ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 11, pp. 1228-1234. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 11, pp. 1529-1537.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-015, September 21, 2008.

Polyamine as a Signaling Molecule for Controlling an Adaptive Mutation

Il Lae Jung and In Gyu Kim*

Department of Radiation Biology, Environmental Radiation Research Center, Korean Atomic Energy Research Institute, P. O. Box 105, Yusong, Daejeon 305-600, Korea; fax: +82-42-861-9560; E-mail: igkim@kaeri.re.kr

Received January 17, 2008
Revision received March 13, 2008

Abstract—In the absence of exogenous polyamines, the polyamine-deficient *Escherichia coli* mutant shows not only a characteristic dual-phase growth with abnormal growth, growth arrest, and normal growth after mutation, but also a higher expression of the SOS genes than the polyamine-proficient wild type. The interval of the growth arrest is inversely regulated in a polyamine concentration-dependent manner. These results indicate that the polyamines can act as a signal not only for provoking an adaptive mutation, but also for hastening generation of an adaptive mutation.

DOI: 10.1134/S0006297908110096

Key words: adaptive mutation, *Escherichia coli*, mutational speed, polyamine, SOS response

The mutational process, which produces genetic variability, is a prerequisite for evolution, and it also participates in other fundamental life processes and a variety of pathological conditions such as aging, cancer, etc. [1-3]. In stressful situations, some *Escherichia coli* cells, as well as yeast, undergo adaptive mutation (also called stationary-phase mutation or selection-induced mutation), in which mutations are formed in stressed or starving, non-growing or slowly growing cells; however, some of the resultant mutants can grow [4-8].

Adaptive mutation is the process for mutational escape from controlled growth, such as in oncogenesis, tumor progression, and resistance to chemotherapeutic drugs; in addition, similar to SOS mutagenesis, adaptive mutation can accelerate mutational speed when evolutionary processes are required [3, 5, 6, 9]. Adaptive mutation has been demonstrated to generate genetic variability upon exposure to a stress and to occur by molecular mechanisms that differ from spontaneous mutation in rapidly growing cells [3, 10, 11], thus suggesting that mutagenic stress responses exist and have the potential to speed up evolution in response to stress [12, 13]. Polyamines (putrescine, spermidine, and spermine) are ubiquitous intracellular polycationic compounds essential for normal cell growth and differentiation; their involvement in growth-related processes has attracted particular interest [14]. Recently, it has been suggested that the abnormal growth of a polyamine-deficient mutant resulted from an oxidative stress, and thus polyamines were closely related with cellular oxidative stresses [15-21].

In this study, we found some unexpected growth properties in a polyamine-deficient mutant JIL601 that have not been shown before. Interestingly, the newly constructed polyamine-deficient mutant showed a phenotype with a "dual-phase growth". In the first growth stage, the cells grew abnormally as described previously, but in the second growth stage they restarted growth and showed normal growth profiles. We also investigated the reason why the dual-phase growth existed, and we suggest that polyamine acts as a signal molecule that controls adaptive mutation and mutational speed.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. A polyamine-deficient *speABC* mutant JIL601 and its isogenic polyamine-proficient wild type QC2461 (MG1655, Δ*lacIZ*) were used in this study. For the β-galactosidase activity assay of *recA-lacZ* expression, JIL970 [QC2461, λ(*recA-lacZ*)] and JIL971 [JIL601, λ(*recA-lacZ*)] were constructed according to the methods previously described [18]. pJIL17 is a ligate of an EcoRV fragment

Abbreviations: LB) Luria–Bertani medium; PBS) phosphate-buffered saline.

* To whom correspondence should be addressed.

from Kohara clone #476 containing the *speC* gene and pBR322.

Cells were grown at 37°C and shaken (200 rpm) for 12-18 h in Luria–Bertani (LB) medium (bacto-tryptone, 10 g/liter; bacto-yeast extract, 5 g/liter; NaCl, 10 g/liter). These cultures were washed with M9, diluted to an initial OD_{600} of 0.005 in minimal M9 medium containing 0.4% glucose (M9/glucose), and then cultivated. For both the elimination of polyamine present in LB and the growth under the polyamine-free condition, we washed the cells, which were grown in polyamine-rich LB medium, with polyamine-free M9 medium (a single washing) and cultivated in the glucose/M9 minimal medium as the times indicated (first starvation), because the initial cell culture medium was polyamine-rich LB and we needed to wash out polyamines that could be carried over from LB medium. To completely remove both intracellular and extracellular polyamines, which possibly remain in the culture medium and inside the cells, we washed the cells with polyamine-free M9 medium again (second washing) and cultivated them in the glucose/M9 minimal medium at the times indicated (second starvation).

Measurement of growth restoration. *Escherichia coli* strains grown in M9/glucose were serially diluted with phosphate-buffered saline (PBS) at each growth time and spread onto an LB agar plate. After overnight cultivation, approximately 100 colonies were selected and patched onto LB plates. These plates were replica-plated onto an M9/glucose agar plate with or without putrescine and then incubated. Cells that grew abnormally on the polyamine-free plate were further investigated for their polyamine requirements. The revision frequency was calculated by dividing the cell numbers not requiring putrescine for normal growth by the total cell number.

Measurement of the polyamine contents and β-galactosidase activity assay. Putrescine and spermidine concentrations were determined by high-performance liquid chromatography (HPLC) as described previously [15]. β-Galactosidase activity was measured by monitoring hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside as described previously [22].

cDNA synthesis and PCR amplification. Total RNA was isolated from cells grown in M9/glucose for 24 h. For generation of the first-strand complementary DNA (cDNA) from the total RNA (2 μg) using a random hexamer, a cDNA synthesis kit (MBI Fermentas Co., USA) was used. Resultant cDNAs served as templates for the polymerase chain reaction (PCR) amplification (25 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min 30 sec) with primers (table).

Western blot analysis. Strains grown for 24 h in M9/glucose were washed twice with PBS. Proteins were separated in 12.5% polyacrylamide gel. After transferring the proteins onto nitrocellulose membranes, the proteins were revealed using a primary antibody (anti-LexA antibody, Cat. No. 46-0710; Invitrogen, USA), secondary antibodies (anti-rabbit antibody), and an electrochemiluminescence detection kit (Amersham, U.K.). The induction ratio was quantified with the Image Gauge Program (Version 3.12; Fuji, Japan).

RESULTS AND DISCUSSION

To remove the intracellular polyamines, we washed the cells (JIL601) grown in the polyamine-rich LB medium with fresh M9 medium, subcultured the cells in the same fresh M9 medium containing glucose as a carbon source (M9/glucose), and then incubated the cells. Although the mutant needs polyamines for normal cell growth, we did not observe significant growth impairment (according to our previous report cited in [17], the final cell yield of the mutant reached the bold dashed line; Fig. 1a, first starvation), and the mutant grew better than in the twice-washed experiment as described below. This finding is due to the fact that, even though the mutant could not synthesize the polyamines because of genetic elimination of the *speABC* genes, polyamines were still

Oligonucleotides used in this study

Gene	Forward primer	Reverse primer
lexA	5'- CCAGGCAACAAGAGGTGTTTGATCTC	5'- TGCTGACGAAGGTCAACGACAATTGG
recA	5'- CAATTTGGTAAAGGCTCCATCATGCG	5'- AGTTGATACCTTCGCCGTAGAGGATCT
uvrD	5'- CTTACCTGCTCGACAGCCTTAATGACA	5'- GTAGATTGACTGGTCGTCATCACCGA
yebG	5'-AATACGTAGTCATTCGTGAGGGCGAAG	5'-ATCTTCCTCTTCGGATTCAGCACCGA
dinB	5'- GTGGATATGGACTGCTTTTTCGC	5'- GCTCGATAATCGCTTCACATTCAG
sulA	5'- ACT TCAGGCTATGCACATCGTTCT	5'- GACGCATAATAAACCCCATAGCGT
recN	5'- TGGCACAACTGACCATCAGCAACTTT	5'- AGCATATCAAGTACGCCGGACAGTTT
dinI	5'- ATGCGAATTGAAGTCACCATAGCG	5'- TATTCGCTGACAAACCAGTCATCG
minD	5'- CGCATTATTGAAGTTACTTCGGGC	5'- ATCGGCGTTAATGTCGAGAAT GAC

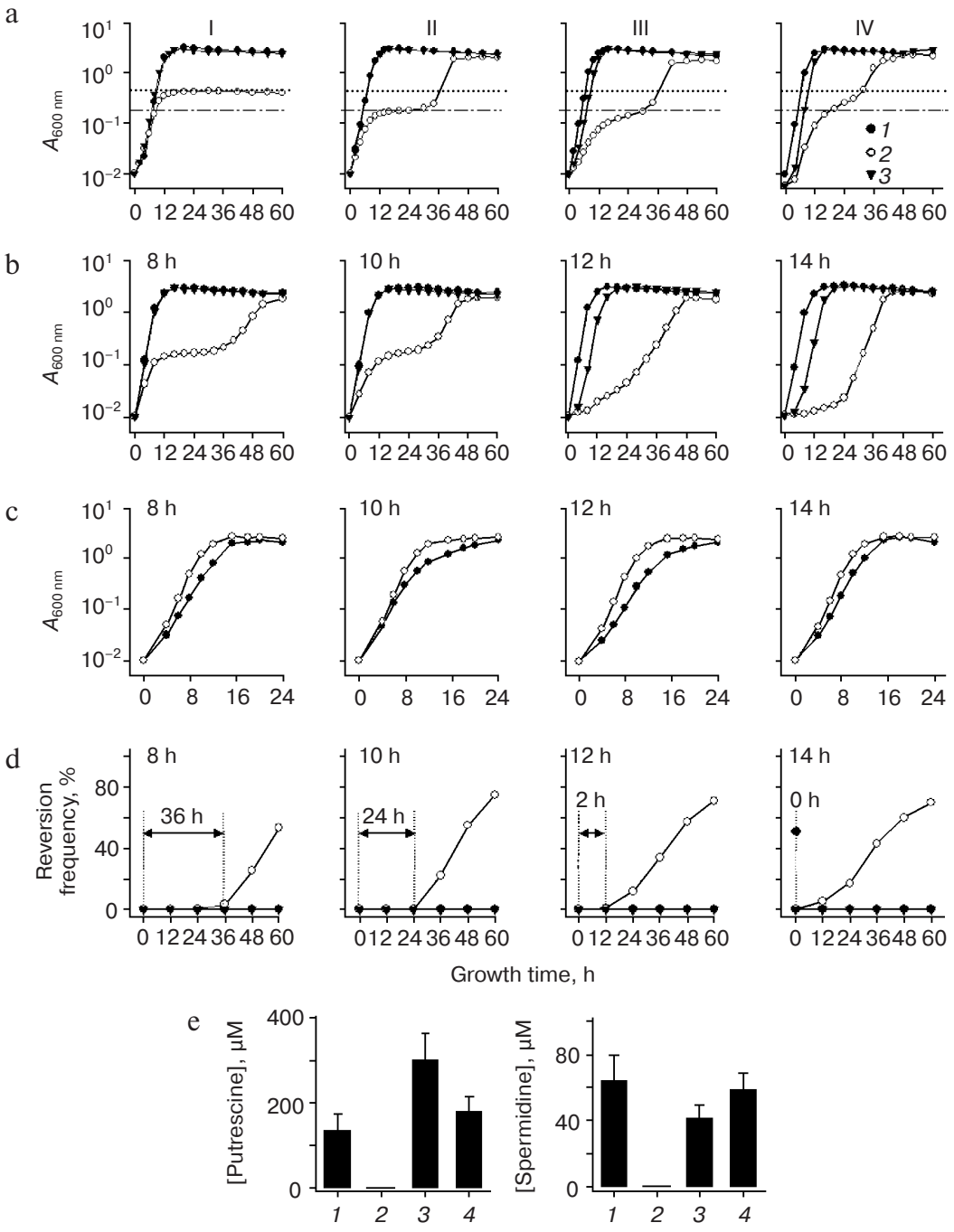

Fig. 1. Polyamine deficiency and dual-phase growth of the polyamine-deficient mutant JIL601. a) Cell growth according to the repeated use of polyamine starvation (I-IV). Cells: *1*) wild type; *2*) JIL601 mutant; *3*) JIL601 mutant in the presence of 1 mM putrescine. b) Growth profiles according to the time of exposure to polyamine starvation. Cells sampled in definite time intervals (8-14 h) since the onset of the second starvation (see Fig. 1a, II) were re-inoculated into fresh M9/glucose medium and cultured. c) Growth profiles of the mutants. The 60-h cultivated cells from each previous seed time (see Fig. 1b) were used as seed cultures and cultivated in the same M9/glucose medium with and without polyamines. d) Determination of growth restoration from each previous time (8-14 h). Cells cultured for 8-14 h in M9/glucose medium (see Figs. 1a (II) and 1b) were sampled and re-cultured in fresh portion of the same medium, and then reversion frequency and time of delay of adaptive mutation were determined. e) Determination of the intracellular polyamine concentrations by HPLC. The 60-h cultivated cells from each seed time (see Fig. 1b) were used for the HPLC analysis. Cells: *1*) wild type; *2*) polyamine-deficient strain JIL601 carrying reverse mutation; *3*) JIL601 cells cultured in the presence of putrescine; *4*) JIL601 cells containing pJIL17 plasmid.

present in the cell as a result of a carryover of the polyamines present in the LB. Thus, only a single washing out was not sufficient for complete removal of the intracellular polyamines.

To completely remove the intracellular polyamines, we again subcultured the once-washed cells grown in the M9/glucose medium for approximately 9-10 h in another fresh M9/glucose medium. In this case, the mutant needed the polyamines for normal cell growth (Fig. 1a, second starvation). To confirm that intracellular polyamines were completely removed by just washing cells two times, we used the cells grown from the two- or three-times subcultured cells as a seed culture for the three- or four-times-washed cultivation, and subcultured the cells from the final cultivation in fresh M9/glucose medium (Fig. 1a, third or fourth starvation). As for the results, each culture showed growth patterns ("two-phase growth pattern") similar to that of twice starvation, indicating that all the intracellular polyamines were completely removed from the mutant when the cells were washed out only twice. We used the twice-starved culture throughout this study.

Next, we subcultured the cells that were incubated for different times (8-14 h) in the twice-starved culture in fresh M9/glucose medium; then we monitored their growth properties. In this case, as the subculture time increased, the cells were exposed a longer time to a stressful polyamine-free condition. In the case of the 8-h seed culture, although the cells showed typical abnormal growth properties for an approximately 36-h cultivation (the first growth phase), surprisingly, the cells resumed their growth after cultivation and reached a final value of OD_{600} similar to that of the mutant grown with a putrescine supplement (the second growth phase, Fig. 1b, 8 h). Moreover, we found that the longer the mutant was exposed to the stressful polyamine-free M9 medium, the quicker was the time to second-growth initiation (Fig. 1b, 10, 12, and 14 h). We call this phenomenon of abnormal cell growth during a definite period, followed by arrest of cell growth and then resumption of normal growth, a "dual-phase growth". This pattern is also shown in Fig. 1a (compare curves in panels II, III, and IV), in which the longer the cell was starved for polyamines, the quicker it resumed its growth.

After a 60-h cultivation of each culture as shown in Fig. 1b, these cells were re-inoculated into fresh M9/glucose medium and their growth was monitored. All the cells grew normally, even in the absence of putrescine, indicating that the mutant cells did not show a polyamine-mutant phenotype; in addition, the dual-phase growth pattern was not shown again, probably because of generation of "mutants" (which are able to grow normally even in the absence of polyamines) by beneficial mutagenesis (Fig. 1c).

We determined the frequency for growth restoration, that is, the percentage ratio of the cells that could grow normally even in the absence of polyamines. The mutants started to appear after approximately 36-h cultivation when the seed culture incubated for 8 h in Fig. 1b was used; the final frequencies for the growth restoration reached 50% (Fig. 1d, 8 h).

However, a mutation was not shown in both the polyamine-proficient wild type and the mutant combined with the polyamine supplement. As the starvation time increased, the mutants appeared sooner (Fig. 1d, 8, 10, 12, and 14 h). Because we doubted whether this property of the mutants not requiring polyamines for normal growth might result from induction of an alternative polyamine biosynthetic pathway, we measured the intracellular polyamine concentrations by HPLC. Although polyamines were detected at normal levels in the wild type, in JIL601 containing pJIL17 (a plasmid containing the normal *speC* gene), and in JIL601 with the polyamine supplement, no polyamines were detectable in the mutant cells, indicating that the normal growth properties of the mutants could not have resulted from polyamine (Fig. 1e).

Various concentrations of polyamines were applied to the mutant strain and its growth properties were examined. Although the mutant grown without polyamines showed the characteristic dual-phase growth after approximately 36-h cultivation, the dual-phase growth was not seen in cells grown above 0.1-μM concentration of polyamines until after 60-h cultivation (Fig. 2a, see color insert). In addition, the final cell yield of the culture grown without a polyamine supplement was greater than that of the culture grown in polyamine concentrations of 0.1 or 0.5 μM. In conclusion, although the growth that preceded the dual-phase growth was inferior, the provision of a lower concentration of polyamines below the threshold value could have caused severe stress in the mutant cells, and prolonged exposure to polyamine-deficient stress could have induced adaptive mutagenesis, with the final growth of the mutated cells being much superior to that of cells grown above the threshold value of the polyamines. In the meantime, an increase in the OD after a dual-phase growth was not the result of cell enlargement, but rather of cell division, suggesting that the cells had divided (Fig. 2b).

On the basis of the above results, we suggest two distinctive evolutionary environments (Fig. 2c). In the first environment, a "mutatable zone" occurred under an unfavorable biosystem (0-0.1 μM polyamine), in which a dual-phase growth led to adaptive mutation as a result of severe stress; thus, some of the beneficial mutants could survive in the unfavorable environment. In the second environment, a "stable zone" occurred under a favorable biosystem (0.5-1000 μM polyamine), in which polyamines were supplied at an amount sufficient for and advantageous to cell growth in a concentration-dependent manner; thus, the cell was highly stable and did not operate the system for adaptive mutation in order to survive.

We examined the relationship between the time for generating mutants and the polyamine concentrations

(Fig. 2d). As expected, as more polyamines were supplied, the time for generating the mutants was further delayed. These results strongly suggest that polyamine supplementation below the minimum concentration (0.2 μM) necessary for normal cell growth could lead to severe stress, causing the generation of mutants so that a cell could survive in that particular stressful situation. In contrast, a higher concentration of polyamines may delay the time for generation of a mutation by lowering the stress level even more, and a sufficient supply (up to 0.2 μM) of polyamines makes the polyamine-deficient mutant grow normally. In conclusion, polyamines may regulate the speed of an adaptive mutation.

A mechanism of an adaptive mutation uses the general DNA damage sensor and repair protein RecA (akin to eukaryotic p53), which activates the global bacterial "SOS" DNA damage response [11, 23, 24]. An intriguing feature of the SOS response is an inducible mutation [13, 25]. Microbial cells under a growth-limiting stress can often generate mutations by promoting genetic changes that can enhance the survival rate of the cells [23].

On the basis of the present experiments in which a polyamine deficiency may have promoted adaptive mutation, we used a reverse transcription assay to examine the transcription level of some SOS genes: *lexA*, *recA-uvrD* (DNA-dependent ATPase I and helicase II, respectively), *recN* (putative enzyme used in a recombination and DNA repair), *yebG* (function unknown, DNA damage-inducible gene in a SOS regulon), *dinI* (DNA damage-inducible protein I), *sulA* (inhibitor of cell division and FtsZ ring formation upon DNA damage/inhibition), *minD* (cell division inhibitor), and *dinB* (DNA polymerase IV, devoid of proofreading). Interestingly, all the SOS genes tested in this study were significantly induced in the mutant, but exogenous putrescine repressed them to a wild-type level (Fig. 3a). We also verified the transcriptional level by constructing a chromosomal *recA-lacZ* fusion, whose expression was higher in the mutant (Fig. 3b).

A central part of the SOS response is derepression of more than 20 genes under transcriptional control of the LexA repressor. However, despite the higher *lexA* transcriptional expression in the mutant compared with the wild type, the expression of the other SOS genes maintained a high level. This lack of accordance might result from abnormal translational processes from the *lexA* messenger RNA (mRNA) to the LexA repressor. To investigate this possible cause, we quantified the intracellular LexA level using a Western blot analysis, in which the LexA was 9-10 times lower in the mutant than in the wild type or in the mutant with a polyamine supplement (Fig. 3c). This discordance between the levels of the transcripts and their products tells us that, although the *lexA* mRNA transcript was high, the LexA protein must be low in the polyamine-deficient mutant because of a malfunction of the translational machinery. In fact, polyamine has been shown to reduce mistranslation [26].

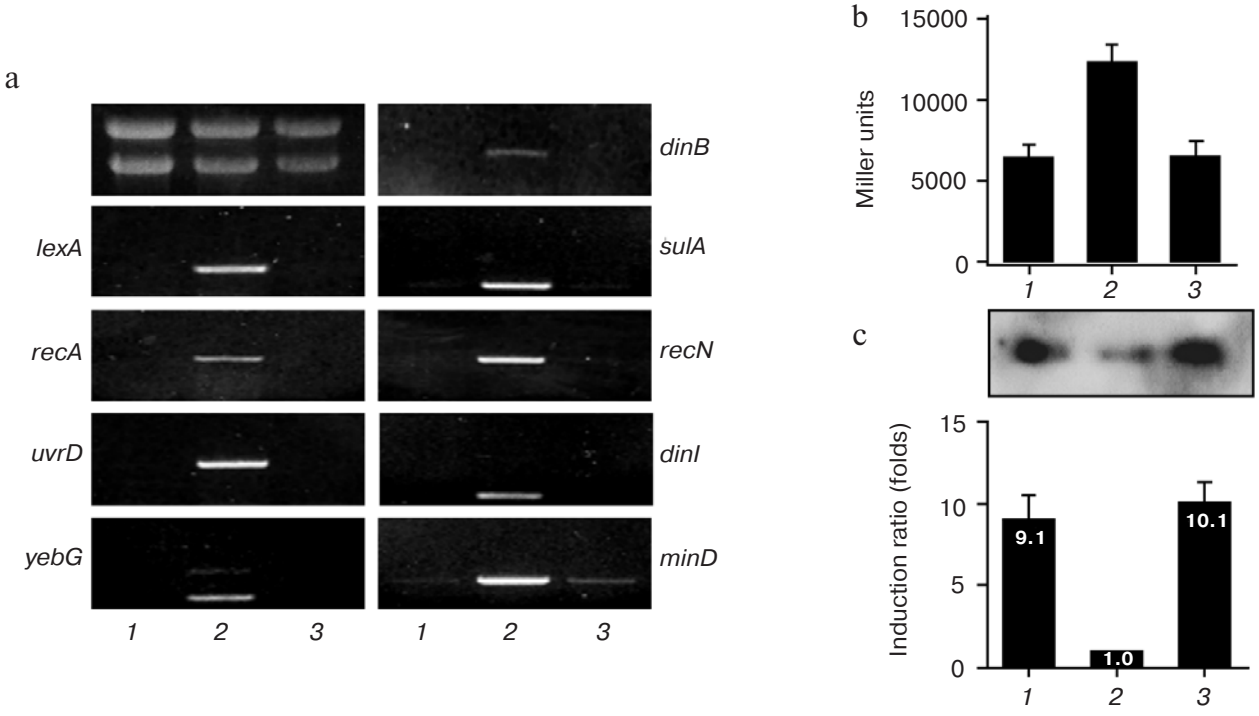

Fig. 3. Determination of transcripts level of some SOS genes and a Western blot of the LexA repressor. a) Reverse transcription analysis. b) β-Galactosidase activities of *recA-lacZ* fusion. c) Western blot analysis of LexA protein. Cells: *1*) wild type; *2*) JIL601 mutant; *3*) JIL601 cells cultured in the presence of putrescine.

We previously found that the polyamine-deficient *E. coli* mutant not only has lower catalase activities [18], but also that polyamines protect against a PQ-induced toxicity [16]. We also suggested that the abnormal growth of the polyamine-deficient *E. coli* mutant partially resulted from oxidative stress-induced damage; thus, the mutant exhibited the requirements for antioxidant or specific nutritional supplements such as amino acids or Tiron during normal aerobic growth [19]. These results indicated that the mutant suffers from severe oxidative stress attributable to polyamine deficiency; therefore, it grows abnormally. Under these severe oxidative stresses, the mutant cells operate a high level of SOS response even in the non-growing stage; thus, the cells can accumulate mutations to survive. After the adaptation period, the mutant cells can survive even in the polyamine-free condition, thus showing a distinctive dual-phase growth phenotype. On the basis of our findings in this study, we propose a new model in which polyamines can act as a signal molecule for controlling the rate and speed of adaptive mutation through the SOS response, which is due to increased intracellular reactive oxidative stress caused by polyamine deficiency. Finally, mutants, in which polyamines are not required for normal cell growth any longer, are generated among the non-growing cells.

After an adaptive mutation process was completed, we further characterized the social characteristics of the mutants, in which two distinct types of mutants were produced. One type is "altruistic", meaning that the mutants could help the growth of the original non-mutagenized polyamine-deficient mutant, which could not adapt to the stressful situation (Fig. 4). This "altruistic" feature may result from a diffusion of some substances or antioxidants such as amino acids, because growth recovery was shown only near the mutants (see box). The other type is "selfish", meaning that the mutants could not help the growth of the polyamine-deficient mutant; therefore, the mutants grew normally and exclusively, but the mutants still grew abnormally.

In conclusion, the theory that polyamines are required for normal cell growth needs amendment and expansion. We suggest that polyamines on "a seesaw" act as signals for deciding whether to grow or to survive. A sufficient provision of polyamines evidently helps normal cell growth and enables a stable preservation of the species. However, insufficient polyamines accelerate adaptive mutation after a definite period of adaptation, thereby turning the recessive cell into a dominant one through an increase in the mutation frequency. Polyamine therefore acts as a signal molecule for regulating the preservation and evolution of the species.

Although higher mutagenesis rates and adaptation mutation are observed in the absence of polyamines, it is also observed as well as in other stressful conditions, such as when bacteria starve for carbon, oxygen, or nutrients.

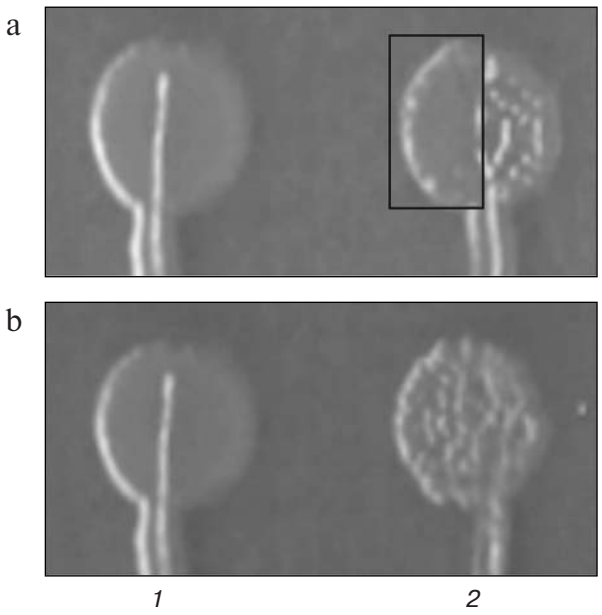

Fig. 4. Two types of mutants generated by an insufficient polyamine supply. Cells were grown in M9/glucose in the absence of polyamines, and two representative mutants (a and b) were selected. These mutants and JIL601 were grown in M9/glucose in the absence of polyamines for 10 h and spotted onto an M9/glucose agar plate. After 2-day incubation, the mutants were photographed. a, b) "Altruistic" and "selfish" types of relations between cells, respectively: *1*, *2*) reverse mutant and polyamine-deficient JIL601 strain colonies, respectively.

However, it is obvious that the polyamine deficiency is one of the factors provoking mutations to escape the stressful condition. Moreover, polyamines are well known as essential components for normal cell growth and differentiation. Thus polyamine deficiency enforces various cellular defects, which is followed by mutation for survival. In conclusion, polyamines act as signaling molecules for provoking and/or hastening an adaptive mutation according to their concentration.

In addition to the above results, we suggest handling the polyamine-deficient mutant of *E. coli* with great care, because the mutant cells are exposed to a severe stressful condition and become highly mutable to survive after only short exposure to a polyamine-deficient condition.

The authors wish to acknowledge the financial support (Nuclear R&D program) of the Ministry of Science and Technology (MOST) of the Republic of Korea.

REFERENCES

1. Bjed, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman, M., Taddei, F., and Matic, I. (2003) *Science*, **300**, 1404-1409.
2. Shankel, D. M., Hartman, P. E., Kada, T., and Hollaender, A. (1987) *Environ. Mutagen.*, **9**, 87-103.

3. Strauss, B. S. (1998) *Genetics*, **148**, 1619-1626.
4. Drake, J. W. (1991) *Annu. Rev. Genet.*, **25**, 125-146.
5. Foster, P. L. (1993) *Annu. Rev. Microbiol.*, **47**, 457-504.
6. Hall, B. G. (1992) *Curr. Opin. Genet. Dev.*, **2**, 943-946.
7. Harris, R. S., Longerich, S., and Rosenberg, S. M. (1994) *Science*, **264**, 258-260.
8. Rosenberg, S. M., and Hastings, P. J. (2003) *Science*, **300**, 1382-1383.
9. Finch, C. E., and Goodman, M. F. (1997) *Trends Neurosci.*, **20**, 501-507.
10. Foster, P. L. (1999) *Annu. Rev. Genet.*, **33**, 57-88.
11. Rosenberg, S. M. (2001) *Nat. Rev. Genet.*, 504-515.
12. Echols, H. (1981) *Cell*, **25**, 1-2.
13. Radman, M. (1975) *Basic Life Sci.*, **5A**, 355-367.
14. Geiger, L. E., and Morris, D. R. (1980) *J. Bacteriol.*, **141**, 1192-1198.
15. Jung, I. L., and Kim, I. G. (2003) *J. Biol. Chem.*, **278**, 22846-22852.
16. Jung, I. L., and Kim, I. G. (2003) *Cell. Biol. Toxicol.*, **19**, 29-41.
17. Jung, I. L., and Kim, I. G. (2003) *Environ. Toxicol. Pharmacol.*, **15**, 19-26.
18. Jung, I. L., and Kim, I. G. (2003) *Biochem. Biophys. Res. Commun.*, **301**, 915-922.
19. Jung, I. L., Oh, T. J., and Kim, I. G. (2003) *Arch. Biochem. Biophys.*, **418**, 125-132.
20. Tkachenko, A. G., Nesterova, L., and Pshenichnov, M. (2001) *Arch. Microbiol.*, **176**, 155-157.
21. Tkachenko, A. G., and Fedotova, M. V. (2007) *Biochemistry (Moscow)*, **72**, 109-116.
22. Miller, J. H. (1972) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) American Society for Microbiology, Washington, DC.
24. Taddei, F., Halliday, J. A., Matic, I., and Radman, M. (1997) *Mol. Gen. Genet.*, **256**, 277-281.
25. Fijalkowska, I. J., Dunn, R. L., and Schaaper, R. M. (1997) *J. Bacteriol.*, **179**, 7435-7445.
26. McMurry, L. M., and Algranati, I. D. (1986) *Eur. J. Biochem.*, **155**, 383-390.