From damaged genome to cell surface: transcriptome changes during bacterial cell death triggered by loss of a restriction–modification gene complex

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ABSTRACT

Genetically programmed cell deaths play important roles in unicellular prokaryotes. In postsegregational killing, loss of a gene complex from a cell leads to its descendants' deaths. With type II restriction–modification gene complexes, such death is triggered by restriction endonuclease's attacks on under-methylated chromosomes. Here, we examined how the *Escherichia coli* transcriptome changes after loss of PaeR7I gene complex. At earlier time points, activation of SOS genes and σ^E-regulon was noticeable. With time, more SOS genes, stress-response genes (including σ^S-regulon, osmotic-, oxidative- and periplasmic-stress genes), biofilm-related genes, and many hitherto uncharacterized genes were induced, and genes for energy metabolism, motility and outer membrane biogenesis were repressed. As expected from the activation of σ^E-regulon, the death was accompanied by cell lysis and release of cellular proteins. Expression of several σ^E-regulon genes indeed led to cell lysis. We hypothesize that some signal was transduced, among multiple genes involved, from the damaged genome to the cell surface and led to its disintegration. These results are discussed in comparison with other forms of programmed deaths in bacteria and eukaryotes.

INTRODUCTION

Programmed cell death is a genetically regulated process of cell suicide that occurs both in eukaryotes and prokaryotes. In multicellular eukaryotes, programmed cell death is used to delete damaged or superfluous cells, which is important to the development and integrity of an organism and its genome. Likewise, programmed cell death processes are beneficial to bacterial populations and genomes (1,2). Bacterial programmed deaths are induced by antibiotics, viral infection, DNA damage, starvation and 'addictive' genetic modules including restriction–modification (RM) systems.

A gene encoding a restriction (R) endonuclease that recognizes and cleaves DNA at a specific sequence is often linked to a gene encoding a modification (M) enzyme that methylates the same sequence to protect it from cleavage. These RM systems are regarded as bacterial tools of defense that attack invading un-methylated DNA, whilst simultaneously protecting the bacterium's own methylated DNA. However, cells that have lost a type II RM gene complex have their newly-replicated, under-methylated chromosomes cleaved by residual levels of restriction endonuclease, leading to cell death (Figure 1) (3–5). This class of programmed deaths, called *postsegregational killing* or *genetic addiction*, might underlie parasitism or symbiosis of genes within a genome (6). Theoretical work has demonstrated that such an addictive gene complex should be able to evolve in a structured habitat (7).

These and other observations suggest that some RM gene complexes behave as selfish mobile genetic elements in the same way as transposons and viral genomes (3,8,9). Several lines of evidence support the mobility and involvement of these complexes in genome rearrangements, which are likely to be related to their attacks on DNA, elaborate regulatory systems in gene expression, and mutual competition (9,10–14). Various adaptations of

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host prokaryotes to this selfish behavior have been observed (9), including the systematic avoidance of some restriction sites by prokaryotic genomes (15,16), which might have evolved through selection by past restriction attacks and/or methylation.

Cellular responses to postsegregational killing were previously analyzed in *Escherichia coli* cells carrying the PaeR7I and EcoRI RM gene complex (3,4,5). Following the loss of this complex, viable cell count increases stopped, cell viability decreased, chromosomes were cleaved, and cells formed long filaments, some of which were multinucleated while others were anucleated. The *E. coli* host induced at least one SOS gene, and the bacterial RecBCD/RecA machinery helped the cells to survive by repairing the cleaved chromosomes (5).

In order to understand this form of cell death, we here followed temporal changes in *E. coli* transcriptome after the loss of an RM gene complex. Our analysis revealed how the cell death process begins with genome damage and culminates in cell-surface disintegration and release of cellular proteins to the environment.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used are listed in Table 1. All the strains were derivatives of *E. coli* K-12. pHSG415 carries a temperature-sensitive replicon from pSC101 (17) and an ampicillin-resistance gene. pTN9 and pTN11 contained PaeR7I r^+m^+ and r^-m^+ genes, respectively, on pHSG415 (3). They were introduced into MG1655 by electroporation. The *rpoE*-plasmid and other plasmids harboring σ^E-regulon genes from the ASKA-clone (18) were introduced into MG1655 or MG1655Δ*htrG* by electroporation. MG1655 *htrG*::Km^r was constructed by P1 transduction using BW25113 *htrG*::Km^r (19) as the donor, and its Km^r gene was then eliminated (20) to generate MG1655Δ*htrG*. MG1655Δ*rpoS* and MG1655 Δ*htrG*Δ*ydhJ*Δ*ydhK*Δ*yaiW*Δ*smpA* were constructed in the same way. MG1655*lexA3*(Ind⁻) was constructed by P1 transduction using SG13171 (21) as the donor, and its *lexA3* mutation was confirmed by dideoxy-sequencing. pBAD33::*paeR7IR* plasmid was constructed by inserting the *paeR7IR* ORF into SmaI site of pBAD33 (22), in which the cloned ORF could be induced by arabinose from P$_{BAD}$. The *paeR7IR* ORF was obtained by PCR using pTN9 as the template and oligo-nucleotides 5′-ggg ggcaatggagaataaatggcac-3′ and 5′-tcagcccagtcgtgccgc ctcggcg-3′ as the primers. The nucleotide sequence and the orientation of the *paeR7IR* ORF on the plasmid were confirmed by dideoxy-sequencing. pBAD33:: *paeR7IR* plasmid was introduced into MG1665, MG1655 *lexA3*(Ind⁻), MG1655Δ*rpoS* and MG1655 Δ*htrG*Δ*ydhJ*Δ*ydhK*Δ*yaiW*Δ*smpA* by electroporation.

Culture conditions

Escherichia coli cells were cultured with constant shaking at 70 r.p.m. in an L-shaped test tube containing 10 ml LB medium using a TN-2612 rocking incubator (Advantec, Dublin, CA, USA), which monitors growth automatically

by measuring OD$_{660}$ every 12 min. Selection was provided with 50 μg/ml ampicillin and 20 μg/ml chloramphenicol as necessary.

Cell death induction by loss of the PaeR7I RM gene complex on ts plasmid

Host cell death was induced by blocking replication of an RM plasmid using temperature-sensitive replication machinery as described previously (3). Cells were cultured at 30°C in LB with 50 μg/ml ampicillin until the OD at 660 nm reached 0.3. This culture was used to inoculate into LB without ampicillin prewarmed to 42°C to block plasmid replication, at an OD$_{660}$ value of 0.01 (Figure 1). The culture at 42°C was diluted 100-fold when its OD$_{660}$ reached 0.5 (2 h after the temperature shift). OD$_{660}$ was monitored (Figure 1D), viable cells were measured as the number of colony formers on LB agar at 30°C (Figure 1C), while the number of colony formers on LB agar with ampicillin at 30°C was taken as the measure of the number of viable cells carrying the plasmid (Figure 1B). Cells were harvested and subjected to transcriptome analysis before (0 h) and 1 h, 1 h 50 min (2 h) and 3 h after the temperature shift. Proteins in the culture medium were analyzed 6 h after the temperature shift. Cells were subjected to microscope analysis 0, 2, 3, 4 and 6 h after the shift (Supplementary Figure 7).

Cell death induction by over-expressing *paeR7IR* gene on pBAD33 plasmid

The *paeR7IR* gene on pBAD33::*paeR7IR* plasmid was induced with arabinose. Cells were cultured at 37°C or 42°C in LB with 20 μg/ml chloramphenicol, and 2% arabinose was added when their OD$_{660}$ reached 0.5 (∼2 h). Viable cells were measured by the number of colony formers on LB agar containing 0.2% glucose at 37°C, 0 h and 1 h after the induction.

Over-expression of *rpoE* and σ^E-regulon genes

The *E. coli* strain carrying the relevant gene under the IPTG inducible promoter was grown at 37°C with chloramphenicol selection for the plasmid. The cells were grown to an OD$_{660}$ value of 0.3 and used to inoculate a main culture at an OD$_{660}$ value of 0.01. Over-expression of the gene was induced by addition of 1 mM IPTG when the OD$_{660}$ reached 0.5. The viable cells were counted at the indicated time intervals. The proteins in the supernatant fraction of the medium were analyzed 3.1 h after IPTG addition for expression of *rpoE*, *htrG* and *yfeK*.

Analysis of proteins released in the medium

The bacterial culture was centrifuged at 17 000*g* for 5 min, the supernatant was collected, and the protein fraction of MW >10 kDa was obtained by concentrating the supernatant using Microcon-10 (Millipore, Billerica, MA, USA). Protein fractions from a 0.2 ml sample of the supernatant of the culture were applied to 10% SDS–PAGE. The protein concentration of the supernatant fraction >10 kDa was determined using Protein Assay

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype	Source/reference
MG1655 (CGSC#6300)	F⁻ λ⁻ *rph*-1	D. Biek (University of Utah, Salt Lake City, UT)
BW25113 *htrG*::Kmʳ	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567Δ(rhaBAD)568 rph–1 htrG*::Kmʳ	Keio Collection (19)
BW25113 *ydhJ*::Kmʳ	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567Δ(rhaBAD)568 rph–1 ydhJ*::Kmʳ	Keio Collection (19)
BW25113 *ydhK*::Kmʳ	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567Δ(rhaBAD)568 rph–1 ydhK*::Kmʳ	Keio Collection (19)
BW25113 *yaiW*::Kmʳ	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567Δ(rhaBAD)568 rph–1 yaiW*::Kmʳ	Keio Collection (19)
BW25113 *smpA*::Kmʳ	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567Δ(rhaBAD)568 rph–1 smpA*::Kmʳ	Keio Collection (19)
BW25113 *rpoS*::Kmʳ	*rrnB3 ΔlacZ4787 hsdR514Δ(araBAD)567Δ(rhaBAD)568 rph–1 rpoS*::Kmʳ	Keio Collection (19)
MG1655Δ*htrG*	as MG1655, but Δ*htrG*	This study
MG1655Δ*rpoS*	as MG1655, but Δ*rpoS*	This study
MG1655Δ*htrG*Δ*ydhJ*Δ*ydhK*Δ *yaiW*Δ*smpA*	as MG1655, but Δ*htrG*Δ*ydhJ*Δ*ydhK*Δ*yaiW*Δ*smpA*	This study
SG13171	F- SA500 *his leu sulA malF*::Tn*10 lexA3 strA*	S. Mizusawa (21)
MG1655*lexA3*(Ind⁻)	as MG1655, but *lexA3*	This study
MG1655/pBAD33::*paeR7IR*	as MG1655, but harboring pBAD33::*paeR7IR*	This study
MG1655*lexA3*(Ind⁻)/pBAD33:: *paeR7IR*	as MG1655*lexA3*(Ind-), but harboring pBAD33::*paeR7IR*	This study
MG1655Δ*rpoS*/pBAD33:: *paeR7IR*	as MG1655Δ*rpoS*, but harboring pBAD33::*paeR7IR*	This study
MG1655Δ*htrG*Δ*ydhJ*Δ*ydhK*Δ *yaiW*Δ*smpA*/pBAD33::*paeR7IR*	as MG1655Δ*htrG*Δ*ydhJ*Δ*ydhK*Δ*yaiW*Δ*smpA*, but harboring pBAD33::*paeR7IR*	This study
pHSG415	temperature sensitive replicon of pSC101, Ampʳ	J. Kato (Tokyo Metropolitan University, Tokyo, Japan)
pTN9	PaeR7I r⁺m⁺ genes on pHSG415	(3)
pTN11	PaeR7I r⁻m⁺ genes on pHSG415	(3)
pCA24N (gfp free)		GFP-free ASKA clone vector (18)
p*rpoE*	*rpoE* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*htrG*	*htrG* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*yfeK*	*yfeK* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*yfeS*	*yfeS* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*ydhK*	*ydhK* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*yaiW*	*yaiW* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*smpA*	*smpA* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*yidR*	*yidR* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
p*yidQ*	*yidQ* gene on pCA24N (gfp free)	GFP-free ASKA clone (18)
pBAD33		National Institue of Genetics (Mishima, Japan) (22)
pBAD33::*paeR7IR*	PaeR7I r⁺ gene on pBAD33	This study

CBB Solution (Nacalai Tesque, Kyoto, Japan) with BAS (Pierce, Rockford, IL, USA) as the standard.

Phase-contrast microscopy observation

An IX-71 light microscope (Olympus, Tokyo, Japan) was used with 100-fold magnification.

Transcriptome analysis

All gene expression measurements were repeated independently. Total RNA was isolated, treated with DNase and used to synthesize cDNA. This was fragmented, labeled and hybridized to an Affymetrix *E. coli* antisense genome array, which was processed to obtain the expression values, following instructions by the manufacturer (http://www.affymetrix.com/index.affx). The statistical significance of differences in gene expression between strains was determined by an ANOVA at a significance level of $P < 0.05$ for each time point. Genes that were reliably differentially expressed between MG1655/pTN9 (r⁺m⁺) and MG1655/pTN11 (r⁻m⁺) were identified by the Student–Newman–Keuls *post hoc* test for each time point. Detailed procedures are provided in the Supplementary Material. Gene expression data and relevant information are deposited in GEO: GSE7326.

RESULTS

Transcriptome analysis: design and reliability

Postsegregational killing of *E. coli* cells was induced by blocking replication of a temperature-sensitive plasmid carrying the PaeR7I RM gene complex (Figure 1) (3,23). At 30°C, the growth of cells with the PaeR7I r⁺m⁺
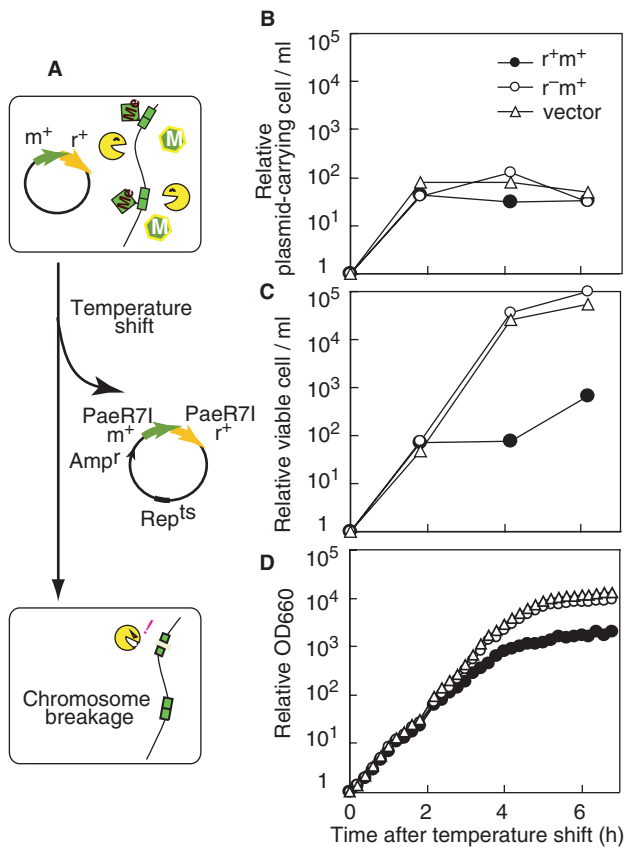
Figure 1. Postsegregational cell killing triggered by loss of PaeR7I RM gene complex. (**A**) Experimental design. Cell death was induced by blocking the replication of a plasmid with temperature-sensitive replication machinery and PaeR7I RM gene complex. (**B**) The plasmid-carrying viable cell counts. (**C**) The viable cell counts. (**D**) OD_{660}. The values are relative to those at the time of the temperature shift (0 h). The culture was diluted 100-fold when its OD_{660} reached 0.5 (2 h after the temperature shift).

*: and protein turnover, chaperones
**: biosynthesis, transport and catabolism

not observed or not enriched
enriched ($0.05 < p$)
significantly enriched ($0.01 < p < 0.05$)
strongly enriched ($p < 0.01$)

Figure 2. Functional classification of genes demonstrating differential expression with respect to r^+/r^-. Genes with a significantly higher expression level in r^+m^+ than r^-m^+ strains (left, reddish color), and a lower expression level in r^+m^+ than r^-m^+ strains (right, greenish color) were categorized by COG codes (http://www.ncbi.nlm.nih.gov/COG/new/) (24,25), with an additional J' category. The colors show the significance of category overrepresentation based on the chi-squared test.

plasmid, as measured by optical density (OD) 660 nm, was indistinguishable from those with the r^- version and vector. Shifting the temperature to 42°C arrested the increase in the number of viable cells carrying the plasmid for all three strains within 2 h (Figure 1B). It also stopped the increase in the number of viable cells for the r^+ strain alone (Figure 1C), which caused a decline in cell viability of the r^+ strain as the cell mass increase did not immediately slow down (Figure 1D). These indicators of cell death were similar to those reported in previous studies (3).

Transcriptome analysis was performed 1, 2 and 3 h after the temperature shift and immediately before the shift (= 0 h) using the Affymetrix *E. coli* antisense genome array, which permits monitoring of transcript abundance from 4375 ORFs. The Spearman's rank correlations of the two independent measurements for each strain and time point were in the range of 0.973 to 0.991 (Supplementary Table 1).

Comparing transcript levels prior to cell death induction (0 h) between the r^+m^+ and r^-m^+ strains identified 412 genes that were significantly increased and 200 that were significantly decreased in the presence of the r^+ gene

(ANOVA, $P < 0.05$). At 1, 2 and 3 h after the shift, 51, 86 and 249 genes, respectively, exhibited a significant r^+-dependent increase in transcript levels, while 26, 174 and 399 genes, respectively, exhibited a r^+-dependent decrease. In total, we identified 1328 genes with statistically different transcript levels associated with the r^+ gene during at least at one time point (Supplementary Table 2). We validated the transcriptional changes of 12 selected genes using quantitative real-time RT–PCR (Supplementary Tables 3 and 4).

To examine how these 1328 genes are distributed with respect to function, we classified them into cluster of orthologous genes (COG) categories (http://www.ncbi.nlm.nih.gov/COG/new/) (24,25) (Figure 2; Supplementary Tables 2 and 5). We also performed a hierarchical analysis according to their temporal expression pattern (Figure 3).

r^+-Specific gene expression under permissive conditions

The transcript levels of several SOS genes (26,27) (*recA, sulA, umuD, ruvA, yebG, dinG* and *dinF*) were elevated ($r^+ > r^-$), as were the genes on e14, a UV-inducible defective prophage (28) (Figure 3, clusters 5, 6). In addition, increased expression was observed for genes involved in recombinational DNA repair [*recA, recR, ruvA,* and

0 1 2 3 (h) **Transcription (K)**   **Translation (J, J')**   **DNA replication, recombination, & repair (L)**   **Signal transduction mechanism (T)**   **Cell wall, membrane biogenesis (M)**   **Energy metabolism (C)**   **Unknown**

Post translational modification (O)   **Cell motility (N)**   **Carbihydrate metabolism (G)**

Stress response (T, O, S)   **Amino acid metabolism (E)**

Nucleotides metabolism (F)

cluster 1

ribosomal protein & RNA
rpsS, R
translation factor
efp, infA, prfA, B
rRNA modification
rluC, prmC, pcnB, dbpA, nrd, pcnB
tRNA modification
trmH, tgt
ARS
gltX, glyQ, poxA

chromosomal replication
holB, dnaB, G
reciombinational repair
recD, Q, R
DNA excision repair
nth

Two-component system
ypdAB, barA, evgA
narLX, rstA, yehTU
yfhK, dcuRS, phoB

polyliposaccharide
rfaB-F, I-K, S, kdsA, B
peptideglycan
mepA, mrcA, oppB-D, F
lipoprotein synthesis & transport
spr, lolC, E
outer membrane protein
ompF
lipidA
kdtA, lpxK
pillin synthesis
fimA, C, G
flagellar synthesis
fliC, D, F-J, L-R
flgE-J, L

electron transfer
nuoI, J

polyaminie synthesis
ansA, speC, G, potA, C, D
amino acid synthesis
proV, W
transporters
ydgI, yeeF, pheP

nucleotide metabolism
purB, D, H, L, U,
pyrC, D, F, H
adk, apt, cmk, gmk, gpt,
uraA, thyA, tdk, tmk

2

sigma
rpoD
RNA polymerase
rpoA, B

ribosomal protein & RNA
rplA, B, D, F, M-O, Q, R, V, Y
rpmB, D, E, H
rpsC-F, H, I, L, N, P, Q, U
translation factor
infB, rplY, rpsP, tsf
rRNA modification
rluA, B, rbfA, rsuA, prmA
rsmB, yebU, deaD
rRNA modification
trmD, truB, dusB, yjbN, yqcB

chromosomal replication
dnaA, priB, yicR, fis
reciombinational repair
exo, endA
DNA excision repair
xseB, xthA

Two-component system
uphA

Chaperons & protein folding
hscA,B (under sigma70)
yegQ

polyliposaccharide
rfaQ
peptideglycan
murC, mipA
outer membrane protein
ompX

lipidA
lpxM

electron transfer
cyoA, C, D, E
carbohydrate catabolism
aceE, F, ackA, ilvB, C
polyaminie synthesis
speDE, potB
transporters
lysP, ycdZ

nucleotide metabolism
carB, cdd, hpt, ndk, upp
purF
nudB, nupG

3

sigma
fliA

rRNA modification
hslR
rlmB

chromosomal replication
mukE, polA
reciombinational repair
recB

RpoE regulon
ydhK, yaiW
Two-component system
yfhA, phoPQ, glnL

pillin synthesis
fimF
flagellar synthesis
fliZ
flgC, D, K, N

electron transfer
nuoE, G, K
carbohydrate catabolism
manY, gatB, C, Y, mtlA,D, gpmI

nucleotide metabolism
codA, B

4

ribosomal protein & RNA
rplE, J-L, U, W, rpmI,
rpsG, J, K, M
ssrA, rrsA, C, G, H
rrfG, rrlA, C, E, G, H
translation factor
infC, fusA, tufA

rRNA modification
yfiF
rRNA modification
miaA, yfhO
tRNA
glnW, gltU-W

Fe-S assmbly
iscR, S, A

phospholipid biosynthesis
fadD, L
outer membrane protein
ompA

oxidative phosphrylation
atpA, B, F, H
carbohydrate catabolism
icd, sucA-D, acnA, sdhB,
narG, mdh

5

sigma
rpoH
predicted transcription factor
yidL, Z

DNA repair, SOS
nrvA, ssb, dinF
UV inducible
hlyE, yafO, N

UV inducible /prophage
ymfG, J-L, N, Q, S, T

Chaperons & protein folding
yegD (predicted)

lipidA
ddg

nucleotide metabolism
nrdA

agaA, cbrA, yaiS,
yidB, L, R, Z, yjbM,
ybfD,M, yqeH

6

ribosomal protein & RNA
rpsV (stress inducible)
UV inducible
recX, rnuC, yebF, G
ycgH, ydjM

DNA repair, SOS
umuC, D, sbmC, nrvB,
recA, sulA, lexA
dinB, D, G, uvrA, B, D

UV inducible /prophage
intE, ymfD, H, M, O, P, R
tfaE, stfE, lit
yfjH, yagM,lar

colanic acid biosynthesis
rcsA, wcaE

Osmotic stress inducible
osmC, Y, otsA, yciEF
Stress inducible (under RpoS)
yjbJ, katE, gadA, yhbO

msyB, setC, wcaE, yaaX, ybbD,
ycaC, yccR, yciE, F, ydiZ, yeaG,
ybiA,B, yncI ygcK, ygdR, yghA, ygiU, yegS
ybgS, yhcO, yncB yiaG, yibG, J, yieM, yjgB, ymgA

7

transcription factor
osmE

DNA repair, SOS
ydjQ

Stress inducible (RpoS)
yhiO, sodB, dsrB
Osmotic stress inducible
otsB

Fe-S assmbly
sufA-D, sufS
Oxidative stress response
yggE, yqhD, ariR, wrbA

dsrB, elaB, glcG, hcaC
ybaY, ybhB, P, yccJ, ycfR,
yegP, yehZ, ygaM, U, W
ygiW, yhiO, yjdI, J,
yqjD, yraR yaeR, yeaQ, ynhG

8

DNA repair, SOS
recN, yebE

Osmotic stress inducible
bdm

colanic acid biosynthesis
wcaF

ydcD, yrhB yadC, K, ybbC, yccE,
ycdU,ymgD, G, yggG, yhbV, ykgI,

9

sigma
rpoE

RpoE regulon
htrG, yfeK, S, rseA, B

anaearobic respiration
fumC

Small molecule metabolism
bioB, F

10

Stress inducible
spy
Osmotic stress inducible
osmB

RpoH regulon
dnaJ, K, grpE, ibpA, B
clpA, B, P, X, groL, S
hslV, htpG, X
lon

aceA, B, K, gltA
carbohydrate catabolism
glpC, F, X
galM, garK, araF
alsC, gatD, pykA
gcvH, P, mglC, srlB

amino acid transporter
adiC

11

sigma
rpoS

carbohydrate catabolism
ppc
nucleotide metabolism
nrdD-F, I, H

r^+/r^- 1/3   3

Figure 3. Hierarchical clustering of genes demonstrating differential expression with respect to r^+/r^-. (Left) The 1328 genes with a statistically significant difference in expression at, at least, one of the four time points were hierarchically clustered according to their temporal expression. The red (higher) and green (lower) colors of the bars represent the transcript ratio of r^+/r^-. (Right) Examples of the genes from each cluster are shown with their functional categories by COG as in Figure 2.

dinG (clusters 1, 5, 6)], many of which overlapped the SOS genes, as well as for those encoding DNases for excision repair [*xseB, nth* and *xthA* (clusters 1, 2)], those with a role in chromosomal replication [*dnaA, priB, holB, yicR* and *fis* (clusters 1, 2)], and those involved in nucleotide metabolism (clusters 1, 2), which might provide precursors for DNA repair/replication and transcription.

Transcripts of many genes encoding ribosomal proteins, rRNAs, translational factors, tRNA and rRNA modification enzymes were increased in the r^+ strain, suggesting activation of translation (Figure 2 and 3, clusters 1, 2 and 4). In relation to this, the transcript levels of *rpoA,* *rpoB* and *rpoD* for RNA polymerase core subunits were moderately increased (cluster 2). Polyamine biosynthesis and transport gene expression was shown to increase (clusters 1, 2), which might also contribute to translation (29).

Central metabolism appeared to be repressed ($r^+ < r^-$), as several genes involved in glycolysis (*fbaB* and *pykA*) and entrance into the TCA cycle (*gltA* and *fumC*) were inactivated. However, the expression of genes that play a role in ATP production (*cyoACDE, cydB* and *atpABHF*) was increased, perhaps for compensation (Figure 2 and 3).

These features were similar to those seen during recovery from UV-irradiation (30), in which it has been

reported that SOS induction was prominent and the expression of many genes involved in replication, nucleotide metabolism, protein translation and polyamine transport increased, while the expression of those associated with central metabolism decreased. Genes that are reported to have increased or decreased transcription by UV exposure are marked in Supplementary Table 2 (30).

Genes related to reactive oxygen were induced, such as *pqiAB* and *sodB*. *pqiAB* is induced by paraquat and other superoxide generators. SodB is an iron-containing super peroxide dismutase protecting cytoplasmic superoxide-sensitive proteins. Genes involved in iron-sulfur cluster assembly (*iscS, iscA, hscBA, iscX, ytfE*) were also induced. Several genes for iron ion transporter (*fepA, fes, cirA, fhuF*) were activated at the same time. A few of these expressional changes were observed under UV irradiation, though not emphasized by the authors (30). Recently, a common process was suggested for cell death by all major classes of bacteriocidal antibiotics, including quinolones driving DNA double-strand breakage, whereby superoxide is formed, releasing iron from iron–sulfur clusters and resulting in hydroxyl radical formation via the Fenton reaction (31). At 0 h, the involvement of this mechanism is suggested, as several superoxide-reactive genes were induced and genes related to iron-sulfur repair and synthesis were activated, which suggests restoration of consumed iron–sulfur cluster. Additionally, lowered expression of several TCA cycle genes and increased expression of electron transfer genes do not conflict with the possible formation of superoxides (31).

We noticed other features not seen in UV-irradiated cells; transcripts of genes under σ^{32} were decreased and expression of the genes related to cell-surface proteins were increased by the r^+ gene (Figures and 2, 3; Supplementary Table 2). The latter includes genes related to envelope formation such as those encoding outer membrane proteins (*ompAX*) and those involved in the synthesis of polyliposaccharide (*rfaCQI* and *kdsAB*), O-antigen (*glf, rfbCB* and *rffG*), peptideglycan (*murC, mipA* and *mrcA*) and lipid A (*lpxKM* and *ddg*) (Figure 3, clusters 1, 2, 4, 5).

These results suggest that, even under the permissive condition, the chromosome is attacked by the restriction endonuclease, and the host cells respond to and overcome damage to maintain their growth.

r^+-Specific gene expression during the death process

One hour after the shift. The r^+-dependent transcsriptome change was relatively small (Figures 2 and 3; Supplementary Tables 2 and 5); however, an r^+-dependent increase of *rpoS* transcript was noticeable (Figure 3; Supplementary Table 2).

Two hour after the shift. The r^+-dependent gene expression pattern was similar to that seen under permissive conditions as a whole (Figures 2 and 3) with the following interesting exceptions: more SOS genes, i.e. *recQ, umuC, sbmC, uvrD* and *recN*, were induced (Figure 3, clusters 6, 8); genes for chromosome replication and nucleotide metabolism became inactive (clusters 1–3); genes involved

in translation and polyamine biosynthesis were not significantly active (clusters 1, 2), and genes encoding transcription factors were activated instead (Figure 2), which may enable a more specific control of gene expression; energy production appeared to be more severely inactivated, as suggested by the transcript decrease in genes throughout the TCA cycle (clusters 4, 10), and genes for ATP generation were not activated (cluster 4); σ^E-regulon genes (cluster 9), categorized in signal transduction by COG were significantly activated (Figures 2 and 3; Supplementary Table 2).

These features might reflect a fundamental difference between the processes of cellular recovery and death. Particularly, of these genes, it is expected that those unaffected by the r^+ gene before the induction of death are directly involved in the death process. Among 198 such genes, *recN, umuC, sbmC, rpoE, htrG, yfeK, yfeS, adiC, codB* and *yhiL* had remarkably higher (> 1.4 fold), and *sucD* and *ydcU* had remarkably lower (< 0.6 fold) transcript levels, respectively, in the r^+ strain (Supplementary Table 2; Figure 3). Actually, several of these genes are related to some features in cell death. RecN is involved in repair of DNA double-strand breaks, which accumulate in the chromosomes after the loss of RM gene complexes (3,5). UmuC delays the resumption of DNA replication after DNA damage (32), in addition to participating in *trans*-lesion DNA synthesis (33). SbmC, an inhibitor of DNA gyrase, can cause cell filamentation and growth defect when over-expressed (34). *sucD* codes for α subunit of succinyl-CoA synthetase, a key enzyme in TCA cycle, decreased expression of which should inactivate host cell growth. The others out of these 12 genes could be somehow involved in the cell death process. Especially, increased transcription of *rpoE* (for $\sigma^E = \sigma^{24}$) and its regulon genes, *htrG* and *yfeKS*, is interesting, because σ^E was suggested to direct dead cell lysis in the early stationary phase (35).

Three hour after the shift. The increase of mass (OD660) of the r^+ cells started slowing down (Figure 1D). SOS induction became more intensive than 2 h after the shift (Figure 2; Figure 3, clusters 5–8). Transcript levels were greatly increased for stress response genes, including σ^S-regulon genes (*yjbJ, yhiO, yhbO, wrbA, sodC, rpsV, katE* and *gadA*), osmotic stress-inducible genes (*bdm, yciEF, osmE*, and its regulon genes, *otsAB, osmC* and *osmY*), periplasmic stress-inducible gene (*spy*), and oxidative stress-inducible genes (*yggE, yqhE, ariR*) (Figure 3, clusters 6–8, 10). Among the σ^S-regulon genes, *sodC* (superoxide dismutase), *katE* (catalase), *wrbA* (quinine oxidoreductase) and *yhbO* are related to oxidative stress, and transcription of the operon for iron-sulfur cluster biogenesis (*sufABCD, sufS*) were increased, both suggesting hydroxyl radical formation (Figure 3, cluster 7).

Genes involved in nucleotide metabolism and chromosome replication were now repressed (Figures 2 and 3). Other genes repressed 3 h after the shift included *fliA*, encoding σ^{28} for motility-related genes, genes for flagellar and pilin synthesis (Figure 3, clusters 1, 3), those involved in energy metabolism, and those for outer membrane biogenesis (clusters 1, 2). Decreased expression of genes
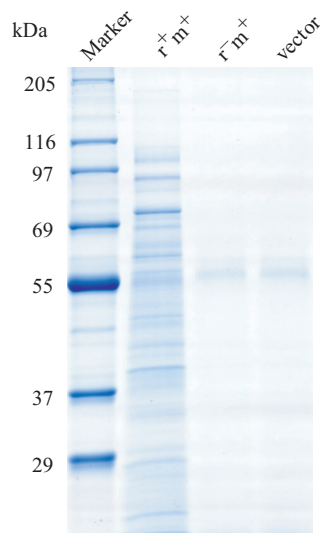
Figure 4. Protein release associated with the RM-mediated cell death. Protein fractions outside of the cells were obtained 6 h after the shift and analyzed by SDS–PAGE. The bacterial culture was centrifuged at 17 000g for 5 min, the supernatant was collected. The protein fraction of MW >10 kDa obtained from 0.2 ml of the supernatant was applied to 10% SDS–PAGE.

for many two-component systems (Figure 2; Figure 3, clusters 1–3) suggested that many signal transduction routes from the cell surface toward the genome have been weakened.

In this way, increased expression was observed for the genes related to spheroplast formation and lysis, *spy* likely encoding a stress protein for spheroplast, and *hlyE* encoding HlyE protein causing lysis of mammalian cells through its pore-forming activity (36), respectively, along with decreased expression for the genes for outer membrane biogenesis. It was impressive that the genes related to biofilm development (37) were activated on the other hand.

The *relA* (Figure 3, cluster 3) and *spoT* (cluster 1) related to nutritional stress were repressed, but no transcriptional changes were observed in *mazEF*, which encodes toxin/antitoxin proteins implicated in programmed death (38).

Overall, the most noticeable was that at 2 h time point, changes were observed mainly in the genes related to chromosome repair, while 3h after the death induction, many genes related to cell surface were affected in addition. It appeared as if the signal of DNA damage was transmitted to cell surface with time (Figure 6).

Comparison with ofloxacin-mediated and ampicillin-mediated deaths

We noticed a resemblance between the present transcriptome and those in dying *E. coli* cells exposed to two different antibiotics: ofloxacin and ampicillin (39) (Figure 6; Supplementary Table 2).

Ofloxacin binds topoisomerases and causes DNA damage. It induces SOS genes in the same manner as the RM gene complexes (39). RM-mediated induction of

SbmC, a DNA gyrase inhibitor, might explain the similarity. DNA gyrase is also the target of Ccd family of the classical proteic toxin–antitoxin type of postsegregational killing gene complexes (40,41). They reduce DNA synthesis, activates the SOS regulon, and induces filamentation, anucleation and, finally, cell death (40).

In contrast, ampicillin inhibits cell-wall synthesis (42) and elicits rapid autolysis. It induces stress response genes including osmotic stress, periplasmic stress and oxidative stress genes (*sodC, katE, yggE, yhbO, wrbA*) and colanic-acid synthesis genes (*rcsA* and *wca*), which are related to biofilm development, as well as many uncharacterized genes, and represses *rfa* genes for lipopolysaccharides (39). These changes characteristic to ampicillin-induced death were also observed in the RM-mediated death at the 3 h time point. Biofilm formation could help the survival of the persisting subpopulation of bacterial cells.

Transcriptional changes common to ofloxacin and ampicillin exposures also overlapped with the r^+-dependent changes. Of the 22 genes induced both by ampicillin and ofloxacin (39), 9 (*rpsV, osmC, ybhB, yciEF, yebE, yjbJ, ykfE* and *dsrB*) were induced by the r^+ gene 3 h after death induction, many of which were related to SOS response and stress responses under σ^S and OsmC. Of the 139 genes repressed by both agents (39), 61 were also repressed by r^+ 3 h after the temperature shift. Among these were many genes related to protein synthesis, motility, energy metabolism, envelope biogenesis and chromosome replication (clusters 1 and 3) (Supplementary Table 2). These expressional changes could trigger common processes in dying bacteria.

Thus, the transcriptome profile of RM-mediated death combines the transcriptome features of oflaxacin-induced and ampicillin-induced deaths, which would be consistent with involvement of DNA damage and its transduction to cell-surface alteration (Figure 6).

Cell lysis and protein release into the medium

Our transcriptome analysis revealed elevated expression of *rpoE* and σ^E-regulon genes (see above). As σ^E was suggested to direct stationary phase lysis (35), we investigated whether cell lysis occurs in the present RM-mediated death. We noted that the increase in OD slowed down in cells that had lost the r^+ plasmid (Figure 1D).

Cell lysis was observed microscopically (Supplementary Figure 7). After filamentation as reported for the EcoRI gene complex (4), cells with reduced density and cell debris aggregates were observed (Supplementary Figure 7). We also observed the appearance of cells with small buds (Supplementary Figure 7, 6 h, third photograph), which are similar to those found after penicillin treatment (43). This provided another line of evidence for the similarity between RM-mediated death and the penicillin/ampicillin-mediated death.

We found r^+-dependent accumulation of proteins in the medium (Figure 4). We observed a 6.6-fold higher protein (>10 kDa) concentration in the medium with the

r$^+$ strain than with the r$^-$ strain. These results suggested that the r$^+$ cells released their proteins upon lysis.

Expression of σ^E-regulon genes leading to death, lysis and protein release

We next investigated activity of those individual σ^E-regulon genes with elevated expression during the RM-mediated death, by induction with IPTG from ASKA-plasmids (18). We had found that *htrG*, *yfeK* and *yfeS* shared a similar transcriptional pattern with
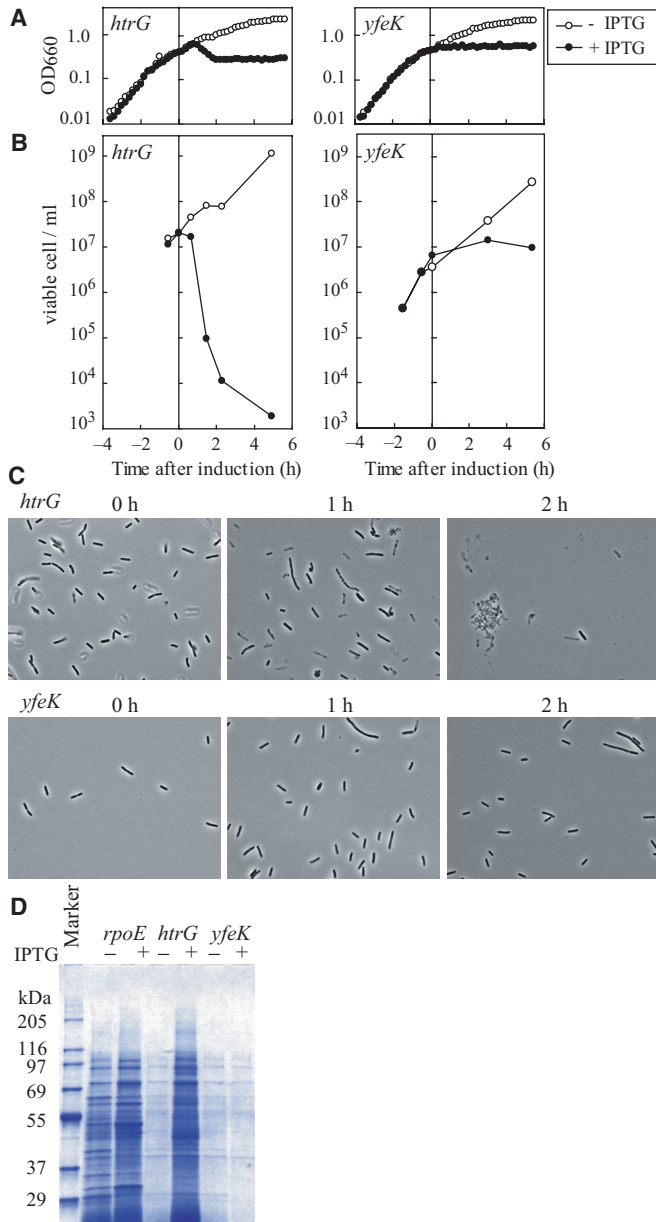
Figure 5. Effects of over-expression of σ^E regulon genes. (**A**) OD$_{660}$. (**B**) Viable cell counts. (**C**) Cell morphology. (**D**) Proteins outside of the cells were analyzed 3.1 h after IPTG addition for expression of *rpoE*, *htrG* and *yfeK*. The bacterial culture was centrifuged at 17 000*g* for 5 min, the supernatant was collected. The protein fraction of MW >10 kDa obtained from 0.2 ml of the supernatant was applied to 10% SDS–PAGE.

rpoE: activation of which was significant 2 h after the shift (Figure 3, cluster 9). Cell lysis began within 1 h of *htrG* induction, after which the OD dropped to a lower level (Figure 5A), while cell viability eventually decreased 10 000-fold (Figure 5B). Cell budding and aggregation of lysed cells were observed microscopically 1 h after induction, and most of the cells had aggregated by 2 h after induction (Figure 5C). Proteins accumulated in the medium, as shown by SDS–PAGE (Figure 5D), and the protein concentration was elevated 5.1-fold there. Immediately following *yfeK* induction, the OD and viable cell counts stopped increasing and remained constant thereafter. Lysed cells were not seen microscopically, although cells of various lengths were observed, suggesting that cell division might have been affected, as seen after (within 2 h) the RM-mediated death induction (Supplementary Figure 7). Accumulation of proteins in the medium was not detected (Figure 5). Over-expression of *yfeS*, located downstream of *yfeK*, did not affect OD or cell viability (data not shown).

Induction of *rpoE* led to cell lysis (Figure 5D), as previously reported (35). The σ^E-mediated lysis is presumably achieved through combined effect of multiple σ^E-regulon genes, as a Δ*htrG* mutation did not significantly alter the decline of OD upon *rpoE* induction (data not shown). Actually, among the σ^E-regulon genes that were up-regulated 1 h (*ydhK* and *yaiW*) or 3 h (*smpA*, *yidQ*, and *yidR*) after the temperature shift, induction of *ydhK*, *yaiW*, *smpA* and *yidQ* led to decrease in cell viability (Supplementary Figure 8). Induction of *yaiW* and *smpA* caused decrease in OD, though less dramatically than *htrG* over-expression, suggesting lysis (Supplementary Figure 8).

According to a database (GenoBase ver.6, http://ecoli. aist-nara.ac.jp/), out of those 8 σ^E-regulon genes mentioned here (*htrG, yfeK, yfeS, ydhK, yaiW, smpA, yidQ* and *yidR*), growth on LB agar plates after IPTG induction of the 6 genes except for *yidQ* and *yidR* has been examined, and almost no growth was observed with all 6 genes. Our results agreed with their observations except for *yfeS*, over-expression of which did not affect the host growth in the liquid medium.

DISCUSSION

Our main interest is to elucidate genetic processes of the death/lysis triggered by DNA damage. In *E. coli*, DNA damage is sensed by RecA protein, which induces SOS genes. Activating the *sulA* gene, one of the SOS genes, causes inhibition of cell division. If the DNA damage is not repaired, the cell will die and will be lysed. This mechanism is supposed to be important to elimination of defective cells as well as to aid repair. Though several genes responsible for death or lysis such as *hip* (high persistence) genes, *amiB* (encoding autolysin) (1) and *rpoE* have been reported (35), pathway from DNA damage to death/lysis has remained elusive.

Here, we analyzed the temporal changes of the transcriptome in cell death triggered with DNA damage by the disturbance of the RM gene complex in *E. coli*.
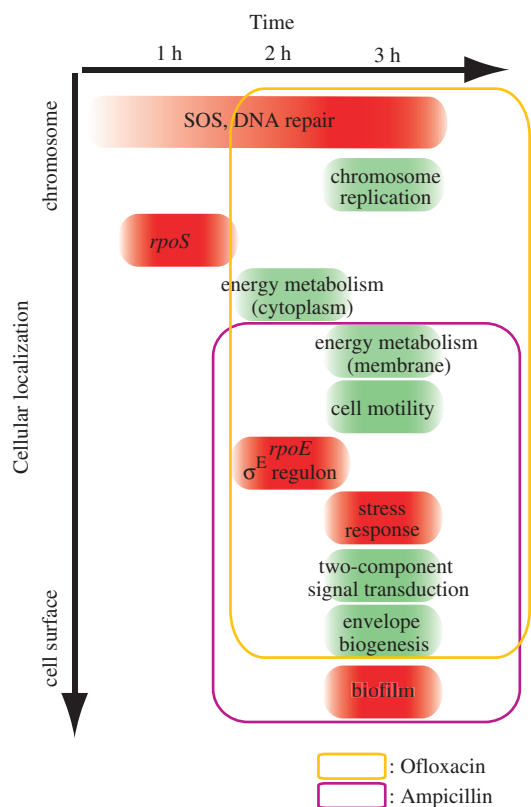
Figure 6. Summary of the transcriptome changes during the death process following the loss of RM gene complex. Induction (red) and repression (green) are shown. Transcriptome features reported for ofloxacin-induced (yellow) and ampicillin-induced (purple) deaths are indicated.

Our results suggest the following events during the death process: restriction endonuclease-mediated chromosomal DNA cleavage; SOS induction; attempts to repair DNA damage; DNA gyrase inhibition; induction of the *rpoE* regulon; periplasm changes; induction of stress responses, including periplasmic, osmotic and oxidative stress responses; repression of chromosome replication; inactivation of energy metabolism and cell motility; disintegration of the membrane, followed by lysis and release of cellular protein contents into the environment (Figure 6). Although some of these steps have been observed in other forms of bacterial cell death (35,39,40,41), to our knowledge, this work is the first to suggest that signal of chromosomal DNA damage might be transmitted to cell surface via changes in gene expression.

Among these transcriptional changes, activation of σ^E regulon was especially interesting. σ^E controls the envelope stress response and is also involved in dead cell lysis in stationary phase in *E. coli* (35). As expected from the induction of the *rpoE* gene, the present RM-mediated cell death was accompanied by lysis. In addition, σ^E regulon is supposed to be essential for the integrity of the outer membrane, monitoring and responding to imbalance in the components of lipopolysaccharides and outer membrane proteins to maintain envelope homeostasis (44). We demonstrated that over-expression of σ^E-regulon genes activated during the death process,

including *htrG*, lead to cell lysis. *htrG* product is predicted as an outer membrane protein (27). We do not yet know whether increased expression of *htrG* and other envelope-related genes under σ^E control causes disintegration of the outer membrane as reported with some outer membrane components (45).

Our results demonstrate that RM-mediated postsegregational cell killing is accompanied by cell lysis and release of cellular proteins into the environment. It would be of interest to determine whether proteins from these dead cells serve as nutrients for the surviving cells that retain the RM gene complex as observed in starving *Bacillus subtilis* (46) and other bacteria (47,48).

To understand the death mechanism, determining direct trigger of each transcriptional change in the sequence of events seen in our analysis (Figure 6) is a challenge. A recent study suggested that all major classes of bacteriocidal antibiotics, including quinolones driving DNA double-strand breakage, may cause cell death by a common mechanism generating hydroxyl oxide (31). Some features of our transcriptome changes, such as induction of oxidative stress response and iron-sulfur cluster assembly genes, suggest that this mechanism might be triggered by the RM-mediated chromosomal DNA damage. The hydroxyl radical is supposed to damage DNA and proteins, and induce SOS response and other cellular processes, thereby the signal of chromosomal DNA damage might be amplified and conveyed. It is also notable that reactive oxygen formation does not necessarily lead to cell death if repair is sufficient, as we saw at 0 h.

To elucidate the death pathway, we examined possible involvement of SOS induction, stress response, and σ^E-regulon induction (Figure 6) in the death process. We constructed mutants altered in these and tested them for cell death. In the present transcriptome analysis, asynchrony and heterogeneity in the population could have arisen because we used a ts plasmid and a temperature shift procedure to trigger loss of the plasmid to induce cell death. However, it would be safely said that chromosomal damage was induced in the population as a whole because a majority of the cells in the r$^+$ population showed filamentous morphology (4) (Compare Figure 1C and D; Supplementary Figure 8). We used the arabinose inducible promoter for this mutant analysis (22) in order to realize more synchronized death induction than in the temperature shift protocol in the transcriptome experiments. Host cell death/lysis was induced by over-expressing *paeR7IR* gene on pBAD33 plasmid (37). In MG1655, cell viability decreased 100-fold and 1000-fold at 37°C and 42°C, respectively, one h after the *paeR7IR* induction. We examined MG1655*lexA3*(Ind$^-$), which carries an uncleavable LexA protein, MG1665$\Delta rpoS$, and MG1655$\Delta htrG\Delta ydhJ\Delta ydhK\Delta yaiW\Delta smpA$, in which the σ^E-regulon genes activated during the RM-mediated death and shown to cause cell death/lysis upon over-expression were all deleted. Significant difference in cell viability or lysis, as detected by the accumulation of proteins in the medium, were not observed in any of these mutants compared to MG1655, both at 37°C and 42°C (data not shown). This result suggests that the cell death

would be realized by multiple pathways. Presence of multiple pathways would be a feature of programmed deaths in unicellular organisms because a single pathway should be easily blocked by a mutation that allows survival of such a 'cheater' mutant lineage (49).

The death phenomenon would be further complicated by the presence of other sorts of subpopulations. In bacteria, existence of persister cells, which constitute a small part of the populations treated with a range of deleterious factors such as UV irradiation and antibiotics (1), has been known. *E. coli* stationary phase culture has also been reported to contain more than 20 subpopulations, in which presence of cells on various pathways toward different cell fates such as dormancy and cell death has been suggested (50). The cell-to-cell communication in such a heterogeneous population may be important to the death process (51,52). We need to remember that such heterogeneity in the cell fates, survival or death, would be another feature of the programmed deaths in unicellular microorganisms.

In our transcriptome, some sets of genes were possibly activated or inactivated in only a subset of the population. 3 h after the cell death induction, for example, viability of the population began to decline. The resulting transcriptome may have represented only the surviving cells present in the population, which could even include cells with different states, dying and surviving. This would be one reason why genes related to protecting mechanisms were especially enriched among the activated genes.

E. coli cells might have multiple mechanisms which could cause cell death/lysis when activated, and also have protecting mechanisms for overcoming them. To elucidate the pathways as well as the branch points of death and survival is an interesting issue. For this purpose, separating each subpopulation or single cells to analyze temporal changes in transcription is to be addressed.

In RM-mediated cell death in *E. coli*, involvement of reactive oxygen is suggested. Reactive oxygen species is known to play an important role in eukaryotic apoptosis. Budding yeast shows up-regulation of the genes related to mitochondria, oxidative stress, membrane and cell wall synthesis in addition to DNA damage response, DNA repair and cell cycle regulation after mutational arrest of chromosome replication (53). Reactive oxygen species is involved also in *S. pombe* apoptosis (54). Ionizing radiation induces stress response genes and redox-related genes in *C. elegans* apoptosis (55) and genes for DNA repair genes and redox-related genes in *Drosophila* (56). The production of reactive oxygen species has been linked with virtually all sorts of plant stress responses and may serve as a signal to initiate cell death pathways in plants as well (57). Thus, the process of radical formation and the subsequent DNA damage by reactive oxygen species seem common from bacteria to eukaryotes.

Further comparisons with related forms of death and a detailed analysis of each step of the death pathway are necessary to understand the mechanism and biological significance of the cell death in the simplest forms of life.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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