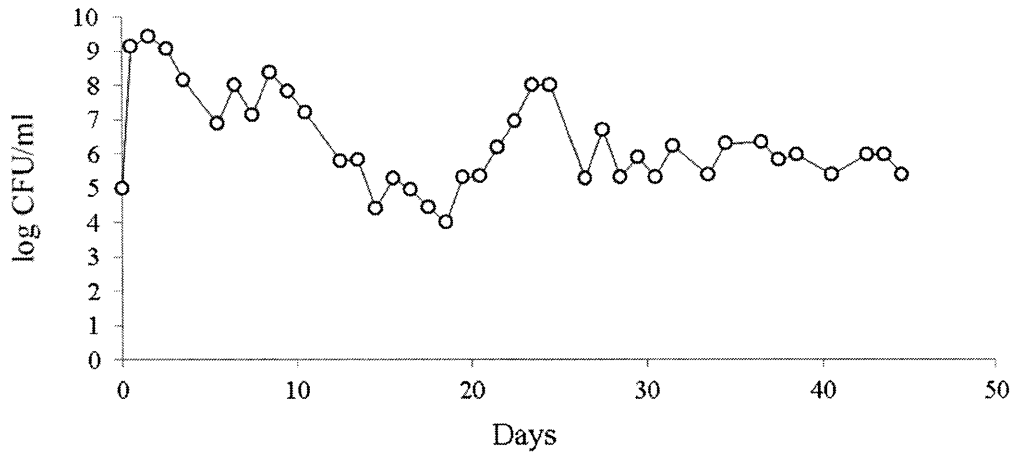


Fig. 3.1 Survivability of *rpoS* strain in long-term cultivation
W3110 *rpoS*::*tet* cells were aerobically grown in LB medium at 37 °C under the condition as described in Materials and Methods. At the times indicated, CFU were determined.

A stable long-term stationary phase in W3110 was observed at 33°C, 36°C and 37°C but not at a temperature of more than 38°C (Fig. 3.2 and data not shown), indicating that temperature is one of critical factors for survival through the long-term stationary phase.

The first large decline in CFU probably as the death phase was observed after about 3 days and 2 days under the conditions of 33°C and 36°C, respectively, in W3110 (Fig. 3.2). The peaks after the first decline were observed at about 14 days and 9 days at the temperatures of 33°C 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 with that of the previous report (Navarro *et al.*, 2010). The difference in peak position between 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.

3.3.3 Necessity of MicA and RybB as a key factor in σ^E-dependent cell lysis for long-term stationary phase

σ^E-dependent cell lysis may contribute to the elimination of damaged cells as a portion of VBNC cells, which seem to be exposed to stresses including oxidative stress, and such a lysis event occurs after the beginning of the stationary phase (Murata *et al.*, 2012; Noor *et al.*, 2009b). MicA and RybB as sRNA 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 in 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. 3.2). As a result, it was found that the CFU of *micA*::*kan* and *rybB*::*kan* became non-detectable at around the peak of the

putative first GASP population in the parental strain. Notably, a peak or shoulder in both mutants was observed at about 7days at the temperatures of 33°C 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 gives rise to accumulation of

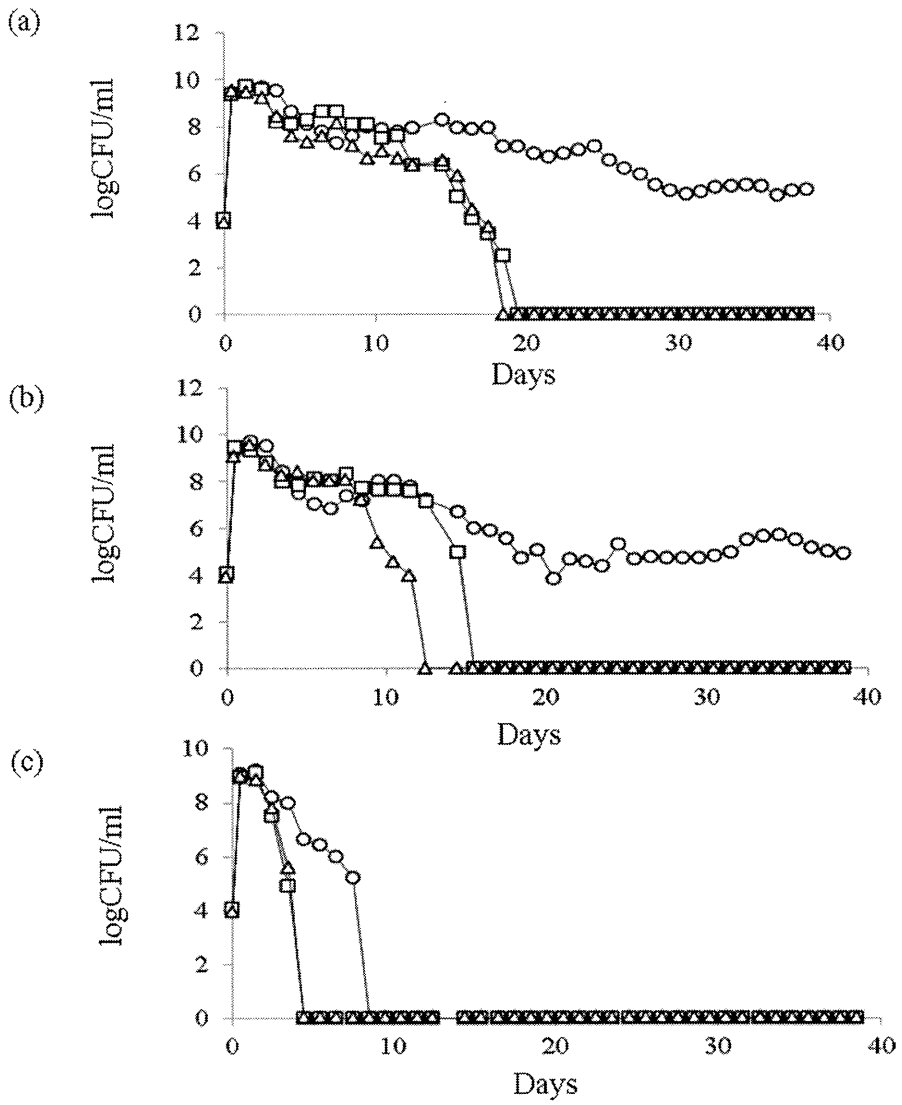


Fig. 3.2 Comparison of *micA, rybB* and wild-type strains in long-term cultivation at different temperatures

W3110 (circles), W3110 *micA*::*kan* (squares) and W3110 *rybB*::*kan* (triangles) cells were grown as described in the legend of Fig. 3.1 except for different temperatures, 33°C (a), 36°C (b) and 42°C (c), and CFU were determined at the times indicated.

damaged cells and increase in mutation frequency. We thus determined CFU on streptomycin- and rifampicin-containing plates with prolonged cultivation (Fig. 3-3). CFU of the *micA*- and *rybB*-disrupted strains on both drug-containing plates dramatically increased just before the time when CFU on drug-free plates became undetectable. The apparent mutation frequency at the peaks in the *micA*- and *rybB*-disrupted strains was two to three orders of magnitude higher than that in the parental strain: 5.8×10^{-2} in *micA* in streptomycin, 2.4×10^{-2} in *rybB* in streptomycin, 3.7×10^{-2} in *micA* in rifampicin and 1.2×10^{-2} in *rybB* in rifampicin. Notably, the mutation frequency of the parental strain in the long-term stationary seems to be much higher than that in exponential phase, which is consistent with results in previous studies (Finkel, 2006). Therefore, it is likely that the accumulation of mutations is linked with 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.

3.4 DISCUSSION

The first GASP mutation, which was identified in the *rpoS* gene, resulted in extension of the C-terminal region of RpoS (Zambrano and Kolter, 1996), conferring enhanced catabolization ability of amino acids (Zinser and Kolter, 2004; Zinser and Kolter, 1999). Some *rpoS*-down mutations similarly exhibit a GASP phenotype (Zambrano *et al.*, 1993; Zinser and Kolter , 2000) but *rpoS* null alleles was reported to express no GASP phenotype (Zambrano *et al.*, 1993). On the other hand, the occurrence of *rpoS*-independent GASP has been found (Martínez-García *et al.*, 2003). In this study, we suggest that a 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 (Figs. 3.1 and 3.2). At least the first and second main peaks after the death phase in the growth curves appeared at the time of period similar to those in previous studies, which inform that aged cells in the period around the peak defeated unaged cells in competition experiments (Finkel, 2006; Martínez-García *et al.*, 2003). These results may suggest population takeovers by acquisition of mutations of not only *rpoS* but also other genes. Interestingly, when examined at different temperatures, the peak position in CFU was greatly shifted (Fig. 3.2). Considering the evidence that oxidative stress increases as temperature rises (Noor *et al.*, 2009b), 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 reduction in CFU as was observed in the parental strain after the stationary phase but lost CFU at the beginning of the long-term stationary phase under cultivation conditions at 33°C, 36°C or 37°C (Figs. 3.2 and 3.3). MicA and RybB, which are members of the cascade of σ^E-dependent cell lysis (Murata *et al.*, 2012), are involved as sRNAs in the negative regulation of *ompA* and *ompC*/*ompW*, respectively (Vogel and Papenfort, 2006; Udekwu and Wagner, 2007) but are not related to the repairing response under the control of σ^E. Disruption of *micA* and *rybB* results in hampering of the σ^E-dependent cell lysis, and enhanced expression of both genes causes an increase in cell lysis (Murata *et al.*, 2012). These lines of evidence indicate the possibility

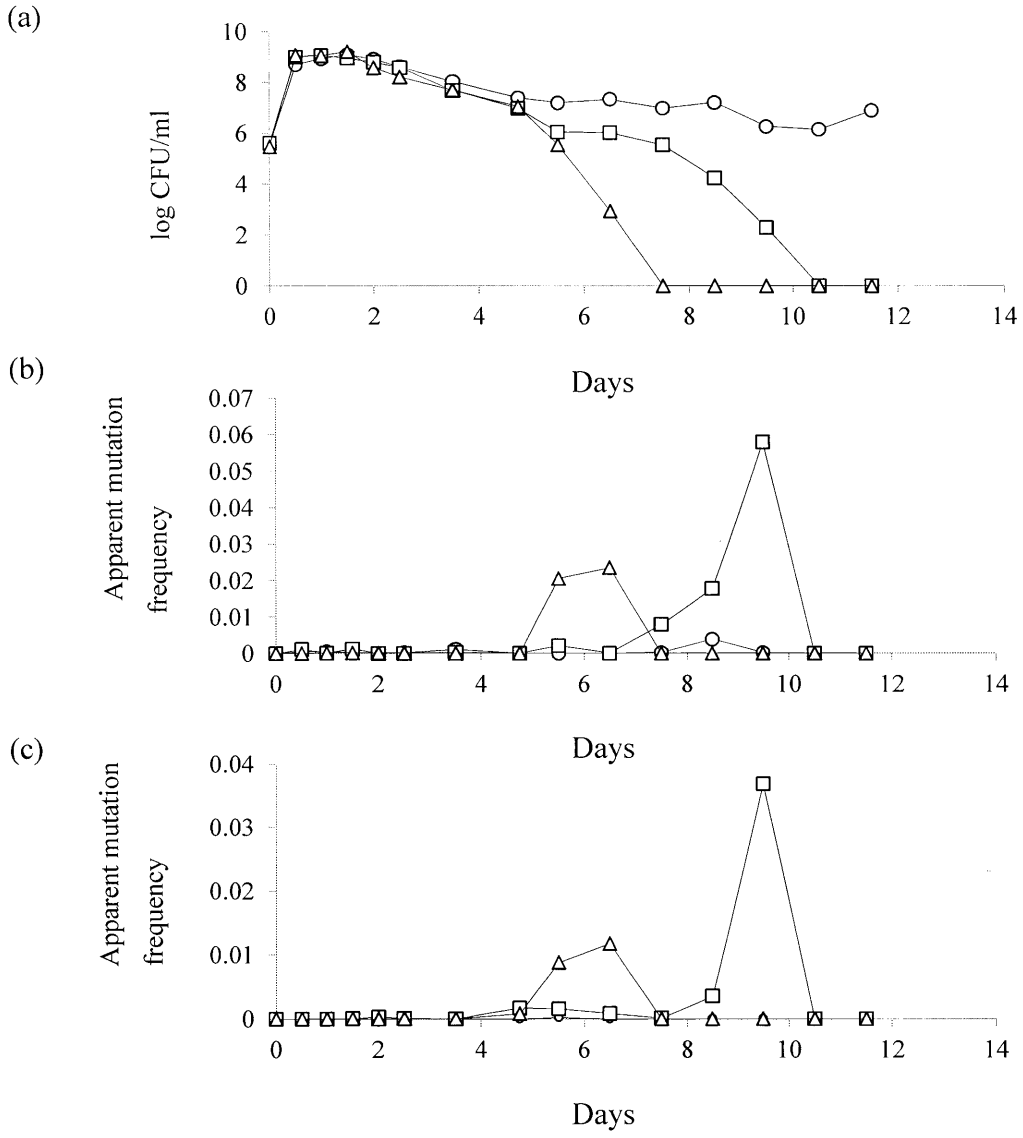


Fig. 3.3 Spontaneous drug-resistant mutants from *micA, rybB* and wild-type strains in long-term cultivation

W3110 (circles), W3110 *micA*::*kan* (squares) and W3110 *rybB*::*kan* (triangles) cells were grown as described in the legend of Fig. 3.1. 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.

that the σ^E-dependent cell lysis is essential for continuous survival in the long-term stationary phase. In addition, the mutation frequency based on the number of spontaneous drug-resistant mutants 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 to prevent rapid accumulation of mutations over the capacity in the population. On the other hand, a number of different mutations in the defective mutant of the cell lysis pathway would be acutely accumulated, and eventually VAC cells would catastrophically decrease in the population. Consistently, 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 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 the additional role for providing nutrients for next generation.

Our data presented here and previous data from other research groups (Zambrano and Kolter, 1996; Navarro *et al.*, 2010; Finkel, 2006) suggest that the survival of cells in the long-term stationary depends on the appearance of new and advantageous mutations. Cells with advantageous mutation over other cells have the chance to 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 for elucidation of the mechanism of dynamic population takeovers in the long-term stationary phase.

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要旨

大腸菌におけるσ^E依存性溶菌の分子機構と長期定常期での生理学的役割
(Molecular mechanism of σ^E-dependent cell lysis and its physiological role at long-term stationary phase in *Escherichia coli*)

原核生物におけるσ因子は、RNAポリメラーゼサブユニットの１つであり、遺伝子上のプロモータ配列の認識に関与することで環境に応じた遺伝子発現を可能にする。中でもσ^Eはペリプラズムにおけるストレスに由来する異常タンパク質を感知し、シャペロンやプロテアーゼを動員することで修復処理に関与することが知られている。σ^Eの新たな機能として、障害により生育不能となった細胞の溶解除去に関わる可能性が提唱されてきたが、その機構は不明であった。また、σ^E活性変異株で外膜タンパク質（OMP）であるOmpA, OmpC, OmpWのタンパク質レベルでの発現が低下するとの報告があったが、その原因についても不明である。

本研究では、まず、σ^E依存性の溶菌プロセスの直接の原因はOMPの発現低下によるもの、さらにOMPの発現低下はsmall noncoding RNA (sRNA)であるMicAとRybBによるOMPの翻訳抑制の結果であることを明らかにした。増殖定常期初期において大多数の大腸菌はviable-but-non-culturable (VBNC) 状態となり、σ^E活性型となった細胞は溶解へと導かれる。一方、MicAとRybBはそれぞれOmpAとOmpCやOmpWの翻訳を抑制することが報告されている。そこで、両sRNAがσ^E依存性溶菌に関連するか否かを検討するため、*micA*破壊株および*rybB*破壊株における濁度の変化や培地中へ放出されたタンパク量を指標に検討を行った。親株に比べ、両sRNA破壊株ではほぼ完全に溶菌が抑制された。逆に*micA*および*rybB*過剰発現株、あるいは*ompA*, *ompC*, *ompW*破壊株では溶菌が顕著に引き起こされた。また、*rpoE*過剰発現株に観察されるゴースト形状はMg^{2+}存在下で抑制されることが明らかになった。以上の結果より、σ^E依存性溶菌過程は　$\sigma^E \rightarrow$ *micA*, *rybB*発現の増加→OMP発現の減少→外膜の損傷へと進行すると推定した。

σ^E依存性溶菌は、増殖定常期初期に酸化ストレスなどにより引き起こされる障害細胞の除去に作用することが示唆されているが、定常期後期以降の長期定常期で働いているか否か不明である。そこで、*micA*破壊株と*rybB*破壊株を用いて両遺伝子破壊の長期生存への影響を検討した。また、σ^Eを誘導する酸化ストレスの影響を見るため、培養温度による違いも検討した。親株では約40日以上生存したが、*micA*破壊株および*rybB*破壊株では生存期間が大幅に短縮された。この傾向は高温になるとより顕著であった。長期定常期における集団の存続に必要とされるGrowth Advantage in Stationary Phase (GASP)の影響を検討するため、同時に自然薬剤耐性変異株の出現頻度を指標に変異率を比較したところ、２つのsRNA破壊株は親株に比べ高い変異率を示した。また変異率のピーク直後に生菌数が検出不能なまでに低下し

た。これらの結果より、*micA*破壊株や*rybB*破壊株では急激な変異細胞の増加によってGASPを維持できなくなったと推測した。

以上の研究は、σ^E依存性溶菌の分子機構を明らかにするとともに、σ^E依存性溶菌が細胞集団から過剰な変異細胞の除去を行うことによって安定な長期定常期を確立する役割を果たすことを示唆した。このように本研究は定常期以降の微生物生理学を解明する上で重要な情報を提供する。

LIST OF PUBLICATIONS

1. Masayuki Murata, Noor Rashed, Hiroshi Nagamitsu, Shuhei Tanaka and Mamoru Yamada: Novel pathway directed by σ^E to cause cell lysis in *Escherichia coli*. *Genes to Cells* Vol. 17(2012), No3 pp. 234-247

(For CHAPTER 2)

2. Hiroshi Nagamitsu, Masayuki Murata, Tomoyuki Kosaka, Junpei Kawaguchi, Hirotada Mori and Mamoru Yamada: Crucial roles of MicA and RybB as vital factors for σ^E-dependent cell lysis in *Escherichia coli* long-term stationary phase *Journal of Molecular Microbiology and Biotechnology* Vol. 23(2013), No3 pp.227-232

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