ABSTRACT

Programmed cell death (PCD) under certain conditions is one of the features of bacterial altruism. Given the bacterial diversity and varied life style, different PCD mechanisms must be operational that remain largely unexplored. We describe restriction endonuclease (REase) mediated cell death by an apoptotic pathway, beneficial for isogenic bacterial communities. Cell death is pronounced in stationary phase and when the enzyme exhibits promiscuous DNA cleavage activity. We have elucidated the molecular mechanism of REase mediated cell killing and demonstrate that released nutrients from dying cells support the growth of the remaining cells in the population. These findings illustrate a new intracellular moonlighting role for REases which are otherwise established host defence arsenals. REase induced PCD appears to be a cellular design to replenish nutrients for cells undergoing starvation stress and the phenomenon could be wide spread in bacteria, given the abundance of restriction–modification (R–M) systems in the microbial population.

INTRODUCTION

Microorganisms have evolved various strategies that allow them to inhabit almost any niche. Metabolic flexibility and the capacity for adaptation to different environmental cues underscore their success. Ability to respond as a population to changing environmental conditions is another crucial factor for their survival. Bacteria employ a variety of developmental programs for their diverse social behavior (1). Although best exemplified in organisms such as Myxococcus xanthus (2), Bacillus subtilis, Pseudomonas aeruginosa, Vibrio cholerae and a few other species, it is now apparent that a large number of bacteria belonging to different groups exhibit community behavior under certain conditions and circumstances (1,3). Well studied quorum sensing and biofilm formation often exhibit interlinked features of bacterial social life (3,4). The studies on switch from planktonic to multicellular lifestyle and the associated altered gene expression pattern have led to a paradigm shift in our understanding of the social behavior in bacteria (4). Under certain hostile conditions, bacteria undergo programmed cell death (PCD), defined as the death of any cell which is mediated by an intracellular program (5,6). Different factors and conditions are likely to be associated with PCD and only a few of them are well documented (7). The death of mother cell during sporulation in Bacillus and cell lysis during Myxococcus fruiting body development are well studied examples. PCD mediated by toxin and antitoxin modules under stressful conditions and antibiotics action are other emerging examples. To better understand the biological significance of PCD, it is important to investigate different molecular mediators involved in the process. Here, we describe restriction endonuclease (REase) mediated PCD and its likely benefit for the bacterial population.

R–M systems are ubiquitous and diverse, serving as innate immunity component of bacteria by targeting the invading genomes. It is also apparent that cellular defence by R–M systems is not an infallible mechanism to counter invading bacteriophages. Phages elaborate diverse anti-restriction strategies; thus, host and virus are continuously engaged in the co-evolutionary arms race (8). Given their wide distribution and the presence of several enzymes in many genomes, REases are implicated to have other cellular roles (9). These functions range from genetic exchange between the bacteria through DNA uptake, homologous recombination (10), nutrition for viral propagation (11) and virulence. Many of the R–M systems also appear to exhibit selfish behavior (12).

We have considered a new intracellular role for REases due to the intrinsic promiscuity exhibited by a number of R–M systems (9). Would the inherent promiscuous nature of REases have consequences for host cell survival under certain conditions? Our studies provide evidence for REase mediated altruistic behavior in bacteria. Endonuclease triggered DNA damage leads to cellular apoptosis which appears to provide benefit for the survival of the rest of the population. Such a moonlighting function for these enzymes could have far reaching implications in community behavior of bacteria.

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MATeRIAls ANd Methods

Bacterial Strains

Escherichia coli (E. coli) MG1655 strain was transformed with low copy plasmid pACYC184 (13) harbouring M.KpnI (M), entire KpnI R–M (WT) (14) or KpnI R–M with high fidelity mutation (D163I) in R gene (HF). The genes (r.KpnI and m.KpnI) are expressed from their own endogenous promoters. The strains and genotypes of bacteria and plasmids used in this study are listed in Supplementary Table S1.

Confocal microscopy and FACS analysis

To examine the cells using confocal microscopy, DiBAC4(3) (Sigma) or the Live/Dead kit was used. The Live/Dead kit (Invitrogen) contains Propidium iodide (PI) and Syto9. PI stained dead cells and gives green fluorescence upon binding to nucleic acid. E. coli MG1655 cells harbouring WT, HF and M were grown to different time points. The cells were pelleted and washed twice with 1× PBS (phosphate buffered saline). To analyse cell morphology, the samples were stained with 0.1 mg/ml 4′-6-diamidino-2-phenylindole (DAPI) staining (cells appear in blue color). For DiBAC4(3) staining (Invitrogen), 10 μl of DiBAC4(3) (1 mg/ml) in ethanol was added. For the Live/Dead staining, 10 μl of a 1:1 mixture of PI and Syto9 was used. Samples were incubated for 15 min at room temperature and washed twice in 1× PBS. The cells were visualized in a ZEISS LSM-710 confocal microscope under a 100× objective. In order to observe DiBAC4(3) (green) and Syto9 (green), the argon laser with excitation at 488 nm and emission at 515 nm was used. To observe PI (red), a HeNe laser with excitation at 543 nm and emission at 530 nm was used. To compensate for the overlapped wavelength between Syto9 and PI, a sequential scanning was carried out. The total number of bacterial cells (n = 800) were counted for the quantification of PI staining. Fraction of PI stained cells/10 000 or DiBAC4(3) staining cells/5000 cells were analysed using fluorescence intensity by FACS and results were plotted using FCS Express V3 software. All the experiments were repeated at least three times independently and error bars indicate standard deviation (SD).

Real-time PCR analysis

Cells were grown to different growth phases (exponential phase: 6 h; stationary phase: 16 h) RNA extraction was carried out by using RNA protect bacteria reagent (Qiagen) and RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. RNA concentration and purity (A260/280) was measured using Nano Drop ND-1000 spectrophotometer. Ten microgram of total RNA was subjected to DNase I treatment (Roche) as per manufacturer’s instructions followed by cDNA synthesis using an ABI high capacity cDNA synthesis kit. Two microgram of DNase I treated RNA and random primers were used to synthesize cDNA. For quantitative real-time PCR (qPCR), cDNA was diluted 10-fold and quantitation was carried out in 10 μl reaction using SYBR Green Master Mix (Fermentas) in a 7500 Fast Real-Time PCR system (Applied Biosystems). The qPCR cycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 57°C for 30 s and 72°C for 20 s. All reactions were repeated at least three times independently to ensure the reproducibility of the results. idrF transcript levels were used as an endogenous control for RNA levels in the samples (15). Relative mRNA levels of genes in WT and HF were calculated after normalizing with mRNA level from M. Graphs were plotted as a ratio of relative mRNA levels in stationary phase versus exponential phase. Statistical significance was calculated using t-test. The primers used in this study are listed in Supplementary Table S2A.

Detection of OH* formation

To detect OH*, the molecule hydroxyphenyl fluorescein (HPF), which fluoresces only when it reacts with OH* was employed. E. coli MG1655 cells harbouring WT, HF or M were grown; cells were pelleted and washed twice with 1× PBS. HPF was added to the samples to a final concentration of 10 μM and cells were incubated at the room temperature for 1 h, washed twice with 1× PBS (pH 7.2). The fraction of HPF stained cells/5000 cells was analysed using fluorescence intensity by FACS with BD FACSVerse™ machine with a 488-nm argon laser for excitation and a 530±15-nm emission filter, and the results were plotted using FCS Express V3 software. Each experiment was repeated at least three times and error bars indicate SD.

Complex I and complex II activity of oxidative respiration

Cells were grown to exponential phase (6 h) and stationary phase (16 h), aliquots were taken at different time intervals. Cell pellets were resuspended in 50 mM morpholineethanesulfonic acid (MES) buffer (pH 6.0) with 10% glycerol and 1 mg/ml lysozyme and incubated with vigorous shaking at room temperature for 5 min, sonicated for 10 s twice. Cell extracts prepared were used for NADH dehydrogenase I activity as described (16). To prepare membrane fraction, whole-cell extract was centrifuged (45 000 g, 2 h) and the pellet was suspended in the same buffer. This membrane fraction was used to test succinate dehydrogenase activity (16). Protein concentrations were determined by Bradford method using BSA as a standard. NADH dehydrogenase total activity (Respiratory Complex I + NdhII) was assayed spectrophotometrically at 30°C by following the absorbance at 340 nm (εNADH = 6.22 mM−1. cm−1), in a reaction mixture containing 50 mM MES (pH 6.0), 10% glycerol, 200 μM NADH. Succinate dehydrogenase activity was assayed from the membrane fraction by monitoring dichlorophenol indophenol (DCPIP) reduction. The activity was determined spectrophotometrically at 30°C by following the phenazine ethosulfate (PES)-coupled reduction of DCPIP at 600 nm, in a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 4 mM succinate, 1 mM KCN, 400 μM PES and 50 μM DCPIP (16). These data represent the results from three independent experiments and error bars indicate SD. Statistical significance was calculated using two way ANOVA coupled with Bonferroni’s post-test and is represented by an asterisk (*) [P-value, P < 0.001 (***)] and P > 0.05 (not significant—ns)].

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Western blot analysis

Briefly, *Klebsiella pneumoniae* (*K. pneumoniae*) OK8 cell lysate or *E. coli* MG1655 cells expressing both MTase and REase from their respective endogenous promoters (WT) were grown to different growth phases. Cells were harvested by centrifugation, resuspended in 3 ml of extraction buffer [10 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM 2-mercaptoethanol], and disrupted by sonication. Cell debris was removed by centrifugation. Equal amounts (250 μg) of total cell lysate were resolved by SDS–12% PAGE and detected by immunoblotting with respective polyclonal antibodies. R.KpnI polyclonal antibody was generated from mice whereas M.KpnI and Ribosomal recycling factor (RRF) polyclonal antibodies were generated from rabbit, respectively. RRF was used as an endogenous loading control and the experiments were repeated three times.

Long term growth experiments

Overnight grown cultures of WT, HF and M were subcultured and then diluted to O.D 0.1. For long-term growth studies, individual cells expressing WT, HF or M were grown till 144 h and CFU analysis was carried out by plating on solid agar containing Chloramphenicol (25 μg/ml) at different time points. Graphs were plotted as CFU/ml against time and error bars indicate SD. Results are presented from three independent experiments.

Conditioned medium experiments

Filter-sterilized conditioned medium was prepared following the protocol described previously (17). LB medium (50 ml) was inoculated with cells from a fresh overnight culture of WT, HF and M in 250-ml Erlenmeyer flasks at 37°C with vigorous aeration. After 5 days (120 h), cells were pelleted and the supernatant was collected. The supernatant was filtered through a 0.2 μm NYL filter unit (Nalgene) to remove intact cells. This conditioned medium was utilized for the growth of new culture. Freshly grown *E. coli* MG1655 (pACYC 184) cells of 1 × 10⁸ CFU/ml were inoculated into the conditioned medium and aliquots were taken at different time points. O.D. was monitored at 595 nm and CFU analysis was carried out at different time points. All experiments were repeated 5 times and data are presented from three independent biological replicates. Error bars indicate SD.

Statistical analysis

Levels of significance for comparison among the samples were determined by Student’s *t* test distribution and analysis of variance (ANOVA) (18). The represented graphical data are expressed as the SD from three independent experiments unless mentioned otherwise. *P*-values ≤0.05 were considered to be significant. Prism 5.0 software (Graph Pad Software, Inc., USA) was used for all the statistical analysis.

RESULTS

REase mediated bacterial cell death

The primary determinants of the cell fate in bacterial populations are not completely uncovered. We hypothesized that R–M systems might be important contributors of cell fate under certain circumstances. A Type II R–M system comprises two components, a methyltransferase (MTase), an epigenetic determinant and a REase, a DNA cleavage factor. We have chosen well studied KpnI R–M system for the present analysis. The genes for REase and MTase are arranged divergently in the KpnI R–M locus and the intergenic region contains all the regulatory elements required for the expression of both the genes (Figure 1A) (19). The MTase of the KpnI R–M system is an N⁰-adenine methyltransferase and is highly sequence specific (20). The REase exhibits a high degree of promiscuous activity but its single amino acid variant (D163I) shows high fidelity compared to WT, i.e. cleaves only at canonical sites (20–22). *E. coli* MG1655 cells harbouring either entire KpnI R–M system (WT) or D163I mutation in the R gene along with M (HF) or the MTase alone (M) in a low copy number plasmid were employed to understand the role of MTase and REase, if any, in cellular physiology. The cells harbouring M alone did not show significant difference in growth compared to vector containing cells. Surprisingly, the viability assays showed that the WT had reduced growth compared to the other two strains (Supplementary Figure S1). In the microscopic analysis, a subpopulation of cells appeared to be dead (PI stained cells – red color) (Figure 1B). The death was more pronounced in cells expressing WT compared to others at late logarithmic and stationary phases (compare Figure 1C and D). FACS analysis also revealed a higher percentage of dead cell population in REase expressing cells during stationary phase (Figure 1D). Microscopic analysis of cells expressing HindIII R–M system also showed similar results (Supplementary Figure S2). The REase expressing cells showed filamentation and increased dead cells compared to MTase alone cells.

REase induces extensive DNA damage

From the above results, we surmise that REase catalysed extensive DNA cleavage would have caused the cell death. DNA damage, if any, caused by REase would have also induced SOS response, leading to RecA activation and LexA proteolysis. We measured the SOS response in the WT, HF and M cells using P<sub>amiD</sub>:lacZ fusion as a reporter (23). In the stationary phase, WT activated the highest SOS response (measured as β-galactosidase activity) when compared to HF or M cells (Figure 2A). This suggested that the WT R.KpnI cleaved the DNA at the non-cognate sites resulting in increased accumulation of double-stranded breaks leading to SOS induction. Further, the genes under the SOS regulon, viz., *recA*, *lexA*, *sulA* and *recN* were up-regulated in WT by 2.5–4.5-fold compared to HF (values normalized to levels in M) indicating that a functional SOS response pathway was activated (Figure 2B). Similarly, in *K. pneumoniae* OK8 itself, we found the difference in the expression of genes belonging to SOS regulon (Supplementary Figure S3). We hypothesized that the observed SOS response could be due to altered levels of MTase and REase. Expression analysis (both at mRNA and protein level) showed that indeed the levels of REase was more than MTase (Figures 2C–D and 3A–B) at stationary phase, suggesting that the increase in REase level induces the SOS response. Although,
Figure 1. REase mediated cell death in bacteria. (A) Organization of KpnI R–M system and the arrangement of R and M genes. (B–D) E. coli cells expressing WT, HF or M were grown to different time points and stained with propidium iodide (PI) and Syto9. The fluorescence intensity of PI and Syto9 in the cells was analysed by microscopy and FACS. (B) Confocal microscopy images of WT, HF and M. Cells stained with PI are shown in red color (dead cells) and with Syto9 are indicated by green color (live cells). (C) The number of PI positive cells/800 bacteria from various fields was determined at different time points by confocal microscopic analysis and the percentage of PI stained cells was plotted against time. (D) For FACS analysis, cells with PI intensity >10^3 were considered positive. Percentage of PI stained cells/10,000 cells was calculated and plotted against time. All the data are representative of three independent experiments and error bars indicate SD.

mRNA level is not significantly different in this low copy number plasmid experimental set up when the cells transit from exponential to stationary phase, a substantial difference in the levels of the two proteins is apparent. However, in K. pneumoniae OK8 (having genomic copy of the R–M system), increased expression of REase mRNA and protein was seen. DNA cleavage assay showed promiscuous activity of the enzyme (Supplementary Figure S4); both canonical and non-canonical sites were cleaved efficiently by the REase.

Endonuclease mediated bacterial apoptosis

Cell death observed above could be a natural process accelerated or by the induction of a specific program of cascading events seen during PCD. During PCD, bacterial cells are known to undergo DNA damage, cell filamentation, membrane depolarization and hydroxyl radical formation (7). Microscopic analysis showed that a sub-population of cells were filamented in WT (Figure 3C). Further, DNA fragmentation was visualized by analyzing the genomic DNA
Figure 2. REase induces SOS response by DNA damage: (A) E. coli AP1 strain with a chromosomally placed dinD::lacZ promoter fusion carrying plasmids (WT, HF or M) were grown at 37°C in LB broth to different growth phases. The cultures were diluted to OD$_{600}$ 0.3 and the β-galactosidase activity was measured as described previously by Miller. Miller units (in units per milliliter) were calculated and the results were plotted. Data are represented as SD from three independent experiments. Statistical significance was calculated using two way ANOVA coupled with Bonferroni’s post-test and is represented by an asterisk (*) [P-value, P < 0.001 (***)]. (B) Real-time PCR analysis was carried out to quantify recA, lexA, sulA and recN mRNA levels at exponential phase (6 h) and stationary phase (16 h). idnT transcript levels were used as an endogenous control. Relative mRNA levels of genes in WT and HF were calculated after normalizing with mRNA levels from M. Graph is plotted as a ratio of relative mRNA levels in stationary versus exponential phase. Statistical significance was calculated by unpaired t-test (*) P-value <0.05. (C) Cellular mRNA levels of the r.kpnI and m.kpnI were determined in E. coli MG1655 expressing WT or HF at different growth phases. idnT transcript levels were used as an endogenous control. Relative mRNA levels of genes in WT and HF were calculated after normalizing with mRNA levels from early exponential phase. Statistical significance was calculated using two way ANOVA coupled with Bonferroni’s post-test and is represented by an asterisk (*) [P < 0.05 (*) and P > 0.05 (not significant—ns)]. (D) Cellular mRNA levels of the r.kpnI and m.kpnI was determined in K. pneumoniae OK8 at different growth phases. 16S RNA transcript levels were used as an endogenous control for RNA levels in the samples. Relative mRNA levels of genes were calculated after normalizing with mRNA levels from early exponential phase. Experiments were carried out three times independently and error bars represent SD. Statistical significance was calculated by unpaired t-test and (*) P-value P < 0.05.
Figure 3. REase induces apoptotic like death. Expression levels of R.KpnI and M.KpnI were analysed from *E. coli* MG1655 and *K. pneumoniae* from different growth phases (0.3 O.D.—early exponential phase, 0.6 O.D.—exponential phase and 1.2 O.D.—stationary phase). Ribosomal recycling factor (RRF) was used as an endogenous loading control. Blots were probed with respective polyclonal antibodies. Western blot showing the growth phase dependent expression of R.KpnI and M.KpnI from (A) *E. coli* MG1655 cells expressing both MTase and REase from their respective endogenous promoters (WT). (B) Lysates *K. pneumoniae* OK8 cells expressing endogenous R.KpnI and M.KpnI were used to carry immunoblotting. *E. coli* cells harbouring WT, HF or M were grown till stationary phase (16 h). (C) Cell morphology was visualized using confocal microscopy. Cultures were pelleted and washed with 1× PBS and cells were incubated with DAPI (shown in blue) for 15 min. (D) To measure the membrane depolarization, cells were stained with DiBAC4(3). Microscopy images of WT, HF and M cells stained with DiBAC4(3) are shown in green. (E) DiBAC4(3) stained cells /5000 cells were quantified using fluorescence intensity as determined by flow cytometry. The fraction of cells in which membrane depolarization had occurred was determined as >10^3 DiBAC4(3) intensity. The experiment was repeated three times independently and error bars indicate SD. (F) The hydroxyl radical (OH\*) formation was determined using flow cytometry. Graphs were plotted by considering HPF stained cells/5000 cells. All experiments were carried out three times independently and error bars indicate SD. Significant differences were observed between WT and HF or M cells as calculated by using one way ANOVA coupled with multiple comparison post-test and is represented by an asterisk (*) [P-value, *P* < 0.001 (***), *P* < 0.05 (**)].
(Supplementary Figure S5). Membrane depolarization was analyzed using DiBAC4(3), a voltage-sensitive fluorescent dye (24). A sub-population of WT exhibited enhanced fluorescence (Figure 3D). Quantification by FACS revealed that >2-fold of WT cells displayed DiBAC4(3) fluorescence (Figure 3E), i.e. more membrane depolarization compared to cells carrying HF or M. Membrane depolarization causes reactive oxygen species (ROS) production leading to apoptosis (25). Indeed, an enhanced formation of hydroxyl radical (OH*) was observed (Figure 3F). All these results indicate that REase induced DNA damage stimulates apoptotic-like response. We observed 6-fold increase in the rpoE expression in WT cells during stationary phase (Supplementary Figure S6). The sigma E is known to direct the expression of genes specific for cell lysis (26).

Different mechanisms of PCD in bacteria have been connected to different cellular phenomena (5,27). The three major PCD pathways described to date are mazEF mediated death (28), thymine less death (TLD) (29) and apoptotic-like death (ALD) (30,31). The mazEF, one of the well-studied TA system, induces PCD in most of the population by increasing the synthesis of ‘death proteins’ (28). ALD is an extreme DNA damage induced death pathway and is mainly dependent on RecA (31). TLD pathway is mediated by increasing the synthesis of ‘death proteins’ (28). ALD is an extreme DNA damage induced death pathway and is mainly dependent on RecA (31). TLD pathway is mediated by increasing the synthesis of ‘death proteins’ (28).

EDIN is known to direct the expression of genes specific for cell lysis (26). Among these, we considered a possibility that the PCD observed in the present study is occurred through the ALD pathway, given the extent of DNA damage and induction of SOS response (see Figure 2). Compared to the canonical SOS pathway, a unique set of genes termed as extensive damage induced (Edin) genes are induced during ALD (30). Indeed, expression analysis of a representative subset of Edin genes, viz. sdhA, uvrA, oppB and aceA showed a 1.2–2.8-fold up-regulation in WT compared to M alone (Figure 4A). However, analysis of expression of genes specific to either mazEF system (yfdD, yfbU and yafQ) or TLD (yebG, ycgB) mediated pathways did not show any appreciable change (Supplementary Figure S7). Notably, earlier studies have revealed that sdhA and oppB responded to both ALD and TLD pathways but in opposite manner; they were up-regulated in the former and the expression decreased in the latter pathway (29,30). From the data, it appears that REase mediated cell death follows ALD pathway. Further, during ALD, decrease in the activity of complex II of the oxidative respiration was demonstrated (30). In our studies, the activities of both the complexes (I and II) of oxidative respiration were reduced at stationary phase compared to log phase in REase expressing cells (Figure 4B). Together, these studies demonstrate that REase harbouring cells induced PCD in bacteria through the ALD pathway.

**REase induced cell death benefits population at large**

In bacterial species where PCD has been studied, it is apparent that the evolutionarily conserved processes primarily contribute for population benefit. For example, under conditions of nutrient starvation, *M. xanthus* cells undergo PCD as a mechanism to release nutrients for the remaining clonal cells for the development of myxospores (32). In contrast, the ‘cannibalistic’ *B. subtilis* resorts to feeding on their siblings in order to delay the onset of sporulation (33). These and other examples show the importance of PCD for increasing the fitness of a population as a whole. Would the REase mediated PCD confer any such benefit to bacteria? Comparison of growth of WT, HF and M cells (Supplementary Figure S1) revealed the reduced net growth of WT, correlating with the cell killing observed in the stationary phase (see Figure 1), implying that WT cells could be fitness compromised. However, when cultures were grown for longer periods, i.e. beyond 72 h, surprisingly, more viable cells could be scored with WT compared to the others (Figure 5A).

The survival of the WT in the long-term growth could be due to the nutrients released from the dying cells that would allow the clonal sibling survival, prolonging life span of the population and contributing to the fitness under nutrient limiting conditions. This premise was tested by the following experiments. Conditioned media was prepared from the cultures of WT, HF and M cells and tested whether media can support the growth of freshly inoculated new culture. Supplementary Figure S8 shows the schematic of preparation of conditioned medium (17). Briefly, conditioned media was prepared by filter sterilization of supernatants collected from 5-day-old cultures. When a freshly grown culture of *E. coli* MG1655 cells was inoculated into the conditioned medium obtained from WT, HF and M cells, an increase in the CFU (3 × 10^3 CFU/ml) was observed in the medium collected from WT compared to the other two (Figure 5B and C). To understand the basis for the enhanced growth, the nature of contents in the conditioned medium was analysed. Upon treatment with DNase I and RNase A, a substantial increase in the CFU was observed in case of WT (Supplementary Figure S9). Increased protein content (determined by SDS-PAGE and protein estimation) in the conditioned medium also supported the growth. Heat treatment of the medium curtailed the growth of the bacteria (Supplementary Figure S9). We interpret all these results as follows. REase mediated apoptotic-like death results in the release of DNA, RNA, proteins and other cellular essential components. This would facilitate the survival of the other starving cells as they can take up these nutrients. Thus, the rest of the bacterial population would benefit from the REase induced death of some cells.

**DISCUSSION**

Normally, REases as components of R–M system provide immunity to the modified host genome, destroying the incoming DNA (34). However, the occurrence of a large number of enzymes and the inherent promiscuity exhibited by many of them (35), could be suggestive of their participation in other cellular functions (9). Here, we show that REases can cause bacterial cell death. DNA fragmentation catalysed by the enzyme initiates a cascade of reactions and events ultimately leading to cellular apoptosis. DNA damage induces the expression of SOS and Edin genes, which subsequently decrease the complex I and II activities of the respiratory chain, leading to membrane depolarization and generation of hydroxyl radicals causing cellular destruction. The features of the REase mediated PCD matches with the ALD pathway (31). Importantly, the nutrients released from the dying cells provide growth benefit to the remaining
Figure 4. Expression analysis of extensive damage induced (Edin) genes and activities of complex I and II of respiratory chain. Cultures of WT, HF and M were grown till stationary phase (16 h). (A) Total RNA was extracted from the cells, and quantitative real-time PCR was carried out to quantify mRNA levels of Edin genes (aceA, sdhA, oppB and uvrA). idnT transcript levels were used as an endogenous control. Relative mRNA levels of genes in WT and HF were calculated after normalizing with mRNA level from M cells. The experiments were carried out three times independently and error bars represent SD. Statistical significance was carried out by *t*-test and indicated by (*) *P*-value ≤ 0.05. (B) Activities of complex I and II were determined in WT, HF and M. Cells were grown to different growth phases (exponential phase: 6 h; stationary phase: 16 h). Enzymatic activities were determined as described in Materials and Methods. Total activity (NADH dehydrogenase I) was determined by measuring the reduction levels of NADH at 340 nm. Complex II activity (succinate dehydrogenase) was determined by measuring the reduced levels of dichlorophenolindophenol (DCPIP) and absorbance was monitored at 600 nm. These data represent the results of three independent experiments and error bars indicate SD. Statistical significance was calculated using two-way ANOVA coupled with Bonferroni's post-test and is represented by an asterisk (*) [*P*-value, *P* < 0.001 (***)) and *P* > 0.05 (not significant—ns)].
Figure 5. Long term growth studies of WT, HF and M cells and growth of MG1655 cells in conditioned medium. (A) E. coli cells harbouring WT, HF and M were grown for 144 h at 37°C. Samples were collected at different time intervals as indicated and CFU analysis was carried out. The graph was plotted as CFU/ml against time. (B and C) Growth studies of E. coli MG1655 (pACYC184) cells in conditioned medium. Conditioned medium was prepared from 5 days old LB grown cultures of WT, HF and M as described in Materials and Methods. MG1655 (pACYC184) cells were inoculated into conditioned medium generated from WT, HF or M cells and grown till 24 h. Growth was monitored by optical density by measuring absorbance (B) O.D. at 595 nm and (C) CFU analysis was carried out by plating on LB agar with chloramphenicol. Data is presented from three independent experiments and error bar indicates SD. Statistical significance was calculated using one-way ANOVA coupled with multiple comparison post-test and is represented by an asterisk (***) [P-value and *P* < 0.0012 (***)].

members of the community (Figure 5). Thus, death of some members of the population appears to allow the survival of a subpopulation that eventually may form a nucleus for renewed growth when growth conditions become favourable with the availability of the nutrients.

While it is clear that PCD benefits multicellular organisms (36), the selective advantage of promoting cell death is less apparent in bacteria. However, several studies have emerged that demonstrate the benefit of induced cell death for the community in bacterial populations (5,27). During B. subtilis sporulation, the mother cell undergoes autolysis through PCD to release nutrients for spore maturation (27). M. xanthus developmental paradigm is yet another well studied PCD. During the early steps of fruiting body formation and under nutrient depletion, a population of cells undergoes altruistic cell lysis to release the contents which feed the remaining cells that differentiate into myxospores. Similarly, mazEF mediated PCD appears to benefit bacterial community, as it is involved in the development of biofilms (37) and release of virulence factors (38). Likewise, REase induced cell death described here seems to release nutrients to benefit the remaining members of the population. PCD at late stationary phase thus appears to provide survival advantage for the population at large. Exponentially growing cells undergo a major transition when they enter stationary phase. In stationary phase, not only the nutrients
Figure 6. Model: REase mediated cell death for community benefit. At late stationary phase or upon nutrient depletion, there is a change in the nucleoid architecture and metabolic status of bacterial cells. It has also been reported that there is an increase in Mn2+ levels and a decrease in polyamines which decorate and protect the genome from different hazardous molecules. REase mediated DNA cleavage (both at recognition or canonical and non-canonical sequences) induces double-stranded breaks leading to extreme DNA damage inside the cell. This might further result in the activation of rpoE regulon and lysis of stationary phase cells. This process releases nutrients into the environment which can be taken up by the neighbouring cells, thus enhancing the survival of the population as a whole.

are limited, the metabolic status of the cell is also altered. Alteration in gene expression and nucleoid associated protein (NAPs) profiles, increase in Mn2+ concentration, decrease in polyamines and possibly changes in several other factors accompany the transition from exponential phase to stationary phase (39–42). The concentration of polyamines and NAPs that protect the genome in exponentially growing cells is significantly reduced at stationary phase (14,41), rendering the genome more exposed and vulnerable to attack by its own nuclease. Reduced supercoiling and decompacted nucleoid are characteristics seen in stationary phase cells (41).

The increased expression of the REase in the stationary phase (Figures 2C–D and 3A–B) and its enhanced promiscuous activity in the presence of Mn2+ (20,22) would lead to higher susceptibility of the genome for DNA damage. Indeed, we find increased level of Mn2+ concentration in cells in stationary phase and high promiscuous activity of the enzyme in the presence of Mn2+ (Supplementary Figure S10). Thus, change in the intracellular environment, chromosomal dynamics, REase/MTase ratio and increased promiscuity of the enzyme would lead to enhanced REase induced cell death (Figure 6). Genomes of E. coli and K. pneumoniae have about 500 and 900 canonical (GGTACC) sites respectively for R.KpnI (43) (www.rebase.neb.com) and a much larger number of non-canonical sites. Extensive cleavage at the non-canonical sites would result in massive genome destruction which would be beyond the capacity of SOS mechanism to repair and resurrect the cell, leading to ALD and consequent cell death. Such an extensive intracellular damage would result in activation of the rpoE regulon (Supplementary Figure S6), and the expression of RpoE regulated genes involved in cell death, followed by lysis (26). This process would liberate nutrients, which can be taken up by neighbouring desperately starving cells thereby enhancing the survival of the population as a whole. Prolonging the life span of the population gives an opportunity for these cells to thrive if nutrient availability is restored. Thus, it appears that the promiscuity built into a site-specific enzyme is a cellular design to induce apoptosis when needed.

The PCDs described so far have similarities and differences. Orchestrated, temporally regulated gene expressions coupled to specific signalling cascades are hallmarks of sporulation events occurring under starvation stress (27). In contrast, mazEF mediated cell death occurs only during exponential phase and not during stationary phase. An extracellular death factor, a small peptide is shown to enhance mazEF mediated PCD in exponential phase (44) which has
been not substantiated further. Our extensive experiments aimed to identify such signals of apoptosis have also not lead to the identification of any candidate molecules. In the hindsight, it seems unlikely that such a signal would be required in the present scenario given that the changes in the intracellular environment and internal dynamics lead the exposure of the target (genome) to the nuclease. We surmise that the REase induced apoptosis could be an intracellular built in program. The stochastic events in gene expression in a bacterial cell have been established (45).

An exciting possibility is that REase mediated cell death is not confined to one R–M system. Although, originally REases were considered to exquisitely site- specific, now, it is evident that a large number of them are promiscuous in their DNA cleavage characteristics (14,22,35,46). The ‘star’ activity exhibited by a number of REases is due to their promiscuous cleavage activity (21). From the present studies with HF enzyme and the studies with EcoRV (47), it is clear that even the REases which confine to site- specific cleavage could end up damaging the self DNA. However, DNA damage inflicted by enzymes having ‘star’ activity such as EcoRI (47) would be more severe as in the case of Knpl. Moreover, the experiments described with yet another R–M system substantiate the idea that REases can cause cell death (Supplementary Figure S2). Induction of SOS response has been examined in the case of MboII R–M system (48) which exhibits star activity. It is likely that the MboII also induces PCD. All these observations are pointers that many REases can engage themselves in more robust PCD under conditions requiring such behavior to support the survival of the population. Another factor which could contribute to cell death is the imbalance in intracellular concentration of REase and MTase. In spite of being organized together and in close proximity to each other, REase and MTase are often subjected to differential regulation at transcription and post transcriptional stages including protein turn over (49). In many cases, a transcription regulator encoded by C gene controls the expression of R and M genes in opposite fashion (50). Further, the expression of many REases may change significantly during the stationary phase as seen in the present study. REase mediated apoptosis thus would be yet another aspect of altruistic as well as social behavior of eubacteria. This new intracellular role also appears to be a cellular design to provide nutrients for the cells under starvation stress. Given the large repertoire, diversity and promiscuity seen in REases, the phenomenon is likely to be wide spread adding another facet to bacterial cell biology.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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