MECHANISM OF CELL DEATH BY POLYAMINE ACCUMULATION

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ABSTRACT

The mechanism of cell death by polyamine accumulation has been studied. The accumulation of spermidine leads to a decrease in cell viability and to a decrease in the synthesis of ribosome modulation factor (RMF) and of the cation-selective porin OmpC. RMF is involved in stabilization of ribosomes during the transition from exponential growth to the stationary phase in *Escherichia coli*, and it is necessary for cell viability. The enhancement of cell death by OmpC deficiency caused by RMF deficiency was investigated. The *ompC* mutant by itself did not affect protein synthesis or cell viability, but the double *rmf ompC* mutant produced a much larger decrease in protein synthesis and cell viability than did the single *rmf* mutant. There was also a decrease in the amount of ribosomes and in the Mg^{2+} content in the double *rmf ompC* mutant, and cell viability could be partially restored by the addition of Mg^{2+} to the growth medium. RMF deficiency was found to inhibit the synthesis of another cation-selective porin OmpF. Thus, the double *rmf ompC* mutant is deficient in both OmpC and OmpF, which probably accounts for the pronounced decrease in Mg^{2+} uptake in this mutant. The results indicate that both RMF and Mg^{2+}, acting through stabilization of ribosomes, are important for cell viability at the stationary growth phase.

The mechanism of cell death caused by spermidine over-accumulation was further examined by using the spermidine acetyltransferase (SAT) gene-deficient mutant CAG2242. The growth

of *E. coli* CAG2242 in LB medium was normal in the presence and absence of 2 mM or 4 mM spermidine. However, the cell viability at 24 h after the onset of cell growth decreased greatly by the addition of 4 mM spermidine. The addition of 2 mM spermidine also caused a decrease in cell viability but the effect on cell viability was slower than that of 4 mM spermidine, which correlated to the amount spermidine accumulated in the cells. The accumulation of spermidine caused an increase in guanosine 3',5'-bispyrophosphate (ppGpp) synthesis, which its synthesis depends on activities of the products of two genes, *relA* and *spoT*, at stationary phase of growth. Whereas ppGpp level in cells grown in the absence of spermidine was decreased.

The decrease in cell viability of the transformant of *E. coli* CAG2242 with the pALS10 plasmid containing the full length *relA* clone under Ptac promoter was observed when 1 mM IPTG was added at the stationary phase. The accumulation of spermidine also caused a decrease in the syntheses of σ^s, the stationary phase specific RNA polymerase σ factor, RMF and OmpC which are essential for viability. The results suggested that accumulated spermidine caused the accumulation of ppGpp which leads to the inhibition of ribosome function. Therefore, the synthesis of essential factors for viability at the stationary phase was also inhibited resulting in cell death.

INTRODUCTION

Polyamines are polycationic compounds that are present in all living organism. The most common polyamines are putrescine (1,4-diaminobutane), spermidine and spermine (1-4). Prokaryotic *Escherichia coli* cell has a high content of putrescine and spermidine, which no detectable amount of spermine (5). While eukaryotic cells have a relatively high content of spermidine and spermine (6).

The general pathway for polyamine biosynthesis is illustrated in Fig.1. In mammalian cells and fungi the first, and at this stage rate-limiting, step is the decarboxylation of ornithine to form putrescine, catalyzed by ornithine decarboxylase (ODC) (4). Many micro-organisms (2,3) and higher plants (7,8) synthesize putrescine by at least two routes, either from ornithine though the activity of ODC, or via agmatine. Agmatine is formed by the decarboxylation of arginine by arginine decarboxylase, then hydrolyzed to be putrescine by agmatinase, with the removal of urea. Some organism, e.g. *E. coli*, possess both of these pathways (2). In plants, there is an additional routes to form putrescine from agmatine. Agmatine is hydrolyzed by agmatine iminohydrolase to yield ammonia and *N*-carbamoylputrescine, which is catalyzed by *N*-carbamoyl-putrescine amidohydrolase to give ammonia, carbon dioxide and putrescine.

Most organisms, spermidine is synthesized from putrescine by addition of aminopropyl group donated by decarboxylated *S*-adenosylmethionine, a reaction catalyzed by spermidine synthase, an

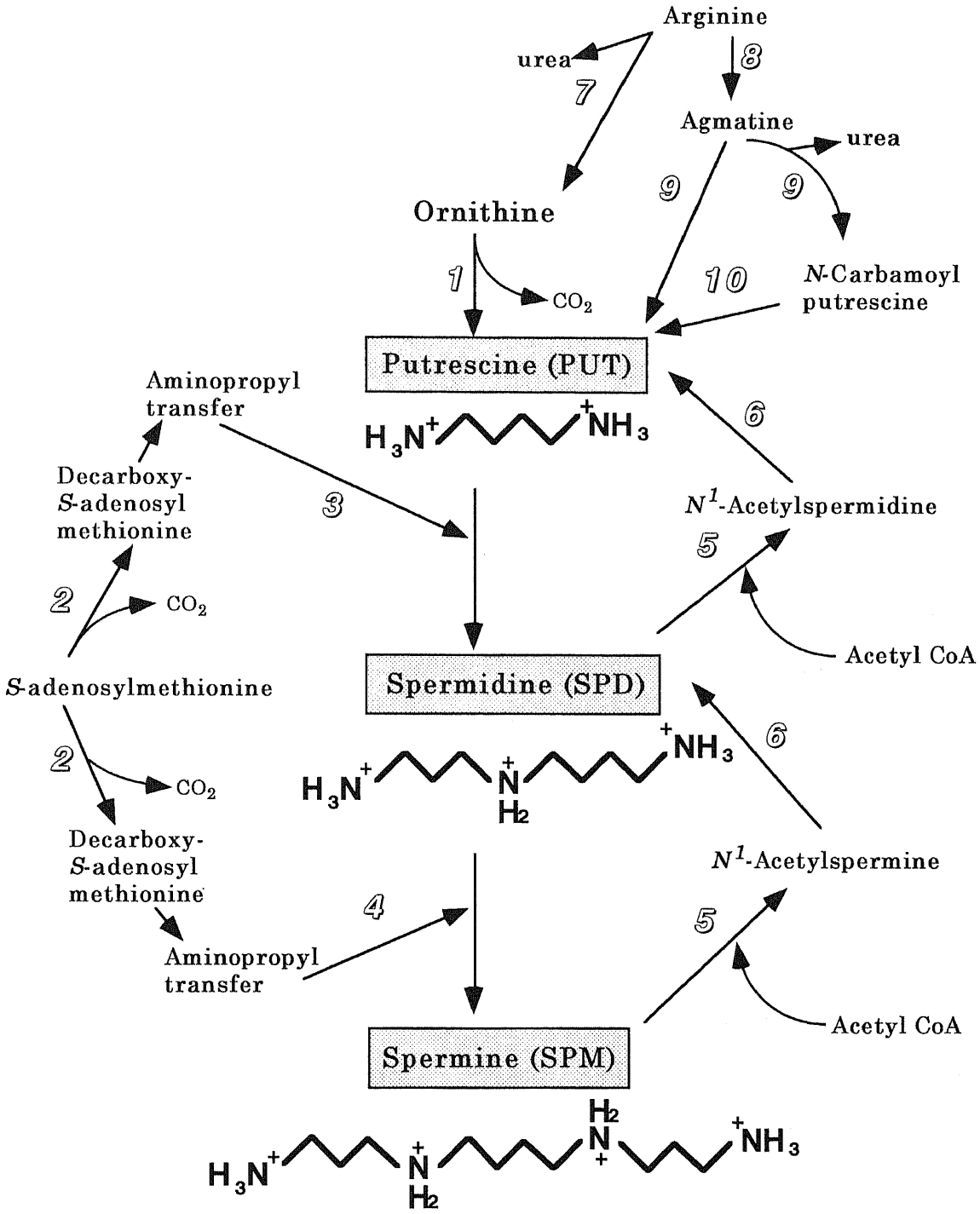

Fig. 1. General pathway for polyamine biosynthesis.
The enzymes involved are: *1*, ornithine decarboxylase (ODC); *2*, *S*-adenosylmethionine decarboxylase (SAMDC); *3*, spermidine synthase; *4*, spermine synthase; *5*, spermidine/spermine N^1-acetyltransferase; *6*, polyamine oxidase; *7*, arginase; *8*, arginine decarboxylase; *9*, agmatine deiminase; *10*, *N*-carbamoylputrescine amidase.

aminopropyl-transferase. Then, the addition of a second aminopropyl moiety to spermidine, catalyzed by a different aminopropyltransferase termed spermine synthase, forms spermine (9). The source of the aminopropyl group also comes from decarboxylated *S*-adenosyl-methionine. The synthesis of spermidine and spermine depends on the availability of the aminopropyl donor, hence *S*-adenosylmethionine decarboxylase (SAMDC) is also rate-limiting in polyamine biosynthesis.

The aminopropyltransferase reactions which form spermidine and spermine are effectively irreversible but these polyamines can be converted back into putrescine by the combined actions of two enzymes, spermidine/ spermine-N^1-acetyltransferase (N^1-SAT) and polyamine oxidase (Fig.1). N^1-SAT catalyzes the conversion of spermine into N^1-acetylspermine which is then degraded by polyamine oxidase to form spermidine and 3-acetamidopropanal. Similarly, spermidine is a substrate for N^1-SAT to form putrescine and 3-acetamidopropanal. Tissue polyamine oxidase activity is usually sufficient to ensure that intracellular level of N^1-acetylspermine are below the limits of detection, thus the activity of N^1-SAT regulates the rate of polyamine degradation. N^1-SAT is ubiquitous in mammalian cells but not present in plants (10). A similar enzyme has been found in *E. coli* (2). The acetylpolyamine pathway permits cells to adjust polyamine levels and to dispose of excess spermidine and spermine (5).

Therefore, there are three key enzymes that regulate polyamine biosynthesis : ODC, SAMDC and N^1-SAT. Besides the biosynthesis and degradation, intracellular polyamine level is also regulated by the

uptake system. Polyamine-specific transport systems are distributed widely in a variety of living cells from animal cells to bacteria (11). They are not only activated by cellular responses to proliferative stimuli (12), but are also controlled by several factors including polyamines themselves (13).

Polyamine have been implicated in numerous biological reactions. For instance, interaction with nucleic acids and phospholipids (14-15). Polyamines promote the aggregation of ribosome subunits and protect against their dissociation (16-19), involve in protein synthesis at either transcriptional or translational stages (20), stabilize membranes (22,23). Recently, it has been found that polyamines have important roles in neurophysiology (24-26).

Hence, it is clear that polyamines are essential for normal growth. The studies in the author's laboratory were found that a decrease in polyamine content of *E. coli* caused a decrease in the rate of cell proliferation and protein synthesis (17,27,28). Furthermore, over-accumulation of polyamines inhibited protein synthesis and cell growth (29,30) strongly indicating that an optimal concentration of polyamines was necessary for cell growth process.

Bacterial *E. coli* cells in the natural environment, they hardly encounter the condition that permit period of exponential (logarithmic) phase. Rather, bacterial growth is characterized by long periods of nutritional deprivation punctuated by short periods that allow fast growth (31). Experimentally, upon depletion of essential nutrients from medium, the growth rate of bacteria culture slow down and

eventually reaches zero. At this point the culture has entered, defined as stationary phase (32). In this stage of growth, bacteria can remain viable during prolonged periods of starvation and exit stationary phase and return to exponential phase cell cycle when starvation is relieved. Thus, when bacteria enter the stationary phase their response involved drastic changes in cellular physiology and morphology. *E. coli* become smaller, develop a spherical rather than a rod-shape morphology. Their cytoplasm is condensed whereas the volume of periplasm increases, membrane and cell envelope composition are altered, and the nucleoid condenses. Stationary phase cells are resistant to multiple stresses and survive long-term starvation. The synthesis of a set of stationary phase or postexponential proteins is induced, which has a role in stress protection. All of this phenotypes are dependent on an intact *rpoS* allele (33). The *rpoS* -encoded sigma factor, σ^{s} (σ^{38}), has been identified as a central regulator for many stationary-phase-responsive genes. Some stationary phase-specific genes are not dependent on *rpoS* (Table 1). *rpoS* seems to require guanosine 3',5'-bispyrophosphate (ppGpp) for the expression (34).

The cellular level of ppGpp increases in response to starvation for amino acids and sources of carbon, nitrogen and phosphate. The accumulation of ppGpp depends on activities of the products of two genes, *relA* and *spoT*. During amino acid starvation, the ppGpp synthetic activity of RelA protein is activated by uncharged tRNA. The accumulation of ppGpp in response to energy source starvation occurs

Table 1 Some stationary-phase-activated gene in *E. coli* and *S. typhimurium* (34)

Gene or operon	Genetic map position (min)	Gene product (s)	Physiological function
Regulated by σ^s			
ftsQAZ	2.3	Cell division proteins	Septum formation
bolA	10.0	Regulatory protein	Morphogene ; controls synthesis of PBP6[a]
appY	13.0	Regulatory protein	Controls expression of hya and cyxAB-appA
dps (pexB)	18.0	DNA-binding protein	DNA protection, control of gene expression
treA (osmA)	26.0	Periplasmic trehalase	Growth on trehalose in high-osmolarity medium
osmB	28.0	Outer membrane lipoprotein	Cell aggregation
katE	37.8	Catalase HPII[b]	Protection against H_2O_2
proU (proVWX)	57.7	Glycine betaine and proline transport system	Osmoprotection
katG	89.2	Catalase HPI[b]	Protection against H_2O_2
Not regulated by σ^s			
rmf	21.8	Ribosome modulation factor	Dimerization of ribosomes
hns (osmZ)	27.6	Histone-like protein (H-NS or H1)	Global gene regulation and chromosome organization
rpoS	58.9	σ subunit of RNA polymerase (σ^s)	Control of stationary-phase-induced and osmoregulated genes
sspAB	69.5	Stringent starvation protein (SspA)	Global gene regulation
rpoH	76.4	σ subunit of RNA polymerase (σ^{32})	Control of heat shock genes

[a] PBP6, penicillin-binding protein 6
[b] HPI and HPII, hydroperoxidase I and II

by blocking degradation of ppGpp catalyzed by a ppGpp 3'-pyrophosphohydrolase encode by the *spoT* gene (35). ppGpp thus appears to be a general starvation signal and it was found that ppGpp has a major regulation role in the activation of σ^s synthesis occurring early in the transition from exponential growth to stationary phase (36).

In this present study, an interest in how polyamine accumulation affects cell death during stationary phase arises because polyamines are now considered essential on cellular growth processes. Many researchers have examined numerous polyamine biosynthetic inhibitors in clinical use, especially in cancer chemoprevention (37). However, the precise mechanisms at molecular level have not been clearly known yet.

To gain understanding of the mechanism of polyamine accumulation on cell death, the study was employed the bacterial *E. coli* system as a model. Although, bacteria differ from fungi and mammals in the metabolism of polyamines, indeed, resemble plants in some respects (5). The study of polyamine accumulation in bacteria could provide some valuable information that may help to understand the mechanism in other cellular systems.

As the accumulation of spermidine in *E. coli speG* mutant caused a decrease in cell viability at the stationary phase of growth, the synthesis of some proteins was remarkably inhibited, i.e. ribosome modulation factor (RMF) and outer membrane protein C (OmpC) (30). It is of interest to perceive whether cell death would occur when *E. coli* cells lack these two proteins. Therefore, the relationship between cell

death and the deficiency of RMF and OmpC was investigated by using *rmf* and/or *ompC* mutant as described in Part I of this study.

In addition, Igarashi *et al.*(38) found that the synthesis of ppGpp is stimulated by spermidine, and ppGpp has been known to be a key regulator of σ^s factor (36) which has an important role for cell survival during stationary phase (33). To further clarify the mechanism by which spermidine accumulation caused cell death at the stationary phase, the promissing factors which is stimulated by spermidine, i.e. ppGpp was also examined in Part II. of this study.

PART I

Enhancement of Cell Death Due to Decrease in Mg^{2+} Uptake by OmpC (Cation-Selective Porin) Deficiency in RMF (Ribosome Modulation Factor)-Deficient Mutant

During the transition from the exponential to the stationary growth phase of *E. coli*, 70S ribosomes are converted to 100S dimers (39). A small basic protein of 55 amino acids, ribosome modulation factor (RMF), is uniquely associated with 100S dimers, and the RMF mRNA begins to appear when *E. coli* cells enter the stationary phase. RMF has been found to convert 70S ribosomes into the more stable protease- and nuclease-resistant 100S dimers *in vitro*, and to inhibit protein synthesis (40). It is possible that 100S dimers are involved in the selective translation of stationary phase specific mRNAs, although this has yet to be determined. When the *rmf* gene was disrupted, cell viability of the *rmf* mutant decreased at the stationary phase, suggesting that the dimerization of ribosomes is essential for viability at this phase (41).

Fukuchi *et al.* (42) isolated *speG* gene coding for spermidine acetyltransferase (SAT) which catalyzed the first step of polyamine degradation in *E. coli* and purified SAT enzyme to help understanding of the characteristic of *E. coli* polyamine degradation. Moreover, to understand the physiological function of SAT in *E. coli*, the study was done in *speG* mutant and the cloned *speG* gene (30). In a *speG* mutant, it was found that addition of spermidine to the medium caused a

decrease in cell viability at the late stationary phase due to accumulation of spermidine. The accumulation of spermidine caused a decrease in protein synthesis but not in DNA and RNA synthesis at the stationary phase. The synthesis of several kinds of proteins was particularly inhibited. They included RMF and OmpC protein, a cation selective porin protein in the outer membrane (43).

To determine whether there would be the relationship between cell death and the deficiency of RMF and OmpC, the study was done by making *rmf* and/or *ompC* mutants and examining the cell viability during stationary phase of growth. The profile of ribosomes and outer membrane protein were investigated. Cellular contents of cations, and polyamines were also determined.

Materials and Methods

Bacterial strains and culture conditions

E. coli C600 (*supE44 hsdR thi thr leu lacY1 tonA21*) was grown in a modified LB medium (0.8 g tryptone, 0.4 g yeast extract, 0.5 g NaCl per liter) (44). The plasmid pT3101 containing *rmf::Cm* was prepared as described (41). The plasmid pMAN006 containing *ompC* (45) was kindly provided by Dr. T. Mizuno (Nagoya University), and the *Hind*III-*Sal*I fragment was inserted into the same restriction site of pBluescript SK^+ (Stratagene) (pBS*ompC*). After the pBS*ompC* was digested with *Bgl*II, the Km^r gene (1.3 kb *Bam*HI fragment) of pUC4K (Pharmacia LKB Biotechnology Inc.) was inserted into the cut site

(pBS*ompC::Km*). *E. coli* C600 *rmf::Cm*, *E. coli* C600 *ompC::Km*, and *E. coli* C600 *rmf::Cm ompC::Km* were prepared by P1*kc* transduction as described previously (46). Chloramphenicol (30 μg/ml) and/or kanamycin (50 μg/ml) were added to the medium during the culture of the mutants. Cell growth was followed by measuring absorbance at 540 nm. Cell viability was determined by counting colony numbers grown on LB-containing 1.5% agar plates at 37 °C (44).

Western blot analysis

Antibody for RMF was made by injecting 1 mg RMF with Freund's complete adjuvant to a rabbit. Rabbit antibodies for OmpC and OmpF were kindly supplied by Dr. H. Mori (Kyoto University). Western blotting was performed by the method of Nielsen *et al.* (47).

Preparation of outer membrane proteins and their separation on 8M urea-SDS-polyacrylamide gel electrophoresis

The outer membrane proteins were analyzed according to the method of Matsuyama *et al.* (45). *E. coli* cells were disrupted by sonication. After centrifugation at 1,500 x g for 10 min to remove unbroken cells, cell envelopes were suspended in 2% Triton X-100-10 mM Na-phosphate buffer, pH 7.2, and incubated for 37 °C for 15 min. The insoluble fractions were recovered by centrifugation at 100,000 x g for 30 min. They were washed once with 2 ml of 10 mM Na-phosphate buffer, pH 7.2, and used as the outer membrane proteins. The

proteins (3 μg) were preheated in 1% SDS-1% 2-mercaptoethanol at 100 °C for 5 min and analyzed on 8M urea-SDS-polyacrylamide gel electrophoresis. OmpC, OmpF and OmpA were stained with Coomassie Brilliant Blue R250.

Measurement of protein synthesis in *E. coli*

At 24 h after the onset of cell growth, cell suspension (2.5 ml) was labeled with 0.37 MBq of [^{35}S]methionine (10 mM) in M9 medium (44). At designated time, hot trichloroacetic acid insoluble radioactivity was counted using 0.6 ml aliquots for measurement of [^{35}S]methionine incorporated into protein.

Sucrose density gradient centrifugation of ribosomes

The 30,000 x g supernatant (1 ml) was prepared as described previously (48) using 20 ml cell suspension. Briefly, after harvesting the cell, cells were suspended in 1 ml of the buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 100 mM ammonium acetate and 6 mM 2-mercaptoethanol and 2 μg/ml of DNaseI. Cells were then broken by French Press at the pressure of 20,000 and centrifuged at 30,000 x g. The 30,000 x g supernatant (0.25 ml) was loaded on a 5 ml linear sucrose gradient (5-20%) containing 20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, and 100 mM ammonium acetate. The tubes were centrifuged for 80 min at 40,000 rpm in a Hitachi P55ST2. The sucrose gradients were fractionated into 31-32 tubes. Absorbance at 260 nm was measured after 4-fold dilution of each

fraction.

Measurement of protein, RNA, polyamines, ATP and cations

E. coli cells were harvested either at the logarithmic phase of growth (A_{540} = 0.5) or at 24 h after the onset of cell growth. Protein was determined by the method of Lowry *et al.* (49). RNA was measured by the method of Ceriotti (50) with some modification. RNA solution was incubated in 0.3 N KOH for 18 h at 37 °C, then RNA reaction with 10% orcinol, $FeCl_3$-HCL (0.75%) was performed at 100 °C for 20 min after addition of HCl (final concentration of 0.3 N) and perchloric acid (final concentration of 6%). The formed coloring solution was read on a Hitachi U-1100 Spectrophotometer at 670 nm against a blank treated in the same manner. For measurement of polyamines, ATP and cations, cell suspension (10 ml) was overlaid on a mixture of 0.9 ml of silicone oil (Toray SH-550) and 0.15 ml of mineral oil (Sigma light white oil) in a 15-ml centrifuge tube, and the cells were immediately centrifuged through the oil at 1,500 x g for 20 min (51). After the supernatant and oil were removed stepwise, the pellets were suspended in 0.5 ml of 5% trichloroacetic acid. The resulting was centrifuged at 6,000 x g for 5 min. The supernatant thus obtained was used for measurement of polyamines, ATP and cations.

Polyamine levels were determined by high pressure liquid chromato-graphy as described by Igarashi *et al.* (52) using a TSK gel IEX215 column (4 x 80 mm) heated to 50 °C was mounted. The flow

rate of the buffer (0.35 M citric acid buffer [pH 5.35], 2 M NaCl, 20% methanol) was 0.42 ml/min. Detection of polyamines was by fluorescence intensity after reaction of the column effluent at 50 °C with an *o*-phthaladehyde solution containing 0.06% *o*-phthaldehyde, 0.4 M boric acid (pH 10.4), 0.1% Brij 35, and 37 mM 2-mercaptoethanol. The flow rate of *o*-phthaladehyde solution was 0.4 ml/min, and fluorescence was measured at an excitation wavelength of 336 nm and emission wavelength of 470 nm. The retention times for putrescine, spermidine and spermine were 6, 12, and 24 min, respectively.

ATP was determined as described by Kimmich *et al* (53). The 100 μl-aliquot of 5% trichloroacetic acid extracted samples was immediately neutralized with 50 mM K_2HPO_4 - 1 M KOH. The ATP in samples was measured by reacting with luciferin-luciferase (Sigma Chemical Co.) in the assay buffer containing 5 mM sodium arsenate, 4 mM $MgSO_4$ and 20 mM sodium glycylglycine against ATP standard. The luminescence was determined by TD-20/20 Luminometer (Turner Designs, CA.)

Mg^{2+} and K^+ were analyzed by means of atomic absorption spectroscopy (Hitachi Z-8000).

Mg^{2+} uptake by intact cells

E. coli cells were cultured in M9 medium supplemented with 10 μM Mg^{2+} for 24 h to deplete the Mg^{2+} content in *E. coli* cells. After harvesting the cells, Mg^{2+} uptake was measured at 30 °C in M9 medium supplemented with 50 μM Mg^{2+}. The Mg^{2+} content in *E. coli* cells was

measured as described above.

Results

Enhancement of cell death by OmpC deficiency in RMF-deficient mutant

To study whether OmpC deficiency enhances cell death caused by RMF deficiency, OmpC-deficient (*ompC*) and RMF-deficient (*rmf*) mutants as well as a mutant deficient in both OmpC and RMF (*rmf ompC*) were made by using *E. coli* C600 as the parent strain. The deficiency of OmpC and RMF was confirmed by Western blot analysis (Fig. 2A). Cell viability did not change in OmpC-deficient cells, but cell viability was reduced in RMF-deficient cells as reported previously (41). There was a further decrease in cell viability in the double *rmf ompC* mutant (Fig. 2B).

Correlation between the decrease in cell viability and the decrease in protein synthesis

Macromolecular synthetic activity of the mutants was measured at 24 h after the onset of cell growth. Protein synthetic activity of the double *rmf ompC* mutant was greatly decreased compared to the parent strain (see Fig. 5) or the single *ompC* or *rmf* mutants. DNA and RNA synthetic activities of the double mutant were not decreased significantly. The ribosome pattern was then analyzed by sucrose gradient centrifugation (Fig. 3). Using gradients with an equal

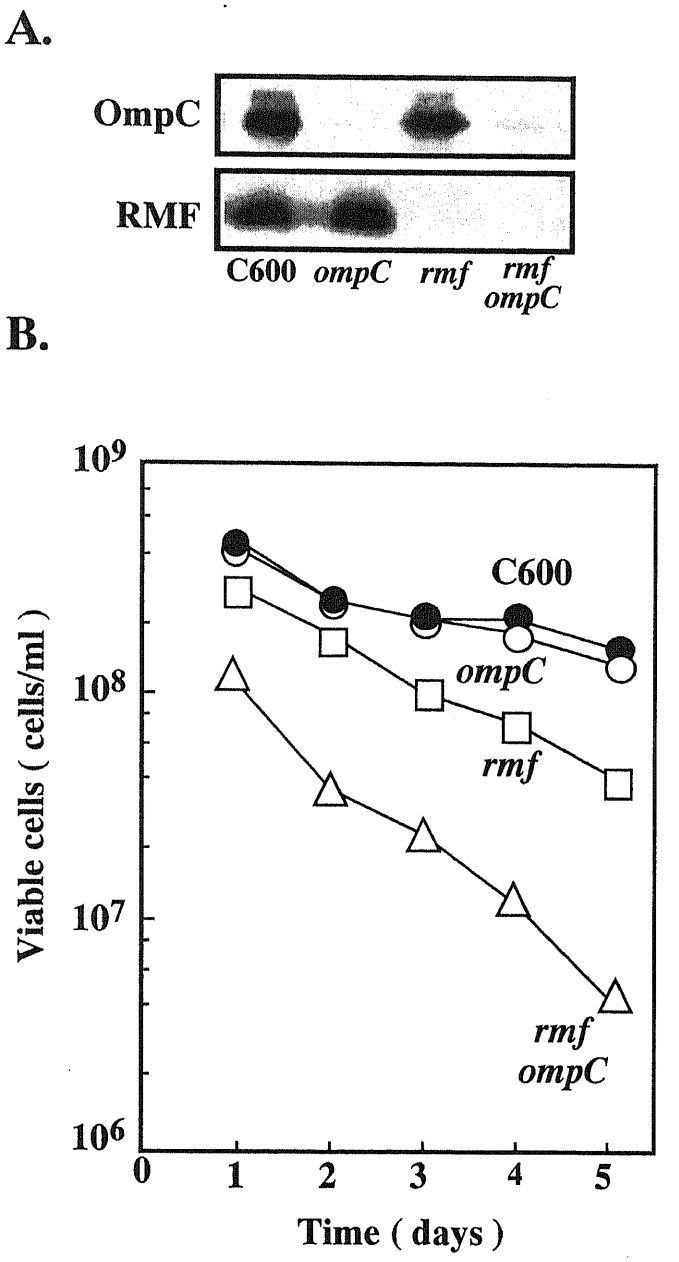

Fig. 2. Cell viability of various mutants. A. Western blot analysis of OmpC and RMF. Western blot analysis was performed using a cell lysate (5 μg protein for OmpC and 30 μg protein for RMF) of *E. coli* cultured for 24 h. B. Cell viability. Each value is the average of duplicate determinations. ●, *E. coli* C600; ○, *ompC* mutant; □, *rmf* mutant; △, double *rmf ompC* mutant.

Table 2 Amount of protein, RNA and outer membrane protein (OMP) in various mutants cultured for 24 h

Strain	Protein[a] (mg)	RNA[a] (mg)	OMP (mg)
C600	4.61± 0.45[b]	1.50± 0.19	0.29± 0.06
ompC	4.01± 0.58	1.54± 0.14	0.20± 0.02
rmf	4.54± 0.60	1.36± 0.08	0.26± 0.06
rmf ompC	4.06± 0.57	0.58± 0.09	0.15± 0.05

[a] The amount in 10 ml culture

[b] Values are mean±S.D. of triplicate determination

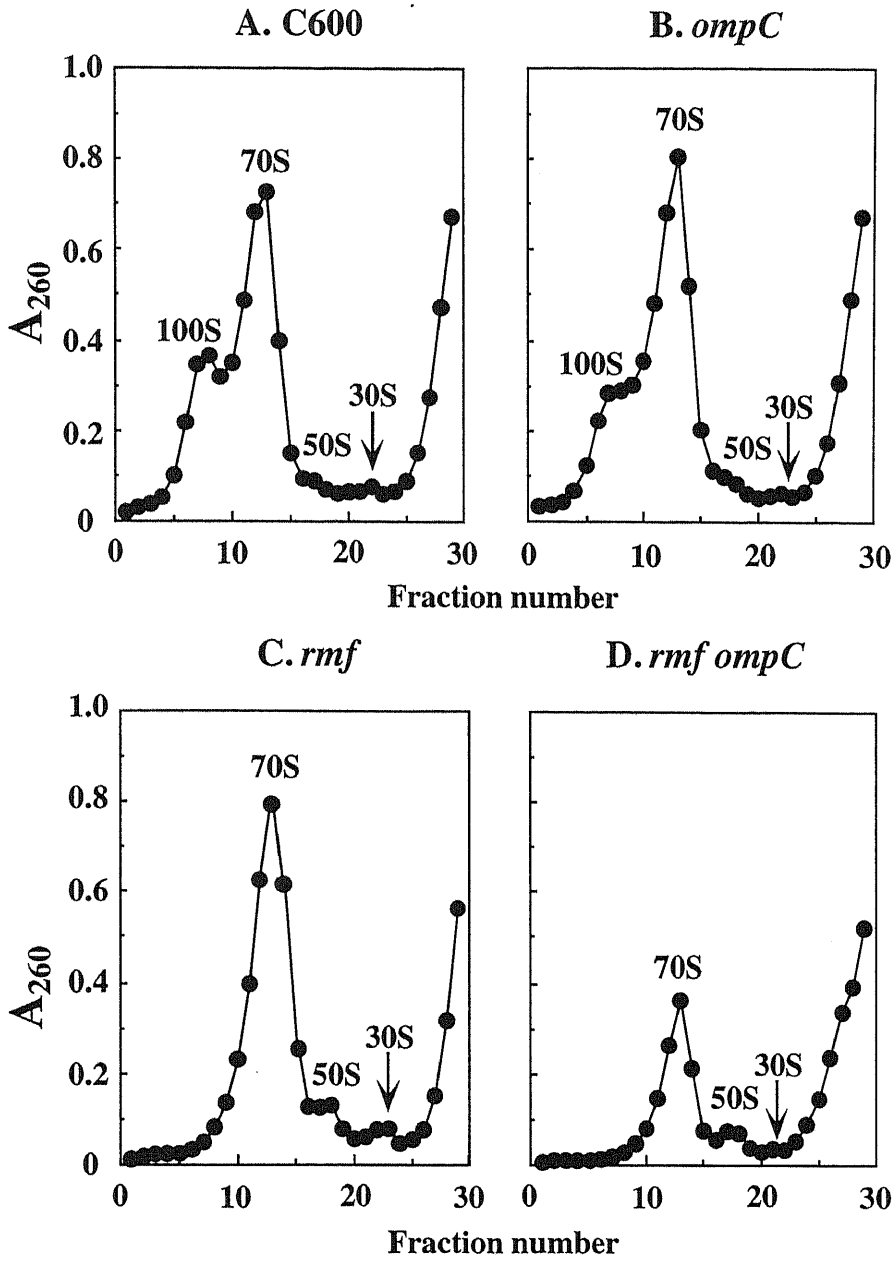

Fig. 3. Sucrose density gradient centrifugation of ribosomes obtained from various mutants. *E. coli* cells (A. C600; B. *ompC* mutant; C. *rmf* mutant; D. double *rmf ompC* mutant) were harvested at 24 h after the onset of cell growth, and sucrose density gradient centrifugation was performed using 0.25 ml of the 30,000 x g supernatant.

volume of cell lysate from each strain, 100S ribosomes were observed in the parent strain and the *ompC* mutant but not in the *rmf* mutant or the double *rmf ompC* mutant. Moreover, the amount of ribosomes (100S, 70S, 50S and 30S) in the double mutant (Fig. 3D) was lower than that in the parent strain and the single mutants (Figs. 3A, 3B and 3C). The total amount of RNA and protein in the cell lysate from the mutants were also measured. As shown in Table 2, the amount of RNA in the double *rmf ompC* mutant was much lower than that in the parent strain and the single mutants. However, the amount of protein in the double mutant was not altered compared to the parent strain and the single mutants. The ATP level was also measured and found that there was unchanged in the mutants. The results suggested that the decrease in protein synthetic activity of the double mutant at 24 h after the onset of cell growth is mainly due to a decrease in the amount of ribosomes.

Recovery of cell viability by Mg^{2+}

Since OmpC is a cation-selective porin, the levels of Mg^{2+}, K^+, and polyamines, which are necessary for the activity and stabilization of ribosomes, were measured in cells at the logarithmic and the late stationary phases. Upon entry to the late stationary phase, the K^+ content decreased markedly for all the strains examined, but at the late stationary phase, the K^+ content in the double *rmf ompC* mutant was higher than that in the parent strain and the single mutants (Fig. 4). In contrast, the cellular Mg^{2+} and polyamine content in the double *rmf ompC* mutant was lower than that in the parent strain and the single

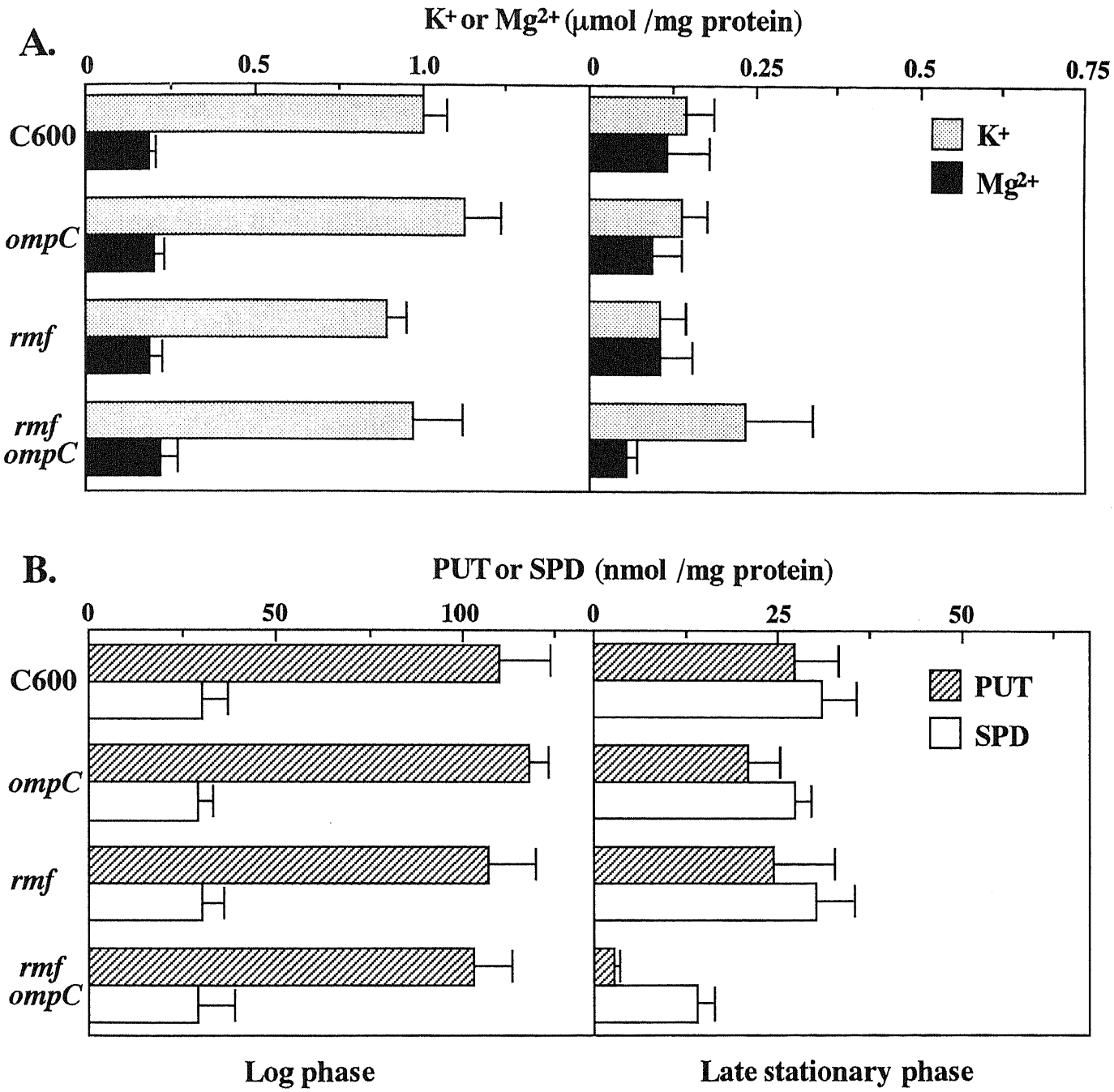

Fig. 4. Contents of K^+ and Mg^{2+} (A) and polyamines (B) in various mutants. Cells were harvested at $A_{540} = 0.5$ (log phase) or at 24 h after the onset of cell growth (late stationary phase). Each value is mean ± S. D. of triplicate determinations.

mutants at the late stationary phase (Fig. 4).

Because a decrease in Mg^{2+} and polyamines in the double *rmf ompC* mutant was observed, Mg^{2+}, spermidine, or putrescine was added to the medium to determine if cell viability would recover. Viability of the double mutant was not recovered by the addition of 10 to 30 mM polyamines. When 30 mM Mg^{2+} was added to the medium, viability of the double *rmf ompC* mutant was increased (Fig. 5A). In the presence of 30 mM Mg^{2+}, there were more viable *rmf ompC* cells over 1 to 5 days, with about a 10-fold increase on day 5, compared with cells grown in the absence of Mg^{2+}. The Mg^{2+} content in the double mutant cultured with 30 mM Mg^{2+} increased by 2-fold on day 1, and was nearly equal to the Mg^{2+} content in the parent strain cultured in the presence or absence of 30 mM Mg^{2+}.

Protein synthetic activity was then measured using cells cultured for 24 h. The activity was restored by the addition of 30 mM Mg^{2+} and was nearly equal to that of the parent strain (Fig. 5B). The amount of RNA also increased after culturing the double mutant with 30 mM Mg^{2+}, suggesting reduction in the degradation of ribosomes. Moreover, an increase in the amount of 70S ribosomes was also observed in cells grown in the presence of 30 mM Mg^{2+}.

Decrease in OmpF in the double *rmf ompC* mutant

Next, to determine whether RMF influenced the synthesis of the cation-selective porin proteins the 4 strains were cultured and harvested at 24 h after the onset of cell growth. The outer membrane

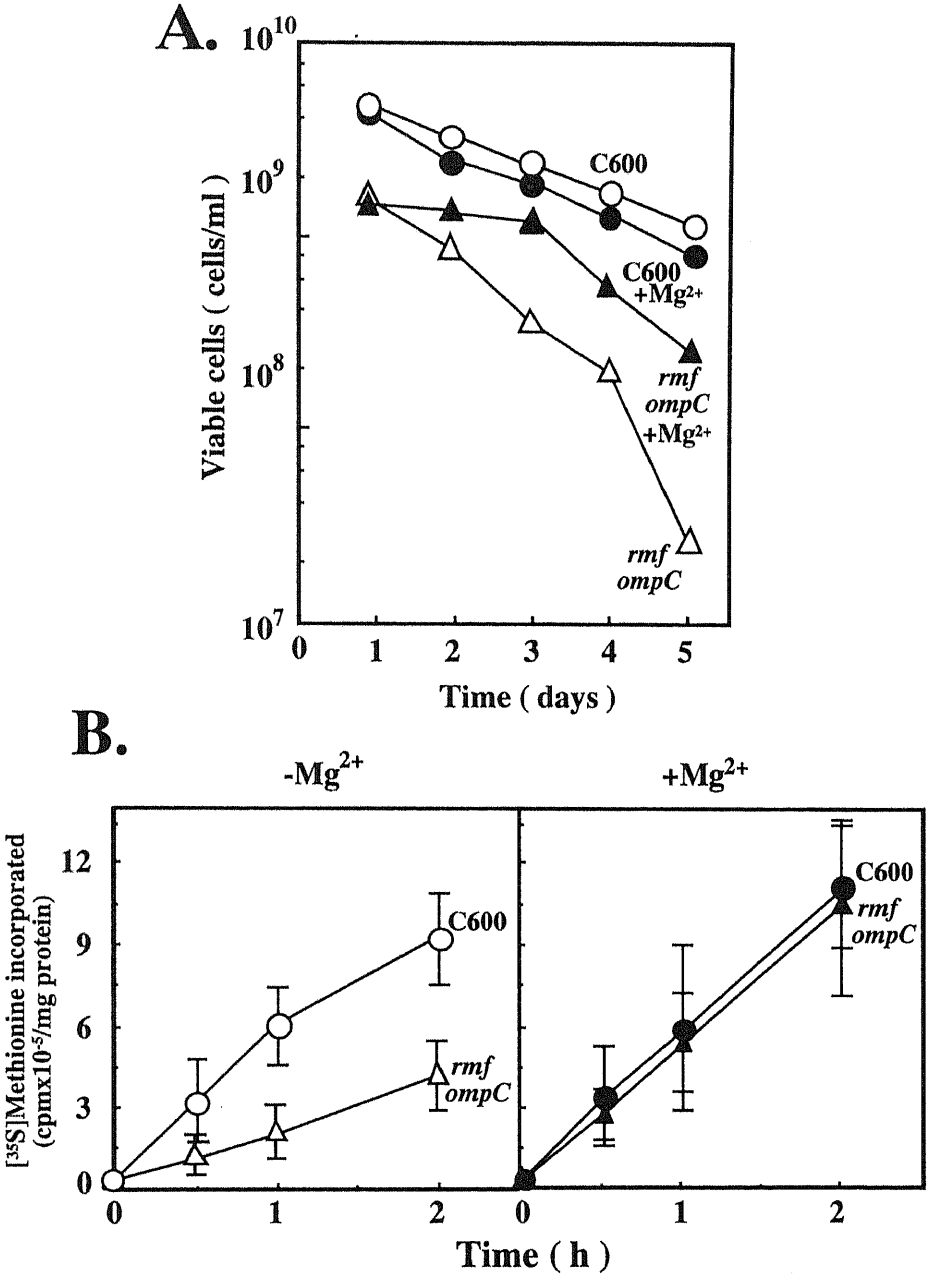

Fig. 5. Recovery of cell viability (A) and protein synthesis (B) by Mg^{2+}. ○ and △, *E. coli* C600 and the double mutant cultured in the modified LB medium; ● and ▲, *E. coli* C600 and the double mutant cultured in the modified LB medium containing 30 mM Mg^{2+}. A. Each value is the average of duplicate determinations. B. Protein synthetic activities are shown as mean ± S. D. of triplicate determinations.

protein pattern was determined by 8 M urea SDS-PAGE, as shown in Fig. 6. The parent strain synthesized more OmpF than OmpC at the late stationary phase of growth. The *ompC* mutant synthesized only OmpF, but the *rmf* mutant mainly synthesized OmpC at the late stationary phase. In the double *rmf ompC* mutant, which lacks OmpC, the synthesis of OmpF was also strongly inhibited. Furthermore, the total amount of outer membrane proteins in the double mutant was less than that in the parent strain and the single mutants (Table 2). These results suggested that ribosome-associated RMF may be involved in the recognition of several specific types of mRNA on ribosomes.

Decrease in Mg^{2+} uptake activity in the double *rmf ompC* mutant

The investigation was further done to examine whether Mg^{2+} transport was decreased in the double *rmf ompC* mutant, because OmpC and OmpF are constituents of the cation selective porins (43) and the Mg^{2+} and putrescine content in the double mutant was greatly reduced compared to wild-type (Fig. 4). As shown in Fig. 7, Mg^{2+} transport activity in the double mutant was decreased compared to the activity in the parent strain and the single mutants. Similarly, putrescine transport was greatly decreased in the double mutant.

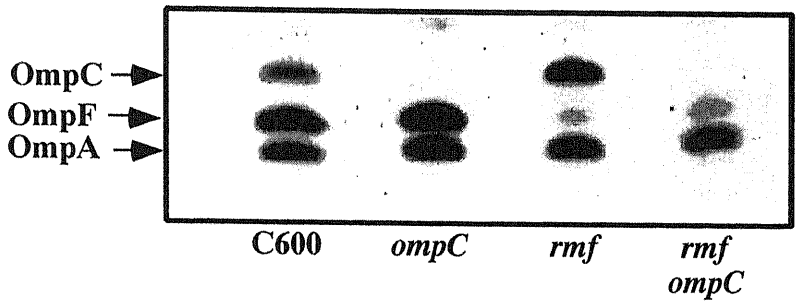

Fig. 6. Profiles of outer membrane proteins of various mutants. *E. coli* C600 and its mutants were harvested at 24 h after the onset of cell growth. Arrows indicate the positions of OmpC, OmpF and OmpA.

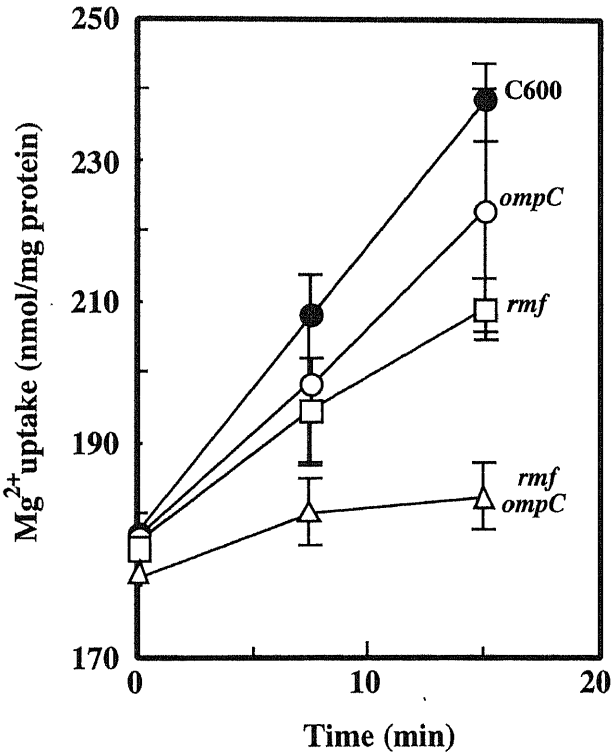

Fig. 7. Mg $^{2+}$uptake activity of various mutants. Data are shown as mean ± S. D. of triplicate determinations. ●, *E. coli* C600; ○, *ompC* mutant; □, *rmf* mutant; △, double *rmf ompC* mutant.

Discussion

The study how OmpC deficiency enhanced cell death caused by RMF deficiency was undertaken which could be expressed as shown in Fig. 8, and found that in a double *rmf ompC* mutant, a decrease in both Mg^{2+} and polyamine contents was observed. Moreover, a decrease in Mg^{2+}, but not polyamines, was the main reason for the increase in cell death caused by OmpC deficiency, since the addition of Mg^{2+} to the medium recovered cell viability. Addition of polyamines or K^+ did not recover cell viability, consistent with reports that those cations cannot maintain ribosome activity without Mg^{2+} (54, 55).

Mg^{2+} is known to be actively transported in *E. coli* (56). It was found that the level of OmpF was reduced in the *rmf ompC* double mutant. A reduced influx of Mg^{2+} from the outside into the periplasmic space through the OmpC and OmpF porins is probably responsible for the reduced transport of Mg^{2+}. It has been reported that a double *ompC ompF* mutant decreased cell viability, although the mechanism was not studied (57). The results suggested that the mechanism of decreased viability in the *ompC ompF* mutant involves a decrease in cellular Mg^{2+} content, similar to that seen in the *rmf ompC* mutant.

Although Mg^{2+}, K^+ and polyamines are necessary for maximal protein synthesis (48, 58), only Mg^{2+} is essential for the activity and stabilization of ribosomes (54, 55). RMF is also involved in the stabilization of ribosomes (30). Features common to both Mg^{2+} and to RMF include their ability to shift the equilibrium of ribosomes from 30S

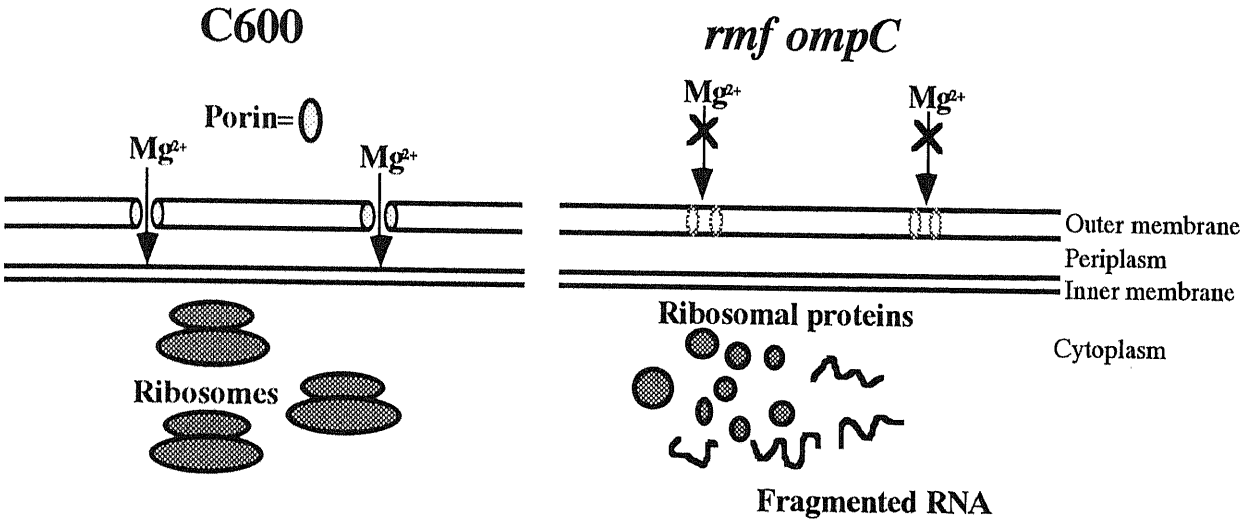

Fig. 8. Provisional model for the enhancement of cell death due to a decrease in Mg^{2+} uptake caused by RMF deficiency. In *E. coli* Mg^{2+} goes through outer membrane via cation-selective porins and Mg^{2+} is actively transported at the inner membrane to cytoplasm where Mg^{2+} is functionally to stabilize ribosomes. Moreover, at the stationary phase, RMF also protects ribosomes from the degradation. Thus, cells can maintain viable. In the double *rmf ompC* mutant, the level of OmpF was decreased. A reduced influx of Mg^{2+} from outside into the periplasm through OmpC and OmpF porins may reduce the transport of Mg^{2+}. Simultaneously, *rmf ompC* mutant lacks RMF to protect ribosome. The ribosomes are then degraded greatly in the consequence of cell death.

and 50S subunits to 70S ribosomes and to 100S dimers. Addition of Mg^{2+} to the RMF-deficient mutant caused a shift from 30S and 50S subunits to 70S ribosomes, and cell viability was partially recovered, presumably due to the enhanced stability of ribosomes. The results indicate that both RMF and Mg^{2+}, acting through stabilization of ribosomes, are important for cell viability at the stationary phase.

In addition to the effects of RMF on the stability of ribosomes, the result from this study was shown that the synthesis of some proteins such as OmpF was actually stimulated by RMF, although total protein synthesis was inhibited by RMF (40). Thus, it could be proposed that RMF modulates the synthesis of certain proteins at the translational level, which is a novel mechanism for the action of this modulatory factor. It is known that the synthesis of RNA polymerase σ^s is involved in cell viability at the late stationary phase (59-61). Thus, the synthesis of proteins that are important for cell viability in the late stationary phase may be regulated by both RNA polymerase σ^s and RMF.

PART II

Stimulation of ppGpp Synthesis by Polyamine Causes Cell Death

According to the effect of polyamine accumulation on the inhibition of RMF and OmpC proteins, results in a decrease in cell viability. The study was undertaken and revealed in Part I that the cell death could be enhanced by OmpC, the cation-selective porin, deficiency in RMF-deficient mutant via a decrease in Mg^{2+} uptake (62). It was clear that RMF is important for cell survival during the entry into the stationary phase. In addition, RMF may contribute the influence on many genes which function together to prolong the viability.

As mention above that the inhibition effect of polyamine accumulation on RMF and as well as OmpC was clarified. To further elucidate the mechanism of cell death in *E.coli* , another factor, i.e. ppGpp, was pointed. Since, ppGpp is a small molecule implicated in the regulation of large number of distinct physiological processes in *E. coli* (63, 64). Moreover, the participation of spermidine as a stimulator of ppGpp formation has been reported by Igarashi *et al* (38). However, the effect of spermidine accumulation on ppGpp synthesis has not been explored yet. Thus, the cell death caused by spermidine accumulation may be involved in an alteration of ppGpp level. In order to test this hypothesis, the investigations of Part II were carried out.

Materials and Methods

Bacterial strains, plasmids and culture conditions

E. coli CAG2242 (*speG supE44 hsdR thi thr leu lacY1 tonA21*) was grown in a modified LB medium (0.8 g tryptone, 0.4 g yeast extract, 0.5 g NaCl per liter) (44), supplemented with 1 mM sodium phosphate, pH 7.4. The plasmids pALS10 and pALS14 were kindly provided by Dr. M. Cashel (National Institute of Health, MD.). The plasmid pALS10 contains a full length *relA* gene clone under a conditional Ptac promoter, such that transcription is controlled by IPTG (isopropyl-1-thio-β-D-galactopyranoside) while pALS14 is a C-terminal truncated, inactive *relA* control (64). *E. coli* CAG2242 cells transformed with pALS10 or pALS14 were grown in the presence of ampicillin (100 μg/ml). Cell growth and viability were determined as described in Part. I.

Measurement of ^{32}P-labeled nucleotides

For the determination of the effect of spermidine accumulation on ppGpp formation, *E. coli* CAG2242 cells were cultured in the presence and absence of 2 mM or 4 mM spermidine, and uniformly labeled with [^{32}P]orthophosphoric acid at 100 μCi/ml. When the density of the culture was reached at A_{540} nm the range of 0.4-0.5 or at the indicated times, cells from the radioactive culture were collected and washed with MOPS (morpholinepropane-suffonic acid) medium (66), then extracted with cold 1 M formic acid for 30 min at 0 °C. After 30-min extraction period, the formic acid suspension was centrifuged

and the supernatant was added with the mixture of 400 mM sodium tungstate, 500 mM tetraethyammonium hydrochloride, and 500 mM procaine hydrochloride in the ratio 5:4:1 to precipitate the inorganic phosphate along with the acid-insoluble debris. After centrifugation, the supernatant extract was saved and the pellet was discarded. The supernatant was resolved by polyethylenimine (PEI)-cellulose (E. Merck) thin-layer chromatography developed with 1.5 M KH_2PO_4 (pH 3.4) (67). Nucleotides were quantified with Fujix Bas-2000II imaging system-Bastation software (Fuji Photo Film).

For the determination of the effect of the accumulated ppGpp on cell death, cultures of the transformants *E. coli* CAG2242 with pALS10 and pALS14 were labeled with [^{32}P]orthophosphoric acid at 100 μCi/ml. When cells entered the stationary phase (~6h after the onset of cell growth) , 1 mM IPTG was added to the cultures. At the designated times, nucleotide samples were extracted and quantified as described above. The cell growth and viability were followed in unlabeled parallel cultures.

Results

Decrease in cell viability due to the overaccumulation of spermidine

To ensure and confirm the effect of spermidine over-accumulation on cell viability, a SAT- deficient mutant, *E. coli* CAG2242 cells were grown in the presence and absence of 2 mM or

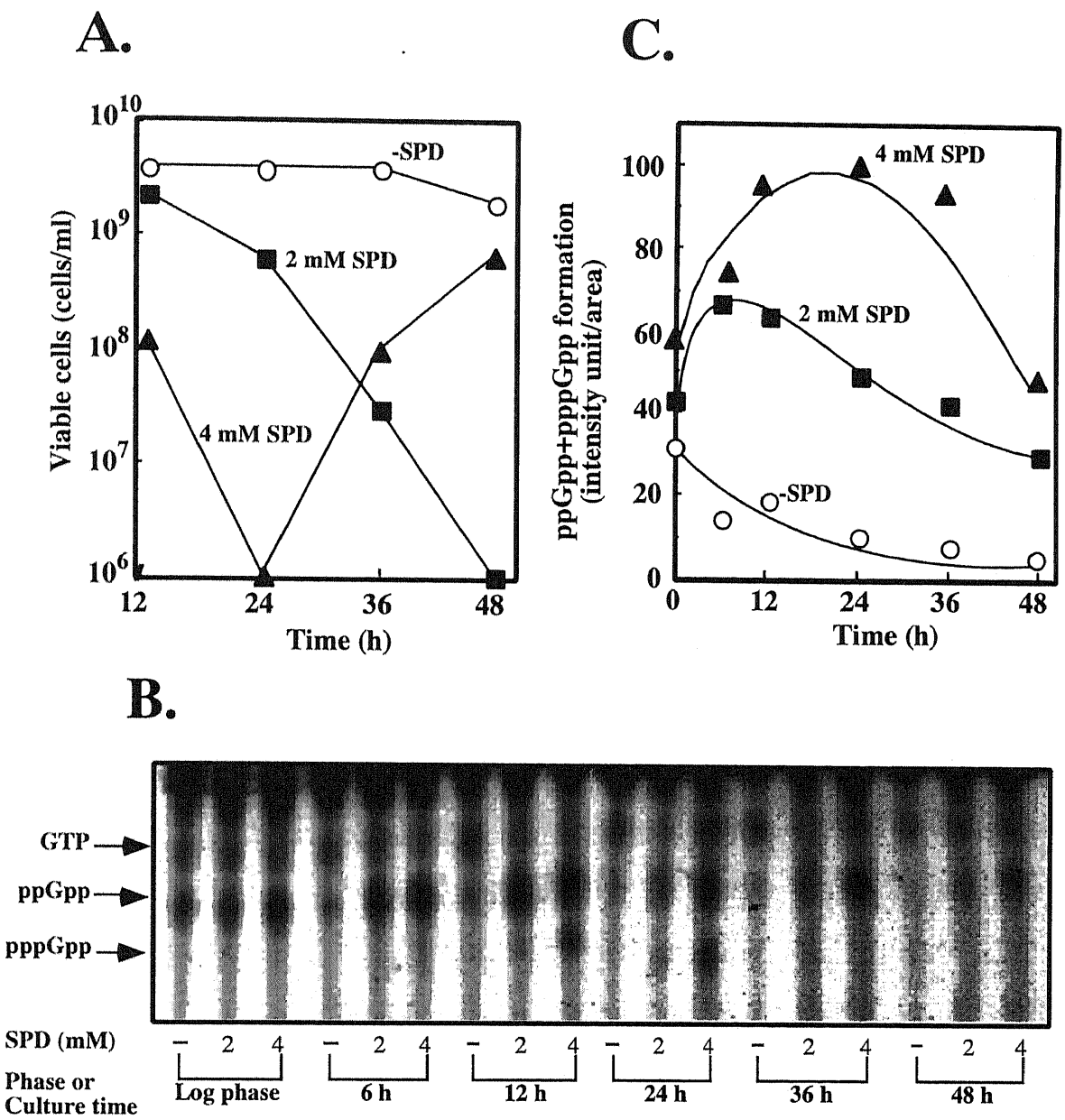

Fig. 9. Effect of spermidine on cell viability (A) and (p)ppGpp formation (B,C). *E. coli* CAG2242 grown in the presence and absence of 2 or 4 mM spermidine (SPD) were labeled with [^{32}P]orthophosphoric acid in modified LB medium. Cell viability (A) was followed in unlabeled parallel cultures. Cells were harvested at A_{540} = 0.5 (log phase) or at the times shown in the figure. The labeled nucleotides were analyzed by TLC (B), and quantified by densitometry. The ratio of pppGpp+ ppGpp formed were shown (C), which time = 0 was a result of nucleotides extracted from log phase cultures.

4 mM spermidine, which the concentration of spermidine was higher than that in the previous report (30). The cell growth of *E. coli* CAG2242 in the presence or absence of spermidine was nearly equal. Then, the effect of spermidine on cell viability was examined. As shown in Fig. 9A, the cells grown without spermidine were viable over 48 h of the observation time. But the viability of cells grown with spermidine either 2 mM or 4 mM was decreased, which was much drastically in cells grown with 4 mM spermidine. After 24 h of the onset of cell growth, the recovery of viability of cells grown in 4 mM spermidine was observed.

Next, polyamine contents in *E. coli* cells at the logarithmic and stationary phases were measured (Fig. 10). The cellular putrescine content (Fig.10A) at the logarithmic phase was higher than that at the stationary phase which was gradually decreased in cells grown either with or without spermidine. The putrescine content in cells cultured without spermidine was higher than cells cultured with spermidine. The spermidine content (Fig. 10B) in cells grown without spermidine at the logarithmic phase was not much higher than that at the stationary phase. Clearly, spermidine was overaccumulated in *E. coli* CAG2242 cells grown in the presence of spermidine, either 2 or 4 mM. After 24 h of the onset of cell growth where the recovery of viability was observed, correspondingly spermidine content in the cells grown in 4 mM spermidine was also decreased. The results revealed that the overaccumulation of spermidine caused a decrease in cell viability of *E. coli* cells, in the other word, spermidine overaccumulation caused

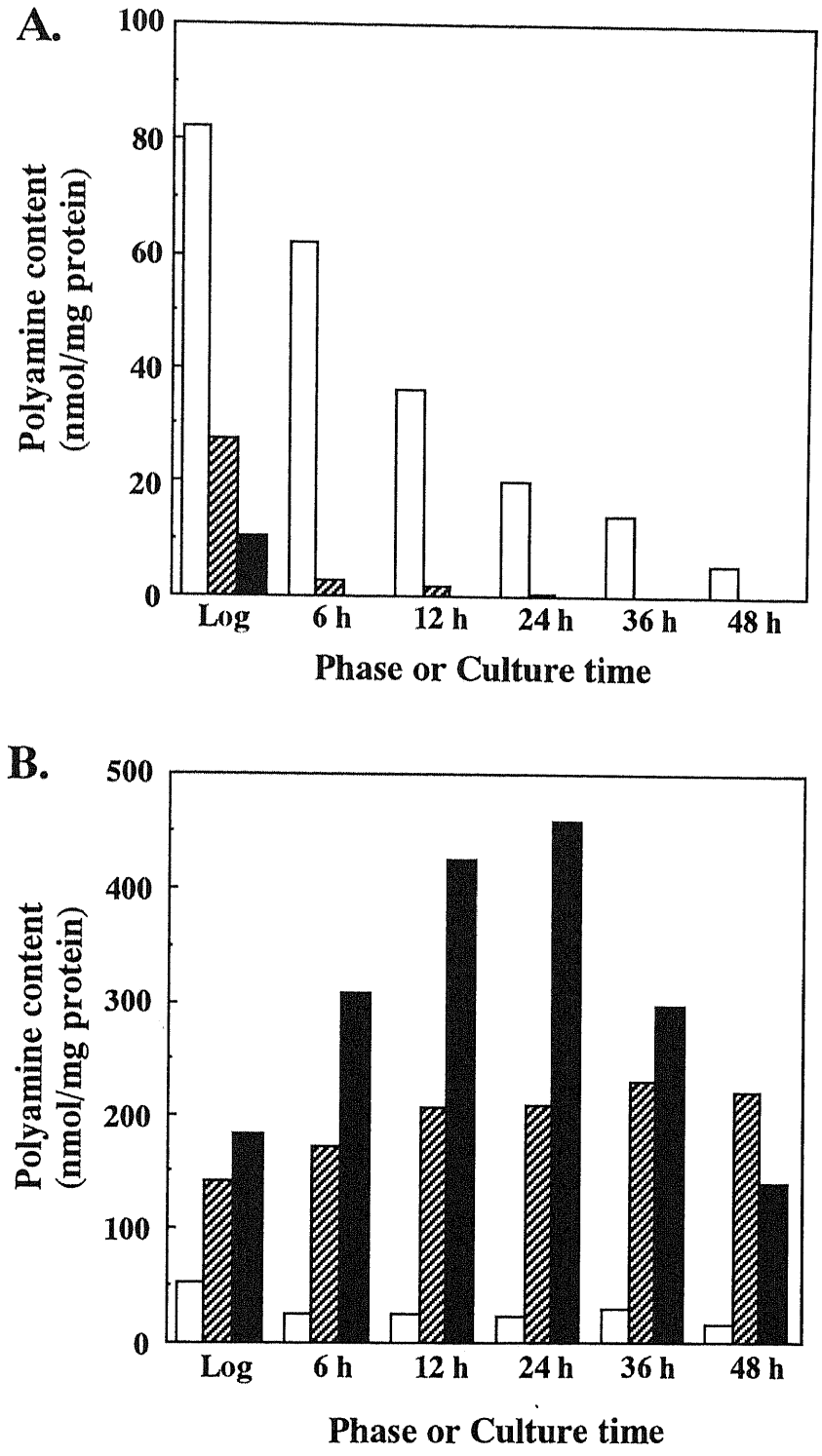

Fig. 10. Effect of spermidine on cellular putrescine (A) or spermidine (B) contents. *E. coli* CAG2242 grown in the absence (□) and presence of 2 mM (▨) or 4 mM (■) spermidine were harvested at the times shown in the figure and polyamines were analyzed by HPLC.

E. coli cell death. However, it has not been clarified the mechanism by which *E. coli* grown in the presence of 4 mM spermidine recovered the viability after 24 h of the onset of cell growth. It was also noticed that when the recovery of viability occurred, cellular spermidine was also decreased.

Correlation between the decrease in cell viability and the increase in ppGpp synthesis

To determine whether spermidine accumulation would cause ppGpp accumulation at either the logarithmic phase or stationary phase, *E. coli* CAG2242 grown in the presence and absence of 2 mM or 4 mM spermidine were labeled with [^{32}P]orthophosphoric acid. At the indicated times, a formic extract was prepared and nucleotides were separated by ascending chromatography on PEI-cellulose plates. The synthesis of (p)ppGpp at the logarithmic phase in cells grown in the presence and absence of spermidine was not different significantly (Fig. 9B and 9C). Upon the entry into the stationary phase, the synthesis of (p)ppGpp in cells grown without spermidine was decreased markedly compared to that in cells grown with spermidine. Furthermore, the increase in (p)ppGpp synthesis in cells grown with spermidine was observed, which more significantly in cells grown with 4 mM spermidine. The results suggested that spermidine accumulation leads to the stimulation of (p)ppGpp formation.

Because the accumulation of spermidine causes not only a decrease in cell viability but also an increase in (p)ppGpp synthesis.

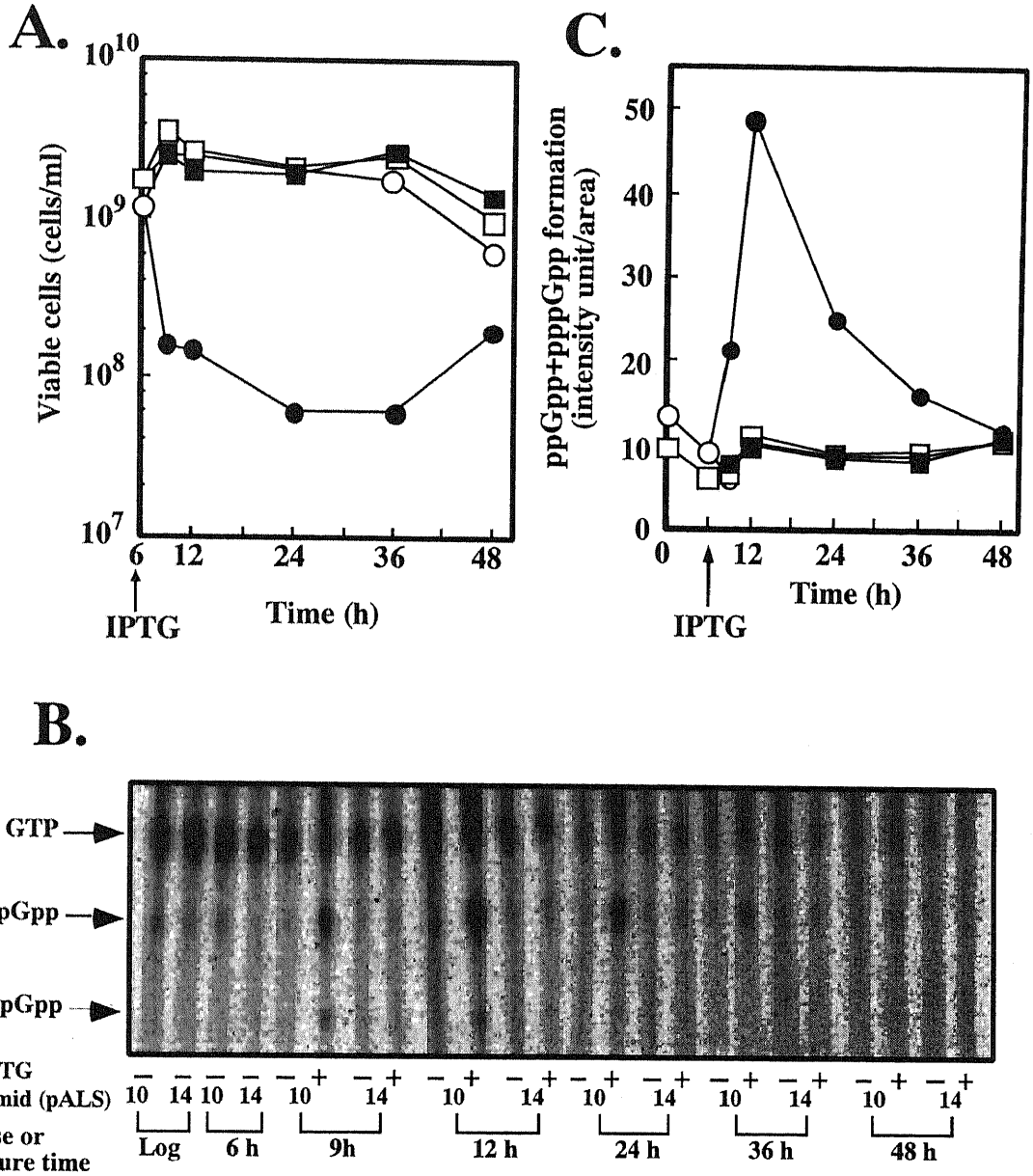

Fig. 11. Effect of ppGpp on cell viability at the stationary phase. Cultures of *E. coli* CAG2242 containing plasmid pALS10 (○) or pALS14 (□) were labeled with [^{32}P]orthophosphoric acid in modified LB medium. Cell viability (A) was followed in unlabeled parallel cultures. Cells were harvested at A_{540} = 0.5 (log phase) and 6 h after onset of growth. After entry into stationary phase (6 h), 1 mM IPTG was added (indicated with *arrow*) to cultures containing pALS10 (●) and pALS14 (■) and aliquots were removed at the times shown in the figure. The labeled nucleotides were analyzed by TLC (B), and quantified by densitometry. The ratio of pppGpp+ppGpp formed were shown (C), which time = 0 was a result of nucleotides extracted from log phase cultures.

Thus, the increase of ppGpp signaling at the stationary phase of growth may bring about the *E. coli* cell death. To determine whether the elevated synthesis of ppGpp at the stationary phase would be a cause of cell death, the investigation was done by using transformants *E. coli* CAG2242 with pALS10, containing the full length of *relA* gene clone under Ptac promoter, and pALS14, a control plasmid. To induce the synthesis of ppGpp at the stationary phase of growth, IPTG was added to the medium when cells entered the stationary phase. Then, the cell viability and ppGpp synthesis were measured. As shown in Fig. 11, the cell viability of *E. coli* CAG2242/pALS10 grown without IPTG was not decreased obviously and ppGpp was not accumulated. In contrast, by the addition of IPTG, the cell viability of *E. coli* CAG2242/pALS10 was decreased which correlated to the increase in ppGpp formation. The change in cell viability and ppGpp synthesis was not observed in *E. coli* CAG2242/pALS14. The results strongly indicated that an increase of ppGpp at the stationary phase causes cell death.

Decrease in stationary phase-essential proteins due to the elevated ppGpp formation caused by spermidine accumulation and is lethal

It has been reported that ppGpp is a positive regulator of σ^s synthesis (36). σ^s is a stationary phase-specific σ factor, product of *rpoS* gene. σ^s factor has been well known to be necessary for cell viability at the stationary phase (31, 33, 34).

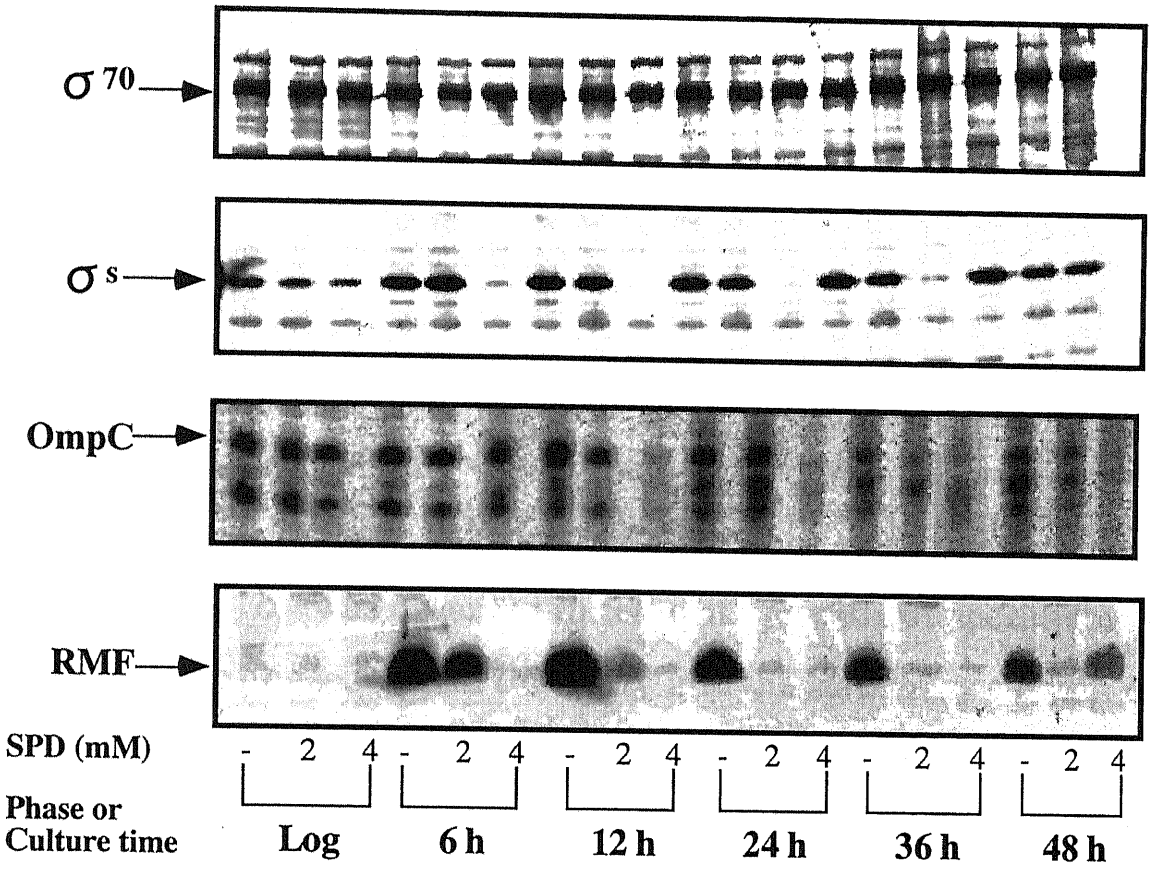

Fig. 12. Western blot analysis of σ^{70}, σ^{s} and RMF, and 8 M urea SDS-PAGE of OmpC. *E. coli* CAG2242 cultured in the presence and absence of 2 or 4 mM spermidine. Cells were harvested at $A_{540} = 0.5$ (log phase) or at the times shown in the figure. Western blot analyses were performed using 5 μg protein for σ^{70}, σ^{s} and 30 μg protein for RMF. 8 M urea SDS-PAGE was done by using 3 μg of outer membrane fraction for OmpC determination.

Another protein which is a stationary phase-specific and essential for cell viability is RMF (41), and it was found that spermidine accumulation causes a decrease in the synthesis of OmpC (30). Then, to further study the mechanism of cell death caused by spermidine accumulation through ppGpp accumulation, the synthesis of σ^s, RMF and OmpC was also determined. Fig. 12 shows the effect of spermidine accumulation on σ^{70}, σ^s, OmpC and RMF judging by 8M urea SDS-PAGE and western blot analysis. The cells grown in the presence and absence of spermidine, the expression of the housekeeping σ^{70} factor was similar either at the logarithmic phase or stationary phase. A basal level of σ^s was observed in cells with or without spermidine at the logarithmic phase. Upon the entry into the stationary phase, σ^s level of the cells grown without spermidine was increased while that of the cells grown with spermidine was not. The inhibitory effect on the synthesis of σ^s of 4 mM spermidine was more than that of 2 mM spermidine, i.e. dose-dependent. After 24 h, the synthesis of σ^s in cells grown with 4 mM spermidine was observed again, correlated with the recovery of the viability (Fig. 9A). Similarly, the effect of spermidine accumulation on the synthesis of OmpC and RMF was also observed, which the effect on RMF was more drastically.

In addition, the effect of accumulated ppGpp on σ^s and RMF was also examined by using *E. coli* CAG2242/pALS10 and CAG2242/pALS14. As shown in Fig. 13, when both cells were grown at the logarithmic phase, a basal level of σ^s was observed, but RMF

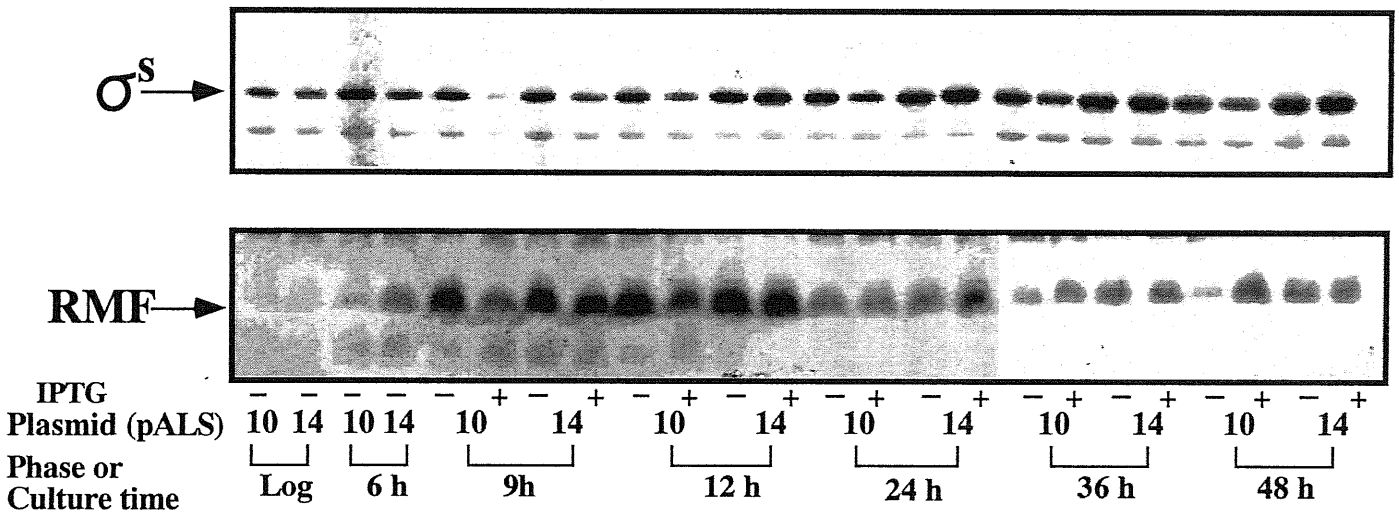

Fig. 13. Western blot analysis of σ^s and RMF. *E. coli* CAG2242/pALS10 and CAG2242/pALS14 were cultured and harvested at $A_{540} = 0.5$ (log phase) or at the times shown in the figure. After harvesting samples at 6 h, 1 mM IPTG was added to cultures and then periodically harvesting over 48 h after onset of cell growth.

was not synthsized. At 6 h after the onset of cell growth (before IPTG addition), The synthesis of σ^s in *E. coli* CAG2242/pALS10 more than in CAG2242/pALS14 which was opposite to the RMF synthesis. After IPTG addition, the syntheses of σ^s and RMF in CAG2242/pALS10 were markedly decreased which correlated to the loss of viability and the increase of ppGpp level (Fig. 11). Besides, at 48 h after the onset of cell growth where the viability of CAG2242/pALS10 was slightly increased and ppGpp level was declined, the syntheses of σ^s and especially RMF were reverse. The results indicated that the cell death caused by spermidine accumulation at the stationary phase is modulated by the increase of ppGpp formation.

Discussion

The effect of spermidine accumulation on ppGpp synthesis resulting in *E. coli* cell death has been studied. It was found that the accumulated spermidine decreased the cell viability and increased (p)ppGpp level at the stationary phase of cell growth. It could be inferred that the decrease of ppGpp at the stationary phase is an important signal to keep cells to be viable. Since the elevated ppGpp level either by spermidine accumulation (Fig. 9) or by an inducible multicopy plasmid which encoded an active RelA protein (Fig. 11) at stationary phase causes cell death. It has been reported that the high level of ppGpp leads to the cessation of growth (68) and lethality (69-71) because of its inhibition effect on ribosome and protein syntheses

(65, 68-72). Furthermore, the inhibition of σ^s and RMF, which are known to be essential for cell viability at the stationary phase of growth, was occurred. The inhibition of OmpC synthesis was also observed which significantly in the high concentration of spermidine addition. Because the synthesis of RMF is not regulated by σ^s (39, 41), the decrease of RMF is not due to the inhibition of σ^s. Therefore, ppGpp may serve as modulator for the synthesis of both σ^s and RMF.

One point of this study should be mentioned that the further investigations will be necessary to provide an explanation for the recovery of viability which was found (Fig. 9A) in *E. coli* cells grown with a high concentration of spermidine. The spermidine content in those cells was also decreased (Fig. 10). It has been known that *E. coli* possess another pathway to metabolize spermidine apart from the metabolism by SAT, i.e. via the conjugation with glutathione by glutathionylspermidine (GSP) synthetase (73, 74). This enzyme owns a second catalytic activity, glutathionylspermidine hydrolysis. Thus, it has been called as GSP synthetase/amidase, which its optimal pH of synthetase activity is 6.8 while the optimal pH of amidase activity is higher (75). Moreover, glutathionylspermidine is found to accumulate in stationary cultures of *E. coli* B in which the medium pH is 6 (76). Therefore, it may be possible that to struggle with the stress caused by spermidine accumulation, *E. coli* GSP synthetase activity would be induced and glutathionylspermidine may be exported by carrier proteins (77). Although there have been no reports of this class of transporter protein in *E. coli* compared to the transporters found in

eukaryotic and plant cells (78, 79).

Based on the observations mentioned above, a tentative model which attempts to explain how polyamine accumulation influences ppGpp synthesis and causes cell death is presented (Fig. 14). The intracellular concentration of ppGpp is controlled at the level of both its synthesis and degradation which can be described as a guanine nucleotide cycle (35, 80). First, the guanosine 5'-triphosphate 3'-diphosphate (pppGpp), a precursor molecule of ppGpp, is formed by the transfer of pyrophosphoryl group from ATP to GTP by ppGpp synthetase I and II. *E. coli* ppGpp synthetase I is a product of *relA* gene which operates on ribosomes whereas ppGpp synthetase II is a product of *spoT* gene which is ribosome-independent, but catalyze the same reaction. Then, pppGpp is degraded to ppGpp by guanosine pentaphosphohydrolase and ppGpp is degraded to GDP by 3'-pyrophosphohydrolase which is another function of *spoT* gene product. When *E. coli* cells grown in the logarithmic phase, they produce a basal level of ppGpp to operate the synthesis of σ^s and RMF which is mainly necessary for cell viability during the stationary phase. After σ^s and RMF are synthesized and functional to maintain the cell survival, the ppGpp signaling is declined. Hence, if polyamine is accumulated which may act at ribosome since polyamines exist as a polyamine-RNA complex in cells (81, 82) , the synthesis of ppGpp is stimulated and accumulated although cells enter the stationary phase. Because of the high level of ppGpp, it suppresses the ribosome function and as well as protein synthetic activity. Then, σ^s and RMF cannot be synthesized

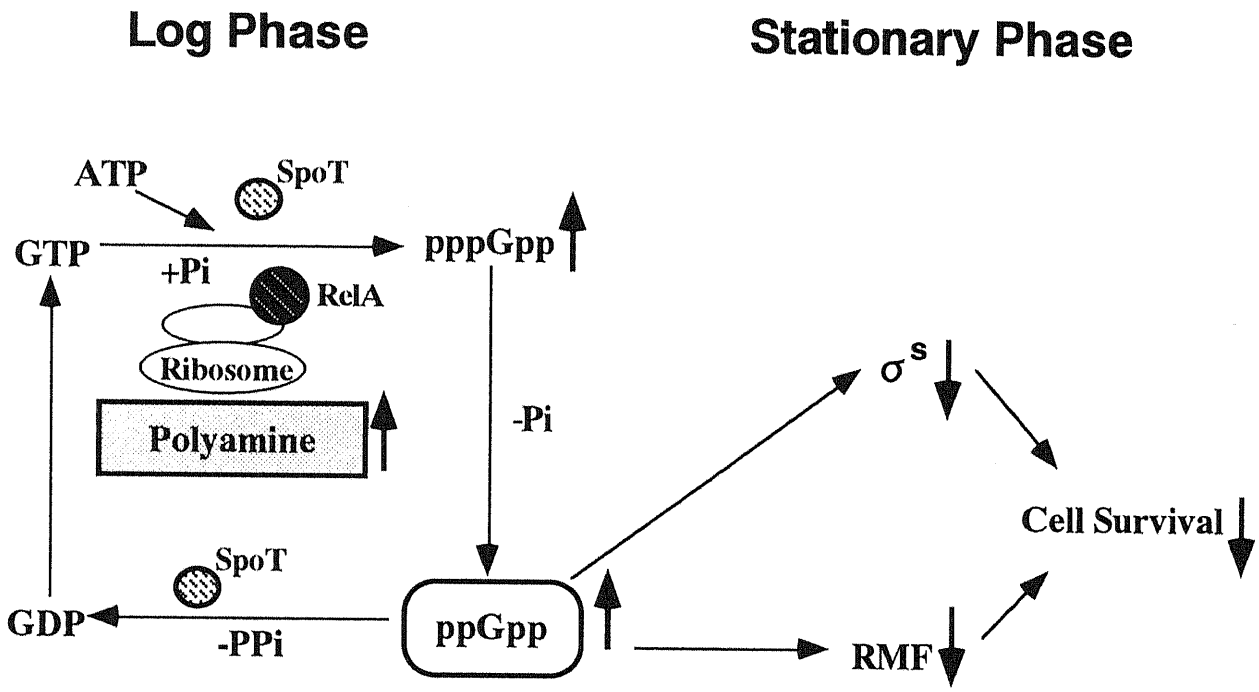

Fig. 14. Model for polyamine-induced ppGpp formation which causes *E. coli* cell death at the stationary phase.

properly through the stationary phase, in consequence of *E. coli* cell death. In this point of view, polyamine and ppGpp may share the common feature, that is, both have the optimal concentration to maintain appropriate physiological functions of cells.

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LIST OF PUBLICATIONS

Apirakaramwong, A., Fukuchi, J., Kashiwagi, K., Kakinuma, Y., Ito, E., Ishihama, A., and Igarashi, K. : Enhancement of cell death due to decrease in Mg^{2+} uptake by OmpC (cation-selective porin) deficiency in ribosome modulation factor-deficient mutant. *Biochem. Biophys. Res. Commun.* **251**, 482-487 (1998)

THESIS COMMITEE

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