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Bacterial persisters tolerate antibiotics by not producing hydroxyl radicals

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ABSTRACT

In a phenomenon called persistence, small numbers of bacterial cells survive even after exposure to antibiotics. Recently, bactericidal antibiotics have been demonstrated to kill bacteria by increasing the levels of hydroxyl radicals inside cells. In the present study, we report a direct correlation between intracellular hydroxyl radical formation and bacterial persistence. By conducting flow cytometric analysis in a three-dimensional space, we resolved distinct bacterial populations in terms of intracellular hydroxyl radical levels, morphology and viability. We determined that, upon antibiotic treatment, a small sub-population of *Escherichia coli* survivors do not overproduce hydroxyl radicals and maintain normal morphology, whereas most bacterial cells were killed by accumulating hydroxyl radicals and displayed filamentous morphology. Our results suggest that bacterial persisters can be formed once they have transient defects in mediating reactions involved in the hydroxyl radical formation pathway. Thus, it is highly probable that persisters do not share a common mechanism but each persister cell respond to antibiotics in different ways, while they all commonly show lowered hydroxyl radical formation and enhanced tolerance to antibiotics.

1. Introduction

Within a population of bacteria, a sub-population of multidrug-tolerant cells exists. These cells, termed persisters, are believed to be in a state of dormancy [1], which enables them to tolerate antibiotics. Upon reinoculation of cells that survived from antibiotics, the persisters give rise to new populations that have the same vulnerability to antibiotics as the ancestral population. Unlike antibiotic-resistant cells, the persisters are not genetically different from normal antibiotic-sensitive cells but are phenotypic variants of the wild-type cells [2,3].

This non-inherited bacterial resistance to antibiotics, called persistence, can be considered as an insurance policy that permits survival of a sub-population from an antibiotics encounter [4]. Alternatively, persistence may be a social trait of bacteria that benefits other individuals [5]. Paradoxically, this phenomenon can be disastrous to humans because it disarms antibiotics which have been the strongest weapon that humans have developed to cure bacterial infection. Some well-known examples are tuberculosis, syphilis, typhoid fever and gastric ulcer. The pathogens linger in the host for long periods of time in spite of prolonged antibiotics treatment. More importantly, persisters are a potential source for the emergence of inheritable antibiotics resistance [6]. Thus, the problems posed by persistence are no less intractable than those by the resistance [2,7–11].

Although persistence was first described ~70 years ago, its mechanism still remains unknown. A major hurdle for studying persistence is in the fact that bacterial persisters are formed at a very low rate (i.e. $\sim 10^{-6}$) [7]. Moreover, the persisters, which are phenotypic variants of the normal population, have a transient nature. While it is still a mystery how persisters survive from an antibiotic encounter, several studies have suggested that toxin–antitoxin (TA) modules are important for persister formation. In the 1980s, the *hip* (high frequency of persisters) mutant was identified [12]. The *hipA7* allelic strain produced 1000× more persisters than the wild-type strain. The *hipBA* operon was shown to act as a TA module, in which the HipA toxin is tightly regulated by the HipB antitoxin [13]. Lewis and colleagues proposed that various TA modules such as HipBA and RelBE could lead to multidrug tolerance on the basis of microarray analysis of the *Escherichia coli* transcriptome [14,15]. Recently, a mechanism for HipA-mediated persistence and its neutralization by HipB was suggested based on the HipA and HipA–HipB–DNA crystal structures [16]. However, the DnaJ, PmrC and DskS proteins, which are unrelated to TA modules, changed persister frequency [17]. Moreover, several genes that do not show direct relevance to TA modules but display

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altered persister formation were identified from genetic screens [18–20]. Thus, persistence may be a result of the coincidence of many different events.

Kohanski et al. suggested an intriguing mechanism of antibiotics for killing bacteria [21]. They showed that three major bactericidal antibiotics stimulate hydroxyl radical formation in bacteria, and this toxic chemical contributes to the killing efficiency of lethal antibiotics. This process was accompanied by hyperactivation of NADH dehydrogenases and a depletion of NADH. Based on the results, they concluded that generation of hydroxyl radicals is a common mechanism of bacterial cell death caused by antibiotics. Hydroxyurea, which is an inhibitor of class I ribonucleotide reductase, also induced hydroxyl radical-mediated cell death in *E. coli* [22]. Based on these findings, we questioned whether a lack of hydroxyl radical generation inside a bacterial cell upon antibiotic treatment could lead to the cell becoming a persister. Consistent with this idea, our experiments using flow cytometer and fluorescence microscopy reveal that upon bactericidal antibiotic treatment, the majority of *E. coli* cells which are killed display morphological changes (i.e. filamentation) and intracellular accumulation of hydroxyl radicals, whereas persisters maintain normal morphology and do not overproduce hydroxyl radicals. Based on the result, we conclude that persisters do not share a common mechanism of antibiotic-tolerance because hydroxyl radical formation can be blocked in various ways.

2. Materials and methods

2.1. Bacterial strains and chemicals

E. coli K12 BW25113 was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). All antibiotics were purchased from Sigma. The following concentrations of antibiotics were used: 10 μg/ml ampicillin, 250 ng/ml norfloxacin. 5 μM hydroxyphenyl fluorescein (HPF) and 1 μg/ml propidium iodide (PI) were used to analyze persister cells by flow cytometry and confocal microscope.

2.2. Determination of colony forming unit (cfu) in a liquid culture

Cells were pre-cultured in 10 ml LB overnight. The pre-culture was inoculated into fresh LB (50 ml) and cultured at 37 °C until the cfu reached $\sim 1 \times 10^8$/ml. 3 ml of bacterial culture was transferred to a 14 ml polypropylene round-bottom tube. After adding antibiotics at the indicated concentrations, bacterial cells were removed at the indicated times. The cells were washed twice with phosphate buffered saline (PBS), serially diluted and plated onto LB agar plates. After incubating the plates overnight, visible colonies were counted to determine cfu.

2.3. Flow cytometry analysis

To observe hydroxyl radical formation and morphological changes of *E. coli* upon antibiotic treatment, 3 ml of bacterial culture ($\sim 10^8$ cells) was transferred to a 14 ml round-bottom tube. After adding indicated concentrations of antibiotics and HPF, bacterial cells were incubated at 37 °C for 3 h. Cells (200 μl) were removed at the indicated times and washed twice and diluted with phosphate buffered saline (PBS). The diluted cells were stained with propidium iodide (PI) for 10 min and analyzed with a FACS Calibur flow cytometer.

2.4. Confocal microscopy

The relationship between hydroxyl radical formation and bacterial cell death was examined by fluorescence microscopy. A bacterial culture (100 μl) treated with antibiotics and HPF was washed twice with PBS. Washed cells were stained in a dark chamber with PI for 10 min. The stained cells (5 μl) were dropped onto an L-lysine coated slide glass and analyzed by a Zeiss LSM510 Meta DuoScan Spectral Confocal microscope. Emission laser length was 488 nm, and HPF and PI were detected by the green/red filter.

3. Results and discussion

We initially observed the formation of bacterial persisters. When *E. coli* cells were treated with ampicillin (Amp) or norfloxacin (Nor), they were rapidly killed: the colony forming unit (cfu) was reduced by a factor of ~3 during the first 2 h of antibiotic treatment (Fig. 1A). However, due to the formation of persisters [2,6], the cell death rate was reduced after 2 h of antibiotic treatment. Decimal reduction time (D), which is the time required to reduce cfu by a factor of 10 and is the inverse slope when *y*-axis is in common logarithmic scale, was 1 h for the fast cell death period while it was prolonged to ~5 h during persister death period, indicating that *E. coli* cells tolerate antibiotics.

Some bactericidal antibiotics have been demonstrated to kill bacteria by promoting generation of hydroxyl radicals inside cells [21]. Therefore, we hypothesized that antibiotics might fail to provoke formation of hydroxyl radicals in bacterial persisters. To explore this idea, we employed hydroxyphenyl fluorescein (HPF)-assisted flow cytometry because HPF precisely detects hydroxyl radicals generated inside bacterial cells [21]. Consistent with the previous report [21], we found that the levels of hydroxyl radicals commonly increased in *E. coli* cells exposed to either of the bactericidal antibiotics Amp or Nor (Fig. 1B).

However, we could not observe resolvable distinct sub-populations using this simple HPF-assisted flow cytometric technique. To acquire more precise information about persisters, we tested whether cell elongation, which is a well-known post-antibiotic effect (Fig. 1C), could be employed as another parameter to judge the persistence. We filtered antibiotic-treated cells using a filter membrane with 5 μm pore size, and the persister fraction among the cells in the filtrate was compared with that in the filter cake. The total number of cells was counted by using a hematocytometer on a light microscope, and the number of persisters was counted based on colony forming units on an LB plate. After filtration, the persister fraction in the filtrate was ~40× higher than that of the cells in the filter cake (Fig. 1D). Micrographs taken at each step clearly show that rod-shaped cells are enriched in the filtrate (Fig. 1G) while most cells in the filter cake are elongated (Fig. 1F). This result suggests that persistence is closely correlated to cell elongation in a certain degree. Cell elongation can be simply measured using forward (FSC) and side scatter (SSC) of a flow cytometer.

To better resolve bacterial sub-populations using a flow cytometer, we also employed a dye propidium iodide (PI) as a cell death indicator. PI is commonly used to identify dead cells in a population, since PI intercalates into DNA but is membrane-impermeant and generally excluded from viable cells. Using these three parameters (HPF, PI and FSC/SSC), we examined populations of *E. coli* cells that were grown in the presence or absence of antibiotics. When bacterial cells were analyzed without treatment of antibiotics, both HPF and PI intensities were low, indicating that most cells are viable and hydroxyl radical formation is minimal (Fig. 2A). In contrast, the intensities of these two dyes dramatically increased upon antibiotics treatment (Fig. 2A). This positive relationship between HPF and PI intensities supports the idea that hydroxyl radical formation is the cause of bacterial cell death.

Next, we resolved the same bacterial population in an FSC–SSC plane. Relatively low FSC and SSC values flocked in a narrow area for untreated cells (Fig. 2B), indicating that the bacterial population is morphologically homogeneous, with rod-shaped bacterial cells

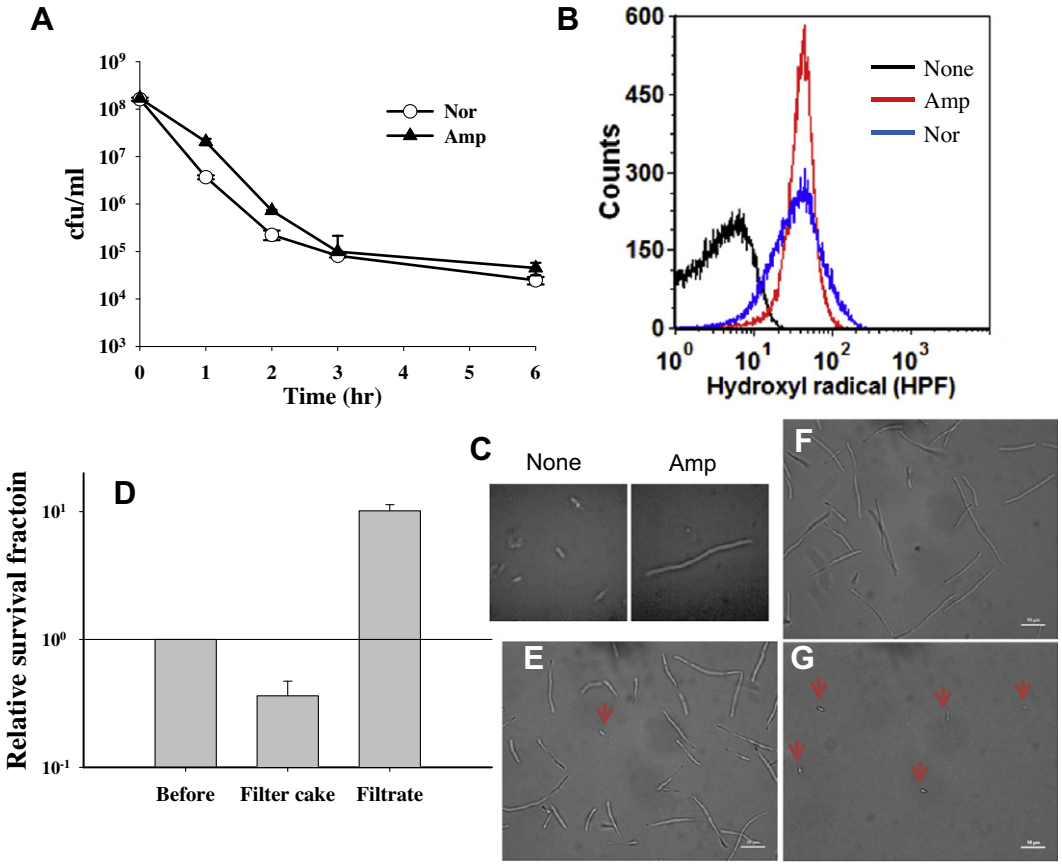

Fig. 1. Formation of persisters, hydroxyl radicals, and morphological changes upon antibiotic treatment. (A) Persister formation. (B) Flow cytometric analysis of hydroxyl radical contents using HPF. (C) Filamention of *E. coli* after Amp treatment. (D) Filtration of Amp-treated cells. After treating *E. coli* with 5 μg/ml Amp for 3 h, cells were filtered using the membrane with 5 μm pore size and colony forming units were counted. (E) Total cells before filtration are largely elongated but contain a few rod-shaped cells (arrows). (F) Morphology of cells in the filter cake. (G) Filtrate exhibited higher frequency of rod-shaped cells compared to cells before filtration.

(Fig. 2B). Both FSC and SSC values increased upon antibiotic treatment as a result of cell filamentation. While antibiotics treatment shifted the majority of the bacterial population to the area with high FSC and SSC values (Fig. 2B), a small fraction of cells remained with the same FSC and SSC values as those of untreated cells (Fig. 2B, indicated as gate 1). When the cells in gate 1 were analyzed for hydroxyl radical contents (Fig. 2C) and cell death (Fig. 2D), they showed a much lesser increase in both HPF and PI values than the majority of the population.

Fluorescence microscopy conducted on bacterial cells that were stained with HPF and PI further supports the flow cytometry data. Most of the *E. coli* cells were elongated and overproduced hydroxyl radicals upon antibiotic treatment, and the micrographs for HPF and PI signals are superimposed perfectly (Fig. 3). Furthermore, we obtained a few images containing several rod-shaped cells out of tens of micrographs, and these cells were not stained by HPF or PI (Fig. 3 and S1, indicated as arrows). Cumulatively, our results suggest that bacterial persisters are the cells that do not overproduce hydroxyl radicals and retain normal morphology while the dead cells contain high levels of hydroxyl radicals and display filamentous morphology.

Next, we traced the time-dependent changes of morphology, hydroxyl radical formation, and cell death by measuring FSC, HPF, and PI values (Fig. 4A). In general, all values gradually increased as a function of time after antibiotic treatment. However, these three indices did not change simultaneously, and a time-dependent order was apparent (Fig. 4A). Upon antibiotic addition, the center of the cell population shifted first toward a higher FSC value in the FSC–HPF plane. Next, a period of HPF changes followed with relatively small FSC value changes. In the PI–HPF plane, HPF first increased followed by an increase in PI value. To clearly show the sequence of these antibiotic responses, the average FSC, HPF and PI values of 10,000 cells near the center of the population were calculated. The average values were plotted in FSC–HPF and PI–HPF planes and then extrapolated using a single exponential function or Monod kinetics-like saturation function (Fig. 4B and C). The vectors, which linearly connect the values obtained at each time point on the extrapolated lines, clearly showed such a time-dependent response tendency. Each vector was separated into two designated components to represent the rate of each physiological change as the length of the arrow. During the first 1 h time period of Amp treatment (shown in solid arrows), the increase in FSC value was much bigger than those of HPF. During later time periods, hydroxyl radical formation surpassed the rate of filamentation (Fig. 4B). Similarly, cell death rate was accelerated during the final time period, which followed initial overproduction of hydroxyl radicals (Fig. 4C). These results clearly indicate that cell elongation takes place first and hydroxyl radical accumulates later upon antibiotics treatment, with cell death occurring last. This order of response was common between the antibiotics tested (Fig. S2).

Hydroxyl radical is extremely deleterious leading to bacterial cell death and is commonly induced by bactericidal antibiotics regardless of drug-target interaction. In the present study, we showed that persisters, which tolerate bactericidal antibiotics, do

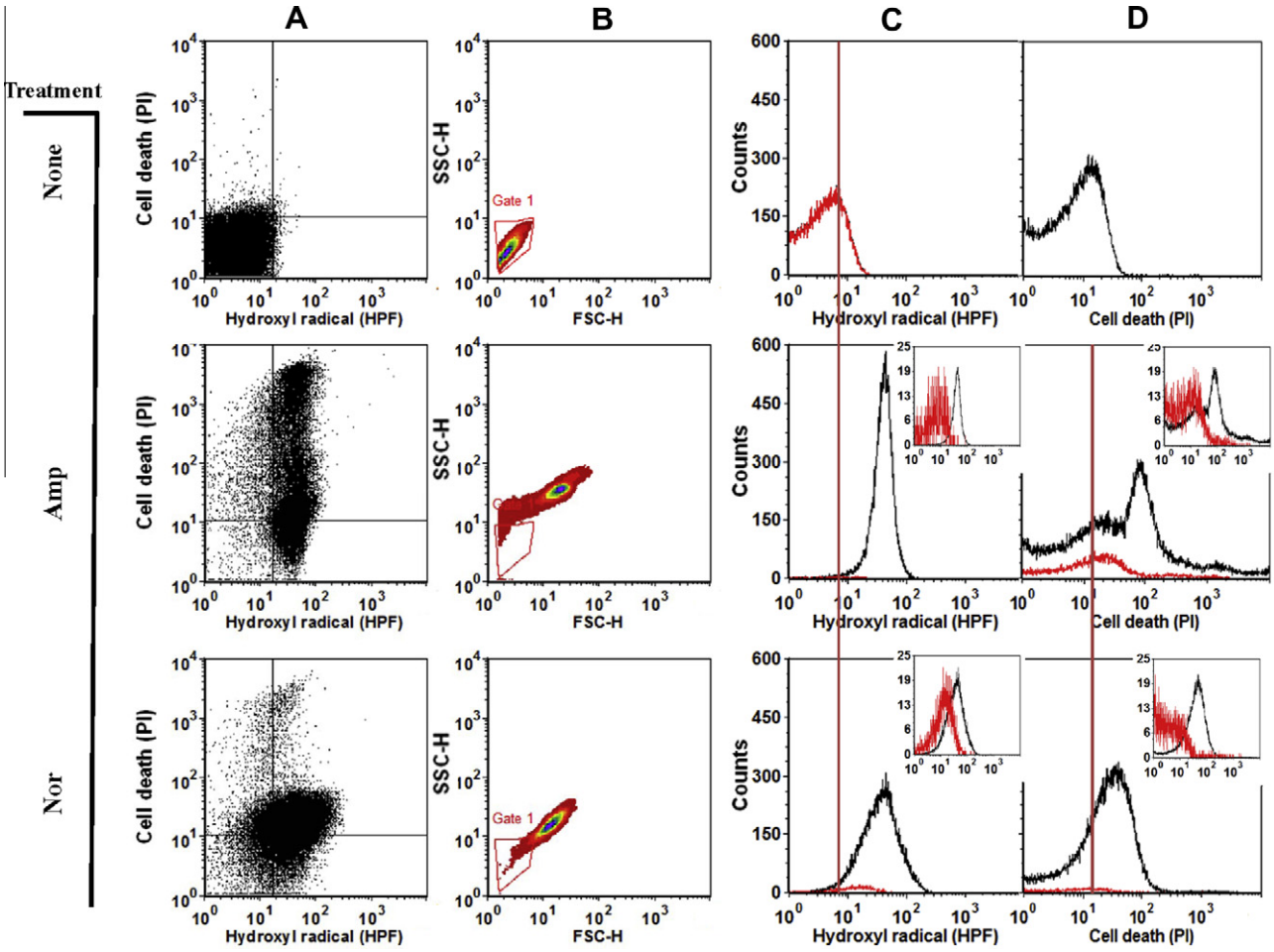

Fig. 2. Flow cytometric analysis of post-antibiotic responses. PI, HPF and FSC (or SSC) were employed as the indicators of cell death, hydroxyl radical contents, and cell elongation, respectively. (A) Post-antibiotic responses of a cell population were resolved in an HPF–PI plane. A positive relationship between each index indicates that cells with higher hydroxyl radical contents have a high probability of being dead cells. (B) Antibiotic-treated cells were resolved in an FSC–SSC plane. The area with the same FSC and SSC values as those of untreated cells (gate 1) is also shown for the antibiotic-treated cells. The cells in gate 1 (red lines in C and D) were analyzed separately from total cells (black lines in C and D). (C) Hydroxyl radical contents of the cells in gate 1 (red lines) were compared with total cells (black lines). Because there were very small numbers of cells in gate 1, the bottom region of the *y*-axis was magnified by normalizing total cell number with respect to the cell number in gate 1 (insets). Insets indicate that the cells in gate 1 exhibit much lower hydroxyl radical contents compared to the majority of cells. (D) Using the same method as (C), the cells in gate 1 were analyzed using PI. Insets indicate that the cells in gate 1 exhibit much less cell death compared to the majority of cells. Samples were subjected to flow cytometric analysis after 3 h antibiotic treatment. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

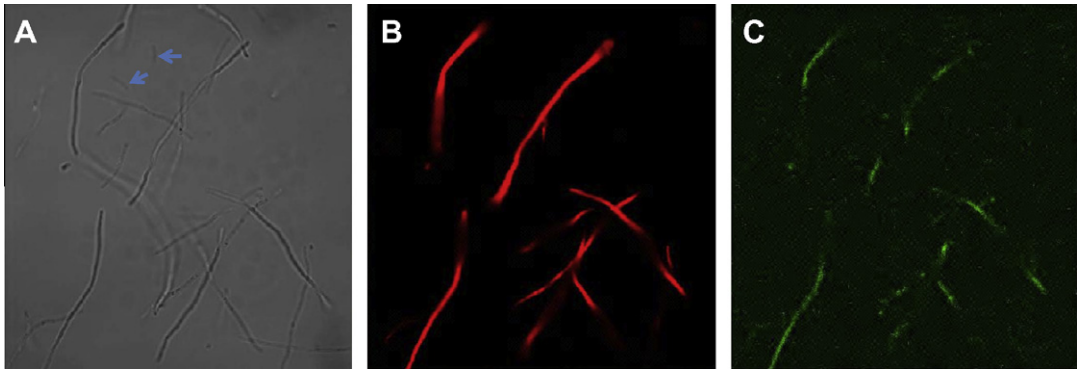

Fig. 3. Fluorescence micrographs of antibiotics-treated cells. After staining the antibiotic-treated cells with HPF and PI, cells were observed using bright field (A) red filter (B) and green filter (C). Due to low persistence frequency only two pictures contained rod-shaped cells out of tens of micrographs. The other picture is shown in Fig. S1. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

not overproduce hydroxyl radicals while most normal cells are killed by the antibiotic-induced hydroxyl radicals. Results suggest that persisters phenotypically fail to operate the reactions involved in hydroxyl radical formation pathways, of which major steps include transient depletion of NADH, destabilization of iron–sulfur clusters and Fenton-reaction. Blocking any of these steps led to cell

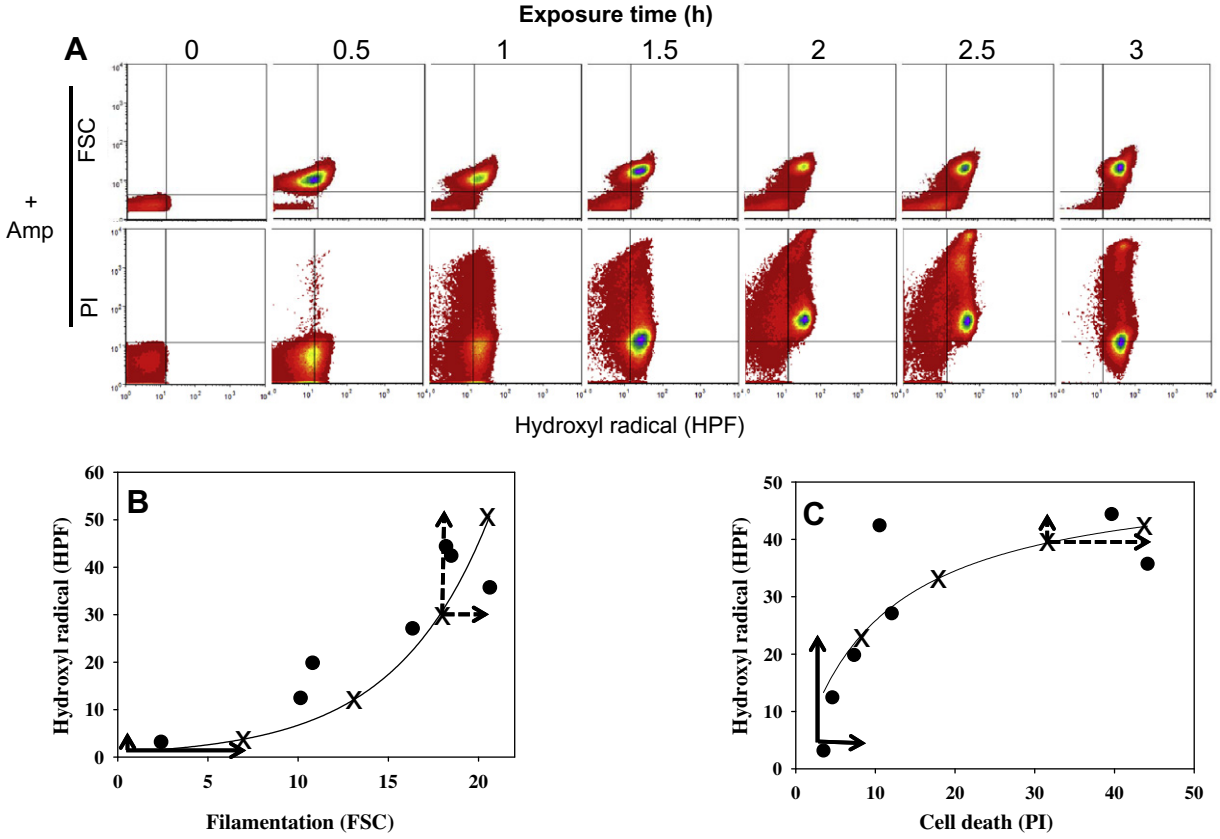

Fig. 4. Time-dependent hydroxyl radical formation, filamention and cell death responses to Amp treatment. (A) Time-dependent flow cytograms of *E. coli* cells that were resolved in the HPF–FSC planes (upper panels) and HPF–PI planes (lower panels) after treatment with 5 μg/ml Amp. The average FSC value in a panel was obtained by averaging 10,000 cells near the center of cell spots in the panel. After obtaining average values for HPF and PI for each panel, the average numbers were plotted in a FSC–HPF plane (B) and PI–HPF plane (C). In the FSC–HPF plane, spots were extrapolated using a single exponential curve. Monod kinetics-like function was employed to extrapolate spots in the PI–HPF plane. After dividing the lines into four sections (designated as *x*), every two dots were connected to derive a vector. In the figures, only the first and the last vectors are shown. When a vector is separated into each component, the length of arrow denotes the tendency of that specific physiological change. The same analysis for nor-treated *E. coli* is shown in Fig. S2.

survival against antibiotics [21]. Thus, it is highly probable that persisters are not a homogeneous population with a defect of single chemical reaction but are heterogeneous mixture of phenotypic variants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.063.

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