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Viable but non-culturable and persistence describe the same bacterial stress state

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Summary

Bacteria are often thought of as having two dormant phenotypes: the viable but non-culturable (VBNC) state and the persister state. Here we investigate the relatedness of the two stress-induced phenotypes at the single-cell level and examine cell morphology and quantify cell resuscitation. Using the classic starvation conditions to create VBNC cells, we found that the majority of the remaining *Escherichia coli* population are spherical, have empty cytosol and fail to resuscitate; however, some of the spherical cells resuscitate immediately (most probably those with dense cytosol). Critically, all the culturable cells in this starved population became persister cells within 14 days of starvation. We found that the persister cells initially are rod-like, have clear but limited membrane damage, can resuscitate immediately and gradually become spherical by aging. After 24 h, only rod-shaped persister cells survive, and all the spherical cells lyse. Both cell populations formed under the VBNC-inducing conditions and the persister conditions are metabolically inactive. Therefore, the bacterial population consists of dead cells and persister cells in the VBNC-inducing conditions; that is, the non-lysed particles that do not resuscitate are dead, and the dormant cells that resuscitate are persister cells. Hence, 'VBNC' and 'persister' describe the same dormant phenotype.

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Introduction

Bacteria have elegant ways to survive during stress (Storz and Hengge, 2010; Requena, 2012), such as that arising from inevitable nutrient depletion as well as antibiotic exposure, and two distinct phenotypes have been described in which the cells enter a non-heritable, reversible, dormant state: viable but non-culturable (VBNC) cells (Xu *et al.*, 1982) and persister cells (Hobby *et al.*, 1942). In what have become known as persister cells, Hobby *et al.* (1942) determined that 1% of *Staphylococcus aureus* cells are not killed by penicillin and that these antibiotic-tolerant cells are metabolically inactive. Forty years later, VBNC cells were first described as those *Escherichia coli* and *Vibrio cholerae* cells that are present after an extended period (2 weeks) in salt water microcosms that are not culturable on selective and non-selective media upon which they are usually capable of growth (Xu *et al.*, 1982); however, a few stimuli such as nutrients and temperature shifts serve to resuscitate VBNC cells (Li *et al.*, 2014).

The two states of dormancy have much in common. Both persisters (Mulcahy *et al.*, 2010) and VBNCs (Li *et al.*, 2014) have been linked to chronic infections, both occur in biofilms (Spoering and Lewis, 2001; Li *et al.*, 2014), and both cell types have been generated by more than one kind of stress; for example, oxidative and acid stress (Hong *et al.*, 2012; Li *et al.*, 2014). Furthermore, the genetic basis for both cell types is not well characterized. For persisters, toxin/antitoxin systems (Moyed and Bertrand, 1983; Shah *et al.*, 2006), the alarmone guanosine tetraphosphate (ppGpp) (Korch *et al.*, 2003) and the stationary-phase sigma factor RpoS (Hong *et al.*, 2012) clearly play a major role in their formation; however, many systematic studies such as transposon-sequencing (Shan *et al.*, 2015), protein expression (Spoering *et al.*, 2006) and gene knockouts (Hu and Coates, 2005; Hansen *et al.*, 2008) have not yielded significant additional insights into persister cell formation, and it appears any toxic protein that slows bacterial growth induces persistence, even in the absence of ppGpp (Chowdhury *et al.*, 2016). For VBNC cells, RpoS (Boaretti *et al.*, 2003; Kusumoto *et al.*, 2012), the transcriptional regulator OxyR that controls genes related to oxidative stress (Li *et al.*, 2014) and toxin/antitoxin systems (Ayrapetyan *et al.*, 2015a) have been

linked to VBNC cells. Hence, it has been suggested that these two survival states may be part of a 'dormancy continuum' (Ayrapetyan *et al.*, 2015a); that is, the two kinds of resting states may be related with VBNCs as the more dormant of the two states. The key feature that has been suggested to distinguish persister cells from VBNC cells is that VBNC cells are reported to not be resuscitated under normal conditions while persister cells can be easily converted to normal cells that are sensitive to antibiotics or other stresses (Ayrapetyan *et al.*, 2015a).

VBNC cells and persister cells share many similar features, and they co-exist (Orman and Brynildsen, 2013; Ayrapetyan *et al.*, 2015b; Goncalves and de Carvalho, 2016; Saito *et al.*, 2017); however, no studies have been performed to compare these two stages of dormancy based on their physiology and morphology. Here, we demonstrate that the viable fraction of VBNC cells generated from nutrient depletion are persister cells by comparing their antibiotic tolerance, rates of resuscitation, morphology and metabolic activity. The remainder and vast majority of the cells generated under VBNC-inducing conditions are dead. Hence, the dormant cell phenotype known as VBNC is the same as that known as persister cells.

Results

We hypothesized that VBNC cells are related to persister cells, so we generated VBNC cells from *E. coli* K-12 using long-term nutrient depletion (prolonged exposure to 0.85% NaCl buffer), a well-known method to form VBNC cells (Ohtomo and Saito, 2001; Muela *et al.*, 2008), and studied the cell population under these VBNC-inducing conditions in a temporal fashion (over 7 weeks) by measuring the number of total cells, culturable cells and persister cells as well as by characterizing their morphology. Total cells were visualized using a haemocytometer with membrane stain Syto 9, and propidium iodide (PI) was used to indicate cells with damaged membranes. However, we found later that PI was not effective in indicating the dead cells in the VBNC population (below). Culturable cells were determined by colony forming units (CFU) without antibiotic treatment, and persister cells were determined by counting CFU after antibiotic treatment. To enable a comparison to the cell population in the VBNC-inducing conditions, we generated persister cells in high numbers by pre-treating cells with rifampicin (Kwan *et al.*, 2013). We have verified seven ways that these rifampicin pre-treated cells are persister cells (multidrug tolerance, easy conversion from persister state to non-persister state, metabolic dormancy, no change in the minimum inhibitory antibiotic concentration compared with exponential cells, no resistance phenotype, similar morphology to natural persisters and similar resuscitation as natural persisters) (Kim and Wood, 2018). Critically, this method of creating a high concentration of persister cells, by pretreating with one of the compounds we utilized for *E. coli*, carbonyl cyanide *m*-chlorophenylhydrazone (Kwan *et al.*, 2013), has been validated for *Pseudomonas aeruginosa* and *S. aureus* (Grassi *et al.*, 2017); pretreating with rifampicin and tetracyline to generate *E. coli* persisters has also been verified by an independent group (Cui *et al.*, 2018). We also compared the rates at which individual cells resuscitate by using agarose pads and light microscopy as well as compared cell morphology via transmission electron microscopy (TEM).

The small, culturable fraction formed under VBNC-inducing conditions are persister cells

In an *E. coli* population with an initial size of 2×10^8 total cells ml^{-1} (viable + non-viable cells) (Fig. 1) as determined using a haemocytometer to ensure all particles were counted (Supporting Information Fig. S1), we found that the number of culturable cells decreased dramatically from 10^8 to 10^3 cells ml^{-1} in 7 weeks (Fig. 1). The number of total cells stained by PI was less than 4%. Critically, the number of persister cells (i.e., those that survive antibiotic treatment) increased from 10^3 to 10^5 cells ml^{-1} in 2 weeks (Fig. 1), and all the culturable cells under the VBNC-inducing conditions become persister cells; hence, all the culturable cells under the VBNC-inducing conditions became persister cells and remained persister cells for 7 weeks (when the experiment was stopped). This result indicates that starvation induces persister cell formation and that all the culturable cells in the VBNC-induced condition phenotypically behave exactly like persister cells after 2 weeks.

To ensure this result was not an artefact of using a laboratory strain (*E. coli* K-12), we performed the same

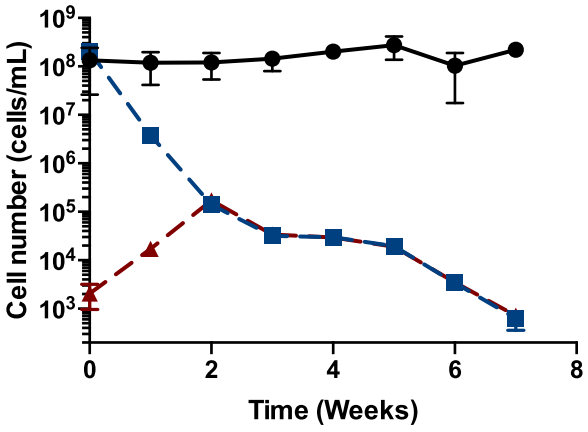

Fig. 1. Temporal change in VBNC culturable and persister cells. The total *E. coli* K-12 cell population is indicated by black circles and was determined using a haemocytometer with membrane stain Syto 9. The culturable cell number is indicated by blue squares and was determined by colonies formed on plates. Persister cells are indicated by red triangles and were determined by colonies formed on plates after ampicillin treatment for 3 h.

starvation experiment with the environmental isolate *E. coli* O157:H7 EDL933 (EHEC) (Perna *et al*., 2001). As with *E. coli* K-12, we found that all the culturable cells under the VBNC-inducing conditions become persister cells in 2 weeks (Supporting Information Fig. S2).

The small, culturable fraction formed under VBNC-inducing conditions resuscitate like persister cells

Persister and VBNC cells share many features like tolerance toward many stress factors (e.g., antibiotics, heat, acid) (Ayrapetyan *et al*., 2015a), induction by various environmental stresses (Ayrapetyan *et al*., 2015a; Harms *et al*., 2016), and dormancy in stressful environments (Bigger, 1944; Xu *et al*., 1982). However, unlike what has been reported for VBNC cells, persister cells can easily be resuscitated by providing nutrients when the stress is removed (Ayrapetyan *et al*., 2015a). To confirm that the live cell population created under the VBNC conditions, that we found to be antibiotic tolerant (Fig. 1), resuscitate like persister cells, the resuscitation rate of individual cells on agarose pads was determined.

The positive control, exponential cells, began cell division immediately (Supporting Information Movie S1), which indicates, that there are no delays or artefacts affecting growth inherent with our agarose pad method. The persister cells resuscitated with various waking times (0–6 h) (Supporting Information Movie S2), and had a similar growth rate as exponential cells, which means that persister cells convert to normal growing cells upon waking (data not shown). In contrast to persister and exponential cells, on the agarose pads, most of the cells under the VBNC-inducing condition were small, not dense and spherical (Supporting Information Movie S3). After 5 weeks, the total number of viable cells in the population grown under the VBNC-inducing conditions did not change appreciably compared with 0–4 weeks, which indicates that this morphologically abnormal cell population is the major cell phenotype. Critically, this cell spherical phenotype found under the VBNC-inducing conditions did not resuscitate in 72 h on the agar pads (Supporting Information Movie S3).

Since the number of persister cells formed under the VBNC-inducing conditions was very low after 5 weeks (about 10^4 cells ml^{-1}, Fig. 1), 2-week-old VBNC cultures that were pre-treated with ampicillin were examined, and it was found that prior to dividing, some spherical cells became rod-shaped (Supporting Information Fig. S3, blue arrows) while some did not revive (Supporting Information Fig. S3, red arrows). Similarly, rods formed under the VBNC-induced condition revived immediately (Supporting Information Fig. S3, green arrows).

The small, culturable fraction formed under VBNC-inducing conditions and persister cells have similar morphology

Cells formed under VBNC-inducing conditions cells are frequently spherical (Du *et al*., 2007; Zeng *et al*., 2013), exponential-phase *E. coli* cells are rods; and persister cells have not been characterized well via microscopy due to the difficulty in getting populations with significant fractions of persister cells. To explore further the relationship between the cell population formed under VBNC-inducing conditions and persister cells, we utilized TEM. The TEM images confirm that exponentially growing cells (positive control) are rod shaped and healthy (i.e., dense cytosol, Fig. 2); hence, healthy cells have dense cytosol with this visualization method (Robinow and Kellenberger, 1994). Critically, the majority of the cells (5 weeks) formed under VBNC-inducing conditions have empty cytosol, and they have either intact or damaged membranes (Fig. 2; Supporting Information Fig. S4). The empty region in the cytosol might explain why most VBNC cells do not resume normal growth: they are dead, since empty cytosol is a sign of cell death (Szostak *et al*., 1996; Amara *et al*., 2013). Using the TEM images, the viability of cells formed under the VBNC-inducing condition was estimated by counting cells with dense cytosol as live cells and cells with empty cytosol as dead: from our images, 99.5% of the total VBNC cell-like particles have the characteristics of dead cells (Table 1).

TEM images of younger cells (2 weeks) formed under the VBNC-inducing conditions showed two types of spherical cells: those with dense cytosol and those with empty cytosol (Supporting Information Fig. S5). This suggests that the spherical cells that do not resuscitate are dead (empty cytosol), whereas spherical cells with dense cytosol resuscitate. These morphological changes (empty cytosol and dwarfing) of cells under VBNC-inducing conditions have been seen in the plant pathogen, *Ralstonia solanacearum*, which indicates that our findings apply to environmental isolates (Um *et al*., 2013).

Newly-formed persister cells (3 h) are also rod shaped, but a few of them are clearly injured with damaged membranes (Fig. 2; Supporting Information Fig. S6). Additionally, some of the persister cells are spherical after 3 h. Critically, a few of the VBNC-like (spherical/empty cytosol) shaped cells were also present in the persister cell population after 3 h (Fig. 2) This result suggests that perhaps the newly-formed persister cells undergo a morphological change as they age; hence, we monitored the shape of persister cells via fluorescent microscopy with FM 4-64 staining (which helped visualize the cells). As expected, the exponential cells were cylindrical (positive control) but rifampicin-induced persister cells had shorter cell lengths after 3 h (Fig. 3A); this reduction in cell volume increased with age for persister cells (Fig. 3A). Based on

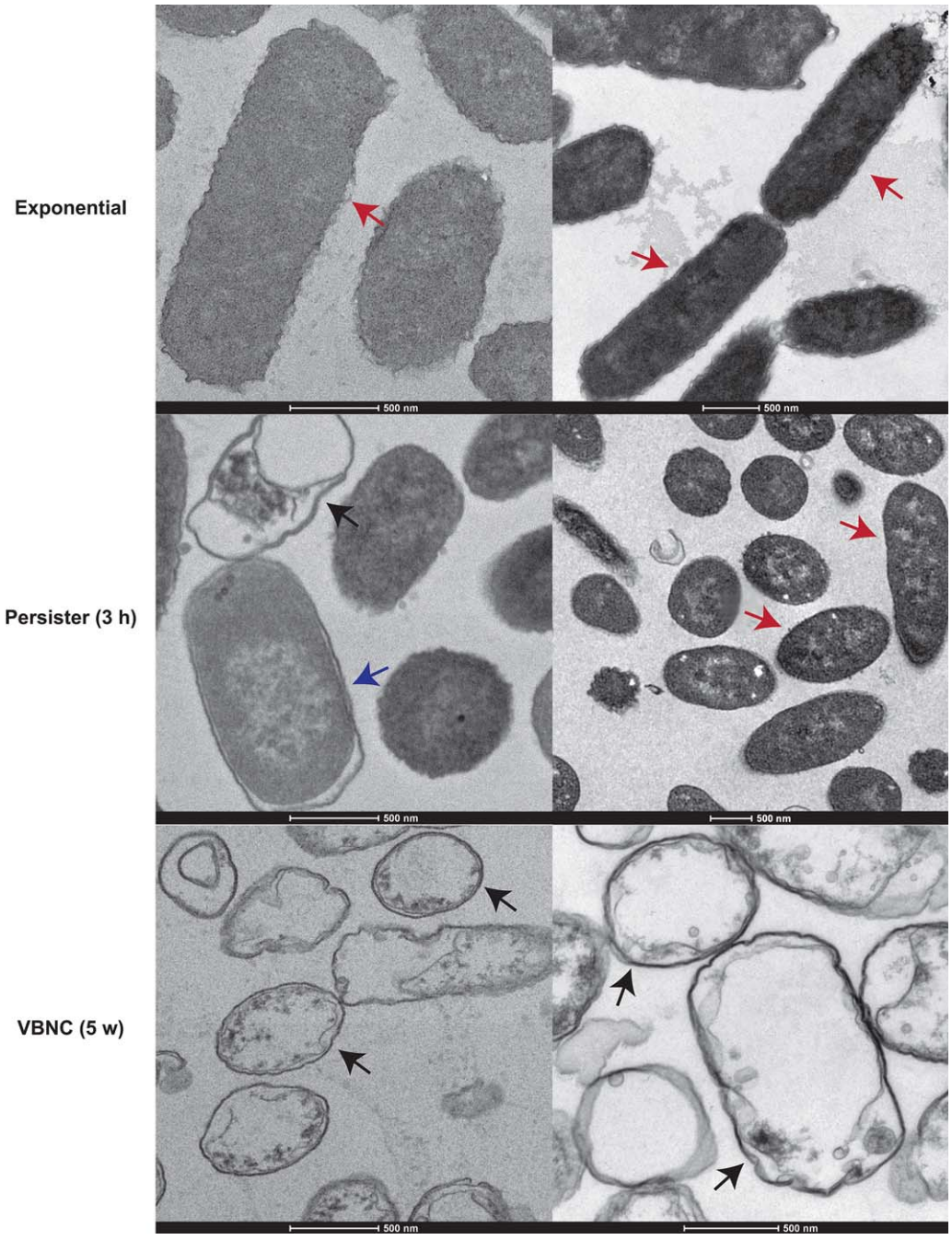

Fig. 2. TEM images of exponential, persister and VBNC cells. Exponentially grown *E. coli* K-12 cells, rifampicin-induced persister cells after 3 h ampicillin treatment, and 5 week (5 w) old VBNC cells (after ampicillin treatment for 3 h) in saline solution were visualized by TEM. Red arrows indicate cells with dense cytosol and intact membranes, blue arrows indicate cells in the intermediate stage of material loss, and black arrows indicate cells with empty cytosol but intact membranes. Scale bar indicates 500 nm.

this observation, cell roundness was calculated. The average roundness, which indicates how closely the shape of a cell approaches that of a circle, of exponential cells was 0.4 while the average roundness of persister cells was 0.6 after 3 h (Fig. 3B); hence, persister cells were more spherical than exponential cells and began to appear more similar to VBNC cells as they aged. The spherical shape for the persister cells from 3 to 9 h increased as evidenced by the shift to the right (increasing roundness) of the cell population average (Fig. 3B).

After 24 h, rod-shape persister cells remained but there was the accumulation of cell membrane debris as

Table 1. Comparison of the viability of each *E. coli* K-12 cell type via TEM and PI staining method.

Techniques	Cell type	Total (*n*)	Dense cytosol/live (%)	Empty cytosol/dead (%)
TEM	**Exponential**	239	100	0
	Persister (3 h)	252	95 ± 1	6 ± 1
	VBNC	272	0.7 ± 0.2	99.4 ± 0.2
Flow cytometry	**Exponential**	100 000	99.2 ± 0.4	0.8 ± 0.4
	Persister (3 h)	100 000	94 ± 2	6 ± 2
	VBNC	100 000	86 ± 2	14 ± 2

visualized by FM 4-64 staining since the population of persisters usually decreases (Supporting Information Fig. S7); hence, the persister cells that became spherical died and lysed, much like the cells formed under the VBNC-inducing conditions as demonstrated by TEM (Fig. 2). We hypothesized that the empty spheres in the persister culture were dead cells and only the rod-shape persister cells could resuscitate; that is, cells in the persister cell culture

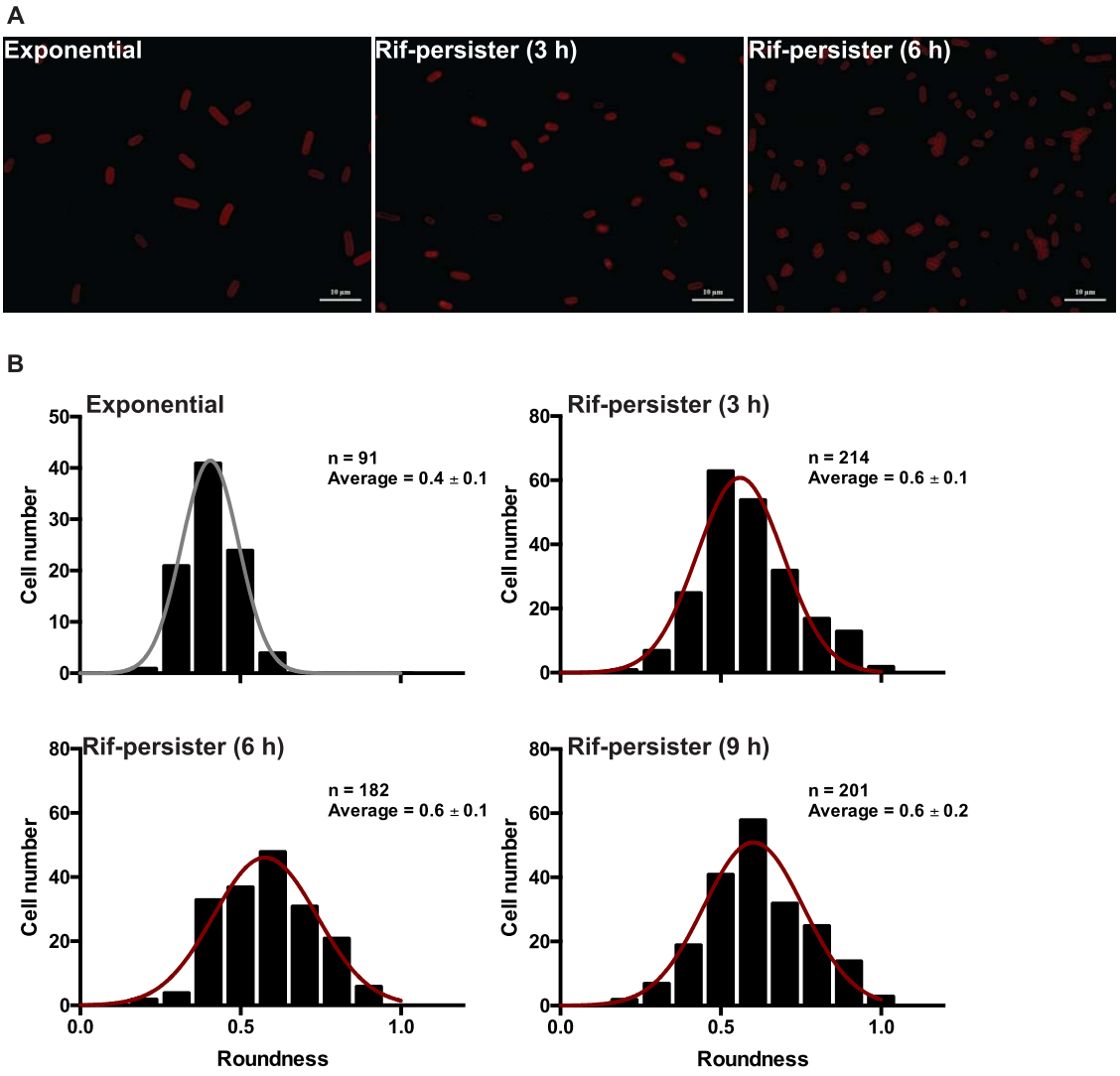

Fig. 3. Aging persister cells become spherical. Rifampicin-induced *E. coli* K-12 persister cells in LB in the presence of 100 μg ml^{-1} of ampicillin were observed after 3, 6, 9 and 12 h via fluorescence microscopy with FM 4–64 staining.
A. Representative microscopic images for exponentially growing cells and aging persister cells.
B. Calculation of cell roundness by ImageJ. Compared with exponential cells, persister cells have increasing roundness with age (distribution of roundness shifts to right).

slowly die over 24 h in the presence of ampicillin. We tested this hypothesis by measuring the viability of persister cells and found that in 24 h, the viable persister cell fraction decreased by 70-fold (Supporting Information Fig. S8), which is much slower than the death rate of exponential cell by ampicillin (10 000-fold lower than exponential cell death in 24 h). Therefore, the persister population is reduced due to cell death over 24 h.

TEM images of fresh persister cells (3 h) (Supporting Information Fig. S6) and old persister (24 h) cells (Supporting Information Fig. S9) showed both the 3-h and 24-h persister cells were smaller compared with exponentially-growing cells (Fig. 2) which agrees with FM 4-64 results (Fig. 3). Moreover, the spheres seen in the fresh persister population had dense (full) cytosol. Since, most of the old persister cells (24 h) are spherical with empty cytosol (Supporting Information Fig. S9), they resemble cells of the VBNC cultures (Supporting Information Figs. S4 and S5); however, some rod-shaped cells with full cytosol remain in the 24 h persister culture (Supporting Information Fig. S9), and it is these rod-like cells that remain after 24 h that were seen to revive on agarose pads like fresh persister resuscitation (Supporting Information Fig. S10). Hence, persister cells that are aged in the presence of antibiotic appear the same as cells formed under the VBNC-inducing conditions treated with ampicillin.

The small, culturable fraction formed under VBNC-inducing conditions and persister cells are metabolically inactive

VBNC cells (Oliver, 2005; Nowakowska and Oliver, 2013) and persister cells (Kim and Wood, 2016; 2017) are both dormant. To investigate the metabolic activity of the cells formed under VBNC-inducing conditions and persister cells used in this study, redox sensor green, which indicates cellular metabolic activity, and PI, which indicates membrane damage, were utilized with flow cytometry so that the signals could be quantified. In terms of metabolic activity, both the 5-week-old cells formed under the VBNC-inducing conditions and the 3-h persister cells had low metabolism similar to that of the dead cell control (Fig. 4A). Note this lack of metabolic activity in persister cells as demonstrated by redox sensor green staining has been confirmed by us (Kim and Wood, 2018). Perhaps the persister cells and cells formed under VBNC-inducing conditions appear to have lower metabolic signal than the dead cells since their membrane structures are altered as we showed with TEM; however, the crux is the two cells type have similar, low metabolism. As a positive control, there was high metabolic activity with the exponential cells, as expected (Fig. 4A), and unstained exponential cells (negative control) had the expected low signal (Fig. 4A).

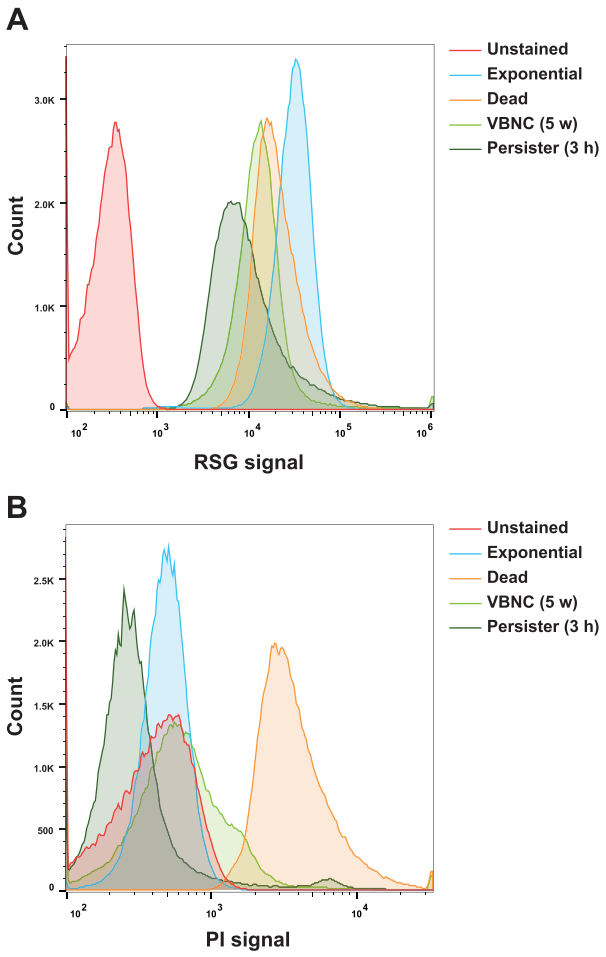

Fig. 4. Metabolic activity of persister and VBNC cells. Metabolic activity of exponentially-growing *E. coli* K-12 cells, persister cells (3 h), and VBNC cells (5 weeks: '5 w', ampicillin-treated for 3 h) as determined by the BacLight RedoxSensor Green (RSG) Vitality Kit and flow cytometry.
A. Metabolic activity of each cell culture based on the RSG signal.
B. Cell viability of each cultures based on PI signal. As controls, unstained exponential cells and dead cells (70% isopropanol treatment for 1 h) were employed.

As with the fluorescence microscopy data where PI staining indicated little change in the total number of live cells ($<$ 4%), only 15% of cell population in the VBNC culture appeared dead using PI staining (Fig. 4B). However, the TEM results indicate that a majority of cells ($>$ 99%) formed under the VBNC-inducing conditions have lost their cellular mass and are dead (Supporting Information Fig. S4). As controls, the dead cell fraction of exponentially growing cells was 0.5% and the fraction of dead cells in the isopropanol-treated dead cell control was 99.3% (Fig. 4B). These conflicting results of the TEM images showing nearly all the cells are dead (i.e., have empty cytosol) and PI staining indicating most cells are viable indicate that PI staining works in many situations but unfortunately, not for VBNC cultures where it has been used previously

(Cunningham *et al*., 2009; Su *et al*., 2016). To confirm the majority of the starving cells lack cytosolic compounds, we assayed for double-stranded DNA by staining with PicoGreen and found that DNA decreases by an order of magnitude per cell in 2 weeks (Supporting Information Table S1).

Discussion

Although many similarities exist in the published descriptions of VBNC and persister cells (the main distinction is that VBNC cells are not culturable), these two phenotypes have not been compared experimentally. By assaying for persister cells in the VBNC population, we discovered here that the viable cells formed under the VBNC-inducing conditions are persister cells (tolerant to antibiotics) (Fig. 1); hence, persister cells may be formed from nutritive stress, and the culturable fraction of the cells formed under the VBNC-inducing conditions are persister cells. For the cell population previously known as VBNC, most of the cells are dead as evidenced by their empty cytosol (Fig. 2; Supporting Information Fig. S4). Additionally, we also found that there are some spherical cells in the persister culture after 3 h that lack cellular material (Fig. 2; Supporting Information Fig. S6), and that persister cells become spherical as they age and have an increasing fraction with empty cytosol (Fig. 3; Supporting Information Fig. S9). Furthermore, the persister cell population is reduced dramatically over 24 h (Supporting Information Figs. S6–S8). Hence, cultures of 24-hour-old persisters and cells formed under VBNC-inducing conditions have the same morphology and both cultures have live cells (dense cytosol) that are found in a background of dead cells, and it is these intact *E. coli* cells that resuscitate. We also found both the culturable fraction of the cells formed under VBNC-inducing conditions and persister cells have low metabolic activity (Fig. 4) and can resuscitate immediately; note we have demonstrated that for persister cells, resuscitation is a function of their ribosome content (Kim and Wood, 2018). Therefore, the viable cells formed under the VBNC-inducing conditions appear to be the same as persister cells based on antibiotic tolerance, morphology, resuscitation rates and metabolic activity. We suggest then that the terms VBNC and persistence describe the same phenotype for dormant cells and that the term VBNC should be replaced with persister cells since VBNC cells do not represent a separate cell phenotype. In addition, since persister cells have been found in nearly all strains that have been tested (Van den Bergh *et al*., 2017), and we have shown that VBNC cells are persister cells in *E. coli*, our results suggest that what have been characterized previously as VBNC cells for non-laboratory strains should be persister cells as well. Corroborating this expectation, we found the natural isolate *E. coli* O157:H7 also formed persister cells under VBNC-inducing conditions (Supporting Information Fig. S2). Hence, our results suggest the persister phenotype is more prevalent than generally recognized since nearly all cells in the environment experience nutrient limitations.

One non-intuitive finding here is that most of the 'VBNC' cells shrink and are dead since they contain little cytosolic content (i.e., proteins, Fig. 2); yet, they do not stain with PI, which indicates their membranes are not visibly damaged. This lack of staining by PI has led many groups to surmise that these cell remnants are alive and are difficult to culture; however, TEM clearly indicates they are dead. What remains unclear is how the cellular contents are lost without damaging the membranes. The loss of cell content does fit well with the fact that others have found that cells shrink and become spherical [e.g., *Salmonella typhi* (Zeng *et al*., 2013), *Edwardsiella tarda* (Du *et al*., 2007)] as we have demonstrated with aging persister cells (Figs. 2 and 3). Perhaps this loss of cell content occurs as the persister cells age through blebbing as seen in the presence of β-lactams (DeLoney and Schiller, 1999) which would keep the membrane intact. Regardless of the aging mechanism, stressed cells die, and the remaining viable fraction are persister cells.

Material and methods

Bacterial strain and growth conditions

E. coli K-12 BW25113 (Baba *et al*., 2006) and *E. coli* O157:H7 EDL933 (EHEC) (Perna *et al*., 2001) were used in this study. All experiments were conducted at 37°C in saline (0.85% NaCl) for VBNC cultures and lysogeny broth (LB) (Sambrook *et al*., 1989) for persister cells.

Total, viable and antibiotic-tolerant cells in VBNC cultures

To generate VBNC cells, overnight LB cultures were inoculated into 25 ml fresh LB using a 1:1000 dilution and incubated until the turbidity at 600 nm reached 3. Cells (5 ml) were harvested by centrifugation at 3500 × g, 4°C for 10 min and the cell pellet was diluted 1:10 into saline (0.85% NaCl, 50 ml) and incubated at 37°C, 250 rpm. Every week, cell viability and total cell counts were determined with a hemocytometer after staining with Syto9 (Molecular Probes, Eugene, OR) and PI (Molecular Probes, Eugene, OR) and visualizing cells with a fluorescence microscope (Zeiss Axioscope.A1) with 400× magnification (40×, 10×), bright field, FITC and PI channel (exposure time ~ 2000 ms). The images were superimposed and counted manually. The culturable cell number in the VBNC populations were determined by counting colonies on LB agar plates after 16 h. To determine the number of antibiotic-tolerant cells (i.e., persister cells) in the VBNC population, 25 ml were harvested, the cell pellet was resuspended into 25 ml of LB containing ampicillin (100 μg ml^{-1}) for 3 h to remove the antibiotic-sensitive cells (which lyse) (Shah *et al*., 2006), centrifuged, washed by phosphate buffered saline (PBS) twice, and plated onto LB agar for 16 h.

Persister and VBNC cell resuscitation

To generate persister cells, 25 ml of exponentially-growing cells (turbidity at 600 nm ~ 0.8) were treated with rifampicin (100 μg ml^{-1}) for 30 min (Kwan *et al.*, 2013) and harvested by centrifugation (3500 × g, 4°C for 10 min). The cell pellets were resuspended in 25 ml of LB containing ampicillin (100 μg ml^{-1}) and incubated for 3 h to remove non-persisters via lysis.

For resuscitation, 1 ml was taken from the exponential, persister and VBNC cultures and washed by PBS twice and plated onto agarose gel pads for microscopy. For the VBNC culture (2 or 5 weeks), cells were harvested by centrifugation and resuspended in LB containing ampicillin for 1 or 3 h to remove antibiotic-sensitive cells before the PBS wash. The agarose gel pads were prepared with LB with 1.5% low melting temperature agarose (Nusieve GTG agarose – BMB # 50081); the melted LB agarose was poured into a template made from five glass slides (75 × 25 × 1 mm). The pad was covered by another slide glass and held together with a 50 g weight for 30 min to solidify. Samples (5 μl) were added to the agarose gel pad, covered by a coverslip and sealed by nail polish to prevent evaporation. Cell growth at 37°C on the agarose gel pad was observed every 20 min for up to 24 h by light microscopy (Zeiss Axioscope.A1, bl_ph channel at 1000 ms exposure). During the observations, the microscope was placed in a vinyl glove box (Coy Labs) warmed by an anaerobic chamber heater (Coy Labs, 8535-025) to maintain 37°C.

Transmission electron microscopy imaging

Samples (1 ml) of exponential and persister cells (3 and 24 h) were pelleted by centrifugation at 17 000 × g for 2 min, washed twice with 1 ml of normal saline and resuspended in 1 ml of normal saline. For VBNC cultures (5 weeks), 10 ml of cells were treated with ampicillin (100 μg ml^{-1}) in LB for 3 h at 37°C to remove exponentially growing cells, pelleted, washed once with 1 ml of normal saline and resuspended in 1 ml of normal saline (10-fold concentration). In brief, for double staining (McAnulty *et al.*, 2017), cells were pelleted and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1–12 h. Pellets were washed three times for 5 min with 0.1 M sodium cacodylate followed by secondary fixation with 1% osmium tetroxide for 1 h in the dark at room temperature. Next, samples were washed three times for 5 min with 0.1 M sodium cacodylate and for 5 min with water followed by en bloc staining with 2% uranyl acetate for 1–12 h. Samples were then dehydrated by using a series of ethanol washes (50%, 70%, 85% and 95%, 3 × 100%), washed three times for 5 min with acetone and embedded in Epon-Araldite (Ted Pella, Redding, CA). After the blocks had cured, they were sliced using an ultramicrotome and 70 nm thin slices were collected on TEM grids (formvar/carbon coated grid) and post-stained with uranyl acetate and lead citrate. Sample grids were imaged by a Tecnai G2 Lab6 TEM at 200 kV.

Flow cytometry

With exponential cells, rifampicin-induced persister cells (3 h) and VBNC cells (5 weeks old), metabolic activity was measured by flow cytometry. Cells were pelleted and washed twice with PBS. For the VBNC culture, to remove the exponentially growing cells, ampicillin (100 μg ml^{-1}) in LB was utilized for 3 h before staining. For the dead cells (negative control), 1 ml of exponential culture was centrifuged, resuspended in 70% isopropanol and incubated for 1 h at room temperature. The redox sensor and PI stains (BacLight™ RedoxSensor™ Green Vitality Kit, Thermo Fisher Scientific, Waltham, MA) were used with samples incubated at 37°C for 10 min with light protection. The fluorescence signal was analyzed by flow cytometry (Beckman Coulter FC500) using the FL1 (525 ± 40 nm) and FL3 channels (620 ± 20 nm).

Tracing morphological changes during persister aging

At each time (3, 6, 9 and 24 h), 1–3 ml of exponential and rifampicin-induced persister cell cultures were harvested by centrifugation at 17 000 × g for 1 min and resuspended in 1 ml of PBS. Morphological changes were observed by utilizing FM 4-64 fluorescent dye (10 μg ml^{-1}, Thermo Fisher Scientific), which stains cellular membranes, via fluorescence microscopy for improving the resolution to allow better observation of the cell size (Zeiss Axio.A1, Blight field, 1000 ms exposure and FM 4-64 filter 10 000 ms exposure). Cell roundness was calculated by ImageJ as $4 \times \frac{[\text{Area}]}{\pi \times [\text{Major Axis}]^2}$.

DNA assay

At each time point (0, 1, 2 and 3 weeks), 500 μl of the VBNC culture was sonicated for 30 sec (Sonic Dismembrator F60, Thermo Fisher Scientific, Waltham, MA) on ice to lyse completely the cells and the insoluble cellular debris was removed by centrifuging at 13 000 rpm for 1 min. The supernatants (100 μl) were mixed with the same volume of the double-strand DNA stain PicoGreen reagent (1:1). The mixtures were incubated in the dark room for 5 min and the fluorescence signals were measured at 480 nm excitation and 520 nm emission via a spectrophotometer (TECAN infinite M200PRO). The DNA amount in single cells were quantified based on a standard curve and normalized by the total cell number as determined by a haemocytometer.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Movie S1. Cell division of exponentially-growing cells on an agarose gel pad. Exponential-phase *E. coli* K-12 cells growing on an agarose gel pad at 37°C demonstrating that all cells grow immediately by cell division. Images were taken every 20 min, and the scale bar indicates 10 μm.
Movie S2. Persister cell resuscitation on an agarose gel pad. Resuscitation of rifampicin-induced *E. coli* K-12 persister cells on an agarose gel pad at 37°C demonstrating immediate resuscitation. Green arrows indicate cells that resuscitate immediately and blue arrows indicate cells that have delayed resuscitation. Images were taken every 20 min, and the scale bar indicates 10 μm.
Movie S3. VBNC cell resuscitation on an agarose gel pad. Resuscitation of *E. coli* K-12 VBNC cells (5 weeks) on an agarose gel pad at 37°C demonstrating no resuscitation. Images were taken every 1 h, and the scale bar indicates 10 μm. Red arrows indicate spherical cells that did not resuscitate.
Table S1. Loss of DNA per cell in *E. coli* K-12 cells formed under VBNC-inducing conditions.
Figure S1. **Counting total *E. coli* K-12 cell numbers in VBNC cultures using a hemocytometer.** The cells in 19 squares (0. 05 mm* 0. 05 mm * 0. 1 mm = 2. 50E-04 μL) were counted using Syto 9 and PI staining. Total cell number was averaged based on the equation = [(Average cell number in the square * 1000) x 2. 50E-04] * dilution factor.
Figure S2. **Temporal change in VBNC culturable and persister cells of the environmental isolate *E. coli* O157: H7 EDL933.** The total cell population is indicated by black circles and was determined via a hemocytometer with membrane stain Syto 9. The culturable cell number is indicated by blue squares and was determined by colonies formed on plates. Persister cells are indicated by red triangles and were determined by colonies formed on plates after ampicillin treatment for 3 h.
Figure S3. **Resuscitation of morphologically-different *E. coli* K-12 VBNC cells (2 week old, ampicillin-treated at 100 μg/mL for 1 h).** 2 week old cells formed under VBNC-inducing conditions were treated with ampicillin to lyse non-persister cells. The rod-shape cells (green arrows) resuscitated, some spherical cells (red arrows) did not resuscitate, and some spherical cells (blue arrows) resuscitated. Scale bar indicates 10 μm.
Figure S4. **TEM image of a 5 week-old *E. coli* K-12 VBNC culture (ampicillintreated for 3 h).** 5 week old cells formed under VBNC-inducing conditions were treated with ampicillin to lyse non-persister cells. Most VBNC cells had empty cytosol (black arrows) and some of the population had cytosol with cellular material reduced (blue arrows). Scale bar indicates 1 μm.
Figure S5. **Two types of spherical cells in *E. coli* K-12 VBNC cultures (2 week old, ampicillin-treated at 100 μg/mL for 3 h)**. Green arrows indicate spherical cells with dense cytosol. Red arrows indicate spherical cells with empty cytosol. Scale bar indicates 1 μm.
Figure S6. **TEM image of fresh *E. coli* K-12 persister cells after 3 h in LB medium with ampicillin (100 μg/mL).** Exponentially-growing cells were treated by rifampicin to form persister cells and ampicillin was used for 3 h to lyse non-persister cells. Most persister cells had dense cytosol and intact membranes (green arrows) while a few of the cells had VBNC-like empty cytosol (red arrow). Image is a larger field of view for the cells of **Fig. 2.** Scale bar indicates 1 μm.
Figure S7. ***E. coli* K-12 persister cell morphology after 24 h in LB medium with ampicillin (100 μg/mL).** Rifampicin-induced persister cells were cultured 24 hours in the presence of ampicillin and observed via fluorescence microscope by staining with membrane dye FM 4-64. Yellow arrows indicate representative cells that survive after the 24 h ampicillin treatment, and pink arrows indicate membrane-related debris. Scale bar indicates 10 μm.
Figure S8. ***E. coli* K-12 persister cell viability during aging.** The viability of rifampicin-induced persister cells in the presence of ampicillin was determined by plating. After

24 h, the viable persister cell number was reduced 70-fold, which is much lower than the cell death of exponentially-growing cells (reduction of 10^6-fold in 24 h, data not shown).

Figure S9. **TEM image of aged *E. coli* K-12 persister cells after 24 h in LB medium with ampicillin (100 μg/mL).** Exponentially-growing cells were treated by rifampicin to form persister cells and ampicillin was used to lyse non-persister cells. Most cells (black arrows) had empty cytosol and intact or damaged membranes like the VBNC culture. A few cells had dense cytosol and were rod-shape (red arrow). Scale bar indicates 1 μm.

Figure S10. **Resuscitation of aged *E. coli* K-12 persister cells after 24 h in LB medium with ampicillin (100 μg/mL).** Exponentially-growing cells were treated by rifampicin to form persister cells and ampicillin was used to lyse non-persister cells. The surviving cells were rod-shaped (green arrows), and they resuscitated immediately by cell elongation then cell division. Scale bar indicates 10 μm.